PROTOCOL OPTIMIZATION OF QPCR FOR ANALYSIS OF THE EFFECTS OF ANTIBIOTICS ON MULTIDRUG-RESISTANT *SALMONELLA* POPULATIONS IN SWINE

An Undergraduate Research Scholars Thesis

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ABSTRACT

Protocol Optimization of qPCR for Analysis of the Effects of Antibiotics on Multidrug-Resistant *Salmonella* Populations in Swine

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Antibiotic resistance is a prominent food safety concern. Antibiotics given to food animals may promote the development of antibiotic resistance within bacterial populations, which then poses a threat to both workers in food animal operations and consumers. A previous study was performed which sought to analyze the effects of ceftiofur and chlortetracycline on pan-susceptible and multidrug-resistant (MDR) strains of *Salmonella* in swine. The purpose of this study was to optimize protocols for quantitative PCR (qPCR) to detect and quantify antibiotic resistance genes, specifically *qnrB19* and $bla_{\text{SHV-12}}$, from swine fecal samples. These antibiotic resistance genes were chosen in order to uniquely identify the MDR *Salmonella* strains that were used to challenge the swine. The *invA* gene was also analyzed to determine the total number of *Salmonella* within the fecal samples, allowing for comparison between pan-susceptible and MDR *Salmonella* quantities. The protocols created for *qnrB19* and $bla_{\text{SHV-12}}$ were successfully optimized and are ready for use in sample analysis, but further work still remains to be done on the protocol for the *invA* gene.

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Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

The fecal samples analyzed/used for this study were provided by a study funded by that National Pork Board (15-072). Thank you to Dr. Franklin Lopez and Dr. Javier Vinasco-Torres for their efforts in collecting these samples.

The *invA* primer and probe sequences depicted in this study were sourced from a 2009 study published by González-Escalona, N., Hammack, T. S., Russell, M., et al.[17]

All other work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

MDR	Multi-Drug Resistant
qPCR	Quantitative PCR
S. Derby	*Salmonella enterica* serovar Derby
S. Senftenberg	*Salmonella enterica* serovar Senftenberg
AMR	Antimicrobial resistance

1. INTRODUCTION

1.1 Introduction

Antibiotics used in food animal operations have been shown to promote antibiotic resistance in bacterial strains, which later poses a food safety risk to human consumers and operation workers.[1] Antibiotics are used in swine operations to control, prevent, and treat disease. Antibiotics commonly used in the swine industry include ceftiofur,[2] chlortetracycline,[3] amoxicillin,[4] and enrofloxacin.[5] Antibiotics have been found to promote antibiotic resistance in swine, selecting for antimicrobial resistance (AMR) in bacterial strains and promoting their growth while eliminating susceptible strains.[6],[7] Use of these antibiotics have been positively correlated with increases in AMR genes such as bla_{CMY}, *aac(3)-VI*, *aadA*, $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}. *tetA*, *tetC*, *qepA*, *qnrB*, *qnrS*, *ermA*, *ermT*, *cfxA*, *cepA*, *cblA*, *hla*, *eta*, *etb*.[8],[9],[10],[11],[12] By monitoring how these genes are selected for when exposed to antibiotics, we have the ability to more accurately predict what kind of food safety risk may be brought about by antibiotic use in swine operations. This allows operation workers and veterinarians to make more accurate judgement calls in any given situation where antibiotics may be necessitated; the potential harm to the operation can be weighed against the potential harm to the workers and consumers. One such method of observation is qPCR.

Quantitative PCR (qPCR) is a form of gene detection that allows the user to observe the amplification in real time.[13] While traditional end-point PCR is effective for confirming whether or not a gene is present within a sample, qPCR allows for the user to quantify how much of the gene is present by seeing how it amplifies compared to standards with known quantities.[13] Additionally, it offers much greater discrimination between gene quantities than

can be found with end-point PCR, allowing for more precise measurements of gene quantity.[13] However, it is limited by factors such as the specificity of its reagents.[13] Primer sequences are used to amplify specific regions of the target genome, but if those sequences do not correspond to regions specific to that gene and instead correspond to gene sequences found in multiple genomes then results may be confounded by an inability to discriminate between products.[13] Despite this, it remains an efficient tool in the use gene amplification, and is of great use in the detection of microbial genetics.[13]

1.2 Preliminary Swine Trials

A previous project was performed within the lab wherein 32 swine were challenged orally with both pan-susceptible and multidrug-resistant (MDR) strains of *Salmonella enterica* serovar Derby (*S.* Derby) and intradermally with both pan-susceptible and MDR strains of *Salmonella enterica* serovar Senftenberg (*S.* Senftenberg). The swine were then treated with antibiotics after the bacteria were given time to colonize the swine. Fecal samples were collected on every day of the trial and lymph nodes were collected at the end of the trial following euthanasia. We hypothesize that the use of antibiotics will decrease the *Salmonella* population; however, the proportion of MDR resistant *Salmonella* will increase.

The purpose of this study was to optimize protocols for qPCR analysis of the presence of the *qnrB19* and *bla*$_{\text{SHV-12}}$ genes within the fecal samples collected from this previous study to detect and quantify the MDR resistant *Salmonella* challenge strains. A qPCR protocol for the *invA* gene was also optimized to quantify the total amount of *Salmonella* in the fecal samples.[14]

2. MATERIALS AND METHODS

2.1 Study Design

In the initial trial, 32 swine were challenged orally with both pan-susceptible and multidrug-resistant (MDR) strains of *Salmonella enterica* serovar Derby (*S.* Derby) and intradermally with both pan-susceptible and MDR strains of *Salmonella enterica* serovar Senftenberg (*S.* Senftenberg) following a 2 week quarantine period. The *S.* Derby MDR strain uniquely displayed the antimicrobial resistance gene *qnrB19* while the *S.* Senftenberg MDR strain uniquely displayed the antimicrobial resistance gene $bla_{\text{SHV-12}}$ (Table 2.1). The animals were challenged on Day 1 and Day 3 of the study. Animals were then treated with either ceftiofur, chlortetracycline, both antibiotics, or neither antibiotic in order to determine the effects of these antibiotics on the presence of pan-susceptible and MDR strains of *Salmonella* within the feces and lymph nodes of the swine. Antibiotic treatments were initiated on Day 5, with ceftiofur being given as an intramuscular injection behind the ear at a dose of 2.27 mg/lb, and chlortetracycline being given as a top dressing in feed from Day 5 through Day 18 at a dose of 400g/ton. Fecal samples were collected on every day of the trial until the swine were euthanized on Day 19.

Table 2.1: Antimicrobial resistance phenotypes and genotypes determined by whole genome sequencing of the four Salmonella strains used in the swine challenge study

***Salmonella* strains**	**Phenotype**	**Genotype**
Senftenberg B58HEB1.1	Pan-susceptible	*aph(3')-I*
Senftenberg A40HEB1.1	MDR	*aac(6')*, *aac(6')-I*, *aac(6')-IIc*, *aadA*, *aph(3')-I*, bla_{SHV}, bla_{TEM}, *cat*, *dfr*A, *ere*(A), *str*A *str*B *sul*1, *sul*2, *tet*(A), *tet*(D)
Derby A1CEB1.1	Pan-susceptible	
Derby C79C1	MDR	*aadA*, *qnr*B, *sul*1, *tet*(A)

2.2 DNA Extraction

DNA extractions of positive control *Salmonella* isolates for qPCR were performed on the QIAcube (Qiagen; Hilden, Germany) utilizing the DNeasy Blood and Tissue Mini Kit (50) (Qiagen) plus QIAamp DNA Accessory Set A (Qiagen). Assessment of overall DNA quality was performed on the FLUOstar Omega microplate reader (BMG Labtech; Hopkinton, MA) and assessed for a 260/280 absorbance value between 1.8 and 2.0. A final DNA concentration for each sample was obtained using a Qubit 3 Fluorometer (Thermo Fisher Scientific; Houston, TX). The qPCR reactions were all performed using appropriate concentrations of Invitrogen UltraPure DNase/RNase Free Distilled Water (Thermo Fisher Scientific), Brilliant III Ultra-Fast SYBR Green QPCR Master Mix with Low ROX (Agilent Technologies; Santa Clara, CA) or Brilliant III Ultra-Fast Probe Master Mix with Low ROX (Agilent Technologies) (depending on whether the protocol calls for a fluorescent probe or not), and the appropriate primer set for each gene corresponding to the optimized protocol for each reaction (See below for further details for each

primer set). Standard curve dilutions and qPCR reactions were prepared in the QIAgility (Qiagen) or prepared by hand. The final reaction was performed using the AriaMx Real-time PCR System (Agilent Technologies). Final data analyses were performed using the AriaMx software (Agilent Technologies). The standard curves were analyzed for R^2, slope, and efficiency values. The accepted range values for these are -3.58 to -3.10, 90% to 110%, and >0.99 for slope, efficiency, and R^2 respectively.[15]

2.3 *qnrB19* Primer Set

The *qnrB19* primer set was generated through the use of IDT's PrimerQuest program. The gene[16] was copied in and the 2 Primers + Probe option was selected in order to generate a primer set with probe for increased specificity.

The *qnrB19* reactions were performed using 5-5.6 µL of water (Thermo Fisher Scientific), 10 µL of Brilliant III Ultra-Fast Probe Master Mix with Low ROX (Agilent Technologies), 0.5-2.5 µL of both the forward and reverse *qnrB19* primer sets (Table 2.2) (IDT) (concentration 5µM), 0.4-1 µL of the *qnrB19* probe (Table 2.2) (IDT) (concentration 1-5µM), and 2 µL of DNA template per reaction. Different concentrations and quantities of the primers were assessed to determine the optimal concentration. The reactions were performed in duplicate by hand with 4.0 µL of template and 36.0 µL of Master Mix which was then hand-mixed and separated into 20 µL reactions for analysis.

Table 2.2: The qnrB19 forward and reverse primer and probe sequences

***qnrB19* Forward Primer Sequence**	5´- CGA CGT TCA GTG GTT CAG AT -3´
***qnrB19* Reverse Primer Sequence**	5´- CCT AAC TCC GAA TTG GTC AGA T -3´
***qnrB19* Probe Sequence**	5' - /56-FAM/AA TGT GTG A/ZEN/A GTT TGC TGC TCG CC/3IABkFQ/ -3´

Reactions in the AriaMx (Agilent Technologies) were performed utilizing the Quantitative PCR – Fluorescence Probe program beginning with 3 minutes at 95°C to initiate the reaction, followed by 40 repeating cycles of 5 seconds at 95°C followed by 10 seconds at an annealing temperature ranging from 55°C to 70°C (different temperatures were tested) with measurements taken following each amplification cycle. Different annealing temperatures were assessed to determine the optimal temperature.

2.4 bla_{SHV-12} Primer Set

The primer sets were previously generated by a graduate student within the lab through use of IDT's PrimerQuest program.

The bla_{SHV-12} reactions were performed using 2-3 µL of water (Thermo Fisher Scientific), 10 µL of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix with Low ROX (Agilent Technologies), 2.5-3.0 µL of both the forward and reverse bla_{SHV-12} primer sets (Table 2.3) (IDT) (concentration 5µM), and 2 µL of DNA template per reaction. Different quantities of the primers were assessed to determine the optimal concentration. The reactions were performed in duplicate by hand with 4.0 µL of template and 36.0 µL of Master Mix which was then hand-mixed and separated into 20 µL reactions for analysis.

Table 2.3: The bla_{SHV-12} forward and reverse primer sequences

bla_{SHV-12} Forward Primer Sequence	5′- ATA AGA CCG GAG CTA GCA AAC -3′
bla_{SHV-12} Reverse Primer Sequence	5′- GGC GTA TCC CGC AGA TAA AT -3′

Reactions in the AriaMx (Agilent Technologies) were performed utilizing the Quantitative PCR – DNA Binding Dye Including Standard Melt program beginning with 3 minutes at 95°C to initiate the reaction, followed by 40 repeating cycles of 5 seconds at 95°C

followed by 10 seconds at temperatures ranging from 60°C to 65°C (different temperatures were tested) with measurements taken following each amplification cycle. Different annealing temperatures were assessed to determine the optimal temperature. Following the amplification cycles the reactions were returned to 95°C for 1 minute, cooled to 55°C for 30 seconds, and returned to 95°C for 30 more seconds in order to generate a melting curve. Data was collected along the temperature increase from 55°C to 95°C. The melting curve was analyzed to ensure that only a singular product was generated. If all generated products displayed the same melting curve, then it can be assumed that all generated products were of the same substance, if not concentration.

2.5 *invA* Primer Set

The *invA* reactions were performed using 4.6-5 µL of water (Thermo Fisher Scientific), 10 µL of Brilliant III Ultra-Fast Probe Master Mix with Low ROX (Agilent Technologies), 1 µL of both the forward and reverse *invA* primer sets[8] (Table 2.4) (concentration 0.5µM), 0-0.4 µL of probe[8] (Table 2.4) (concentration 0.5µM), and 2 µL of DNA template per reaction. The reactions were performed in duplicate and at a 1.1X quantity to account for pipetting error within the QIAgility (Qiagen), resulting in final preparations consisting of 4.4 µL of template and 39.6 µL of Master Mix which was then briefly hand-mixed and separated into 20 µL reactions by hand for analysis.

Table 2.4: The invA forward and reverse primer and probe sequences[17]

***invA*_176_F Forward Primer Sequence**	5′- CAA CGT TTC CTG CGG TAC TGT -3′
***invA*_291_R Reverse Primer Sequence**	5′- CCC GAA CGT GGC GAT AAT T -3′
***invA*_FAM_208 Probe Sequence**	5′- /56-FAM/CTC TTT CGT CTG GCA TTA TCG ATC AGT ACC A/3IAbRQSp/ -3′

Reactions in the AriaMx (Agilent Technologies) were performed utilizing the Quantitative PCR – Fluorescence Probe program beginning with 3 minutes at 95°C to initiate the reaction, followed by 40 repeating cycles of 5 seconds at 95°C followed by 10 seconds at 60°C with measurements taken following each amplification cycle.

Two runs were performed using differing control template DNA in order to test if detected issues were resultant from the reagents used. A third test was run using the Quantitative PCR – DNA Binding Dye Including Standard Melt program beginning with 3 minutes at 95°C to initiate the reaction, followed by 40 repeating cycles of 5 seconds at 95°C followed by 10 seconds at 60°C with measurements taken following each amplification cycle. Following the amplification cycles the reactions were returned to 95°C for 1 minute, cooled to 65°C for 30 seconds, and returned to 95°C for 30 more seconds in order to generate a melting curve.

3. RESULTS

3.1 *qnrB19* gene Protocol Optimization

Multiple protocols were tested for the *qnrB19* gene (Table 3.1), including protocols utilizing *qnrB19* primers previously generated in the lab using IDT's PrimerQuest system (unpublished). When no adjusting of annealing temperatures or primer concentration succeeded in eliminating negative control amplification, the current set of primers were generated with a fluorescent probe to improve specificity (Table 2.2). Testing for primer concentration involved multiple trials run simultaneously, with 1 μL of primers and probe providing the most optimized results. The average annealing temperature for the primers and probe was calculated to 58°C, and proved to be the best temperature when testing of other temperatures yielded poorer results. However, negative control amplification persisted, leading to questions about how much contamination was present within the work space.

Table 3.1: All primer/probe quantities, concentrations, and annealing temperatures tested and their resultant R^2, slope, and efficiency values. Those rows without R^2, slope, and efficiency values were positive control tests. The rows marked in red were from a previous primer set that did not produce viable results. This primer set was run with SYBR Green (Agilent Technologies) rather than a probe and was run on the Quantitative PCR – DNA Binding Dye Including Standard Melt program. The rows marked in black were from the primer set and probe which produced optimized results. The highlighted row contains the optimized results.

Forward/Reverse Primers (µL/µM)	Probe (µL/µM)	Annealing Temperature (°C)	R^2	Slope	Efficiency (%)
2.5/5	N/A	60	N/A	N/A	N/A
2.5/5	N/A	60	0.997	-3.142	108.1
2.5/5	N/A	60	0.984	-2.828	125.7
2/5	N/A	60	0.991	-2.840	125.0
1.5/5	N/A	60	0.996	-2.787	128.5
1/5	N/A	60	0.991	-2.670	136.9
1/5	N/A	65	0.998	-3.137	108.3
1/5	N/A	70	0.193	-0.818	1568
0.5/5	N/A	65	0.997	-2.879	122.5
1/5	N/A	62	0.996	-2.697	134.8
1/5	N/A	65	0.998	-2.733	132.3
1/5	N/A	62	0.996	-2.691	135.3
1/5	N/A	62	0.992	-2.933	119.3
1/5	N/A	65	0.997	-2.773	129.4

Table 3.1: Continued

Forward/Reverse Primers (µL/ µM)	**Probe (µL/ µM)**	**Annealing Temperature (°C)**	**R^2**	**Slope**	**Efficiency (%)**
0.5/5	N/A	65	0.993	-2.779	129.0
0.5/5	N/A	55	N/A	N/A	N/A
1/5	1/5	58	N/A	N/A	N/A
1/5	0.4/5	58	N/A	N/A	N/A
1/5	1/1	58	N/A	N/A	N/A
1/5	0.4/1	58	N/A	N/A	N/A
1/5	1/5	58	N/A	N/A	N/A
1/5	1/5	60	N/A	N/A	N/A
1/5	1/5	55	N/A	N/A	N/A
1/5	1/5	58	N/A	N/A	N/A
1/5	1/5	58	0.996	-2.993	115.8
1/5	1/5	60	N/A	N/A	N/A
1/5	1/5	58	0.992	-2.796	127.9
1/5	1/5	62	N/A	N/A	N/A
1/5	1/5	58	N/A	N/A	N/A
1/5	1/5	58	N/A	N/A	N/A
1/5	1/5	58	0.980	-3.180	106.3
1/5	1/5	58	0.999	-3.137	108.3

The final protocol which produced the most optimized results (Figure 3.1) for this primer set was established as 5 µL of water (Thermo Fisher Scientific), 10 µL of Brilliant III Ultra-Fast Probe Master Mix with Low ROX (Agilent Technologies), 1 µL of both the forward and reverse qnrB19 primer sets (Table 2.2) (IDT) (5µM), 1 µL of the qnrB19 probe (Table 2.2) (IDT) (5µM), and 2 µL of DNA template per reaction. The reactions were performed in duplicate by hand due to concerns about potential contamination originating from within the QIAgility (Qiagen). Reactions were prepared in total to 4.0 µL of template and 36.0 µL of Master Mix which was then hand-mixed and separated into 20 µL reactions for analysis. Reactions were performed in the AriaMx (Agilent Technologies) utilizing the Quantitative PCR – Fluorescence Probe program. The final PCR thermal profile began with 3 minutes at 95°C to initiate the reaction, followed by 40 repeating cycles of 5 seconds at 95°C followed by 10 seconds at 58°C with measurements taken following each amplification cycle.

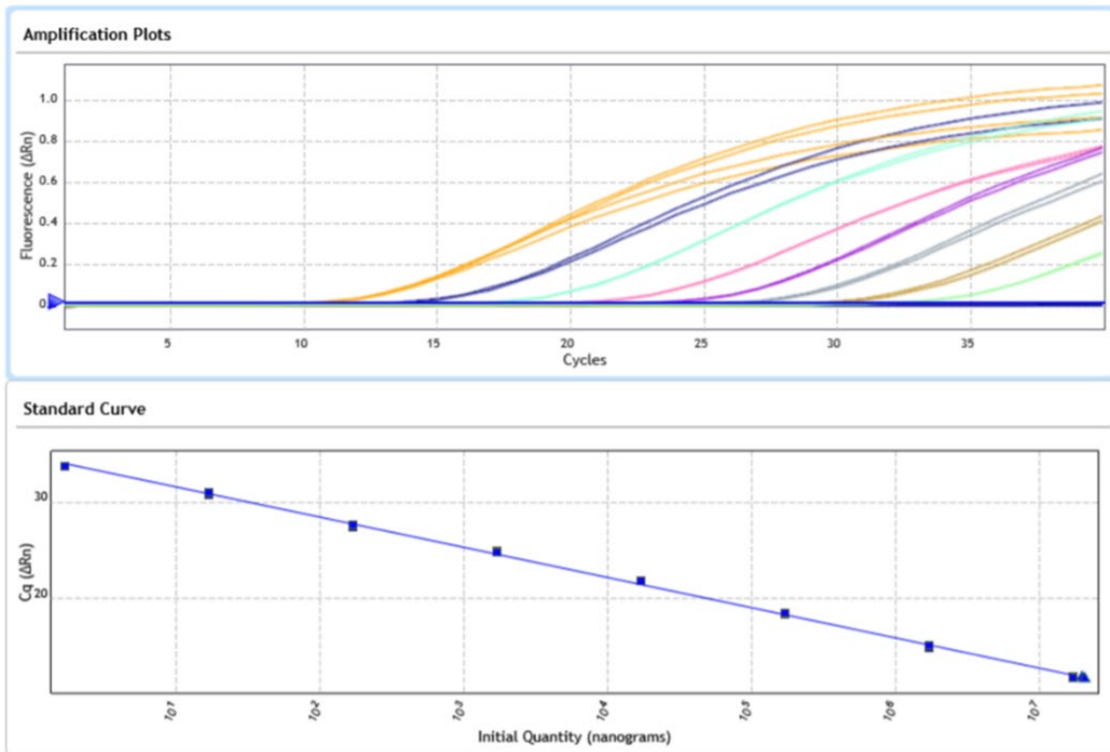

Figure 3.1: The qnrB19 optimized standard curve and amplification plots for the optimized protocol

3.2 *bla*$_{SHV-12}$ gene Protocol Optimization

Multiple protocols were tested for the *bla*$_{SHV-12}$ gene (Table 3.2), with primer concentration beginning at 2.5 µL and an annealing temperature of 60°C, which resulted in near ideal results upon the first run. However, negative control amplification proved to be an issue, and so further adjustment of primer concentrations and annealing temperatures was performed. However, none of these temperatures produced as clean of results as the initial ones tested, and none were successful in eliminating negative control amplification. As such, the same stricter cleaning protocols were implemented for the *bla*$_{SHV-12}$ primers as were used for the *qnrB19* primers which also resulted in the successful generation of a standard curve by hand with the *bla*$_{SHV-12}$ primer set.

*Table 3.2: All primer quantities, concentrations, and annealing temperatures tested and their resultant R^2, slope, and efficiency values. Those rows without R^2, slope, and efficiency values were positive control tests. The row marked with a * are the values gathered from a single curve rather than a duplicate curve like all other trials. This was due to a pipetting error that occurred during the experiment and resulted in one complete and one incomplete standard curve. Only the results from the complete standard curve are recorded here. The highlighted row contains the optimized results.*

Forward/Reverse Primers (µL/µM)	**Annealing Temperature (°C)**	**R^2**	**Slope**	**Efficiency (%)**
2.5/5	60	N/A	N/A	N/A
2.5/5	60	0.997	-3.173	106.6
2.5/5	65	0.996	-3.054	112.5
2.5/5	60	N/A	N/A	N/A
* 2.5/5	60	0.999	-3.569	90.6
2.5/5	60	0.996	-3.328	99.8
2.5/5	60	0.998	-3.266	102.4
3/5	60	0.980	-3.504	92.9

The final protocol which produced the most optimized results (Figure 3.2) for this primer set was established as 3 µL of water (Thermo Fisher Scientific), 10 µL of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix with Low ROX (Agilent Technologies), 2.5 µL of the forward and reverse blaSHV-12 primers (5µM) (IDT), and 2 µL of DNA template per reaction. The reactions were performed in duplicate by hand due to concerns about potential contamination originating from within the QIAgility (Qiagen). Reactions were prepared to a total of 4.0 µL of template and 36.0 µL of Master Mix which was then hand-mixed and separated into 20 µL reactions for analysis. Reactions were performed in the AriaMx (Agilent Technologies) utilizing the Quantitative PCR – DNA Binding Dye Including Standard Melt program. The final PCR thermal profile began with 3 minutes at 95°C to initiate the reaction, followed by 40 repeating cycles of 5 seconds at 95°C followed by 10 seconds at 60°C with measurements taken following each amplification cycle.

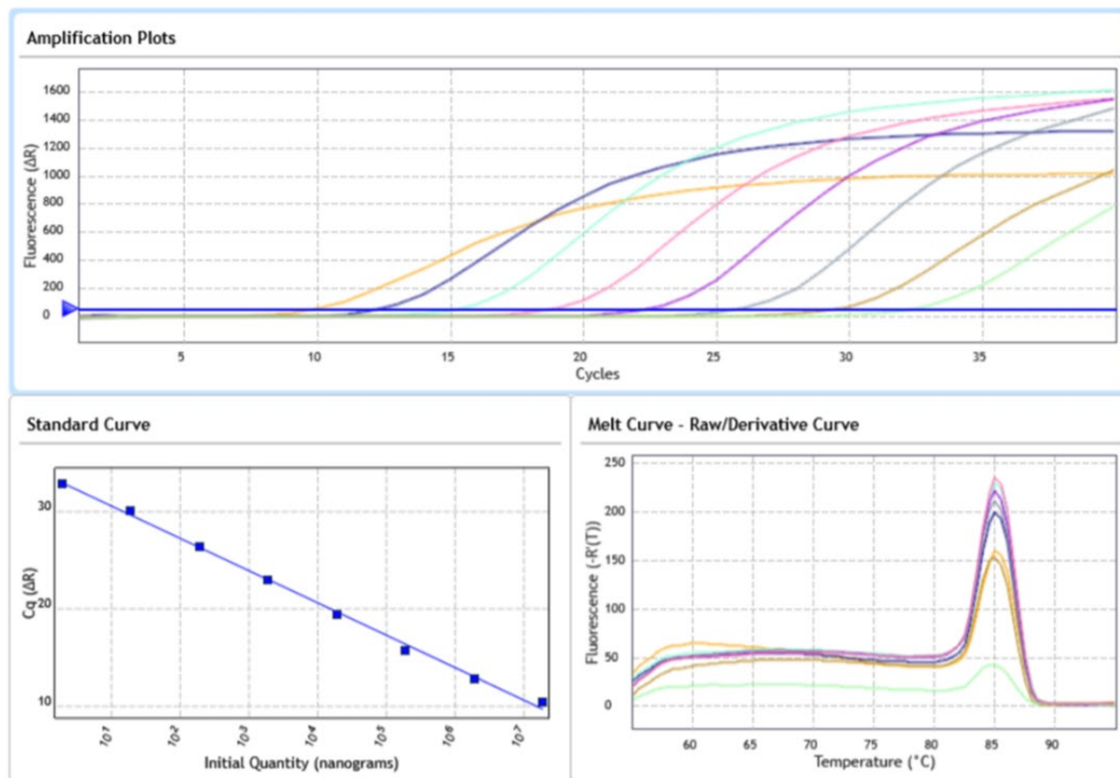

Figure 3.2: The bla_{SHV-12} primers optimized standard curve, amplification plots, and melt curve

3.3 *invA* gene Protocol Optimization

In testing of the *invA* gene, no standard curve was successfully generated with the primer and probe sets using the conditions previously published.[x] However, upon testing of the qPCR product through the use of a gel electrophoresis, product was detected in the positive control samples, with no product detected in the negative control samples (Figure 3.3).

A separate test was run using a generalized fluorescent material rather than a fluorescent probe which did result in amplification detection (Figure 3.4).

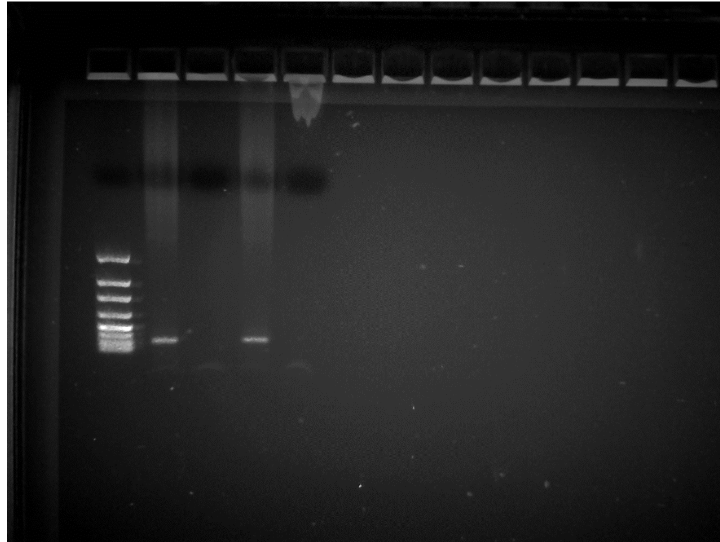

Figure 3.3: Gel electrophoresis of the invA gene positive control test, well 1 is the DNA ladder used for control, wells 2 and 4 are replicate PCR products containing the positive control (DNA from S. Senftenberg) and wells 3 and 5 are replicate PCR products containing the negative control, water

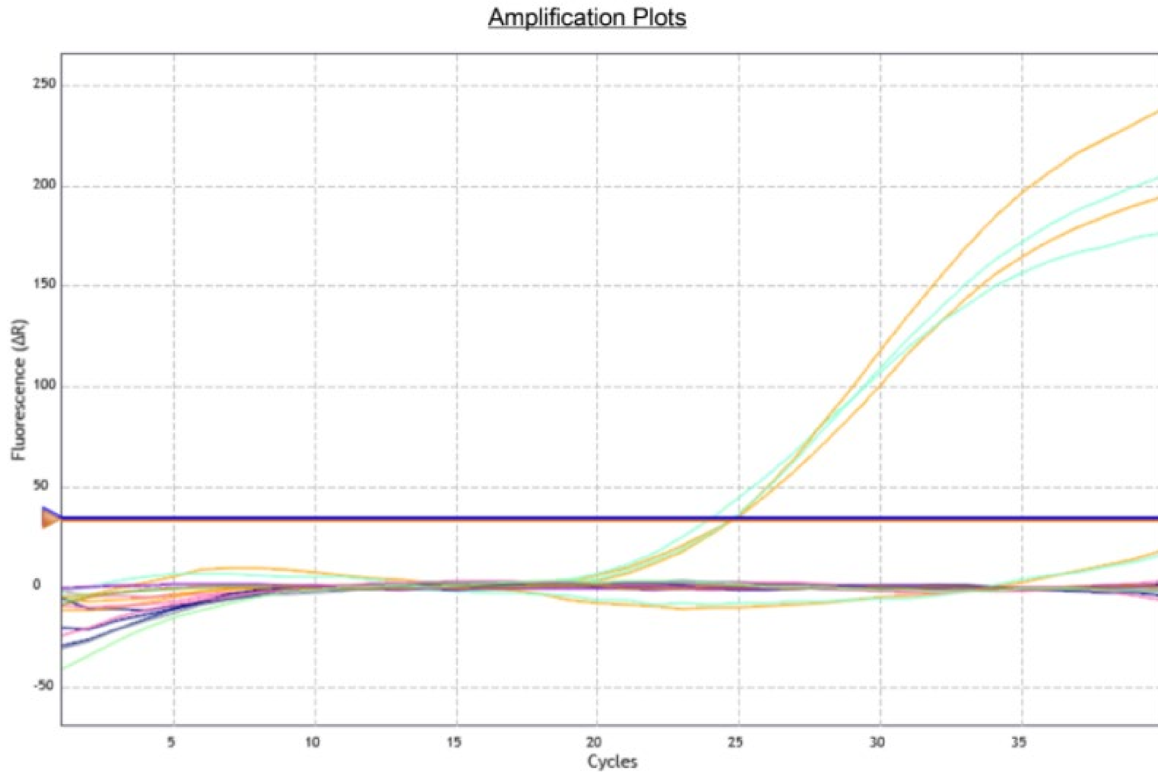

Figure 3.4: Positive test control amplification of two invA positive samples (ATCC 700720, a40heb1.1) utilizing a general fluorescent material rather than the fluorescent probe

3.4 Discussion

The standard curves generated for *qnrB19* and *bla*$_{SHV-12}$ both display results within acceptable ranges for further data analysis. Therefore, these results are applicable to sample testing and will provide acceptable standards against which to compare samples, allowing for gene quantification within samples.

As no standard curve has yet been successfully generated for *invA,* this protocol cannot yet be utilized in sample testing. Possible options for further optimization are discussed in section 4.2.

The primary issue which occurred over the course of both the *qnrB19* and *bla*$_{SHV-12}$ protocol optimization process was negative control amplification. Over the course of both trials,

primer annealing temperatures were adjusted to see if doing so could either eliminate the negative control amplification entirely or push it to a point where it could be cut off from the final results. The standard cycle count for the qPCR programs used was 40, but if the standards could be fully amplified (brought to a plateau) before then, then the remaining cycles would be extraneous data and could be removed. If negative control amplification could be pushed to this region then it would become negligible, but this was not achieved. This showed some promise, with the negative control amplifying later in both trials, but also had negative effects on the R^2, slope, and efficiency values of the generated standard curves, pushing the values beyond the acceptable ranges. The accepted range values for these are -3.58 to -3.10, 90% to 110%, and >0.99 for slope, efficiency, and R^2, respectively.[15] The individual dilutions of the standard curve also changed with the temperature adjustments, with standards amplifying later or earlier depending on the direction of adjustment, but never separating the lower standards from the negative control to a point where the negative control could be cut off.

The R^2 value indicates how well the data points fit to the linear regression generated by the software based off of the data set, indicating how uniform the dilutions were in the reaction. The ideal value is 1, indicating a perfect fit and complete uniformity across the dilutions. The slope is the slope of the linear regression calculated from the data set, with the ideal slope being -3.3, but values within the accepted range being used for publication. The efficiency indicates how much of the reaction amplified in early amplification cycles continued to amplify in subsequent cycles, with the ideal being 100% indicating that all the reaction continued.[15]

The eventual solution to eliminating amplification of the negative control was a stricter cleaning protocol, with heavy decontamination of both work surfaces and tools used during the experiments. These surfaces were decontaminated with the use of DNA Away (Thermo Fisher

Scientific) and 70% ethanol (100% ethanol from Sigma-Aldrich; St. Louis, MO; combined with deionized water). The surface would be thoroughly coated with DNA Away (Thermo Fisher Scientific), left to sit for 10-15 seconds, and then wiped down. Subsequently, the surface would be sprayed down with ethanol (Sigma-Aldrich) and wiped immediately after. The DNA Away (Thermo Fisher Scientific) would eliminate any amplicons which may contaminate the negative control and the ethanol (Sigma-Aldrich) would remove the DNA Away (Thermo Fisher Scientific) to prevent degradation of the experiment. Materials used in the experiment were also replaced before the final protocols were created, both to reduce the risk of contamination and ensure that all materials utilized were fresh and would remove any error brought on by their degradation over time. While this protocol helped to reduce amplification within the QIAgility (Qiagen), some inconsistency still remained, leading to concerns about aerosols or amplicons present in parts of the machine not cleanable during normal operation. This led to the production of a standard curve by hand using the same cleaning protocol in order to reduce the chance of contamination. These modifications resulted in successful results for generating standard curves for both *qnrB19* and $bla_{\text{SHV-12}}$ that were free of negative control amplification and had R^2, slope, and efficiency values within the acceptable ranges for publication.

The final protocols were performed by hand as issues concerning contamination and pipetting error within the QIAgility (Qiagen) were raised. In previous trials the machine generated differing volumes of product despite being programmed to dispense the same amount in each well. The final trials were performed by hand to remove this issue, as although the QIAgility (Qiagen) offers greater consistency in results, the need to remove the pipetting errors outweighed any human error performing the experiment by hand may generate.

The concern of contamination was also raised. Despite thorough decontamination and careful handling of products, some negative controls generated within the QIAgility (Qiagen) still displayed amplification, while those generated by hand did not. This indicated that either aerosols were being generated during the reaction which contaminated results or that some amplicons were present in areas that were unable to be reached for cleaning during routine operation. As such, performing the experiments by hand also assisted to eradicate this issue.

The *invA* gene protocol was not successfully optimized. The primary issue with this gene was the lack of detection. Upon completion of the experiment, it appeared that no standard curve had been generated, however, upon running the PCR products through a gel electrophoresis, it became apparent that product had been produced in the positive controls and no product had been produced in the negative controls. This indicated that the issue lay not within the actual reaction, but the detection of the reaction's occurrence. This was further reinforced by the presence of product when the trial was later run using a general fluorescent material and removing the probe from the reaction, indicating that the probe was the likely issue. As the protocol has not been fully optimized yet it cannot be said for sure that this is the problem, but current evidence points to this conclusion.

4. CONCLUSION

4.1 Conclusion

Despite initial difficulties with negative control amplification, sufficiently intense cleaning of materials and workspace to remove possible contaminants has proved successful in eliminating negative control amplification. In addition, replacing the water for every reaction has also ensured that as many possible sources of contamination are removed as is reasonably feasible.

The successful elimination of negative control amplification has resulted in standard curve generation with acceptable slope, efficiency, and R^2 values for the genes *qnrB19* and *bla*$_{\text{SHV-12}}$ which can later be used for sample testing. Protocols have not yet been optimized for standard curve generation of the *invA* gene; however, preliminary results have shown successful amplification of positive controls and no amplification of negative controls as is desired.

4.2 Future Plans

Future plans include acquisition of a new probe for the *invA* primer set. It is possible that the age of the utilized probes, having been ordered in 2019, may be a contributing factor in the failure to detect amplification. If the present fluorescent issue can be explained via the age and potential degradation of the current materials then use of a freshly created probe should allow for detection of standard curve amplification and resolve the current issue. If the new probe fails to fluoresce then a new probe entirely may have to be designed or another alternate route may be considered. If the curve fluoresces but is not within acceptable ranges for data analysis the protocol may have to be further adjusted.

If the fresh *invA* probe allows for the generation of an acceptable standard curve then sample testing for this gene will begin immediately.

New standard curves for *qnrB19* and *bla*$_{SHV-12}$ will also be generated to ensure that all materials in use are fresh. Assuming the standard curves remain within the previously described acceptable ranges, sample testing for these genes will begin immediately. Generation of new standard curves for *qnrB19* and *bla*$_{SHV-12}$ will also confirm the repeatability of the optimized protocol.

In conclusion, the protocols for the *qnrB19* gene and *bla*$_{SHV-12}$ gene were successfully optimized with new primer generation, annealing temperature adjustment, and stricter cleaning protocols. The protocol for the *invA* gene was not optimized, but future plans, such as ordering fresh probe, include possible routes for fixing the detection issues. Fecal sample testing will begin for the *qnrB19* and *bla*$_{SHV-12}$ genes as those protocols are already optimized, and sample testing for the *invA* gene will also begin as soon as the protocol is optimized.

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