Development of TaqMan® Probe-based RT-qPCR Diagnostic Assay for Rapid Detection of Typhoid Fever

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Dedication

To Humanity,

In the presence of suffering, resilience shines brightest. This thesis is dedicated to every person battling diseases and bacterial infections. Your strength fuels the pursuit of knowledge and discovery. May this humble contribution be a step towards a healthier, brighter future. Together, let us envision a world where compassion and science unite to alleviate the burdens of illness.

With deepest empathy,

Saiqa Aslam

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Saiqa Aslam

Table of Contents

List of Figures

List of Tables

Abstract

Typhoid fever is an important worldwide contributor to illness and death, with an approximate annual prevalence of nearly 9-11 million cases, leading to approximately 110,000 deaths annually worldwide. According to WHO, Pakistan has the highest rate of typhoid fever among south Asian countries with 0.1 million cases annually. Typhoid fever is confirmed by laboratory diagnosis. Despite being recognized for over a century, typhoid fever lacks a definitive test diagnostic biomarker. Blood culture is used as a gold standard for diagnosis, but the sensitivity of blood culture is less than 50%. Bone marrow culture is not used because of the invasive nature. The Widal test quantifies the antibodies produced against flagella and LPS in the serum of patients suspected of having typhoid fever by agglutinating *S. typhi* cells. The earlier use of antibiotic medication can provide a false negative result in the Widal test. Diagnostic methods based on real time PCR have overcome these drawbacks of conventional methods by providing increased sensitivity for the detection of *Salmonella typhi*. Several genes have been targeted for the detection of *Salmonella typhi* in previous studies. This study is focused on developing a TaqMan® probe-based Real-time multiplex PCR Assay for the detection of *Salmonella typhi* by targeting ttr, tviB, and staG genes. The assay was validated with bacterial and blood samples from suspected typhoid patients. All the cultured bacterial samples were positive by the developed Assay making the assay 100% sensitive and specific. In addition, out of 300 blood samples collected from suspected typhoid patients, 48 were positive by developed assay. Using blood culture as a standard diagnostic assay, developed PCR assay showed 100% sensitivity and 95% specificity. But to find the exact sensitivity and specificity, the assay needs to be compared with developed real-time PCR diagnostic kit.

Chapter 1

Introduction

1.1 Introduction

Salmonella enterica serovar *typhi* (*S. typhi*) is responsible for typhoid fever. The bacteria is considered to be transmitted through fecal-oral route [1]. The disease is characterized by noticeable signs such as high-grade fever, extreme fatigue, discomfort, headache, and gastrointestinal issues such as diarrhea, constipation, or stomach pain. The onset of symptoms usually occurs between 1 to 2 weeks following exposure, with a possible range of 3 to 60 days. A fever with a gradual yet steady rise in temperature, reaching 39°C–40°C after 5–7 days, is common. The majority of patients at this stage are administered antimicrobials as outpatients and will experience a full recovery. In the smaller number of patients who do not respond well to treatment, the illness persists beyond the initial week, characterized by ongoing fever, debilitation, loss of weight, and cognitive impairment. Complications including encephalopathy, gastrointestinal bleeding, hepatitis, intestinal perforation, and nephritis may arise, necessitating hospitalization [2].

Typhoid fever continues to be a major public health concern worldwide, especially in developing countries, where residents frequently lack adequate water, sanitation, and hygiene facilities. *Salmonella* enterica serovar *typhi* (*S. typhi*) is known to be present only in humans. While cases are ultimately transmitted between individuals, exposure can occur either directly or indirectly due to the dispersal of the bacteria in food or water [3]. The global incidence of typhoid fever reached 14.3 million cases, resulting in 135.9 thousand casualties. South Asia contributed to a significant majority of this burden, with a share of 69.6% [1, 4]. Typhoid fever has become prevalent in developing countries in Asia, such as Pakistan, because of insufficient sanitation and hygiene practices, as well as limited availability of clean drinking water [5, 6].

Merely 20% of Pakistan's human population has the privilege of accessing potable water, while the remaining 80% are compelled to rely on polluted water due to the scarcity of clean, adequate supplies of drinking water according to a recently conducted study. Typhoid perforation is a fatal ailment in Pakistan, particularly in the southwestern region of Sindh Province. The South Asian study, referred to as SEAP, covered many different countries and sites. The first phase of SEAP, conducted from 2012 to 2015, was aimed at collecting data for future surveillance and documenting the clinical characteristics of typhoid fever. The study included historical evidence on patterns of antimicrobial resistance (AMR), the severity of illnesses, and the associated burden. The study found that children aged 5 to 15 accounted for the highest proportion (52%) of *S. typhi*

cases requiring hospitalization, and 52% of the *S. typhi* isolates showed resistance to multiple drugs (MDR) [7].

Despite the high prevalence of typhoid fever, clinical diagnosis cannot rely solely on signs and symptoms. The fever is distinguished by nonspecific symptoms that resemble other febrile conditions such as influenza, dengue, and malaria [8]. Medical professionals prefer utilizing laboratory techniques, specifically serological testing, for the diagnosis of enteric fever. The suggested serological assays for initial diagnosis of typhoid are the Widal test, *typhi* dot test, and Tubex-M test [9]. However, it is recommended to utilize blood, bone marrow, or other body samples for the definitive diagnosis of enteric fever. The bone marrow culture demonstrates the highest level of sensitivity, at 96%, for detecting *Salmonella* species compared to other samples taken from the body. Blood culture is considered the definitive method for confirming the diagnosis of typhoid fever due to its lower invasiveness compared to bone marrow culture, despite the fact that bone marrow culture exhibits the highest level of sensitivity. The culture sensitivity of blood culture decreased from 60% to approximately 30% in patients who had previously taken antimicrobial drugs and those who had experienced symptoms for less than one week [9-11].

An issue of global significance is the increasing resistance of typhoidal *Salmonella* to antibiotics. The primary therapeutic approach for typhoid fever was the administration of antimicrobial agents such as co-trimoxazole, amoxicillin, and chloramphenicol. Nevertheless, a significant proportion of the global population, exceeding one-third, exhibits resistance to these pharmaceuticals [11]. Patients with multidrug resistance (MDR) include individuals who demonstrate resistance to co-trimoxazole, chloramphenicol, and amoxicillin. Since the emergence of multi-drug resistant (MDR) strains of *S.typhi* in the late 1980s and early 1990s, the preferred treatment has been the use of second and third generation cephalosporins and fluoroquinolones [12]. However, there are concerns over the possibility of treatment failure due to the emergence of extensive drug resistance (XDR) and fluoroquinolone resistance (FQR) [13, 14]. The initial reports of the extensively drug-resistant (XDR) strains originated from southern areas of Pakistan in November 2016. Azithromycin and carbapenems (Meropenem or Imipenem) are the only drugs effective against XDR enteric fever[14-16]. It is challenging to differentiate enteric fever from dengue, malaria, and influenza because of the non-specific clinical symptoms [16, 17]. While blood culture is the most effective approach to confirm diagnosis of typhoid, a systematic analysis of published research found that blood culture has a sensitivity of 61% [11]. In locations where a disease is commonly

found, the accuracy of blood culture is typically lower than the specified value, and there is often a lack of appropriate facilities to perform these tests. Culture-based techniques also necessitate the presence of living bacteria in detectable amounts during specimen sampling, which has been a significant obstacle in detecting *Salmonella*. In addition, it takes 7 days for a blood culture to report results. Point-of-care serological fast assays are accessible, although they have limited diagnostic precision [11].

Due to the limited number of treatment options and blood culture being the only available diagnostic method in spite of having low sensitivity, there is a need to develop rapid and reliable methods for typhoid fever diagnosis which have good sensitivity and specificity. Multiple studies have reported the use of qPCR diagnostic method for the identification of *S. typhi* [18]. These studies have targeted several genes for detection including ttr, *fliC-d, hilA, viaB,* and staG. In comparison with blood culture, molecular techniques eliminate the need of viable bacteria because DNA can be extracted from dead microbes too for performing PCR making them more sensitive, rapid, and reliable methods for detection.

In this study, we used a multiplex qPCR assay targeting ttr, tviB, and staG genes for the detection of typhoid fever. This study is focused on developing TaqMan® probe-based RT-qPCR diagnostic assay for rapid detection of *Salmonella typhi*.

1.2 Objectives

1. Development and validation of PCR Assay for detection of *Salmonella typhi*
2. Evaluation of the sensitivity and specificity of PCR Assay
3. Designing and upscaling of synthetic positive control

Chapter 2

Literature Review

2.1 Typhoid Fever

Typhoid fever can also be referred to as enteric fever. This is a potentially widespread and complicated disease that has been a significant issue for public health, particularly in developing countries. The causative agent of typhoid fever is *Salmonella typhi* [19]. *Salmonella* is a genus consisting of two species, *Salmonella enterica* and *enteritidis*, which have been categorized using a thorough investigation conducted by multiplex quantitative polymerase chain reaction (PCR) [20, 21]. *Salmonella typhi* belongs to the *Salmonella enterica* serotype. *Salmonella typhi* is spread through the fecal-oral route. The transmission of bacteria occurs exclusively between infected individuals, as humans are the only potential host. Poultry and eggs are the most common sources of *Salmonella*. A study conducted in China on the prevalence of *Salmonella* strains in chicken slaughterhouses revealed that 57% of the samples tested positive through whole genome sequencing [22]. The indigenous microorganisms inhabiting the gastrointestinal tract provide a protective barrier against infection. The administration of antibiotics, such as streptomycin, eradicate the indigenous microbial community, hence increasing its susceptibility to invasion. Malnutrition reduces the presence of healthy gut bacteria, hence increasing vulnerability to this infection. Therefore, the utilization of antibiotics that target a wide range of bacteria and inadequate dietary intake significantly increases the occurrence of typhoid fever.

2.2 Symptoms of typhoid fever

Symptoms of typhoid fever include high fever which lasts for several days, abdominal pain, fatigue, nausea, constipation, loss of appetite, cough, headache, and diarrhea [23]. A limited number of patients notice rashes on the skin. Untreated cases may lead to complications and can even cause death.

2.3 Prevalence of typhoid fever

Typhoid fever is an important worldwide contributor to illness and death, with an approximate annual prevalence of 12-33 million cases and 216,000-600,000 deaths [24]. According to the latest estimates in 2019, the number of typhoid fever cases each year is nearly 9 million, leading to approximately 110,000 deaths annually [25]. In 2018, Pakistan had the highest incidence of Typhoid among South Asian countries, with a rate of 493.5 cases per 100,000 people [26]. The number of cases in Pakistan experienced a significant surge following the emergence of a new

widespread outbreak of extensively drug-resistant (XDR) typhoidal strain in 2016 in Hyderabad, with the province of Sindh being the most heavily impacted [26].

Following the outbreak of Covid-19 in Pakistan, healthcare workers redirected their main focus towards controlling the transmission of Covid-19 [27]. The spread of Typhoid was significantly impacted, as demonstrated by the substantial rise in XDR cases. Specifically, in Karachi, the number of reported cases increased from 14,360 between January 2017 and June 2021 to 864 new cases within the period of June to August 14, 2021, alone. This is highlighted by the surge in the number of new XDR cases, which reached a total of 52 during the week immediately preceding August 14 [24]. A study by Ishtiaq A et al., reported the monthly prevalence of typhoid fever during the period of April- July 2022, according to which the frequency of typhoid positive cases was 66.9%. The frequency was higher in rural population as compared to the urban population. In addition, ratio of typhoid fever in females was more than males [28].

2.4 Complications of typhoid

2.4.1 Intestinal complications

The predominant consequences of typhoid fever concern the abdominal organ system, which can be attributed to the manner in which the infection is acquired and the pathophysiological impact on the small intestine [29]. Perforation of the terminal ileum, accompanied by the highest concentration of Peyer's patches, is frequently observed within 3-4 weeks of disease [30]. In most cases, surgical intervention is necessary [24]. Liver involvement is nearly ubiquitous. According to a study, 85% of the cases exhibited elevated levels of alanine transaminase (ALT), whereas 75% of the cases showed elevated levels of aspartate transaminase (AST). In addition, 2% of the individuals exhibited hepatomegaly, whereas jaundice was observed in 13% of the cases [31]. Nevertheless, there have been documented instances of notable increases in AST and ALT levels, resembling symptoms of acute viral hepatitis, as well as cases of acute liver failure [32].

The proposed mechanism for development of acute pancreatitis involves the reflux of *S. typhi*, which contains bile, into the pancreatic duct, causing immediate injury [32]. Additionally, the potential causes include the direct impact of bacterial toxins and the subsequent response of the host's immune system. In individuals with typhoid fever, it is frequently observed that amylase and lipase levels are increased, even in the absence of any clinical signs of pancreatitis. Das et al. conducted a study on 47 patients with enteric fever and confirmed that 44% of the subjects had

increased levels of serum enzymes. However, none of them developed acute pancreatitis, indicating that elevated enzyme levels did not have a negative impact on the outcome [33].

2.4.2 Extra-intestinal Complications

Typhoid fever has been associated with several extra-intestinal effects, encompassing nearly every organ system. Central nervous system complications are the most prevalent, occurring in 3-35% of cases, followed by pulmonary complications ranging from 1-6% and cardiovascular system issues reported to be 1-5%. Additional rare complications include disorders related to soft tissues, joints, and bones, as well as the genitourinary system. *Salmonella typhi* bacteremia is mainly responsible for extra-intestinal complications. Figure 2.1 shows the infection process of *Salmonella typhi* along with severe complications.

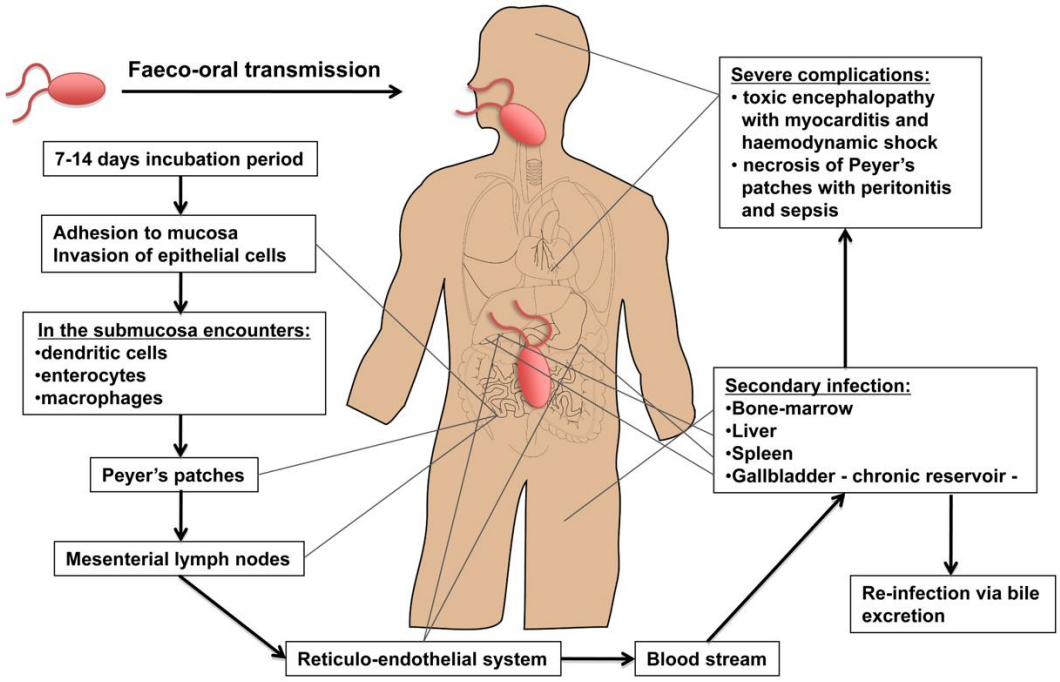

Figure 1 Infection process of Salmonella typhi with complications.

2.4.3 Chronic carriers

Typhoid fever is transmitted through the fecal-oral route by consuming food or drink that has been contaminated with infected excretion from humans. Most patients recover completely from acute typhoid fever after receiving an effective dosage of antibiotic treatment. Approximately 10% of patients still excrete *S. typhi* in their stool for a few weeks throughout the recovery phase after acute infection. About 1-5% of patients continue to excrete *S. typhi* in their stool for over a year.

The individuals in this group are referred to as chronic carriers[34]. The etiology of persistent colonization is not adequately comprehended. The biliary system and gallbladder seem to be primarily the organs where *S. typhi* resides in chronic carriers. In fact, individuals with gallstones tend to be more prone to becoming carriers[35]. *S. typhi* is a pathogen that can only infect humans. As a result, persistent carriage is crucial for sustaining the source of infection in humans. Asymptomatic individuals who carry the disease chronically inadvertently spread it to others through the contamination of food and drink with feces. An early and well-known case illustrating this phenomenon is that of Mary Mallon, sometimes known as 'typhoid Mary', who worked as a chef in New York during the 1950s. Despite showing no symptoms, she transmitted typhoid fever to at least 54 individuals [36]. Subsequently, forensic epidemiology has provided evidence of numerous similar instances. One notable example is the case of Mr. N, known as the Folkestone milker, who transmitted infections to more than 200 individuals over several years through contaminated milk [37]. The 2003 WHO recommendations for enteric fever recommend the use of amoxicillin, co-trimoxazole, or ciprofloxacin as therapeutic options for persistent carriage of typhoid fever.

Chronic carriage not only poses a public health issue, but also increases the individual's likelihood of developing malignancy, including gallbladder cancer [38]. A recent meta-analysis found that individuals carrying *S. typhi* have a 4.28 times greater chance of developing gallbladder cancer. WHO recommendations for enteric fever recommend the use of amoxicillin, co-trimoxazole, or ciprofloxacin as therapeutic options for persistent carriage of typhoid fever. Fig 2 illustrates the mechanism of biofilm formation of *S. typhi* in chronic carriers.

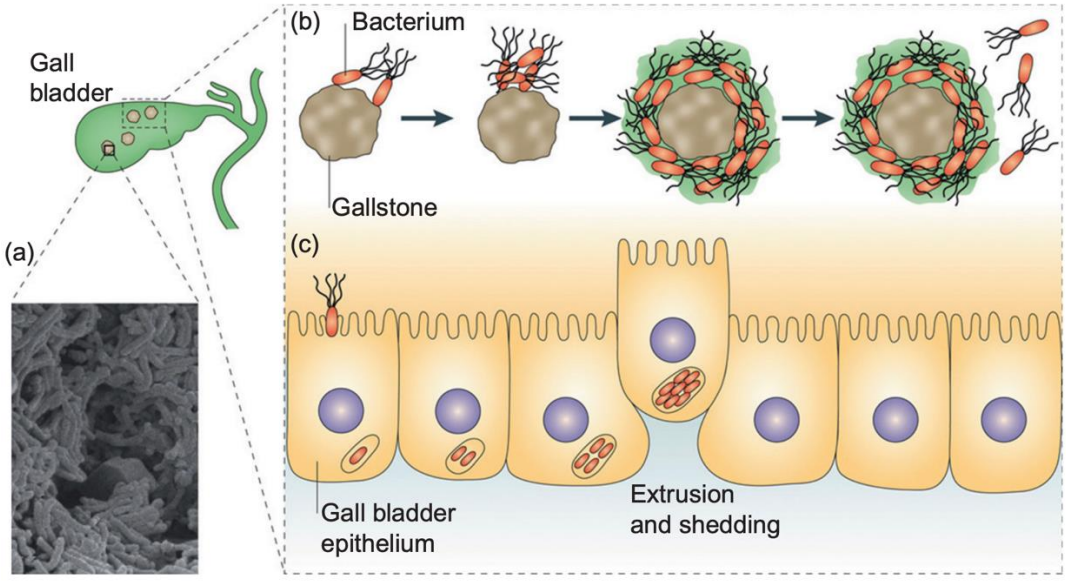

Figure 2 Biofilm formation of S. typhi on gall stones in chronic carriers.

2.5 Treatment

Antibiotics are used as the primary and essential form of treatment. The administration of the medication has been hindered by the emergence of multidrug resistant strains in numerous endemic regions, particularly in south-east Asia and India. The choice of treatment modality is determined by severity of the disease, its length, extent of spread, and any associated consequences.

2.5.1 Antibiotic treatment

Timely administration of the appropriate antibiotic treatment protects against adverse effects of typhoid fever. The selection of the initial drug regimen depends on the sensitivity of the strains.

First-Line Antimicrobials

Amoxicillin, trimethoprim-sulfamethoxazole, and chloramphenicol are all used for the treatment of adults with fully susceptible cases. Amoxicillin should be taken orally at a dose of 750mg four times daily for approximately two weeks. Trimethoprim-sulfamethoxazole should be taken at a dose of 160mg twice daily for two weeks. Chloramphenicol should be taken at a dose of 500mg four times daily for two to three weeks. However, these treatments are facing resistance [39].

Third generation Antimicrobials

Fluoroquinolones are generally the most optimal treatment option in majority of regions.

Fluoroquinolones can be given empirically based on clinical suspicion in urgent cases where quick treatment is necessary, even before the results of the diagnostic culture test are available. Fluoroquinolones effectively treat around 98% of cases with recurrence and result in fecal carriage rates of less than 2%. Ciprofloxacin, administered orally at a dosage of 500 mg twice a day for the duration of 5-7 days, is the most efficacious treatment. Normal cases can be treated at home with antibiotics and medications that reduce fever. Hospitalization is recommended for those experiencing severe problems such as vomiting, diarrhea, and abdominal pain.

Continuation of additional therapy and administration of parenteral antibiotics, specifically third generation cephalosporins should be maintained until 5 days following complete recovery. Endemic areas have witnessed the emergence of extremely drug-resistant (XDR) and multidrug-resistant (MDR) strains. Bacteria's intracellular nature provides protection against antibiotics that act outside of the cell [40]. For cases of multi-drug resistant (MDR) infections, the most effective treatment options include third generation cephalosporins (such as ceftriaxone, cefotaxime, and oral cefixime at a dosage of 2g once daily for a duration of 2 weeks) and azithromycin. Ciprofloxacin can be used as an alternate treatment. The efficacy of these treatments is approximately 5% to 10%, with recurrence rates ranging from 3% to 6%. These medicines effectively resolve fever within a week and have a fecal carriage rate of less than 3%. The inclusion of azithromycin and cefixime decreases the failure rate and shortens the length of hospital stay [40].

2.6 Current Diagnostic strategies for typhoid

Typhoid fever is confirmed by laboratory diagnosis. Despite being recognized for over a century, typhoid fever lacks a definitive test diagnostic biomarker [41]. Currently available diagnostic strategies with their limitations are explained in detail below.

2.6.1 Culture-based Diagnosis

To definitively diagnose enteric fever, it is necessary to isolate bacteria from either the blood or bone marrow [42]. Culture remains the primary method for diagnosis since it enables us to identify the bacterium responsible for the onset of disease in a specific location and determine its antibiotic resistance genes [43]. While the approach demonstrates perfect specificity, it is deficient in terms of sensitivity. The typical sensitivity of blood cultures is approximately 50%, while that of bone marrow cultures is around 80%. This sensitivity is directly related to the amount of viable bacteria

present in the blood (less than or equal to 1 colony-forming unit per milliliter) and in the bone marrow (around 10 colony-forming units per milliliter) [45] [46] [47].

Procedure

The conventional procedure is placing the sample in a controlled environment at a temperature of 37 °C and observing it for the presence of bacterial growth for a minimum duration of one week. Typically, to diagnose bacterial infections, positive cultures are grown at a temperature of 37 °C for 24 hours [44]. Nonselective media, which may include TSB or blood agar, are employed for isolating pathogenic microbes in blood samples, which are expected to be devoid of any microorganisms in healthy persons. XLD and MacConkey agar are mostly used as selective media for *S. typhi* [48].

Efforts for enhancing sensitivity of culture-based diagnosis

Diverse approaches have been used to improve diagnosis by culture-based techniques. For instance, the addition of bile salt or ox bile to culture media has led to a higher frequency of bacterial isolation in a reduced amount of time. To be more precise, the contents of bile inhibit the ability of blood to kill bacteria and cause the breakdown of blood cells, which then release bacteria.

Challenges with culture-based diagnosis

There are other difficulties linked to blood-culture-based diagnostic techniques. Quite simply, when it comes to diagnosing younger children with bacterial infections within the first two weeks, smaller blood samples (2-4 mL) are used compared to the larger samples (5-10 mL) used for older children and adults. This difference in blood volume may contribute to the underdiagnosis of infections in younger populations. The use of antibiotics before diagnosis, which is still prevalent in places with high disease prevalence, also interferes with the diagnosis based on culture. To address this difficulty, bone marrow samples are utilized instead of blood for culture-based diagnosis, as there are less chances for microbes in bone marrow to get eliminated by antibiotic treatment [49]. Therefore, bone marrow culture is regarded as the most reliable method for diagnosing enteric fever in regions where the disease is common. Nevertheless, this approach requires an invasive technique for gathering samples and necessitates specialized expertise and equipment for implementation [50].

In addition to isolating bacteria using bone marrow and blood, other samples including duodenal bile, urine, rose spot, and stool are sometimes utilized for isolating *S. typhi* by culturing techniques. The sensitivity of rose spot culture is approximately 60%, making it a noninvasive treatment.

However, the incidence of these spots is rather uncommon among patients with enteric fever, ranging from 1% to 30%. The diagnostic usefulness of duodenal aspirate culture surpasses that of stool culture; however, its utilization is hindered by the test's limited tolerance, especially in children. The positive results obtained from these additional biological samples just indicate the presence of active disease, which might be attributed to the high prevalence of chronic carriage in locations where the disease is common [51]. Hence, it is imperative to interpret a positive outcome in conjunction with other tests.

As previously mentioned, a diagnosis of enteric fever using bacterial culture is considered the most reliable method. This approach also enables testing for antibiotic sensitivity, which is crucial for choosing an appropriate antibiotic treatment plan [52]. The key challenges associated with this approach involve a prolonged waiting period for isolation of bacteria, as well as the requirement for adequate laboratory facilities, which may not be readily available in regions affected by the disease. Hence there is a need to develop alternative diagnostic assays.

2.6.2 Serological Diagnosis

The identification of *S. enterica* serovars can be determined through serological identification, which is based on the Kauffman-White classification system. Presently, the existing serological assays lack the ability to accurately identify typhoid fever (with a specificity of less than 100%) due to the presence of common antigens among several *Salmonella* serovars. Several antigens are common to various *Salmonella* serovars [53]. Serological tests offer a simple and rapid method, making them exceedingly helpful for promptly addressing disease in economically poor regions with high rates of occurrence.

Widal Test

Widal test quantifies the antibodies produced against flagella and LPS in the serum of patients suspected of having typhoid fever by agglutinating *S. typhi* cells. It is simple, cost-effective, and rapid. A serum sample collected during the acute phase of an illness and another sample collected during the convalescent period, approximately 10 days later, showing a four-fold rise in antibody concentration, suggests a positive outcome. However, in practice, it is common to utilize only one serum sample during the acute period, leading to potential misinterpretation. This is due to the fact that other types of *Enterobacteriaceae* and diseases like malaria can produce antigenic determinants that have a similar reaction to *S. typhi* [53]. Therefore, it is recommended to initially determine the baseline titre with the first test and then observe any rise in the second test. ELISA-

based studies provide higher sensitivity compared to agglutination tests, but they share the same limitations of specificity as the Widal test. The presence of antibodies against the Vi antigen typically emerges too late in the course of the illness to be diagnostically valuable. However, the identification of the Vi antigen has been suggested as a means of detecting individuals who are chronically carrying the infection.

Widal test can be performed using two methods: slide test and tube test. The tube test requires a duration of 6 hours; however, the slide test can be completed within a range of 2 to 5 minutes. Nevertheless, the tube test surpasses the slide test in terms of quality and should be prioritized above the slide test. The tube test has the capability to detect the antibody at a concentration of 1:1,280, but the slide test can only detect it up to a concentration of 1:320. As a standard procedure, blood culture is the preferred method, whereas serological tests should be limited wherever feasible [54].

Limitations of Widal Test

1. In countries where a disease is commonly seen, such as Pakistan and India, the population develops a certain level of antibodies as a result of recurrent exposure to *Salmonella* infection. Therefore, it is challenging to interpret Widal titers unless the baseline levels of these antibodies are known.
2. The earlier use of antibiotic medication can provide a false negative result in the Widal test.
3. *Salmonella typhi* possesses similar "O" and "H" antigens to other *Salmonella* serotypes and has overlapping epitopes with other *Enterobacteriaceae*. As a result, there is a possibility of obtaining a false-positive test result.
4. It can yield false-positive results in cases of malaria, typhus, sepsis caused by other pathogens, cirrhosis, and other conditions.
5. It exhibits a moderate level of sensitivity and specificity. Even in cases where blood cultures have confirmed the presence of infection, it may still yield negative results in approximately 30% of cases [54].

Tubex and Typhidot Test

Many rapid diagnostic tests (RDTs) have been developed to quickly diagnose typhoid fever in locations where the disease is common. These tests evaluate the presence of antigens specific to typhoid fever [47]. Tubex is capable of identifying anti-O9 IgM antibodies in *S. typhi*. Typhidot is capable of identifying IgG and IgM antibodies that target a 50-kDa protein in the outer membrane

of *S. typhi*. Detection of IgM is the optimal indicator for identifying recent infection in individuals who have neither been exposed to *S. typhi* nor have received the Ty21a live attenuated vaccine [55]. Detection of IgG indicates the possibility of reinfection in individuals who have recovered, infection in individuals who have been vaccinated, or the presence of bacteria in individuals who show no symptoms [56].

Limitations of Typhidot Test

1. Typhidot test is unable to distinguish between cases in the acute phase and those in the recovery phase.
2. Current reinfection or previous infection with *S. typhi* might lead to false-positive results, as the subsequent immune response enhances IgG production while IgM becomes undetectable [54].
3. Limitations of Tubex Test
4. The Tubex test is restricted in its use to serum samples that are not hemolyzed or icteric, hence constraining its general use.
5. It is expensive as compared to Widal test and requires expertise.
6. It is challenging to interpret the test results.

2.6.3 Nucleic Acid-based Diagnosis

Due to the limitations associated with conventional diagnostic methods such as culture-based diagnosis and serological diagnosis for typhoid fever, advanced methods demonstrating higher sensitivity and specificity are needed to decrease disease burden. Nucleic acid detection includes the utilization of polymerase chain reaction (PCR) to amplify target DNA sequences from *Salmonella* for the purpose of diagnosis. This procedure requires the extraction of DNA from samples obtained from patients, which is subsequently amplified to target specific DNA sequences unique to *Salmonella*. The frequently used target genes for the diagnosis of typhoid fever comprise cytotoxin (clyA), flagellin (fliC), 16s rRNA, viaB encoding Vi polysaccharide, groEL encoding heat-shock protein, and several other conserved genes [57]. In addition to these genes, ttr, tviB, and staG genes have also been used for the diagnosis of enteric fever in recent studies. The test's correctness is typically determined by comparing its results to those of blood culture, as there is no universally accepted standard reference method. Numerous studies have indicated that the sensitivity can vary between 40-100%, as evidenced by various research findings. On the other

hand, the specificity can approach 100% when the test is carried out under ideal conditions [58, 59].

Target genes for detection

ttr Gene

ttr gene is a conserved gene of *Salmonella* species which encodes tetrathionate reductase enzyme. The gene is located in pathogenicity island 2 of *Salmonella* and plays an important role in the life cycle of *Salmonella*. A recent study has found that the qPCR approach is very sensitive and specific in detecting ttr gene for *Salmonella* in feces and enriched broth medium [60]. But ttr gene does not allow the differentiation between typhoidal and non-typhoidal *Salmonella*.

tviB Gene

The tviB gene, responsible for the production of Vi polysaccharide capsule, is found in *S. typhi* and Para *typhi* C [60].

StaG Gene

Additional genes are necessary to distinguish between *Salmonella* serovars that cause enteric fever. Nga et al. suggested targeting staG gene (often referred to as the STY0201), which encodes a hypothetical fimbrial protein, as an alternative method for detecting *S. typhi* in clinical blood samples [59, 61].

2.6.3.1 Types of PCR used for detection

Multiple technologies, including quantitative real-time PCR (RT-qPCR), conventional PCR, multiplex PCR, nested PCR, and loop-mediated isothermal amplification (LAMP) PCR, have been found to exhibit varying levels of sensitivity. The elimination of human DNA from blood samples and enrichment of blood samples prior to the PCR process resulted in a significant increase in sensitivity [62]. Advantages and disadvantages of PCR assays are mentioned in Table 2.

2.6.3.2 Loop-mediated isothermal amplification (LAMP) PCR

Loop mediated isothermal amplification (LAMP), one of the isothermal amplification techniques, is a fast and less expensive way to diagnose typhoid. LAMP utilizes four complex sets of primers that binds to the target region and forms a stem loop secondary structure that act as binding site for other set of primers to attach and continue the amplification process [63, 64]. It basically consists of two steps, the self- elongation step in which elongation of the template takes place from the stem loop structure that is formed at 3’ end and then the elongation step in which the polymerization from the primers actually begins. LAMP is performed by enzyme Bst, from

Bacillus stearothermophilus and occurs at 55-65 degrees Celsius [65]. Schematic representation of loop-mediated isothermal amplification PCR is illustrated in Figure. 3.

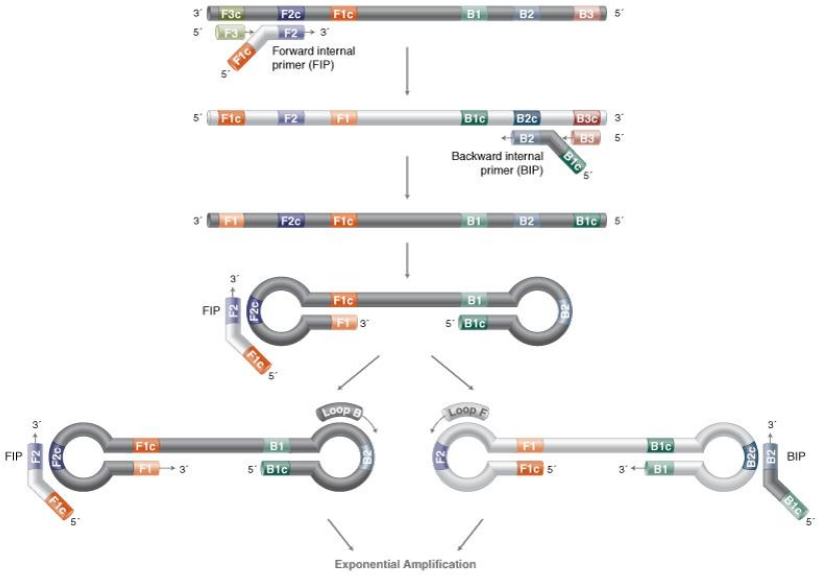

Figure 3 Schematics representation of loop-mediated isothermal amplification PCR.

Advantages of LAMP PCR

Loop-mediated isothermal amplification (LAMP) has recently been employed for the detection of infectious diseases. This method offers several competitive advantages, including visible fluorescence detection results, a closed-tube reaction, less complex conditions, real-time monitoring, high sensitivity, and isothermal amplification [66]. The LAMP primers improve specificity by recognizing six to eight regions of the target gene. The Bst DNA polymerase enables exceptionally efficient amplification across a broad spectrum of isothermal reaction conditions within a time period of 1 hour.

Disadvantages of LAMP PCR

Current LAMP kits lack the ability of multiplexing for detection of different species. Moreover, they produce more false positive results than PCR as well as per sample processing cost is still higher than PCR. Also, tests need to be performed to check the utility and feasibility of LAMP in clinics [67].

Polymerase Chain Reaction

Molecular methods such as PCR developed by Kary Mullis has been used since 1980s for the diagnosis of typhoid. PCR is a laboratory procedure that makes billions of copies of a target region

of DNA by using DNA polymerase to amplify a target fragment of DNA. The PCR reaction consists of Taq polymerase, primers, dNTPs, and template DNA.

PCR Steps

Denaturation

Initially, the reaction mixture is heated at 95 °C to separate the double stranded DNA to form a single stranded template to enable primer annealing.

Annealing

Forward and reverse primers bind to the complementary sequences in the template when the reaction mixture is cooled down to 55-65°C.

Extension

At 72 °C, Taq polymerase extends the primers forming new strands of DNA.

2.6.3.3 Conventional PCR

Various molecular biology techniques for detection of typhoidal bacteria have been developed. Such method of diagnosis is also based on targeting the conserved regions of *S. typhi* such as flagellin gene.

2.6.3.4 Nested PCR

The use of nested polymerase chain reaction (PCR) allows for the rapid amplification and identification of specific gene sequences of *Salmonella* species within a short time period. PCR products from this reaction were detected by a single electrophoretic step. However, this detection method for diagnosis of typhoid is not rapid enough and cannot be applied for routine diagnosis of typhoid. Conventional PCR and ethidium bromide method for product is unreliable for exact quantification of the amplification reaction. Phenol extraction and ethanol precipitation method for preparation of template DNA for PCR might not be considered appropriate because of the hazardous nature of the reagents used, the time and materials required to prepare the template [68].

2.6.3.5 Real-Time PCR

Conventional techniques are time consuming and less sensitive. A molecular biology technique called real-time PCR allows detection and quantitation of the target during each PCR cycle [69]. A reporter molecule generates a fluorescence signal that is used to detect the target. This signal grows with each PCR cycle as the amount of product produced increases.

Real-time PCR can be divided into two types on the basis of genes targeted in a single reaction:

- Singleplex PCR (enables the detection of a single gene in one PCR reaction)

- Multiplex PCR (enables the detection of multiple genes in one PCR reaction)

Multiplex PCR saves time and is cost-effective as compared to single plex PCR [70]. Real-time PCR can also be further divided on the basis of probes/dyes used in assay:

- Real-time PCR using SYBR Green dye
- Real-time PCR using TaqMan Probes

Real-time PCR using SYBR Green dye

An asymmetric cyanin dye, SYBR green that attaches to any double-stranded DNA's minor groove without any specificity to the sequence. The excitation wavelength of SYBR green is 480nm and the emission wavelength is 520 nm which is comparable to fluorescein. During each extension step, it binds to minor groove of each new copy of double stranded DNA. The bound SYBR green produces a 1000-fold greater fluorescent signal as compared to the unbound dye. As a result of this an increase in fluorescent signal produced that can be observed in real time during the amplification step and the signal decreases when DNA is denatured. The fluorescent signal should therefore, be measured at the end of elongation step [71]. The detection mechanism of SYBR Green dye during RT-PCR is illustrated in Figure.4.

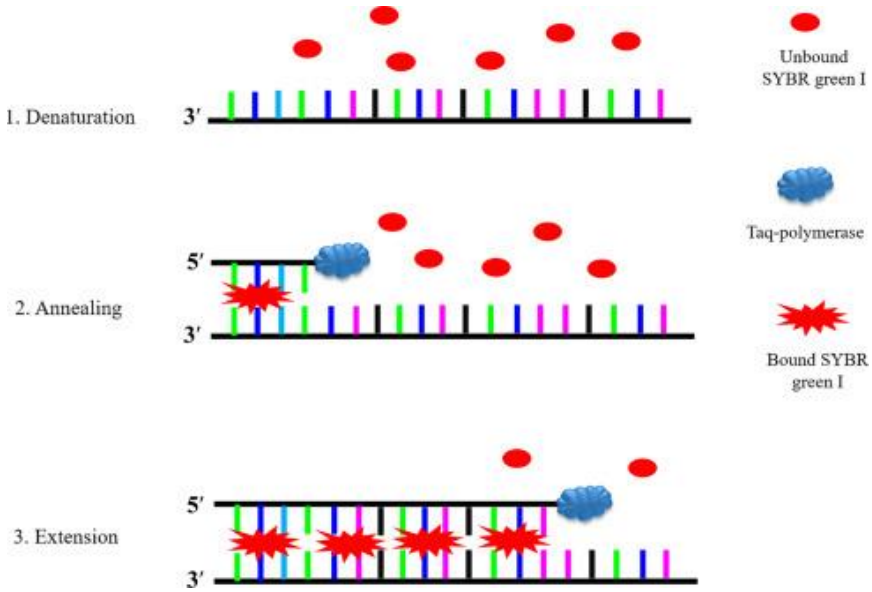

Figure 4 Detection mechanism of SYBR Green dye during RT-PCR

Benefits of using SYBR Green dye

SYBR green based RT-PCR does not require designing an oligo labelled with a fluorescent dye. As a result, primer designing for such type of experimental design is simple as well as the

experimental costs are also minimal specifically when detecting many genes that would not require the designing of multiple probes.

Limitations of SYBR Green dye

SYBR green dye has no specificity to any particular target [72]. This can result in generation of false positive results as it can bind to any double stranded DNA.

Real-time PCR using TaqMan Probes

TaqMan based real time PCR works by using the Taq polymerase 5’ to 3’ nuclease activity to detect the products from PCR. The reaction utilizes hydrolysis probe that is conjugated to different dyes that emit fluorescence at different wavelengths. The probe is designed complementary to a DNA target region that is present in between two PCR primers. The probes have higher Tm as compared to the primers so that they should be hybridized to the target region as the primer extension begins. The 5' and 3' ends of the TaqMan probe are attached to a reporter dye and a quencher dye, respectively. Commonly used dyes for reporter are FAM and VIC while for quencher TAMRA is used.

Other reporter dyes include ROX, cy5, cy3 and HEX and quenchers include BHQ2, and BHQ3. When the probe is bound to the target DNA, the two fluorochromes are present in close proximity to one another, no fluorescence is emitted as the emission from reporter is suppressed by quencher molecule. During extension step, the Taq polymerase begins to elongate the primers. The Taq polymerase then utilizes its 5’ to 3’ exonuclease activity and will start to degrade the probe. As a result, the reporter dye molecule is released from the probe, moves away from the quencher, and begins to emit fluorescent signals indicating the presence of the target sequence [72]. Figure. 4 illustrates detection mechanism of TaqMan Probe during RT-PCR.

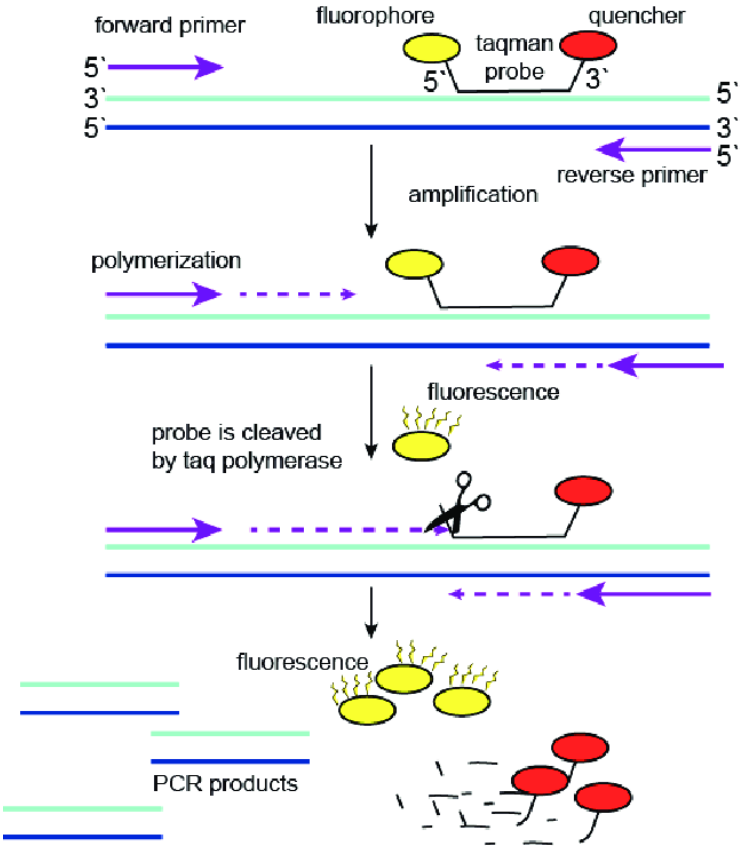

Figure 5 Detection mechanism of TaqMan Probe during RT-PCR

Real-time PCR assay using TaqMan probes for typhoid diagnosis

To improve the diagnosis of typhoid, RT-PCR assay using TaqMan minor groove binding (MGB) probe was developed. The assay targeted 16S rRNA and showed high specificity as compared to the conventional PCR assays [73]. Another study employed TaqMan probes for the detection of *S. typhi* in bile. Probes were designed for the primers targeting oriC, flic-d, STY0201, viaB, phHV, and stoD. The efficiency of PCR assay was recorded between 97 and 98 for different probesets. In addition, sensitivity of PCR assay was reported to be in the range of $10^{\wedge 2}$ to $10^{\wedge 3}$ CFU per ml [73, 74].

Table 1 Advantages and limitations of PCR based assays

Advantages	Limitations
The main benefit of PCR is its high sensitivity and quick response time.	PCR procedures have the drawback of requiring skilled workers.

PCR technologies offer advantages due to their ability to identify *Salmonella*-specific DNA obtained from both viable and non-viable bacteria. Bacteriostasis in the bloodstream can arise as a consequence of antibiotic therapies, a phenomenon that is also prevalent in certain regions with high disease prevalence and/or as a result of the host's immune response.	PCR assays also need specialized equipment for their execution.

Chapter 3

Methodology

3.1 *In-silico* Methodology

3.1.1 Selection of target genes

ttr, tviB, and staG genes were selected for the detection of *Salmonella typhi*. Among all these genes, ttr is present in all *Salmonella* species, tviB is present in *S. typhi* and para *typhi* C, whereas staG gene is conserved in *S. typhi*. Sequences of all the genes were downloaded from NCBI and primers and probes were designed using these sequences. Accession number of *S. typhi* is shown in table 2.

Table 2 Accession numbers of S. typhi and S. Para typhi gene sequences

Strain	Genes	Accession Number
Salmonella typhi	ttr, tviB and staG	AL513382

3.1.2 Designing of Primers and Probes

After obtaining gene sequences from NCBI, primers and probes were designed using PrimerQuest.

Table 3 Sequences of primers designed by PrimerQuest.

Gene	**Sequence 5'-3'**	**Start**	**Stop**	**Length**	**Probe Complementarity**
ttrF	CTGGATATGCGAATGCCGGT	9016	9036	20	Sense
ttrR	GATGAAACCGTCAGCGCACG	9208	9228	20	
tviBF	GCCACTACGATGCGATCATT	1130	1150	20	Sense
tviBR	GCTCAGCCGGAAGAACATAC	1239	1259	20	
staGF	CTCAACGCCGATCACCAGC	438	457	19	Sense
staGR	CGGCGCGAAGTCAGAGTC	550	568	18	

Sequences of probes designed by PrimerQuest are shown in Table 5.

Table 4 Sequences of probes designed by PrimerQuest.

Gene	Sequence 5'-3'	Start	Stop	Length	Probe Complementarity
ttrP	FAM-CCGATGGCCGTGGAGCAGATGAAA-BHQ1	9121	9145	24	Sense
tviBP	HEX-ATGGGAAGTGAGGATATTCGCGGC-BHQ1	1180	1204	24	Sense
staGP	Cy5-CGTCAGCCTGCTCCAGAACAAATGT-BHQ2	437	448	25	Sense

3.1.3 Analysis of Oligonucleotide Properties

The sequence of primer sets and probes were added separately in Oligo Calc software to check the formation of potential hairpins, complementary and self-annealing sites in the primers and probes. The GC content, melting temperatures of the primer set and the probes were also determined using Oligo Calc.

3.1.4 Multiple Primer Analysis

The primers and probe sequences were added in the Thermofisher scientific multiple primer analyzer to determine the presence of dimers and cross complementary regions between the primer- primer, primer- probe and probe- probe sets.

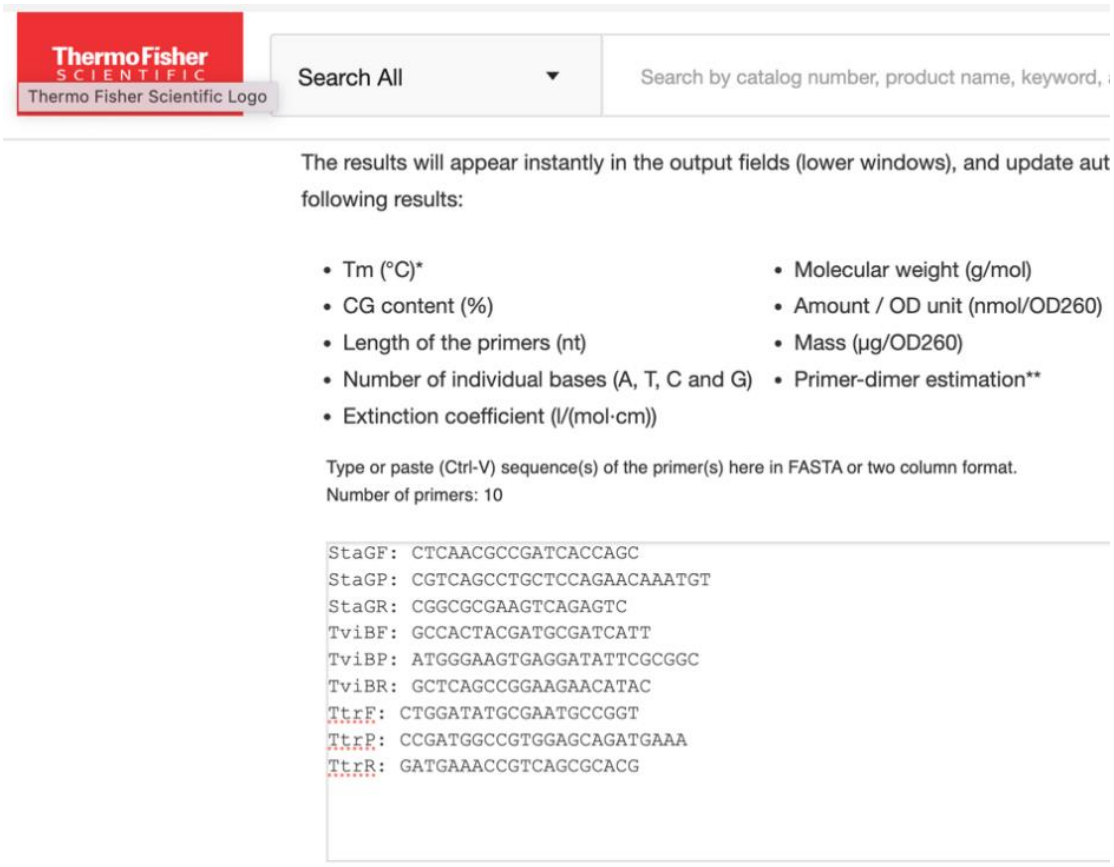

Figure 6 Primer and probe sets added into Thermofischer Scientific multiple primer analyzer.

3.1.5 *In-silico* PCR using SnapGene

In-silico PCRs of all three primer sets (ttr, tviB, and staG) were also run using SnapGene software and the ability of primer set to bind and amplify specific regions of all three genes was determined. FASTA sequences of all three genes previously downloaded from NCBI were uploaded into SnapGene and then *Insilico* PCR was run for each gene with a set of forward and reverse primer.

3.1.6 *In-silico* PCR using *In-silico* PCR amplification software

In-silico PCRs of all three primer sets (ttr, tviB, and staG) were run using *In-silico* PCR amplification software. 45 different *Salmonella* strains are available in *In-silico* PCR amplification software which were all included to determine the ability of primers to amplify only the targeted sequences.

3.1.7 Designing of Synthetic Positive control

Sequences of ttr, tviB, and staG genes were retrieved from NCBI and fragments of DNA containing 50 bp upstream and downstream of forward and reverse primer binding sites were cloned in pUC57 plasmid to be used as positive control in Real Time qPCR.

3.1.8 *In-silico* PCR of Synthetic Positive control

In-silico PCR of pUC57 plasmid containing cloned DNA fragments of ttr, tviB, and staG was performed using SnapGene. The ability of primers to bind and amplify the target regions was determined through *In-silico* PCR.

3.2 *In vitro* Methodology

3.2.1 Sample Collection

A total of 300 whole blood samples from suspected typhoid patients were collected from November 2022 to December 2023 from different hospitals of Pakistan.

3.2.2 Serum Extraction

Fresh whole blood samples collected from suspected patients were centrifuged at 4000 rpm for 5 minutes in refrigerated centrifuge for separation of serum from blood. The separated serum was then used to extract bacterial DNA for Real Time qPCR assay.

3.2.3 DNA Extraction from Serum sample

DNA templates for PCR were prepared by extracting bacterial DNA from serum samples using SYSTAAQ Purification Kit. The following protocol was used for extraction of DNA. 400 µL of lysis buffer, 300 µL of serum and 10 µL of carrier RNA was added into the Eppendorf tube. After being vortexed for roughly ten seconds, it was allowed to incubate for ten minutes. Following incubation, 300 µL of binding buffer was added to the same eppendorf tube and mixed using a vortex mixer. For 30 seconds, the Eppendorf tube was centrifuged at 13000 rpm. Following this, 700 µL of the aforementioned mixture was put into a spin column and centrifuged for 1 minute at 13000 rpm. The collection tube's filtrate was discarded. The spin column was then infused with 500 µL of wash buffer, and it was centrifuged for one minute at 13000 rpm. The washing process was repeated. After that, the spin column was put into an Eppendorf tube, 60 µL of elution buffer was added, and the spin column was centrifuged for 1 minute at 13000 rpm. This extracted DNA was then kept at -20 °C until used for PCR.

3.2.4 DNA QUALITY AND QUANTITY CHECK

Using Nanodrop 2000 (Thermoscientific, USA), the concentration of DNA was measured in ng/ul, and the 260/280 ratio was examined to validate its purity. 260/280 of 1.7- 1.9 is considered good for DNA.

3.2.5 GRADIENT PCR

For primer optimization, a clinically positive sample was processed in order to set the annealing temperature by using gradient PCR. The gradient mode was set in AB Quant Gene Automated Real Time PCR System. Gradient PCR profile is as follow. A starting step of 95 ℃ for 10 min, and 40 cycles of 95℃ for 20 sec, annealing step with temperature ranging from 56-64 ℃ for 25 sec and an extension step of 72 ℃ for 45 sec. An annealing temperature range set for gradient PCR is shown in table 5.

Table 5 Annealing temperature range set for gradient PCR

Temperature Gradient								
56 ℃	57 ℃	58.3 ℃	59.3 ℃	60.4 ℃	61.6 ℃	62.6 ℃	63 ℃	64 ℃

3.2.6 Real Time PCR Assay

For detection of *Salmonella typhi* in clinical samples, RT-qPCR was performed using the AB Quant Gene Automated Real Time PCR System. Reaction mix for the Multiplex PCR experiment was made of Forward and Reverse Primers and probe sets of ttr, tviB, and staG, 2x TaqMan PCR MasterMix (Solarbio Life Sciences), nuclease-free water and template DNA. Components of a 25 ul reaction for the Real time PCR Assay are shown in table 6.

Table 6 Components of a 25 ul reaction for the Real time PCR Assay

Reaction Components		Quantity (ul)
2x TaqMan PCR MasterMix		12.5
ttr	ttrF	0.5
	ttrR	0.5
	ttrP	0.5

tviB	tviBF	0.5
	tviBR	0.5
	tviBP	0.5
staG	staGF	0.5
	staGR	0.5
	staGP	0.5
Nuclease Free water		3
Template DNA		5

The DNA templates were subjected to the following PCR conditions. A starting step of 95 °C for 10 min, and 40 cycles of 95°C for 20 sec, annealing step with temperature ranging from 56-64 °C for 25 sec and an extension step of 72 °C for 45 sec. Each reaction was performed in triplicates. A sample was detected positive by its cycle threshold (Ct) value at which emission of the normalized dye reporter was above the background florescence. Thermal cycling profile for ttr, tviB, and staG is shown in Figure. 7.

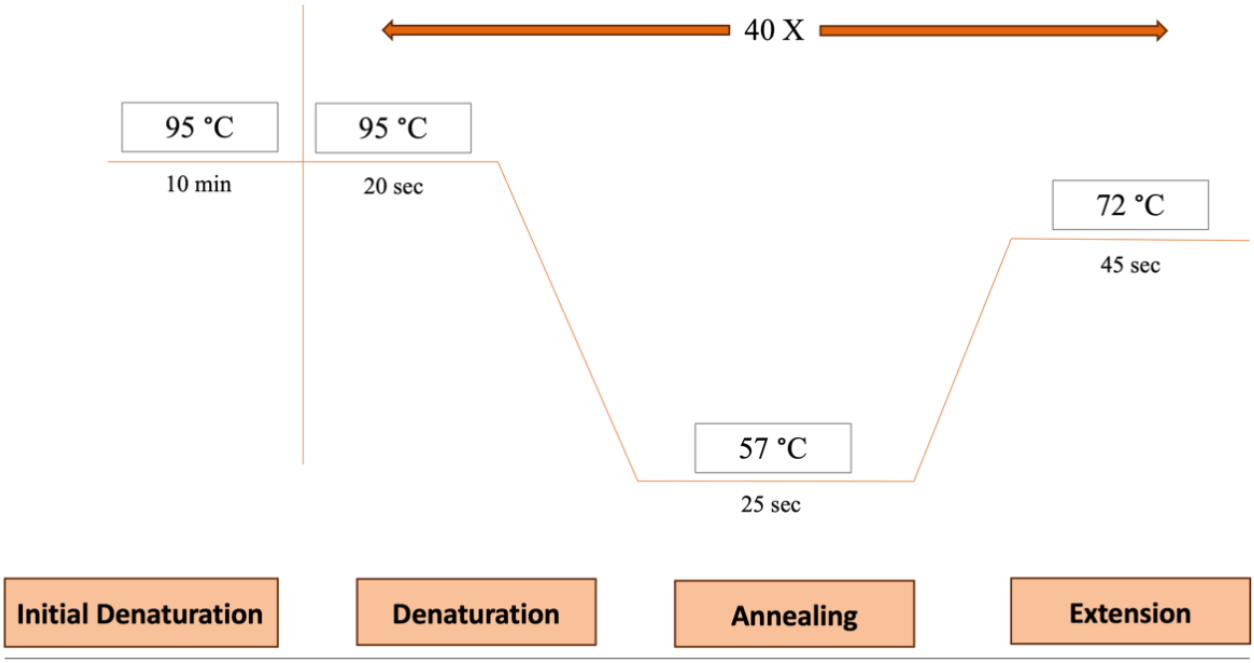

Figure 7 Thermal cycling profile for ttr, tviB, and staG amplification.

3.2.7 Validation of PCR Assay with Bacterial samples

For validation of PCR protocol with bacterial samples, agar plates containing isolated bacterial colonies were obtained from different hospitals of Pakistan. Liquid culture media (LB Broth) was inoculated with one colony of bacteria from agar plates and placed overnight in shaking incubator at 37 °C. DNA was extracted from the cultured bacteria and stored at -20 °C.

3.2.8 Sensitivity Testing of Real Time qPCR Assay

Sensitivity testing of PCR assay was performed using synthetic positive control. The concentration and purity of positive control was checked using Nanodrop 2000 (Thermoscientific, USA). Known DNA concentration (ng/ul) was converted into DNA copy number (DNA copies per microliter) using the following formula:

Number of copies=(ng× [6.022×10^{23}])/(length ×[1×10^{9}]×650)

After calculation of DNA copy number, known concentration of positive control for each gene (ttr, tviB, and staG) was 10-fold serially diluted to contain 1 DNA copy per microliter.

The PCR reaction was further performed for each dilution in triplicates to estimate the assay Limit of Detection. A standard curve was also generated to calculate the PCR efficiency.

The following formula was used to calculate the efficiency of the assay:

Efficiency = -1+10 (-1/slope)

Figure 8 illustrates the principle of 10-fold serial dilution of the extracted DNA with known DNA copy number.

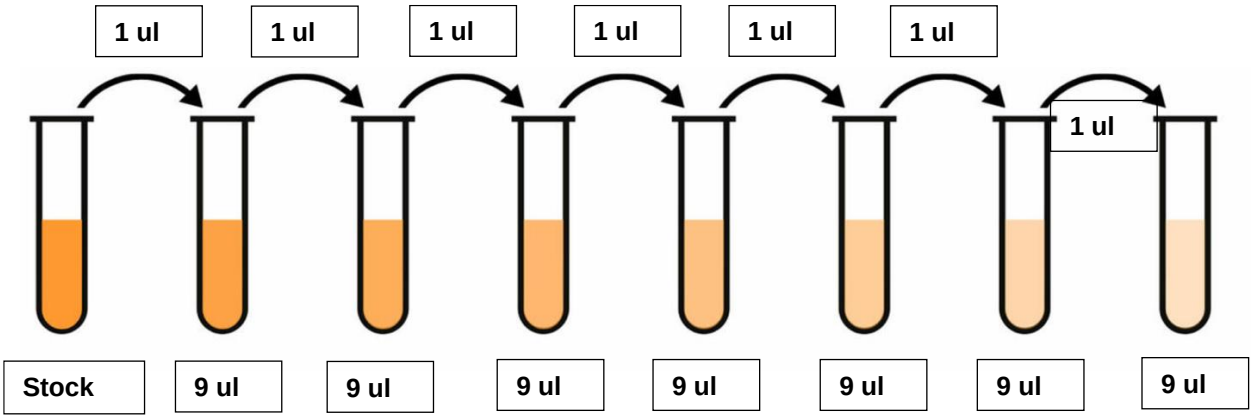

Figure 8 10-fold serial dilution of the extracted DNA with known DNA copy number.

3.2.9 Specificity Testing of Real Time qPCR Assay

To calculate the specificity of primers and probes for the detection of *Salmonella typhi*, real time PCR was performed with the DNA from Human blood and malaria positive blood. Human DNA and malarial DNA was tested using ttr, tviB, and staG primers and probe sets to determine the specificity of the current method. The reaction was performed in triplicates for both human DNA and malarial DNA.

3.2.10 Cloning of Target DNA sequences in pUC57 Plasmid

Target DNA sequences of ttr, tviB, and staG were cloned in pUC57 Plasmid by NovoPro Biosciences Inc.

3.2.11 Amplification of Recombinant pUC57

3.2.11.1 Preparation of Competent DH5α cells

Competent cells were prepared using the following protocol:

A single colony of DH5α strain of E. coli was picked from a cultured plate of cells using a sterile loop and the colony was then inoculated in a flask containing 20ml LB media. After inoculation, the flask was incubated overnight in shaking incubator at 37 °C. Secondary inoculum was prepared after 24 hours by combining 2ml of primary inoculum with 60ml of LB media in another flask. The flask was incubated for 2 hours at 37 °C in shaking incubator. The optical density of secondary inoculum was checked using a spectrophotometer. At OD range of 0.4 to 0.6, cells were considered good for transformation because it indicates the growth phase of bacteria. The inoculum was centrifuged at 4 °C, 6000 rpm for ten minutes by transferring it from flask into chilled centrifuge tubes to obtain cell pellet. After discarding supernatant, cells were resuspended in 200ul of solution A (0.1M CaCl2). Centrifuge tubes were again centrifuged, and the pellet was resuspended in 20ul of Solution B (0.1M CaCl2 + 15% glycerol). Centrifuge tubes were centrifuged at -80 °C to be used for transformation of plasmid.

3.2.11.2 Transformation of Competent DH5α cells

50ul of DH5α competent cells were thawed on ice followed by the addition of 2ul recombinant pUC57 plasmid and incubated on ice for 30 min. After half an hour, heat shock was given to cells at 42 °C in water bath for 90 sec. The cells and plasmid mixture were again placed on ice for 4 min. 1ml of LB broth was added to the mixture and placed in shaking incubator at 37 °C for 1 hour. LB agar plates were made by adding 25ml of agar media having 50ul of ampicillin and

poured onto petri plates. 50ul of transformed cells were spread on ampicillin plates using a sterile spreader and were incubated overnight at 37 °C. Transformed colonies were observed after overnight incubation. The procedure was repeated for all the three recombinant plasmids (ttr-pUC57, tviB- pUC5, and staG- pUC57).

3.2.11.3 Colony PCR

To confirm the uptake of recombinant plasmid, colony PCR was performed using the primer sets of all the three genes (ttr, tviB, and staG). A colony was picked from transformed plate and mixed in 10ul nuclease free water in a centrifuge tube. After gently mixing, centrifuge tube was placed in water bath at 95°C for 10 min. This lysate was used as template DNA in PCR reaction. All the reaction conditions were the same as used for Real Time qPCR.

3.2.11.4 Gel Electrophoresis

Gel electrophoresis of PCR products was performed on 0.5% gel using 50 bp ladder. Gel was run for 47 min at 90V, and the resulting bands were observed under Gel Doc. 0.5% agarose gel was prepared by mixing 1g agarose powder into 50ml 1X TAE buffer. The mixed solution was boiled for 1 to 2 min and then 7ul ethidium bromide was added before pouring it into gel cassette.

3.2.11.5 Plasmid Miniprep

After confirmation of transformation, plasmids (ttr- pUC57, tviB- pUC5, and staG- pUC57) were extracted from transformed colonies using plasmid miniprep kit from Wizbio solutions. For plasmid miniprep, standard protocol provided by the company was used and the extracted plasmid was used as a positive control in Real-time qPCR.

Chapter 4

Results

4.1 *In silico* Analysis

4.1.1 Oligonucleotide Properties Calculator (Oligo Calc)

The results of Oligo Calc for ttr, tviB, and staG primer and probe sequences showed that there are no chances of formation of hairpins, self-complementarity and no self-annealing sites in the primer probe sets. Figure 9 shows the analysis of cross complementarity and hairpin formation in primer and probe sets.

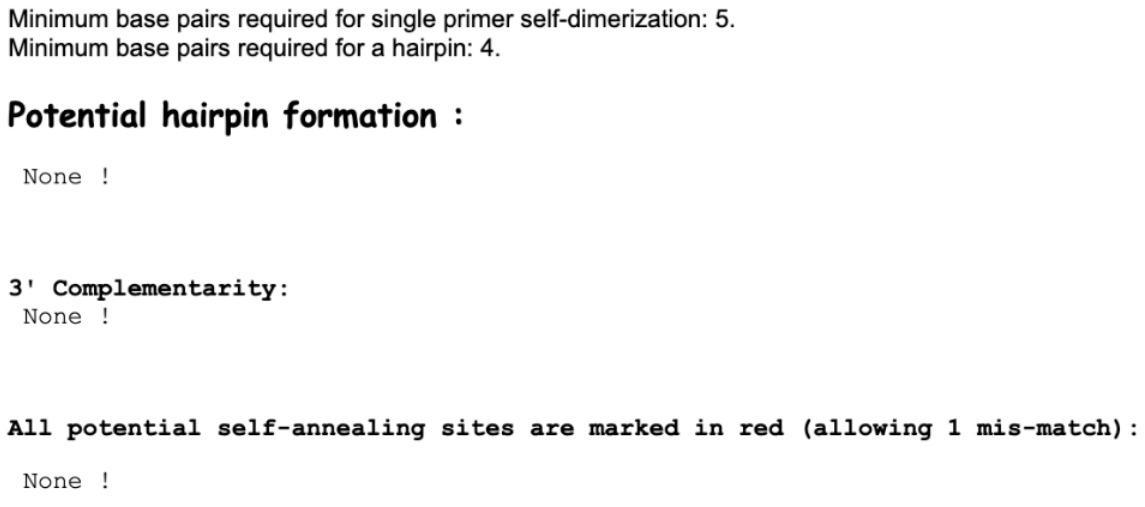

Figure 9 Analysis of cross complementarity and hairpin formation in primer and probe sets.

The GC content, melting temperatures of the primer set and the probes were also determined using Oligo Calc. Melting temperatures and GC percentages of primers and probes are shown in table 7 and 8.

Table 7 Tm and GC percentage of primers calculated by Oligo Calc

Gene	Sequence 5'-3'	Tm (°C)	GC %
ttrF	CTGGATATGCGAATGCCGGT	65	55
ttrR	GATGAAACCGTCAGCGCACG	66	60
tviBF	GCCACTACGATGCGATCATT	62	50
tviBR	GCTCAGCCGGAAGAACATAC	63	55

staGF	CTCAACGCCGATCACCAGC	65	63.2
staGR	CGGCGCGAAGTCAGAGTC	64	66.7

Table 8 Tm and GC percentage of probes calculated by Oligo Calc

Gene	**Sequence 5'-3'**	**Tm (°C)**	**GC %**
ttrP	FAM-CCGATGGCCGTGGAGCAGATGAAA-BHQ1	70	58
tviBP	HEX-ATGGGAAGTGAGGATATTCGCGGC-BHQ1	68	54
staGP	Cy5-CGTCAGCCTGCTCCAGAACAAATGT-BHQ2	69	52

4.1.2 Multiple Primer Analysis

The results from Thermofischer Scientific multiple primer analyzer have confirmed that there no self-dimers and cross dimers in ttr, tviB, and staG primer and probe sets. Figure 10 illustrates results of Thermofischer Scientific multiple primer analyzer showing no dimers and cross complementarity.

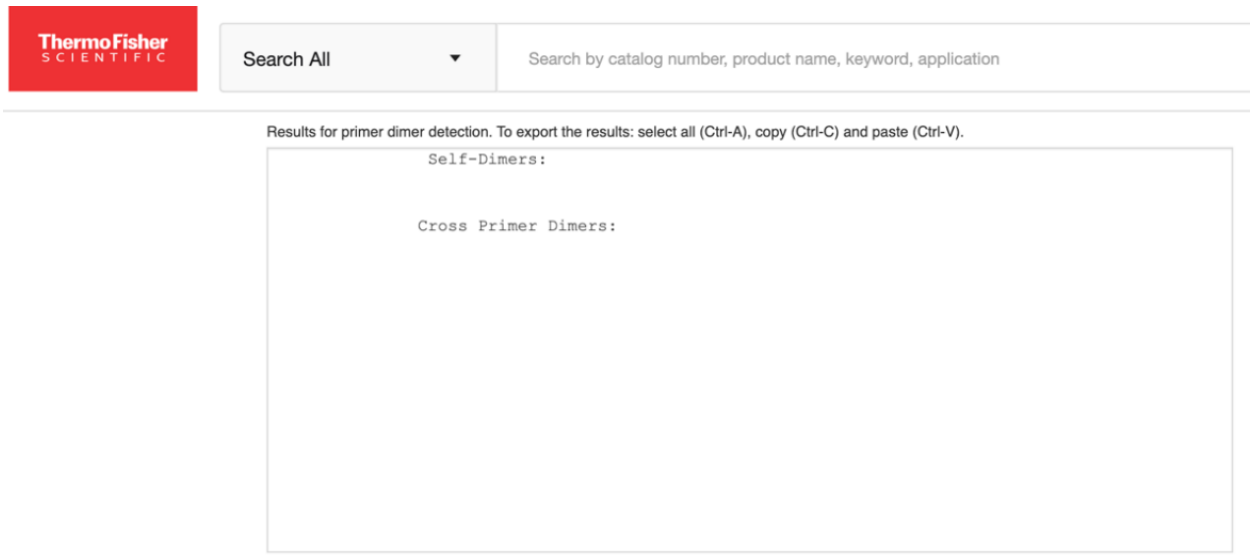

Figure 10 Results of Thermofischer Scientific multiple primer analyzer showing no dimers and cross complementarity.

4.1.3 *In-silico* PCR using SnapGene

The *In-silico* PCR results generated by Snap Gene depicted that forward and reverse primers of ttr, tviB, and staG bind to the target regions on each gene of *Salmonella typhi*. PCR products of ttr, tviB, and staG are 212 bp, 129 bp, and 130 bp respectively. Moreover, by using Snap gene it was confirmed that specific probes for each gene also bind specifically to their target sites.

4.1.3.1 *In-silico* PCR of ttr Gene

Result of *in-silico* PCR of ttr gene with specific primers and probes performed via snap gene is shown in Figure 11.

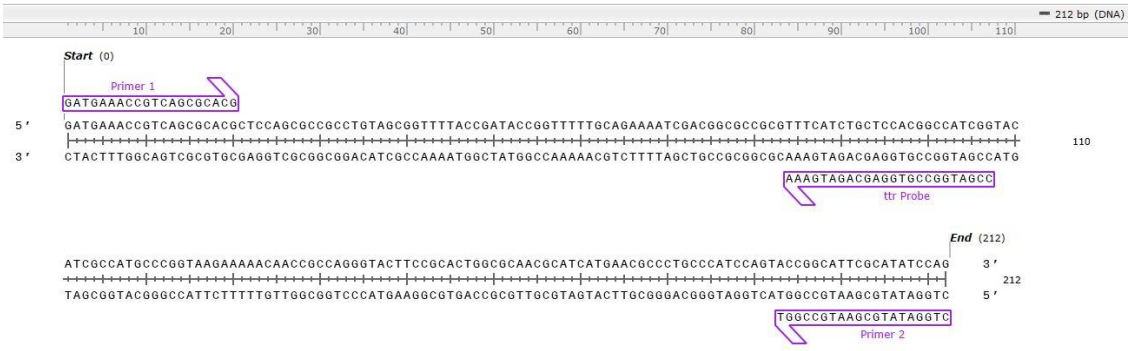

Figure 11 In-silico PCR of ttr gene showing amplification product of 212 bp.

4.1.3.2 In-silico PCR of tviB Gene

Result of in-silico PCR of tviB gene with specific primers and probes performed via snap gene is shown in Figure 12.

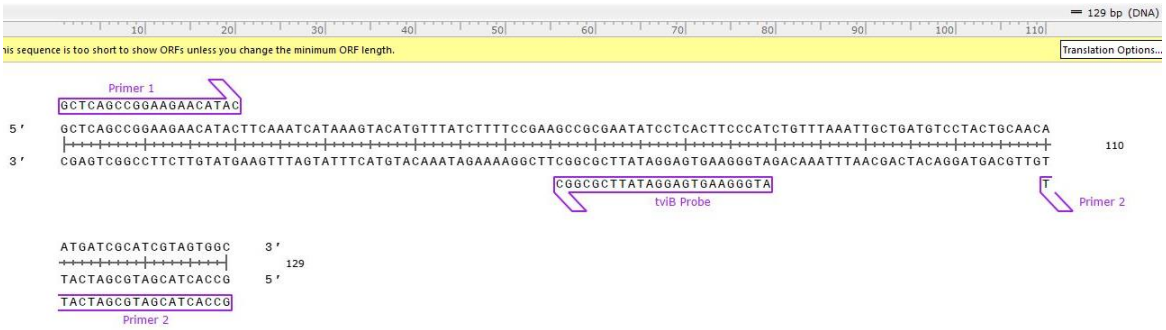

Figure 12 In-silico PCR of tviB gene showing amplification product of 129 bp.

4.1.3.3 *In-silico* PCR of staG Gene

The result of in-silico PCR of staG gene with specific primers and probes performed via snap gene is shown in Figure 13.

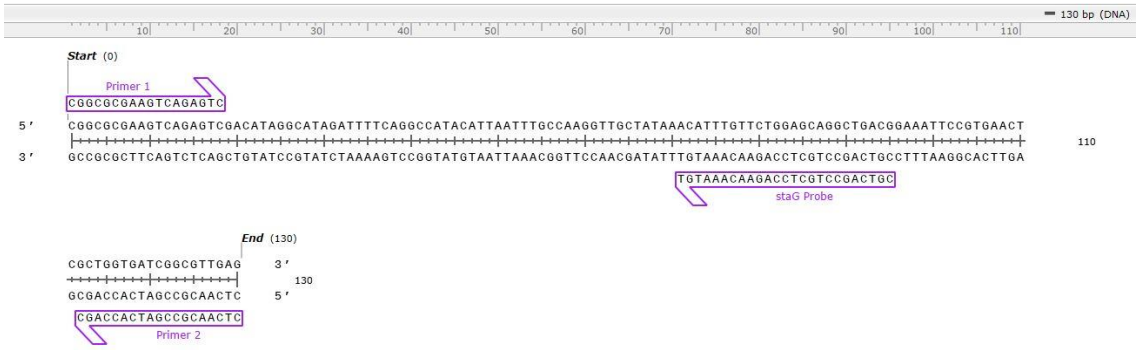

Figure 13 In-silico PCR of staG gene showing amplification product of 130 bp.

4.1.4 In-silico PCR using Salmonella isolates from pubMLST

4.1.4.1 *In-silico* PCR amplification with ttr

In-silico PCR using forward and reverse primers of ttr showed band of 212 base pairs with all 6103 *Salmonella* strains available in database. The result of PCR amplification is shown in figure 14.

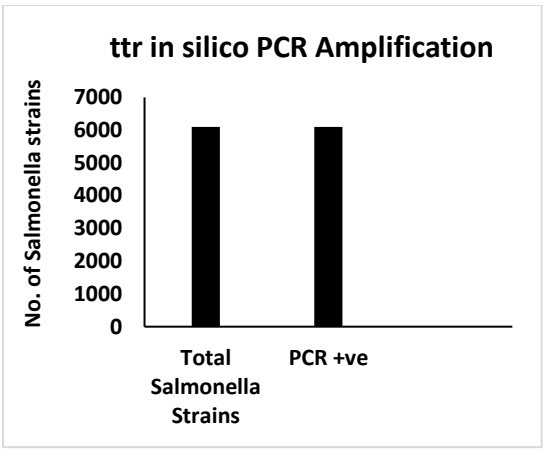

Figure 14 Results of in-silico PCR Amplification with ttr primers.

4.1.4.2 *In-silico* PCR amplification with tviB

In-silico PCR using forward and reverse primers of tviB showed band of 129 base pairs with 928 *Salmonella typhi* strains. Result of PCR amplification is shown in figure 15.

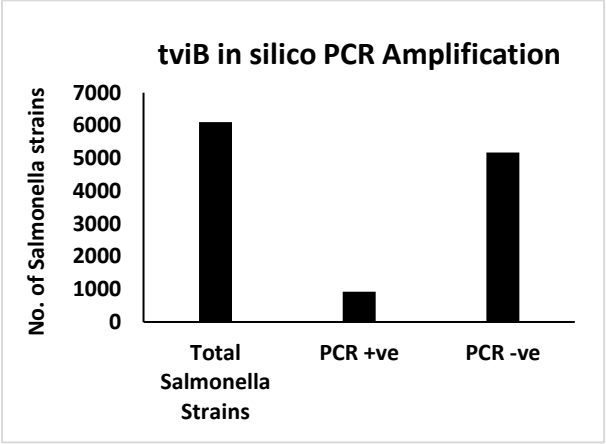

Figure 15 Results of in-silico PCR Amplification with tviB primers.

4.1.4.3 *In-silico* PCR amplification with staG

In-silico PCR using forward and reverse primers of staG showed band of 130 base pairs only with 928 *Salmonella typhi* strains. Result of PCR amplification is shown in Fig 16.

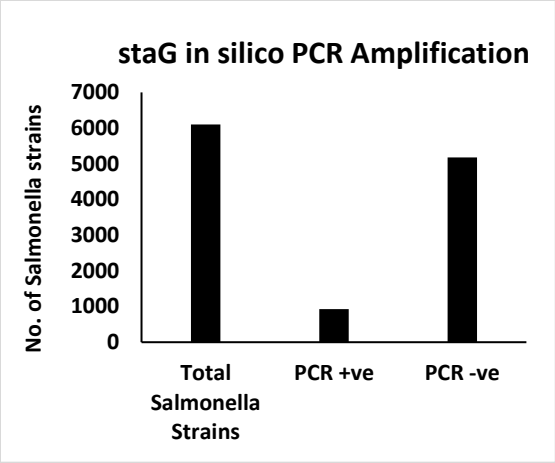

Figure 16 Results of in-silico PCR Amplification with staG primers.

4.1.5 Designing of Synthetic Positive control

Synthetic positive control was designed for each gene separately by cloning the selected sequences of ttr, tviB, and staG in pUC57 plasmid using SnapGene. Plasmid was Digested with BamH1-Kpn1 restriction enzymes for cloning of selected DNA sequence.

4.1.5.1 Cloning of ttr in pUC5

A total of 312 bp DNA fragment of ttr gene was cloned in pUC57 plasmid using SnapGene. BamH1 and Kpn1 restriction sites were used for cloning. Figure 17 illustrates the cloned sequence of ttr in pUC57 plasmid.

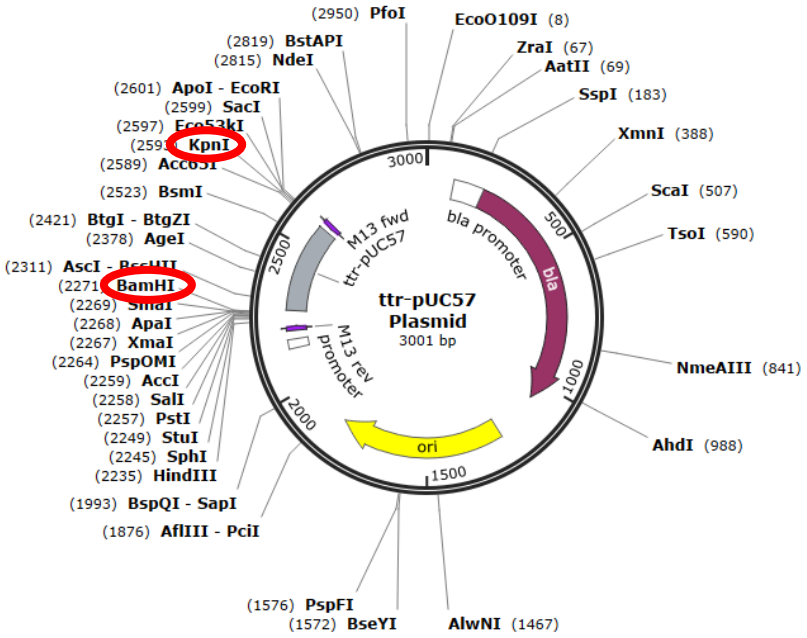

Figure 17 ttr cloned sequence in pUC57 plasmid.

4.1.5.2 Cloning of tviB in pUC57

For cloning of tviB, 227 bp DNA fragment of tviB gene was inserted in pUC57 plasmid using SnapGene. BamH1 and Kpn1 restriction sites were used for cloning. Figure 18 illustrates the cloned sequence of tviB in pUC57 plasmid.

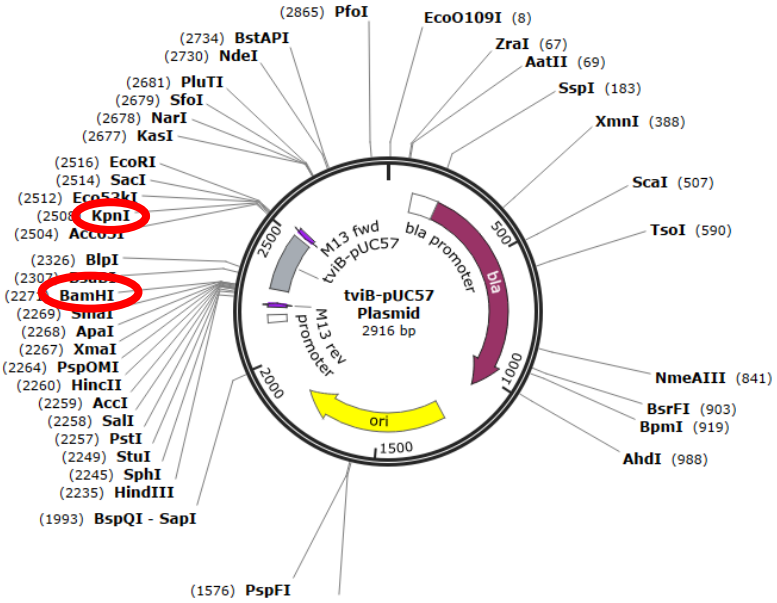

Figure 18 tviB cloned sequence in pUC57 plasmid.

4.1.5.3 Cloning of staG in pUC57

For cloning of staG, 230 bp DNA fragment of staG gene was inserted in pUC57 plasmid using SnapGene. BamH1 and Kpn1 restriction sites were used for cloning. Figure 19 illustrates the cloned sequence of staG in pUC57 plasmid.

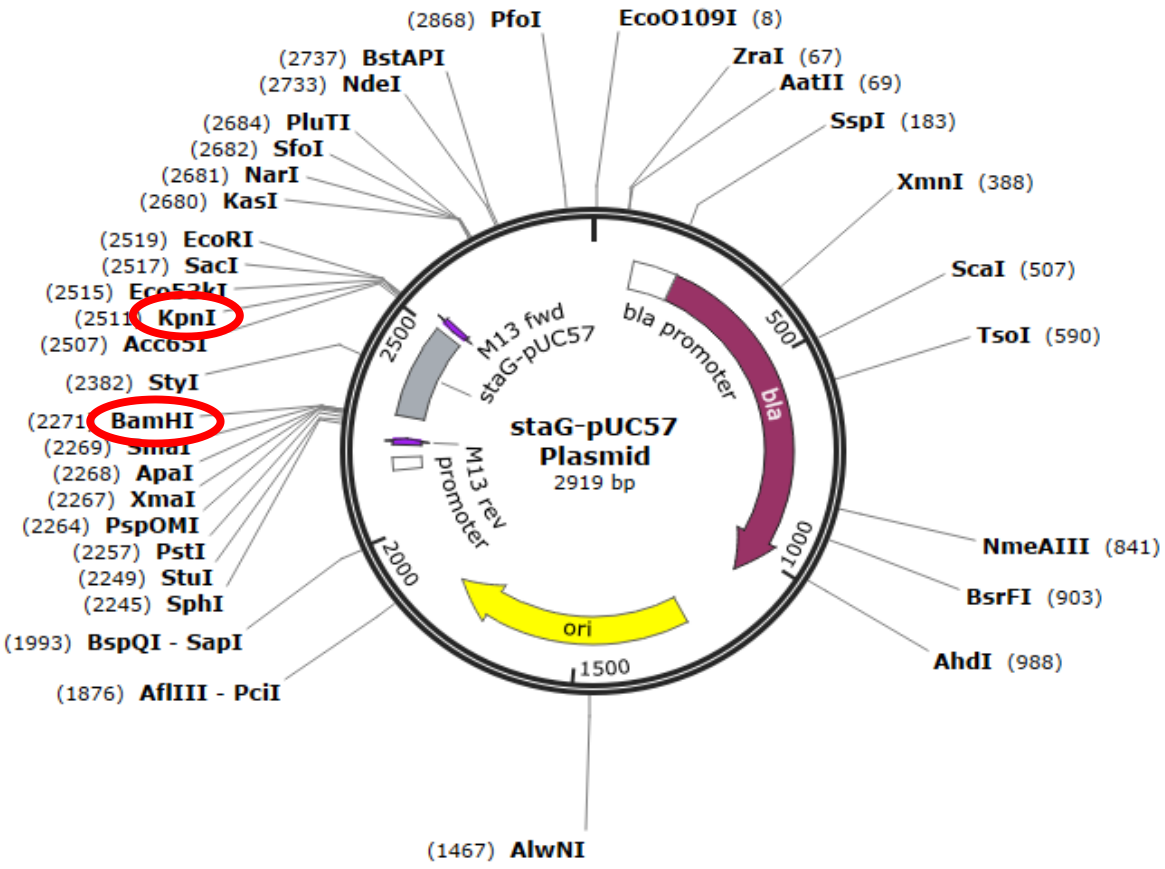

Figure 19 staG cloned sequence in pUC57 plasmid.

4.1.6 *In-silico* PCR of Synthetic Positive control

4.1.6.1 *In-silico* PCR of ttr-pUC57

By performing in-silico PCR of ttr-pUC57 using ttr primers via SnapGene, 212 bp region of plasmid was amplified confirming the specific binding and amplification of primers with synthetic positive control. PCR amplified region of ttr-pUC57 is shown in Figure. 20.

Figure 20 PCR amplified region of ttr-pUC57.

4.1.6.2 In-silico PCR of tviB-pUC57

By performing in-silico PCR of tviB-pUC57 using tviB primers via SnapGene, 129 bp region of plasmid was amplified confirming the specific binding and amplification of primers with synthetic positive control. PCR amplified region of tviB-pUC57 is shown in Figure. 21.

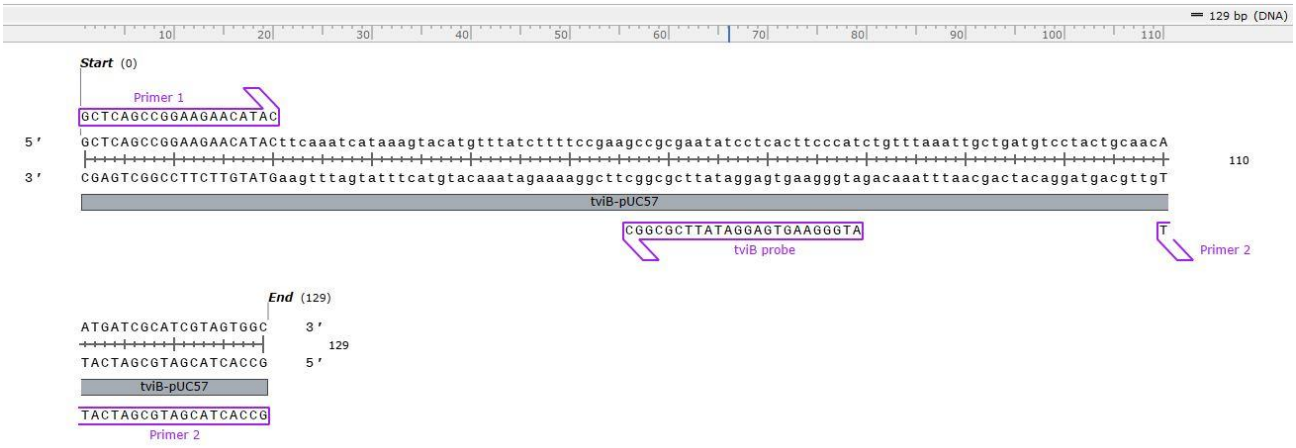

Figure 21 PCR amplified region of tviB-pUC57.

4.1.6.3 In-silico PCR of staG-pUC57

By performing in-silico PCR of staG-pUC57 using staG primers via SnapGene, 130 bp region of plasmid was amplified confirming the specific binding and amplification of primers with synthetic positive control. PCR amplified region of staG -pUC57 is shown in Figure. 22.

Figure 22 PCR amplified region of staG-pUC57

4.2 In vitro Results

4.2.1 Optimization of PCR conditions

Multiplex gradient PCR showed that at all the temperatures between 56-64, all the primer and probe sets were able to detect the presence of *Salmonella typhi* DNA so any of these temperatures can be selected as the annealing temperature of the primers and the probes. Whereas no fluorescence was detected in the no template control (NTC). 57 °C was selected as the annealing temperature of the primers and probe sets.

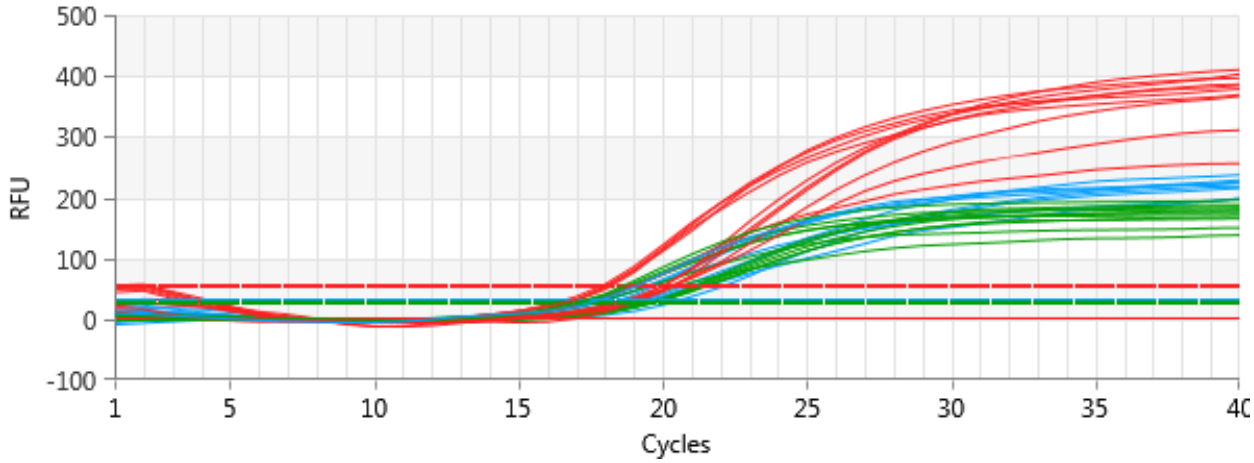

Figure 23 Amplification curve of PCR optimization assay.

4.2.2 RT-qPCR of Bacterial Samples

Multiplex RT-qPCR protocol was validated with 30 bacterial samples, all of which showed positive results with Ct values in the range of 17- 35.

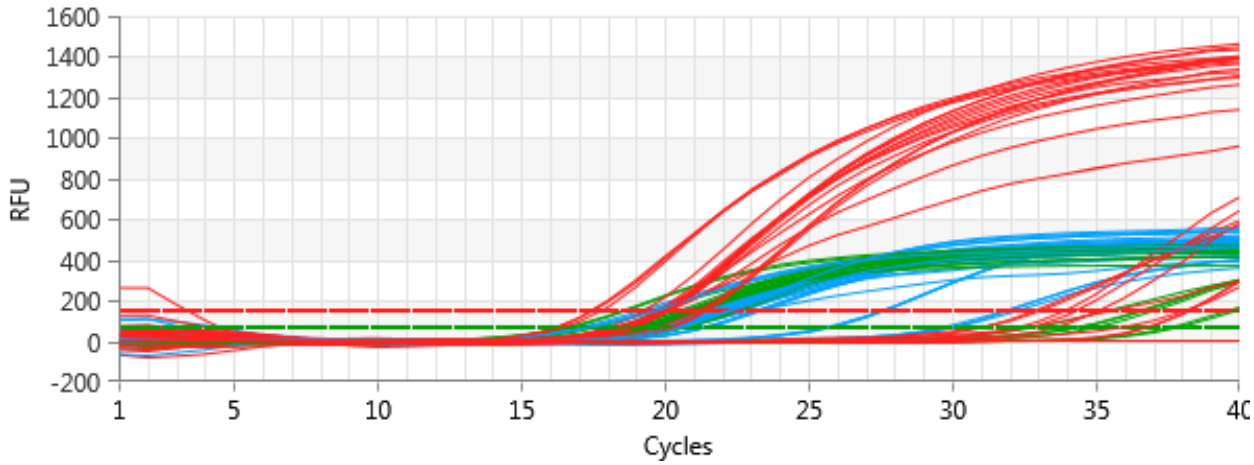

Figure 24 Amplification curve of PCR performed with bacterial samples.

4.2.3 RT-qPCR of Clinical Samples

Out of 300 samples tested, 36 were positive by both PCR and blood culture, 12 were positive by PCR but negative by blood culture and remaining 252 were negative by both PCR and blood culture. The reason behind samples positive by PCR while negative by blood culture could be the presence of bacteria in the samples which could not be cultured but DNA can still be extracted

from limited number of bacteria. Sensitivity and specificity percentage of the Assay was calculated using blood culture as the gold standard by 2 by 2 matrix. The PCR assay has 100% sensitivity while 95% specificity in comparison to the blood culture.

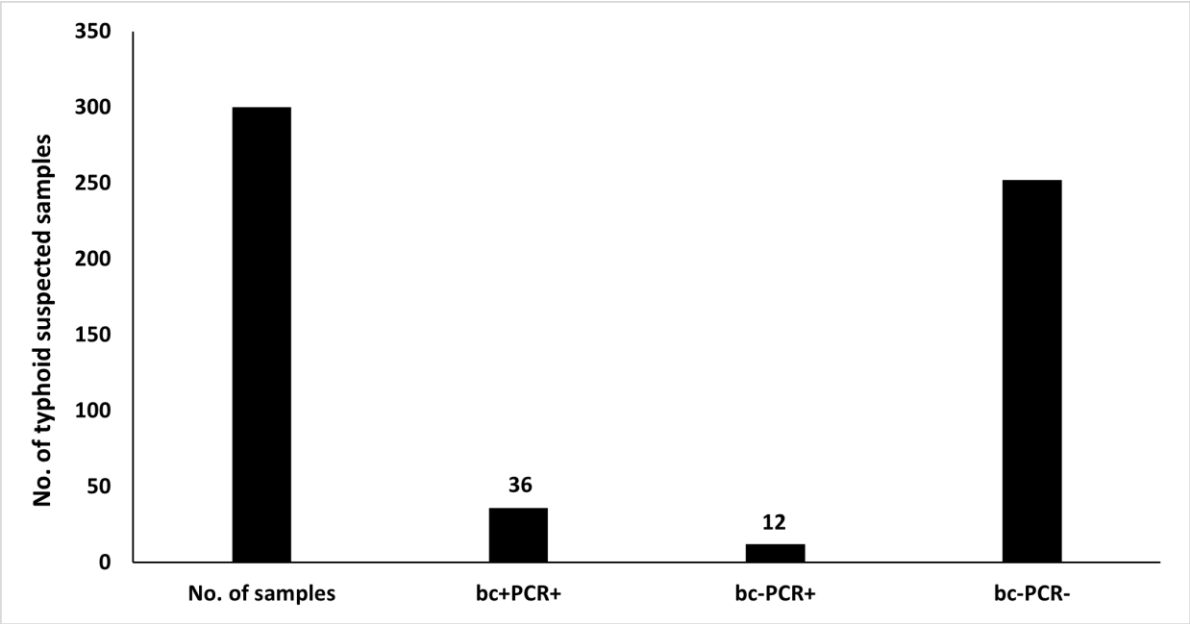

Figure 25 Graph showing comparison of blood culture and PCR results.

Sensitivity and specificity of PCR Assay was calculated using blood culture positive as true positive.

Table 9 Sensitivity and Specificity using Blood Culture as Gold Standard

Sensitivity	Specificity
100%	95%

4.3 Sensitivity Testing

Plasmid pUC57 DNA with target genes was diluted using a ten-fold serial dilution method to achieve a minimum concentration of 2 DNA copies per microliter for sensitivity determination and limit of detection (LOD) assessment. The limit of detection of developed real time assay was 2 DNA copies/ul for *Salmonella typhi*.

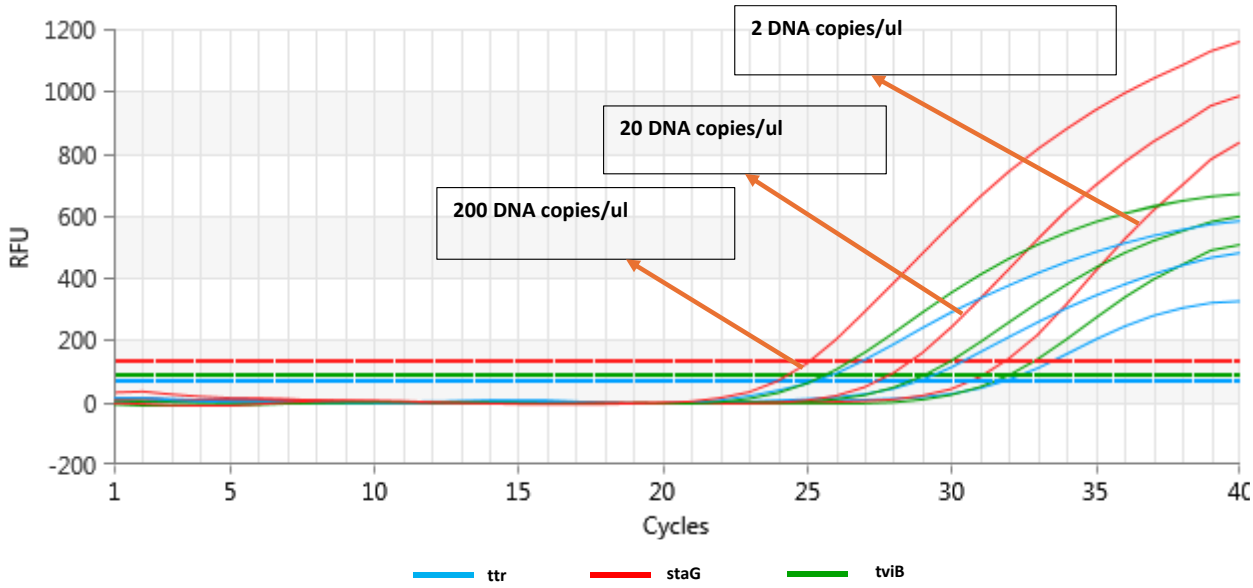

Figure 26 Amplification curve showing LOD of RT-qPCR Assay.

Ct values and log DNA concentration of 10- fold serially diluted DNA were used to generate standard curve to calculate the r2 value and efficiency of the real time PCR assay. Standard curve was generated for primer and probe set of each gene separately.

4.3.1 Standard Curve for ttr primer and probe set

Standard Curve for ttr primer and probe set was generated using the data shown in table 10.

Table 10 DNA concentration and Ct values used for generating ttr standard curve.

Log DNA Concentration (ng/ul)	**Ct values**
485	6.785

48.5	9.809
4.85	12.215
0.485	15.527
0.0485	19.434
0.00485	22.671

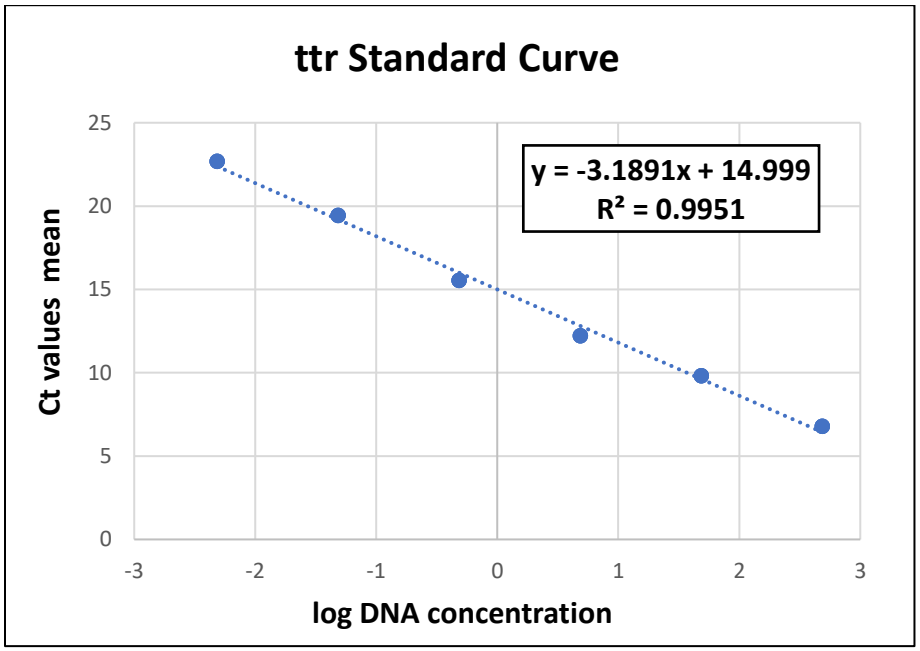

Figure 27Standard Curve for ttr primer and probe set.

By using the slope value, the RT- PCR efficiency was calculated using the following formula:

Efficiency= -1+ 10^(-1/slope)x100

= -1+ 10^(-1/-3.189)x100

= -1+10^(0.313)x100

= -1+2.055x100

= 1.055x100

= 105.5%

The calculated efficiency of PCR is 105.5%.

4.3.2 Standard Curve for tviB primer and probe set

Standard Curve for tviB primer and probe set was generated using the data shown in table 11.

Table 11 DNA concentration and Ct values used for generating tviB standard curve.

Log DNA Concentration (ng/ul)	Ct values
Log (118)	6.707
Log (11.8)	11.824
Log (1.18)	14.051
Log (0.118)	16.918
Log (0.0118)	20.309
Log (0.00118)	23.121

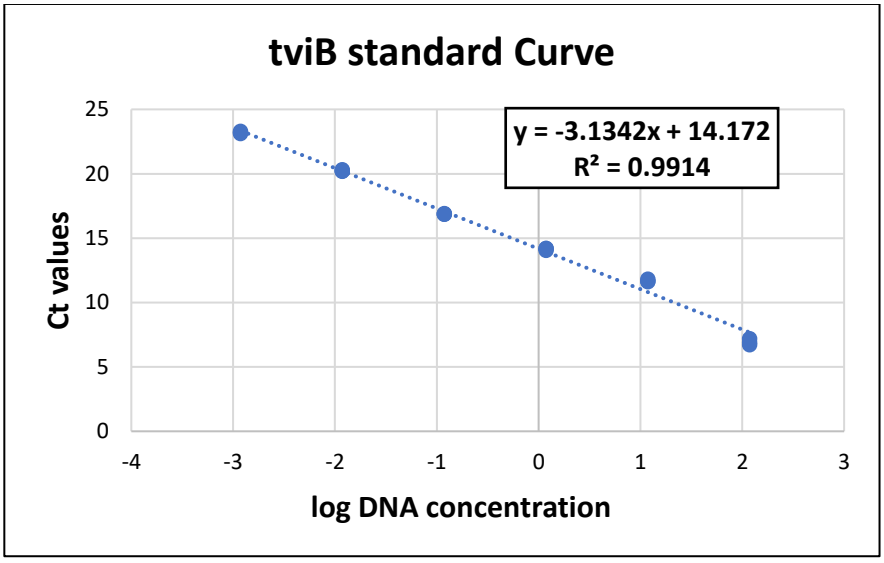

Figure 28 Standard Curve for tviB primer and probe set.

By using the slope value, the RT- PCR efficiency was calculated using the following formula:

Efficiency = -1+ 10^(-1/slope)x100

= -1+ 10^(-1/-3.13)x100

= -1+10^(0.319)x100

= -1+2.08x100

= 1.08x100

= 108%

4.3.3 Standard Curve for staG primer and probe set

Standard Curve for staG primer and probe set was generated using the data shown in table 12.

Table 12 DNA concentration and Ct values used for generating staG standard curve.

Log DNA Concentration	**Ct values**
Log (198.5)	5.387
Log (19.85)	7.590
Log (1.985)	13.309
Log (0.1985)	15.589
Log (0.01985)	18.354
Log (0.001985)	21.645

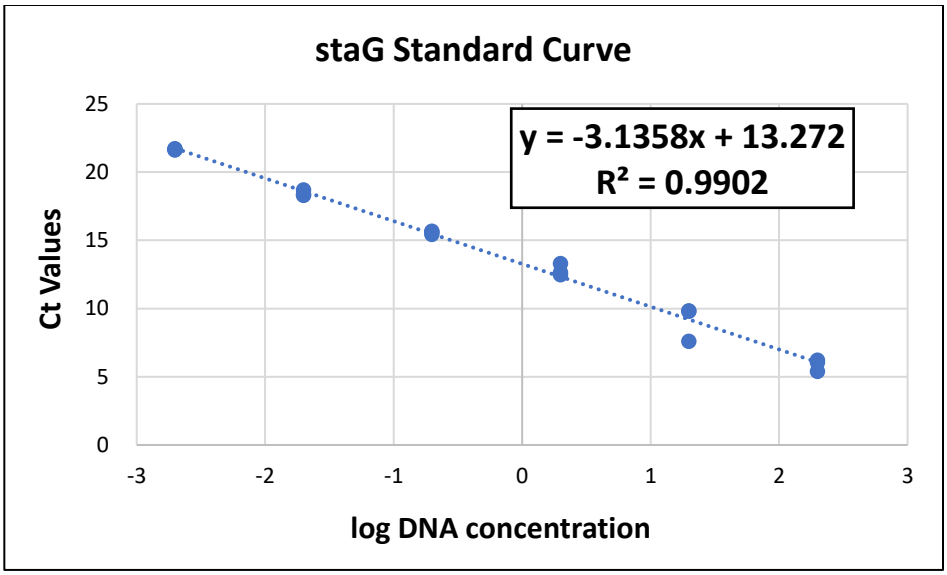

Figure 29 Standard Curve for staG primer and probe set.

By using the slope value, the RT- PCR efficiency was calculated using the following formula:

Efficiency = -1+ 10^(-1/slope)x 100

= -1+ 10^ (-1/-3.135)x 100

= -1+10^ (0.318)x 100

= -1+2.079x 100

= 1.079x 100

= 107.9%

So, the overall efficiency of PCR assay would be in the range of 105-108%.

4.4 Specificity Testing

The PCR assay showed no amplification using ttr, tviB, and staG primer and probe sets for human and malarial DNA samples.

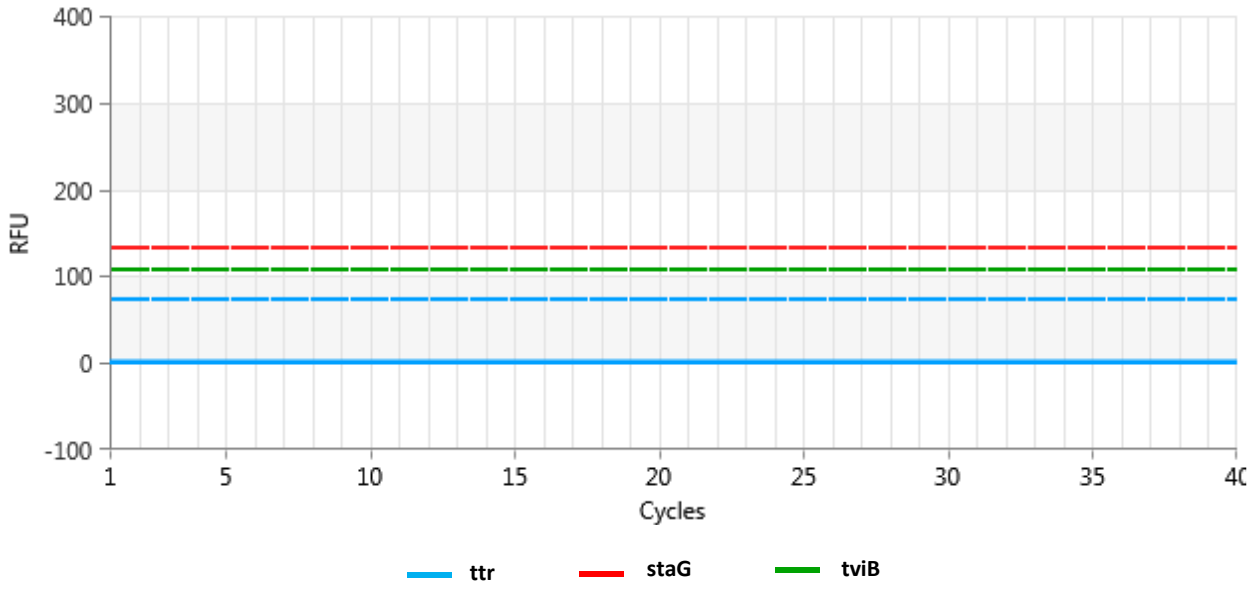

Figure 30 RT-qPCR Amplification curve of specificity Testing.

4.5 Transformation of Competent E.coli DH5α cells

Transformed colonies of ttr- pUC57, tviB- pUC5, and staG- pUC57 plasmids observed on petri plates are shown in Figure 31.

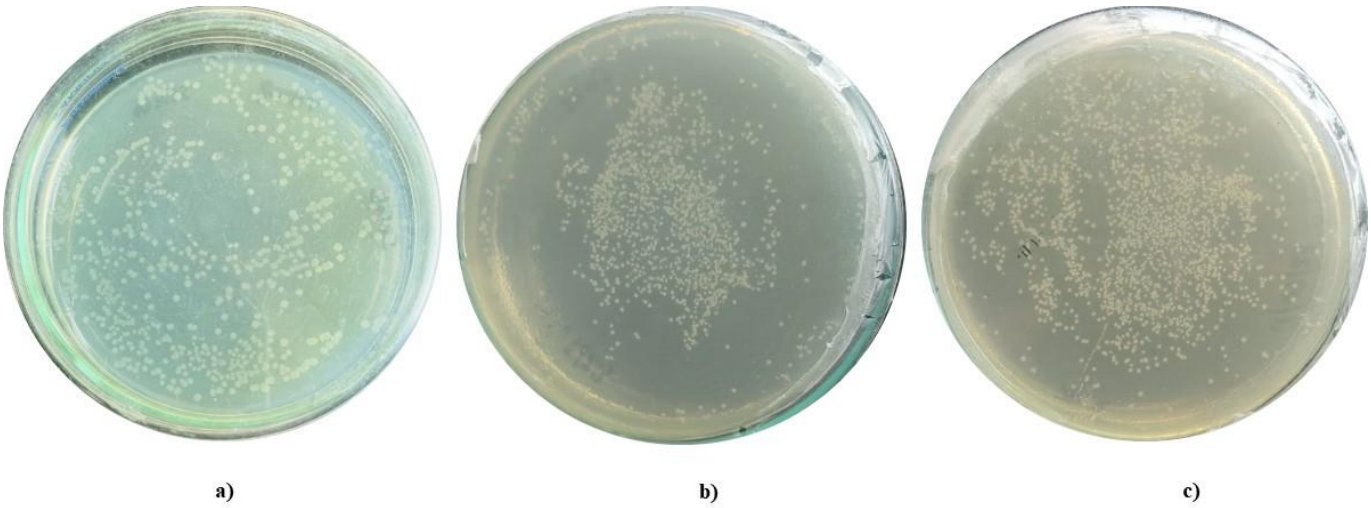

Figure 31 Transformation of DH5-α cells with a) ttr-pUC57, b) tviB-PUC57, and c) staG-pUC57

4.5.1 Gel Electrophoresis

PCR amplification of ttr- pUC57, tviB- pUC5, and staG- pUC57 plasmids gave the expected amplification products of 212 bp, 129 bp and 130 bp respectively. The Gel electrophoresis image of PCR products obtained by colony PCR is shown in figure 32.

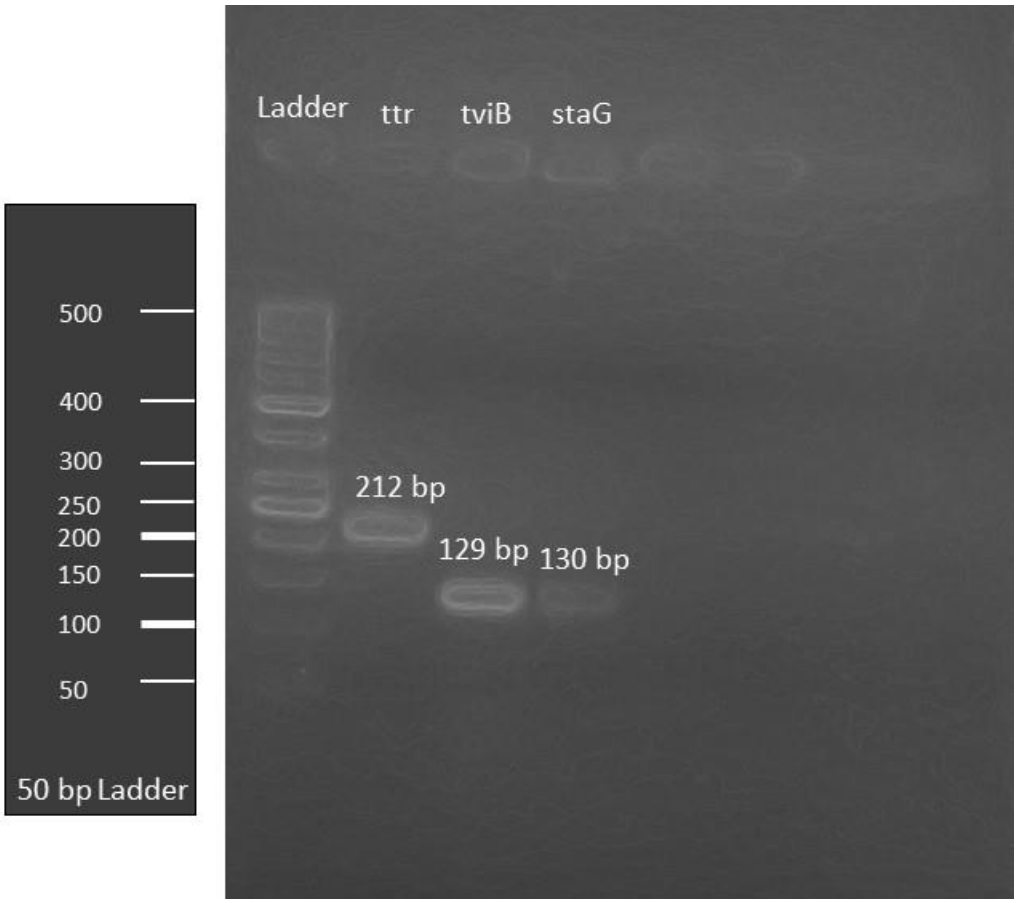

Figure 32 Gel electrophoresis image of PCR products obtained by colony PCR. Lane 1 has 50 bp DNA Ladder, Lane 2 has 212 bp PCR product of ttr, Lane 3 has 129 bp PCR product of tviB, Lane 4 has 130 bp PCR product of staG.

Chapter 5

Discussion

Discussion

Typhoid fever continues to be a major public health concern worldwide, especially in developing countries, where residents frequently lack adequate water, sanitation, and hygiene facilities. Typhoid fever is an important worldwide contributor to illness and death, with an approximate annual prevalence of 12-33 million cases and 216,000-600,000 deaths [24]. According to the latest estimates in 2019, the number of typhoid fever cases each year is nearly 9 million, leading to approximately 110,000 deaths annually. According to WHO, Pakistan has the highest rate of typhoid fever among south Asian countries with 0.1 million cases annually [75].

Typhoid fever is confirmed by laboratory diagnosis. Despite being recognized for over a century, it lacks a definitive test diagnostic biomarker. Blood culture is used as a gold standard for diagnosis but the sensitivity of blood culture is less than 50%. Bone marrow culture is not used because of the invasive nature [76]. Widal test quantifies the antibodies produced against flagella and LPS in the serum of patients suspected of having typhoid fever by agglutinating of *S. typhi* cells. In countries where a disease is commonly seen, such as Pakistan and India, the population develops a certain level of antibodies as a result of recurrent exposure to *Salmonella* infection. The earlier use of antibiotic medication can provide a false negative result in the Widal test [56]. Diagnostic methods based on real time PCR has overcome these drawbacks of conventional methods by providing increased sensitivity for the detection of *Salmonella typhi*. Most of these molecular diagnostic methods are based on targeting the genes unique to *Salmonella typhi* [56].

Among all the reported genes used for *Salmonella* diagnosis, ttr, tviB, and staG is unique to *Salmonella* species. Therefore, in the current, these genes were targeted in a multiplex Real-time PCR Assay for the detection of *Salmonella typhi*. Primers and probes for all three genes were designed and validated in silico to detect typhoid fever. *In silico* PCR confirmed the binding of primers and probes to their target regions with amplification products of 212, 129, and 130 bp of ttr, tviB, and staG respectively.

Currently, primers of all three genes were used for the detection of *Salmonella typhi* only. Out of 300 blood samples collected from suspected typhoid patients, 36 came out positive using the developed Assay. The PCR results were compared with results of blood culture performed in the hospitals. The low positive rate is because samples were collected from suspected typhoid patients which can or cannot have typhoid fever. Overall, real-time PCR Assay is more sensitive as compared to blood culture because DNA can be extracted even from dead bacteria. Using blood

culture as a gold standard, the PCR assay showed 100% sensitivity and 95% specificity. Sensitivity and specificity were calculated using 2 by 2 matrix.

Method developed had a LOD of 2 DNA copies/ μL. The assay also showed good linearity with R2 value of 0.99 and efficiency in the range of 105-108%. The sensitivity of the Assay is similar to already developed Real-time PCR diagnostic kits available in the market.

The specificity of current method was also checked against human DNA and *malarial* DNA as typhoid shares symptoms with malaria. No amplification was observed against malarial DNA indicating that Real time PCR is highly specific to the detection of *Salmonella typhi*.

The real-time PCR's thermal cycling profile took a total of 1 hour and 54 minutes to complete. In comparison to traditional procedures based on nested and conventional PCR, real-time approaches for diagnostic purposes are quick and don't require much processing. They also don't require post-PCR gel electrophoresis for product identification.

The developed test was significantly more costly as compared to SYBR green methods due to use of a TaqMan probe. However, because the cost of the probe is dispersed among hundreds of PCR reactions and therefore has very low cost for a single PCR reaction.

Conclusion

The current study has been designed for the development of real time PCR assay for the detection of typhoid fever. *Salmonella typhi* detection using blood samples from suspected typhoid patients was validated clinically. Using Genomic DNA obtained from suspected typhoid samples, RT-PCR displayed good sensitivity for *Salmonella typhi*. The assay also showed good linearity and is highly specific to the detection of *Salmonella typhi* with no cross reactivity with Human and malarial DNA. The real time PCR assay could help with an early diagnosis in regions where there are high incidence rates of typhoid. It could also help with the protection of the most at-risk groups like children and immunocompromised individuals, in which *Salmonella typhi* can be deadly in a short span of time.

Future Prospects

Although the PCR Assay showed good sensitivity and specificity in comparison with blood culture, the assay needs to be compared with already established real-time PCR based diagnostic kit for further validation.

An internal control needs to be designed for the detection of false negative results in the diagnostic assay at commercial level.

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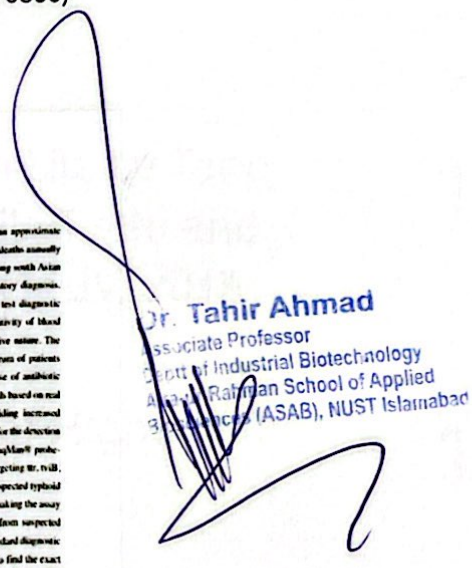

Abstract

Typhoid fever is an important worldwide contributor to illness and death, with an approximate annual prevalence of nearly 9-11 million cases, leading to approximately 110,000 deaths annually worldwide. According to WHO, Pakistan has the highest rate of typhoid fever among south Asian countries with 0.1 million cases annually. Typhoid fever is confirmed by laboratory diagnosis. Despite being recognized for over a century, typhoid fever lacks a definitive test diagnostic biomarker. Blood culture is used as a gold standard for diagnosis, but the sensitivity of blood culture is less than 50%. Bone marrow culture is not used because of the invasive nature. The Widal test quantifies the antibodies produced against flagella and LPS in the serum of patients suspected of having typhoid fever by agglutinating *S. typhi* cells. The earlier use of antibiotic medication can provide a false negative result in the Widal test. Diagnostic methods based on real time PCR have overcome these drawbacks of conventional methods by providing increased sensitivity for the detection of *Salmonella typhi*. Several genes have been targeted for the detection of *Salmonella typhi* in previous studies. This study is focused on developing a TaqMan® probe-based Real-time multiplex PCR Assay for the detection of *Salmonella typhi* by targeting ttr, tviB, and staG genes. The assay was validated with bacterial and blood samples from suspected typhoid patients. All the cultured bacterial samples were positive by the developed Assay making the assay 100% sensitive and specific. In addition, out of 300 blood samples collected from suspected typhoid patients, 48 were positive by developed assay. Using blood culture as a standard diagnostic assay, developed PCR assay showed 100% sensitivity and 95% specificity. But to find the exact sensitivity and specificity, the assay needs to be compared with developed real-time PCR diagnostic kit.

Development of TaqMan® Probe-based RT-qPCR Diagnostic Assay for Rapid Detection of Typhoid Fever

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