THE DEVELOPMENT OF MOLECULAR GENETIC TOOLS FOR DETECTION OF *SALMONELLA* PATHOGEN

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THE DEVELOPMENT OF MOLECULAR GENETIC TOOLS FOR DETECTION OF *SALMONELLA* PATHOGEN

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ABSTRACT

THE DEVELOPMENT OF MOLECULAR GENETIC TOOLS FOR DETECTION OF *SALMONELLA* PATHOGEN

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Although traditional microbiological methods are accepted standard for *Salmonella* detection, they are labor intensive and time consuming. Therefore, for food industry and public health, finding sensitive and rapid methods is required. As a rapid and reliable tool, Real-Time PCR is one of the most common methods in molecular detection and research area.

The aim of the current study is to develop rapid, sensitive and quantitative *Salmonella* detection method using Real-Time PCR technique based on inexpensive, easy to produce, convenient and standardized plasmid based positive control for the first time.

To achieve this, two plasmids were constructed as reference molecules by cloning two most commonly used *Salmonella* specific target regions '*invA* and *ttrRSBC*' into them. Standard curves were constructed for the plasmids and reproducibility, PCR efficiency,

amplification efficiency values were calculated. To illustrate the applicability of the developed method, enriched (as used commonly for *Salmonella* detection with Real-Time PCR) 10^5 to 10^0 CFU/ml level (estimated by standard plate counts before enrichment) *S.* Typhimurium ATCC 14028 cultures were tried to detect and quantify, also compared with traditional culture method. In addition, detection limits of the developed technique were determined by serial dilution of DNA extracted from 10^5 CFU/ml level. The results revealed much faster detection ability of the developed plasmid based *Salmonella* detection method (in comparison to traditional culture method, ISO 6579:2004) allowing quantitative evaluation with perfect reproducibility, sensitivity (except for lower concentrations for *invA* target), detection limit, PCR efficiency, amplification efficiency for both *invA* and *ttrRSBC* targets.

The detection and quantification ability of the method developed by using *S.* Typhimurium ATCC 14028 cultures were tested also with 15 *Salmonella* species using milk as a representative food. The results also revealed much faster (in comparison to traditional culture method, ISO 6579:2004) quantitative detection ability of the developed method.

Thus, the developed method has great potential to be used in food industry for rapid and quantitative *Salmonella* detection.

Keywords: *Salmonella*; Salmonellosis; Milk; *invA*; *ttrRSBC*; Cloning; Real-Time PCR

ÖZ

SALMONELLA PATOJENİNİN BELİRLENMESİ İÇİN MOLEKÜLER GENETİK METOTLARIN GELİŞTİRİLMESİ

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Geleneksel mikrobiyolojik metotlar *Salmonella* belirleniminde standart olarak kabul edilmesine rağmen emek yoğun ve zaman alıcıdır. Bu yüzden, gıda endüstrisi ve halk sağlığı için hassas ve hızlı metotların bulunmasına ihtiyaç vardır. Hızlı ve güvenilir bir cihaz olarak Real-Time PCR, moleküler belirleme ve araştırma alanlarında en yaygın kullanılan metotlardan biridir.

Bu çalışmanın amacı ilk kez ucuz, üretimi kolay, kullanışlı ve standardize plazmid pozitif kontrole dayalı Real-Time PCR tekniğini kullanarak hızlı, hassas ve kantitatif *Salmonella* belirleme metodu geliştirmektir.

Bunu başarmak için, en fazla kullanılan *Salmonella* spesifik hedef bölgeleri '*invA* ve *ttrRSBC*' plazmidlere klonlanarak referans molekülleri olarak iki plazmid dizayn edildi. Plazmidler için standart eğriler oluşturuldu ve tekrarlanabilirlik, PCR verimlilik, amplifikasyon

verimlilik değerleri hesaplandı. Geliştirilen metodun uygulanabilirliğini göstermek için, 10^5-10^0 seviyesinde (standart petri sayımı ile zenginleştirme öncesinde yaklaşık olarak belirlendi) *S.* Typhimurium ATCC 14028 kültürleri tespit edilmeye ve konsantrasyonları belirlenmeye çalışıldı, ayrıca geleneksel kültür metoduyla kıyaslandı. Buna ek olarak, 10^5 kob/ml seviyesinden izole edilen DNA'nın seri dilüsyonu ile geliştirilen tekniğin tespit sınırı belirlendi. Sonuçlar, hem *invA* hemde *ttrRSBC* hedefleri için, plazmid temelli *Salmonella* belirleme metodunun mükemmel tekrarlanabilirliği, hassasiyeti (invA hedefi için düşük konsantrasyonlar hariç), tespit sınırı, PCR ve amplifikasyon verimliliği ile kantitatif değerlendirmeye imkân veren çok daha hızlı belirleme kabiliyetini (ISO 6579:2004 geleneksel kültür metoduna kıyasla) ortaya koymuştur.

S. Typhimurium ATCC 14028 kültürleri ile geliştirilen metodun belirleme ve kantitatif yeterliliği ayrıca 15 farklı *Salmonella* türü ile temsili bir gıda olarak süt kullanılarak test edilmiştir. Bu sonuçlar da geliştirilen metodun çok daha hızlı (ISO 6579:2004 geleneksel kültür metoduna kıyasla) ve kantitatif belirleme kabiliyetini göstermiştir.

Dolayısıyla, geliştirilen metot, hızlı ve kantitatif *Salmonella* belirlenimi için gıda endüstrisinde büyük bir kullanım potansiyeline sahiptir.

Anahtar Kelimeler: *Salmonella*; Salmonellozis; Süt; *invA*; *ttrRSBC*; Klonlama; Real-Time PCR

To My Parents

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INTRODUCTION

1.1 *SALMONELLA* AND SALMONELLOSIS

Salmonella is a genus of gram negative, facultative anaerobic and fermentative, predominantly motile (with peritrichous flagella as seen in Figure 1.1), non-spore forming, short rod shaped (Figure 1.2) bacteria that are indistinguishable from *E. coli* under the microscope or on ordinary nutrient media (Jay et al., 2005; Brands and Alcamo, 2006).

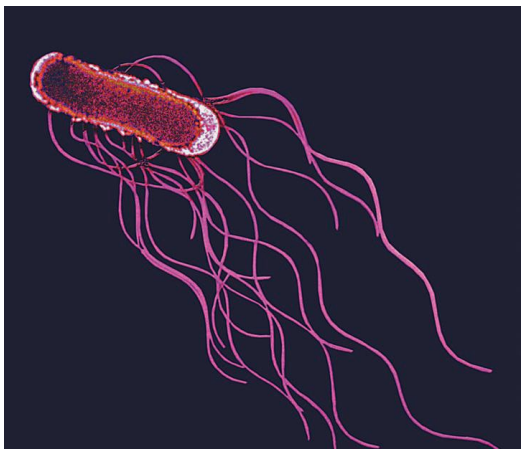


Figure 1.1 Transmission electron micrograph (TEM) of a single *Salmonella* bacterium magnified 13,250 times (Brands and Alcamo, 2006).

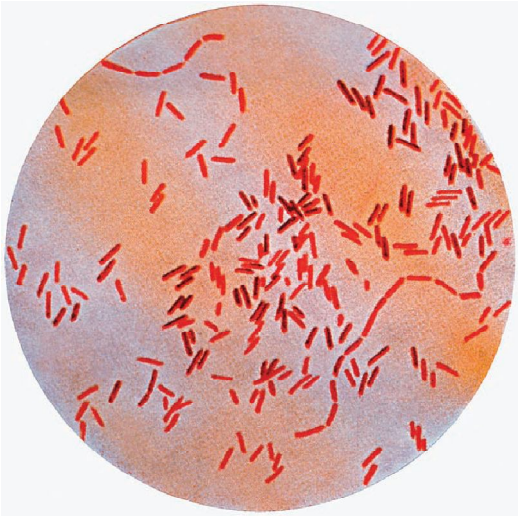


Figure 1.2 The *Salmonella* bacteria shown in this micrograph, stained using the Gram stain procedure, appear as red rods.

The *Salmonella* genus contains two species, enterica and bongori (Brenner et al., 2000). S. enterica is further divided into six subspecies (enterica, salamae, arizonae, diarizonae, houtenae and indica) and contains 2443 serotypes (Mastroeni and Maskell, 2006) out of more than 2,500 *Salmonella* serotypes (serotypes of *Salmonella* are defined based on the antigenic structure of both somatic or cell wall (O) antigens and flagellar (H) antigens) (Chiu et al., 2005; Lim and Thong 2009). Although all members of this genus of gram-negative enteric bacteria are considered to be human pathogens (Jay et al., 2005), most of the salmonellae that cause disease, with some important exceptions, are in the subspecies *Salmonella enterica* subspecies *enterica* (Mastroeni and Maskell, 2006). Salmonellosis caused by *Salmonella* is one of the most important food-borne disease and causes substantial medical and economic burdens worldwide (McClelland et al., 2001; Patel et al., 2006; Verdoy et al., 2012) and the majority of cases of human salmonellosis are due to the consumption of contaminated egg, poultry, pork, beef, milk products,

fruits and vegetables (Icgen et al., 2002; Isiker et al., 2003; Das et al., 2006; Malorny et al., 2008; McCabe et al., 2011).

For epidemiological purposes, the salmonellae can be placed into three groups: 1) Host specific serovars (For example *S*. Typhi, *S*. Paratyphi A, *S*. Paratyphi C for humans; *S*. Gallinarum for fowl; *S*. Abortusovis for sheep). 2) Host-restricted strains associated with one or two closely related host species but may also infrequently cause disease in other hosts (For example *S*. Gallinarum for poultry, *S*. Dublin for cattle and *S*. Choleraesuis for swine but the serovars are potentially capable of infecting humans). 3) Unadapted serovars pathogenic for humans and other animals consist of the ubiquitous *S*. *enterica* serovars, and they usually induce gastroenteritis in a broad range of unrelated host species (For example *S.*Typhimurium and *S.*Enteritidis) (Jay et al., 2005; Mastroeni and Maskell, 2006).

To minimize the risk of *Salmonella* growth, its tolerance to various environmental conditions and optimal growth conditions should be taken into account. The salmonellae are unable to tolerate high temperatures like milk pasteurization temperatures, the pH values below 4.0 and above 9.0, high salt concentrations i.e. above 9%, irradiation at levels 2-5 kGy, water activity (a_w) values below 0.94 (Jay et al., 2005). For optimal growth, *Salmonella* needs neutral pH of 6.6-8.2 and 37 ^{o}C (but has the ability to grow at a wide range of temperatures from 5.3 to 46 ^{o}C) (Jay et al., 2005; Brands and Alcamo, 2006). *Salmonella* also has nutritional requirements for example glucose (a sugar), to grow and divide. If proper temperature, pH, and nutrition are met, *Salmonella* will grow very easily and a human body contains all of these factors (37 ^{o}C, neutral pH and glucose), therefore human body is a good host for *Salmonella* (Brands and Alcamo,

2006). Silva and coworkers (Silva et al., 2009) reported the generation time of *Salmonella* in enteral feeds ranged from 21 to 34.8 min.

Among the gram-negative rods that cause foodborne gastroenteritis, the most important are the members of the genus *Salmonella* (Jay et al, 2005). Figure 1.3 illustrates the route of *Salmonella* ingested through contaminated foods (Brands and Alcamo, 2005) and from the time of ingestion of foods, symptoms usually develop approximately in 12-14 hours and usually persist for 2-3 days (Jay et al., 2005). The bacteria travel to the stomach where the stomach acid does an efficient job of killing the *Salmonella*, but in case of ingestion of bacteria with large initial number some bacteria will still be left alive (Brands and Alcamo, 2005). In stomach, the bacteria can cause gastric problems, Hung and Wang (2004) reported that infection of *Salmonella typhi* produced gastric oxidative stress and hemorrhagic ulcers in rats. The bacteria pass to the intestines from stomach, where they adhere to mucosal cells and then invade the gut epithelium, possibly in the distal ileum (House et al., 2001; Mastroeni and Maskell, 2006). This is such an invasion that once *Salmonella* has entered a cell, the cell dies in about two hours, the cell bursts and all of the *Salmonella* spreads to surrounding cells and the process starts over again (Brands and Alcamo, 2005). The invasion causes typhoid fever include diarrhea and sepsis (results from the spread of bacteria or their products in the bloodstream) (House et al., 2001; Brands and Alcamo, 2005).

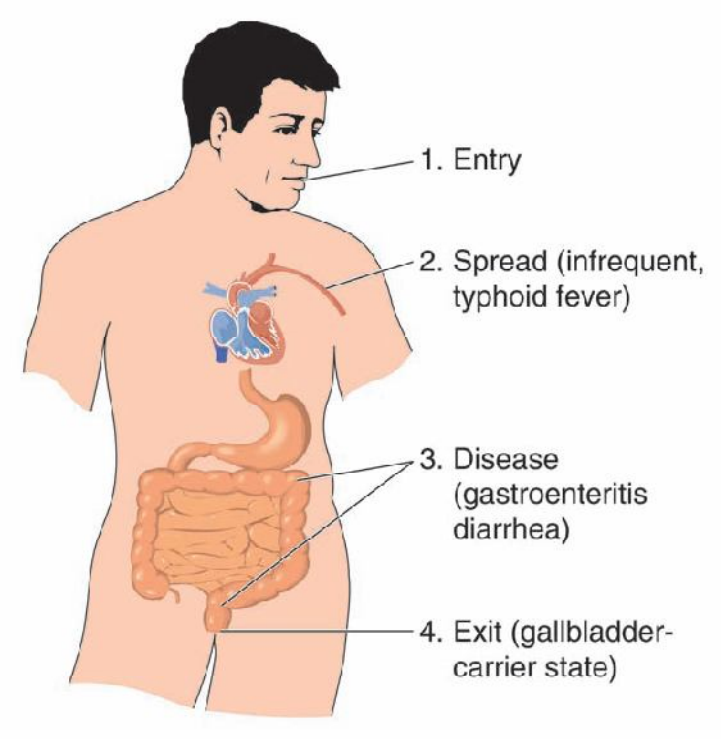


Figure 1.3 The route of *Salmonella* ingested through contaminated foods (Brands and Alcamo, 2005).

Typhoid fever a systemic infection caused by *Salmonella enteric* serotype typhi (*S. typhi*) and a very similar but often less severe disease -paratyphoid A, B and C- is caused by *S. paratyphi* A, B and C (Bhan et al., 2005; Parry, 2005). Nearly 22 million cases of typhoid occur each year with 200,000 deaths globally (Parry, 2005), on the other hand the global incidence of paratyphoid is nearly ¼ of global typhoid incidence (Bhan et al., 2005). The proposed mechanism of typhoid fever is illustrated in Figure 1.4.

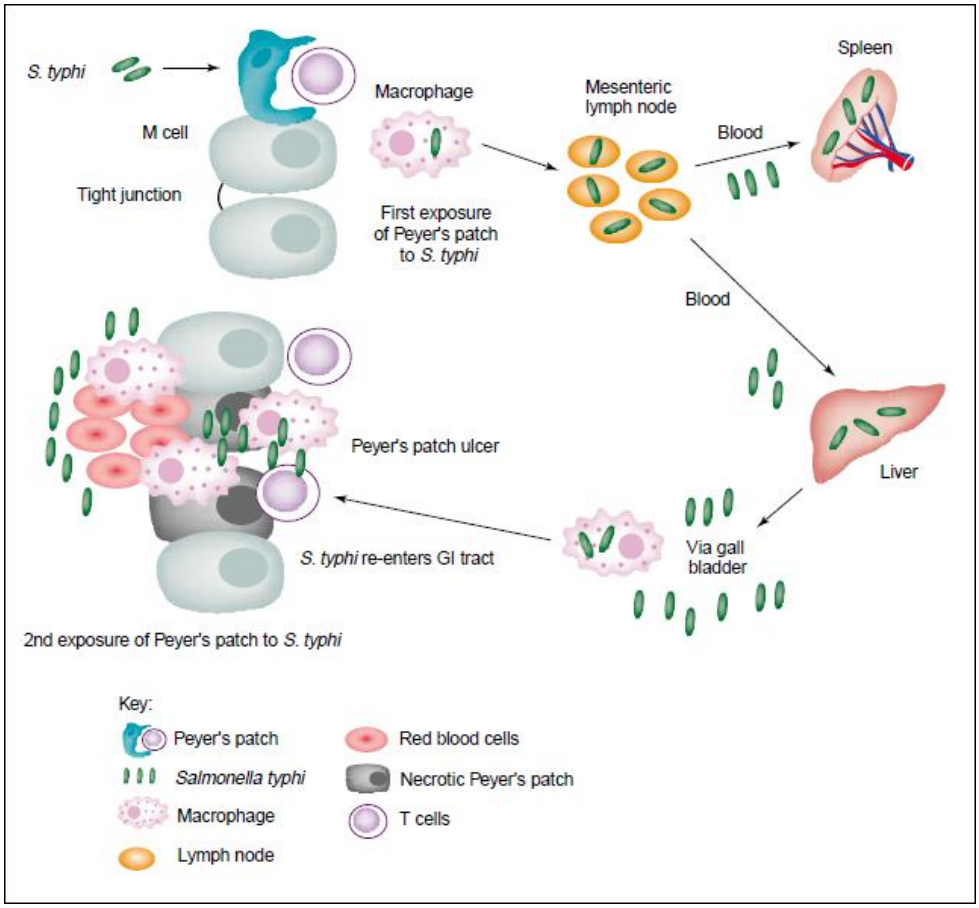


Figure 1.4 Theoretical mechanism of *Salmonella typhi* infection proposed by Everest and coworkers (Everest et al., 2001).

Bacteria infect via the Peyer's patches of the small intestine (Everest et al., 2001) and are transported by the lymphatics to mesenteric lymph nodes (Everest et al., 2001; Parry, 2005). The bacteria spread to the bone marrow (not shown in Figure 1.4), spleen and liver where they multiply (Everest et al., 2001) and secondary invasion occurs, bacteria reenter the intestinal tract via the gall bladder, exposing the Peyer's patches to bacteria second time (Everest et al., 2001). A strong inflammatory response occurs in the ileal Peyer's patches

(Parry, 2005) resulting in necrosis, ulceration, bleeding and in extreme cases full thickness perforation (Everest et al., 2001; Parry, 2005).

When a cell dies due to the *Salmonella* invasion, it releases chemical signals activating immune reactions (Interleukins and cytokines in Figure 1.5) to invading bacteria; to avoid being destroyed the bacteria release a chemical that counteracts and disables macrophages become part of the fluid flowing into the intestinal tract and finally diarrhea occurs (Brands and Alcamo, 2005).

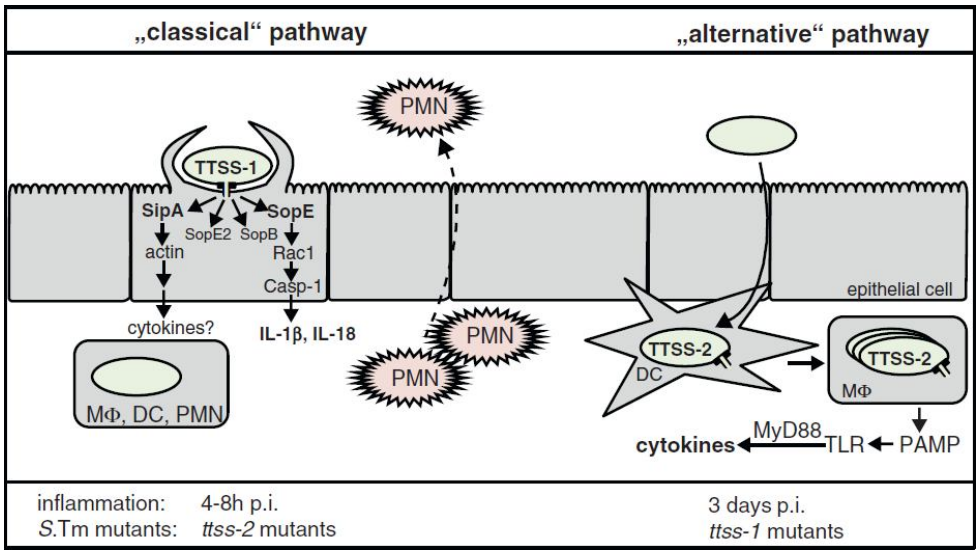


Figure 1.5 Two different mechanisms of *Salmonella* induced gut inflammation proposed by Kaiser and Hardt (2011). a) direct triggering of pro-inflammatroy responses by injection of bacterial virulence factors; b) by entering the mucosa via dendritic cells (CX3CR1+CD11c+ phagocytes), TTSS-2 dependent replication in the mucosal tissue and triggering of innate immune responses. Casp-1: Caspase 1. DC: Dendritic Cells.IL: Interleukin. MyD88: Myeloid differentiation primary response gene. PAMP: Pathogen-associated molecular pattern. PMN: Polymorphonuclear leukocytes. Rac1: A

member of the Rho GTPase family. SipA, SopB, SopE and SopE: Four major effector proteins to invade epithelial cells. TLR: Toll-like Receptor. TTSS: Type three secretion system.

The typhoid may also include sepsis results from the spread of bacteria or their products in the bloodstream as clarified in Figure 1.6. Most cases of Salmonellosis are self-limiting usually associated with non-typhoidal *Salmonella* (Brands and Alcamo, 2005; Mastroeni and Maskell, 2006), so most persons infected by *Salmonella* recover without treatment and the illness usually lasts 4 to 7 days (Brands and Alcamo, 2005; Castillo et al., 2012). However, in severe cases the patients need to be hospitalized for example the patients with enteric fever illustrating sepsis formation (see the Figure 1.6), may die due to the spread of *Salmonella* to other body sites through bloodstream if the patients are not treated promptly with antibiotics (Castillo et al., 2012). Thus, effective antibiotic therapy mostly including ampicillin, gentamicin, trimethoprim/sulfamethoxazole, ciprofloxacin (Brands and Alcamo, 2005) reduces mortality and complications and shortens the duration of the ilness so that successfully treated patients typically respond to antibiotics within 3 to 5 days with resolution of fever and other symptoms (Mastroeni and Maskell, 2006). Also rehydration therapy involving ingesting fluids containing glucose and electrolytes may be required to replace fluids that are lost from diarrhea (Brands and Alcamo, 2005; Mastroeni and Maskell, 2006).

However, overuse of antibiotics causes a longterm problem called antibiotic resistance (the ability of the bacteria to destroy or remain unaffected by a drug used against it) result from mutation or carrying a resistance gene by three different mechanisms as clarified in Figure 1.7 (Brands and Alcamo, 2005).

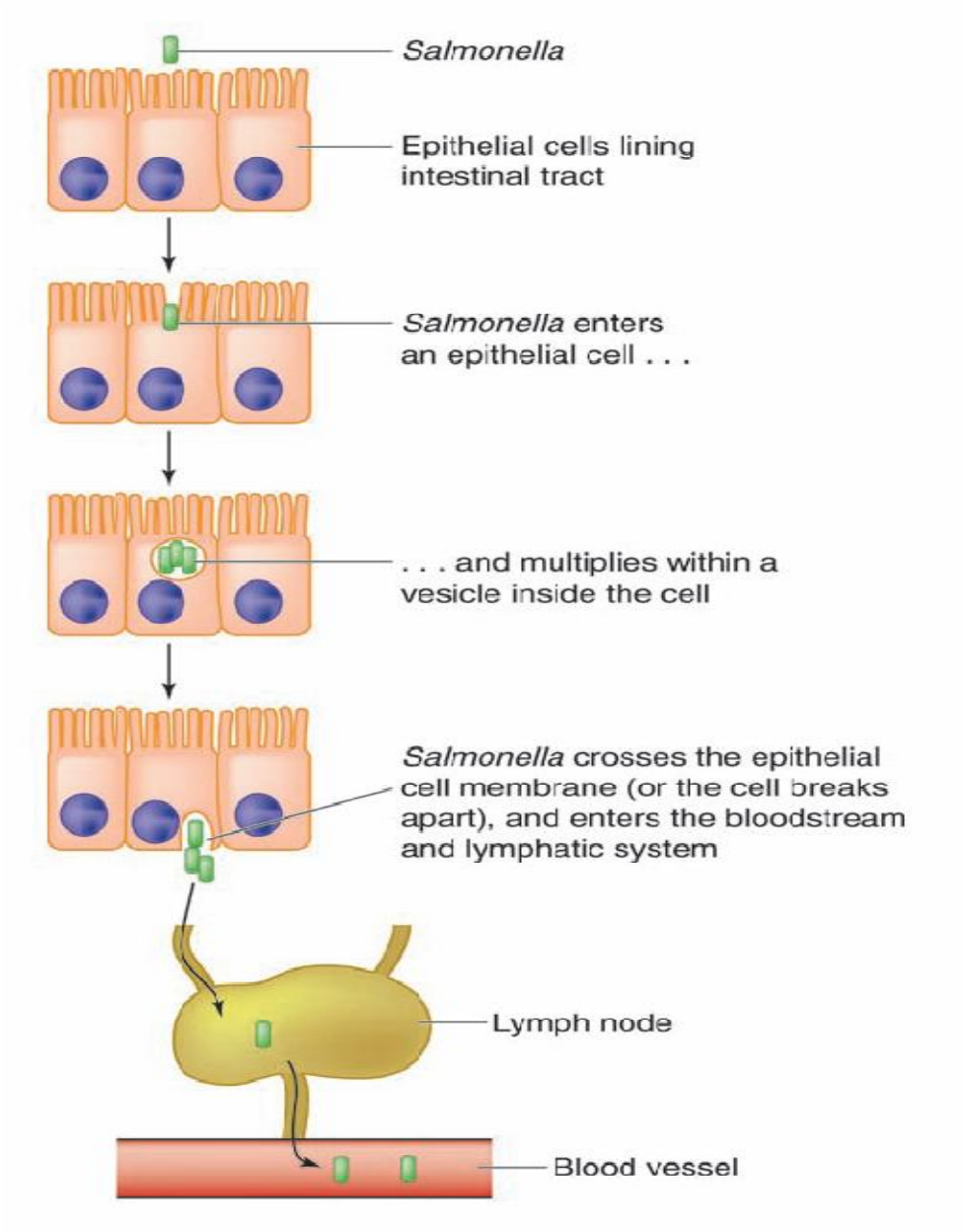


Figure 1.6 Formation of sepsis caused by *Slmonella* infection start with the attachment to the microvilli and end up in a blood vessel (Brands and Acamo, 2005).

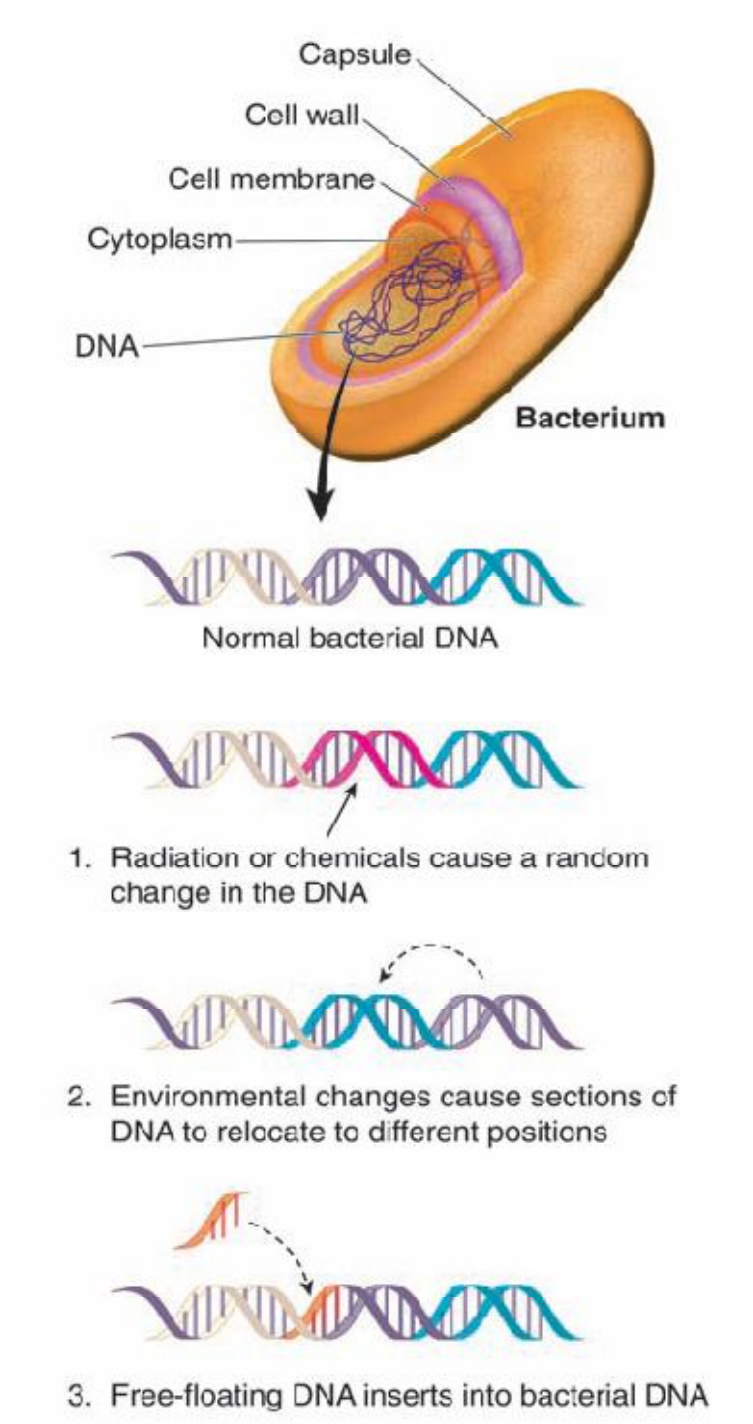


Figure 1.7 Three mechanisms that the bacteria can acquire antibiotic resistance (Brands and Acamo, 2005).

1.2 *SALMONELLA* DETECTION AND REAL-TIME PCR

The detection of pathogenic bacteria is key to the prevention and identification of problems related to health and safety especially for food industry. Therefore, the industry has the biggest portion related to the relative number of works appeared in the literature (Source: ISI Web of Science. ca. 2500 articles found on pathogen detection over the last 20 years) on detection of pathogenic bacteria as illustrated in Figure 1.8 (Lazcka et al., 2007).

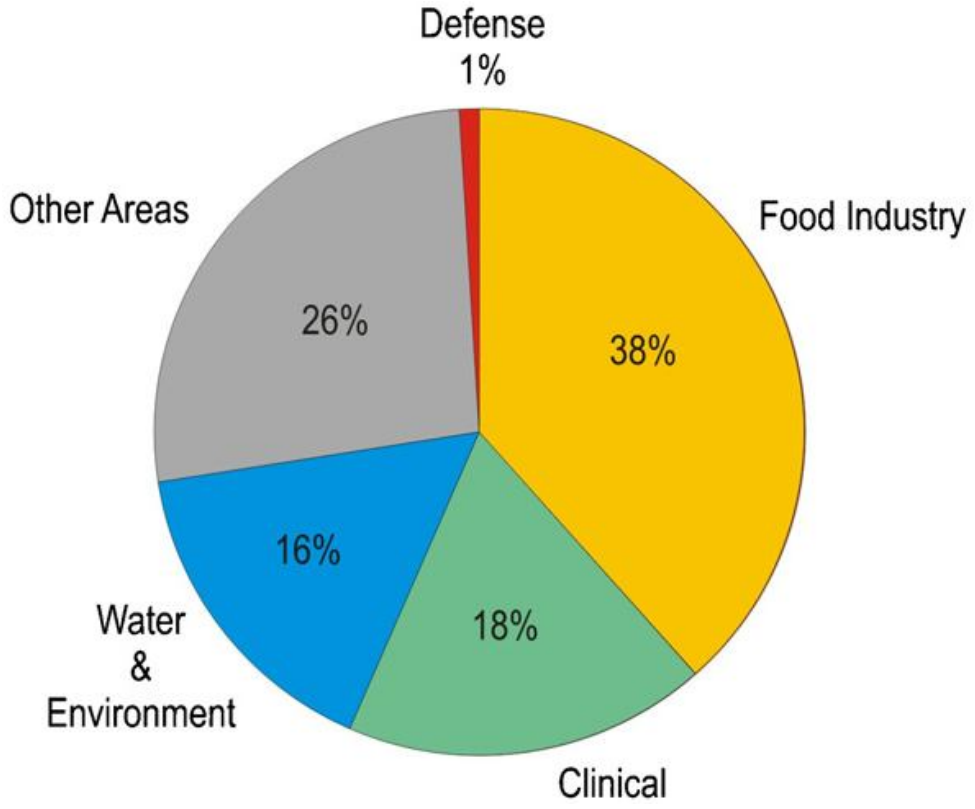


Figure 1.8 Areas of interest for pathogen detection (Lazcka et al., 2007).

Among the gram-negative rods that cause foodborne gastroenteritis, the most important are the members of the genus *Salmonella* (Jay et al, 2005). Parallel to *Salmonella*'s bad reputation, it has the biggest portion related to the relative number of works appeared in the literature (Source: ISI Web of Science. ca. 2500 articles found on pathogen detection over the last 20 years) on rapid detection of pathogenic bacteria as illustrated in Figure 1.9 (Lazcka et al., 2007).

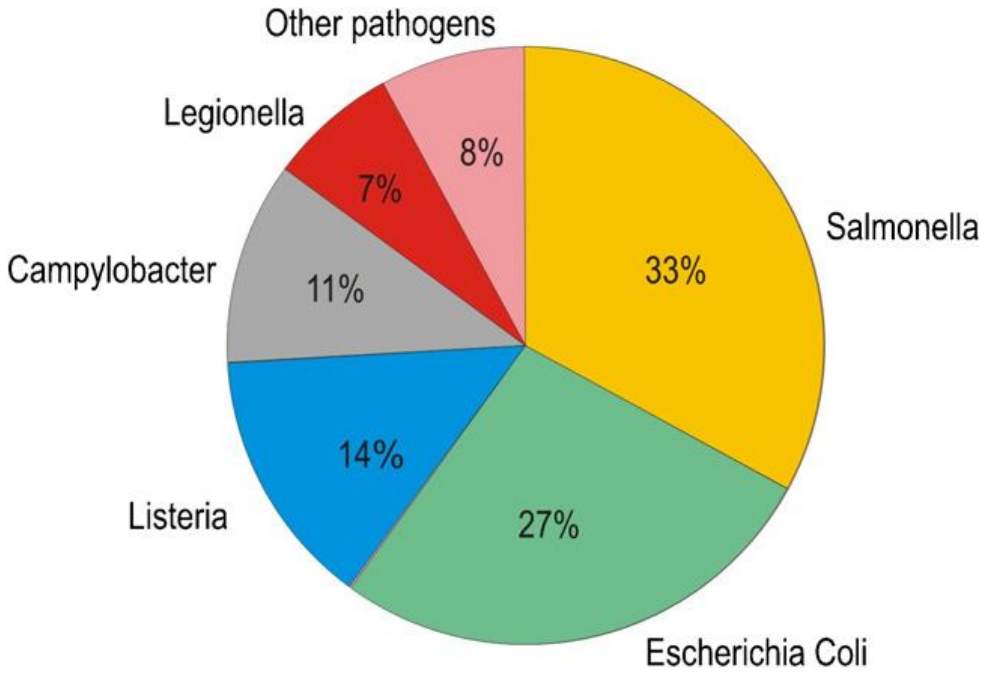


Figure 1.9 Reported detection methods deal with various microorganisms (Lazcka et al., 2007).

Although traditional microbiological methods are accepted standard for *Salmonella* detection (ISO 6579:2004), they are labor intensive and time consuming. These methods consist of series of steps including serological and biochemical tests after enrichment, selective enrichment and selective solid medium. Up to 7 days is required for

intense and detailed analysis (Patel et al., 2006; Hagren et al., 2008; McCabe et al., 2011). Consequently, fast and sensitive detection of pathogen bacteria in food sector is accepted as quality assurance therefore, many researchers have studied to develop rapid methods based mainly on PCR, ELISA, biosensors, electrophoresis techniques and the most popular method is, by far, those based on PCR (Lazcka et al., 2007).

Polymerase chain reaction accelerated pathogen detection substantially, and in some cases it replaced traditional methods for bacterial quantification. Although classical PCR is special for sensitive and optimized conditions, it requires agarose gel electrophoresis study that is not required for evolved form of classical PCR, Real-Time PCR. In addition, latter technique is more sensitive, allows detection of DNA or RNA while reaction is occurring. As a rapid and reliable tool, Real-Time PCR is one of the most common methods in molecular detection and research area including specific detection of *Salmonella* (Malorny et al., 2004).

1.2.1 Real-Time PCR

General quantitative Real-Time PCR detection steps (sample collection, nucleic acid isolation, reverse transcription, and occasionally DNase treatment) were illustrated in Figure 1.10. If the nucleic acid is RNA, before gene expression measurement by Real-Time PCR, mRNA in the sample must be copied into complementary DNA (cDNA) by reverse transcription which is critical step for sensitive and accurate quantification because the amount of cDNA produced by the reverse transcriptase must accurately represent RNA input amounts (Bustin, 2002; Stahlberg et al., 2005). DNA contamination will

result in inaccurate quantification and there is a possibility of RNA degradation in long term storage when treated with DNase, therefore DNase should be added only to the sample to be quantitated (Bustin, 2002).

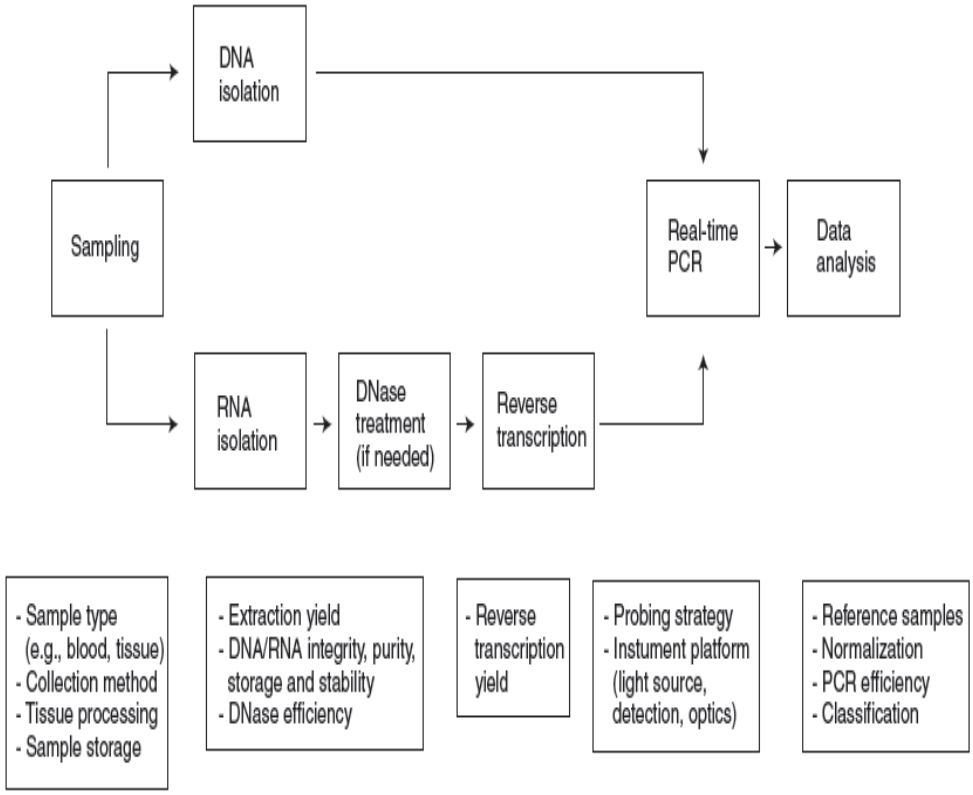


Figure 1.10 Steps in nucleic acid quantification by Real-Time PCR and potential sources of technical variability (Stahlberg et al., 2005).

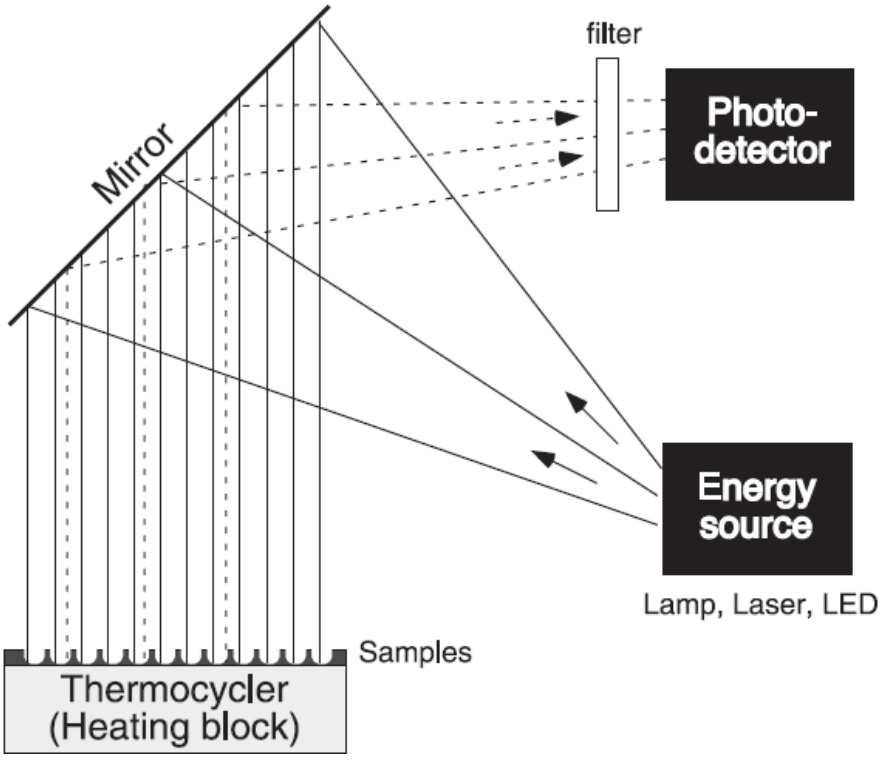


Figure 1.11 Real-Time PCR hardware. LED: Light-emitting diode (Valasek and Repa, 2005).

After DNA isolation or obtaining DNA from mRNA with reverse transcription, amplification curves are obtained for each DNA using Real-Time PCR hardware illustrated in Figure 1.11. DNA in required solution called as master mix is placed in a thermocycler, which controls the temperatures required for each cycle of PCR. The samples are exposed to excitation energy, and the resulting fluorescence is measured by a photodetector with each cycle (Valasek and Repa, 2005).

Master mix includes buffer, two oligonucleotide primers that flank the DNA sequence to be amplified, dNTPs which are the four nucleotide triphosphates, a thermostable polymerase, MgCl2 and also may

include ROX as a passive reference dye, and UNG with dUTP to prevent PCR product contamination (Keer and Birch, 2008; Kubista et al., 2006). The presence of $MgCl_2$ enables efficient hybridization during the rapid cycling conditions used in many instruments, also $MgCl_2$ has impact on both the specificity and yield of PCR (Keer and Birch, 2008). DNA polymerase amplifies specific pieces of DNA using short, sequence-specific oligonucleotides added to the reaction to act as primers and the enzyme has two basic capabilities that make it useful for PCR: 1) it can generate new strands of DNA using a DNA template and primers, and 2) it is resistant to high PCR temperatures e.g. 95 ^{o}C (Valasek and Repa, 2005).

The reaction is performed by temperature cycling includes denaturation, annealing and extension steps as illustrated in Figure 1.12. High temperatures (approximately 95 ^{o}C) are used to separate (melt) the strands of the double helical DNA, then the mixture is cooled to let forward and reverse primers anneal to the template (the annealing temperature depends on the primers, it should be a few degrees below the melting temperature of the primers to form stable complexes with the targeted sequences but not with any other sequences), and finally an optimal temperature for the polymerase is applied to extend the primers by incorporating the dNTPs and thereby first cycle is completed with two double stranded DNA (Kubista et al., 2006; Valasek and Repa, 2005).

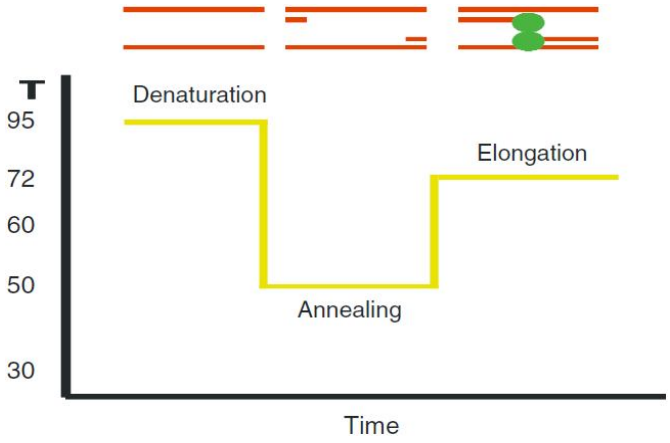


Figure 1.12 The PCR temperature cycle (Kubista et al., 2006).

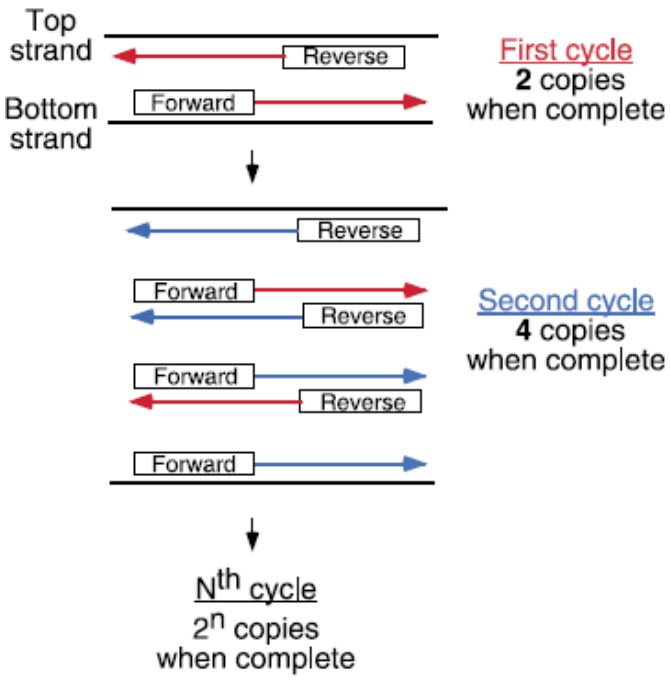


Figure 1.13 Illustration of DNA elongation with PCR cycles (Valasek and Repa, 2005).

The PCR cycle is repeated numerous times, and if the reaction Works with perfect efficiency, 2^n copies of the first DNA fragment are

obtained at the end of Nth cycle as illustated in Figure 1.13 (Valasek and Repa, 2005).

As mentioned above the samples are exposed to excitation energy, the resulting fluorescence is measured by a photodetector with each cycle (Valasek and Repa, 2005). Currently a range of detection strategies are available for Real-Time PCR (Giulietti et al., 2001). Fluorescence signals proportional to the amount of PCR product can be generated by fluorescent dyes like SYBR green I that are specific for double-stranded DNA or by sequence-specific fluorescent oligonucleotides like TaqMan probes, molecular beacons, hybridization probes and scorpion probes (Wilhelm and Pingoud, 2003).

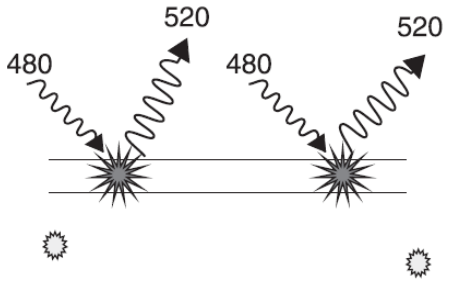


Figure 1.14 Detection based on SYBR green I (Valasek and Repa, 2005).

SYBR green I fluoresces (absorbing light of 480-nm wavelength and emitting light of 520-nm wavelength) when the dye is bound to double stranded DNA (Valasek and Repa, 2005) and this method does not require design a third, modified oligonucleotide or hybridization probe (Ginzinger, 2002).

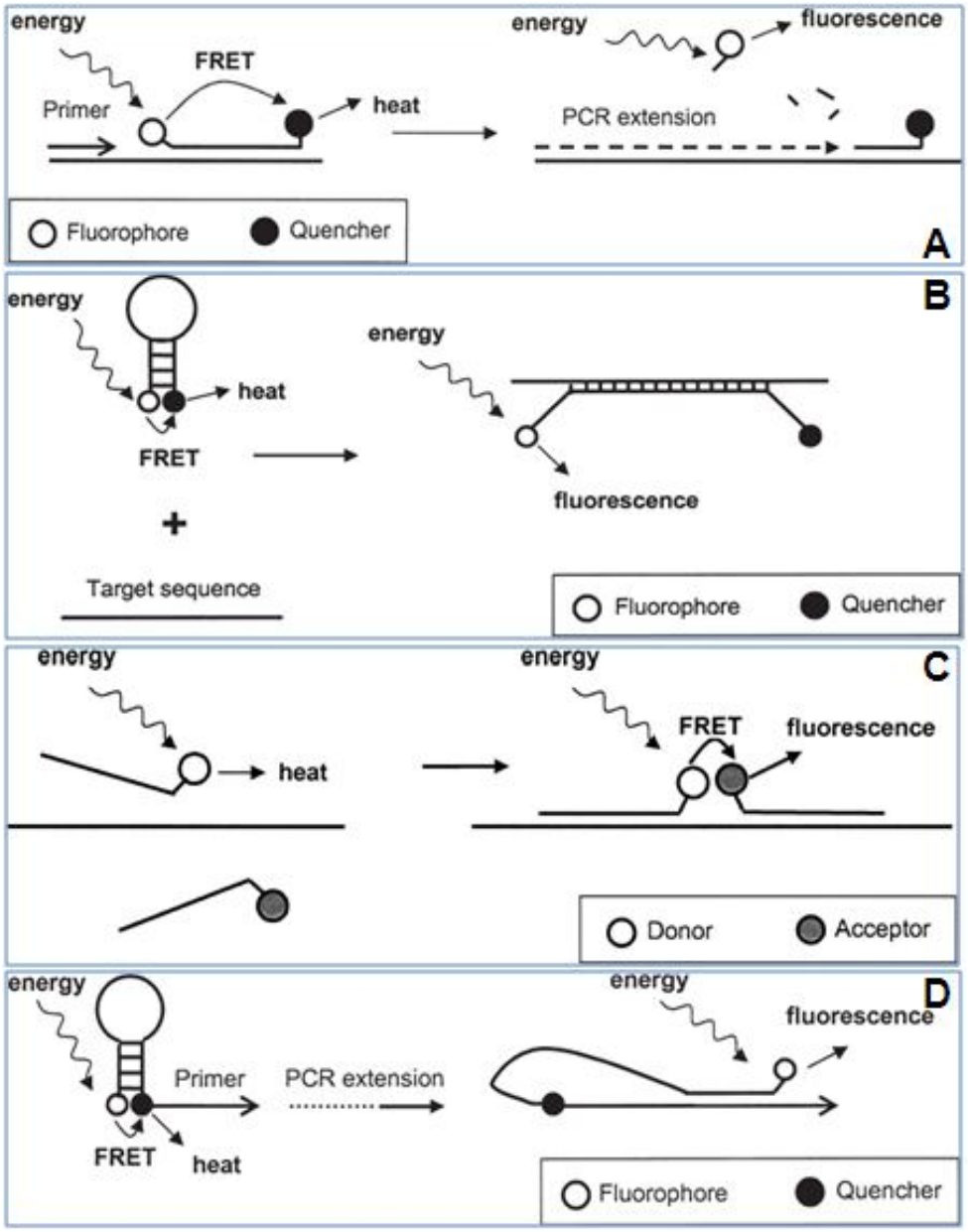


Figure 1.15 Sequence specific detection methods: A) TaqMan Probes B) Molecular Beacons C) Hybridization Probes D) Scorpion Probe FRET: Forster Resonance Energy Transfer (Keer and Birch, 2008).

Although SYBR green I is the cheapest detection method, this detection is nonspesific that is, it can not discriminate between real

template and artifact bands unlike that of sequence specific fluorescent oligonucleotide probes like TaqMan probes or molecular beacons (Giulietti et al., 2001; Ginzinger, 2002; Wilhelm and Pingoud, 2003).

Sequence specific detection formats use compatible fluorophores (reporter fluorophore and quencher fluorophore) (Valasek and Repa, 2005) rely on the transfer of energy (Forster Resonance Energy Transfer 'FRET') between fluorescent reporter and quencher molecules to generate specific signals (Keer and Birch, 2008). Sequence specific detection methods are illustrated in Figure 1.15.

Computer software constructs amplification plot using the fluorescence emission data collected during the PCR amplification (Giulietti et al., 2001). Figure 1.16 illustrates the typical Real-Time PCR amplification plot.

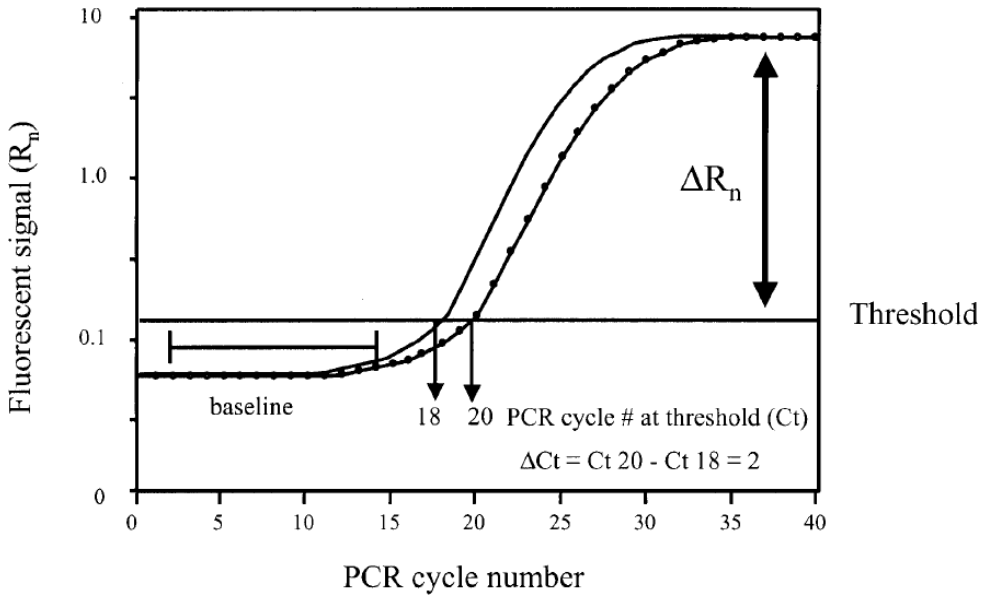


Figure 1.16 A hypothetical Real-Time PCR amplification plot (Ginzinger, 2002).

In amplification plot, the baseline is defined as the PCR cycles in which a signal is beneath the limits of detection of the instrument; a fluorescent signal detected above the threshold is considered a real signal used to define the threshold cycle (Ct) for a sample (Ginzinger, 2002).

The Ct value is inversely proportional to the amount of specific nucleic acid sequence in the original sample (Valasek and Repa, 2005) as illustrated in Figure 1.17 and as a general rule, considering optimal reaction efficiency, 10 fold dilution increases the Ct value in 3.3 cycles. In Real-Time PCR amplification plot final fluorescence is not always proportional to the initial amount of template DNA, while the Ct values are always inversely proportional to the initial number of target genes in the sample (Microbial, http://www.microbial-

systems.com/web/docs/Results_interpretation_guide_ENGv2.pdf, visited on 05.02.2012).

In reality, Real-Time PCR reactions do not maintain perfect efficiency because reactants within the PCR are consumed after many cycles, and the reaction will reach a plateau as can be seen in Figure 1.16 and 1.17 (Valasek and Repa, 2005).

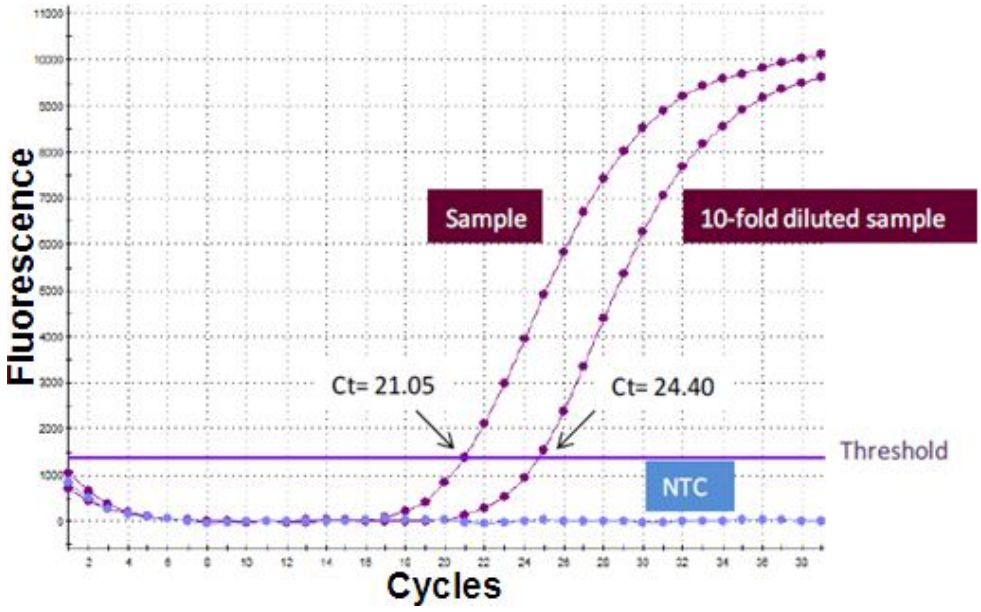


Figure 1.17 Diluted sample crosses the threshold line later than more concentrated sample (NTC is non template control contains no initial template DNA to amplify) (Microbial, http://www.microbial-systems.com/web/docs/Results_interpretation_guide_ENGv2.pdf, visited on 05.02.2012).

For many applications for example scope of this study –quantitative *Salmonella* detection in food samples- a quantitative result is required

(Keer and Birch, 2008). Basic princple of quantitative Real-Time PCR is illustrated in Figure 1.18.

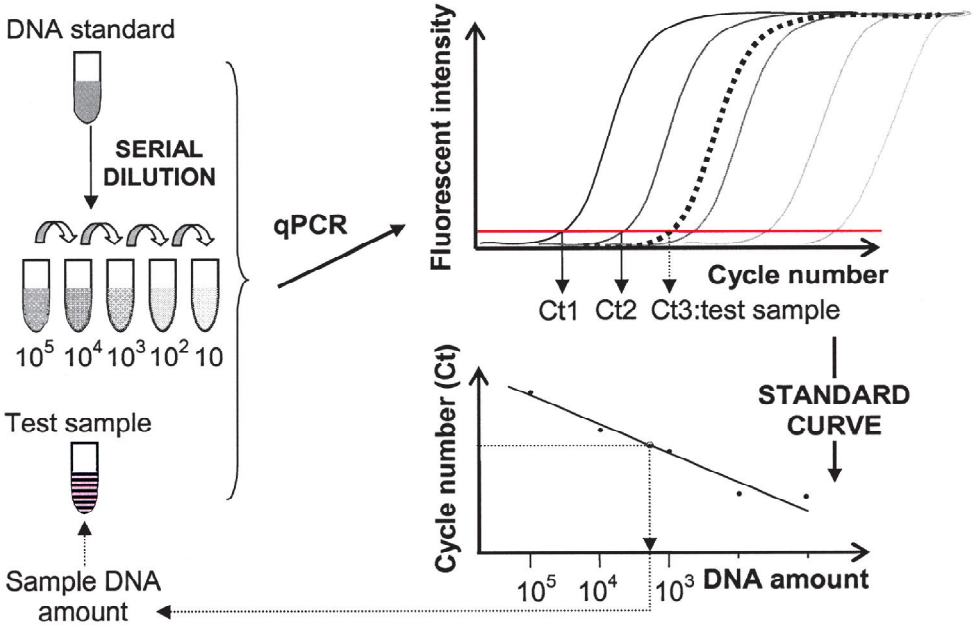


Figure 1.18 Basis of Real-Time PCR using a quantitative standard (Keer and Birch, 2008).

A standard curve is constructed using a range of samples of known target content (serial dilution of known target illustrated in the figure) and the test sample concentration is interpolated from the graph (Keer and Birch, 2008).

Due to the above mentioned superior properties of the technique, detection of *Salmonella* was investigated by many researchers using Real-Time PCR with SYBR green probe (Fukushima et al., 2007; Arrach et al., 2008; Chen et al., 2011; Donhauser et al., 2011) or Taqman probe (Malorny et al., 2004; Josefsen et al, 2007; O'regan et

al., 2008; Woods et al., 2008; Gonzales-Escalona et al., 2009; Lofstrom et al., 2009; Hyeon et al., 2010). In addition, various targets characteristic for *Salmonella* were used for detection of this pathogen: *oriC* (replication origin encoding gene) (Woods et al., 2008), *ompC* (major outer membrane protein gene) (Amavist et al., 2001), *invA* (*Salmonella* invasion protein gene) (Gonzales-Escalona et al., 2009; Chen et al., 2011); *stn* (enterotoxin gene) (Moore and Feist, 2007), *hilA* (type III secretionsystemregulation gene) (McCabe et al., 2011; Donhauser et al., 2011); *iroB* (iron-responsive gene) (Murphy et al., 2007); *aceK* (isocitrate dehydrogenase kinase/phosphatase gene) (O'regan et al., 2008) and *ttrRSBC* locus (on which *ttrA*, *ttrB* and *ttrC* tetrathionate reductase structural genes are located) (Malorny et al., 2004; Josefsen et al, 2007; Lofstrom et al., 2009; Hyeon et al., 2010).

Although wide variety of mentioned studies using different primers and probes reported promising results illustrating Real-Time PCR Technique's potential to replace labor intensive and time consuming traditional methods for *Salmonella* detection (ISO 6579:2004), still there is no internationally accepted standardized protocol for Real-Time PCR based *Salmonella* detection.

1.2.2 Quantification with Plasmid Based Standard

In Real-Time PCR technique, using DNA of known concentrations to create a calibration curve, the precise copy number of a specific nucleic acid sequence can be quantified (Lin et al., 2011). Among DNA standards (plasmid DNA, a PCR amplicon, synthesized oligonucleotide, genomic DNA, or cDNA), plasmid DNA is the most attractive one because it is relatively easy to construct and handle, relatively stable when frozen and can be produced in large quantities,

therefore plasmids can be used as a standard to produce a calibration curve (Burns et al., 2006; Lin et al., 2011; Ustek et al., 2008;). Burns and collaborators (Burns et al., 2006) reported that plasmid calibrants gave equal or better performance characteristics in terms of precision and closeness to the expected value, than their genomic equivalents for the quantification of genetically modified (GM) content of food in experiments performed at three different laboratories within Europe. Thus, plasmid calibrants gained popularity and to date, more than 20 plasmid calibrants have been successfully constructed and used for the detection and quantification of several GM foods (Meng et al., 2012). However, although DNA plasmids as calibrants is becoming essential for the practical quantification of genetically modified organisms (GMOs) (Meng et al., 2012), this method was not applied to the one of the most important issue of food industry –pathogen detection and quantification.

1.3 SALMONELLOSIS AND MILK

Table 1.1 illustrates the average chemical composition of cow milk similar in composition to that of goats and sheep (Jay et al., 2005).

Table 1.1 Average chemical composition (%) of whole bovine milk (Jay et al., 2005).

Water	87.0
Protein	3.5
Fat	3.9
Carbohydrate	4.9
Ash	0.7

Milk protein consists mainly (80-85 %) of casein in α, β, etc. forms and the remaining proteins are found in whey and they include serum albumin, immunoglobulins, α-lactalbumin, etc. Milk carbohydrate is principally lactose and smaller quantities of glucose and citric acid exist also. The fat consists mainly of triglycerides (C14, C16, C18, and C18:1 fatty acids) and it includes smaller quantities of diglycerides and phospholipids. The ash content consists of a relatively high level of Ca^{2+} and a lower level of Fe^{2+}. In addition to nutritious chemical composition, the pH of the milk is around 6.6 and therefore, milk is an ideal medium for heterothrophic microorganisms (Jay et al., 2005).

The major current milkborne bacterial pathogens are *Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella* spp., *Escherichia coli* O157:H7, and *Campylobacter jejuni* and among them *Salmonella* species are perhaps the most frequent pathogens associated with milk and dairy products (Fuquay et al., 2011). Many raw foods of animal origin are frequently contaminated (Brands and Alcamo, 2005), therefore consumption of raw milk is considered a risk factor for enteric infections including salmonellosis (Fuquay et al., 2011), but fortunately, thorough cooking kills *Salmonella* (Brands and Alcamo, 2005). From 1998 to 2005, USA Centers for Disease Control and Prevention identified 45 outbreaks of milkborne diseases result from unpasteurized milk, accounting for 1.007 illnesses, 104 hospitalizations, and two deaths (Omiccioli et al., 2009). Standard pasteurization destroys expected levels of salmonellae (e.g.,<100 cfu ml-1) in milk with a wide margin of safety, however there have been some outbreaks of salmonellosis caused by pasteurized milk, primarily due to inadequate pasteurization and post-processing contamination (Fuquay et al., 2011).

As a rapid and sensitive method for the detection and identification of bacteria, increasingly culture-independent DNA-based technologies are being employed to provide an accurate assessment of milk bacterial composition including *Salmonella* (Quigley et al., In Press). Although extracting DNA from milk in sufficient concentration and purity is of crucial importance due to the fact that milk contains PCR inhibitors such as fats, proteins and calcium (See the Table 1.1 above) causing DNA amplification problems, previous studies have shown the applicability of Real-Time PCR technique for *Salmonella* detection (Omiccioli et al., 2009; Quigley et al., In Press).

1.4 SCOPE AND THE AIM OF THE STUDY

Salmonellosis caused by *Salmonella* is one of the most important food-borne disease and causes substantial medical and economic burdens worldwide (McClelland et al., 2001; Patel et al., 2006; Verdoy et al., 2012) and the majority of cases of human salmonellosis are due to the consumption of contaminated egg, poultry, pork, beef, milk products, fruits and vegetables (Icgen et al., 2002; Isiker et al., 2003; Das et al., 2006; Malorny et al., 2008; McCabe et al., 2011). Reliable intervention methods for inactivating *Salmonella* in food are still being researched and detection systems play an important role in reducing the dissemination of contaminated products. Therefore, for food industry and public health, finding sensitive and rapid methods to detect *Salmonella* is required (Miller et al., 2011).

Although traditional microbiological methods are accepted standard for *Salmonella* detection (ISO 6579:2004), they are labor intensive and time consuming. These methods consist of series of steps including serological and biochemical tests after enrichment, selective

enrichment and selective solid medium. Up to 7 days is required for intense and detailed analysis (Patel et al., 2006; Hagren et al., 2008; McCabe et al., 2011). Consequently, fast and sensitive detection of pathogen bacteria in food sector is accepted as quality assurance and nucleic acid based techniques have been developed for application (O'regan et al., 2008).

Polymerase chain reaction accelerated pathogen detection substantially, and in some cases it replaced traditional methods for bacterial quantification. Although classical PCR is special for sensitive and optimized conditions, it requires agarose gel electrophoresis study that is not required for evolved form of classical PCR, Real-Time PCR. In addition, latter technique is more sensitive, allows detection of DNA or RNA while reaction is occurring. As a rapid and reliable tool, Real-Time PCR is one of the most common methods in molecular detection and research area including specific detection of *Salmonella* (Malorny et al., 2004).

Due to the above mentioned superior properties of the technique, detection of *Salmonella* was investigated by many researchers using Real-Time PCR with SYBR green probe (Fukushima et al., 2007; Arrach et al., 2008; Chen et al., 2011; Donhauser et al., 2011) or Taqman probe (Malorny et al., 2004; Josefsen et al, 2007; O'regan et al., 2008; Woods et al., 2008; Gonzales-Escalona et al., 2009; Lofstrom et al., 2009; Hyeon et al., 2010). In addition, various targets characteristic for *Salmonella* were used for detection of this pathogen: *oriC* (replication origin encoding gene) (Woods et al., 2008), *ompC* (major outer membrane protein gene) (Amavist et al., 2001), *invA* (*Salmonella* invasion protein gene) (Gonzales-Escalona et al., 2009; Chen et al., 2011); *stn* (enterotoxin gene) (Moore and Feist, 2007), *hilA* (type III secretionsystemregulation gene) (McCabe et al., 2011;

Donhauser et al., 2011); *iroB* (iron-responsive gene) (Murphy et al., 2007); *aceK* (isocitrate dehydrogenase kinase/phosphatase gene) (O'regan et al., 2008) and *ttrRSBC* locus (on which *ttrA*, *ttrB* and *ttrC* tetrathionate reductase structural genes are located) (Malorny et al., 2004; Josefsen et al, 2007; Lofstrom et al., 2009; Hyeon et al., 2010).

Although wide variety of mentioned studies using different primers and probes reported promising results illustrating Real-Time PCR Technique's potential to replace labor intensive and time consuming traditional methods for *Salmonella* detection (ISO 6579:2004), still there is no internationally accepted standardized protocol for Real-Time PCR based *Salmonella* detection.

In Real-Time PCR technique, using DNA of known concentrations to create a calibration curve, the precise copy number of a specific nucleic acid sequence can be quantified (Lin et al., 2011). Among DNA standards (plasmid DNA, a PCR amplicon, synthesized oligonucleotide, genomic DNA, or cDNA), plasmid DNA is the most attractive one because it is relatively easy to construct and handle, relatively stable when frozen and can be produced in large quantities, therefore plasmids can be used as a standard to produce a calibration curve (Burns et al., 2006; Ustek et al., 2008; Lin et al., 2011). Burns and collaborators (Burns et al., 2006) reported that plasmid calibrants gave equal or better performance characteristics in terms of precision and closeness to the expected value, than their genomic equivalents for the quantification of genetically modified (GM) content of food in experiments performed at three different laboratories within Europe. Thus, plasmid calibrants gained popularity and to date, more than 20 plasmid calibrants have been successfully constructed and used for the detection and quantificaion of several GM foods (Meng et al., 2012). However, although DNA plasmids as calibrants is becoming

essential for the practical quantification of genetically modified organisms (GMOs) (Meng et al., 2012), this method was not applied to the one of the most important issue of food industry –pathogen detection and quantification.

In the light of promising results of the studies on GMOs (Burns et al., 2006; Lin et al., 2011; Meng et al., 2012) plasmid as a inexpensive, easy to produce, convenient, standardized positive control will contribute to the reliability of Real-Time PCR based *Salmonella* detection and quantification. For this purpose, two plasmids were constructed as reference molecules by cloning two most commonly used *Salmonella* specific target regions '*invA* and *ttrRSBC*' into them to use as standard positive controls for the detection and quantification of *Salmonella*. In this study, the cloned *Salmonella* specific gene regions is reported for the first time as a standard positive control to check Real-Time PCR efficiency, and as a standard positive marker for the quantification in addition to detection of the pathogen.

Many raw foods of animal origin are frequently contaminated (Brands and Alcamo, 2005), therefore consumption of raw milk is considered a risk factor for enteric infections including salmonellosis (Fuquay et al., 2011). From 1998 to 2005, USA Centers for Disease Control and Prevention identified 45 outbreaks of milkborne diseases result from unpasteurized milk, accounting for 1.007 illnesses, 104 hospitalizations, and two deaths (Omiccioli et al., 2009). Standard pasteurization destroys expected levels of salmonellae (e.g.,<100 cfu ml-1) in milk with a wide margin of safety, however there have been some outbreaks of salmonellosis caused by pasteurized milk, primarily due to inadequate pasteurization and post-processing contamination (Fuquay et al., 2011). Salmonellae can enter the food chain at every stage, and therefore occurence of salmonellosis is closely related to

food-processing conditions (Malorny et al., 2008). For this reason, quantitative and cost effective methods that can enumerate low levels of *Salmonella* are essential to identify critical contamination points, to assess the microbiological risks and factors for each processing chain (Malorny et al., 2008; Postollec et al., 2011). However, quantitative microbial risk assessment also providing an estimate of the level of illness that a pathogen can cause in a population is still hampered by the lack of quantitative data and the generation of appropriate data with high sensitivity is a challenge for microbiologists since currently used bacteriological quantitation methodologies are laborious (Forsythe, 2002; Oscar, 2004; Malorny et al., 2008). Nearly 22 million cases of typhoid occur each year with 200,000 deaths globally (Parry, 2005) and this striking statistic illustrate the urgency of quantitative methods.

The developed cost effective and totally quantitative recombinant plasmid based Real Time PCR method having the capability to determine exact copy number of *Salmonella* will provide not only *Salmonella* detection but also risk evaluation ability which is elusive previously.

CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

To test the applicability of plasmids containing *invA* or *ttrRSBC* as positive controls *S.* Typhimurium ATCC 14028 was used as reference strain. As positive controls 15 *Salmonella* serotypes (*S*. Agona,*S*. Anatum, *S*. Bispebjerg, *S*. Coravallis,*S*. Enteritidis, *S*. Infantis,*S*. Kentucky,*S*. Montevideo,*S*. Nchanga,*S*. Salford,*S*. Telaviv,*S*. Senftenberg, *S*. Thompson,*S*. Typhimurium, *S*. Virchow) and as negative controls ATCC strain of 8 different pathogens other than *Salmonella* (*Citrobacter freundii*, *E. coli* O157:H7, *Enterococcus faecalis*, *Listeria innocua*, *Proteus vulgaris*, *Shigella sonnei*, *Staphylococcus auereus*, *Yersinia enterocolitica*) were used to detect and quantify *Salmonella* in milk as a food model

The *S.* Typhimurium ATCC 14028 and negative control pathogens were purchased from Istanbul Hifzissihha Institute (Turkey); positive control pathogens previously isolated from foods in our laboratory (Avsaroglu, 2007) were used and all bacterial strains were kept at -80 ^{o}C until reactivation in Brain Heart Infusion (BHI) or Tryptic Soy Broth (TSB) at 37 ^{o}C.

2.2 PRIMERS AND PROBES

For 284 bp fragment of the *invA* gene:

Salmonella specific 284 bp fragment of the *invA* gene was used as the target sequence. Primers previously suggested as an international standard for PCR based *Salmonella* detection (Malorny et al., 2003) were used:

139 (Forward) (5′-GTG AAA TAA TCG CCA CGT CG GGC AA-3′)

141 (Reverse) (5′-TCA TCG CAC CGT CAA AGG AAC C-3′)

TaqMan® probe mentioned in Hein and coworkers' study (2006) was used:

invA-1(5′-FAM-TTA TTG GCG ATA GCC TGG CGG TGG GTT TTG TTG-TAMRA-3′)

The primers and the TaqMan® probe were purchased from Fermentas (Thermo Fisher Scientific, USA).

For 96 bp fragment of *ttrRSBC* locus:

Previously described primers and probes (Malorny et al., 2004) were used:

ttr-6 (forward) CTCACCAGGAGATTACAACATGG

ttr-4 (reverse) AGCTCAGACCAAAAGTGACCATC

Target probe (ttr-5) FAM-CACCGACGGCGAGACCGACTTT-Dark Quencher

IAC probe Yakima Yellow-CACACGGCGACGCGAACGCTTT-Dark Quencher

The primers, TaqMan® probe and IAC probe were purchased from Fermentas (Thermo Fisher Scientific, USA).

2.3 DNA ISOLATION

Aliquots of 1 ml volumes from bacterial cultures were centrifuged at 6000g for 10 min, and DNA isolated from the pellets using Fermentas Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction.

2.4 PCR

Isolated DNA was amplified using Techne TC-512 Thermo Cycler PCR (Bibby Scientific Limited, UK). PCR solutions were supplied from Fermentas (Thermo Fisher Scientific, USA). 2 µl of DNA was added to PCR mix (50 uM dNTPs, 1.5 mM MgCl2, 10 pmol primers, 0.1 U Taq polymerase). PCR was carried out as follows: one cycle at 98^{o}C for 5 minutes, and 35 cycles; 94^{o}C for 30 seconds, 59^{o}C for 1 minute, and 72^{o}C for 30 seconds, and one final cycle at 72^{o}C for 5 minutes. The PCR products were analyzed on agarose gel in order to determine the target regions and PCR quality.

2.5 GEL ELECTROPHORESIS

Horizontal 2% agarose gel (5x60x3 mm) was prepared with TAE (40 mM Tris-acetate, pH 8.3, 1 mM EDTA) buffer. Electrophoresis was performed at a constant voltage of 130 V. One kb DNA ladder and 10 µl PCR samples were diluted with sample buffer (1% SDS, 50%

Glycerol, 0.05% Bromophenol Blue, pH 8.3) and loaded. PCR fragments recovered from the agarose gel by cutting DNA were eluted from the sliced agarose gel by gel extraction kit (Qiagen Co).

2.6 PLASMID CONSTRUCTION AND SEQUENCING

Fermentas K1214 cloning kit was used (Thermo Fisher Scientific, USA), it carries a single 3' thymidine overhang that can be used for PCR fragment cloning. All amplified fragments cloned into the pTZ57R/T cloning vector (Figure 2.1) according to manufacturer's manual. Briefly, 50 ng of vector DNA and 1 µl of PCR product were incubated with 2x T4 DNA ligase buffer contain 1 µl (3u/µl) T4 DNA ligase at 4^{o}C overnight. 2 µl of ligation reaction and 100 µl of DH5 alpha competent cells heat-shock transformed and spread on agar plates containing 100 µg/ ml ampicillin. Plates were incubated overnight at 37^{o}C. The plasmids were isolated from bacteria using Roche High Pure Plasmid Isolation Kit (Roche Applied Science, Germany) according to manufacturer's manual. To determine whether *invA* or*ttrRSBC* gene were incorporated into isolated plasmids, after PCR reactions having same mixture and reaction conditions as mentioned above, Agarose Gel Electrophoresis was used and confirmed with sequencing (Iontek Co, Turkey).

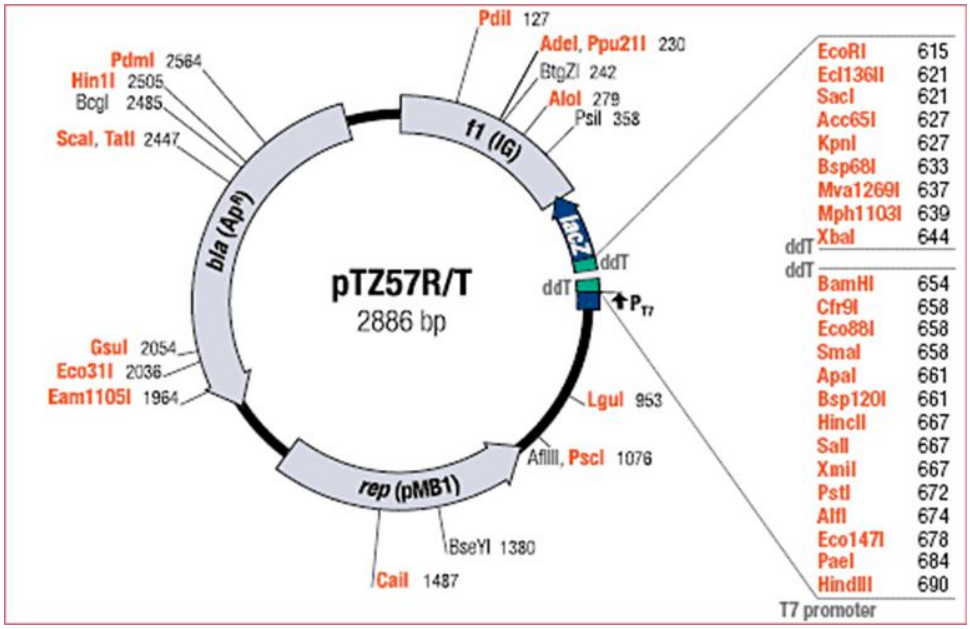


Figure 2.1 pTZ57R/T cloning vector and its restriction map used in this study for Real-Time PCR based quantification and detection of *Salmonella*.
(Thermo Scientific, http://www.fermentas.com/en/products/all/molecular-cloning/kits/k121-instaclone-pcr-cloning Visited on 10.02.2012).

2.7 CALCULATION OF COPY NUMBER OF PLASMIDS CONTAINING *invA* AND PLASMIDS CONTAINING *ttrRSBC*

Isolated plasmids were measured using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and also using much more sensitive device The Qubit 2.0 Fluorometer (Life Technologies Corporation, USA).

Copy number of plasmids containing *invA* (P-invA) and plasmids containing *ttrRSBC* (P-ttrRSBC) were calculated using web calculator

(URI Genomics & Sequencing Center, http://www.uri.edu/research/gsc/resources/cndna.html Visited on 15.01.2012).

Copy number of P-invA: 1.75×10^{10}
Copy number of P-ttrRSBC: 2.32×10^{10}

2.8 REAL-TIME PCR

To confirm precision and reproducibility of Real-Time PCR, standard curves were constructed using P-invA and P-ttrRSBC by diluting them (10^8 to 10^2) in six different runs on different days with two replicates. To construct standard curves for P-invA and P-ttrRSBC, crossing point (Cp) mean values of 12 replicates versus Log calculated copy numbers were plotted. From the slopes of the standard curves, PCR efficiencies and amplification efficiencies for P-invA and P-ttrRSBC were calculated using equations $E = (10^{-1/slope}) - 1$ and $E_{amp}=10^{-1/slope}$ respectively (Gallup and Ackermann, 2006).

To illustrate the applicability of the developed plasmid based positive controls, exact concentrations of 10^5 to 10^0 CFU/ml level (Approximately determined by using traditional microbiological method, ISO 6579: 2004) *S.* Typhimurium ATCC 14028 culture were tried to determineby using the abovementioned calculated copy numbers for the standards. Analysis of enriched culturewas carried out with a manipulation of the procedure described elsewhere (O'Regan et al., 2008) using traditional culture method (ISO 6579:2004) and using developed plasmid based Real-Time PCR method in parallel. Briefly, *S.* Typhimurium ATCC 14028 culture was grown in TSB to optical density (OD_{600nm}) of 0.240 corresponding to 10^8 CFU/ml (Omiccioli et

al., 2009) and 10^5 to 10^0 CFU/ml level concentrations were obtained with serial dilutions in the same medium. 1ml of each level concentration was added to 225 ml of BufferedPeptoneWater (BPW) and incubated at 37 ^oC for 18 h after homogenization for a while. Following pre-enrichment in BPW, traditional cultural method (ISO 6579:2004) contains selective enrichment of 0.1 ml culture in Rappaport Vasilliadis Soya Broth (RVS) for 24 h at 41.5 ^oC and 1 ml culture in Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn) Broth for 24 h at 37 ^oC simultaneously; incubation in XLD and BGA agars for 24 h at 37 ^oC simultaneously; incubation in Tryptone Soy Agar (TSA) for 24 h at 37 ^oC. On the other hand, following enrichment in BPW, the developed plasmid based Real-Time PCR method contains DNA extraction prior to Real-Time PCR analysis. Figure 2.2 illustrates the general steps of traditional culture method and Real-Time PCR assay schematically.

All culture media were from Oxoid (Basingstoke, UK). The correlation of traditional culture method results and the results of the developed plasmid based Real-Time PCR method was investigated after three independent experiments for each method.

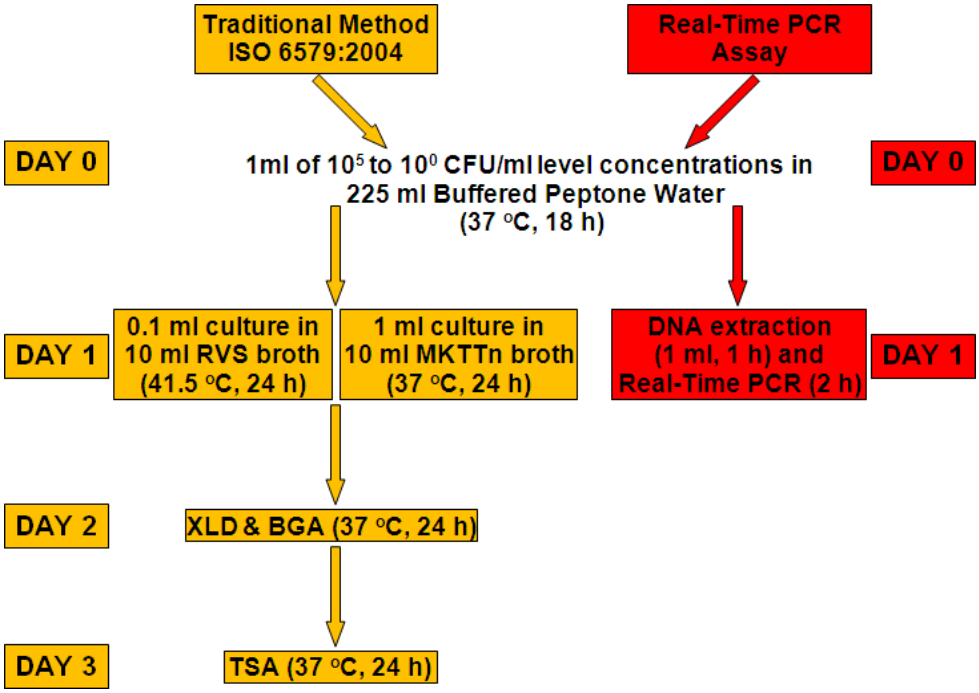


Figure 2.2 Flow diagramof methodology in the traditional cultural method and the Real-Time PCR assay (Adapted fromO'Regan et al., 2008).

In addition, detection limit of the developed technique was determined using diluted isolated DNA (DNA extracted from 10^5 CFU/ml level *S.* Typhimurium ATCC 14028 culture was serially diluted to 10^0 level using nuclease free water).

A 25-µl reaction mixture contained 12.5 µl of Maxima Probe qPCR Master Mix (2x) (MaximaProbeqPCRBuffercontainingKCl, $(NH_4)_2SO_4$, Maxima® Hot Start *Taq*DNA polymerase, dNTPs, dUTP), 0.5µL (10pmol) of each primer (139F and 141R for *invA*; ttr-6F and ttr-4R for *ttrRSBC*), 0.5 µL (10pmol) of each probe (*invA*-1 probe for *invA*; ttr-5 and IAC probes for *ttrRSBC*), 5 µL aliquot of DNA, 150 copies of IACDNA (purified 303-bp PCR product as described previously (Malorny et al., 2004)) for *ttrRSBC*. No template controls that

contained 5 µL of TE buffer instead of DNA were included in each run to detect any PCR fragment contamination. All Real-Time PCR solutions were purchased from Fermentas (Thermo Fisher Scientific, USA).

Real-Time PCR experiments and data analysis were performed using Roche Light Cycler 480 (Roche Diagnostics, Germany). The thermal cycling conditions were 50 ^{o}C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 20 s.

2.9 APPLICATION OF THE DEVELOPED METHOD ONA FOOD MODEL

Raw milk samples were boiled at 90 ^{o}C for 10 minutes and cooled to room temperature. 25 ml of milk samples were artificially contaminated individually with 15 *Salmonella* serotypes at 10^5 to 10^0 CFU/ml level concentrations. 1ml of each level concentration was added to 25 ml milk samples and they were diluted tenfold in BPW (225 ml), then the mixture was incubated at 37 ^{o}C for 18 hafter homogenization for a while. Following pre-enrichment in BPW, traditional cultural method (ISO 6579:2004) contains selective enrichment of 0.1 ml culture in Rappaport Vasilliadis Soya Broth (RVS) for 24 h at 41.5 ^{o}C and 1 ml culture in Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn) Broth for 24 h at 37 ^{o}C simultaneously; incubation in XLD and BGA agars for 24 h at 37 ^{o}C simultaneously; incubation in Tryptone Soy Agar (TSA) for 24 h at 37 ^{o}C. On the other hand, following enrichment in BPW, the developed plasmid based Real-Time PCR method contains DNA extraction prior to Real-Time PCR analysis. Figure 2.3 illustrates the general steps of traditional culture method and Real-Time PCR assay schematically.

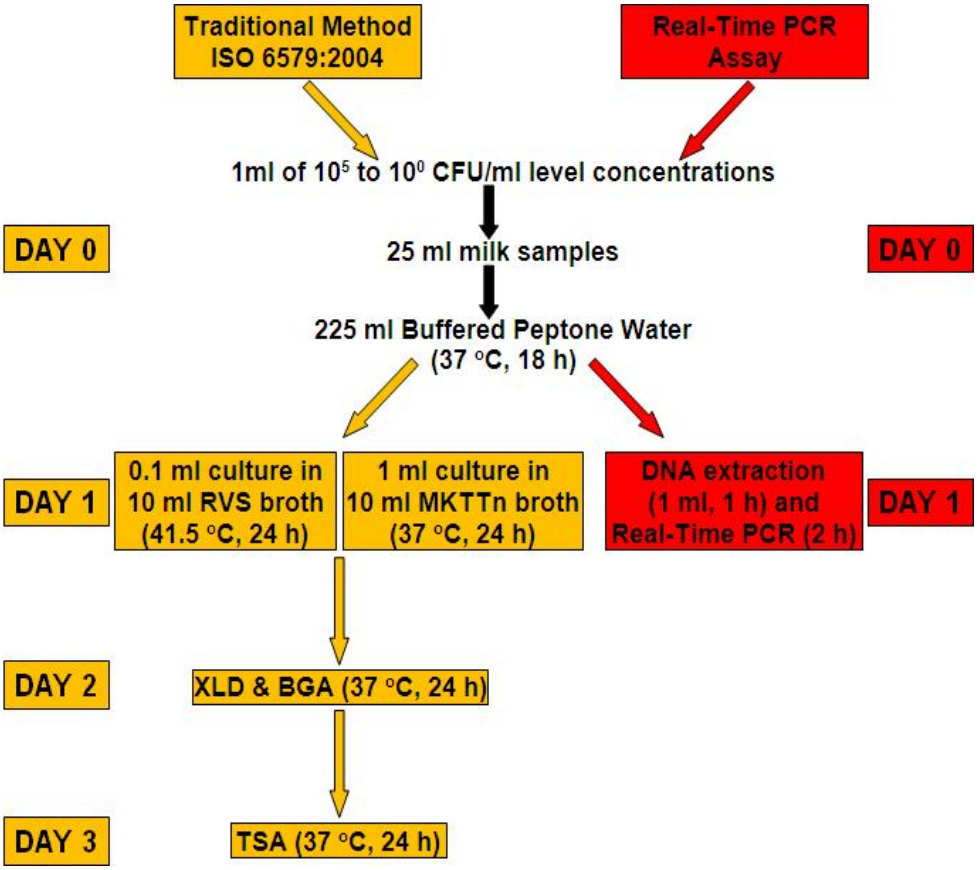


Figure 2.3 Flow diagram of methodology in the traditional cultural method and the Real-Time PCR assay for application of the developed method on milk as a food model (Adapted fromO'Regan et al., 2008).

In addition, for *Salmonella* analysis the developed recombinant plasmid based Real Time PCR mehod was applied to naturally contaminated bulk tank milk samples collected from ten milk vendors throughout Istanbul. The methodology was the same with that of artificially contaminated milk samples except for boiling step.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 CLONING ACHIEVEMENT

All pathogenic *Salmonella* serovars have *invA* gene essential for invading mammalian cells and subsequently cause disease (Gonzales-Escalona et al., 2009), thus it has received great deal of attention from the researchers studying on rapid detection of *Salmonella* with molecular genetic tools and has been extensively used in researches reporting good sensitivity and specificity compared to standard methods (Postollec et al., 2011). Although *invA* gene target is most commonly used for the detection of *Salmonella* in PCR assays (O'regan et al., 2008), instability and natural deletions within *Salmonella* pathogenicity island1 encompassing the *inv*, *spa*, and *hil* loci was shown (Ginocchio et al., 1997; Malorny et al., 2004).

Malorny and coworkers (Malorny et al., 2004) suggested an alternative target '*ttrRSBC*' with high accuracy for various food matrices. The target is responsible for tetrathionate respiration which is significant for *Salmonella* spp. life cycle illustrating genetically stable nature of *ttr* in all *Salmonella* strains (Malorny et al., 2004) contrary to genetic instability of *Salmonella* pathogenicity island1. After Malorny and coworkers (Malorny et al., 2004) *ttrRSBC* was used extensively by other researchers also (Hyeon et al., 2010; Josefsen et al, 2007; Lofstrom et al., 2009; Omiccioli et al., 2009) using various foods.

Two most commonly used *Salmonella* specific target regions were successfully cloned into pTZ57R/T cloning vector for molecular detection of this pathogen. Figure 3.1 shows incorporation of *Salmonella* specific genes '*invA* and *ttrRSBC*' into the vectors. 284 bp *invA* and 96bp *ttrRSBC* gene fragments were obtained followed by agarose gel electrophoresis of the PCR product.

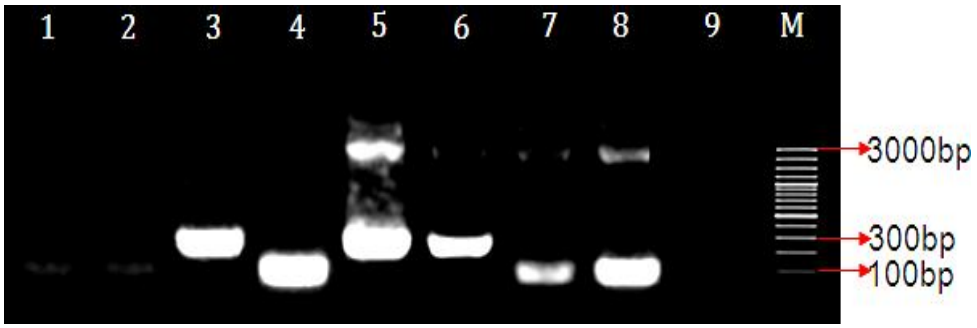


Figure 3.1 Ethidiumbromide stained agarose gel showing PCR from cloned *invA* and *ttrRSBC* regions. **1:** *invA* negative (*invA* forward primer 139F + *invA* reverse primer 141R) **2:** *ttrRSBC* negative (*ttrRSBC* forward primer ttr6F + *ttrRSBC* reverse primer ttr4R) **3:** PCR product of *invA* Primers + Isolated DNA from *S.* Typhimurium ATCC 14028 culture **4:** PCR product of *ttrRSBC* Primers + Isolated DNA from *S.* Typhimurium ATCC 14028 culture **5,6:** PCR product of *invA* Primers + P-invA **7,8:** PCR product of *ttrRSBC* Primers + P-ttrRSBC **9:** *E.coli* O157:H7 as negative control (PCR product of *invA* Primers and *ttrRSBC* Primers + Isolated DNA from *E.coli* O157:H7) **M:** Molecular weight marker

3.2 CONSTRUCTION OF STANDARD CURVES

Standard curves were constructed for P-invA and P-ttrRSBC as shown in Figure 3.3 to use as standard positive controls for the detection and quantification of *Salmonella*. The standard curves were obtained using average of Cp values presented in Table 3.1 belong to amplification plots, average duplicates of the plots are illustrated in Figure 3.2.

To confirm precision and reproducibility of Real-Time PCR, standard curves were constructed using multiple linear regressions corresponding to six different runs on different days with two replicates. Thus copy number Cp values for *invA* or *ttrRSBC* incorporated plasmids are mean values based on 12 replicates.

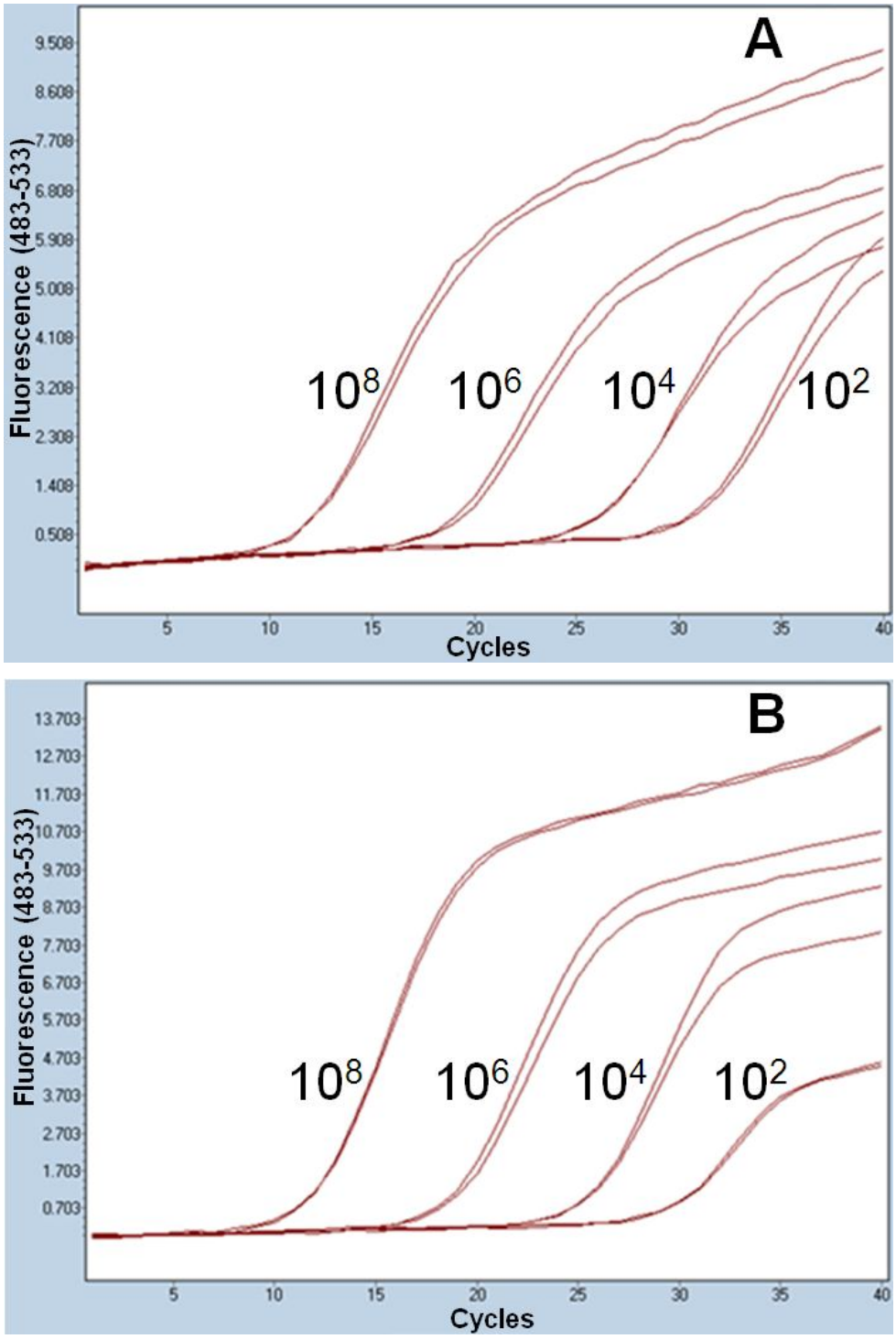


Figure 3.2 Average duplicates of amplification plots of A) P-invA B) P-ttrRSBC.

Table 3.1 Reproducibility of the Real-Time PCR

Copy Number Level	P-invA		P-ttrRSBC	
	Cp	SD	Cp	SD
10^8	12.32	0.08	11.98	0.06
10^6	19.35	0.19	19.23	0.13
10^4	26.18	0.22	25.94	0.18
10^2	32.30	0.66	32.30	0.65

Cp: Crossing point, SD: Standard deviation

Using t test it was investigated whether there is a statistically significant difference between Cp values and copy number values belong to *invA* incorporated plasmids and *ttrRSBC* incorporated plasmids. Two-tailed P values were found as 0.98 and 0.39 for Cp values and copy number values respectively representing statistically insignificant difference ($p<0.05$) for both parameters. Statistical insignificance for Cp values illustrates high reproducibility among Real-Time PCR runs and for copy number values illustrates consistency of cloning protocol.

After obtaining promising statistical analysis results, using the values presented in Table 3.1 standard curves were constructed for *invA* and *ttrRSBC* incorporated plasmids as illustrated in Figure 3.3.

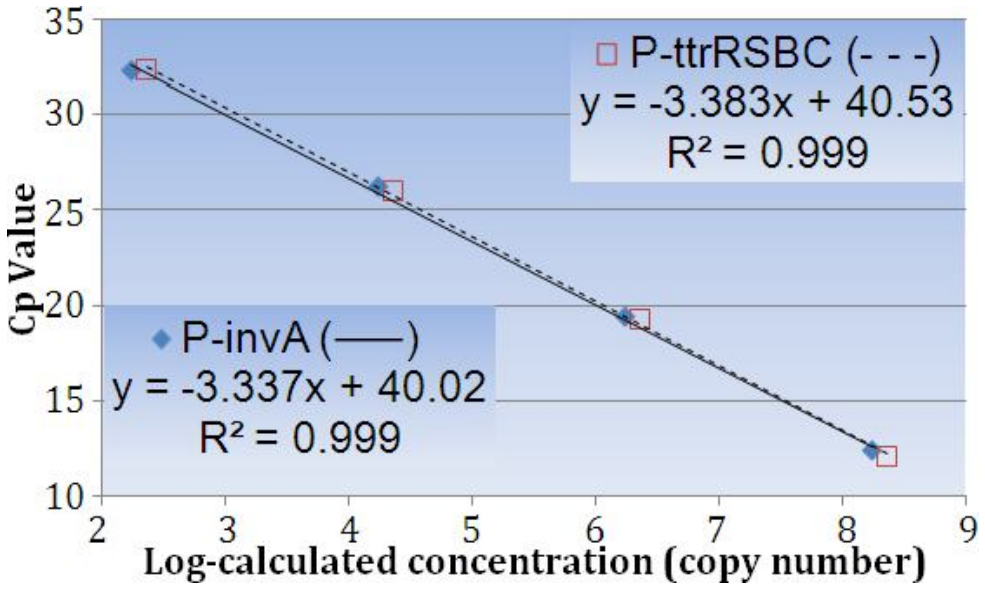


Figure 3.3 Standard curves of P-invA: Plasmids containing *invA* and P-ttrRSBC: Plasmids containing *ttrRSBC*.

In addition to the statistical analysis results mentioned above, linear relationship of both the P-invA and P-ttrRSBC standard curves were 0.999 also illustrating high reproducibility (Zou et al., 2006) of Real-Time PCR for both *invA* and *ttrRSBC* incorporated plasmids.

From the slopes of the constructed standard curves PCR efficiency was calculated using the equation $E = (10^{-1/slope}) -1$ and the efficiencies were found as 99% and 98% for P-invA and P-ttrRSBC respectively. This means amplification efficiencies ($E_{amp}=10^{-1/slope}$) were 1.99 and 1.98 for P-invA and P-ttrRSBC respectively illustrating nearly perfect doubling of template every cycle ($E_{amp}=2$ indicates perfect doubling of template every cycle) (Gallup and Ackermann, 2006).

3.3 APPLICABILITY OF THE DEVELOPED METHOD

After construction of standard curves, to illustrate the applicability of the developed plasmid based positive controls, exact concentrations of 10^5 to 10^0 CFU/ml level (Approximately determined by using traditional microbiological method, ISO 6579:2004) *S.* Typhimurium ATCC 14028 cultures were tried to determine using the calculated copy numbers for the standards. Figure 3.4 illustrates the amplification plots of 10^5 to 10^0 CFU/ml levels (estimated by standard plate counts before enrichment) after 18 h enrichment in BPW.

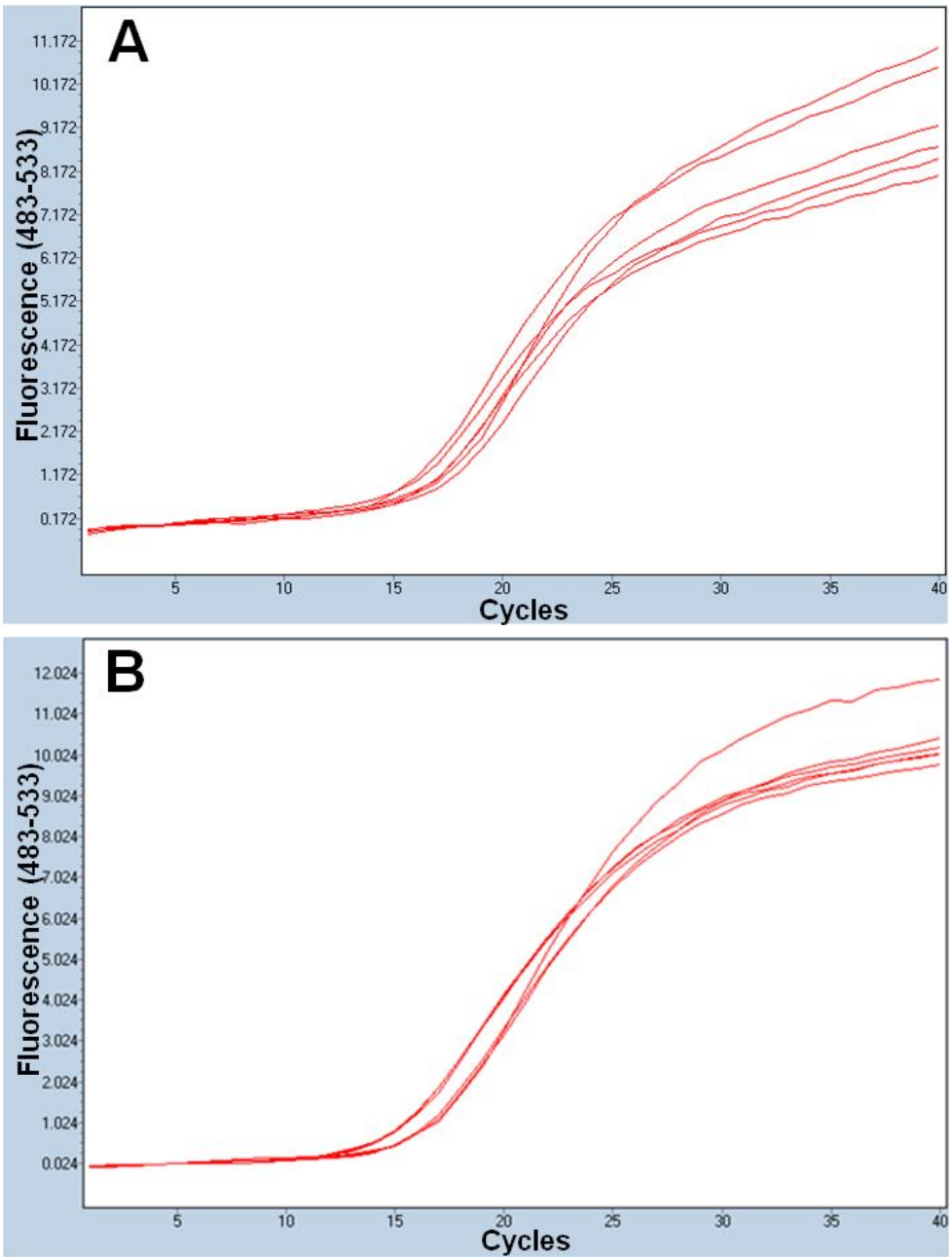


Figure 3.4 Amplification plots of 10^5 to 10^0 CFU/ml levels (estimated by standard plate counts before enrichment) after 18 h enrichment in BPW using A) *invA* target B) *ttrRSBC* target.

Table 3.2 also presents applicability of the developed P-invA and P-ttrRSBC based positive controls for Real-Time PCR to detect and quantify *Salmonella* after enrichment. Cp values of the 10^5 to 10^0 CFU/ml levels (estimated by standard plate counts before enrichment) shown in Figure 3.4 A and B, also their calculated concentrations from constructed standard curves are given in the Table.

Table 3.2 Detection and quantification of *S.* Typhimurium ATCC 14028 after enrichment using traditional culture method and *invA* or *ttrRSBC* incorporated plasmids based Real-Time PCR method.

Estimated Standard Plate Counts Before Enrichment	Traditional Culture Method After Enrichment According to ISO 6579:2004	After 18h Enrichment in BPW			
		P-invA		P-ttrRSBC	
		Cp ± SD	Concentration[a]	Cp ± SD	Concentration[a]
10^5	~10^5	17.63 ± 0.14	5.12x10^6	17.04 ± 0.12	8.78x10^6
10^4	~10^5	15.95 ± 0.10	1.63x10^7	15.27 ± 0.12	2.93x10^7*
10^3	~10^5	16.77 ± 0.15	9.27x10^6	16.17 ± 0.13	1.59x10^7
10^2	~10^5	15.73 ± 0.19	1.90x10^7*	15.04 ± 0.11	3.43x10^7
10^1	~10^5	17.54 ± 0.20	5.45x10^6	16.51 ± 0.13	1.30x10^7
10^0	~10^5	16.24 ± 0.10	1.34x10^7	15.09 ± 0.15	3.31x10^7

[a]Concentration= DNA Copy Number / 225 ml

*The concentrations represent approximately 10^5 CFU/ml used for serial dilution (See Figure3.5 and Table 3.3).

As illustrated in Figure 3.4 and Table 3.2, after enrichment, the developed plasmid based Real-Time PCR method has perfect sensitivity for both *invA* and *ttrRSBC* targets. Detection limit was as little as 1 CFU/225 ml and the proposed detection approach showed 100% concordance with the traditional culture method (ISO 6579:2004) in terms of *Salmonella* detection.

Amplification plots nearly overlap each other also nearly same Cp and concentration values were obtained for all dilution levels with the developed plasmid based *Salmonella* detection method as well as traditional culture method (ISO 6579:2004). This is caused by the fact that cultures with a higher initial concentration reach their stationary phase earlier and are thus longer in this phase (Huber et al., 2010). This situation illustrates the drawback of using enrichment step that it is impossible to quantify initial contaminating amounts (O'Regan et al., 2008; Postollec et al., 2011).

Higher bacterial counts were found with the developed plasmid based Real-Time PCR method than with traditional culture method (ISO 6579:2004) and this situation was most probably caused by much more sensitivity of the developed method: (i) the presence of intact DNA from dead cells which can not be quantified with plate counts, (ii) the presence of viable but non culturable forms, which can not be quantified with plate counts, (iii) one CFU on plate might be generated from more than one cell (Postollec et al., 2011). Above all, the developed method is much faster than traditional culture method (ISO 6579:2004) in addition to its much more sensitivity and much less labor intensive. While traditional culture method (ISO 6579:2004) takes 90h without biochemical and serological test, *invA* or *ttrRSBC* incorporated plasmids based *Salmonella* detection method takes only 21 h (18h enrichment + 1h DNA isolation + 2h Real-Time PCR).

3.4 DETECTION LIMIT OF THE DEVELOPED METHOD

Detection limit of the developed technique was also determined using diluted isolated DNA (DNA extracted from 10^5 CFU/ml level *S.* Typhimurium ATCC 14028 culture was serially diluted to 10^0 level using nuclease free water).

Figure 3.5 illustrates the amplification plots belong to serial dilution of isolated DNA from 10^5 CFU/ml level *S.* Typhimurium ATCC 14028 culture. Table 3.3 presents the Cp values and calculated concentrations of diluted DNAs using constructed standard curves.

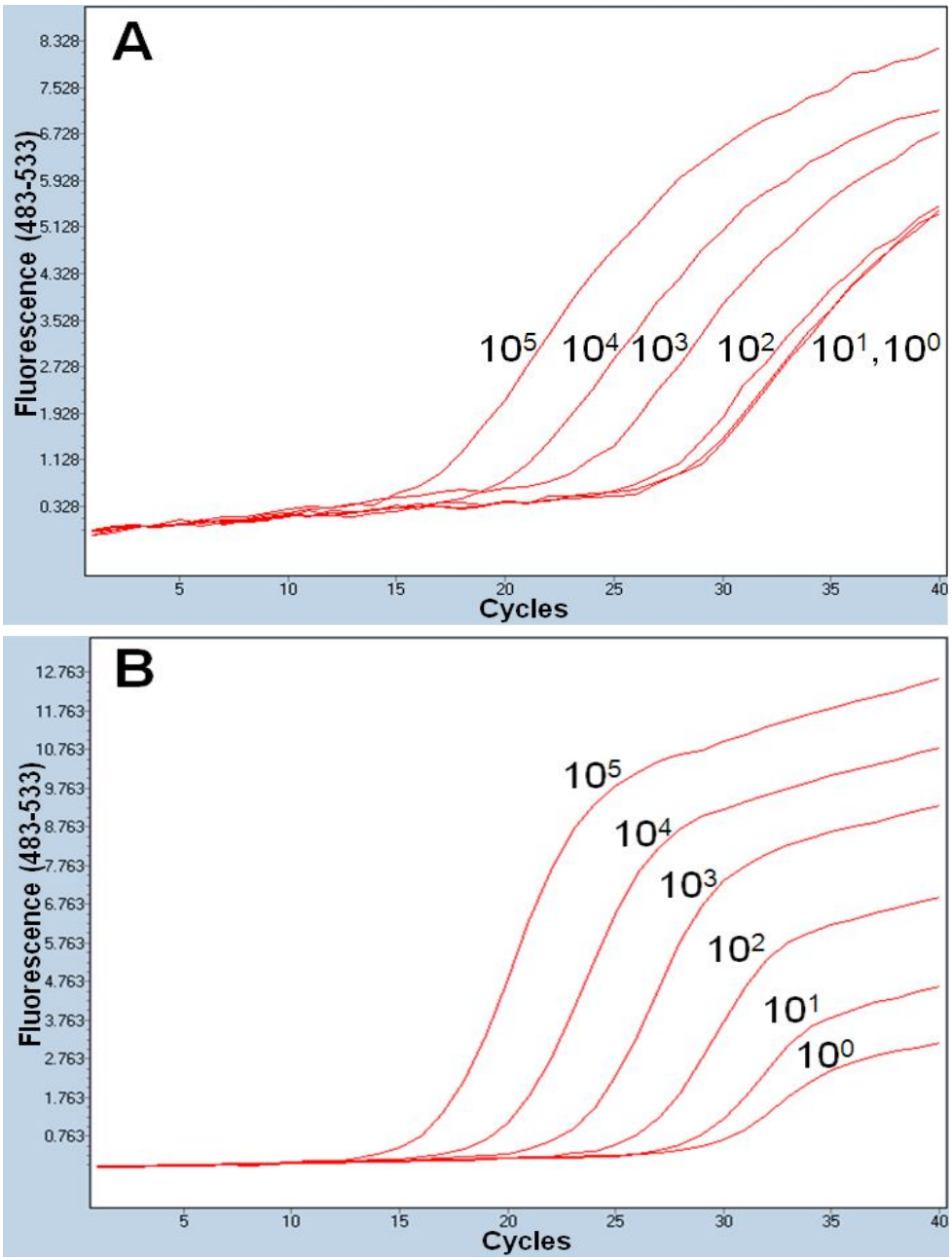


Figure 3.5 Amplification plots obtained by serial dilution of isolated DNA from 10^5 CFU/ml level *S.* Typhimurium ATTCC 14028 culture using A) *invA* target B) *ttrRSBC* target.

Table 3.3 Cp values and calculated concentrations of diluted DNAs using constructed standard curves.

Serial Dilution of DNA Extracted from 10^5 CFU/ml	P-invA		P-ttrRSBC	
	Cp ± SD	Concentration* (DNA Copy Number/ml)	Cp ± SD	Concentration* (DNA Copy Number/ml)
10^5	15.73 ± 0.12	$0.84x10^5$	15.27 ± 0.10	$1.30x10^5$
10^4	20.46 ± 0.17	$7.27x10^3$	20.02 ± 0.13	$1.16x10^4$
10^3	23.70 ± 0.22	$7.77x10^2$	23.30 ± 0.18	$1.24x10^3$
10^2	26.71 ± 0.27	$9.74x10^1$	26.17 ± 0.18	$1.76x10^2$
10^1	27.04 ± 0.22	$7.76x10^1$	28.34 ± 0.21	$4.01x10^1$
10^0	27.05 ± 0.24	$7.70x10^1$	29.34 ± 0.25	$2.03x10^1$

*Concentrations were calculated by dividing the value of each Cp in constructed standard curves by 225.

P-invA and P-ttrRSBC based *Salmonella* detection method has the detection limit of 10^1 CFU/ml for both target (See Figure 3.5 and Table 3.3). However it should be pointed that *ttrRSBC* target is better to discriminate lower concentrations. As can be seen from amplification plots in Figure 3.5 also from Cp and concentration values presented in Table 3.3, for *invA* target, 10^2-10^0 dilutions have nearly overlap each other and have nearly same Cp and concentration values. On the other hand, *ttrRSBC* target allowed more clear discrimination among amplification plots, Cp and concentration values for these dilutions. Genetic instability and natural deletions within *Salmonella* pathogenicity island 1 encompassing the inv, spa and hil loci was

previously reported (Ginocchio et al., 1997; Malorny et al., 2004) and this issue can explain less sensitivity obtained with *invA* target for lower concentrations.

The promising results mentioned above reflects much faster detection ability of the developed plasmid based *Salmonella* detection method (in comparison to traditional culture method, ISO 6579:2004) with perfect reproducibility, sensitivity (except for lower concentrations for *invA* target), detection limit, PCR efficiency, amplification efficiency for both *invA* and *ttrRSBC* targets.

Quantitative and cost-effective methods are essential to estimate the microbiological risks and factors for example storage conditions influencing food safety (Postollec et al., 2011). The developed method allowing quantitative evaluation is crucial in this regard also.

3.5 APPLICATION OF THE DEVELOPED METHOD ONA FOOD MODEL

As a rapid and sensitive method for the detection and identification of bacteria, increasingly culture-independent DNA-based technologies are being employed to provide an accurate assessment of milk bacterial composition including *Salmonella* (Quigley et al., In Press). Although extracting DNA from milk in sufficient concentration and purity is of crucial importance due to the fact that milk contains PCR inhibitors such as fats, proteins and calcium (See the Table 1.1) causing DNA amplification problems, previous studies have shown the applicability of Real-Time PCR technique for *Salmonella* detection (Omiccioli et al., 2009; Quigley et al., In Press).

Therefore, milk is a good candidate as a food model to test the developed rapid, sensitive, efficient, quantitative *Salmonella* detection method based on inexpensive, easy to produce, convenient and standardized plasmid based positive control. The developed quantitative method having the capability to determine exact copy number of *Salmonella* will provide dairy industry not only *Salmonella* detection but also risk evaluation ability.

Commercially available UHT and pasteurized milk samples were firstly used, because UHT and pasteurized milk are already sterile allowing detection and quantification of *Salmonella* artifically added to the milk samples.

There was no bacterial colony formation on petri dishes when different commercially available UHT and pasteurized milk samples from different hypermarkets were used. Thereupon, experiments to test whether there was any problem related to *Salmonella* serotypes previously isolated from foods in our laboratory or bacterial growth medium were carried out. Typical results of these experiments are illustrated in Figure 3.6-3.8.

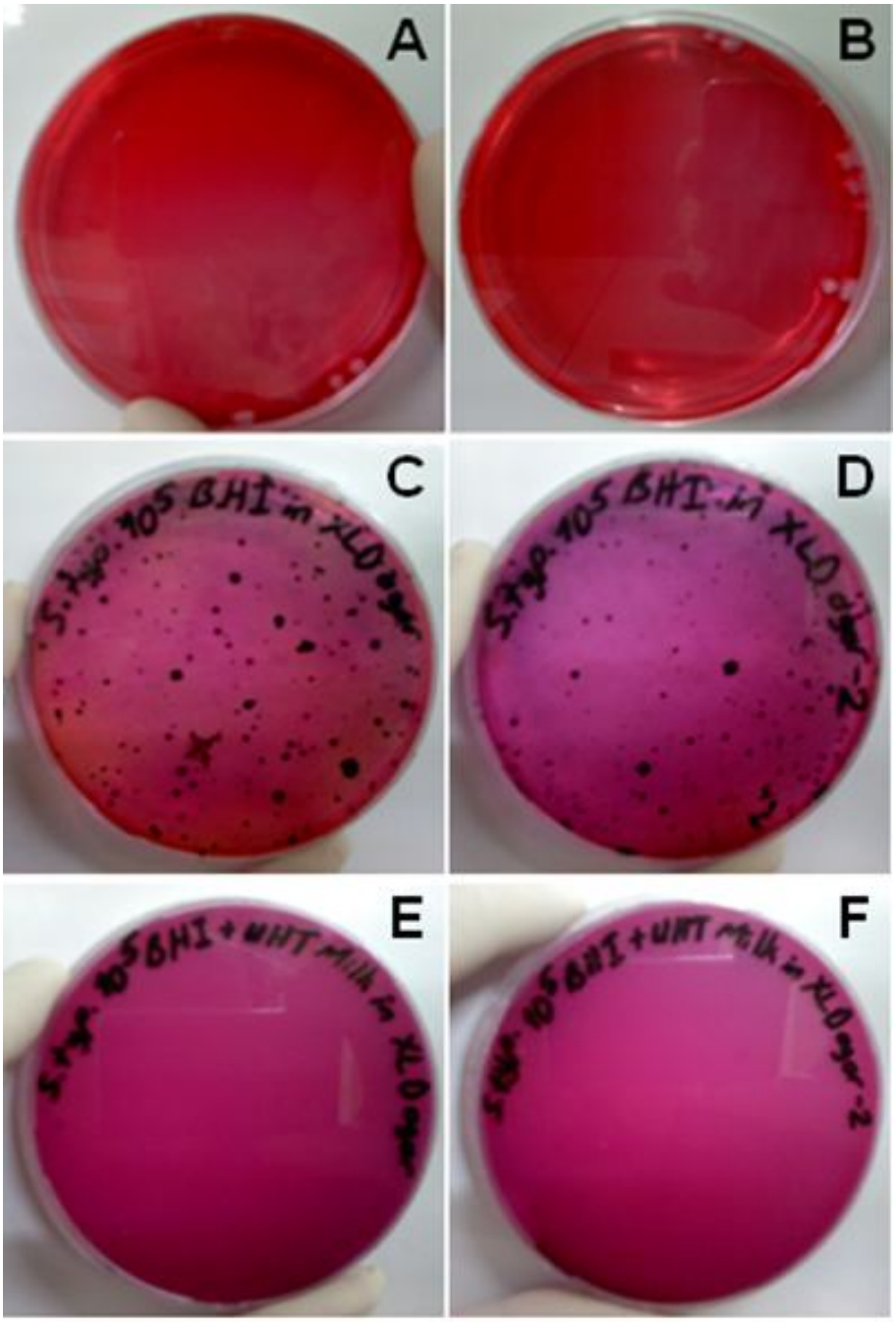


Figure 3.6 Bactericidal activity of UHT milk against *S.* Typhimurium. A,B: BHI (Brain Heart Infusion) broth in XLD agar C,D: *S.* Typhimurium BHI broth culture (10^5 CFU/ml) in XLD agar E,F: *S.* Typhimurium BHI broth culture (10^5 CFU/ml) in XLD agar in the presence of UHT milk.

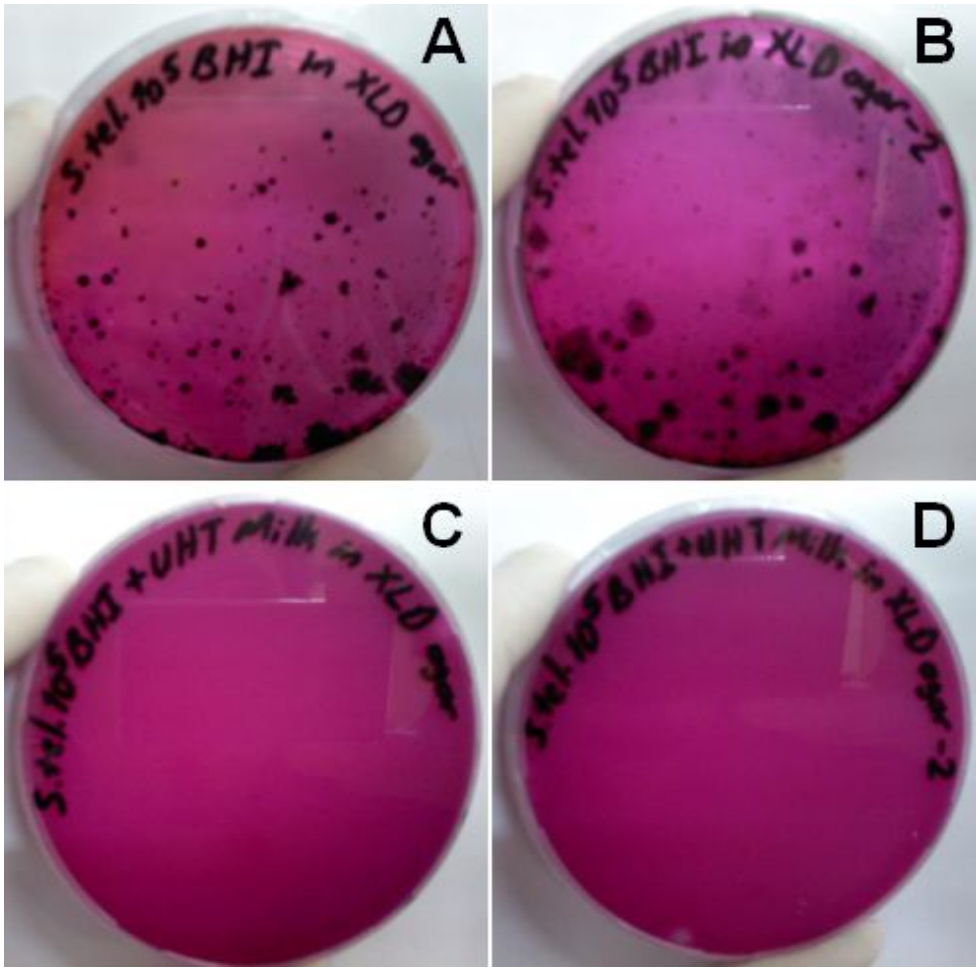


Figure 3.7 Bactericidal activity of UHT milk against *S.* Telaviv. A,B: *S.* Telaviv BHI broth culture (10^5 CFU/ml) in XLD agar C,D: *S.* Telaviv BHI broth culture (10^5 CFU/ml) in XLD agar in the presence of UHT milk.

To test whether there is a problem related to growth medium, 1ml of BHI broth was inoculated in XLD agar and incubated overnight at 37 ^{o}C and there wasn't any bacterial growth in duplicate experiments as expected (See Figure 3.6 A and B).

To test whether there is a problem related to *Salmonella* serotypes previously isolated from foods in our laboratory, *Salmonella* cultures

(S. Typhimurium in Figure 3.6 and S. Telaviv in Figure 3.7) reactivated in BHI broth at 37 ^{o}C. 1 ml of *Salmonella* (10^5 CFU/ml S. Typhimurium in Figure 3.6 and 10^5 CFU/ml S. Telaviv in Figure 3.7) BHI broth cultures were inoculated in 9 ml XLD agar. Petri dishes were incubated overnight at 37 ^{o}C. Extensive bacterial growth was observed in duplicate experiments for both serotypes as expected (See Figure 3.6 C and D; Figure 3.7 A and B).

To test whether bactericidal activity of UHT milk samples against artificially added *Salmonella* serotypes, the experiments mentioned in above paragraph were carried out in the presence of 1ml of UHT milk samples (1ml of *Salmonella* BHI broth culture and 1 ml of UHT milk sample were mixed homogenously then inoculated in XLD agar and petri dishes were incubated overnight at 37 ^{o}C). On the contrary of the experimental results mentioned in above paragraph, there wasn't any bacterial growth in duplicate experiments for both serotypes (See Figure 3.6 E and F; Figure 3.7 C and D).

Most probably antibiotic residues and possible additives in UHT milk samples for longer shelf life kill *Salmonella* serotypes artificially added to the samples.

Pasteurized milk has a much shorter shelf life than UHT milk, therefore most probably it has less amount of antibiotic residues and possible additives according to UHT milk. Using same methodology commercially available pasteurized milk samples were tested for application of the developed method on milk as a food model. As illustrated in Figure 3.8 although there are some black parts (Black color illustrates the *Salmonella* growth in XLD agar) in duplicate experiments for both S. Typhimurium and S. Telaviv serotypes, there

is not any homogen colony formation opposed to extensive bacterial growth in the absence of milk (See Figure 3.6 C and D; 3.7 A and B).

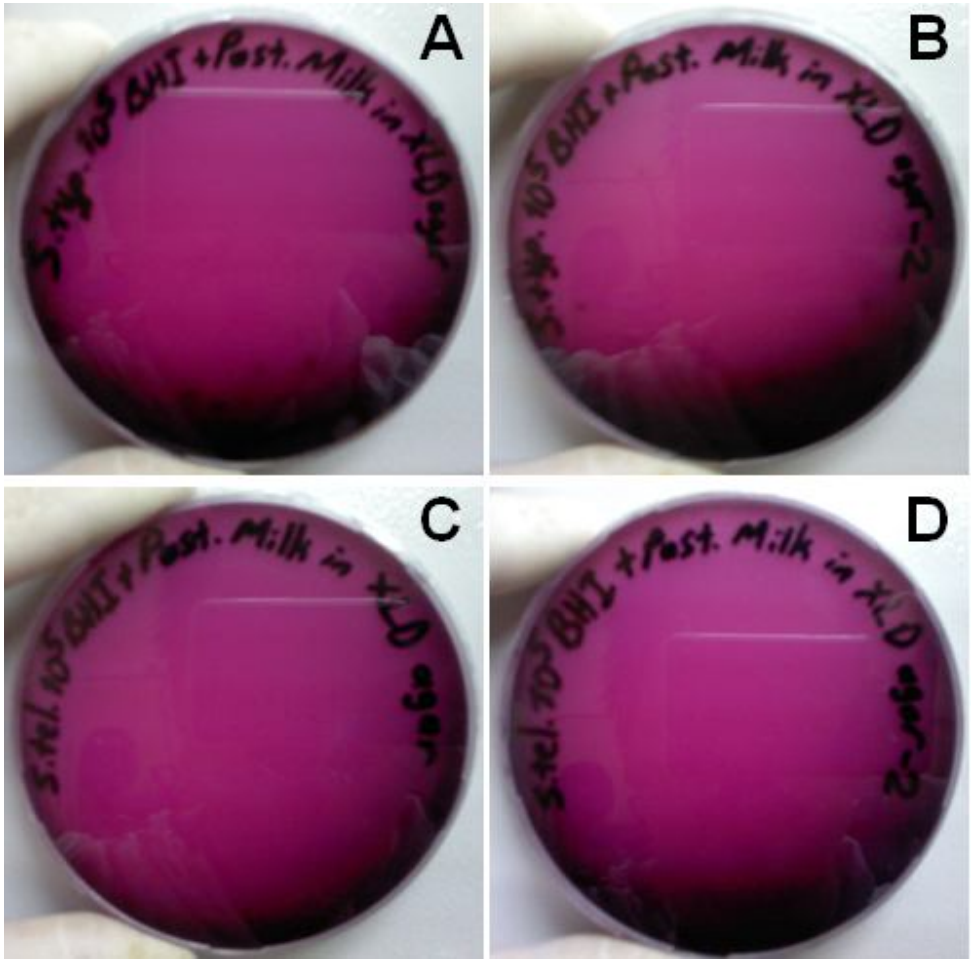


Figure 3.8 Bactericidal activity of pasteurized milk against *S.* Typhimurium and *S.* Telaviv. A,B: *S.* Typhimurium BHI broth culture (10^5 CFU/ml) in XLD agar in the presence of pasteurized milk C,D: *S.* Telaviv BHI broth culture (10^5 CFU/ml) in XLD agar in the presence of pasteurized milk.

In pasteurized milk experiments a little sign of bacterial growth (No sign of bacterial growth in UHT milk experiments) may result from fewer amounts of antibiotic residues and possible additives and this situation illustrates that pasteurized milk samples also have bactericidal activity against artificially added *Salmonella* serotypes. UHT process involves increasing the temperature of the milk to about 140 ^{o}C for one or two seconds while pasteurization process involves temperatures nearly half of UHT temperature (72 ^{o}C for 15 seconds in high temperature short time pasteurization; 62-65 ^{o}C for 30 minutes in low temperature long time pasteurization). Thus, from the nutritional point of view, ingredients of pasteurized milk remains more intact according to UHT milk and this may also contributed a little sign of bacterial growth.

Although *Salmonella* serotypes used in this study gave resistance to some antibiotics (Avsaroglu, 2007), observed bactericidal activity of commercially available UHT and pasteurized milk samples deserves further researches to clarify serotypes' sensitivity to possible residues of other numerous antibiotics which were not tested and/or sensitivity to possible additives.

After observation of bactericidal activity of different commercially available UHT and pasteurized milk samples from different hypermarkets against the *Salmonella* serotypes artificially added to the samples, raw milk samples were used to demonstrate applicability of the developed method to milk as a food model.

Raw milk samples were collected in triplicate from a milk vendor and boiled at 90 ^{o}C for 10 minutes to kill possible pathogens in the samples and thereby, only the *Salmonella* serotypes artificially added to the sterilized samples could be detected and quantified. After

cooling to room temperature, 0.1 ml of *Salmonella* serotypes was added to the 12.5 ml of milk samples and incubated at 37 ^{o}C for 24 h. 1ml of this culture was inoculated to XLD agar and incubated at 37 ^{o}C for 24 h (Figure 3.9 A); 0.1 ml of *Salmonella* milk culture (incubated at 37 ^{o}C for 24 h) was added to 12.5 ml of Buffered Peptone Water and incubated at 37 ^{o}C for 24 h, then 1ml of 10000x diluted culture was inoculated to XLD agar and incubated at 37 ^{o}C for 24 h (Figure 3.9 B). Typical results of these experiments using 10^5 CFU/ml S. Typhimurium are illustrated in Figure 3.9 A and B.

As illustrated in Figure 3.9, boiled raw milk samples have no bactericidal activity against *Salmonella* serotypes artificially added to the samples (Figure 3.9 A), also there is no problem related to methodolgy of standardized method (ISO 6579:2004) involving BPW enrichment (Figure 3.9 B). Thus, collected raw milk samples are ideal candidates to apply developed method after boiling.

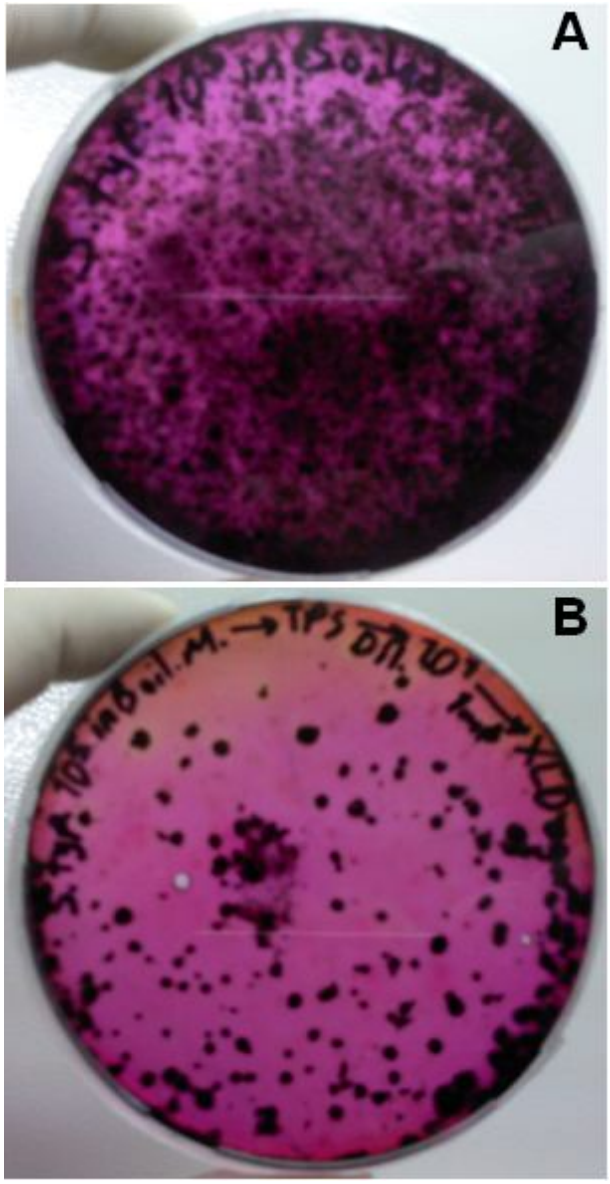


Figure 3.9 Growth of *S.* Typhimurium artificially added to boiled milk. A: *S.* Typhimurium (10^5 CFU/ml) Boiled Milk culture in XLD B: *S.* Typhimurium (10^5 CFU/ml) Boiled Milk culture in Buffered Peptone Water (Illustrated as TPS in the figure) then in XLD agar after 10000x dilution.

To apply the developed method as positive controls 15 *Salmonella* serotypes (*S*. Agona,*S*. Anatum, *S*. Bispebjerg, *S*. Coravallis,*S*.

Enteritidis, *S*. Infantis,*S*. Kentucky,*S*. Montevideo,*S*. Nchanga,*S*. Salford,*S*. Telaviv,*S*. Senftenberg, *S*. Thompson,*S*. Typhimurium, *S*. Virchow) and as negative controls ATCC strain of 8 different pathogensother than *Salmonella* (*Citrobacter freundii*, *E. coli* O157:H7, *Enterococcus faecalis*, *Listeria innocua*, *Proteus vulgaris*, *Shigella sonnei*, *Staphylococcus auereus*, *Yersinia enterocolitica*) were used.

According to a scientific/technical report submitted to European Food Safety Authority (EFSA), a total of 392,485 *Salmonella* cases were reported in the European Union in the study period (2007-2009) and *S.* Enteritidis was the most widely and frequently reported serovar, followed by *S.* Typhimurium and *S.* Virchow (The percentages in reported *Salmonella* cases of the three serovars: 76.3% in 2009, 80.6% in 2008, 81.8% in 2007) (Pires et al., 2011). Thus, the positive controls consist of 15 *Salmonella* serotypes including *S.* Enteritidis, *S.* Typhimurium and *S.* Virchow are good representatives for *Salmonella* study in foods.

Real-Time PCR amplification plots of 10^5 to 10^0 CFU/ml levels belong to the 15 *Salmonella* serotypes are illustrated in Figure 3.10-3.24. To eliminate the problem encountered in part 3.3 (Cultures with a higher initial concentration reach their stationary phase earlier and are thus longer in this phase, therefore amplification plots nearly overlapped each other also nearly same Cp and concentration values were obtained for all dilution levels after enrichment), firstly after enrichment approximately 10^5 CFU/ml level concentrations were determined with standard plate counts for all *Salmonella* serotypes and DNAs extracted from this concentration were serially diluted to 10^0 CFU/ml level using nuclease free water, then with these DNAs Real-Time PCR experiments and analysis were carried out.

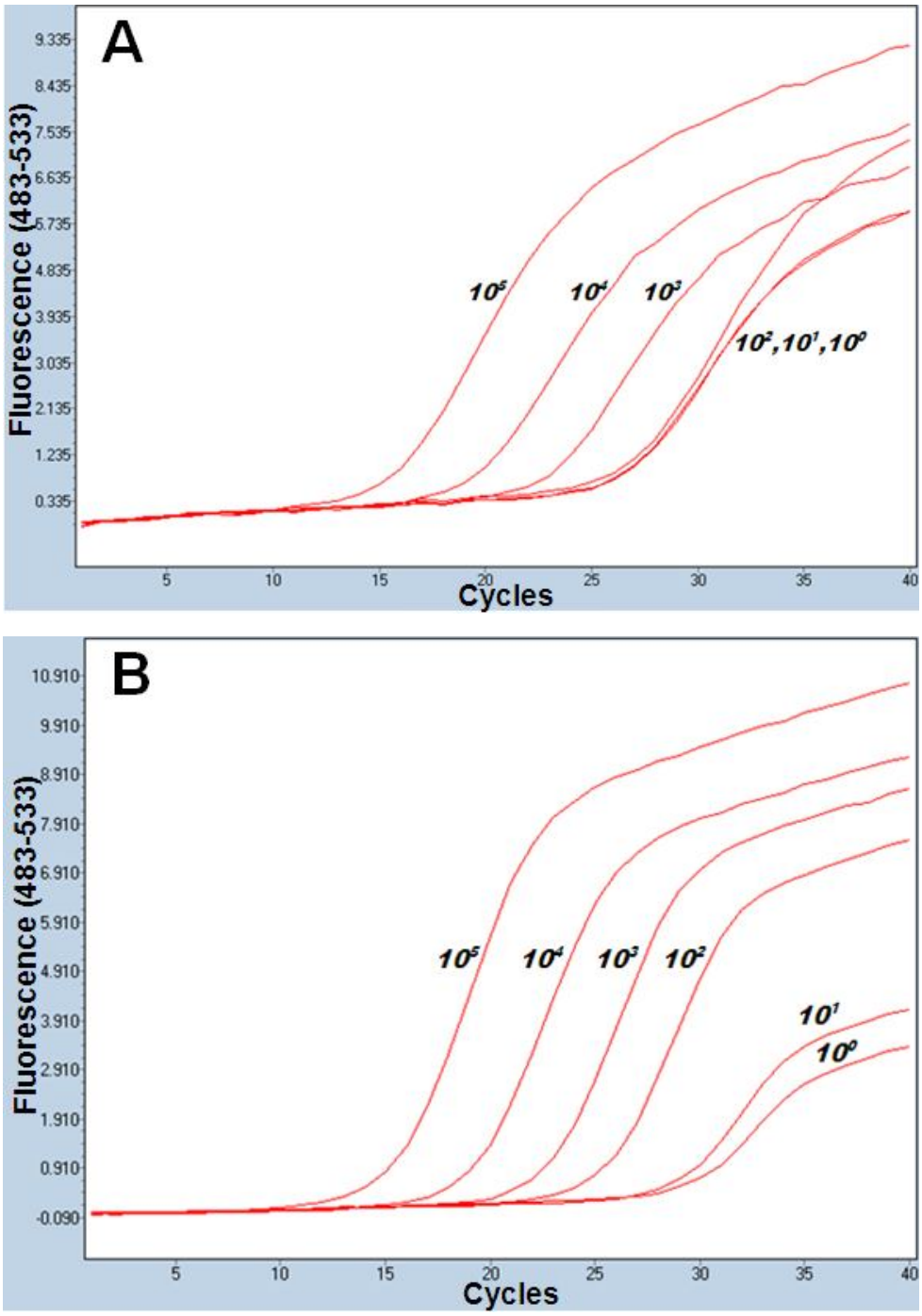


Figure 3.10 Amplification plots of *S*. Agona A) *invA* target B) *ttrRSBC* target.

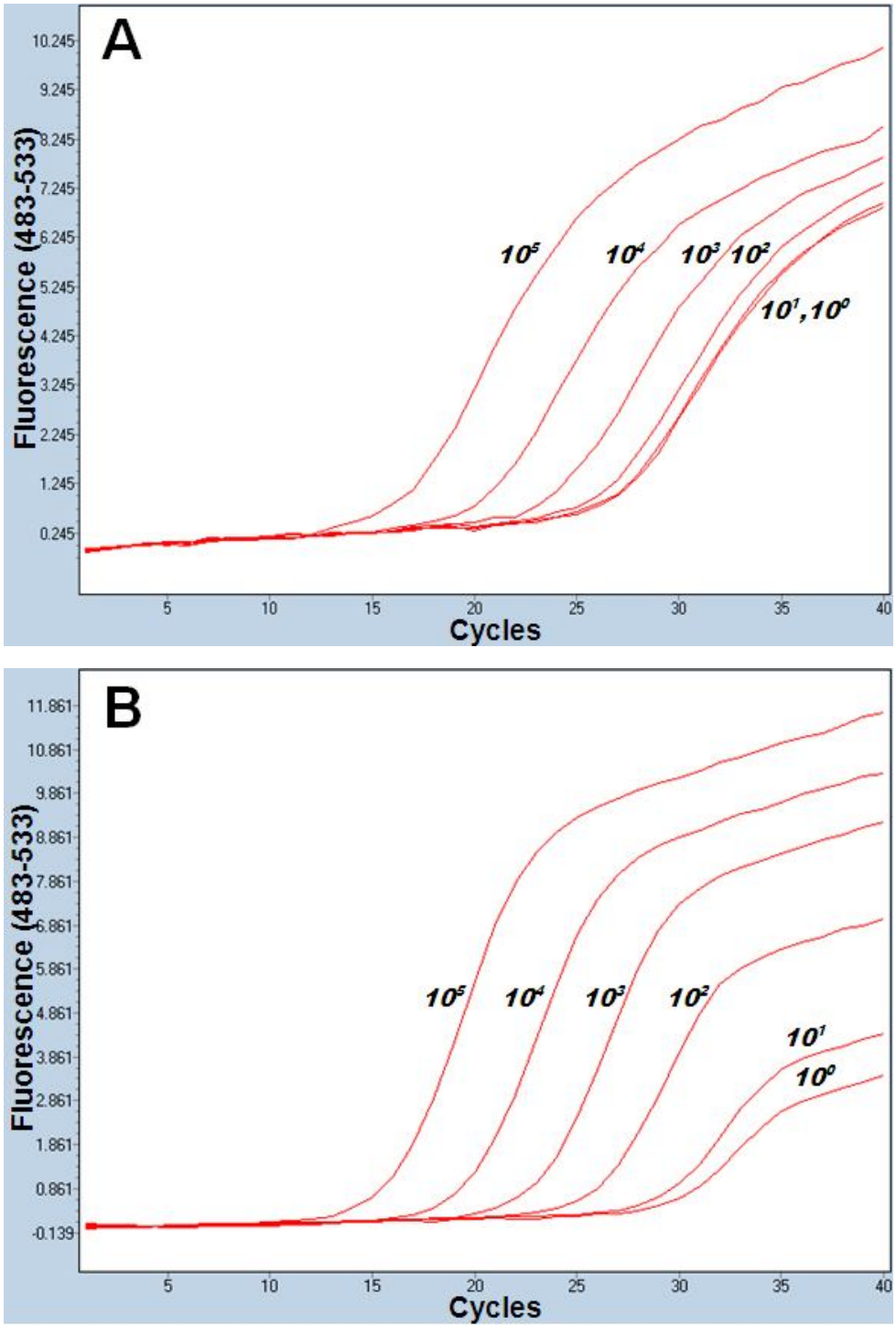


Figure 3.11 Amplification plots of *S*. Anatum A) *invA* target B) *ttrRSBC* target.

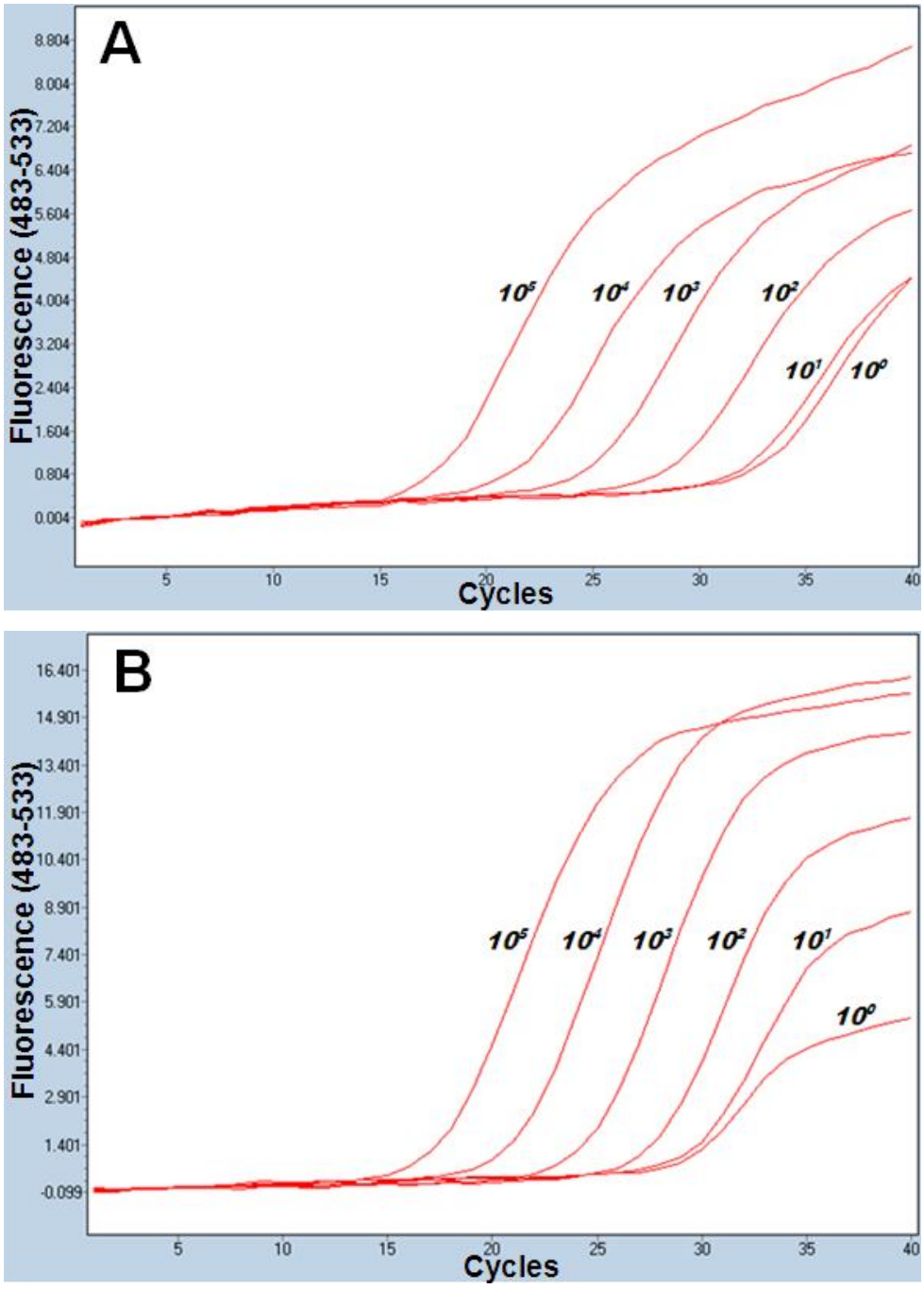


Figure 3.12 Amplification plots of *S*. Bispebjerg A) *invA* target B) *ttrRSBC* target.

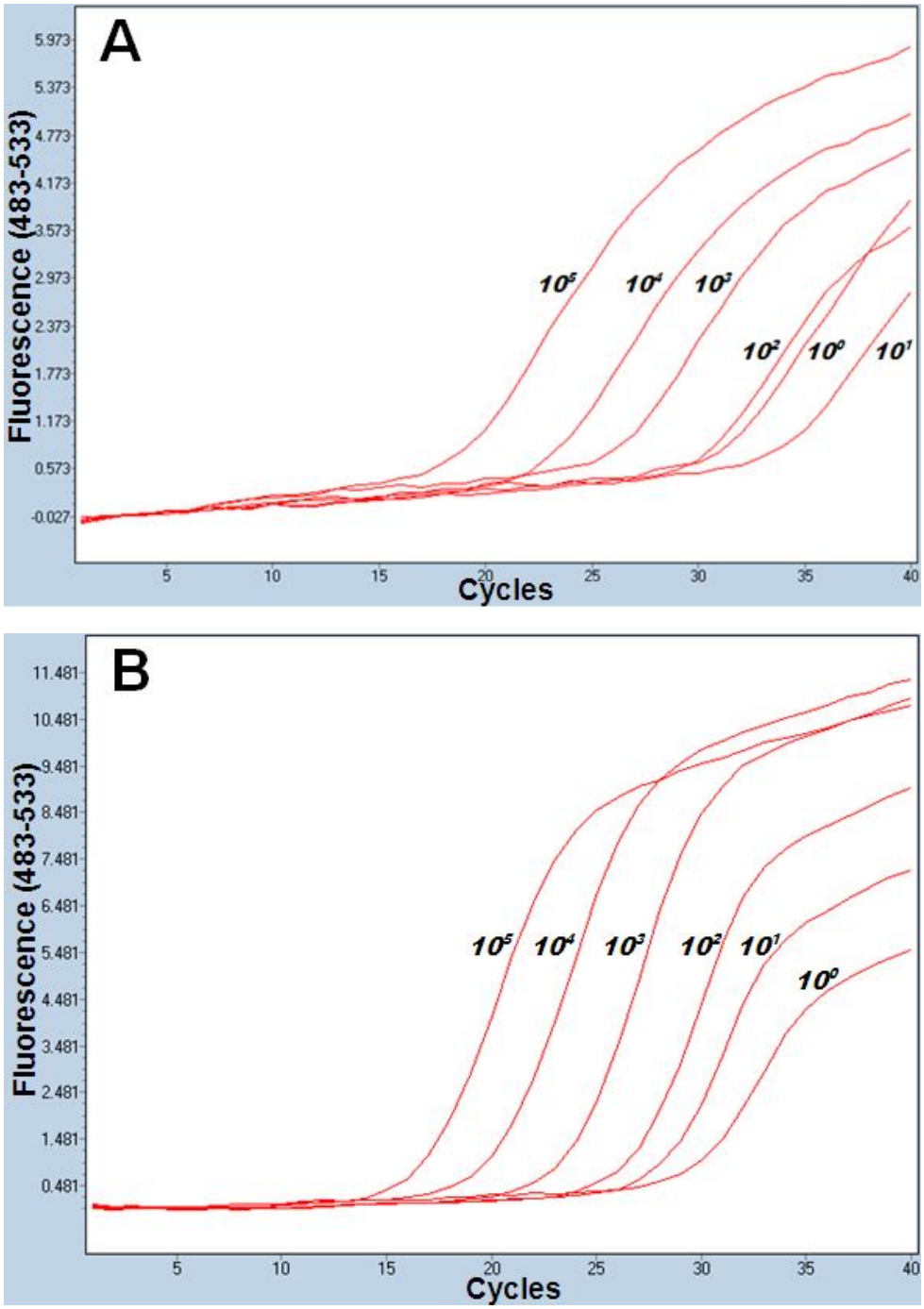


Figure 3.13 Amplification plots of *S*. Corvallis A) *invA* target B) *ttrRSBC* target.

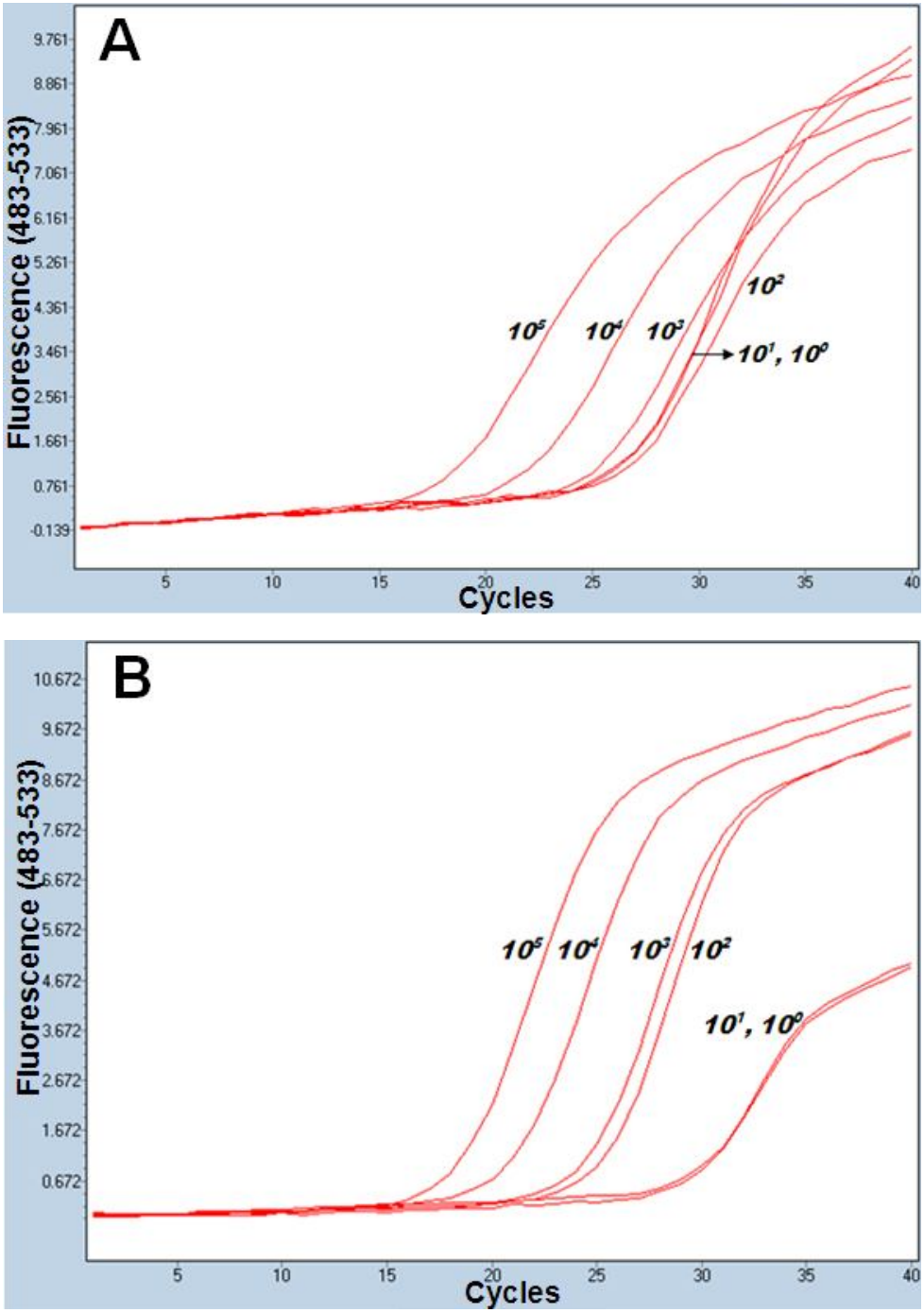


Figure 3.14 Amplification plots of *S*. Enteritidis A) *invA* target B) *ttrRSBC* target.

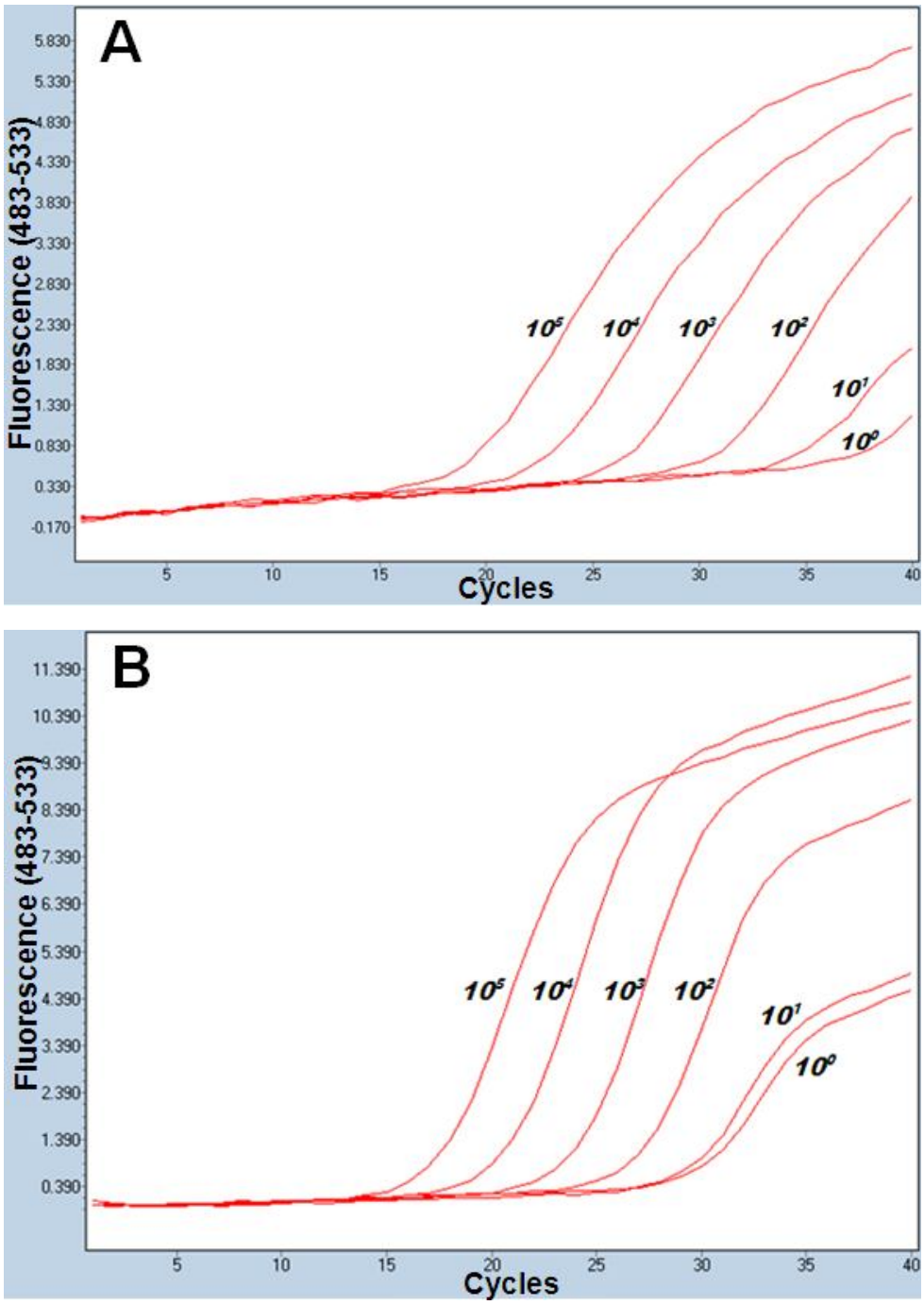


Figure 3.15 Amplification plots of *S*. Infantis A) *invA* target B) *ttrRSBC* target.

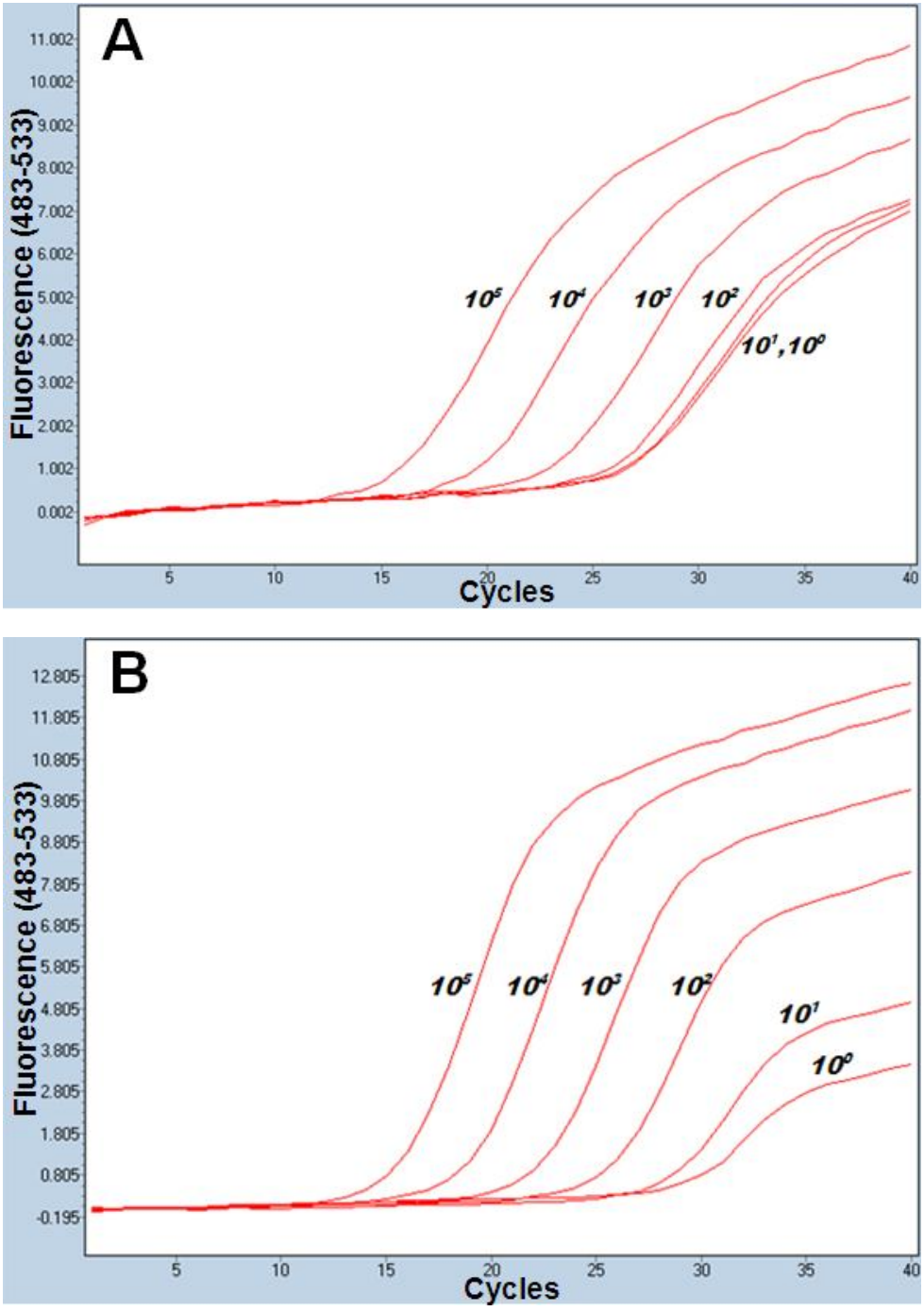


Figure 3.16 Amplification plots of *S*. Kentucky A) *invA* target B) *ttrRSBC* target.

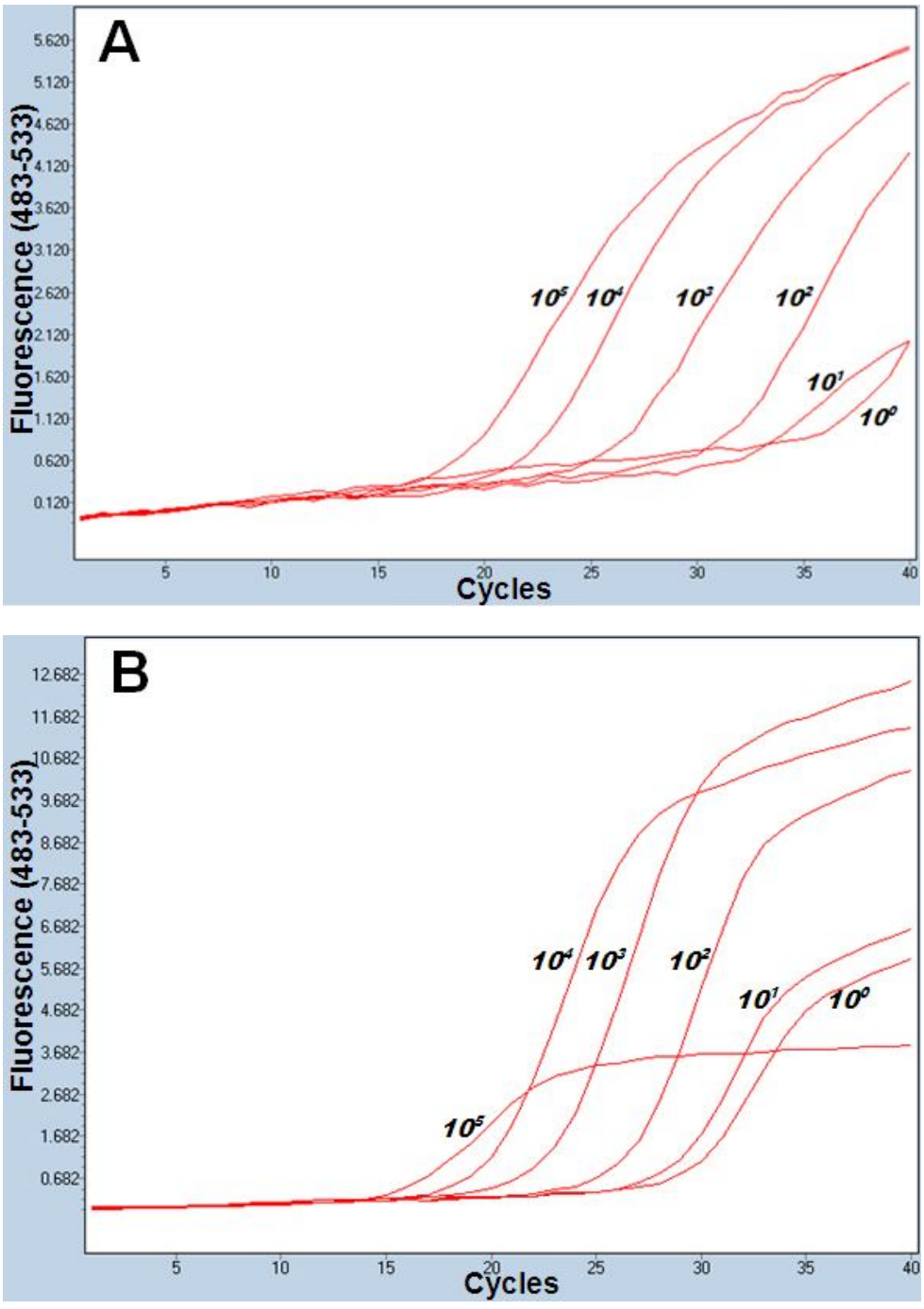


Figure 3.17 Amplification plots of *S*. Montevideo A) *invA* target B) *ttrRSBC* target.

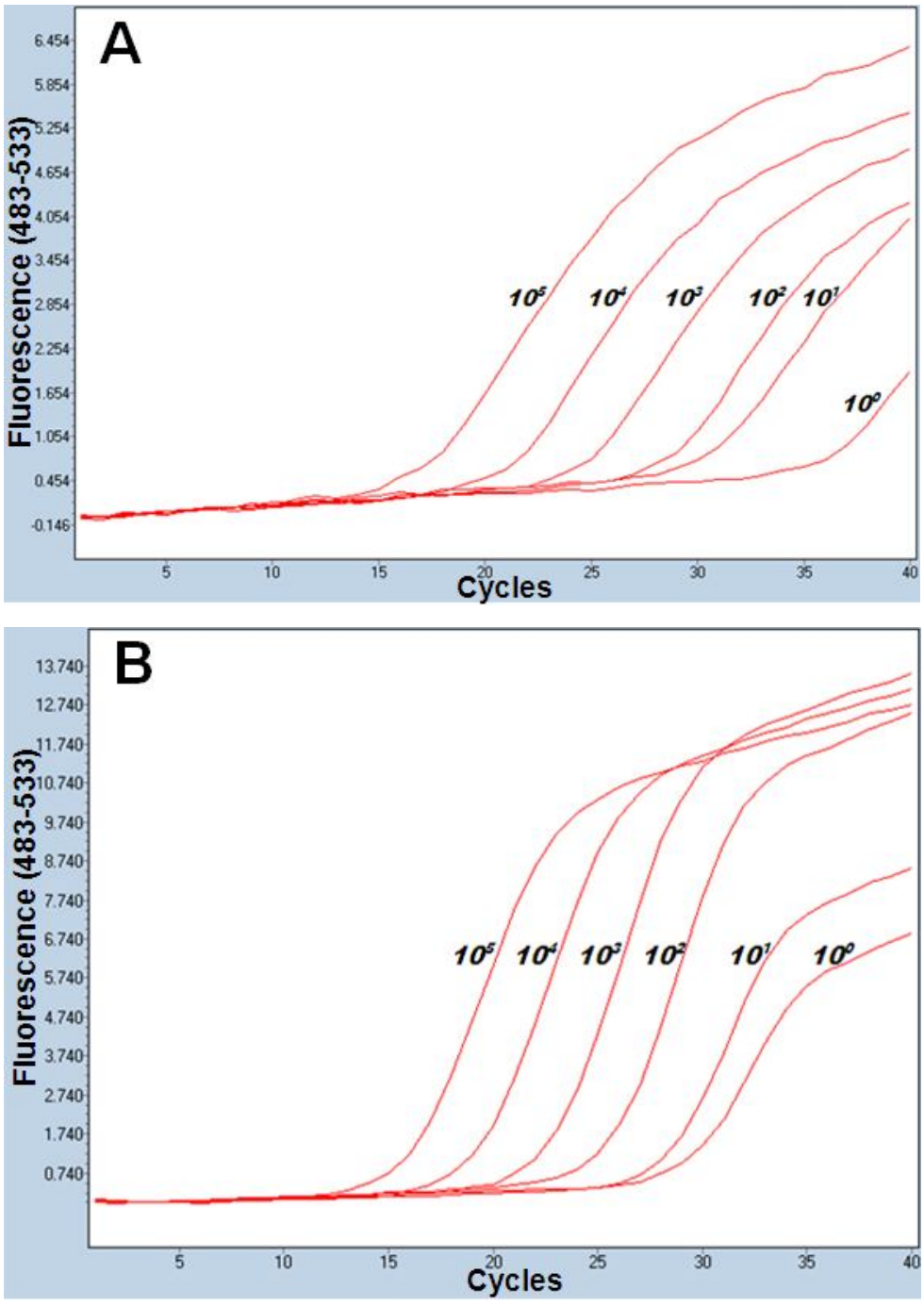


Figure 3.18 Amplification plots of *S*. Nchanga A) *invA* target B) *ttrRSBC* target.

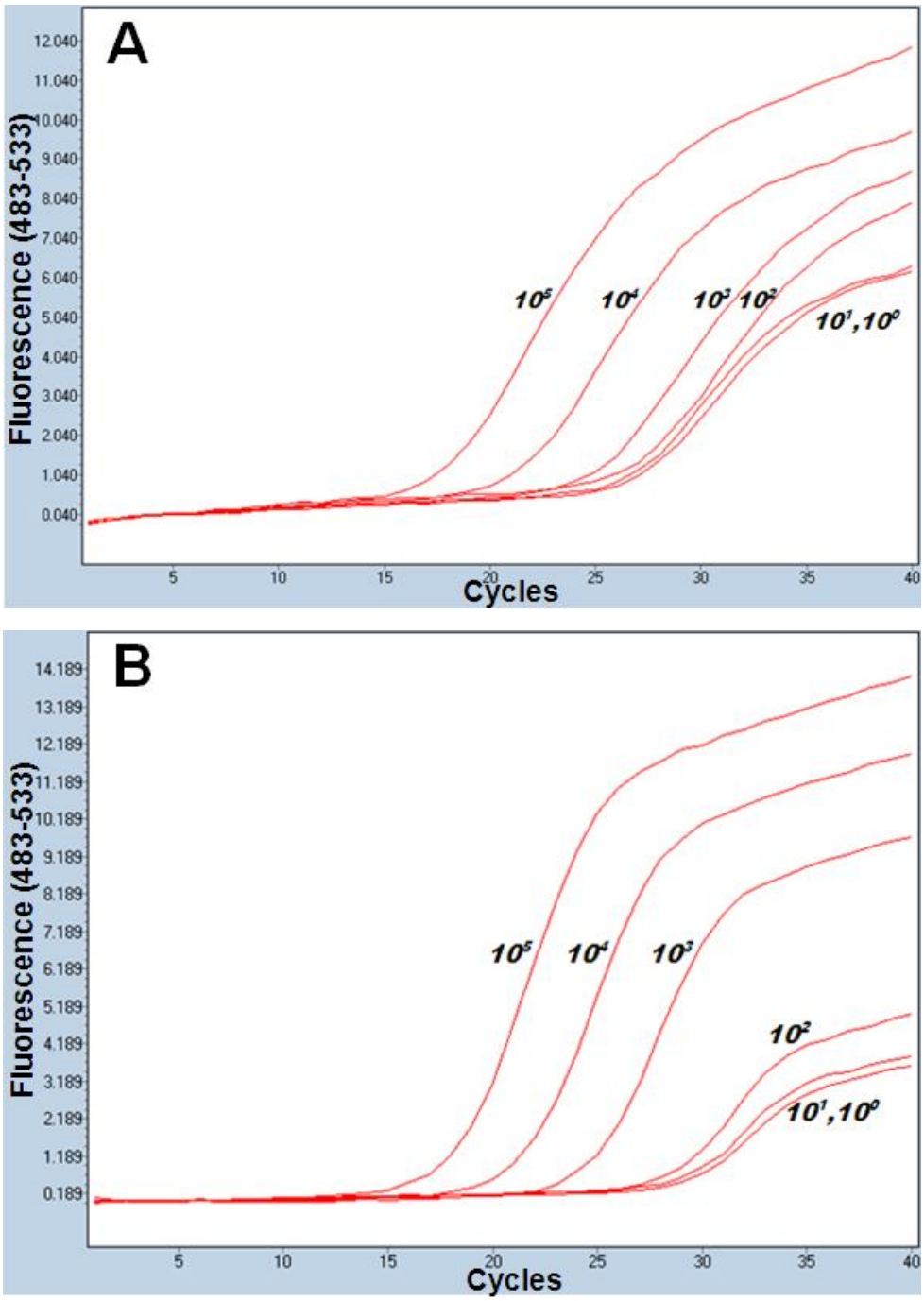


Figure 3.19 Amplification plots of *S*. Salford A) *invA* target B) *ttrRSBC* target.

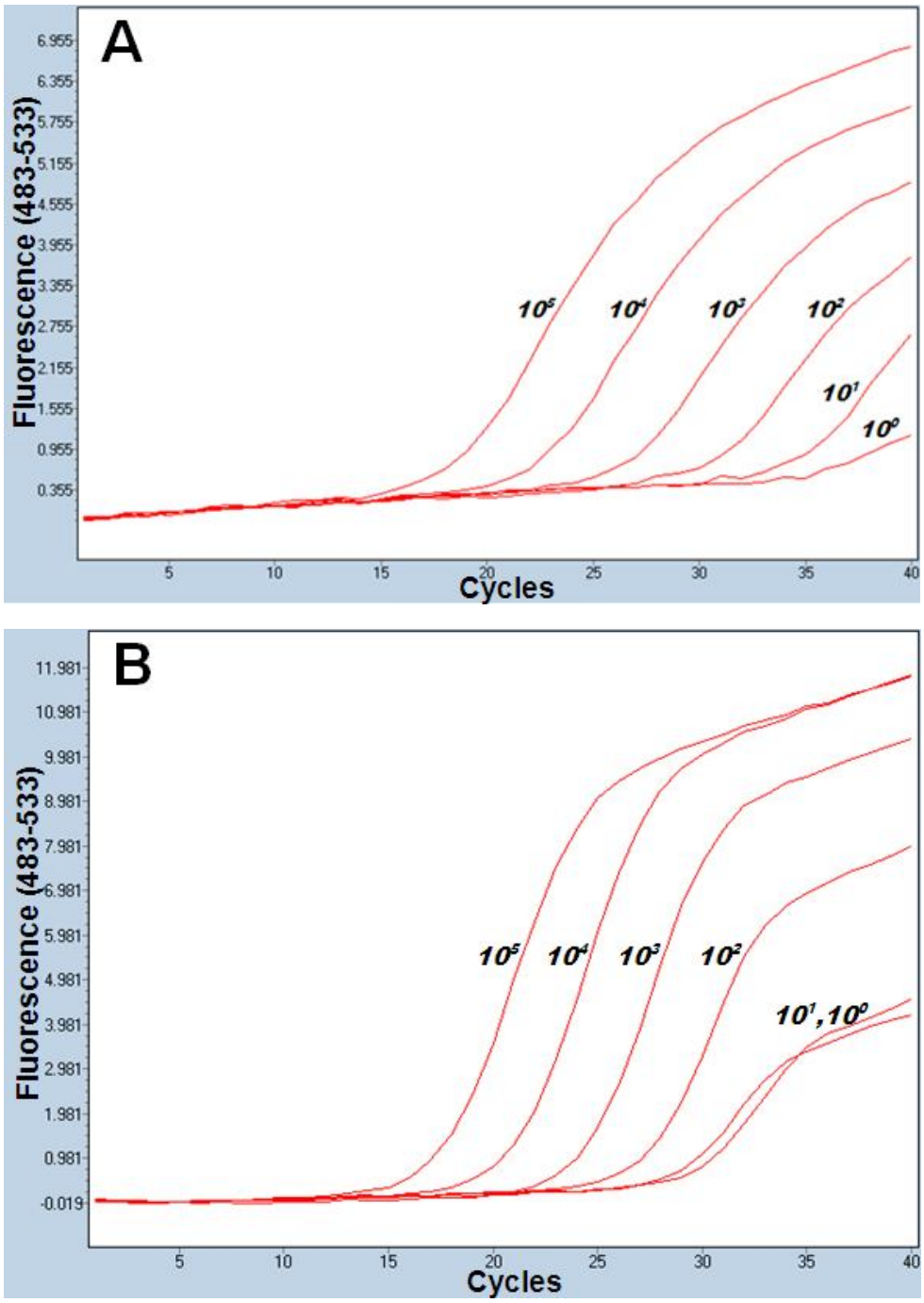


Figure 3.20 Amplification plots of *S*. Senftenberg A) *invA* target B) *ttrRSBC* target.

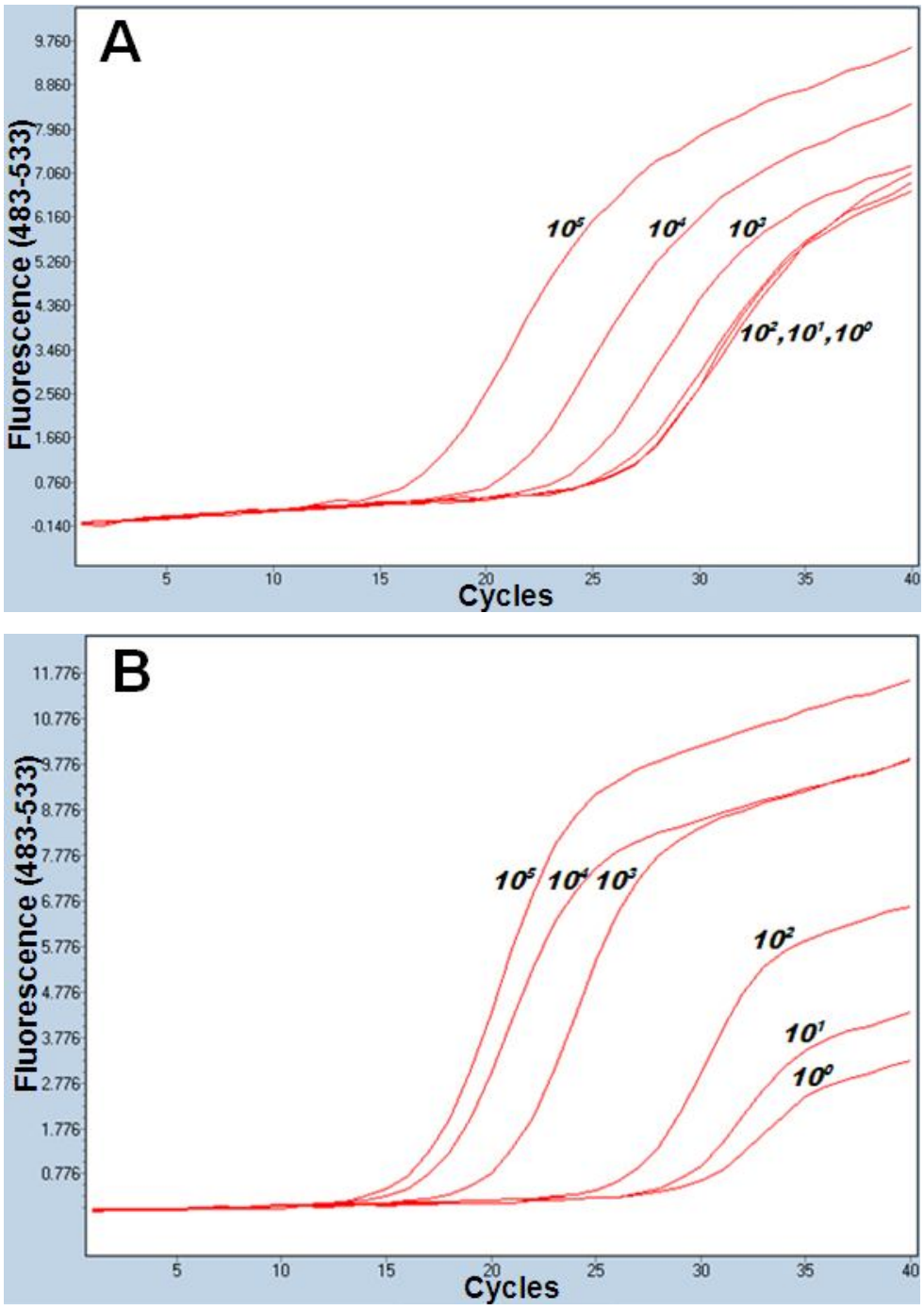


Figure 3.21 Amplification plots of *S*. Telaviv A) *invA* target B) *ttrRSBC* target.

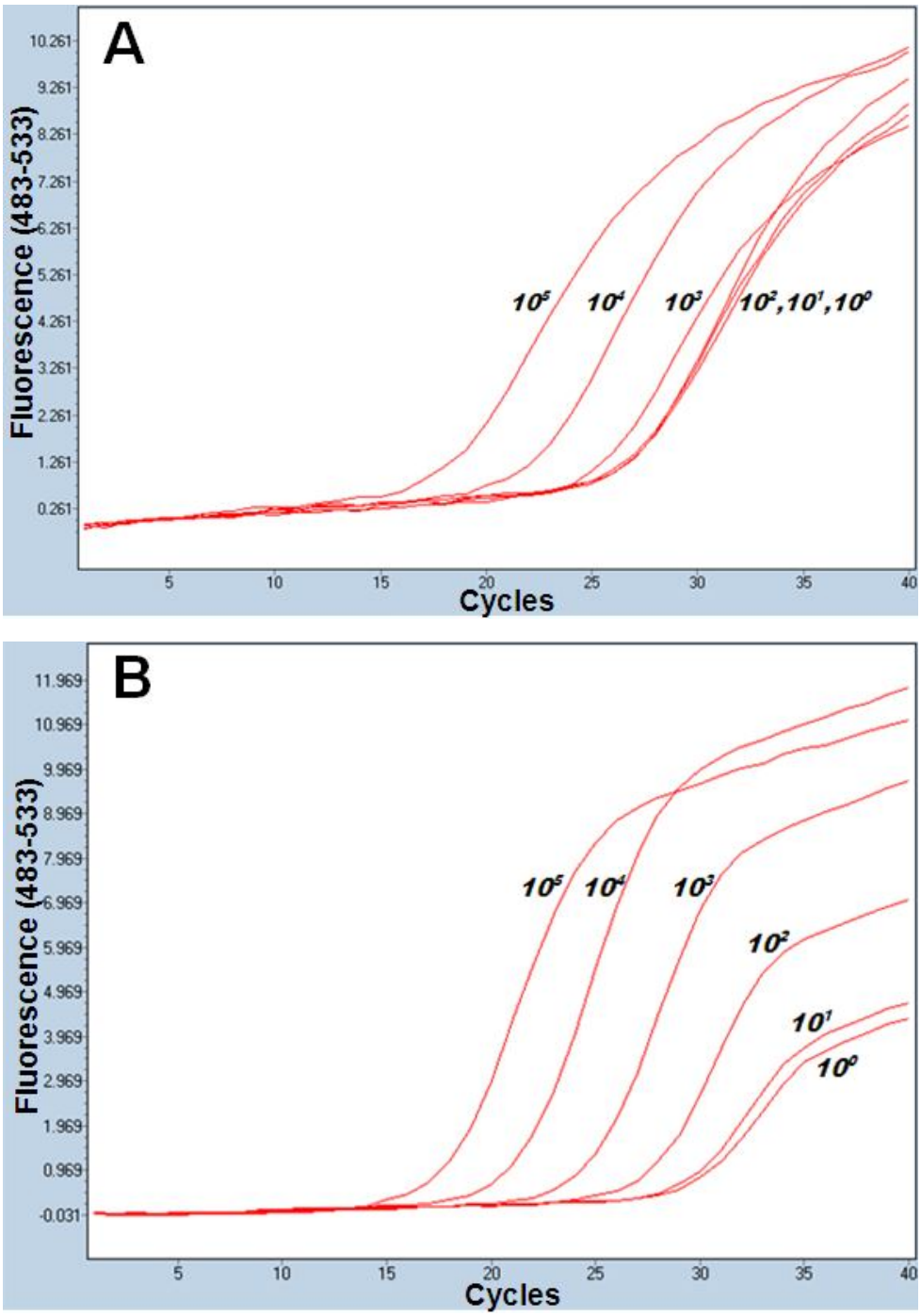


Figure 3.22 Amplification plots of *S*. Thompson A) *invA* target B) *ttrRSBC* target.

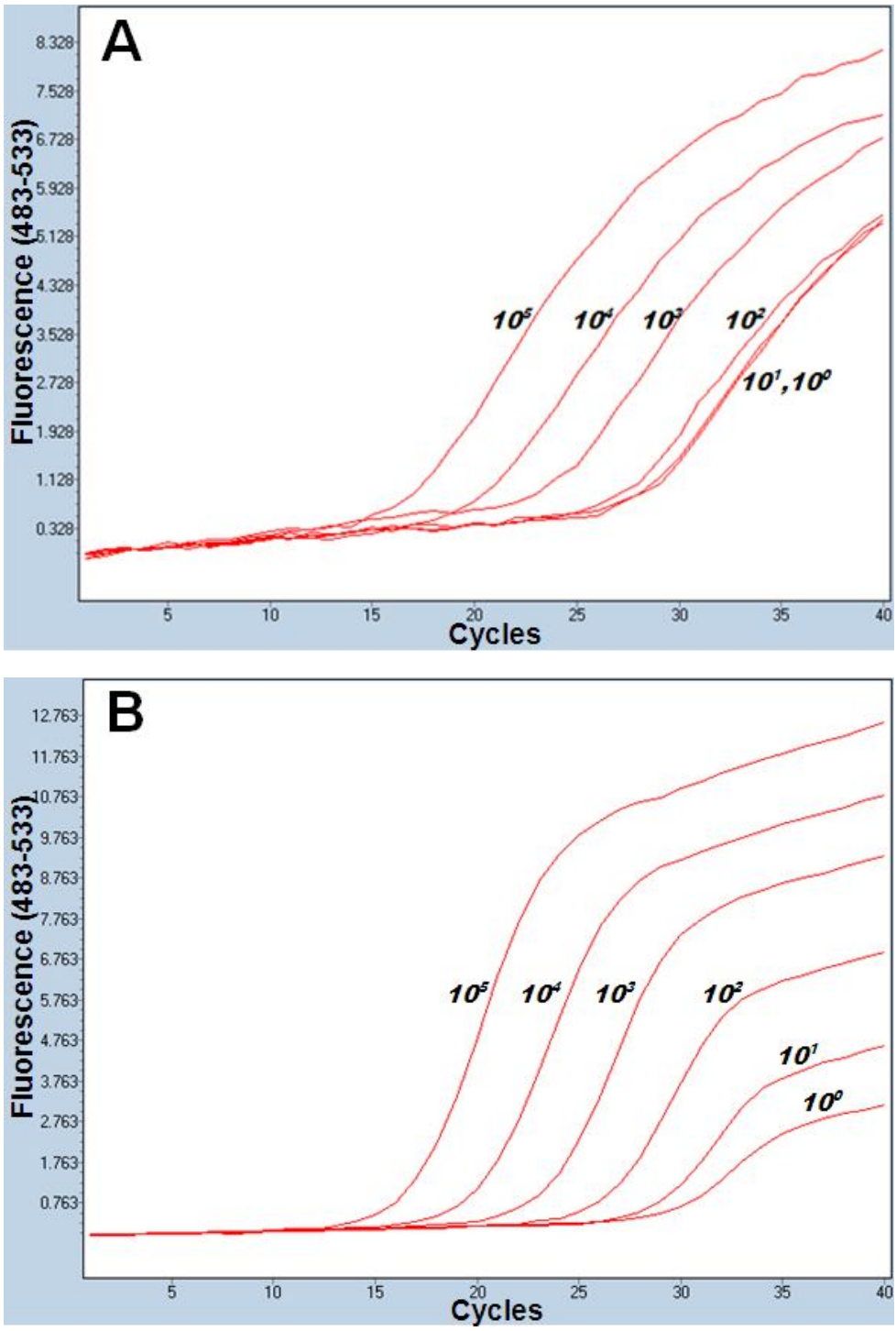


Figure 3.23 Amplification plots of *S*. Typhimurium A) *invA* target B) *ttrRSBC* target.

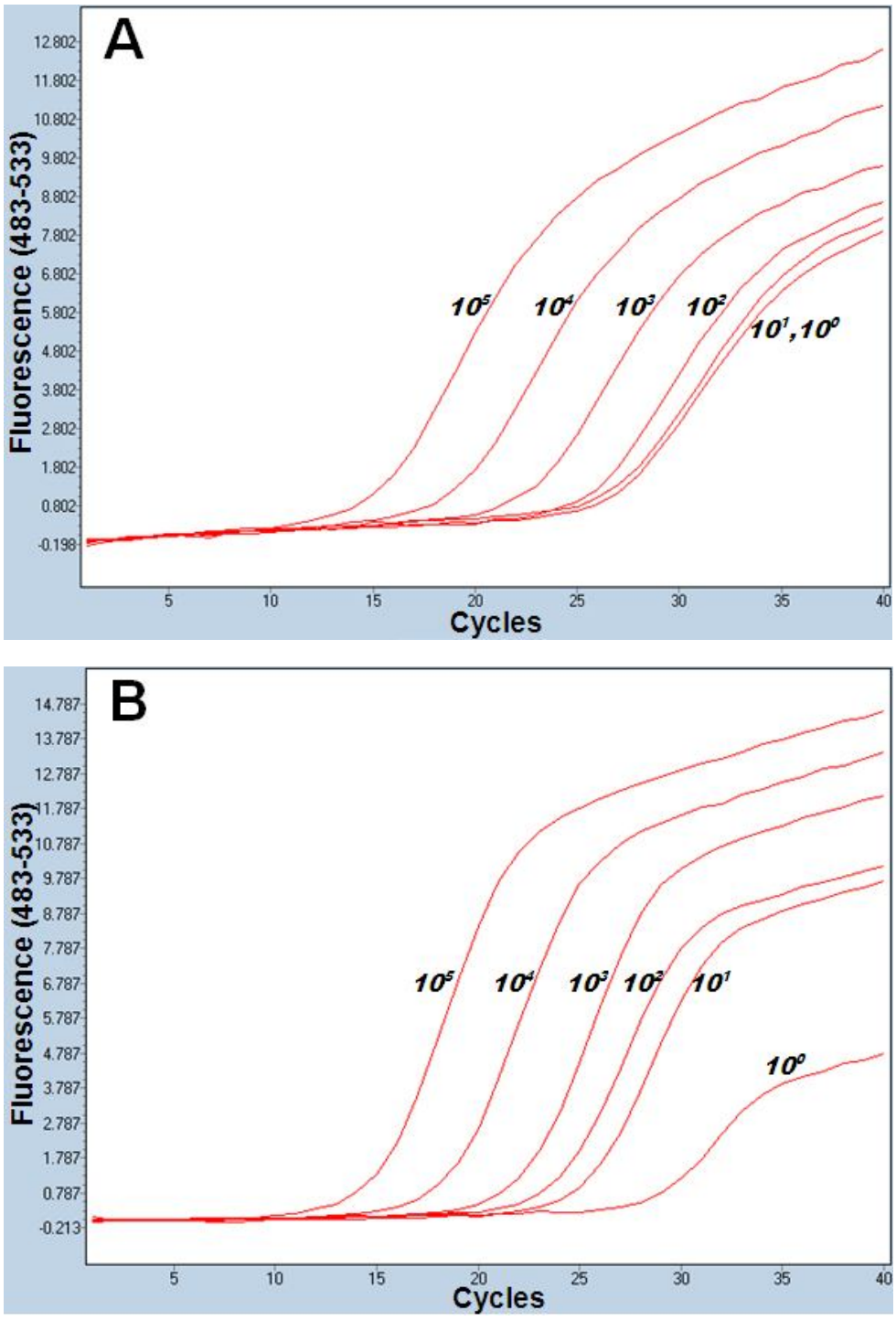


Figure 3.24 Amplification plots of *S*. Virchow A) *invA* target B) *ttrRSBC* target.

By looking roughly at the amplification plots belong to the 15 *Salmonella* serotypes from artificially contaminated milk samples, it can be stated that both *invA* incorporated plasmids and *ttrRSBC* incorporated plasmids are nearly equally effective. However, parallel to the detection limit of the developed method results (see Figure 3.5 and Table 3.3), *ttrRSBC* target is better to discriminate lower concentrations with only two exception *S.* Infantis (see Figure 3.15) and *S.* Senftenberg (see Figure 3.20). As can be seen from amplification plots, for *invA* target all dilutions are discriminated clearly in amplification plots belong to only three serotypes (*S.* Infantis, *S.* Nchanga and *S.* Senftenberg). 10^2 CFU/ml, 10^1CFU/ml and 10^0CFU/ml concentrations are nearly overlap each other in majority of amplification plots (*S.* Agona, *S.* Anatum, *S.* Enteritidis, *S.* Kentucky, *S.* Salford, *S.* Telaviv, *S.* Thompson, *S.* Typhimurium and *S.* Virchow); 10^2 CFU/ml and 10^0CFU/ml concentrations are nearly overlap each other in amplification plots of *S.* Corvallis; 10^1CFU/ml and 10^0CFU/ml concentrations are nearly overlap each other in amplification plots of *S.* Bispebjerg.

On the other hand, for *ttrRSBC* target, overlap occurs between only 10^1CFU/ml and 10^0CFU/ml concentrations in amplification plots of only five serotypes (*S.* Enteritidis, *S.* Infantis, *S.* Salford, *S.* Senftenberg and *S.* Thompson) and there is no overlap containing 10^2 CFU/ml concentration in all amplification plots.

For more precise interpretation, the Cp values belong to the amplification plots, calculated concentrations using constructed satandard curves and statistical analysis of Cp and concentration values are presented in Table 3.4-3.9 for 15 *Salmonella* serotypes.

Table 3.4 Cp values (Mean±SD) belong to the amplification plots of 15 *Salmonella* serotypes for *invA* target

	***invA* Target**					
Serotype	**10^5 CFU/ml**	**10^4 CFU/ml**	**10^3 CFU/ml**	**10^2 CFU/ml**	**10^1 CFU/ml**	**10^0 CFU/ml**
S. Agona	15.87±0.11	19.66±0.18	23.09±0.21	26.67±0.23	26.87±0.24	27.10±0.25
S. Anatum	16.85±0.11	20.63±0.19	23.99±0.19	26.39±0.23	27.15±0.24	27.18±0.26
S. Bispebjerg	17.73±0.12	21.67±0.19	25.11±0.20	28.87±0.24	32.00±0.61	32.91±0.66
S. Corvallis	19.03±0.11	22.95±0.15	26.33±0.22	30.13±0.28	34.46±0.68	31.35±0.65
S. Enteritidis	18.46±0.12	22.21±0.16	25.29±0.21	26.90±0.23	27.00±0.25	27.07±0.24
S. Infantis	19.41±0.12	22.97±0.19	26.58±0.22	31.24±0.29	35.00±0.69	35.00±0.68
S. Kentucky	16.12±0.10	19.77±0.14	23.61±0.20	26.30±0.23	27.09±0.26	27.06±0.25
S. Montevideo	19.02±0.15	22.23±0.20	26.44±0.22	31.77±0.29	32.15±0.63	35.00±0.69
S. Nchanga	17.33±0.13	21.29±0.20	24.70±0.21	28.79±0.24	30.64±0.55	35.00±0.67
S. Salford	18.00±0.12	21.65±0.17	25.20±0.19	27.08±0.24	26.92±0.23	26.72±0.24
S. Senftenberg	18.80±0.13	22.62±0.19	26.83±0.23	30.92±0.29	35.00±0.68	35.00±0.67
S. Telaviv	17.33±0.13	21.30±0.19	24.28±0.20	26.50±0.21	27.09±0.24	27.03±0.24
S. Thompson	18.53±0.10	22.47±0.18	25.34±0.22	26.96±0.23	27.03±0.23	27.10±0.25
S. Typhimurium	16.23±0.14	20.46±0.16	23.70±0.21	26.71±0.23	27.04±0.22	27.05±0.26
S. Virchow	15.47±0.12	19.23±0.16	22.84±0.22	25.83±0.23	27.03±0.23	27.04±0.25

Table 3.5 Cp values (Mean±SD) belong to the amplification plots of 15 *Salmonella* serotypes for *ttrRSBC* target.

	***ttrRSBC* Target**					
Serotype	**10^5 CFU/ml**	**10^4 CFU/ml**	**10^3 CFU/ml**	**10^2 CFU/ml**	**10^1 CFU/ml**	**10^0 CFU/ml**
***S.* Agona**	15.55±0.09	19.11±0.14	22.65±0.18	25.47±0.20	28.70±0.26	29.44±0.30
***S.* Anatum**	15.91±0.08	19.66±0.13	23.03±0.18	25.94±0.20	28.90±0.25	29.62±0.29
***S.* Bispebjerg**	17.66±0.10	21.32±0.15	24.68±0.19	27.66±0.21	29.56±0.27	28.84±0.28
***S.* Corvallis**	16.78±0.09	20.17±0.14	23.66±0.17	26.56±0.22	27.81±0.24	29.53±0.29
***S.* Enteritidis**	18.46±0.11	21.00±0.15	24.53±0.19	25.24±0.20	29.41±0.23	29.48±0.29
***S.* Infantis**	17.47±0.11	20.70±0.16	23.92±0.18	26.96±0.21	28.99±0.23	29.60±0.28
***S.* Kentucky**	15.71±0.09	19.04±0.15	22.44±0.18	25.60±0.20	28.07±0.22	29.00±0.28
***S.* Montevideo**	15.98±0.10	19.97±0.16	23.05±0.18	26.56±0.21	28.31±0.23	29.31±0.29
***S.* Nchanga**	15.93±0.10	19.17±0.15	22.63±019	25.52±0.19	27.89±0.22	28.96±0.28
***S.* Salford**	18.25±0.12	21.35±0.16	24.60±0.19	28.21±0.24	28.89±0.23	29.06±0.28
***S.* Senftenberg**	17.53±0.11	20.92±0.15	24.15±0.17	27.07±0.24	28.49±0.24	29.54±0.29
***S.* Telaviv**	16.87±0.10	17.53±0.14	20.56±0.18	26.83±0.23	28.78±0.24	29.64±0.30
***S.* Thompson**	17.81±0.11	21.25±0.16	24.61±0.18	27.44±0.22	29.02±0.25	29.45±0.29
***S.* Typhimurium**	16.75±0.10	20.02±0.15	23.30±0.17	26.17±0.23	28.34±0.24	29.34±0.28
***S.* Virchow**	14.98±0.08	18.57±0.13	22.10±0.18	25.18±0.20	28.45±0.24	29.79±0.29

As mentioned in Real-Time PCR theoretical explanation part, Ct values (Cp value in Roche Light Cycler 480: Software calculates second derivatives of all amplification plots and determines maximum value called as Cp illustrates the cycle at which maximum increase in fluorescence signal and start of logarithmic phase of PCR occur (Stahlberg et al., 2005)) are always inversely proportional to the concentration (Valasek and Repa, 2005) and theoretically, a PCR reaction proceeding at 100% efficiencywould require 3.3 cycles for 10 fold increase (Microbial, http://www.microbial-systems.com/web/docs/Results_interpretation_guide_ENGv2.pdf, visited on 05.02.2012).

For both *invA* and *ttrRSBC* targets, in amplification plots belong to high concentrations (10^5CFU/ml, 10^4 CFU/ml and 10^3 CFU/ml), differences of Cp values belong to the successive concentrations (A, B and C columns in Table 3.6) are consistent with the theory mentioned above with a few exception. For *invA* target, only three serotypes (*S.* Enteritidis, *S.* Salford and *S.*Thompson) have three abnormal difference values in C column (Differences of Cp values belong to 10^3CFU/ml and 10^2CFU/ml) out of 45 difference values belong to 15 *Salmonella* serotypes for 10^5 CFU/ml, 10^4 CFU/ml and 10^3 CFU/ml concentrations in A, B and C columns in Table 3.6. On the other hand, for *ttrRSBC* target only two serotypes *S.* Telaviv and*S.* Enteritidis, have abnormal difference values in high concentrations. *S.* Telaviv has two abnormal difference values in A (Differences of Cp values belong to 10^5CFU/ml and 10^4CFU/ml) and C columns, while S. Enteritidis has an abnormal difference value in C column out of 45 difference values belong to 15 *Salmonella* serotypes for 10^5 CFU/ml, 10^4 CFU/ml and 10^3 CFU/ml concentrations in A, B and C columns in Table 3.6.

On the contrary, in low concentrations (10^2 CFU/ml, 10^1 CFU/ml and 10^0 CFU/ml) for both *invA* and *ttrRSBC* targets, great majority of the differences of Cp values belong to the successive concentrations (D and E columns in Table 3.6) are inconsistent with the theory. For *invA* target, only *S.* Bispebjerg, *S.* Corvallis, *S.* Infantis and *S.* Senftenberg have Cp difference values in D column (Differences of Cp values belong to 10^2 CFU/ml and 10^1 CFU/ml) close to the theory, while differences of Cp values belong to *S.* Corvallis, *S.* Montevideo and *S.* Nchanga close to the theory in E column (Differences of Cp values belong to 10^1 CFU/ml and 10^0 CFU/ml). For *ttrRSBC* target, Cp difference values of six serotypes (*S.* Agona, *S.* Anatum, *S.* Enteritidis, *S.* Kentucky, *S.* Nchanga and *S.* Virchow) close to the theoretical value in D column, but all of the difference values in E column are inconsistent with the theoretical value.

On the other hand, when the all difference values belong to the successive concentrations for *invA* and *ttrRSBC* targets are compared, it should be stated that the values for *ttrRSBC* target are more homogen and better discrimination capability for low concentrations. Therefore, both *invA* incorporated plasmids and *ttrRSBC* incorporated plasmids have 100 % *Salmonella* detection ability in all concentrations however, *ttrRSBC* incorporated plasmids are better to discriminate thus, they are better to determine exact bacterial number in low concentrations.

Table 3.6 Differences of Cp values belong to successive concentrations of 15 *Salmonella* serotypes for *invA* and *ttrRSBC* targets.
A: Cp of 10^4CFU/ml-10^5CFU/ml B:Cp of 10^3CFU/ml-10^4CFU/ml
C: Cp of 10^2CFU/ml-10^3CFU/ml D:Cp of 10^1CFU/ml-10^2CFU/ml
E: Cp of 10^0CFU/ml-10^1CFU/ml

	***invA* Target**					***ttrRSBC* Target**				
Serotype	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**
***S.* Agona**	3.79	3.43	3.58	0.20	0.23	3.56	3.54	2.82	3.23	0.74
***S.* Anatum**	3.78	3.36	2.40	0.76	0.03	3.75	3.37	2.91	2.96	0.72
***S.* Bispebjerg**	3.94	3.44	3.76	3.13	0.91	3.66	3.36	2.98	1.90	0.72
***S.* Corvallis**	3.92	3.38	3.80	4.33	3.11	3.39	3.49	2.90	1.25	1.72
***S.* Enteritidis**	3.75	3.08	1.61	0.10	0.07	2.54	3.53	0.71	4.17	0.07
***S.* Infantis**	3.56	3.61	1.66	3.76	0.00	3.23	3.22	3.01	2.03	0.61
***S.* Kentucky**	3.65	3.84	2.69	0.79	0.03	3.33	3.40	3.16	2.47	0.93
***S.* Montevideo**	3.21	4.21	5.33	0.38	2.85	3.99	3.08	3.51	1.75	1.00
***S.* Nchanga**	3.96	3.41	4.09	1.65	4.36	3.24	3.46	2.69	2.37	1.07
***S.* Salford**	3.65	3.55	1.88	0.16	0.20	3.10	3.25	3.61	0.68	0.17
***S.* Senftenberg**	3.82	4.21	4.09	4.08	0.00	3.39	3.23	2.92	1.42	1.05
***S.* Telaviv**	3.97	2.98	2.22	0.59	0.06	0.66	3.03	6.27	1.95	0.86
***S.* Thompson**	3.94	2.87	1.62	0.07	0.07	3.44	3.36	2.83	1.58	0.43
***S.* Typhimurium**	4.23	3.24	3.01	0.69	0.35	3.27	3.28	2.87	2.17	1.00
***S.* Virchow**	3.76	3.61	2.99	1.20	0.01	3.59	3.53	3.08	3.27	1.34

Using the Cp values for *invA* and *ttrRSBC* targets presented in Table 3.4 and 3.5 respectively, exact concentrations of 10^5 to 10^0 CFU/ml level (Approximately determined by using traditional microbiological method, ISO 6579:2004) *Salmonella* cultures were calculated using constructed standard curves for *invA* and *ttrRSBC* targets and presented in Table 3.7 and 3.8 respectively.

The tables illustrate that the developed plasmid based Real-Time PCR method has perfect sensitivity for both *invA* and *ttrRSBC* targets for *Salmonella* analysis in milk. The developed method has the detection limit of ~10^1 CFU/ml (The concentration values presented in Table 3.7 and 3.8 illustrateDNA Copy Number / 225ml and 10^3 level was taken into account as minimum concentration because it was minimum calculated concentration for most of the *Salmonella* serotypes with a few exception for *invA* target so they were neglected) for both target. *ttrRSBC* incorporated plasmids are better to discriminate thus, they are better to determine exact bacterial number in low concentrations which may result from genetic instability and natural deletions within *Salmonella* pathogenicity island 1 encompassing the *inv* loci as previously mentioned. The proposed detection approach showed 100% concordance with the traditional culture method (ISO 6579:2004) in terms of *Salmonella* detection. These results are exactly consistent with the results mentioned in part 3.4 and this proves the great potential of the developed method for *Salmonella* detection and quantification in milk as a food model.

Table 3.7 Concentration values (DNA Copy Number/225ml)belong to 15 *Salmonella* serotypes for *invA* target calculated from constructed standard curve using Cp values in Table 3.4.

	invA Target					
Serotype	**10^5 CFU/ml**	**10^4 CFU/ml**	**10^3 CFU/ml**	**10^2 CFU/ml**	**10^1 CFU/ml**	**10^0 CFU/ml**
S. Agona	$1.73x10^7$	$1.26x10^6$	$1.18x10^5$	$1.00x10^4$	$8.72x10^3$	$7.44x10^3$
S. Anatum	$8.78x10^6$	$6.47x10^5$	$6.36x10^4$	$1.21x10^4$	$7.19x10^3$	$7.04x10^3$
S. Bispebjerg	$4.78x10^6$	$3.16x10^5$	$2.94x10^4$	$2.19x10^3$	$2.53x10^2$	$1.35x10^2$
S. Corvallis	$1.95x10^6$	$1.30x10^5$	$1.27x10^4$	$9.20x10^2$	$4.64x10^1$	$3.96x10^2$
S. Enteritidis	$2.89x10^6$	$2.17x10^5$	$2.59x10^4$	$8.54x10^3$	$7.97x10^3$	$7.60x10^3$
S. Infantis	$1.50x10^6$	$1.29x10^5$	$1.07x10^4$	$4.28x10^2$	$3.19x10^1$	$3.19x10^1$
S. Kentucky	$1.45x10^7$	$1.17x10^6$	$8.27x10^4$	$1.29x10^4$	$7.49x10^3$	$7.65x10^3$
S. Montevideo	$1.96x10^6$	$2.14x10^5$	$1.17x10^4$	$2.97x10^2$	$2.28x10^2$	$3.19x10^1$
S. Nchanga	$6.30x10^6$	$4.10x10^5$	$3.90x10^4$	$2.32x10^3$	$6.47x10^2$	$3.19x10^1$
S. Salford	$3.97x10^6$	$3.20x10^5$	$2.76x10^4$	$7.55x10^3$	$8.43x10^3$	$3.67x10^3$
S. Senftenberg	$2.29x10^6$	$1.64x10^5$	$8.97x10^3$	$5.33x10^2$	$3.19x10^1$	$3.19x10^1$
S. Telaviv	$6.30x10^6$	$4.07x10^5$	$5.21x10^4$	$1.13x10^4$	$7.49x10^3$	$7.81x10^3$
S. Thompson	$2.75x10^6$	$1.82x10^5$	$2.51x10^4$	$8.20x10^3$	$7.81x10^3$	$7.44x10^3$
S. Typhimurium	$1.35x10^7$	$7.27x10^5$	$7.77x10^4$	$9.74x10^3$	$7.76x10^3$	$7.70x10^3$
S. Virchow	$2.27x10^7$	$1.70x10^6$	$1.41x10^5$	$1.79x10^4$	$7.81x10^3$	$7.76x10^3$

Table 3.8 Concentration values (DNA Copy Number/225ml) belong to 15 *Salmonella* serotypes for *ttrRSBC* target calculated from constructed standard curve using Cp values in Table 3.5.

	***ttrRSBC* Target**					
Serotype	**10^5 CFU/ml**	**10^4 CFU/ml**	**10^3 CFU/ml**	**10^2 CFU/ml**	**10^1 CFU/ml**	**10^0 CFU/ml**
S. Agona	$2.42x10^7$	$2.62x10^6$	$1.93x10^5$	$2.83x10^4$	$3.14x10^3$	$1.90x10^3$
S. Anatum	$1.89x10^7$	$1.48x10^6$	$1.49x10^5$	$2.05x10^4$	$2.74x10^3$	$1.68x10^3$
S. Bispebjerg	$5.76x10^6$	$4.77x10^5$	$4.84x10^4$	$6.37x10^3$	$1.75x10^3$	$2.85x10^3$
S. Corvallis	$1.05x10^7$	$1.04x10^6$	$9.70x10^4$	$1.35x10^4$	$5.75x10^3$	$1.78x10^3$
S. Enteritidis	$3.34x10^6$	$5.93x10^5$	$5.36x10^4$	$3.31x10^4$	$1.94x10^3$	$1.85x10^3$
S. Infantis	$6.55x10^6$	$6.93x10^5$	$8.12x10^4$	$1.03x10^4$	$2.58x10^3$	$1.70x10^3$
S. Kentucky	$2.17x10^7$	$2.25x10^6$	$2.22x10^5$	$2.59x10^4$	$4.82x10^3$	$2.56x10^3$
S. Montevideo	$1.81x10^7$	$1.20x10^6$	$1.47x10^5$	$1.35x10^4$	$4.09x10^3$	$2.07x10^3$
S. Nchanga	$1.87x10^7$	$1.92x10^6$	$1.83x10^5$	$2.73x10^4$	$5.45x10^3$	$2.63x10^3$
S. Salford	$3.85x10^6$	$4.67x10^5$	$5.11x10^4$	$4.38x10^3$	$2.76x10^3$	$2.46x10^3$
S. Senftenberg	$6.29x10^6$	$6.26x10^5$	$6.95x10^4$	$9.52x10^3$	$3.62x10^3$	$1.77x10^3$
S. Telaviv	$9.86x10^6$	$6.29x10^6$	$8.00x10^5$	$1.12x10^4$	$2.97x10^3$	$1.66x10^3$
S. Thompson	$5.20x10^6$	$5.00x10^5$	$5.08x10^4$	$7.40x10^3$	$2.53x10^3$	$1.88x10^3$
S. Typhimurium	$1.07x10^7$	$1.16x10^6$	$1.24x10^5$	$1.76x10^4$	$4.01x10^3$	$2.03x10^3$
S. Virchow	$3.57x10^7$	$3.10x10^6$	$2.80x10^5$	$3.45x10^4$	$3.72x10^3$	$1.50x10^3$

Using t test it was investigated whether there is statistically significant difference between *Salmonella* detection and quantification ability of *invA* incorporated plasmids and *ttrRSBC* incorporated plasmids. Two-tailed p values for Cp and calculated concentrations belong to 15 *Salmonella* serotypes are presented in Table 3.9.

Table 3.9 Statistical analysis of Cp and concentration values for *invA* and *ttrRSBC* targets belong to 15 *Salmonella* serotypes.

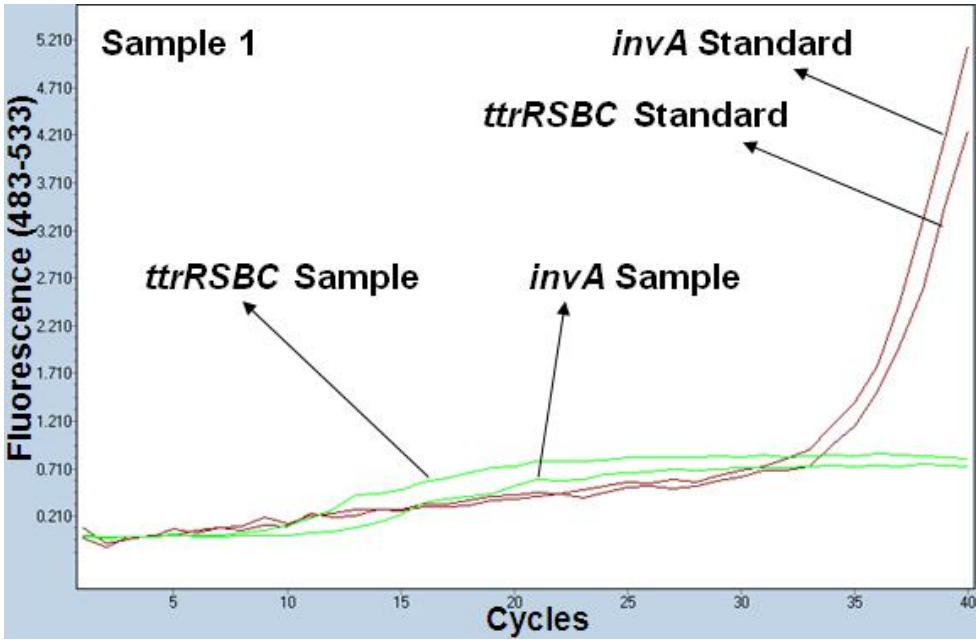
P Value		
Serotype	**Cp**	**Concentration**
***S.* Agona**	0.9265	0.7813
***S.* Anatum**	0.9595	0.6075
***S.* Bispebjerg**	0.6553	0.8773
***S.* Corvallis**	0.3088	0.3836
***S.* Enteritidis**	0.9330	0.8451
***S.* Infantis**	0.2800	0.4077
***S.* Kentucky**	0.9959	0.7494
***S.* Montevideo**	0.2671	0.3588
***S.* Nchanga**	0.4018	0.4842
***S.* Salford**	0.7435	0.9942
***S.* Senftenberg**	0.3064	0.5060
***S.* Telaviv**	0.8493	0.4191
***S.* Thompson**	0.8825	0.6408
***S.* Typhimurium**	0.8695	0.8940
***S.* Virchow**	0.9305	0.7343

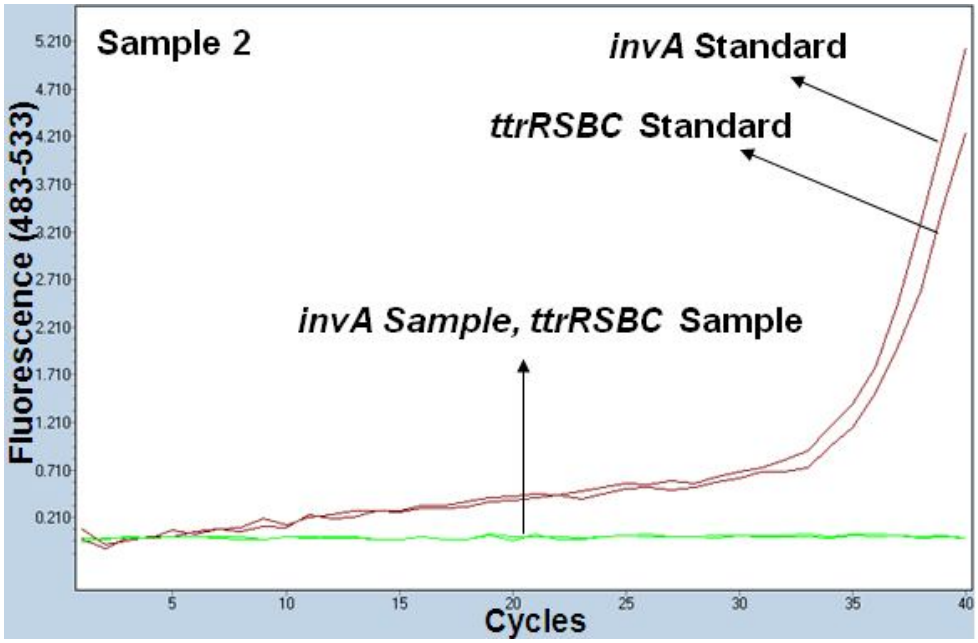
All P values belong to 15 *Salmonella* serotypes for both parameters were found as much greater than 0.05 representing statistically insignificant difference ($p<0.05$) for Cp and concentration parameters, thus representing statistically insignificant difference ($p<0.05$) between *Salmonella* detection and quantification ability of *invA* incorporated plasmids and *ttrRSBC* incorporated plasmids.

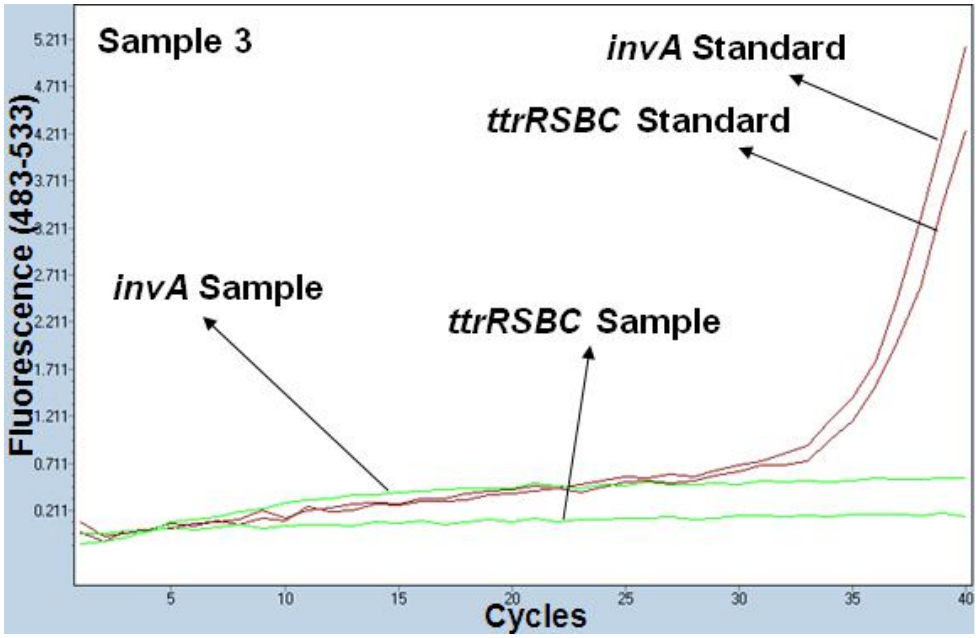
3.6 *SALMONELLA* ANALYSIS OF RAW MILK SAMPLES IN ISTANBUL

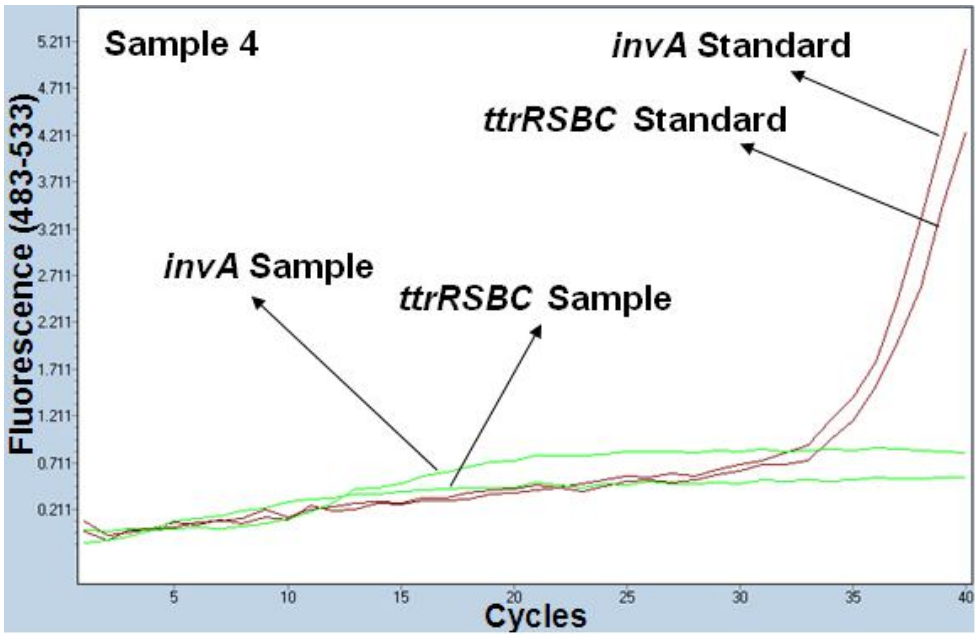
In previous part, the developed recombinant plasmid based Real Time PCR mehod was applied for enumeration of *Salmonella* in artificially contaminated milk samples. In this part, the developed recombinant plasmid based Real Time PCR mehod was applied to naturally contaminated bulk tank milk samples collected from ten milk vendors throughout Istanbul.

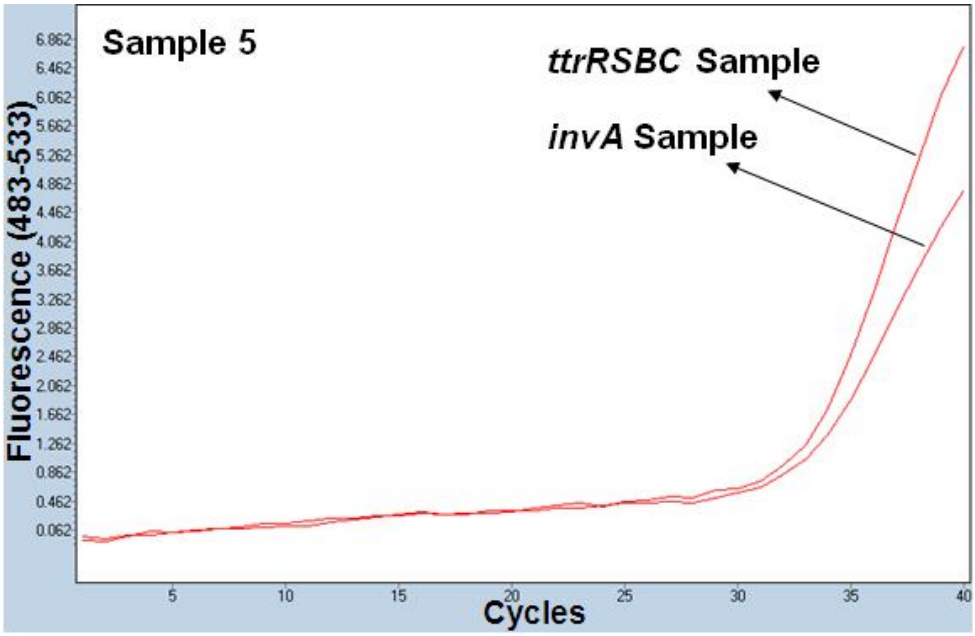
As illustrated in Figure 3.25 two of the ten milk samples were found as positive (5th and 8th samples in the figure).

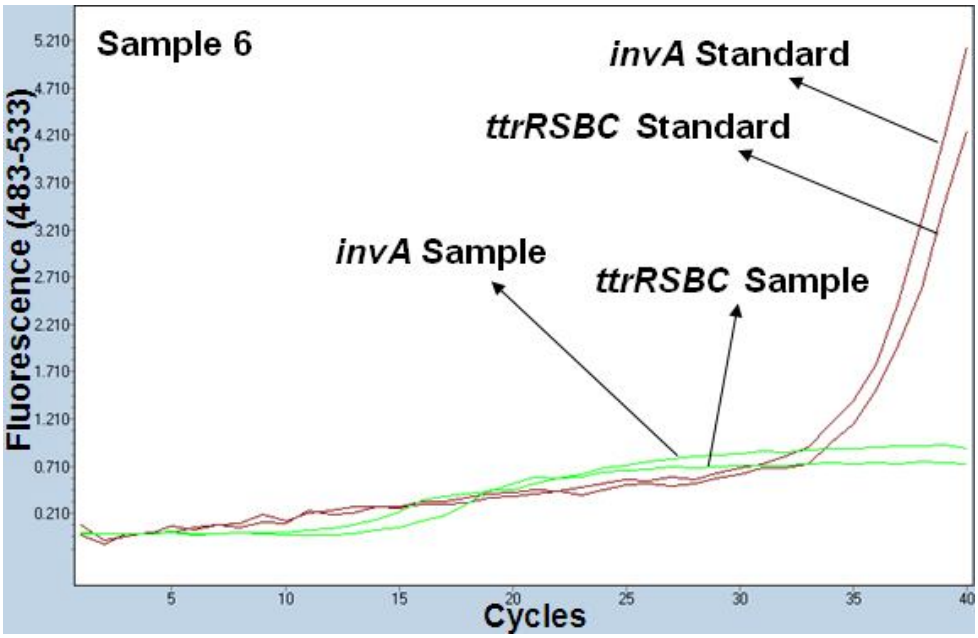

Sample 1
invA Standard
ttrRSBC Standard
ttrRSBC Sample
invA Sample
Fluorescence (483-533)
5.210
4.710
4.210
3.710
3.210
2.710
2.210
1.710
1.210
0.710
0.210
5
10
15
20
25
30
35
40
Cycles

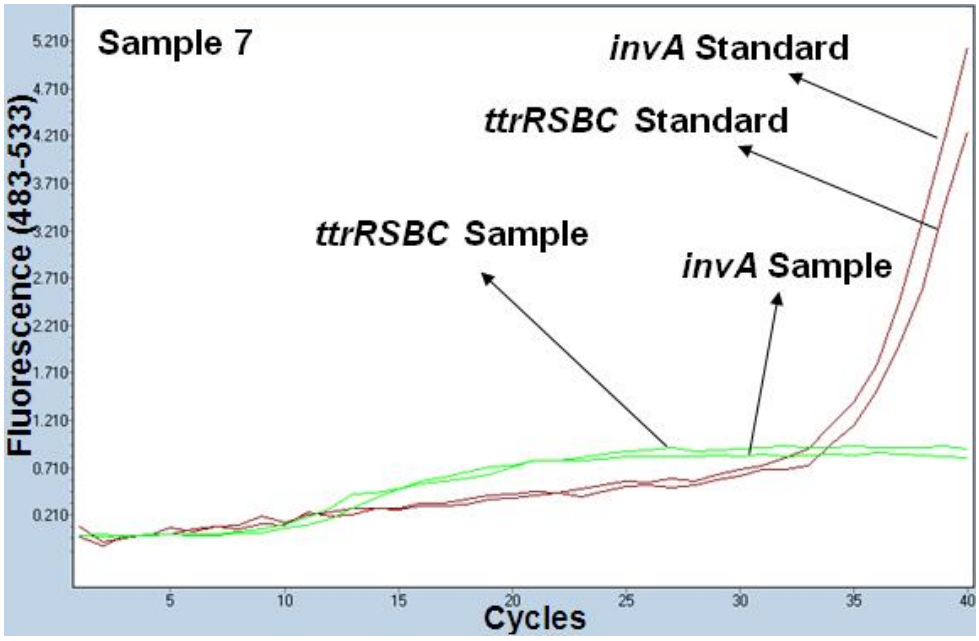

Sample 2
invA Standard
ttrRSBC Standard
invA Sample, ttrRSBC Sample
Fluorescence (483-533)
5.210
4.710
4.210
3.710
3.210
2.710
2.210
1.710
1.210
0.710
0.210
5
10
15
20
25
30
35
40
Cycles

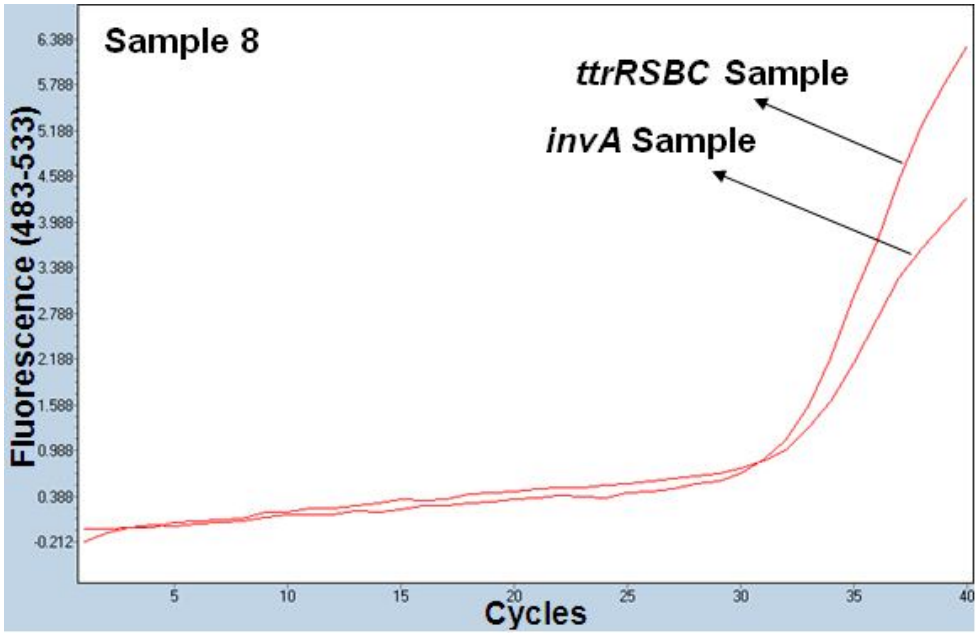

Sample 3
invA Standard
ttrRSBC Standard
invA Sample
ttrRSBC Sample
Fluorescence (483-533)
5.211
4.711
4.211
3.711
3.211
2.711
2.211
1.711
1.211
0.711
0.211
5
10
15
20
25
30
35
40
Cycles


Sample 4
invA Standard
ttrRSBC Standard
invA Sample
ttrRSBC Sample
Fluorescence (483-533)
5.211
4.711
4.211
3.711
3.211
2.711
2.211
1.711
1.211
0.711
0.211
5
10
15
20
25
30
35
40
Cycles


Sample 5
ttrRSBC Sample
invA Sample
Fluorescence (483-533)
6.862
6.462
6.062
5.662
5.262
4.862
4.462
4.062
3.662
3.262
2.862
2.462
2.062
1.662
1.262
0.862
0.462
0.062
5
10
15
20
25
30
35
40
Cycles


Sample 6
invA Standard
ttrRSBC Standard
invA Sample
ttrRSBC Sample
Fluorescence (483-533)
5.210
4.710
4.210
3.710
3.210
2.710
2.210
1.710
1.210
0.710
0.210
5
10
15
20
25
30
35
40
Cycles


Sample 7
invA Standard
ttrRSBC Standard
ttrRSBC Sample
invA Sample
Fluorescence (483-533)
5.210
4.710
4.210
3.710
3.210
2.710
2.210
1.710
1.210
0.710
0.210
5
10
15
20
25
30
35
40
Cycles


Sample 8
ttrRSBC Sample
invA Sample
Fluorescence (483-533)
6.388
5.788
5.188
4.588
3.988
3.388
2.788
2.188
1.588
0.988
0.388
-0.212
5
10
15
20
25
30
35
40
Cycles

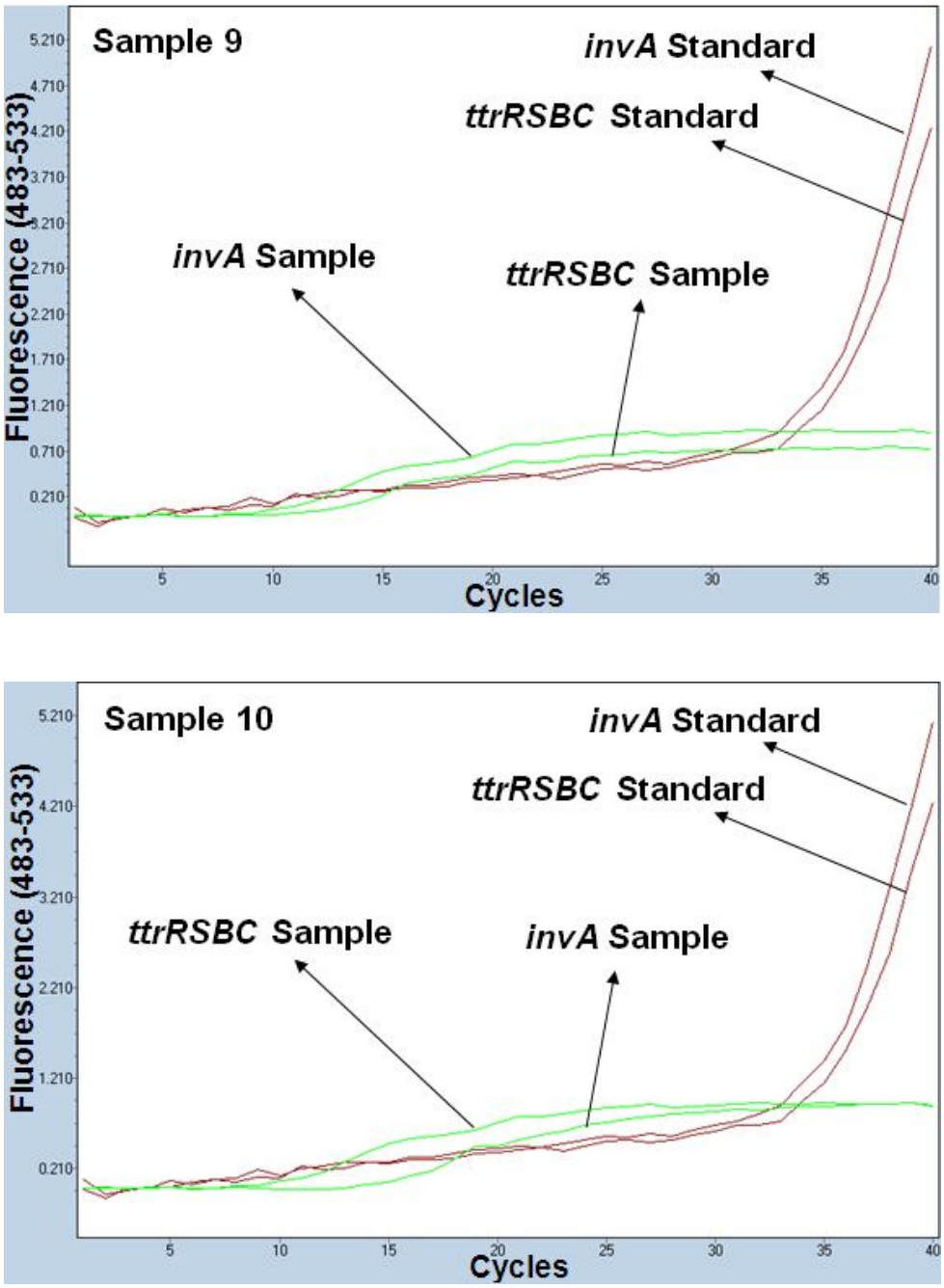


Figure 3.25 Amplification plots belong to *Salmonella* analysis of ten raw milk samples with the developed recombinant plasmid based Real Time PCR mehod.

Table 3.10 Detailed *Salmonella* analysis of ten raw milk samples with the developed recombinant plasmid based Real Time PCR mehod.

Samples	Traditional Culture Method According to ISO 6579:2004	P-invA		P-ttrRSBC	
		Cp	Concentration[a]	Cp	Concentration[a]
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	+	29.07	$1.91x10^3$	28.99	$2.58x10^3$
6	-	-	-	-	-
7	-	-	-	-	-
8	+	28.11	$3.71x10^3$	27.94	$5.27x10^3$
9	-	-	-	-	-
10	-	-	-	-	-

[a]Concentrations were calculated from constructed standard curves using Cp values belong to the samples. Concentration= DNA Copy Number / 225 ml.

Detailed analysis revealed that positive samples (5^{th} and 8^{th} samples) have approximately 10^1 CFU / ml *Salmonlla* concentration.

Successful application of the developed recombinant plasmid based Real Time PCR method to naturally contaminated milk samples reflects great potential of the method for milk industry: much faster detection ability in comparison to traditional culture method ISO 6579:2004 (21 h vs 90 h) with perfect reproducibility, sensitivity (except for lower concentrations for *invA* target), detection limit, PCR efficiency, amplification efficiency for both *invA* and *ttrRSBC* targets. The developed method allowing quantitative evaluation is crucial also to estimate the microbiological risks and factors.

CHAPTER 4

CONCLUSION

In the present study, for the first time it was investigated applicability of plasmids as a inexpensive, easy to produce, convenient, standardized positive control for detection and quantification of most important gram negative bacteria causing foodborne gastroenteritis –*Salmonella* using rapid and reliable Real-Time PCR technique to create an alternative for labor intensive (series steps including serological and biochemical tests after enrichment, selective enrichment and selective solid medium) and time consuming (up to 7 days) internationally accepted traditional microbiological methods (ISO 6579:2004).

Two plasmids were constructed as reference molecules by cloning two most commonly used *Salmonella* specific target regions '*invA* and *ttrRSBC*' into them to use as standard positive controls for Real-Time PCR based detection and quantification of *Salmonella*.

Constructed standard curves for *invA* and *ttrRSBC* incorporated plasmids illustrated perfect reproducibility, consistency, PCR efficiency, amplification efficiency.

Investigation of the detection limit study illustrated that the developed plasmid based Real-Time PCR method has perfect sensitivity for both *invA* and *ttrRSBC* targets. When standardized procedure (ISO 6579:2004) was followed, detection limit was as little as 1 CFU/225 ml

and the proposed detection approach showed 100% concordance with the traditional culture method (ISO 6579:2004) in terms of *Salmonella* detection. Although 100% concordance with the traditional cuture method (ISO 6579:2004) in terms of detection, the developed method allowed higher bacterial counts, this situation was most probably caused by much more sensitivity of the developed method. Above all, the developed method is much faster than traditional culture method (ISO 6579:2004) in addition to its much more sensitivity and much less labor intensive. While traditional culture method (ISO 6579:2004) took 90 h without biochemical and serological test, *invA* or *ttrRSBC* incorporated plasmids based *Salmonella* detection method took only 21 h (18h enrichment + 1h DNA isolation + 2h Real-Time PCR) with absolute quantification ability. When the DNAs were diluted with nuclease free water, P-invA and P-ttrRSBC based *Salmonella* detection method could detect 10^1 CFU/ml, but *ttrRSBC* target was better to discriminate lower concentrations. That is, the developed method has a capability to detect 10 CFU in 1ml for both target without any enrichment step.

invA incorporated plasmids and *ttrRSBC* incorporated plasmids based *Salmonella* detection and quantification method developed by using *S.* Typhimurium ATCC 14028 was tested to detect and quantify artificially added 15 *Salmonella* serotypes in milk as a food model. There was no bacterial colony formation on petri dishes when different commercially available UHT and pasteurized milk samples from different hypermarkets were used due to the possible antibiotic residues and additives acting bactericidal activity against artificially added 15 *Salmonella* serotypes to the samples. Therefore, detection and quantification of artificially added 15 *Salmonella* serotypes in milk as a food model was carried out with raw milk samples after they were boiled. However, observed bactericidal activity of commercially

available UHT and pasteurized milk samples deserve further researches to clarify this activity.

Application of the developed method to milk gave 100% consistent results with that of method standardization with *S.* Typhimurium ATCC 14028. Although in low concentrations *ttrRSBC* incorporated plasmids are better to discriminate thus they are better to determine exact bacterial number, statistically insignificant difference ($p<0.05$) of Cp and concentration parameters for both target belong to 15 *Salmonella* serotypes illustrates statistically insignificant difference ($p<0.05$) between *Salmonella* detection and quantification ability of *invA* incorporated plasmids and *ttrRSBC* incorporated plasmids in milk samples. Thus, the determined detection limit of 10^1 CFU/ml for both target illustrates capability of the developed method to detect 10 CFU in 1ml milk for both target without any enrichment step. And the proposed approach showed 100% concordance with the traditional culture method (ISO 6579:2004) but it was much faster, much more sensitive and much less labor intensive than traditional one in terms of *Salmonella* detection in milk.

Successful application of the developed method to milk reflects great potential of the method for milk industry: much faster detection ability (in comparison to traditional culture method, ISO 6579:2004) with perfect reproducibility, sensitivity (except for lower concentrations for *invA* target), detection limit, PCR efficiency, amplification efficiency for both *invA* and *ttrRSBC* targets. The developed method allowing quantitative evaluation is crucial also to estimate the microbiological risks and factors.

In conclusion, it was developed rapid, sensitive, efficient, quantitative *Salmonella* detection method based on inexpensive, easy to produce,

convenient and standardized plasmid (*invA* incorporated pTZ57R/T plasmids and *ttrRSBC* incorporated pTZ57R/T plasmids) based positive control for the first time and it was illustrated the applicability of the developed method in milk industry with much faster quantification ability according to traditional methods (21 h vs 90 h), perfect reproducibility, sensitivity (except for lower concentrations for *invA* target), detection limit, PCR efficiency, amplification efficiency and above all absolute quantification ability allowing risk assessment. Extracting DNA from milk in sufficient concentration and purity is of crucial importance due to the fact that milk contains PCR inhibitors such as fats, proteins and calcium causing DNA amplification problems. Thus, perfect application of the developed method to milk having problematic ingredients for Real-Time PCR technique proves the applicability of the developed method for all food industry.

The only limitation of this study is contribution of intact DNA from dead cells to quantification. Prolonged persistence of DNA after cell death can result in false-positive identification and overestimation and using selective nucleic acid intercalating dyes like ethidium monoazide and propidium monoazide is one of the alternative approaches to detecting and quantifying viable cells by quantitative PCR (Taskin et al., 2011). RNA is present only in actively growing and replicating cells and unlike DNA, RNA tends to degrade rapidly in dead cells therefore, real-time reverse-transcriptase PCR based methods can be used for determining the presence of live bacterial cells (Sharma, 2006). Thus, these approaches have potential to improve current study further.

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EDUCATION

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MSc	METU, Biotechnology	2005
BSc	METU, Department of Biology Education	2003
High School	Kayseri Sumer High School	1996

WORK EXPERIENCE

Year	Place	Enrollment
2008-2010	TUBITAK	Project Asistantship
2002-2003	Ankara Science College	Intern Biology Teacher

FOREIGN LANGUAGES

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PATENT:

Absolute *Salmonella* Quantification by Recombinant Plasmid Positive Controls Based Real Time PCR. Patent Application in preparation.

PUBLICATIONS:

Refereed Journal Papers (Covered by SCI)

•**Kurtulus Gokduman**, Dilek Avsaroglu, Aris Cakiris, Duran Ustek, G. Candan Gurakan, *Enumeration of Salmonella Bacteria in Milk by Plasmid Positive Controls Based Real-Time PCR*, In preparation.

•**Kurtulus Gokduman**, Dilek Avsaroglu, Aris Cakiris, Duran Ustek, G. Candan Gurakan, *Cloning of invA and ttrRSBC genes as positive controls for quantitative molecular genetic detection of Salmonella*, Submitted to Food Microbiol.

•Feride Severcan, **Kurtulus Gokduman**, Ayca Dogan, Sukran Bolay, Saadet Gokalp, *Effects of In-Office and At-Home Bleaching on Human Enamel and Dentin: An in Vitro Application of Fourier Transform Infrared Study*, Applied Spectroscopy, 2008, Volume 62, Issue 11, pp: 1274-1279.

•Ayca Dogan, **Kurtulus Gokduman**, Sukran Bolay, Feride Severcan, *Evaluation of In-Office Bleaching on Enamel and Dentine: An FTIR Study*, Proceeding of WSEAS Transactions on Biology and Biomedicine, Issue 4, Volume 2, October 2005, pp: 365-369.

Abstract in Refereed Journal (Covered by SCI)

•Feride Severcan, **Kurtulus Gokduman**, Ayca Dogan, Sukran Bolay, Saadet Gokalp, *Comparison of Office and Home Bleaching on Dental Tissue: An FTIR Study,* Cells Tissues Organs, 189, pp: 295-302 (2009).

•Ayca Dogan, **Kurtulus Gokduman**, Sukran Bolay, Saadet Gokalp and Feride Severcan, *FTIR Spectroscopic Characterization of Bleaching Agents on Human Teeth*, Biophysical Journal 88 (1): 55A-56A Suppl.S (2005).

PRESENTATIONS:

Oral

•**Kurtulus Gokduman**, *Evaluation of Office and Home Bleaching on Enamel and Dentine*, Faculty of Medicine, University of Castilla-La Mancha, Albacete, Spain, July 2006.

•**Kurtulus Gokduman**, *Effects of Office and Home Bleaching on Enamel and Dentine*, XVII. National Biophysics Congress, Ege University, Izmir-Turkey, September 2005.

Poster

•**Kurtulus Gokduman**, Ayhan S. Demir, *Development of Biocompatible Nanosized Drug Delivery Systems for Specific Targeting of Topo I Inhibitors*, Poster at XXIII. National Chemistry Congress, Cumhuriyet University, Sivas-Turkey, June 2009.

•**Kurtulus Gokduman**, Ayhan S. Demir, *Development of Biocompatible Nanosized Targeted Drug Delivery Systems for TOPO I Inhibitors*, Poster at XXII. National Chemistry Congress, Eastern Mediterranean University, Magusa-Cyprus, October 2008.

•**Kurtulus Gokduman**, Ayca Dogan, Sukran Bolay, Feride Severcan, *Evaluation of Office and Home Bleaching on Enamel and Dentine: An FTIR Study*, Poster at International University Bremen Summer School (Biosensing with channels: faster, smaller, smarter), Bremen-Germany, 30 July-5 August 2005.

•**Kurtulus Gokduman**, Ayca Dogan, Sukran Bolay, Saadet Gokalp, Feride Severcan, *Effects of Office and Home Bleaching on Enamel and Dentine*, Poster at IX. National Spectroscopy Congress, Bilkent University, Ankara Turkey, June 2005.

•Ayca Dogan, **Kurtulus Gokduman**, Sukran Bolay, Saadet Gokalp, and Feride Severcan, *FTIR Spectroscopic Characterization of Bleaching Agents on Human Teeth*, Poster at Biophysical Society 49th Annual Meeting, Long Beach, CA, February 2005.

HOBBIES:

Basketball; Photograph; Billard; Chess; Traveling; European Cinema; Middle East Technical University Nature Club; Middle East Technical University Archeology Club; Middle East Technical University Chess Club