# PCR PRIMER EFFICACY IN DETERMINING *SALMONELLA* PREVALENCE IN SOUTHERN ARIZONA SHELTER DOGS AND FERAL CATS

By

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## Abstract

*Salmonella* is an enteric pathogen that can be transmitted zoonotically between humans and animals, including companion cats and dogs. One of the virulence factors that enables colonization of the gastrointestinal tract is the type III secretion system, as encoded in *Salmonella* Pathogenicity Island 1 (SP-1), and one of the component proteins of this virulence factor is the Invasive A protein (InvA). InvA is encoded by the *invA* gene, which is highly conserved within *Salmonella* species, so amplification of this gene through polymerase chain reaction (PCR) is often used to detect clinical isolates of *Salmonella* in humans, animals, and contaminated food and water sources. This study utilized the *invA* PCR to determine the prevalence of *Salmonella* in shelter dogs and feral cats. Additionally, PCR detection efficacy was studied by utilizing different primer sets and the additive bovine serum albumin (BSA). Three different primer sets, coined the "MC" primer set, "KC" primer set, and "VO" primer set, were tested. The MC primer set detected the *invA* gene in 16 of 58 canine fecal samples and 6 of 56 feline fecal samples, the KC primer did not detect the *invA* gene in any canine fecal sample, and the VO primer set detected the *invA* gene in 7 of 58 canine fecal samples. It was discovered that the MC primer set was able to detect the *invA* gene at a lower DNA concentration than the other two, which could contribute to the higher frequency of positive results obtained with that primer set. The effects of BSA addition were observed in sample groupings from shelter dogs and feral cats that were initially positive or negative for the *invA* gene in their first PCR, where the presence of BSA influenced PCR results from the original samples. The addition of BSA caused 1 sample from both the canine and feline fecal samples that had initially returned as negative to be positive for the *invA* gene; however, 1 sample from both the canine and feline fecal samples that had initially returned as positive was not positive in the PCR with BSA. PCR is a sensitive

and specific tool to confirm the presence of *invA*, as indicative of *Salmonella*, and appropriate adjustments, regarding the primer set characteristics and reaction mixture additives, should be considered. Optimizing the PCR conditions will maximize the reliability of PCR as a diagnostic tool to confirm *Salmonella* diagnoses and to guide treatment and preventative strategies.

## Introduction

*Salmonella* is a Gram-negative bacterium transmitted through the consumption of fecal-contaminated water, meats, or fresh produce (1). There are 2,637 serovars under the species *Salmonella enterica*, including *Salmonella enterica* subsp. *enterica* serovar Typhimurium that can be found in a variety of warm-blooded animals, which facilitates dissemination between humans and animals (2). Once ingested, *Salmonella* colonizes the distal ileum and proximal colon, where it can invade the enteric cells, using its type III secretion system from SP-1, which injects effector proteins across the two bacterial membranes and into the cytoplasm of the host cell (3, 4). *Salmonella* toxins associated with diarrhea production are enterotoxin and cytotoxin (5). Enterotoxin induces accumulation of fluid in the ileum, and cytotoxin inhibits protein synthesis and leads to intestinal mucosal damage (6). Serovar Typhimurium produces non-typhoidal disease referred to as salmonellosis that is characterized by symptoms of diarrhea, stomach cramps, nausea, vomiting, and loss of appetite (7). Non-typhoidal salmonellosis can be self-limiting, where complications due to the disease are treated with ciprofloxacin; however, resistance to this has been emerging in *Salmonella* species (8). CDC estimates there are 1.3 million cases of salmonellosis, 12,500 hospitalizations, and 238 deaths every year in the U.S. as of 2025 (9). The actual disease burden is likely underestimated due to the self-limiting nature of salmonellosis (10).

Due to contamination being the source of transmission, the disease can be controlled by increasing preventative measures, including the improvement of assessment tools for patients, food, and water sources. *Salmonella* is commonly diagnosed with the Widal test method that detects O and H antigens in a relatively rapid and inexpensive manner, but the test can cross-agglutinate with other pathogens, so false-negatives and false-positives have been reported (11, 12). Enzyme-linked immunosorbent assay (ELISA) is another detection method that uses IgM and IgG antibodies against *Salmonella* surface molecules (11). ELISA is reported to have high sensitivity and specificity to *Salmonella* surface antigens, but the expense of the materials may be a concern in areas of limited resources (11, 13). Polymerase chain reaction (PCR) is a sensitive and specific diagnostic tool, in which a segment of DNA extracted from various sources can be amplified and visualized on an agarose gel where subsequent sequencing of the gene can be used to confirm presence. The invasive A (*invA*) gene is widely used to detect *Salmonella* via PCR as this protein is highly conserved within the type III secretion system in *Salmonella* (4). Non-typhoidal *Salmonella* species are predominantly found in animals, and shelter pets tend to have higher rates of prevalence than household pets. A study in 2017 found the prevalence of *Salmonella* to be less than 1% in household cats and 2.5% in household dogs as collected from 11 geographically dispersed veterinary testing laboratories between 2012 and 2014 (14). Meanwhile, the prevalence of *Salmonella* in diarrheic and non-diarrheic shelter cats in Florida was reported in 2012 to be 6% and 4%, respectively, and a study in 2020 reported the prevalence of *Salmonella* in shelter dogs in Texas to be 4.9% (15, 16). It is critical to refine PCR diagnoses to accurately detect *Salmonella* in animals to identify them as possible sources of bacterial shedding, proceed with appropriate treatment to limit zoonotic transmission to humans and, and mitigate contamination of water and food sources.

## Methods

The fecal samples were collected from shelter dogs in the Pima Animal Care Center and from Trap Neuter Release (TNR) feral cats through the Southern Arizona Humane Society under University of Arizona IACUC Protocol 2021-0746. These samples underwent DNA extraction using the DNeasy PowerSoil Pro Kit (QIAGEN Inc., Germantown, Maryland) according to the manufacturer's instructions. Isolated DNA was stored at -20°C for the duration of this study. The DNA extracts from canine and feline fecal samples were used in PCR amplification of the *invA* gene with three separate primer sets to analyze their results for primer efficacy in detecting *invA* in *Salmonella* (Figure 1).

The first primer set applied in this research, coined the "MC" primer set, was developed by a previous study that identified the pathogens *Giardia lamblia, Entamoeba histolytica*, and *Salmonella* in drinking water sources with a multiplex PCR (17). The forward and reverse primers in the MC primer set were ordered and diluted from a concentration of 100 μM to 10 μM to reduce the possibility of primers binding to each other, which would decrease the likelihood of primer annealing to the DNA template. The MC primer set amplifies a fragment length of 293 base pairs (bp), relatively upstream in the *invA* gene (Figure 2). The second primer set, coined the "KC" primer set, used in this experiment was originally designed in 1992 and notably, it has been used in food safety detection of *Salmonella*, including a study of the molecular characterization of *Salmonella* in ready-to-eat foods in 2022 and the prevalence of *Salmonella* in chicken carcasses from a retail grocery store in 2024 (18, 19, 20) . The third primer set, coined the "VO" primer set, was designed by the lab manager of the Dr. Kerry Cooper Laboratory. The KC and VO primer sets amplify a fragment length of 284 bp, where the VO primer set amplifies a fragment more upstream to the fragment amplified by the KC primer set with a 24 bp space

between the VO reverse primer and KC forward primer (Figure 2). The process of dilution was performed on the second and third primer sets as it was for the first primer set. The sequences for the forward and reverse primer sequences for each primer set, and their amplification conditions are found in Table 1.

The PCR mixture is composed of 12.5 μL of GoTaq Master Mix (Promega Cooperation, Madison, WI, contains Taq polymerase, dNTPs, $MgCl_2$, and reaction buffer), 9.5 μL of nuclease-free water, 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), and 1 μL of DNA template from the fecal sample DNA extract. *Salmonella* Typhimurium DNA served as the positive control, and nuclease-free water was used for the non-template control in each set of PCR reactions. Each PCR reaction was performed for 16-20 fecal samples at a time in a thermal cycler, with one negative control per five fecal samples and one positive control in every round.

Overall, the project consists of an analysis of 58 fecal samples from shelter dogs and 56 fecal samples from feral cats. Canine fecal samples underwent individual PCRs with each primer set and feline fecal samples were analyzed through PCR with only the MC primer set. The same reaction mixture and amplification conditions were used for the feral cat samples as for the shelter dog samples. The PCR samples were observed on a 1.5% agarose gel for a 293 bp band for the MC primer set and for a 284 bp band for the KC and VO primer sets, indicating the presence of the *invA* gene in the fecal samples (Figure 1).

## Results

The experiment began by performing a PCR with the MC primer set on an *S.* Typhimurium DNA extract at its original concentration and at a 1:10 dilution to confirm the functionality of the primer set. The resultant gel is displayed in Figure 3, and due to the similar band intensity between the samples at the original concentration and at the 1:10 dilutions, the

1:10 dilution was used as the positive control in all the sample preparations. The experiment proceeded by performing a PCR on 58 canine fecal samples with the MC primer set at the amplification conditions listed in Table 1. After PCR amplification, each PCR reaction was loaded into a 1.5% agarose gel to visualize and assess the results for each of the samples. Samples were considered *invA* positive if they displayed a 293 bp fragment that matched the band for the positive control, based on the comparison to the 100 bp ladder scale of the gel. In total, 16 of the 58 (27.6%) fecal samples were positive for the *invA* gene with the MC primer set, as shown in Table 2.

To validate fecal samples that appeared as positive for the *invA* gene with the MC primer set, 4 of the 16 positive samples were submitted for sequencing at Arizona Genetics Core. The protocol for PCR clean-up was followed, according to the QIAquick PCR and Gel Cleanup Kit (QIAGEN Inc., Germantown, Maryland), to extract the DNA from the reaction mixture of the 4 samples after amplification. The sequences for the 4 samples did not return as the *invA* gene, but rather, the majority of the nucleotides in the sequences appeared as "N," indicating that the submitted DNA was of poor quality. This led to the concern of the MC primer set producing false positive results for the *invA* gene due to possible cross-reactivity of the primers with other antigenic genes that would appear as a band in the gel lanes. To confirm the positive results from the MC primer set as the *invA* gene, the KC primer set was used on the same 58 fecal samples to determine possible alignments between the results of the KC primer set and those of the MC primer set. The same reaction mixture was prepared for each of the 58 fecal samples as allocated in the MC primer set reactions, except KC FWD and KC RVS primers replaced the MC FWD and MC RVS primers. The prepared reaction mixtures were performed at the amplification conditions for the KC primer set stated in Table 1. After PCR amplification, each PCR reaction

was loaded into a 1.5% agarose gel to visualize and assess the results of the samples. Samples were considered *invA* positive if they displayed a 284 bp fragment that matched the band for the positive control, based on the comparison to the 100 bp ladder scale of the gel. The KC primer set did not produce any positives from the canine fecal samples, indicating that *invA* DNA could not be detected. Overall, there was no alignment of the PCR results between the KC primer set and the MC primer set, which suggested a differing efficacy between the primer sets that impacted the ability to amplify the *invA* gene in fecal samples.

The discrepancy in the results between the two primer sets inspired the decision to sequence the positive *Salmonella* controls with both the MC primer set and KC primer set. The results were visualized with a 1.5% agarose gel, and the PCR product bands were gel extracted, according to the QIAquick PCR and Gel Cleanup Kit (QIAGEN Inc., Germantown, Maryland). The DNA extracts from both the MC positive control and the KC positive control were submitted for Sanger sequencing to the Arizona Genetics Core. The Sanger sequencing results were assessed through a NCBI Nucleotide BLAST, and the sequenced PCR product for both MC and KC primer sets aligned with a portion of the *invA* gene in *Salmonella enterica* (Figures 4 and 5). These results confirm that both the MC and KC primer sets do detect the *invA* gene.

Once it was confirmed that both the MC and KC primer sets correctly returned positive results for the *invA* gene, a PCR was conducted with the MC primer set on two of previously positive fecal samples from the first MC primer set PCR. The PCR was performed in triplicate for both samples by preparing three PCR reactions for each sample to increase the final DNA concentration. The bands for the *invA* gene appeared in the lanes for both fecal samples as it had previously, and then a DNA gel extraction with the QIAquick PCR and Gel Cleanup Kit

(QIAGEN Inc., Germantown, Maryland) for both samples was performed. Once the DNA extracts were prepared, they were submitted for Sanger sequencing and those sequences were subsequently BLASTed against the non-redundant database of the NCBI Nucleotide BLAST website. Both sample sequences returned as having aligned with a fragment of the *invA* gene in *Salmonella enterica* subspecies *enterica*.

The sequencing results for canine fecal samples aligning with the *invA* gene provided confirmation to determine the DNA detection limits of the MC and KC primer sets as a possible point of difference between the primer sets. The DNA concentration of the original *S.* Typhimurium DNA extract was measured with a Qubit fluorometer using broad range limit and the resulting concentration was determined to be 14.3 ng/µL. A 10-fold dilution series was made, ranging from the original concentration (14.3 ng/µL) of the DNA extract to a dilution of $10^{-6}$ (0.0000143 ng/µL). The dilutions acted as DNA templates, and they were added to their own PCR mixture with either the MC or KC primer set. The PCR was performed using the conditions listed in Table 1, according to the primer set, then a 1.5% agarose gel was used to visualize the reactions. It appeared that both primer sets amplified the *invA* gene at a dilution of $10^{-3}$ (0.0143 ng/µL), but the MC primer set was able to amplify the *invA* gene at the subsequent dilution of $10^{-4}$ (0.00143 ng/µL).

The VO primer set was introduced into this project to qualify the results from the MC and KC primer sets. The VO primer set was used on the same 58 canine fecal samples and its forward and reverse sequences and PCR amplification conditions are listed in Table 1. After amplification with the VO primer set, the reactions were visualized on a 1.5% agarose gel, and it

appeared that 7 of the 58 (12.1%) fecal samples returned as positive for the *invA* gene. Four of these 7 (57.1%) positive samples overlapped with the positive results of the MC primer set.

The project progressed from detecting the *invA* gene in shelter dog fecal samples, indicating the presence of *Salmonella*, to testing feral cat fecal samples. A PCR with the MC primer set was performed on 56 feral cat fecal samples, and 6 (10.7%) samples returned as positive for the *invA* gene, as confirmed by gel visualization. These results opposed the sequencing results for these feral cat samples, which were completed with MinION in 2024 and confirmed the presence of *Salmonella* in the majority of the samples. Due to this contrast, the DNA concentrations of a select few of the feral cat samples were read with a Qubit fluorometer to possibly correlate the minimal *invA* gene positive results to a lower DNA concentration in the DNA extracts. All the tested DNA extracts had high DNA concentrations, ranging from 462 ng/µL to 1580 ng/µL.

Considering that the MC primer set was initially designed to detect *Salmonella* in water samples, the fecal samples as the source of the DNA extracts were acknowledged as a variable in the frequency of positive results through PCR. Fecal samples tend to have higher levels of inorganic materials and inhibitors compared to water samples, which could interfere with Taq polymerase interacting with the DNA template during PCR (21). As a final assessment of primer efficacy of the MC primer set, a PCR with bovine serum albumin (BSA) was performed on 3 previously positive samples from shelter dogs, 3 previously negative samples from shelter dogs, 3 previously positive samples from feral cats, and 3 previously negative samples from feral cats. BSA was added to interact with possibly interfering inorganic molecules and other inhibitors in the fecal samples and enhance polymerase activity. The reaction mixture was composed of 12.5

µL of GoTaq Master Mix (Promega Cooperation, Madison, WI, contains Taq polymerase, dNTPs, $MgCl_2$, and reaction buffer), 8.5 µL of nuclease-free water, 1 µL of BSA, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), and 1 µL of the DNA template from the fecal sample DNA extract. The PCR was performed under the conditions listed in Table 1 for the MC primer set, then a 1.5% agarose gel was used to visualize the reactions. The results from this gel are summarized in Table 3, and there were notable differences in the number of previously positive samples that returned as positive in the PCR with BSA. One sample from both the canine and feline fecal samples that had initially returned as negative was positive for the *invA* gene in the PCR with BSA; however, 1 sample from both the canine and feline fecal samples that had initially returned as positive was not positive in the PCR with BSA. This suggested that BSA can impact the number of positive results when added to the PCR mixtures as it increased the amplification of a previously negative sample in both the canine and feline groups, while decreasing the amplification of a sample that was previously positive.

## Discussion

This research indicates that there is variability between primer sets in detecting the *invA* gene, and the possibility of improving *Salmonella* diagnosis to extend beyond confirmation with the *invA* gene. It has also offered insight into the distinctions between primer sets as dependent on the source of the DNA extract. This is demonstrated as the MC primer set was designed to screen for *Salmonella* in water samples, compared to its application in this study to fecal samples. This can be considered if the fecal samples contain more inorganic materials and inhibitors than the water samples, as the inorganic molecules and PCR inhibitors would inhibit Taq polymerase from interacting with the DNA template (21). BSA is noted to increase the efficiency of PCR by reducing interference by inorganic materials and tends to be more reliable

than other additives, such as glycerol and dimethyl sulfoxide (DMSO), but the results of this study reveal a possible variable effect induced by BSA (22).

Another note to consider is primer strength and the ability of certain primers to detect DNA at lower concentrations within a sample based on their intended design. Two components of primer strength are GC content and melting temperature, as stated in Table 1, where GC content greater than 50% may increase primer binding to non-target templates (23). The MC primer set has the lowest GC content compared to the other primer sets, which may influence its lower melting temperature and its ability to detect the *invA* gene at a higher frequency. Primer efficacy is also influenced by the differing DNA detection limits of the MC primer set and the KC primer set as the MC primer set was able to target the *invA* gene in fecal samples at a lower DNA concentration than the KC primer set. This may also contribute to the differing results between the primer sets.

The original DNA concentration of the DNA extract is an additional factor to consider in PCR. The high DNA concentrations of the feline fecal samples refutes the possibility that a low DNA concentration could have accounted for the low number of positives in the PCR results. This supports the reasoning that the samples may not have had as much *Salmonella* as was previously detected by sequencing, or that PCR may not be the most effective method to identify *Salmonella* in fecal samples. High DNA concentration in the feline fecal samples could also indicate that DNA from other organisms interfered with the *invA* PCR. Another possibility is that the DNA extracts from which the DNA templates were pipetted were not completely homogenous with DNA, therefore, a mechanical error in not vortexing the DNA extracts prior to adding them to the PCR mixture may have played a role in the results.

In general, the *invA* gene may not be the most reliable gene to detect *Salmonella* as a study confirmed several *invA* primer sets to be producing false-positive results with bacteria in the *Salmonella, Citrobacter,* and *Enterobacter* genera (24). Even though the sequencing results of this study confirmed the alignment of the *invA* amplified sequences from canine fecal samples and feline fecal samples with *Salmonella,* the possibility of the *invA* primers binding to DNA templates in other bacteria is concerning as the MC, KC, and VO primer sets were not tested with other bacterial species. Another study performed an *in-situ* analysis of *invA* primers that resulted in a false detection rate of 87.5% as the *invA* gene was detected in bacterial species other than *Salmonella* (25). The researchers cited multiple events of horizontal gene transfer of SP-1 into other genera as possible evidence for *invA* detection in different bacteria (25). This would indicate that the *invA* gene is not uniquely conserved to *Salmonella* and that amplification of other genes in *Salmonella* would be better suited to PCR detection to limit cross-reactivity with other genera. To increase the reliability of *Salmonella* detection, PCR with culturing and multiplex PCR for simultaneous detection of multiple genes within a single reaction mixture should be considered. Another option is to detect *Salmonella* positives through PCR, then culture those presumptive positives on selective media for *Salmonella*. By ensuring accurate detection, this can increase awareness of the actual prevalence of *Salmonella in* shelter dogs and feral cats and encourage preventative measures to reduce transmission between humans and animals.

## Tables

**Table 1:** Overview of each primer set

| Primer Set | Sequence | GC Content (%) | Melting Temperature (℃) | Size of Fragment Amplified (bp*) | Amplification Conditions |
|---|---|---|---|---|---|
| **MC primer set (17)** | Forward (FWD) - GTT GTC TTC TCT ATT GTC ACC GT | FWD - 43.5% | FWD - 58.4℃ | 293 bp | Initial denaturation - 95℃ for 5 min<br>35 cycles of:<br>• Denaturation at 95℃ for 30 sec<br>• Primer annealing at 55℃ for 30 sec<br>• Elongation at 72℃ for 60 sec<br>Final elongation of 72 for 7 min |
| | Reverse (RVS) - CGA AAT ACC GCC AAT AAA GTT CAG | RVS - 41.7% | RVS - 58.7℃ | | |
| **KC primer set (18)** | FWD - GTG AAA TTA TCG CCA CGT TCG GGC AA | FWD - 50.0% | FWD - 61.8℃ | 284 bp | Initial denaturation - 94℃ for 3 min<br>35 cycles of:<br>• Denaturation at 94℃ for 1 min<br>• Primer annealing at 60℃ for 1 min<br>• Elongation at 72℃ for 1 min<br>Final elongation of 72 for 7 min |
| | RVS - TCA TCG CAC CGT CAA AGG AAC C | RVS - 54.5% | RVS - 59.9℃ | | |
| **VO primer set** | FWD - ACA GTG CTC GTT TAC GAC CTG AAT | FWD - 45.8% | FWD - 62.9℃ | 284 bp | Initial denaturation - 94℃ for 3 min<br>35 cycles of:<br>• Denaturation at 94℃ for 1 min<br>• Primer annealing at 60℃ for 1 min<br>• Elongation at 72℃ for 1 min<br>Final elongation of 72℃ for 7 min |
| | RVS - AGA CGA CTG GTA CTG ATC GAT AAT | RVS - 41.7% | RVS - 61.2℃ | | |

*bp = base pairs

**Table 2:** Summary of PCR results from different *invA* gene primer sets

| Primer Set | Frequency of Positive *invA* Results from Shelter Dog Samples | Frequency of Positive *invA* Results from Feral Cat Samples |
|---|---|---|
| MC Primer Set | 16/58 (27.6%) | 6/56 (10.7%) |
| KC Primer Set | 0/58 | ND* |
| VO Primer Set | 7/58 (12.1%) | ND* |

*Not done (ND) = samples were not tested with these primer sets

**Table 3:** Bovine serum albumin addition PCR results

| | # of PCR positive results of previously positive samples with BSA | # of PCR positive results of previously negative samples with BSA |
|---|---|---|
| Shelter Dogs | 2/3 | 1/3 |
| Feral Cats | 2/3 | 1/3 |

## Figures

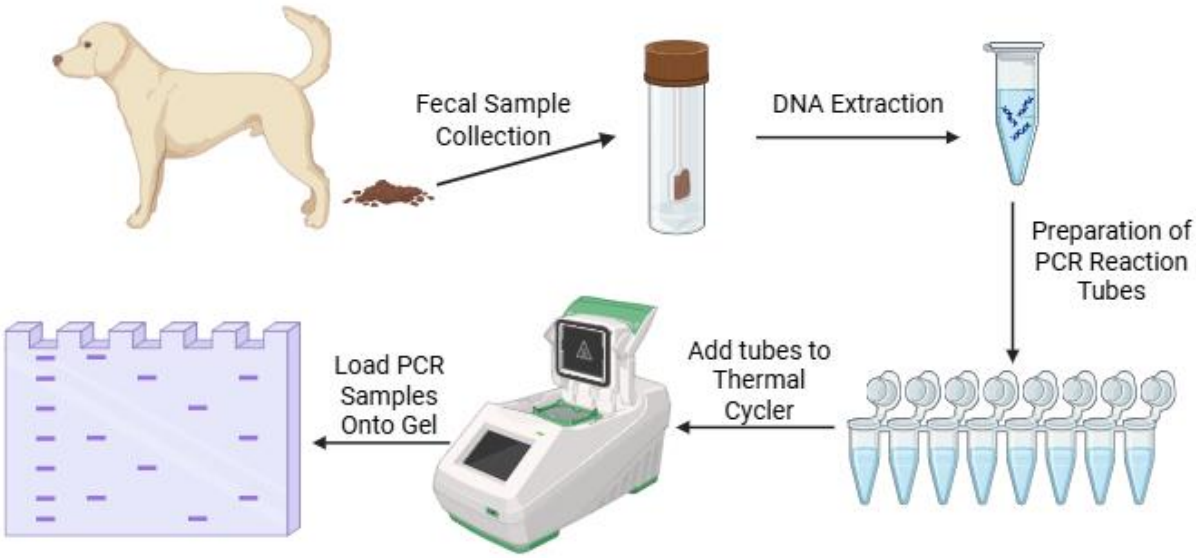


**Figure 1:** Overview of DNA extraction and PCR amplification from fecal samples of shelter dogs and feral cats. Created in BioRender

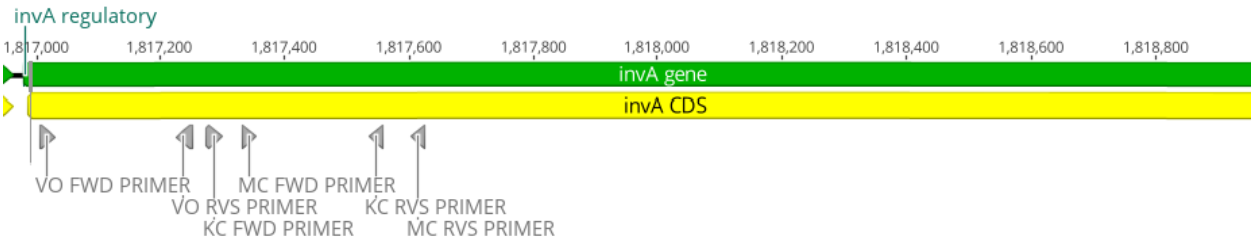

**Figure 2:** Geneious display of MC, KC, and VO forward and reverse primers within the *invA* gene of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2, complete genome

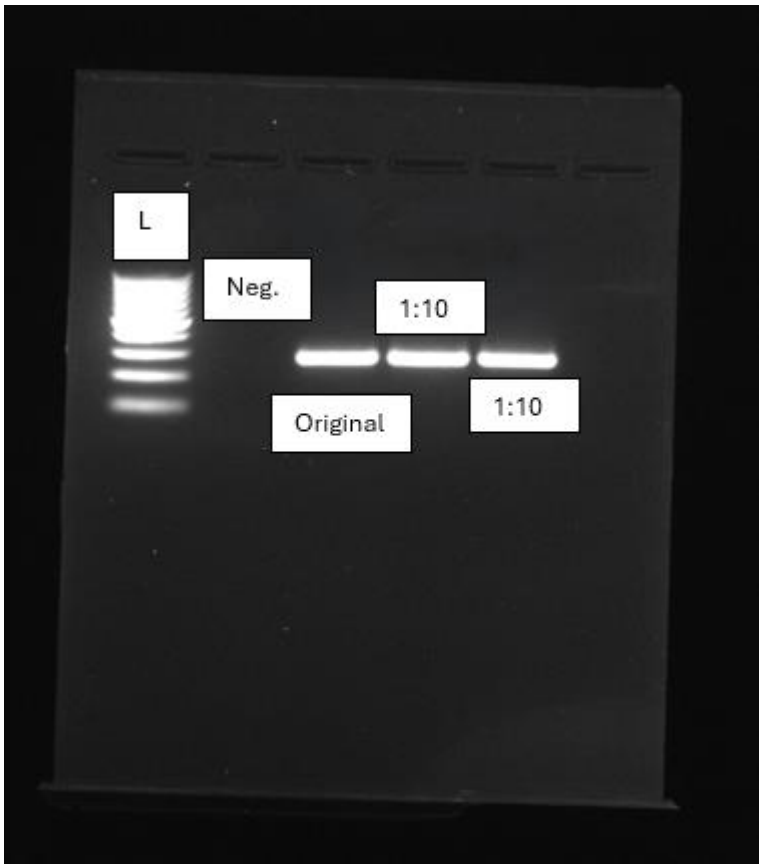


**Figure 3:** Gel of Dilution Series of Positive Control *S*. Typhimurium DNA Extract (L = Ladder, Neg. = Negative control, Original = Original *S*. Typhimurium DNA extract, 1:10 = 1:10 dilution of DNA extract)

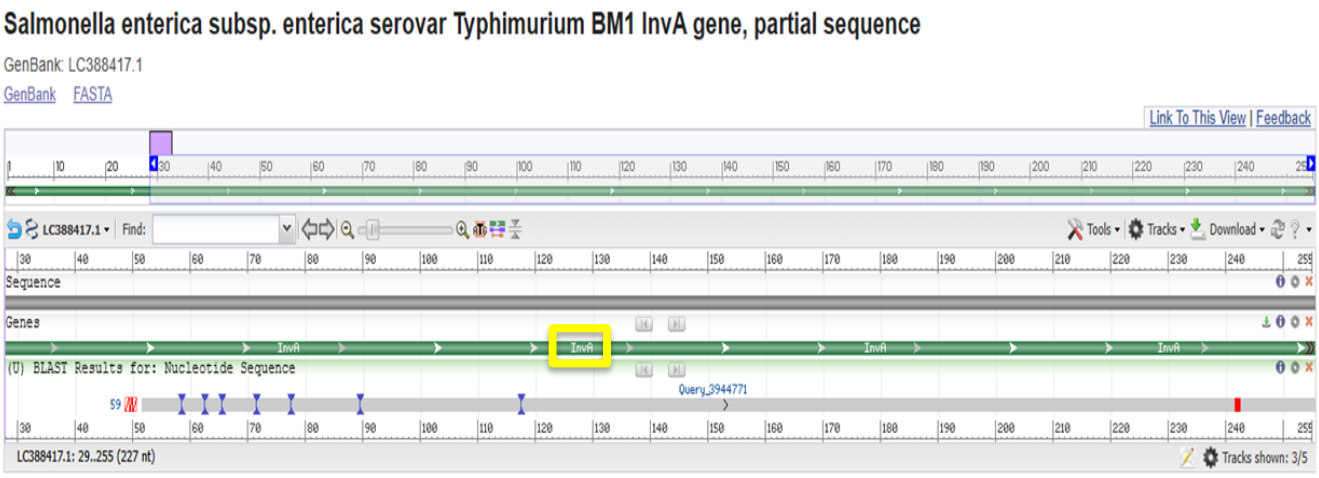


**Figure 4:** Alignment of positive control sequence amplified with the KC primer set with *S.* Typhimurium *invA* gene (bracketed in yellow)

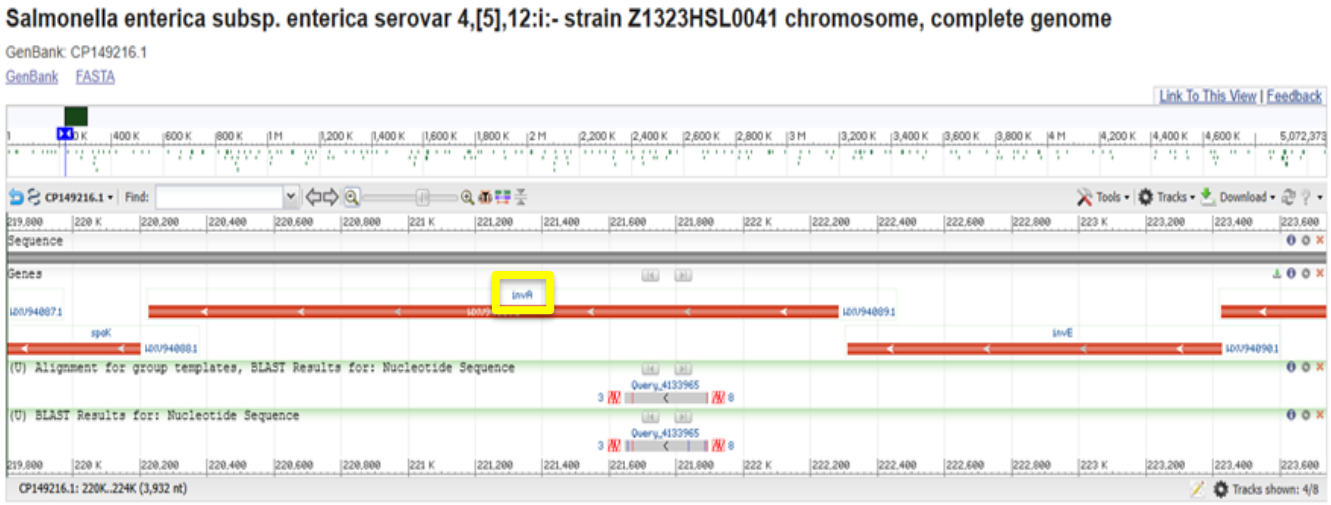


**Figure 5:** Alignment of positive control sequence amplified with the MC primer set with *S.* Typhimurium *invA* gene (bracketed in yellow)

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