COMPARISON OF CONVENTIONAL CULTURE METHODS AND THE POLYMERASE CHAIN REACTION FOR THE DETECTION OF *Shigella* spp. ON TOMATO SURFACES

By

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Isolation of *Shigella* spp. from food is very difficult due to the lack of appropriate selective media and the fastidious nature of Shigellae. Nucleic acid-based detection methods such as the polymerase chain reaction (PCR) have recently been developed for the detection of *Shigella* spp. with greater specificity and sensitivity than conventional culture methods. In this study, artificially inoculated *S. boydii* UI02 or *S. sonnei* UI05 was recovered from tomato surfaces using a phosphate buffer rinse and vigorous shaking/hand manipulation. Detection of inocula was evaluated by enrichment protocols of the U.S. Food and Drug Administration's (1998) Bacteriological Analytical Manual (FDA BAM), the Compendium of Methods for the Microbiological Examination of Food (CMMEF), enrichment in *Enterobacteriaceae* Enrichment (EE) broth supplemented with 1.0 µg/ml novobiocin and incubated at 42ºC), and FTA® filtration/ nested PCR. Conventional culture enrichments were repeated using enrichments supplemented with

50µg/ml rifampicin (rif+) to exclude natural tomato microflora and rifampicin-adapted inocula. Additionally, enrichments were plated on *Shigella* Plating Medium (SPM), *Salmonella-Shigella* agar (SSA) and MacConkey agar (MAC) in order to compare isolation rates of *S. boydii* UI02 and *S. sonnei* UI05 among the three plating media.

The lowest detection levels (LDLs) of enrichment procedures in the presence of natural tomato microflora were >5.3 x 10^5 CFU/tomato (all three methods) for *S. boydii* UI02; and 1.9 x 10^1 (FDA BAM), 1.5 x 10^3 (CMMEF), and 1.1 x 10^1 CFU/tomato (EE broth) for *S. sonnei* UI05. There were no significant differences ($\alpha = 0.05$) between the FDA BAM and the CMMEF for the isolation of *S. boydii* UI02, and no significant differences ($\alpha = 0.05$) among any of the enrichment methods for the isolation of *S. sonnei* UI05. LDLs from enrichment procedures where background microflora was excluded were 6.3 x 10^0 CFU/tomato (FDA BAM rif+ and CMMEF rif+) and >5.3 x 10^5 CFU/tomato (EE broth rif+) for *S. boydii* UI02; and 1.9 x 10^1 CFU/tomato (FDA BAM rif+ and CMMEF rif+), and 1.1 x 10^1 CFU/tomato (EE broth rif+) for *S. sonnei* UI05. The LDL of the FTA® filtration/ nested PCR method was 6.2 x 10^0 CFU/tomato for *S. boydii* UI02 and 7.4 x 10^0 CFU/tomato for *S. sonnei* UI05. The FTA® filtration/ nested PCR method was significantly better than enrichment protocols of the CMMEF ($P = 0.010$) and in EE broth ($P < 0.001$) for the detection of *S. boydii* UI02; however it was not significantly better than the FDA BAM ($P = 0.177$). The FTA® filtration/ nested PCR method was significantly better than enrichment protocols of all three conventional culture methods ($P < 0.001$) for the detection of *S. sonnei* UI05. EE broth was found to be inhibitory to *S. boydii* UI02. Furthermore, there were no significant differences ($\alpha = 0.05$) among SPM, SSA and MAC for the isolation of *S. boydii* UI02 or *S. sonnei* UI05.

CHAPTER 1
INTRODUCTION

Foodborne illness associated with the consumption of fresh produce has increased during recent years in the Unites States. Although this increase of illness can be partially attributed to increased consumption of fresh produce, the increased demand for minimally processed fruits and vegetables and the growth in global food trade have also contributed (Tauxe et al., 1997). Between 1987 and 1997, the total fresh produce market increased from $34.8 billion to $70.8 billion in retail and foodservice sales (Kaufman et al., 2000). During the same time period, U.S. imports of fruits and vegetables have grown from $2.0 billion in sales to $4.1 billion (Kaufman et al., 2000). Imported fruits and vegetables represent an increased potential for foodborne illness, especially if grown under poor production standards or mishandled during a long distribution cycle (Food and Drug Administration (FDA), 2001d).

Coinciding with the increase of fresh produce consumption, the incidence of foodborne shigellosis has also increased. Previously thought to be primarily a water-borne pathogen, foodborne outbreaks of *Shigella* spp. are increasing, most recently involving fresh parsley, iceburg lettuce, and a bean salad containing parsley and cilantro. Of the pathogens under surveillance by the U.S. Centers for Disease Control and Prevention's (CDC) Emerging Infections Program, Foodborne Diseases Active Surveillance Network (FoodNet), *Shigella* spp. accounted for 18.2% of laboratory confirmed cases during the year 2000. *Shigella* spp. are the leading causative agents of

foodborne illness among children age 1 to 4 years at 29.1 cases per 100,000 (CDC, 2002b), with a national incidence rate of 3.8 cases per 100,000 (CDC, 2002a).

Produce related illnesses caused by *Shigella* spp. can be reduced with the use of rapid detection methods and the proper sampling/testing of imported and domestic produce. Historically, conventional culture methods such as the U.S. FDA's (1998) Bacteriological Analytical Manual (BAM) and the *Shigella* culture method published in the Compendium of Methods for the Microbiological Examination of Foods have been employed to detect *Shigella* spp. in food products. These methods make use of selective media and biochemical tests, which require several days to complete. Furthermore, the selective media available are not able to eliminate closely related organisms such as *Enterobacter* spp., *Klebsiella* spp., and *Citrobacter* spp. which tend to out-compete *Shigella* spp. More modern microbiological methods, such as DNA hydridization and the polymerase chain reaction (PCR), have focused on the detection of DNA segments unique to *Shigella* spp. and enteroinvasive *E. coli*. These assays are usually completed in one or two working days, thereby allowing a more rapid determination of contaminated product over conventional culture methods. The early detection and identification of a contaminated product, especially during an outbreak, can significantly limit the number of illnesses. Furthermore, the elimination of background microflora is not necessary in DNA-based assays, allowing for the detection of low populations of *Shigella* spp. among high levels of potentially competitive background.

The primary problem associated with PCR methods for the detection of *Shigella* spp. and other human pathogens is that no colony is isolated; therefore no further characterization can be performed. To overcome this problem, FDA is investigating a

new method for the detection of *Shigella* spp. in foods that incorporates both conventional culture methods and PCR techniques (Dr. Keith Lampel, FDA, personal communication). In the proposed method, a conventional culture method involving an enrichment step followed by plating with selective media is initiated simultaneously with a nested PCR method. The nested PCR method involves two successive PCR reactions in which diluted product from the first reaction is used as template for the second reaction. Template preparation procedures for the nested PCR include a double filtration (size exclusion/FTA®) technique from which FTA® filter punches are taken and used as template for the first PCR reaction. According to the proposed method, enrichment procedures to isolate *Shigella* spp. would only be carried forth if positive amplification were observed in the first PCR reaction. The proposed method would thereby incorporate the rapid detection capabilities of PCR with the ability to isolate a colony of conventional plating techniques.

The objectives of this study were as follows:

1. To compare conventional culture methods and the FTA® filtration/nested PCR method for the detection of *Shigella* spp. on tomato surfaces.
2. To compare enrichment in *Enterobacteriaceae* Enrichment broth to enrichment by the FDA BAM and CMMEF methods.
3. To compare *Shigella* Plating Medium to MacConkey and *Salmonella-Shigella* agar for the isolation of *Shigella* spp. on tomato surfaces.

CHAPTER 2
LITERATURE REVIEW

General Tomato Background

Tomatoes, although commonly classified as a vegetable, are actually a fruit since they are the ripened ovary of the tomato plant. Tomatoes are grown for one of two distinct markets: the fresh market or the processing market. The fresh and processing tomato markets can be distinguished by four general characteristics. First, tomato varieties grown for the processing market tend to have higher percentages soluble solids in order to efficiently make products like tomato paste (Economic Research Service, U.S. Department of Agriculture (ERS), 2003). Second, most tomatoes grown for processing are produced under contract between the grower and processing firms (ERS, 2003). Third, fresh-market tomatoes are all hand picked while processing tomatoes are machine harvested (ERS, 2003). Finally, fresh market tomato prices are higher and more variable due to larger production costs and greater market uncertainty (ERS, 2003). Most of the fresh tomatoes produced in the United States are grown in Florida (~ 40%) and California (~ 27%) (Sargent, 1998). Fresh market tomatoes are available year round because imports from Mexico supplement winter decreases in domestic tomato production (ERS, 2003). In 1999, the Americans ate 4.8 billion pounds of fresh tomatoes, or 17.8 pounds per person (ERS, 2003).

Tomatoes provide a rich source of vitamins, minerals, carotenoids, and other phytochemicals (Florida Tomato Committee (FTC), 2003). One medium, fresh tomato (5.2 oz) provides 40% of the U.S. recommended daily allowance of vitamin C and 20%

of the vitamin A (ERS, 2003). Lycopene, an antioxidant found almost exclusively in tomatoes, is under current investigation as a reducer of risk for several types of cancer (FTC, 2003). Additionally, current research is also investigating a link between lycopene-rich diets and reduced incidence of heart disease (FTC, 2003).

Use of Tomatoes as a Model for Detection of *Shigella* spp. from Fruits and Vegetables

When choosing the tomato as a model for recovery of *Shigella* spp. from fruits and vegetables, the surface characteristics must be considered. The waxy surface of tomatoes is very smooth, unlike the corky surfaces of cantaloupes or potatoes, and without the invaginations found on oranges. Such surface irregularities make the recovery of inouclum difficult and more inconsistent. Furthermore, tomatoes do not contain epidermal or peridermal pores, such as stoma or lenticels, respectively, which allow gas exchange and also may allow internalization of bacterial pathogens. For these reasons, bacteria used to artificially inoculate tomatoes may be recovered with greater efficiency than from other fruits and vegetables. For purposes of this study, the inoculation of waxy surfaces of tomatoes was performed to reduce the number of variables associated with other surfaces, such as bacterial internalization and inefficient surface removal, thus yielding a better comparison of methods.

General Characteristics of *Shigella*

Shigella, the causative agent of shigellosis or "bacillary dysentery," was first discovered over 100 years ago by a Japanese scientist Kiyoshi Shiga (Anonymous, 2002). Shigellae are members of the family *Enterobacteriaceae*, and are nearly genetically identical to *Escherichia coli* (*E. coli*) and are closely related to *Salmonella* and *Citrobacter* spp. (American Public Health Association (APHA), 2001). Shigellae are characterized as Gram-negative, facultatively anaerobic, non-sporulating, non-motile

rods. Typically, species of *Shigella* do not ferment lactose, are lysine-decarboxylase negative, are acetate and mucate negative, and do not produce gas from glucose, although several exceptions exist (Echeverria et al., 1991).

There are four serogroups of *Shigella*: *S. dysenteriae* (serogroup A) serotype 1 – 15, *S. flexneri* (serogroup B) serotype 1 – 8 (9 subtypes), *S. boydii* (serogroup C) serotype 1 – 19, and *S. sonnei* (serogroup D) serotype 1. Serogroups of *Shigella* can be differentiated by their biochemical traits and antigenic properties (CDC, 2003); however they can also be differentiated by their epidemiology (Ingersoll et al., 2002). *S. dysenteriae* is the serogroup primarily associated with epidemics (Ingersoll et al., 2002); *S. dysenteriae* type 1 is associated with the highest case fatality rate of all *Shigella* serogroups at 5-15% (CDC, 2003). *S. flexneri* is the predominate group found in areas of endemic infection, while *S. sonnei* is the group implicated in source outbreaks in developed countries (Hale, 1991). *S. boydii* has been associated with food imported from Central and South America and is rarely isolated in North America.

Shigella, although classically thought of as a waterborne pathogen, has been involved in an increasing number of food-borne outbreaks (Smith, 1987). Food products associated with *Shigella* outbreaks are most commonly subjected to hand processing or preparation, limited heat treatment, or served/delivered raw to the consumer (Wu et al., 2000). Examples of food products from which *Shigella* spp. have been isolated include potato salad, ground beef, bean dip, raw oysters, fish, and raw vegetables.

The infective dose for *Shigella* spp. is reported to be very low; ingestion of 1.0 x 10^1 cells of *S. dysenteriae* is sufficient for infection, while the other serogroups require ingestion of 1.0 x 10^2 to 1.0 x 10^4 cells (cited in Muriana, 2002). The low infective dose

associated with *Shigella* spp. results in common spread of the disease through person to person contact. Typical symptoms of infection include bloody diarrhea, abdominal pain, fever, and malaise. Seizures in children with shigellosis have been reported in 5.4% of cases (Galanakis, 2002). Late complications of *S. dysenteriae* serotype 1 infections can include hemolytic uremic syndrome (HUS), while *S. flexneri* infections can result in development of Reiter's syndrome, especially in persons with the genetic marker HLA-B27 (CDC, 2003). Reiter's syndrome is characterized by joint pain, eye irritation, and painful urination (CDC, 2003).

Recent Outbreaks Involving *Shigella* spp.

In recent years, there has been an increasing number of *Shigella* outbreaks involving produce and prepared foods. In 1989, German potato salad was implicated in an outbreak of a multi-antibiotic resistant strain of *S. flexneri* aboard a cruise ship (Lew et al., 1991). In 1994, iceburg lettuce was implicated in an outbreak of *S. sonnei* which affected people from six Northern European countries (Long et al., 2002). In 1998, uncooked, chopped, curly parsley was implicated in a multi-state outbreak of *S. sonnei* (Morbidity and Mortality Weekly Report (MMWR), 1999). The source of this outbreak was traced back to a Mexican farm. In 1999, bean salad which contained parsley and cilantro was implicated in a Chicago area foodborne outbreak of *S. boydii* serotype 18. In 2000, a nationally distributed five layer bean dip was implicated in another multi-state outbreak of *S. sonnei* (MMWR, 2000).

Prevalence of *Shigella* spp. on Produce

In response to President Clinton's National Food Safety Initiative (January 1997) and Produce & Imported Foods Safety Initiative (October 1997), the FDA has begun investigating the presence of human pathogens on produce. In March 1999, FDA initiated

a field assignment entitled "FDA Survey of Imported Fresh Produce" to collect data on the incidence and extent of pathogen contamination on selected imported produce (FDA, 2001b). The survey analyzed broccoli, cantaloupe, celery, cilantro, culantro, loose-leaf lettuce, parsley, scallions (green onions), strawberries, and tomatoes for *E. coli* O157:H7 and *Salmonella*. All commodities except cilantro, culantro, loose-leaf lettuce, and strawberries were analyzed for *Shigella*. For those commodities tested, contamination with *Shigella* spp. was observed at the following rates: 0.9% (9/1003) of all commodities tested, 2.0% (3/151) cantaloupe samples, 2.4% (2/84) celery samples, 0.9% (1/116) lettuce samples, 1.2% (1/84) parsley samples, and 1.1% (2/180) scallion samples.

In May 2000, FDA initiated its "Survey of Domestic Fresh Produce" to focus on high-volume domestic produce that is generally consumed raw (FDA, 2001a). This survey included the following commodities: cantaloupe, celery, cilantro, green onions, loose-leaf lettuce, parsley, strawberries, and tomatoes. All commodities were to be analyzed for the presence of *E. coli* O157:H7 and *Salmonella*, while all except strawberries were to be tested for *Shigella*. At time of publication, only interim results from analysis of 767 of the required 1000 samples had been released. As with the 1999 Imported Fresh Produce Survey, 0.9% (6/646) of the commodities tested were contaminated with *Shigella*, 0.9% (1/115) cantaloupe samples, 1.6% (1/62) cilantro samples, 4.1% (3/73) green onion samples, and 1.6% (1/64) parsley samples.

In January 2001, FDA announced another survey entitled "FDA Survey of Imported Fresh Produce: Imported Produce Assignment FY 2001." The focus of the study was to examine further the presence of *E. coli* O157:H7, *Salmonella*, and *Shigella*, on cilantro, culantro, cantaloupe, and tomatoes based on high rates of pathogens from

previous surveys (FDA, 2001c). At time of publication, results from this survey were not available.

Survival Characteristics of *Shigella*

The ability of *Shigella* to survive is dependent, in part, upon pH, temperature, and salt concentration of its environment. In a study using *S. flexneri*, Zaika (2001) demonstrated the effects of temperature and pH on survival. A strain of *S. flexneri* was cultured in brain heart infusion broth (BHI) and subjected to various incubation temperatures (4, 12, 19, 28, and 37°C) and pH conditions (pH 2, 3, 4, 5). In general, survival was enhanced by lower temperatures and increased pH for all experiments. Results of this study indicated that *S. flexneri* has acid resistance and suggest that foods of pH 5 or lower stored at or below room temperature may permit survival of the organism over long periods of time in sufficient numbers to cause illness (Zaika, 2001). Zaika (2002a) also investigated survival characteristics of *S. flexneri* as affected by NaCl. *S. flexneri* was able to tolerate and survive in levels of NaCl (1 – 6%) commonly found in food items such as pickled vegetables, caviar, pickled herring, dry cured ham, and certain cheeses for two weeks to two months (Zaika, 2002a). Survival of *S. flexneri* in the presence of organic acids (citric, malic, and tartaric acid), commonly found in fruits and vegetables, and fermentation acids (acetic and lactic acid), commonly used as preservatives, was studied (Zaika, 2002b). *S. flexneri* was cultured with each acid (plus an HCl control) at 0.04 M in BHI adjusted to pH 4, and incubated at various temperatures (4, 19, 28, and 37°C). As seen in other experiments, survival increased as temperature decreased (Zaika, 2002b). At 4°C, *S. flexneri* survived in the presence of all the acids tested for > 55 days (Zaika, 2002b).

In water alone, *Shigella* spp. can survive with little decline in population levels. Rafii and Lunsford (1997) inoculated *S. flexneri* into distilled water. The initial count of 2.8 x 10^8 CFU/ml was only decreased to 9.2 x 10^7 CFU/ml after storage at 4°C for 26 days. The high survival rate of *S. flexneri* in water supports the historical association of shigellosis outbreaks with water sources.

Shigella spp. can survive for extended periods of time on raw vegetable surfaces. Wu et al. (2000) studied survival of *S. sonnei* on whole and chopped parsley leaves. When held at 21°C, *S. sonnei* was able to grow on chopped parsley at a rate similar to that which occurs in nutritious liquid medium (Wu et al., 2000). At 4°C, populations declined on both chopped and whole parsley throughout the 14 day storage period, however the pathogen survived regardless of initial population (Wu et al., 2000). Rafii and Lunsford (1997) studied the survival of *S. flexneri* on raw cabbage, onion, and green pepper held at 4°C. Although the population decreased, *S. flexneri* survived storage at 4°C for 12 days (at which time sampling was terminated due to spoilage) on the onion and green pepper at levels of 2.10 x 10^5 and 2.2 x 10^4 CFU/g, respectively (Rafii and Lunsford, 1997). *S. flexneri* continued to survive on the cabbage after 26 days at 1.13 x 10^3 CFU/g. These studies demonstrate how *Shigella* spp. can survive on refrigerated raw vegetables for periods of time that exceed the expected shelf life (Wu et al., 2000).

Several studies have demonstrated the ability of *Shigella* spp. to survive in low pH foods at low temperature storage. Bagamboula et al. (2002) demonstrated the ability of *S. sonnei* and *S. flexneri* to survive in apple juice (pH 3.3-3.4) and tomato juice (pH 3.9-4.1) held at 7°C for 14 days. No reduction was noted in the tomato juice, while only 1.2 to 3.1 $\log_{10}$ reduction was observed in the apple juice during the 14 day study. Rafii and

Lunsford (1997) observed the ability of *S. flexneri* to survive in carrot salad (pH 2.7 – 2.9), potato salad (pH 3.3 – 4.4), coleslaw (pH 4.1 – 4.2), and crab salad (pH 4.4 – 4.5) held at 4°C. Sampling was terminated at day 11 for the carrot and the potato salad, at which time *S. flexneri* counts decreased from an initial 4.3×10^6 to 4.2×10^2 CFU/g and from 1.32×10^6 to 8.5×10^2 CFU/g, respectively. Sampling of the coleslaw and the crab salads ceased due to product spoilage on days 13 and 20, respectively, while *S. flexneri* counts were 2.16×10^4 and 2.4×10^5, respectively. These studies indicate that inoculated Shigellae were not rapidly killed by normal microflora, low pH (Rafii and Lunsford, 1997), or low temperature.

Intracellular Activity of *Shigella* spp.

Invasion of Epithelial Cells

Invasion of epithelial cells by Shigellae involves four steps: entry into epithelial cells, intracellular multiplication, intra- and intercellular spreading, and killing of the host cell (Sansonetti, 1991). The invasion process is controlled by a 220-kDa plasmid. The plasmid contains invasion plasmid antigen (*ipa*) genes which encode four highly immunogenic polypeptides; IpaA, IpaB, IpaC, and IpaD. Studies in which Tn5 insertions affecting the expression of the *ipa* genes reveal that expression of *ipaB*, *ipaC*, and *ipaD* is strongly associated with entry, while *ipaA* is not (Sansonetti, 1991).

Invasion is also mediated by the *virF* gene, which is located on the virulence plasmid, and the *virR* gene, which is located on the chromosome. The *virF* gene encodes a 30-kDa protein that positively regulates the expression of the *ipa* genes and a plasmid gene *icsA* (also known as *virG*), which encodes intra- and intercellular spread. Environmental factors which affect the expression of *virF* are not known. The *virR* gene is a repressor of the plasmid invasion genes in a temperature-dependant manner

(Sansonetti, 1991). When Shigellae are grown at 30°C they do not express any of the Ipa polypeptides and are therefore non-invasive, however, Shigellae grown at 37°C are fully invasive and all plasmid polypeptides are encoded (Sansonetti, 1991).

Gaining entry into epithelial cells

Once ingested, Shigellae move through the gastrointestinal tract to the colon, where they translocate the epithelial barrier via M cells that overlay the solitary lymphoid nodules (Suzuki and Sasakawa, 2001). Upon reaching the underlying M cells, *Shigella* infects the macrophages and induces cell death (Suzuki and Sasakawa, 2001). Infected macrophages release interleukin-1β, which elicits a strong inflammatory response (Zychlinsky et al., 1994). Once released from the macrophage, *Shigella* will enter the epithelial cells, also called enterocytes, which predominately line the colon via membrane ruffling and macropinocytosis. Epithelial cells produce inflammatory cytokines in response to bacterial invasion, therefore increasing inflammation of the colon (Suzuki and Sasakawa, 2001).

Macropinocytosis involves the cell extending its membrane as formations known as pseudopodia, which will engulf large volumes and close around them forming a vacuole. In order for macropinocytosis to occur, actin must be polymerized and myosin, an actin binding protein, must be present (Stendahl et al., 1980). Common stimuli that induce macropinocytosis include cytokines and bacterial antigens.

Intracellular multiplication

Shigellae immediately disrupt phagocytic vacuoles allowing entry into the host cell cytoplasm. Once in the cytoplasm, Shigellae multiply rapidly. Sansonetti et al. (1986)

observed the generation time for *S. flexneri* in HeLa cells to be approximately 40 minutes.

Sustaining efficient intracellular growth requires the acquisition of host cell nutrients. Although production of Shiga toxin facilitates the availability of host cell nutrients, no relation between its production and intracellular growth rates can be observed (Fontaine et al., 1988). Likewise, no relation between Shiga-like toxin production and intracellular growth rate can be observed (Sansonetti et al., 1986; Clerc et al., 1987).

Since little free iron exists within mammalian host cells, *Shigella* spp. must also express high-affinity iron acquisition systems. In order to obtain iron, *Shigella* spp. synthesize and transport the siderophores aerobactin and enterobactin (Vokes et al., 1999) and utilize a receptor/transport system in which iron is obtained from heme. Siderophores (aerobactin and enterobactin) are low molecular weight iron binding compounds that remove iron from host proteins. Enterobactin is produced by some but not all *Shigella* spp. (Perry and San Clemente, 1979; Payne, 1984) while aerobactin is synthesized by *S. flexneri* and *S. boydii* (Lawlor and Payne, 1984) and some *S. sonnei* (Payne, 1988). Headley et al. (1997) demonstrated that aerobactin systems, although active in extracellular environments, are not expressed intracellularly. This suggests that siderophore-independent iron acquisition systems can provide essential iron during intracellular multiplication (Headley et al., 1997).

Intra- and intercellular spreading (actin-based motility)

The capacity for *Shigella* to spread intracellularly and infect adjacent cells is critical in the infection process (Sansonetti, 1991). Intra- and intercellular spreading is controlled by the *icsA* (*virG*) gene located on the virulence plasmid. The *icsA* gene

encodes the protein IcsA, which enables actin-based motility (Bernardini et al., 1989) and intercellular spread (Makino et al., 1986). IcsA is a surface-exposed outer membrane protein consisting of three distinctive domains: a 52 amino acid N-terminal signal sequence, a 706 amino acid α-domain, and a 344 amino acid C-terminal β-core (Goldberg et al., 1993; Lett et al., 1989; Suzuki et al., 1995). The α-domain is the exposed portion and the β-core is embedded in the outer membrane (Suzuki et al., 1995).

IcsA is distributed at one pole of the outer membrane surface. This asymmetrical distribution allows the polar formation of actin tails, and thus polar movement of *Shigella* within host cell cytoplasm. The polar localization of IcsA is primarily affected by two events: (i) the rate of diffusion of outer membrane IcsA (Sandlin et al., 1995; 1996; Sandlin and Maurelli, 1999; Robbins et al., 2001) and (ii) the specific cleavage of IcsA by the protease IcsP (SopA) (d'Hauteville et al., 1996; Egile et al., 1997; Steinhauer et al., 1999). Rate of diffusion of outer membrane IcsA is directly affected by the O side chains of the membrane lipopolysaccharide (LPS). Sandlin et al. (1996) demonstrated this relationship with a *S. flexneri* LPS mutant, BS520 which does not make any O-antigen. As compared with a wild-type strain of *S. flexneri*, which polymerized actin at one pole, the LPS mutant strain polymerized actin in a non-polar fashion. Expression of LPS that does not have any O side chains causes an even distribution of IcsA over the entire outer membrane (Sandlin et al., 1995; 1996; Monack and Theriot, 2001). Composition of the C-terminal one-third of the IcsA α domain is also required for polar localization as well as polar movement of *S. flexneri* (Suzuki et al., 1996). A *S. flexneri* mutant, in which a segment of this section was deleted, was unable to polymerize actin in a polar fashion or move unidirectionally (Suzuki et al., 1996).

The *icsP* gene encodes the outer membrane protease IcsP (also called SopA) which cleaves laterally diffused IcsA, thus promoting polar localization (Egile et al., 1997). An *E. coli* K-12 strain, engineered to express the *icsA* gene, was shown to diffuse IcsA along its outer membrane (Monack and Theriot, 2001). When the same *E. coli* K-12 strain was engineered to express the *icsP* gene with the *icsA* gene, the number of bacteria which polymerized actin at one pole increased (Monack and Theriot, 2001).

The N-terminal two-thirds of the IcsA α domain is essential for mediating actin assembly in *Shigella* host cells (Suzuki and Sasakawa, 2001). This portion of IcsA contains six glycine rich repeats which interact with the Wiskott-Aldrich syndrome protein (N-WASP). N-WASP is composed of distinct domains: PH, a pleckstrin homology domain; IQ, a calmodulin binding domain; GBD, a GTPase binding domain that binds Cdc42; PRR, a proline-rich region; V, a G-actin-binding veroprolin homology domain; C, cofilin homology domain; A, a C-terminal acidic amino acid segment (cited in Suzuki and Sasakawa, 2001; Miki et al., 1996). The VCA domain of N-WASP activates and interacts with the Arp2/3 complex. The IcsA--N-WASP--Arp2/3 complex mediates rapid actin filament growth at the barbed end, including cross-linking between the elongated actin filaments (Suzuki and Sasakawa, 2001). The resulting network of actin filaments allows *Shigella* to gain a propulsive force with which to move in the cytoplasm of host cells (Suzuki and Sasakawa, 2001).

Cdc42 (an N-WASP activator), profilin (an actin monomer-binding protein), and cofilin (which depolymerizes actin) also have roles in efficient actin assembly. Cdc42 binds to the GBD domain of N-WASP preventing the intramolecular interaction between the C-terminal acidic amino acids and the basic amino acids of the GBD, thereby forcing

the N-WASP complex to unfold to its activated form (Suzuki and Sasakawa, 2001). Cdc42 independent activation of the N-WASP complex by IcsA has been reported for actin-based motility in *Shigella*, however efficient entry into cells was reported as Cdc42 dependent (Shibata et al., 2002). The role of Cdc42 in invasion and motility is still somewhat controversial. Profilin delivers monomeric actin to sites of actin assembly (Goldberg, 2001). Although profilin has been shown not to be absolutely essential for actin based motility, it is required for maximum rates of movement (Loisel et al., 1999). Cofilin generates actin monomers from the filamentous actin. By disassembling unneeded actin filaments within the tail, cofilin might work to free up actin for incorporation into newly generated filaments (Goldberg, 2001).

Intercellular spreading is dependent upon an actin-based motility mechanism (Fig. 2-2) (Monack and Theriot, 2001). *Shigella* cells first form a membrane bound protrusion into an adjacent cell. This protrusion must distend two membranes: one from the donor cell, and another from the recipient cell (Parsot and Sansonetti, 1996). As the protrusion pushes further into the recipient cell, it is taken up by the recipient cell resulting in the bacteria enclosed in a double-membrane vacuole (Monack and Theriot, 2001). Intercellular spread is completed when *Shigella* rapidly escapes from the double-membrane vacuole, releasing it into the cytosol of the secondary cell. Monack and Theriot (2001) observed intercellular spread of an *E. coli* K-12 strain expressing the *icsA* and *icsP* genes in HeLa cells. As expected, they found the *E. coli* K-12 strain spread to adjacent cells and enclosed in a double-membrane vacuole as well as free in the cytosol of the adjacent cells.

Early host cell apoptosis

The early killing of host cells by *Shigella* is mediated by the virulence plasmid. Sansonetti (1991) demonstrated the inability of non-invasive *S. flexneri* to kill host cells whereas the invasive species killed efficiently and rapidly. Non-invasive strains can not kill host cells, since this requires that the *Shigella* be intracellular. Early killing of host cells involves metabolic events which rapidly drop the intracellular concentration of ATP, increase pyruvate, and arrest lactate production (Sansonetti and Mounier, 1987). Interestingly, Shiga toxin, a potent cytotoxin produced by *S. dysenteriae* serotype 1, does not play a role in the early killing of host cells. Fontaine et al. (1988) constructed a Tox^- mutant strain of *S. dysenteriae* serotype 1 and found that the mutant killed as efficiently as the wild-type strain.

Toxins Produced by *Shigella* spp.

Shiga toxin

S. dysenteriae type 1 strains produce a potent toxin known as Shiga toxin (STX). Although the toxin is not necessary to sustain an infection, its expression increases the severity of disease. Three biologic activities associated with STX are cytotoxicity, enterotoxicity, and neurotoxicity, while the one known biochemical effect is the inhibition of protein synthesis (Donohue-Rolfe et al, 1991). STX is considered the prototype to a family of toxins known as Shiga-like toxins (SLT), which are similar in structure and function, and share the same receptor sites. Perhaps the most widely known human pathogen that produces SLT's is *E. coli* O157:H7, which has two toxin variants (SLT I and SLT II).

STX is composed of two polypeptides: an A subunit (32,225 MW) and five B subunits (7,691 MW, each) (Donohue-Rolfe et al., 1991). The B subunits mediate

binding to cell surface receptors, which have been identified as glycolipids containing terminal galactose-α(1-4)galactose disaccharides such as galabiosylceramide and globotriasylceramide (Gb3) (Brown et al., 1991; Keusch et al., 1991). The A subunit, once inside the host cell cytoplasm, acts enzymatically to cleave the N-glycosidic bond of adenine at nucleotide position 4324 in the 28S rRNA of the 60S ribosomal unit (Donohue-Rolfe et al., 1991).

Shigella enterotoxins 1 and 2

Recently, two enterotoxins, shigella enterotoxin 1 (SHET 1) and shigella enterotoxin 2 (SHET 2), have been characterized and are believed to play a role in the clinical manifestation of shigellosis (Yavzori et al., 2002). SHET 1, which is chromosomally encoded, was only prevalent in isolates of *S. flexneri* 2a. SHET 2, however, is encoded on the large virulence plasmid, and was detected in all *Shigella* isolates tested except several isolates which lost their plasmid.

Traditional Microbiological Media for the Isolation/Detection of *Shigella*

Traditional microbiological techniques make use of selective media for the enrichment/isolation of *Shigella* spp. Many variants of enrichment and plating media have been investigated for optimal recovery, often with conflicting results between laboratories and sample types. Due to the lack of appropriate selective media and the presence of *Shigella* spp. in relatively low population, background microflora tends to out-compete *Shigella* spp. when isolation is attempted from a food product. Unless media can be developed that are both specific and sensitive for *Shigella* spp. regardless of potential background microflora, isolation by traditional microbiological methods will always be suspect.

Early enrichment of *Shigella* spp. was attempted using Selenite-F (SF) or Tetrathionate (TT) broth. These broths were originally designed for the isolation of salmonellae, but due to the lack of specific enrichment media for Shigellae they were used as all-purpose enteric enrichment broths (Taylor and Schelhart, 1969). Sodium selenite, although selective for salmonellae, is toxic to *Shigella* spp. (and most enterics), therefore its use in enrichment procedures for *Shigella* spp. has been terminated. TT is a peptone base broth with bile salts and sodium thiosulfate, which inhibit most Gram-positives and *Enterobacteriaceae*. Gram-negative (GN) broth is a peptone-based broth with glucose and mannitol. The concentration of mannitol in GN broth is higher than glucose to promote mannitol fermentors, like *Shigella* spp. Both TT broth and GN broth contain bile salts, which can be inhibitory to stressed cultures. Furthermore, GN broth contains sodium deoxycholate, which has been shown to inhibit heat-stressed Shigellae (Uyttendaele et al, 2001). Currently, enrichment procedures use a low carbohydrate medium, *Shigella* broth (SB) with addition of novobiocin, for the detection/isolation of *Shigella* spp. (APHA, 2001; FDA BAM). Acids produced by other *Enterobacteriaceae* during the fermentation of carbohydrates have been reported to be toxic to Shigellae (Mehlman et al., 1985); however, other studies have shown the acid tolerance of *Shigella* spp. to grow at a pH of 4.5 to 4.75 (Bagamboula et al., 2002) and to survive at a pH of 4.0 (Zaika, 2002). Since SB contains very little carbohydrate, the effect of a low pH environment on the enrichment of *Shigella* spp is limited when SB is used. SB is also less stringent than TT broth and GN broth since it contains neither bile salts nor sodium deoxycholate. In a recent study investigating enrichment media for detection of Shigellae, SB, GN broth, tryptic soy broth, and *Enterobacteriaceae* Enrichment (EE) broth with the

addition of novobiocin were compared (Uyttendaele et al., 2001). When incubated in GN broth, *Shigella* spp. were unable grow to comparable levels as observed in SB and EE broths, even though EE broth contains bile salts.

In order to increase the specificity of enrichment media, elevated incubation temperatures and anaerobic atmospheric conditions are recommended by the FDA BAM. In a competitive inoculation study, cultures incubated in SB and EE broth at 42ºC eliminated competitors such as *Aeromonas* and *Erwinia* while incubation at 37ºC did not (Uyttendaele et al., 2001).

Due to the fastidious nature of Shigellae, multiple plating media of different selectivity should be used to increase the chances of isolation. The most common low selectivity media used for plating *Shigella* spp. is MacConkey Agar (MAC); while Eosin methylene blue (EMB) or Tergitol-7 (T7) agars could also be used. Since differentiation is based on lactose fermentation, non-lactose competitors make detection of *Shigella* spp. on MAC very difficult (Uyttendaele et al., 2001). On MAC, *Shigella* spp. are translucent and slightly pink, with and without rough edges. *Shigella* spp. produce colorless colonies on EMB and bluish colonies on the yellowish-green T7 agar (APHA, 2001). A more recently developed low selectivity, differential medium is *Shigella* Plating Medium (SPM) (RF Laboratories, West Chicago, IL). *Shigella* spp. are white to clear on SPM. Intermediate selectivity media useful in isolating *Shigella* spp. are desoxycholate citrate agar (DCA) and xylose lysine desoxycholate agar (XLD). *Shigella* spp. produce colorless colonies on both DCA and XLD. Bhat and Rajan (1975) reported XLD superior to DCA for the isolation of *Shigella* spp. since DCA required a 48 hour incubation to show clear colony morphology as opposed to overnight incubation for XLD. A problem with XLD is

that D-xylose, which serves as a differentiating agent, is fermented by some strains of *S. boydii* while most *Shigella* spp. do not ferment xylose (APHA, 2001). This can cause some strains of *Shigella* to be missed if only XLD is used as a plating media. Highly selective media for *Shigella* spp. include *Salmonella-Shigella* agar (SSA) and Hektoen Enteric agar (HEA). A problem associated with SSA and HEA is that they may be too stringent for some strains of *Shigella* spp., especially if the culture is stressed (APHA, 2001; Uyttendaele et al., 2001). *Shigella* spp. produce colorless, translucent colonies on SSA and green colonies on HEA.

U.S. Food and Drug Administration (1998) Bacteriological Analytical Manual

The FDA BAM outlines a conventional culture method for the isolation and detection of *Shigella* spp. from food. A 25 g sample is transferred to 225 ml of *Shigella* broth (SB) to which novobiocin (0.5 µg/ml for *S. sonnei*; 3.0 µg/ml for other *Shigella* spp.) has been added. Samples are held at room temperature for 10 minutes and periodically shaken. Sample supernatants are transferred to an Erlenmeyer flask and the pH adjusted to 7.0 ± 0.2 with sterile 1 N NaOH or 1 N HCl. Flasks are incubated anaerobically for 20 hours (44ºC for *S. sonnei*; 42ºC for all other *Shigella* spp.). After incubation, enrichment culture is used to streak a MAC plate. Confirmation of suspicious colonies involves tests for motility, H_2S, gas formation, lysine decarboxylase, and fermentation of sucrose or lactose. All isolates showing any of these characteristics are discarded. Isolates negative for all confirmatory tests are tested for further biochemical reactions including adonitol, inositol, lactose, potassium cyanide, malonate, citrate, salicin, and methyl red. Shigellae are negative for all except methyl red. Antisera agglutination is then used to identify any culture displaying typical *Shigella* characteristics.

The effectiveness of the FDA (1992) BAM method for *Shigella* spp. was evaluated by June et al. (1993). The 1992 FDA BAM procedures were the same as the 1998 FDA BAM procedures with respect to the isolation and detection of *Shigella* spp. Two strains of *S. sonnei*, strains 9290 and 25931, were inoculated on potato salad, chicken salad, cooked shrimp salad, lettuce, raw ground beef, and raw oysters. The lowest number of unstressed cells of strain 9290 recovered from a 25 g food sample were: 1.0×10^{0} for potato salad, 8.5×10^{-1} for chicken salad, 8.8×10^{-1} for cooked shrimp salad, 7.6×10^{-1} for lettuce, 8.4×10^{1} for raw ground beef, and 8.5×10^{2} for raw oysters. The lowest number of unstressed cells of strain 25931 recovered from a 25 g food sample were: 5.3×10^{-1} for potato salad, 6.6×10^{-1} for chicken salad, 1.6×10^{2} for cooked shrimp salad, 4.2×10^{-1} for lettuce, 6.9×10^{4} for raw ground beef, and 5.4×10^{2} for raw oysters. The recovery of 8.4×10^{0} CFU/25 g for strain 9290 versus 6.9×10^{4} CFU/25 g for strain 25931 from raw ground beef samples suggests high strain variability, which can complicate recovery methods. Chilled stressed cells for both strains were recovered with similar results for all 6 foods. Since the infective dose of *Shigella* spp. is has low as 10 cells per person, the 1992 FDA BAM was considered ineffective for the evaluation of raw ground beef and raw oysters.

Compendium of Methods for the Microbiological Examination of Foods

Another method for the isolation/detection of *Shigella* spp. from foods is described in the Compendium of Methods for the Microbiological Examination of Foods (CMMEF) (APHA, 4th Edition, Chapter 38). This method calls for the enrichment of 25 g sample in either 225 ml of SB or GN broth, both with the optional addition of novobiocin (0.3 µg/ml for *S. sonnei*; 3.0 µg/ml for all other *Shigella* spp.) After holding at room temperature for 10 minutes, enrichment samples are incubated for 16 to 20 hours

at 37ºC. The CMMEF suggests that 2 to 3 plates of various selective media be used to streak the enriched cultures; MAC for low selectivity, XLD for intermediate selectivity, and HEA for high selectivity. Suspicious colonies are tested for motility, with all non-motile isolates identified by biochemical and serological tests.

Nucleic Acid-Based Detection of *Shigella* spp. in Foods

The essential principle of nucleic acid based detection methods is the specific formation of double stranded nucleic acid molecules from two complementary, single stranded molecules under defined physical and chemical conditions (Olsen et al, 1995). There are basically two types of nucleic acid assays: hybridization assays and the polymerase chain reaction (PCR).

Colony Hybridization Assays

In colony hybridization assays, a culture sample is spread-plated on appropriate media. Following incubation, the colonial pattern is transferred to a solid support (usually a membrane or paper filter) by pressing the support onto the agar surface (FDA, 1998). Cells are lysed in situ by a combination of high pH and temperature (0.5 M NaOH and/or steam or microwave irradiation), which also denatures and affixes the DNA to the support (FDA, 1998). Solid supports are incubated in solution containing labeled probes (^{32}P- or enzyme-label) to allow the probes to attach to their target DNA. Unbound probe is removed by washing the probe-target complexes on the support at an appropriate temperature and salt concentration (FDA, 1998). A signal is then generated using the label attached to the probe to identify positive colonies. Colony hybridization assays are useful when further characterization of positive colonies is required (Olsen et al., 1995).

PCR: The Basics

PCR amplifies regions of DNA by annealing specific primers to single stranded DNA (ssDNA) and rebuilding the double stranded molecule using a polymerase enzyme. Typical reactions are performed in a mixture of water, dNTPs, PCR buffer, primers, taq polymerase, and a DNA template. After initial denaturation, PCR subjects reaction mixtures to approximately 30 cycles of denaturation, annealing, and extension. Denaturation occurs at 94ºC and involves the unwinding of double stranded DNA (dsDNA) to ssDNA. Annealing, typically at 50-70ºC, is where the single stranded primers attach to the ssDNA at their specific sites. Extension occurs at 72ºC which is the optimal temperature for most taq polymerases. During extension, the polymerase enzyme interacts with the primer/ssDNA complex and rebuilds the dsDNA molecule using the dNTPs. As the cycling continues, the number of dsDNA copies doubles with each cycle. After the final cycle, most PCR reactions include a final extension step which allows the completion of any incomplete reactions. In theory, PCR can amplify a single copy of target DNA to over a million copies in a 30 cycle reaction.

Bacterial DNA Template Preparation for PCR

The failure or success of PCR greatly depends on the effectiveness of the DNA extraction method to provide adequate amounts of purified DNA. Early PCR used "crude" extracts of DNA obtained by boiling a culture/sample, centrifuging the cell material, and using the supernatant as DNA template. DNA templates prepared as crude extracts are often contaminated with high amounts of protein and contain very low concentrations of target DNA.

The most common way to purify and concentrate DNA samples is to perform a phenol, phenol-chloroform, or guanidine isothionate extraction (purification) followed by

ethanol precipitation (concentration). Other methods for DNA purification/concentration include the use of anion exchange resin (DNA affinity) columns or various filtration techniques. DNA affinity columns require elution by high salt and large fragments of DNA >20,000 bp can stick to the column (Millipore, 2003). Several filtration techniques are available for isolating DNA including size exclusion, glass fiber filters, and FTA filter paper. FTA filtration involves a chemically treated filter which can trap bacterial cells, lyse the cellular membrane, and bind bacterial DNA. Several PCR methods have been developed which can amplify DNA directly off the filter paper (Lampel et al., 2000; Orlandi and Lampel, 2000).

Nested PCR

Nested PCR refers to a two-step PCR technique in which the PCR product from the first reaction is diluted and used as template in the second PCR reaction. In true nested PCR, the first reaction uses an external set of primers (P1 and P2) to amplify a target region in a gene of interest. The second PCR reaction uses an internal primer set, (P3 and P4) to amplify a region from within the product of the first reaction. Semi-nested PCR is the same as nested PCR with the exception that instead of using two internal primers in the second PCR reaction, one internal (P3) and one external primer (P1 or P2) are used. The advantage of nested or semi-nested PCR is that greater specificity and sensitivity can be achieved. Protocols for the analysis of *Shigella* spp. in development at FDA utilize a nested PCR (Dr. Keith Lampel, personal communication). In the protocol, positive amplification in the first reaction serves as presumptive detection while positive amplification in the second reaction serves as confirmation of *Shigella* spp.

PCR for the Detection of *Shigella* sp.

PCR assays for *Shigella* spp. have targeted the invasion associated locus (*ial*) (Islam and Lindberg, 1992; Lindqvist, 1999), the *virA* gene (Vantarakis et al., 2000; Villalobo and Torres, 1998) or the *ipaH* gene (Sethabutr et al., 1993; Sethabutr et al., 2000). All three of these targets detect all four serogroups of *Shigella* and enteroinvasive *E. coli*. The *ial* and *virA* gene are located on the virulence plasmid, while the *ipaH* is encoded multiple times on the plasmid and on the chromosome (Jin et al., 2002). Since detection of the *ipaH* gene is possible in the event of losing the plasmid, it is a very attractive target for PCR assays. Although few studies have used PCR methods in the examination of food for *Shigella* spp., they have demonstrated higher sensitivity than conventional culture methods.

Vantarakis et al. (2000) developed a multiplex PCR method to detect both *Salmonella* spp. and *Shigella* spp. in mussels. Artificially inoculated *S. typhimurium* and *S. dysenteriae* were recovered by homogenizing mussel meat with peptone water. DNA from an aliquot of the homogenate was purified using a guanidine isothionate method and concentrated via ethanol precipitation. Amplification of *Shigella* spp. and *Salmonella* spp. DNA targeted the *virA* and *invA* genes, respectively. The multiplex PCR was able to detect *S. dystenteriae* at 1.0 x 10^3 CFU/ml homogenate with no pre-enrichment, and 1.0 x 10^1 -1.0 x 10^2 CFU/ml homogenate following 22 hour incubation in buffered peptone water.

Villalobo and Torres (1998) investigated PCR for the detection of *Shigella* spp. in mayonnaise. *S. dysenteriae* type 1 DNA was isolated from artificially contaminated mayonnaise samples by homogenizing in buffered peptone water, lysing cells with detergent, extracting with phenol-chloroform, and precipitating with ethanol.

Amplification targeted the *virA* gene and was multiplexed with a region of the 16srDNA. This detection method was able to detect *S. dysenteriae* type 1 at 1.0 x 10^2 -1.0 x 10^3 CFU/ml homogenate.

In a study by Lindqvist (1999), a nested PCR method was compared to a conventional culture method (NMKL no. 151 1995) for detection of *Shigella* spp. Recovery of DNA from spiked lettuce, shrimp, milk, and blue cheese samples was accomplished by homogenizing with physiological saline, buoyant density centrifugation (to separate components based on density), and boiling at 96-98ºC for 10 minutes. Amplification targeted internal regions of the *ial*. Single PCR, using the external primer sets only, was only able to detect *S. flexneri* in aqueous solution at 0.5-1.0 x 10^5 CFU/ml, however the nested PCR was able to detect 1.0 x 10^3 CFU/ml. The nested PCR assay in combination with buoyant density centrifugation was able to detect *S. flexneri* inoculated onto all four foods at 1.0 x 10^1 CFU/g (Lindqvist, 1999).

Theron et al. (2001) investigated a semi-nested PCR method for the detection of *Shigella* spp. in spiked environmental water samples. *S. flexneri* was inoculated into sterile and non-sterile environmental water samples. Dilutions of the water samples were made and bacterial cells from each dilution were harvested by centrifugation and resuspended in GN broth. After incubation at 37ºC for 6 hours, bacterial cells were washed twice in distilled water and lysed by heating at 100ºC for 10 minutes. Lysate supernatant was used as DNA template for semi-nested PCR. Amplification targeted the *ipaH* gene. The detection limits of the various environmental water samples were 2 x 10^3 cfu/ml for well water, 1.4 x 10^1 CFU/ml for lake water, 5.8 x 10^2 CFU/ml for river water, 6.1 x 10^2 CFU/ml for treated sewage water, and 1.1 x 10^1 CFU/ml for tap water.

Variability in results among the water samples was attributed to the presence of humic substances which serve as PCR inhibitors. Pre-enrichment in GN broth served to dilute these PCR inhibitors while allowing the *S. flexneri* to multiply, thereby increasing the concentration of target DNA.

Real-time PCR: The Future for the Detection of *Shigella* spp. in Food

Real-time PCR utilizes a fluorescently labeled oligonucleotide probe or a nonspecifically binding intercalating dye which allows for detection of generated product after each cycle of the PCR reaction. Total assay time is greatly reduced as compared to conventional PCR as there is no need for post-reaction analysis. The ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) runs in a 96-well format and ABI offers support in primer and probe development (Applied Biosystems, 2003). Furthermore, the Taqman® Universal Master Mix can be utilized to reduce laboratory preparation, contamination risks, and assay to assay variation. The Smart Cycler® II System (Cepheid, Sunnyvale, CA) offers more flexibility with its sixteen ICORE® (Intelligent Cooling/Heating Optical Reaction) modules (Cepheid 2003). The Smart Cycler's® software allows separate experiments with unique cycling protocols to be carried out and analyzed simultaneously. The LightCycler® (Roche Applied Science, Indianapolis, IN) provides the fastest PCR results by use of its unique capillary sample tubes format. Air heating of the 32 capillary tubes, as compared to a thermal block in other units, allows for rapid PCR cycling and a faster overall assay (Roche Applied Science, 2003). A 30-40 cycle PCR reaction can usually be completed in 20 to 30 minutes with the LightCycler®. All real-time PCR systems provide quantitative results based on assay specific standard curves or the incorporation of internal controls.

CHAPTER 3
METHODS AND MATERIALS

Preliminary Trials

Preparation of Microbiological Media

Wild strain cultures were grown in Tryptic Soy Broth (TSB) (Difco, Sparks, MD) and maintained on Tryptic Soy Agar (TSA) (Difco) slants. Adapted strains were cultured and maintained using TSB and TSA supplemented with 80 µg/ml rifampicin (TSB-R80 and TSA-R80, respectively). All dilutions and tomato rinses were performed using Phosphate Buffered Saline (PBS) prepared using PBS tablets (ICN Biomedicals Inc., Aurora, OH).

Enrichment media

Shigella broth was prepared according to the U.S. Food and Drug Administration's (1998) Bacteriological Analytical Manual (FDA BAM) and supplemented with novobiocin at 3.0 µg/ml (SB3.0), 0.5 µg/ml (SB0.5), or 0.3 µg/ml (SB0.3). EE Broth (Difco) was prepared as directed by manufacturer's instructions and supplemented with novobiocin at 1.0 µg/ml (EE1.0). For trials involving antibiotic supplemented enrichment media, rifampicin was added at 50 µg/ml (SB3.0-R50, SB0.5-R50, SB0.3-R50, and EE1.0-R50). When necessary, the pH was adjusted using filter sterilized 1N NaOH. Enrichment media were prepared fresh for each experiment.

Solid media

MacConkey Agar (MAC) (Difco), *Salmonella-Shigella* Agar (SSA) (Difco), *Shigella* Plating Medium (SPM) (RF Laboratories, West Chicago, IL), Triple Sugar Iron

(TSI) (Difco), Lysine Iron Agar (LIA) (Difco) and Motility Medium (MM) (Difco) were all prepared according to manufacturers instructions. When necessary, the pH was adjusted using filter sterilized 1N NaOH. MAC, SSA, and SPM were each poured into one compartment of a three-compartment Petri dish (Tri-Plate). TSI and LIA were prepared as slants and MM was prepared according to the FDA BAM.

Acquisition and Maintenance of *Shigella* cultures

Outbreak strains of *Shigella sonnei* and *Shigella boydii* were obtained from Dr. Hans Blaschek's Laboratory at the University of Illinois, Department of Food Science and Human Nutrition. The *Shigella boydii* serotype 18 strain, encoded UI02, was isolated from a person involved in a bean salad outbreak in Chicago in March of 1999. The *Shigella sonnei* strain, encoded UI05, was isolated from a patient during an outbreak. Both strains, UI02 and UI05, originated from the State of Illinois Department of Public Health, Chicago. In addition, a strain of *Shigella boydii* serotype 18 (ATCC 35966) (encoded UI01), a human isolate of *Shigella sonnei* (encoded UI03) from an outbreak involving bean dip, and a human isolate of *Shigella sonnei* (encoded UI04) from an outbreak involving cilantro were also obtained. Strains UI03 and UI04 originated from the Enteric Bacteriology Unit, Microbial Diseases Laboratory, State of California Department of Health Services.

Additional *Shigella* cultures were obtained to provide additional positive controls and to facilitate representation of each of the four serogroups. *Shigella dysenteriae* serotype 1 (ATCC 9361) and *Shigella sonnei* (ATCC 9290) were purchased from the American Type Culture Collection (ATCC). A strain of *Shigella flexneri* was obtained from Dr. Linda Harris' Laboratory at the University of California, Davis, Department of

Food Science and Technology. This strain originated from Dr. Keith Lampel at the U.S. Food and Drug Administration.

Upon receipt, each strain was grown in 10 ml TSB at 37°C (30 rpm) overnight. Overnight cultures were plated for isolation onto MAC and incubated overnight at 37°C. Overnight plates were examined for typical growth. One typical colony of each strain was transferred to a TSA slant and stored at 4°C. Another typical colony was transferred per product instructions to Protect ™ Bacterial Preservers (Scientific Device Laboratory, Inc., Des Plaines, IL) and stored at -76°C.

Adaptation of Cultures to Rifampicin

Adaptation of Shigellae to the bactericidal agent rifampicin was accomplished by challenging cultures in enrichment broth with low doses and increasing the dosage with each successive 24 hr transfer. A 10,000 ppm (1%) stock solution of rifampicin was prepared by dissolving 2.0 g rifampicin (Fisher # BP267925, Fisher Scientific, Pittsburg, PA) in 200 ml deionized water. This stock solution was then filter sterilized and stored in the dark at room temperature.

Stock cultures were grown overnight in 10 ml TSB (37°C, 30 rpm). Overnight cultures were transferred to 10 ml TSB with the progression of 2.5, 5.0, 10, 25, 40, 60, and 80 ppm rifampicin (TSB-R2.5, TSB-R5.0, TSB-R10, TSB-R25, TSB-R40, TSB-R60, and TSB-R80, respectively) and grown overnight (37°C, 30 rpm). Once the cultures were adapted to 80 ppm rifampicin, cultures were grown overnight (37°C, 30 rpm) three consecutive times in TSB-R80 to ensure well adapted populations.

Once adaptation was complete, the final overnight adapted culture was plated for isolation onto MAC-R80 and incubated overnight at 37°C. One typical colony from the

overnight MAC-R80 plate was transferred to a TSA-R80 slant and stored at 4°C. Another typical colony was transferred per product instructions to a Protect ™ Bacterial Preserver and stored at minus 76°C.

Preparation of Optical Density Standard Curve

For all standard curve studies, non-adapted cultures were grown using TSB and TSA. All adapted cultures were grown using TSB-R80 and TSA-R80. Procedures given below are worded for un-adapted cultures.

A sterile wooden stick was used to transfer stock culture from a TSA or TSA-R80 slant into a 10 ml TSB tube. The tube was incubated overnight (37°C, 30 rpm). A 10 µl aliquot of the first overnight culture was transferred to fresh 10 ml TSB and grown overnight (37°C, 30 rpm). Also from the first overnight culture, 10 µl was used to inoculate 100 ml TSB in an Erlenmeyer flask. This flask was incubated (37°C, 30 rpm) and observed every 30 minutes to determine the lag time associated with each strain. End of lag phase was determined by visible growth (cloudiness) in the TSB flask.

A 10 µl aliquot of the second overnight culture was transferred to fresh 10 ml TSB and grown for 18 hours (37°C, 30 rpm). A 10 µL aliquot of this 18 hour culture was then transferred into each of three Erlenmeyer flasks containing 100 ml TSB to provide three replicates. Each flask was labeled 1, 2, or 3 and inoculated five minutes apart in numerical order and incubated (37°C, 30 rpm).

After the pre-determined lag phase was complete, sampling began in 30 minute intervals. At each sampling, 1.25 ml of culture was transferred to a disposable cuvette and the absorbance was read at 600 nm using a spectrophotometer (Shimadzu Scientific Instruments, model UV-1201). Additionally, 1 ml of the culture was used to prepare

serial dilutions using PBS. Appropriate dilutions were pour plated with TSA and incubated 24 to 48 hours at 37°C.

TSA plates were analyzed for growth and plates containing colonies in the countable range (25-250) were counted. Replicate results were averaged yielding one count of colony forming units (CFU) per ml for each sampling. Replicate results of absorbance values were also averaged. Using Excel software (Microsoft, Redmon, WA), a graph of the absorbance versus $\log_{10}$ CFU/ml was prepared. The linear range was identified and subjected to linear regression analysis to form a standard curve. This standard curve was then used to estimate CFU/ml by means of absorbance at 600 nm in TSB.

DNA Extraction of Stock *Shigella* Cultures

Stock *Shigella* cultures used in this study are listed in Table 3-1. DNA was extracted from stock *Shigella* cultures via the DNeasy® Tissue Kit (Qiagen, Valencia, CA). Stock cultures were grown overnight in 10 ml TSB (37°C, 30 rpm). Overnight cultures were plated for isolation onto MAC and incubated overnight at 37°C. Overnight plates were examined for typical growth and one typical colony was transferred to 10 ml TSB and grown overnight (37°C, 30 rpm).

A 1 ml aliquot of overnight culture was transferred to a clean, sterile 1.5 ml microcentrifuge tube and centrifuged for 10 minutes at 7,500 rpm. Supernatant was discarded and the resulting pellet was re-suspended in 180 µl Buffer ATL (supplied with DNeasy® Tissue Kit). DNA extraction continued from this point from step 2 of the DNeasy® Protocol for Animal Tissues as per product literature. Final DNA elution was performed twice; once with 200 µl Buffer AE (supplied with DNeasy® Tissue Kit), and a

Table 3-1. Stock *Shigella* cultures. DNA from each stock *Shigella* spp. was extracted from a 1 ml aliquot of an overnight culture in TSB (37ºC, 30 rpm) using a DNeasy® Tissue Kit. Extracted DNA was transferred to a clean, sterile 1.5 microcentrifuge tube and stored at minus 20ºC until use. Extracted DNA was used to evaluate specificity of each primer set.

DNA Code	Culture	Origin
01	*Shigella boydii* serotype 18	ATCC 35966
02	*Shigella boydii* serotype 18	Outbreak isolate
03	*Shigella sonnei*	Patient isolate
04	*Shigella sonnei*	Patient isolate
05	*Shigella sonnei*	Outbreak isolate
06	*Shigella sonnei*	ATCC 9290
07	*Shigella flexneri*	FDA, Dr. Keith Lampel
08	*Shigella dysenteriae* serotype 1	ATCC 9361

second time with 50 µl Buffer AE. This yields a final elution of 250 µl DNA template in one 1.5 ml microcentrifuge tube. DNA templates for stock *Shigella* cultures were stored at minus 20°C.

Crude DNA Extraction of Stock Non-*Shigella* Cultures

Stock non-*Shigella* cultures used as negative controls in this study are listed in Table 3-2. DNA from non-*Shigella* stock cultures was extracted to provide negative control templates. Stock cultures frozen on Protect ™ Bacterial Preservers were retrieved from minus 76°C storage and thawed. One bead was aseptically transferred from the Protect ™ Bacterial Preserver into 10 ml TSB and grown overnight (37°C, 30 rpm). Overnight cultures were plated for isolation on an appropriate selective and differential medium and incubated overnight at 37°C. Overnight plates were observed for typical colony morphologies. One typical colony was transferred to 10 ml TSB and grown overnight (37°C, 30 rpm).

A 1 ml aliquot of the overnight culture was transferred to a clean, sterile 1.5 ml microcentrifuge tube and centrifuged for 10 minutes at 7,500 rpm. Supernatant was

Table 3-2. Stock non-*Shigella* cultures. DNA from each of the non-*Shigella* spp. cultures was extracted by boiling a 1 ml aliquot of an overnight culture for 10 minutes, then centrifuging away any cell wall material. Supernatants were transferred to clean, sterial 1.5 ml microcentrifuge tubes and used to test the specificity of each primer set.

DNA Code	Culture	Origin
09	*Salmonella* Agona	Dr. Harris, UC Davis
10	*Salmonella* Gaminara	Dr. Harris, UC Davis
11	*Salmonella* Montevideo	Dr. Harris, UC Davis
12	*Salmonella* Michigan	Dr. Harris, UC Davis
13	*Salmonella* Poona	Dr. Harris, UC Davis
14	*Salmonella* Enteritidis	Dr. Rodrick, UF
15	*Salmonella* Typhimurium	ABC Research
16	*Escherichia coli* O157:H7	Deibel Laboratories
17	*Escherichia coli* O157:H7	Deibel Laboratories
18	*Escherichia coli* O157:H7	Deibel Laboratories
19	*Escherichia coli* O157:H7	Deibel Laboratories
20	*Escherichia coli* JM109	Dr. Wright, UF
21	*Escherichia coli* K12	Dr. Wright, UF
22	*Escherichia coli* (ATCC 25922)	ATCC
23	*Citrobacter freundii*	Cantaloupe isolate
24	*Klebsiella pneumoniae*	Soil isolate
25	*Klebsiella ozoanae*	Cantaloupe isolate
26	*Enterobacter cloacae*	Cantaloupe isolate

discarded and the resulting pellet was re-suspended in 200 µl double de-ionized, sterilized water (hereby referred to as PCR water). Samples were then boiled for 10 minutes in a dry bath incubator (Fisher Scientific, IsoTemp 125D). Supernatant (DNA template) was aseptically transferred to a clean, sterile 1.5 ml microcentrifuge tube and stored at minus 20°C. Pellet was discarded.

Acquisition and Maintenance of PCR Primers for the Detection of *Shigella*

Primers were synthesized by Sigma Genosys (The Woodlands, TX). Upon receipt, primers were reconstituted with 10% TE Buffer (1.0 mM Tris-Cl, 0.1 mM EDTA pH 8.0) to yield a 100 mM stock solution. The working solution was a 1:10 dilution of the stock solution with PCR water. Primer sets investigated in this study are shown in Table 3-3.

Table 3-3. Primers for the detection of *Shigella* spp. All primers sets below are specific for *Shigella* spp. and EIEC. Primer set 01-001 amplifies a 620 bp fragment of the *ipaH* gene. Primer set 01-002 amplifies a 215 bp fragment of the *virA* gene. Primer sets 01-003 and 01-004 are a nested primer set where 01-003 is the internal primer amplifying a 217 bp region of the invasion associated locus (ial) and 01-004 is the external primer amplifying a 320 bp fragment of the ial. Primer set 01-005 serves as an internal primer to set 01-001 and amplifies a 290 bp fragment of the *ipaH* gene.

Primer Code	Primer Sequence	Source
01-001F	5' gtt cct tga ccg cct ttc cga tac cgt c 3'	Sethabutr et al., 2000
01-001R	5' gcc ggt cag cca ccc tct gag agt ac 3'	Sethabutr et al., 2000
01-002F	5' ctg cat tct ggc aat ctc ttc aca tc 3'	Villalobo and Torres, 1998
01-002R	5' tga tga gct aac ttc gta agc cct cc 3'	Villalobo and Torres, 1998
01-003F	5' ttt tta att aag agt ggg gtt tga 3'	Lindqvist, 1999
01-003R	5' gaa cct atg tct acc tta cca gaa gt 3'	Lindqvist, 1999
01-004F	5' ctg gta ggt atg gtg agg 3'	Frankel et al., 1990
01-004R	5' cca ggc caa caa tta ttt cc 3'	Frankel et al., 1990
01-005F	5' cca ctg aga gct gtg agg 3'	Lampel (unpublished 2002)
01-005R	5' tgt cac tcc cga cac gcc 3'	Lampel (unpublished 2002)

Specificity of Primers

The specificity of each primer set was tested against DNA from each strain of *Shigella* (Table 3-1) and a battery of non-*Shigella* DNA (Table 3-2). Amplification of target DNA sequences was performed in a 25 µl reaction mixture in clean, sterile 0.2 ml polypropylene microcentrifuge tubes. The reaction mixture consisted of 15.75 µl PCR water, 2.5 µl 10x PCR Buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.3), 2.5 µl dNTP mixture (2.5 mM each), 1.0 µl forward primer, 1.0 µl reverse primer, 0.25 µl taq polymerase (TaKaRa Taq Hot Start Version, Shiga, Japan), and 2 µl DNA template. The thermocycler used to perform the PCR reaction was an Eppendorf Mastercycler gradient (Brinkmann-Eppendorf, Westbury, NY). Temperature programs used to evaluate each primer set are given in Table 3-4.

Analysis of PCR Product by Gel Electrophoresis

DNA amplicons were analyzed by gel electrophoresis. Gel used in all experiments

Table 3-4. Temperature programs for PCR primers. All temperature programs were obtained from the sources of the primer sets listed above in Table 3-3.

Primer Set	Initial Heating	Denaturation	Annealing	Extension	Number of Cycles	Final Extension	Hold
01-001	94°C 10 min	94°C 15 sec	60°C 30 sec	72°C 45 sec	30	72°C 2 min	4°C
01-002	94°C 10 min	94°C 45 sec	65°C 30 sec	72°C 30 sec	35	72°C 10 min	4°C
01-003	94°C 5 min	94°C 30 sec	60°C 1 min	72°C 30 sec	30	72°C 10 min	4°C
01-004	94°C 5 min	94°C 30 sec	60°C 1 min	72°C 30 sec	30	72°C 10 min	4°C
01-005	94°C 10 min	94°C 15 sec	60°C 15 sec	72°C 15 sec	30	72°C 2 min	4°C

was 3% NuSeive® GTG® Agarose (BioWhittaker Molecular Applications, Rockland, MA). Electrophoresis was performed on Thermo EC gel trays (model CSSU78115, Holbrook, NY) powered with a Thermo EC power supply (model EC105). Gel trays were leveled using a Thermo EC leveling platform (model CSSLP78). Gels were stained with ethidium bromide (FisherBiotech, BP1302-10). DNA amplicons and DNA markers (ΦX174/*Hin*f I, Promega, Madison, WI) were visualized by UV transilluminator (Spectroline® TE-312S, Westbury NY). Images of DNA amplicon bands were captured using a photodocumentation handheld camera (Fisherbiotech, FB-PDC-34) loaded with Polaroid Type 667 high-speed print film (Fisher Scientific, 04-441-91).

Inoculated Studies

Acquisition of Tomato

Tomatoes of the Florida cultivar 47 were obtained from a nearby packinghouse (DiMare, Palmetto, FL). Tomatoes were pulled prior to the wash/wax line in order to retain the normal bacterial population. Upon arrival, tomatoes were stored at 13ºC until

use. Only fully green or tomatoes with less than 50% red color were used. All tomatoes with greater than 50% red color were discarded.

Inoculum Preparation

Three days prior to each experiment, stock culture stored on TSA-R80 slants at 4ºC was grown (37ºC, 30 rpm) in a 10 ml tube of TSB-R80. Overnight transfers were performed using 10 ml tubes of TSB-R80 each day. On the day of the experiment, an 18-hour culture (late stationary phase) was centrifuged (4,000 x g for 10 minutes) and washed twice with PBS. The washed culture was serially diluted using 9 ml tubes of PBS. Appropriate dilutions were pour-plated with TSA-R80 to confirm cell titer.

Inoculation of Tomatoes and Subsequent Recovery

Prior to each experiment, tomatoes were removed from cold storage and allowed to warm to room temperature (~21ºC). Large plastic trays were sanitized using reagent alcohol, 70% v/v (ethanol – 63% v/v, methanol – 3.5% v/v, isopropanol – 3.5% v/v, and water – balance) (LabChem Inc., Pittsburg, PA) prior to each experiment. Tomatoes were placed on the tray stem scar down. Using an Eppendorf Repeater Micropipette, ten 10 µL aliquots of the appropriate dilution were spot inoculated around the blossom scar of each tomato without inoculating directly on the blossom scar (Figure 3-1). *S. sonnei* was inoculated onto tomato surfaces at levels of 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU/tomato. *S. boydii* was inoculated onto tomato surfaces at levels of 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU/tomato. Inoculated tomatoes were allowed to air dry completely prior to continuing with recovery.

Figure 3-1. Spot inoculation of tomatoes. Tomatoes were inoculated with ten 10 µl spots around but not touching the blossom scar. Inocula were allowed to air dry prior to recovery.

After inocula were completely dry, tomatoes were transferred aseptically to a sterile stomacher bag containing 100 ml PBS (Figure 3-2). Each bag was sealed using stomacher bag clips. Inoculum was recovered by methods similar to that of Beuchat et al. (2001), modified to include vigorous shaking and hand rub/manipulation (15 seconds shake/ 15 seconds rub/ 15 seconds shake).

Experimental Design

Thirty tomatoes were prepared at each inoculation level and rinsed as described above. Ten tomato rinses were analyzed for *S. boydii* UI02 and *S. sonnei* UI05 using standard enrichment media, ten were analyzed using enrichment media supplemented with rifampicin, and ten were analyzed using the polymerase chain reaction (PCR). For standard enrichment, 25 ml of the tomato rinse was transferred to each of three 18 oz Whirl-Pak® bags (Nasco, Modesto, CA) containing 225 ml of appropriate enrichment

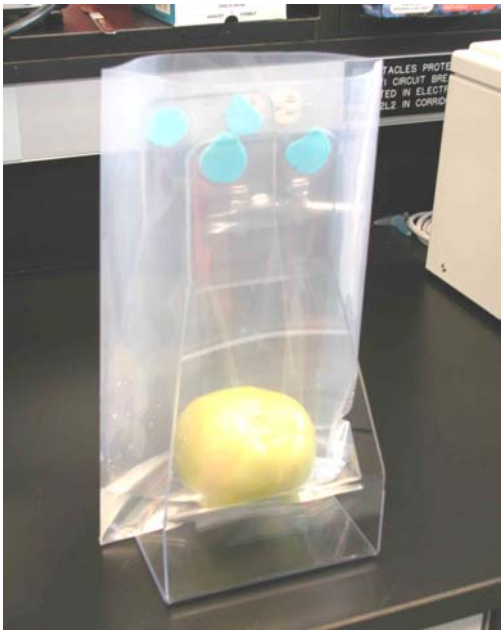

Figure 3-2. Stomacher bag with inoculated tomato. Inoculated tomatoes were aseptically transferred to a sterile stomacher bag which contained 100 ml PBS. The bag was sealed using stomacher bag clips and the inoculum was recovered using a vigorous shaking and hand rub/manipulation method (15 seconds shake/ 15 seconds rub/ 15 seconds shake).

media. Inoculation studies involving *S. sonnei* utilized enrichments in SB0.3 (44ºC, anaerobically) (FDA BAM), SB0.5 (37ºC) (CMMEF), and EE1.0 (42ºC) (EE Broth). Inoculation studies involving *S. boydii* utilized enrichments in SB3.0 (42ºC, anaerobically) (FDA BAM), SB3.0 (37ºC) (CMMEF), and EE1.0 (42ºC) (EE Broth). Anaerobic conditions were generated using the Pack-Anaero anaerobic gas generating system (Mitsubishi Gas Chemical Company, Inc. (MGC), Japan) and 7.0 Liter Pack-Rectangular Jars (MGC). For antibiotic supplemented enrichment, SB0.3-R50, SB0.5-R50, SB3.0-R50, and EE1.0-R50 were used in place of standard enrichment media and incubated at the same conditions. After 24 hours, each enrichment bag was mixed and sterile wooden sticks were used to streak the enrichment for isolation on each compartment of a Tri-Plate. Tri-Plates were incubated overnight (37ºC).

Confirmation of Typical Colonies on Tri-Plates

Typical *Shigella* isolates from all plating media on Tri-Plates were carried through the following confirmation process. TSI and LIA slants were inoculated with suspect colonies and incubated overnight (37ºC). TSI and LIA slants demonstrating typical reactions for *Shigella* were used to inoculate MM and a 10 ml tube of TSB which were then incubated overnight (37ºC). Growth in TSB from samples demonstrating no motility in MM was streaked for isolation on MAC and incubated overnight (37ºC). Biochemical reactions were tested using the BBL® Enterotube™ II (Becton Dickinson, Sparks, MD). Enterotubes were incubated overnight (37ºC) and positive reactions were read according to manufacturer's instructions.

Assembly of Tandem Filter Funnels

Two Whatman® 25 mm disposable filter funnels with grade 4 filters (Whatman®, Clifton, NJ) were assembled in tandem (Figure 3-2) with the following modifications. In the top filter funnel, the grade 4 filter was aseptically lifted and a 25 mm diameter VWR 413 filter was placed underneath. The threading on the funnel was lined with a layer of Parafilm "M" laboratory film (American National Can™, Chicago, IL). In the bottom filter funnel, the grade 4 filter was replaced with a 25 mm FTA® filter, aseptically cut from a FTA® Classic Card (Whatman®, Cat, No. WB12 0205). The FTA® filter was covered with a 25 mm diameter plastic shield, which had a 6 mm hole punched in its center. The plastic shield was aseptically cut from a plastic weight boat. The top filter funnel was inserted into the top of the bottom filter funnel and the joint was sealed with Parafilm.

Tomato rinses to be analyzed by PCR were transferred to Oxford 4 oz. specimen cups (Cat # OX-067, International BioProducts, Bothell, WA) to facilitate easy pouring.

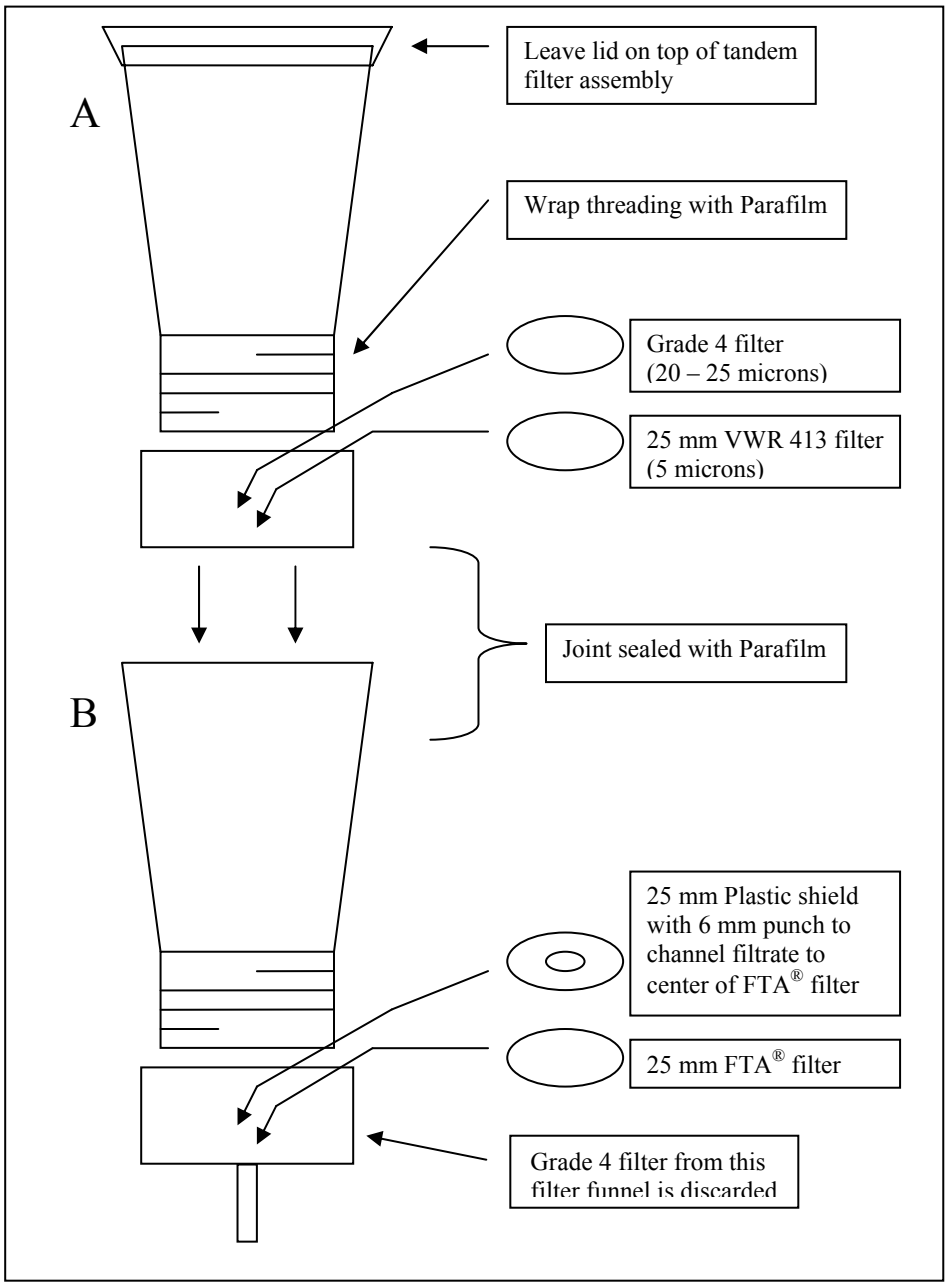

Figure 3-2. Assembly of tandem filter funnels. (A) Top filters are for size exclusion, (B) bottom FTA® filter is for trapping bacteria, lysing bacterial cell walls, and binding bacterial DNA.

A ring stand equipped with a clamp was used to secure a 500 ml Pyrex vacuum flask. The side arm of the vacuum flask was connected to a water trap consisting of a 500 ml side arm flask filled with Drierite® (anhydrous calcium sulfate) (W.A. Hammond Drierite Company Ltd., Xenia, OH) which was then connected to a vacuum pump (Emerson,

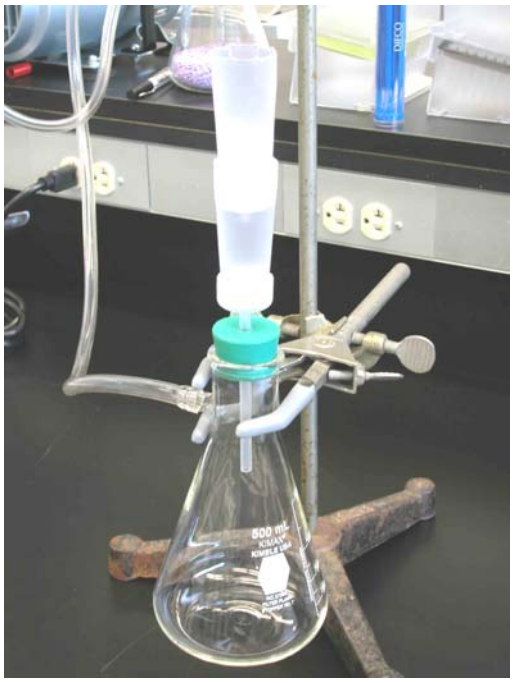

Figure 3-3. Vacuum flask apparatus with tandem filter funnels. Filter funnels are attached the top of a 500 ml filter flask via a plastic stem (supplied with the filter funnels) inserted through the center of a number 7 rubber stopper. The filter flask is attached via Tygon® tubing to another filter flask which is set up as a water trap. The water trap filter flask is connected to a vacuum pump. Filtration of tomato rinses were facilitated by vacuum at 400 mm Hg.

model SA55NX6TE-4870, St. Louis, MO) using Tygon® tubing. The top of the flask was sealed with a No. 7 rubber stopper in which a 6 mm hole had been bored through the center, through which a 4 inch plastic stem (supplied with the filter funnels) had been inserted.

Tomato rinse was filtered through the tandem filter funnel via vacuum pressure (400 mm Hg). After all of the rinse had passed through the FTA® filter, the vacuum pressure was turned off and the tandem filter funnel removed from the plastic stem. The FTA® filter and plastic shield was aseptically removed and placed in one compartment of a three compartment Petri dish. FTA® filters and shields were allowed to air dry overnight with the shield faced down. Using the holes in the plastic shields as a guide, 6

mm punches were aseptically taken from each FTA® filter and placed in a clean, sterile 1.5 microcentrifuge tube. FTA® punches were washed twice with 500 ml FTA® Purification Reagent (Whatman®, Clifton, NJ), then twice with 500 ml TE Buffer. Washed punches were aseptically transferred to Petri dishes and dried in a 37ºC incubator with the lids slightly open.

Nested PCR Amplification of *ipaH* gene for Detection of *Shigella*

Dry, washed FTA® punches were transferred to 0.5 ml microcentrifuge tubes and subjected to a nested, two step PCR reaction. The PCR step 1 reaction used FTA® punches as DNA template submerged in 200 µl of PCR step1 reaction mix. The PCR step 1 reaction mix consisted of 142 µl PCR water, 20 µl 10x HotMaster Taq Buffer (with 25 mM Mg^{2+}, pH 8.5), 20 µl dNTP mixture (2.5 mM each), 8.0 µl primer 01-001F, 8.0 µl reverse primer, 2 µl HotMaster Taq Polymerase (Eppendorf, Hamburg, Germany). Prior to PCR, 0.5 microcentrifuge tubes were centrifuged (30 seconds at 15,000 x g) to ensure the FTA® punch was completely submerged in step 1 reaction mix. The cycling conditions for step 1 were: initial denaturation at 94ºC for 10 min; 30 cycles of denaturation at 94ºC for 15 sec, annealing at 60ºC for 30 sec, and elongation at 72ºC for 45 sec; and a final elongation at 72ºC for 2 minutes.

PCR step 2 amplified a segment from within the target DNA segment of step 1. A 1 µl aliquot from the PCR step 1 product was diluted using 99 µl PCR water. Amplification in PCR step 2 was performed in 25 µl reactions in clean, sterile 0.2 ml microcentrifuge tubes. The step 2 reaction mixture consisted of 15.75 µl PCR water, 2.5 µl 10x PCR Buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.3), 2.5 µl dNTP mixture (2.5 mM each), 1.0 µl primer 01-005F, 1.0 µl primer 01-005R, 0.25 µl HotMaster taq polymerase, and 2 µl DNA template (from the 1:100 dilution of step 1 product). The

cycling conditions for step 2 were: initial denaturation at 94ºC for 10 min; 30 cycles of denaturation at 94ºC for 15 sec, annealing at 60ºC for 15 sec, and elongation at 72ºC for 15 sec; and a final elongation at 72ºC for 2 minutes.

DNA amplicons were analyzed by gel electrophoresis as described above. The amplicon in step 1 was 620 bp and the amplicon from step 2 was 290 bp.

Recording of Data and Statistical Evaluation

All results from inoculation studies involving *S. boydii* UI02 and *S. sonnei* UI05 were recorded as either positive or negative for detection. Positive enrichment was scored based on the isolation of inoculated *S. boydii* UI02 or *S. sonnei* UI05 by at least one of the three plating media. Positive isolation by plating media resulted from typical reactions for *Shigella* spp. in all confirmation steps and identification from Enterotubes. Positive detection by PCR methods resulted from amplification of a single band of the appropriate base pairs.

Logistical regression models were constructed using the R software (R Development Core Team, Version 1.7.0 Patched) to identify significant differences between isolation of *Shigella* spp. by PCR methods and enrichment methods and isolation of *Shigella* spp. on the three plating media. Models for evaluating PCR methods and enrichments were constructed (without an intercept) with covariate factors for enrichments/PCR and inoculation levels. Models for evaluating plating media were constructed (without an intercept) using plating media as covariates. Multiple comparisons were performed using the Bonferroni method since this method is conservative in its estimates of significance compared to other multiple comparison methods. P values of < 0.05 were considered significant.

CHAPTER 4
RESULTS

This study consisted of two phases of research. The first phase consisted of preliminary trials involving the preparation of growth curves, optical density standard curves, and testing the specificity of each set of primers against a DNA library of positive and negative controls. The second phase consisted of inoculation studies where tomatoes were spot inoculated with *S. boydii* UI02 and *S. sonnei* UI05 at pre-determined levels. Recovery/detection of the inocula was tested using conventional culture methods, conventional culture methods with rifampicin supplemented enrichment, and a newly developed FTA® filtration/ nested PCR method.

Preliminary Trials

Growth Curves and Optical Density Standard Curves

Growth curves and optical density (O.D.) standard curves were prepared for *S. boydii* UI02 – wild strain, *S. sonnei* UI05 – wild strain, and *S. sonnei* 9290 – rifampicin adapted. Since the growth curve and O.D. standard curve for *S. sonnei* 9290 – wild strain was similar to that of *S. sonnei* UI05 – wild strain, results for the 9290 wild strain are not shown. Results from growth curve and O.D. standard curve data were used to cultivate consistent inocula, thereby reducing variability in later recovery studies on tomatoes.

S. boydii UI02 – wild strain

The growth curve for *S. boydii* UI02 – wild strain demonstrates that stationary phase was reached in approximately 8 hours (Figure 4.1). A lag phase of approximately 4 hours was observed prior to exponential growth.

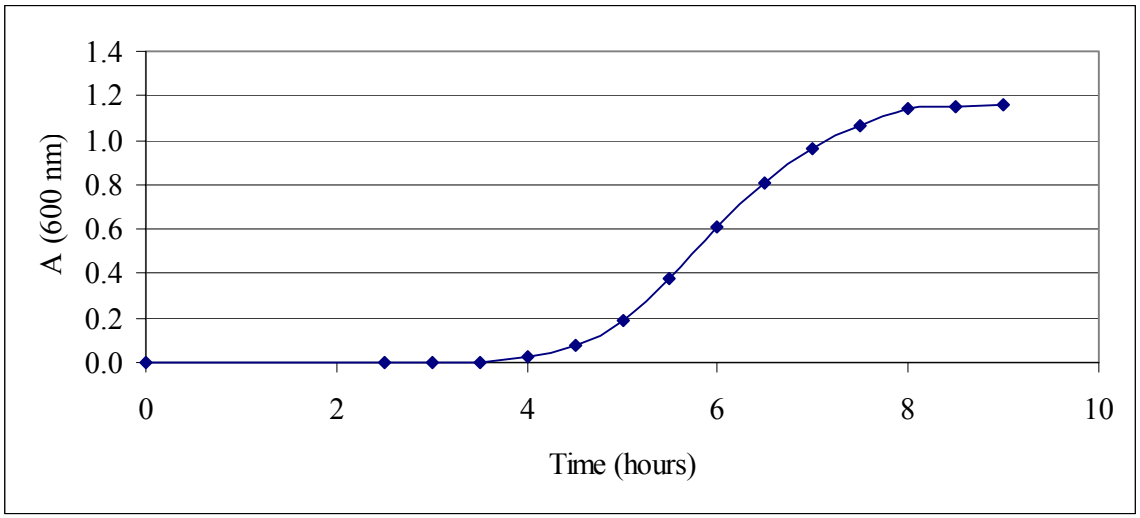

Figure 4-1. Growth curve: *S. boydii* UI02 – wild strain. Each of three 100 ml TSB microcosms were inoculated with 10 µl aliquots of an 18-hour *S. boydii* UI02 culture. The microcosms were incubated (37ºC, 30 rpm) and sampled at appropriate time intervals. At each time of sampling, the absorbance at 600 nm (A 600 nm) was measured spectrophotometrically. Shown above is the average A 600 nm of the three trials plotted against time.

An O.D. standard curve for *S. boydii* UI02 – wild strain was prepared. The cell titer in which the relationship between $\log_{10}$ CFU/ml and absorbance at 600 nm (A 600 nm) showed linearity ($R^2 = 0.9926$; Figure 4-2) was approximately 7.03×10^7 to 3.18×10^8 CFU/ml.

<u>*S. sonnei* UI05 – wild strain</u>

The growth curve prepared for *S. sonnei* UI05 – wild strain demonstrates stationary phase was reached in approximately 6 ½ hours (Figure 4-3), with an initial lag phase of approximately 3 hours.

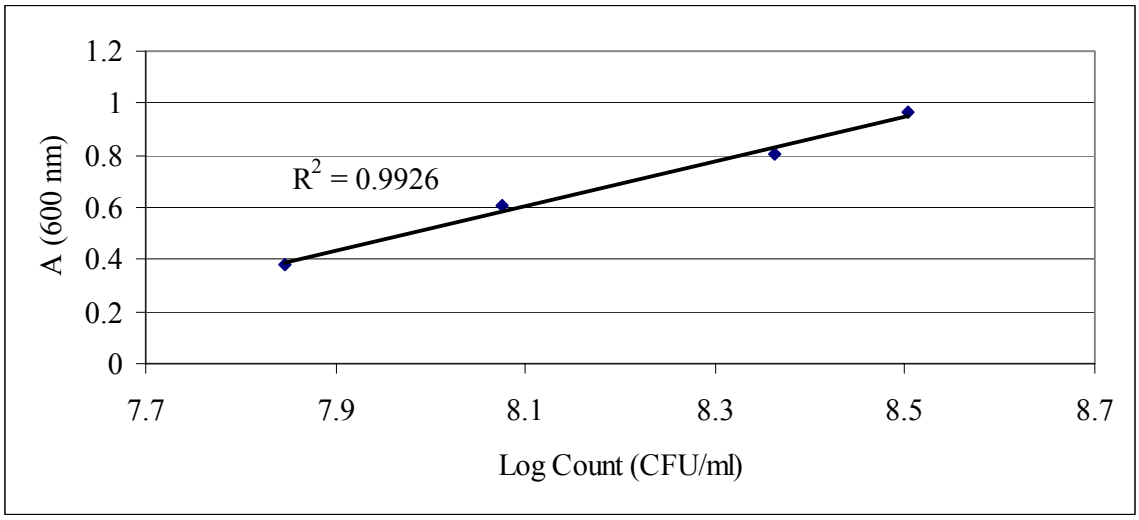

Figure 4-2. Optical density standard curve for *S. boydii* UI02 – wild strain. Each of three 100 ml TSB microcosms were inoculated with 10 µl aliquots of an 18-hour *S. boydii* UI02 culture. The microcosms were incubated (37ºC, 30 rpm) and sampled at appropriate time intervals. At each time of sampling, appropriate serial dilutions in PBS were pour-plated using TSA and incubated overnight (37ºC). Shown above is the average absorbance at 600 nm (A 600 nm) of the three trials (from Fig 4-1) plotted against the $\log_{10}$ CFU/ml for the data points which form a linear relationship.

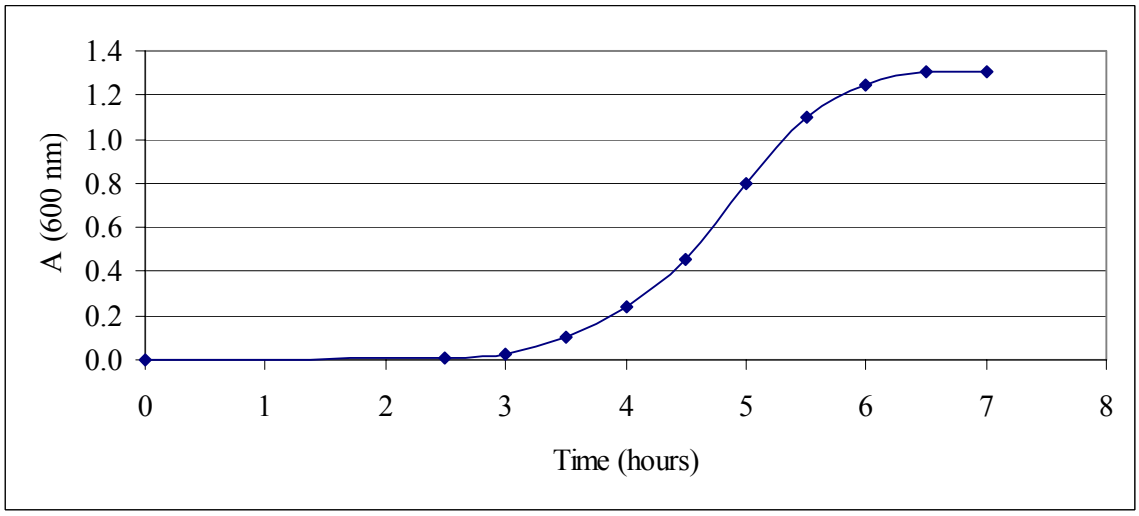

Figure 4-3. Growth curve: *S. sonnei* UI05 – wild strain. Each of three 100 ml TSB microcosms were inoculated with 10 µl aliquots of an 18-hour *S. sonnei* UI05 culture. The microcosms were incubated (37ºC, 30 rpm) and sampled at appropriate time intervals. At each time of sampling, the absorbance at 600 nm (A 600 nm) was measured spectrophotometrically. Shown above is the average A 600 nm of the three trials plotted against time.

An O.D. standard curve for *S. sonnei* UI05 – wild strain was prepared. The cell titer in which the relationship between $\log_{10}$ CFU/ml and absorbance at 600 nm (A 600 nm) showed linearity ($R^2 = 0.9833$; Figure 4-4) was approximately 4.13×10^7 to 6.43×10^8 CFU/ml.

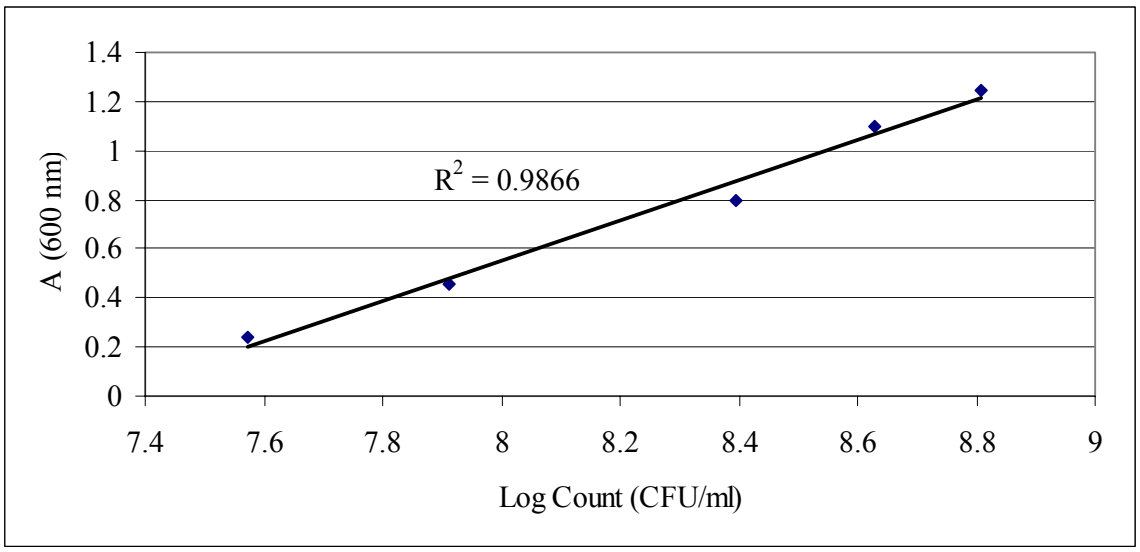

Figure 4-4. Standard curve of O.D. (600 nm) of *S. sonnei* UI05 – wild strain compared to log plate count. Each of three 100 ml TSB microcosms were inoculated with 10 µl aliquots of an 18-hour *S. sonnei* UI05 culture. The microcosms were incubated (37ºC, 30 rpm) and sampled at appropriate time intervals. At each time of sampling, appropriate serial dilutions in PBS were pour-plated using TSA and incubated overnight (37ºC). Shown above is the average absorbance at 600 nm (A 600 nm) of the three trials (from Fig 4-1) plotted against the $\log_{10}$ CFU/ml for the data points which form a linear relationship.

<u>*S. sonnei* 9290 – rifampicin adapted strain</u>

The growth curve for *S. sonnei* 9290 – rifampicin adapted strain demonstrates stationary phase was reached in approximately 8 ½ hours (Figure 4-5), with an initial lag phase of approximately 5 hours.

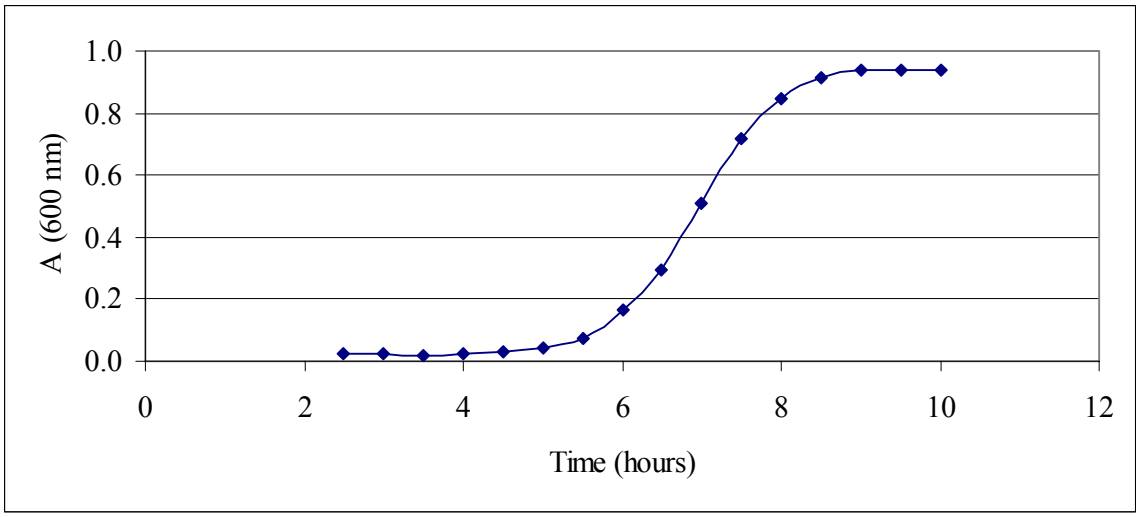

Figure 4-5. Growth curve: *S. sonnei* 9290 – rifampicin adapted strain. Each of three 100 ml TSB-R50 microcosms were inoculated with 10 µl aliquots of an 18-hour *S. sonnei* 9290 culture. The microcosms were incubated (37ºC, 30 rpm) and sampled at appropriate time intervals. At each time of sampling, the absorbance at 600 nm (A 600 nm) was measured spectrophotometrically. Shown above is the average A 600 nm of the three trials plotted against time.

An O.D. standard curve for *S sonnei* 9290 – rifampicin adapted strain was prepared. The cell titer in which the relationship between $\log_{10}$ CFU/ml and absorbance at 600 nm (A 600 nm) showed linearity ($R^2 = 0.9944$; Figure 4-6) was approximately 3.26 x 10^7 to 8.20 x 10^8 CFU/ml.

Primer Specificity

Each primer set (Table 3-3) was tested against DNA templates from both stock *Shigella* spp. (Table 3-1) and closely related microorganisms (Table 3-2). Primer set 01-001, which amplifies a 620 bp region of the *ipaH* gene of *Shigella* spp. and enteroinvasive *E. coli* (EIEC), successfully amplified DNA from all eight strains of *Shigella*. Primer set 01-002, which targets a 215 bp region of the *virA* gene of *Shigella* spp. and EIEC, successfully amplified DNA from *S. boydii* UI02, *S. flexneri*, and *S. dysenteriae* ATCC 9361, however it did not amplify a product from DNA from any of the

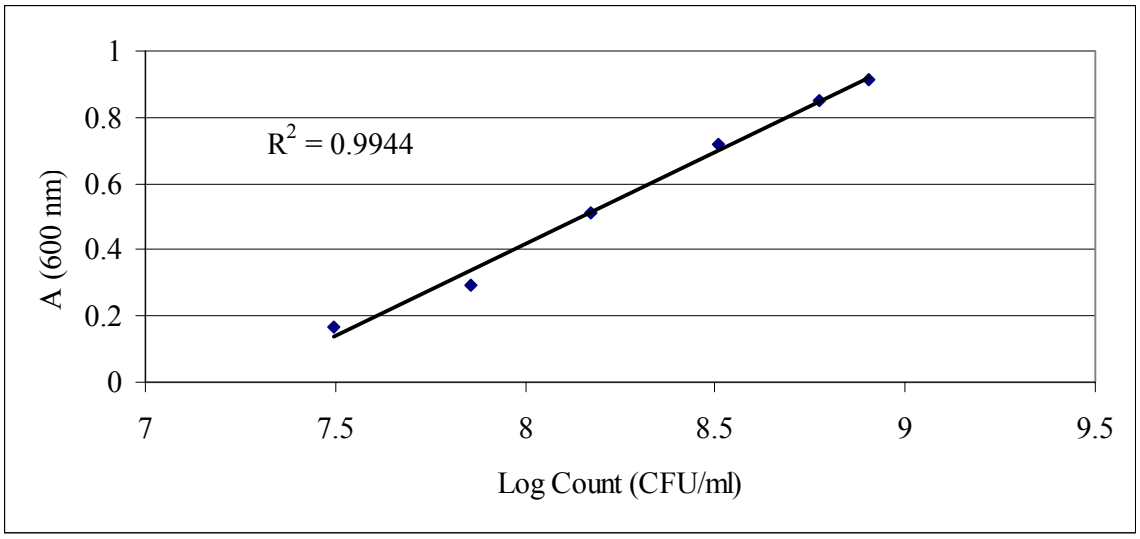

Figure 4-6. Standard curve of O.D. (600 nm) of *S. sonnei* 9290 – rifampicin adapted strain compared to log plate count. Each of three 100 ml TSB-R50 microcosms were inoculated with 10 µl aliquots of an 18-hour *S. boydii* UI02 culture. The microcosms were incubated (37ºC, 30 rpm) and sampled at appropriate time intervals. At each time of sampling, appropriate serial dilutions in PBS were pour-plated using TSA-R50 and incubated overnight (37ºC). Shown above is the average absorbance at 600 nm (A 600 nm) of the three trials (from Fig 4-1) plotted against the $\log_{10}$ CFU/ml for the data points which form a linear relationship.

four *S. sonnei* strains or *S. boydii* serogroup 18 (ATCC 35966). Primer set 01-004 and 01-003, which amplify a 320 bp region of the invasion associated locus (*ial*) and a 217 bp region from within that 320 bp region, respectively, successfully amplified a product from the DNA of *S. boydii* UI02 only, while producing no product from *S. sonnei* UI05, *S. flexneri*, and *S. dysenteriae*. Primer sets 01-004 and 01-003 were not tested against DNA from the other four strains of *Shigella*. Primer set 01-005, which amplifies a 290 bp region from within the region amplified by primer set 01-001, was successful in amplifying DNA from all eight strains of *Shigella* tested.

Primer sets 01-001, 01-002, and 01-005 did not amplify DNA from any of the non-*Shigella* DNA (Table 3-2). Primer sets 01-004 and 01-003 were only tested against non-

Shigella DNA of *Salmonella* Gaminara and *Salmonella* Typhimurium, producing no amplification in either sample.

Inoculated Studies

Detection of *Shigella* spp. by Conventional Culture Methods

Tomato rinses were enriched according to protocols of the FDA BAM, the CMMEF, and in EE broth as described by Uyttendaele et al. (2000). Overnight enrichments were then plated using SSA, MAC, and SPM. For all conventional culture trials, 10 replicates were analyzed at each inoculation level. Inoculation levels were verified by pour plating serial dilutions in triplicate with TSA-R50. Recovery results for all conventional culture method experiments are expressed as "percent recovery," defined as the number of tomatoes which tested positive for *Shigella* spp. out of the total number tested.

S. boydii UI02 was not recovered by conventional culture methods from any samples inoculated at 10^6, 10^5, or 10^4 CFU/tomato. For this reason, trials at the lower inoculation levels were not performed for *S. boydii* UI02.

Figure 4-7 demonstrates the percent recovery of *S. sonnei* UI05 by conventional culture methods. At inoculation levels of 10^5, 10^4, and 10^3 CFU/tomato, 10% recovery of *S. sonnei* UI05 was observed by CMMEF enrichment and plating on SSA and MAC. When these enrichments were plated on SPM however, 20% recovery was observed from inoculation levels of 10^5 and 10^3 CFU/tomato, and 10% recovery from 10^4 CFU/tomato. No recovery of *S. sonnei* UI05 was observed with CMMEF enrichment of inoculation levels of 10^2, 10^1, or 10^0 CFU/tomato.

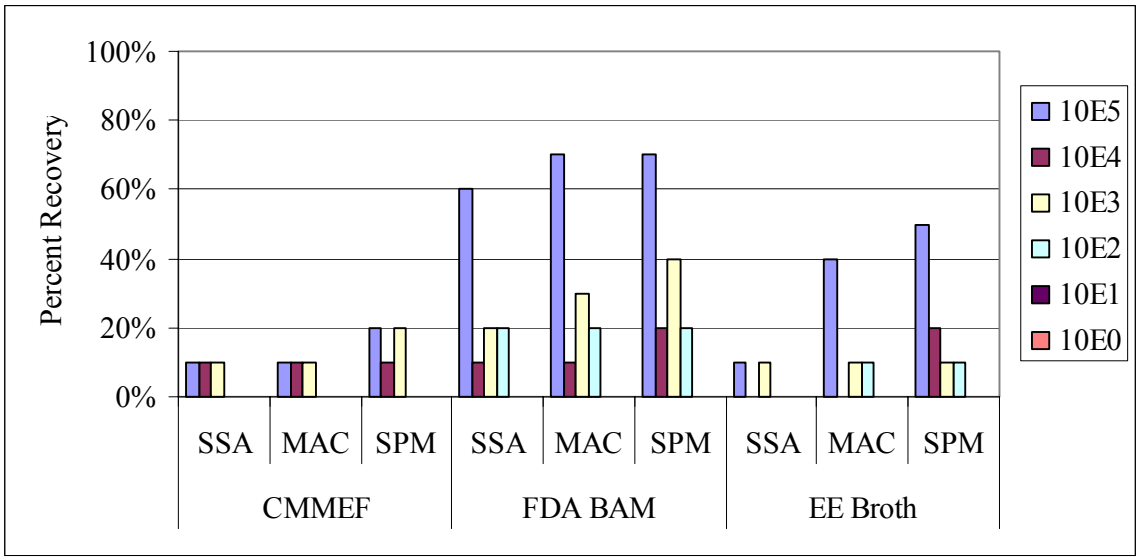

Figure 4-7. Recovery of *S. sonnei* UI05 by conventional culture methods. Tomatoes were inoculated with *S. sonnei* UI05 at levels of 10E5 to 10E0. Inocula was recovered via a 100 ml PBS rinse using a shake-rub-shake method as described in Chapter 3. Tomato rinses were enriched by *Shigella* culture methods of the Compendium of Methods for the Microbiological Examination of Foods (CMMEF), the FDA Bacteriological Analytical Manual (FDA BAM), and in EE broth with 1.0 µg/ml novobiocin at 42ºC. Percent recovery is calculated as the number of tomatoes from which *S. sonnei* UI05 was isolated over the total number of tomatoes sampled for each enrichment.

S. sonnei UI05 recovered by enrichment by the FDA BAM is also shown in Figure 4-7. *S. sonnei* UI05 inoculated at 10^5 CFU/tomato was recovered at 60%, 70%, and 70% when plated on SSA, MAC, and SPM, respectively. When inoculated at 10^4 CFU/tomato, *S. sonnei* UI05 was recovered at 10%, 10%, and 20% when plated on SSA, MAC, and SPM, respectively. At inoculation levels of 10^3 CFU/tomato, recovery rates of 20%, 30%, and 40% were observed when plated on SSA, MAC, and SPM, respectively. At an inoculation of 10^2 CFU/tomato, *S. sonnei* UI05 was recovered from 20% of tomatoes when plated on SSA, MAC, or SPM. *S. sonnei* UI05 was not recovered when inoculated at levels of 10^1 or 10^0 CFU/tomato.

Results from enrichment of *S. sonnei* UI05 in EE broth as described by Uyttendaele et al. (2000) is also shown in Figure 4-7. *S. sonnei* UI05 inoculated at 10^5 CFU/tomato was recovered from 10%, 40%, and 50% of tomatoes when enrichments were plated on SSA, MAC, and SPM, respectively. With an inoculation of 10^4 CFU/tomato, *S. sonnei* UI05 was only recovered when plated on SPM (20%). When inoculated at 10^3 CFU/tomato, *S. sonnei* UI05 was recovered from 10% of tomatoes when plated on SSA, MAC, or SPM. When inoculated at 10^2 CFU/tomato, *S. sonnei* UI05 was recovered from 10% of tomatoes when plated on MAC and SPM, but in none when plated on SSA. No recovery of *S. sonnei* UI05 was observed when inoculated at levels of 10^1 or 10^0 CFU/tomato.

Detection of *Shigella* spp. by Conventional Culture Methods with Rifampicin Supplemented Enrichment

Conventional culture methods (FDA BAM, the CMMEF, and in EE broth as described by Uyttendaele et al. (2000)) were repeated using enrichments supplemented with 50µg/ml rifampicin to exclude natural tomato microflora and rifampicin-adapted inocula. Overnight enrichments were plated using SSA, MAC, and SPM. For all conventional culture trials with rifampicin supplemented enrichment, 10 replicates were analyzed at each inoculation level. Inoculation levels were verified by pour plating serial dilutions in triplicate with TSA-R50. Recovery results for all conventional culture method experiments are expressed as "percent recovery," defined as the number of tomatoes which tested positive for *Shigella* spp. out of the total number tested.

Results for the recovery of *S. boydii* UI02 using conventional culture methods where enrichments were supplemented with 50 µg/ml rifampicin (rif+) are shown in Figure 4-8. Supplemented enrichment according to the CMMEF (rif+) protocol resulted

in 100% recovery of *S. boydii* UI02 from tomatoes inoculated at 10^6, 10^5, and 10^3 CFU/tomato on all three plating media. *S. boydii* UI02 inoculated at 10^4 CFU/tomato was recovered from 40%, 80%, and 70% of tomatoes when plated on SSA, MAC, and SPM, respectively. At an inoculation level of 10^2 CFU/tomato, *S. boydii* UI02 was recovered from 60% of tomatoes when plated on SSA, and 50% when plated using MAC or SPM. When inoculated at 10^1 CFU/tomato, *S. boydii* UI02 was recovered from 10% regardless of which plating medium was used.

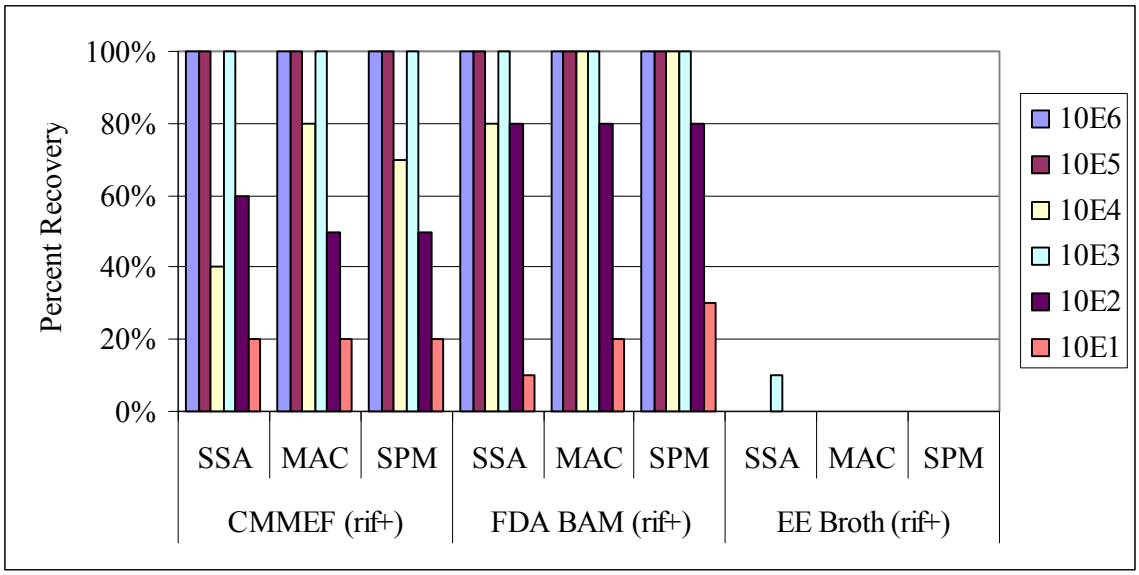

Figure 4-8. Recovery of *S. boydii* UI02 by conventional culture methods with rifampicin supplemented enrichment. Tomatoes were inoculated with rifampicin adapted *S. boydii* UI02 at levels of 10E6 to 10E1. Inocula were recovered via a 100 ml PBS rinse using a shake-rub-shake method as described in Chapter 3. Tomato rinses were enriched by *Shigella* culture methods of the Compendium of Methods for the Microbiological Examination of Foods (CMMEF), the FDA Bacteriological Analytical Manual (FDA BAM), and in EE broth with 1.0 µg/ml novobiocin at 42ºC. All enrichments were supplemented with 50 µg/ml rifampicin (rif+)to screen out natural background tomato microflora. Percent recovery is calculated as the number of tomatoes from which *S. boydii* UI02 was isolated over the total number of tomatoes sampled for each enrichment.

Results from the enrichment of *S. boydii* UI02 by the FDA BAM (rif+) protocol are shown in Figure 4-8. 100% recovery of *S. boydii* UI02 was achieved from tomatoes

inoculated at 10^6, 10^5, 10^4, and 10^3 CFU/tomato on all three plating media, except for tomatoes inoculated at 10^4 CFU/tomato and plated on SSA, which were recovered at 90%. At an inoculation level of 10^2 CFU/tomato, *S. boydii* UI02 was recovered from 90% of tomatoes on all three plating media. When inoculated at 10^1 CFU/tomato, *S. boydii* UI02 was recovered from 10%, 20%, and 30% of tomatoes when plated using SSA, MAC, and SPM, respectively.

Figure 4-8 shows that enrichment in EE broth (rif+) as described by Uyttendaele et al. (2000) resulted in almost no recovery of *S. boydii* UI02. Only one tomato from the 10^3 CFU/tomato inoculation tested positive for *S. boydii* UI02 when plated on SSA.

Results for the recovery of *S. sonnei* UI05 using conventional culture methods where enrichments were supplemented with 50 µg/ml rifampicin (rif+) are shown in Figure 4-9. Supplemented enrichment of *S. sonnei* UI05 according to the CMMEF (rif+) protocol resulted in 100% recovery when inoculated at 10^5 CFU/tomato, 90% recovery when inoculated at 10^4 CFU/tomato, and 80% recovery when inoculated at 10^3 CFU/tomato and plated on SSA, MAC, or SPM. When *S. sonnei* UI05 was inoculated at 10^2 CFU/tomato, recovery was 20%, 30%, and 50% when plated on SSA, MAC, and SPM, respectively. At an inoculation level of 10^1 CFU/tomato, *S. sonnei* UI05 was not recovered when plated on SSA, and recovered from 20% of tomatoes when plated on MAC or SPM. No *S. sonnei* UI05 was recovered from inoculation levels of 10^0 CFU/tomato.

Results from the enrichment of *S. sonnei* UI05 by the FDA BAM (rif+) protocol are shown in Figure 4-9. 100% recovery was achieved from tomatoes inoculated at 10^5 CFU/tomato when plated SSA, MAC, or SPM. At an inoculation level of 10^4

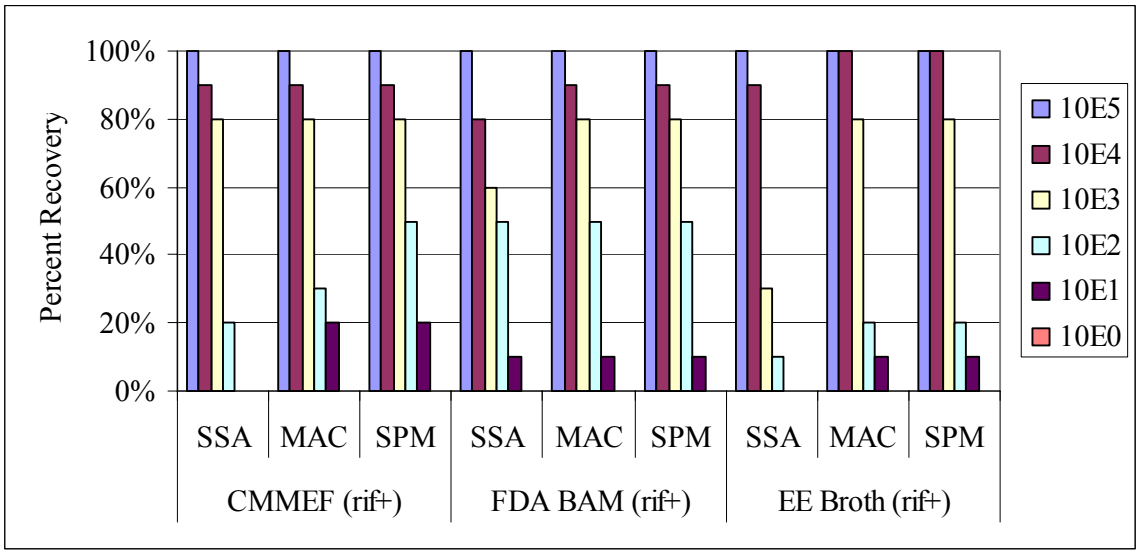

Figure 4-9. Recovery of *S. sonnei* UI05 by conventional culture methods with rifampicin supplemented enrichment. Tomatoes were inoculated with rifampicin adapted *S. sonnei* UI05 at levels of 10E5 to 10E0. Inocula were recovered via a 100 ml PBS rinse using a shake-rub-shake method as described in Chapter 3. Tomato rinses were enriched by *Shigella* culture methods of the Compendium of Methods for the Microbiological Examination of Foods (CMMEF), the FDA Bacteriological Analytical Manual (FDA BAM), and in EE broth with 1.0 µg/ml novobiocin at 42ºC. All enrichments were supplemented with 50 µg/ml rifampicin (rif+) to screen out natural background tomato microflora. Percent recovery is calculated as the number of tomatoes from which *S. sonnei* UI05 was isolated over the total number of tomatoes sampled for each enrichment.

CFU/tomato, *S. sonnei* UI05 was recovered from 80% of tomatoes when plated on SSA, and 90% of tomatoes when plated on MAC or SPM. When inoculated with 10^3 CFU/tomato, *S. sonnei* UI05 was recovered from 60% of tomatoes when plated on SSA, and 80% of tomatoes when plated on MAC or SPM. For inoculation levels of 10^2 and 10^1 CFU/tomato, *S. sonnei* UI05 was recovered from 50% and 10% of tomatoes, respectively, when plated on SSA, MAC, or SPM. No *S. sonnei* UI05 was recovered when inoculated at 10^0 CFU/tomato.

Results from the enrichment of *S. sonnei* UI05 in EE broth (rif+) as described by Uyttendaele et al. (2000) are shown in Figure 4-9. 100% recovery was observed at

inoculation levels of 10^5 CFU/tomato when plated on SSA, MAC, or SPM. At inoculation levels of 10^4 CFU/tomato, *S. sonnei* UI05 was recovered from 90% of tomatoes when plated on SSA, and 100% of tomatoes when plated on MAC or SPM. When inoculated at a level of 10^3 CFU/tomato, *S. sonnei* UI05 was recovered from 30% of tomatoes when plated on SSA and 80% of tomatoes when plated on MAC or SPM. At inoculation levels of 10^2 CFU/tomato, *S. sonnei* UI05 was recovered from 10% of tomatoes when plated on SSA and 20% of tomatoes when plated on MAC or SPM. No *S. sonnei* UI05 was recovered from tomatoes inoculated with 10^1 CFU/tomato when plated on SSA, however *S. sonnei* UI05 was recovered from 10% of tomatoes when plated on MAC or SPM. No *S. sonnei* UI05 was recovered when inoculated at 10^0 CFU/tomato.

Lowest Detection Levels of Conventional Culture Methods

Results were reported based on the initial inoculation, however only 25 ml of the 100 ml tomato rinse was enriched by each protocol, therefore only a fourth of the inocula could have theoretically been enriched by each protocol. When reporting the lowest detection level (LDL) for conventional enrichments, corrections for the distribution of inoculum have been made. For example, results reported for *S. sonnei* UI05 at 10^5 CFU/tomato were reported for an initial inoculation of 6.1×10^5 CFU/tomato, but each enrichment procedure actually reflected a theoretical cell titer of 1.5×10^5 CFU, assuming all of the inoculum was recovered and distributed evenly in the rinse. LDLs from the enrichment procedures are reported as the lowest inoculation that resulted in isolation of *Shigella* spp. in at least one out of the 10 replicates. Conventional enrichment procedures in the presence of natural tomato microflora resulted in no recovery of *S boydii* UI02 (LDL $>5.3 \times 10^5$ CFU/tomato); however LDLs for *S. sonnei* UI05 were 1.9×10^2 (FDA BAM), 1.5×10^3 (CMMEF), and 1.1×10^2 CFU/tomato (EE broth). For

enrichment procedures supplemented with rifampicin (rif+) to exclude natural tomato microflora, LDLs were: 6.3 x 10^0 CFU/tomato (FDA BAM rif+ and CMMEF rif+) and >5.3 x 10^5 CFU/tomato in EE broth rif+ for *S. boydii* UI02; and 1.9 x 10^1 (FDA BAM rif+ and CMMEF rif+) and 1.1 x 10^1 CFU/tomato (EE broth rif+) for *S. sonnei* UI05.

Table 4-1. Lowest detection levels (LDLs) of conventional culture methods. Tomatoes were inoculated with various levels of *S. boydii* UI02 or *S. sonnei* UI05. Inocula were allowed to air dry before tomatoes were rinsed in 100 ml PBS using a shake-rub-shake method. A 25 ml aliquot of the tomato rinse was transferred to 225 ml of the appropriate enrichment broth and incubated according *Shigella* culture methods found in the Compendium of Methods for the Microbiological Examination of Foods (CMMEF), the U.S. FDA's Bacteriological Analytical Manual (FDA BAM) or in EE Broth as described by Uyttendaele et al. (2000). Enrichment procedures were repeated using rifampicin adapted strains and supplemented enrichment (rif+). LDLs were determined by calculating the inoculum applied to the respective tomato, assuming 100% of the inoculum was recovered in the PBS rinse, and adjusting for how much of that inoculum went into each enrichment.

Enrichment Procedure	*S. boydii* UI02 (CFU/tomato)	*S. sonnei* UI05 (CFU/tomato)
CMMEF	>5.3 x 10^5	1.5 x 10^3
FDA BAM	>5.3 x 10^5	1.9 x 10^2
EE Broth	>5.3 x 10^5	1.1 x 10^2
CMMEF rif+	6.3 x 10^0	1.9 x 10^1
FDA BAM rif+	6.3 x 10^0	1.9 x 10^1
EE Broth rif+	>5.3 x 10^5	1.1 x 10^1

Additionally, the lowest detection level in which *Shigella* spp. was isolated in 100% of the replicates (LDL100) was also determined for each enrichment procedure. In studies involving conventional enrichment procedures in the presence of natural tomato microflora, LDL100s were not achieved in this study, however from the results it can be stated that the LDL100s of *S. boydii* UI02 and *S. sonnei* UI05 are >5.3 x 10^5 CFU/tomato and >1.5 x 10^5 CFU/tomato, respectively. For trials involving rifampicin supplemented enrichments, the LDL100s were: 6.3 x 10^2 CFU/tomato (FDA BAM and CMMEF) and >5.3 x 10^5 CFU/tomato (EE broth) for *S. boydii* UI02; and 1.5 x 10^5 CFU/tomato (FDA

BAM and CMMEF), and 1.5 x 10^4 CFU/tomato (EE broth) for *S. sonnei* UI05. The high inoculation levels required to achieve the LDL100 in conventional culture methods demonstrates the need for better methods with which to evaluate food products for *Shigella* spp.

Table 4-2. Lowest detection levels in which *S. boydii* UI02 or *S. sonnei* UI05 was isolated in 100% of replicates (LDL100s) of conventional culture methods. Tomatoes were inoculated with various levels of *S. boydii* UI02 or *S. sonnei* UI05. Inocula were allowed to air dry before tomatoes were rinsed in 100 ml PBS using a shake-rub-shake method. A 25 ml aliquot of the tomato rinse was transferred to 225 ml of the appropriate enrichment broth and incubated according *Shigella* culture methods found in the Compendium of Methods for the Microbiological Examination of Foods (CMMEF), the U.S. FDA's Bacteriological Analytical Manual (FDA BAM) or in EE Broth as described by Uyttendaele et al. (2000). Enrichment procedures were repeated using rifampicin adapted strains and supplemented enrichment (rif+). LDL100s were determined by calculating the inoculum applied to the respective tomato, assuming 100% of the inoculum was recovered in the PBS rinse, and adjusting for how much of that inoculum went into each enrichment.

Enrichment Procedure	*S. boydii* UI02 (CFU/tomato)	*S. sonnei* UI05 (CFU/tomato)
CMMEF	>5.3 x 10^5	>1.5 x 10^5
FDA BAM	>5.3 x 10^5	>1.5 x 10^5
EE Broth	>5.3 x 10^5	>1.5 x 10^5
CMMEF rif+	6.3 x 10^2	1.5 x 10^5
FDA BAM rif+	6.3 x 10^2	1.5 x 10^5
EE Broth rif+	>5.3 x 10^5	1.5 x 10^4

Detection of *Shigella* spp. by FTA® Filtration / Nested PCR

FTA® filtration/ nested PCR was used to detect inoculated *S. boydii* UI02 and *S. sonnei* UI05 on inoculated tomatoes. Positive detection was recorded if a single band of 290 bp was produced in the second step of the nested PCR. Inoculation levels were verified by pour plating serial dilutions in triplicate with TSA-R50.

Figure 4-10 shows representative gels from the first step of the nested PCRs. Positive amplification in the first PCR produced a very faint band of 620 bp (Figure 4-10A) from samples inoculated at 10^5 and 10^4 CFU/tomato with either *S. boydii* UI02 or *S.*

sonnei UI05 and no bands (Figure 4-10B) from samples inoculated at 10^3, 10^2, 10^1 and 10^0 CFU/tomato.

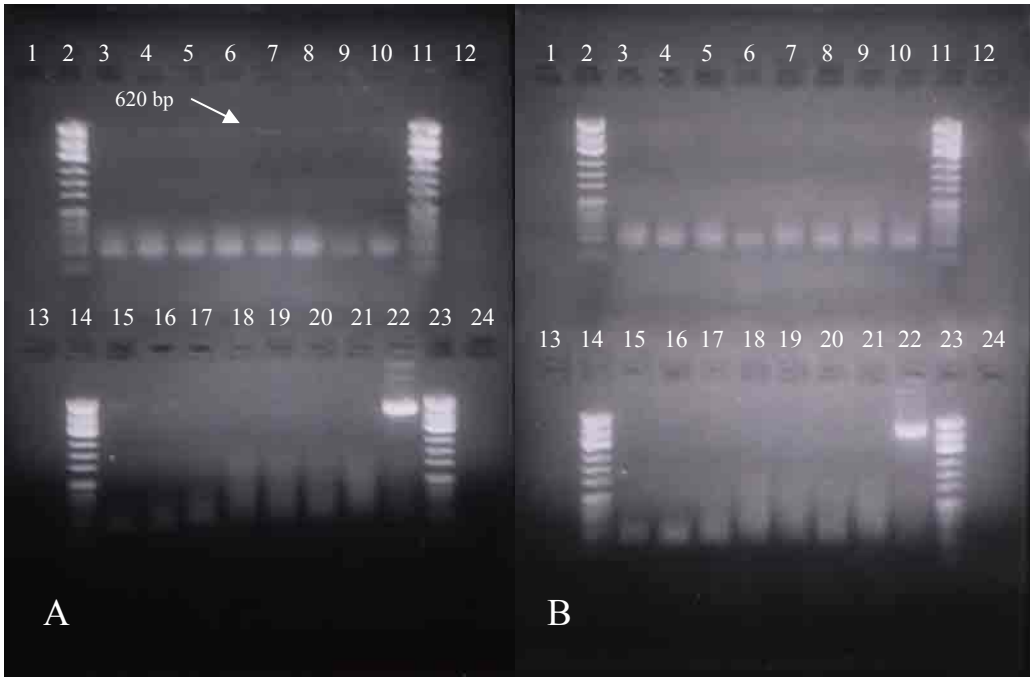

Figure 4-10. Representative gels from the first step nested PCR. *S. boydii* UI02 inoculated at (A) 10^4 CFU/tomato and (B) 10^2 CFU/tomato. Lanes assignments for both gels are: 1, 12, 13, and 24 – empty; 2, 11, 14, and 23 – DNA ladder; 3-10, 15, and 16 – sample lanes; 17 – tomato control; 18 – filter control; 19 – PBS control; 20 – water control; 21 – negative control; 22 – positive control.

Figure 4-11 shows a representative gel from the second step of the nested PCRs. Positive amplification in the second step PCR resulted in a single band of 290 bp.

The results shown in Figure 4-12 demonstrate that *S. boydii* UI02 was detected at rates of 100% regardless of inoculation level. *S. sonnei* UI05 was detected in 100% of samples inoculated at levels of 10^5, 10^4, and 10^3 CFU/tomato. When inoculated at 10^2, 10^1, and 10^0 CFU/tomato, *S. sonnei* UI05 was detected on 90%, 40%, and 30% of the tomatoes, respectively.

Figure 4-11. Representative gel from the second step nested PCR. *S. boydii* UI02 inoculated at 10^4 CFU/tomato. Lanes assignments are: 1, 12, 13, and 24 – empty; 2, 11, 14, and 23 – DNA ladder; 3-10, 15, and 16 – sample lanes; 17 – tomato control; 18 – filter control; 19 – PBS control; 20 – water control; 21 – negative control; 22 – positive control.

Lowest Detection Levels of the FTA® Filtration/ Nested PCR Method

Results were reported based on initial inoculation levels. Since the entire tomato rinse was evaluated by FTA® filtration/ nested PCR, no corrections for the distribution of inoculum was required. Reported results assumed recovery of 100% of the inoculum from the tomato surface. LDLs of the FTA® filtration/ nested PCR method for the detection of S. *boydii* UI02 and *S. sonnei* UI05 were 6.2 x 10^0 CFU/tomato and 7.4 x 10^0 CFU/tomato, respectively (Table 4-3). In comparison, the LDL100s for *S. boydii* UI02 and *S. sonnei* UI05 were 6.2 x 10^0 CFU/tomato and 6.1 x 10^3 CFU/tomato, respectively (Table 4-3).

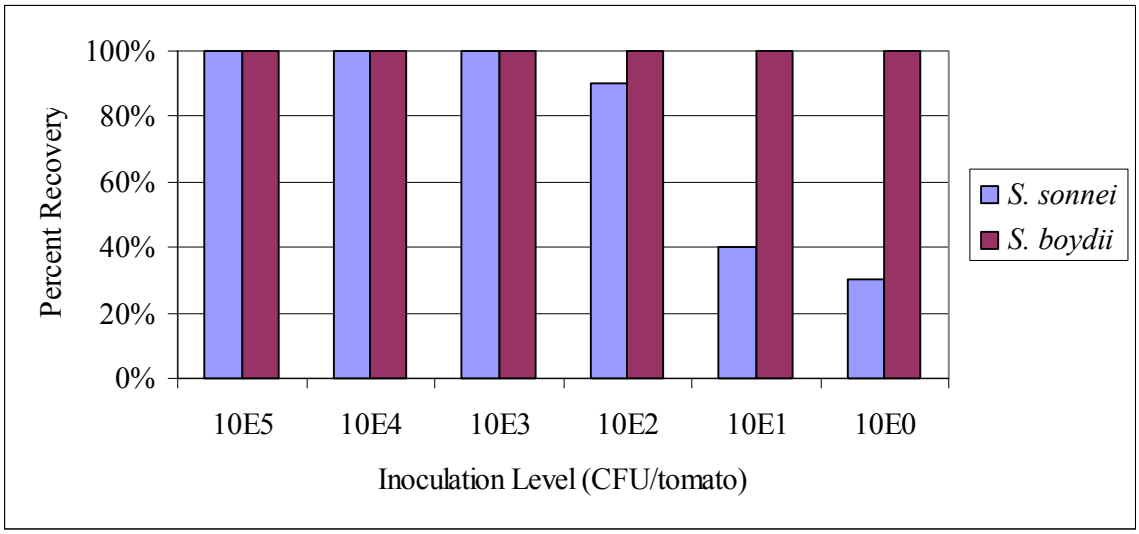

Figure 4-12. Detection of *S. boydii* UI02 and *S. sonnei* UI05 by FTA® filtration/ nested PCR. Tomatoes were inoculated with *S. boydii* UI02 or *S. sonnei* UI05 at levels of 10E5 to 10E0. Inocula were recovered via a 100 ml PBS rinse using a shake-rub-shake method as described in Chapter 3. Tomato rinses were filtered using tandem filter funnels as described in Chapter 3. FTA punches were used as DNA template in the first step of the nested PCR. Positive detection was based on visualization of a 290 bp band from the second step of the nested PCR. Percent recovery is calculated as the number of tomatoes from which *S. boydii* UI02 or *S. sonnei* UI05 was isolated over the total number of tomatoes sampled.

Table 4-3. Lowest detection levels and lowest detection levels in which *S. boydii* UI02 or *S. sonnei* UI05 was isolated in 100% of replicates of FTA® filtration/ nested PCR. Tomatoes were inoculated with *S. boydii* UI02 or *S. sonnei* UI05 at levels of 10E5 to 10E0. Inocula were recovered via a 100 ml PBS rinse using a shake-rub-shake method as described in Chapter 3. Tomato rinses were filtered using tandem filter funnels as described in Chapter 3. FTA punches were used as DNA template in the first step of the nested PCR. Positive detection was based on visualization of a 290 bp band in the second step of the nested PCR.

	S. boydii UI02 (CFU/tomato)	*S. sonnei* UI05 (CFU/tomato)
LDL	6.2×10^0	7.4×10^0
LDL100	6.2×10^0	6.1×10^3

CHAPTER 5
DISCUSSION AND CONCLUSION

Foodborne shigellosis has been on the rise in recent years. Several outbreaks involving fresh produce have demonstrated a need for a better method of evaluating produce for *Shigella* spp. This study investigated conventional culture methods and a newly developed FTA® filtration/ nested PCR method for the detection of *Shigella* spp. on tomato surfaces. *S. boydii* UI02 and *S. sonnei* UI05 were inoculated onto tomatoes at levels of 10^6 through 10^0 CFU/tomato and 10^5 through 10^0 CFU/tomato, respectively. Recovery of *Shigella* from tomatoes was performed by placing inoculated tomatoes in 100 ml PBS and applying a shake/rub/shake method as previously described. Tomato rinses were then analyzed by enrichment procedures as found in the U.S. Food and Drug Administration's (1998) Bacteriological Analytical Manual (FDA BAM), the Compendium of Methods for the Microbiological Examination of Food (CMMEF), and by EE broth as described by Uyttendaele et al. (2000). *Shigella* Plating Medium (SPM) was evaluated for the isolation of *Shigella* spp. against *Salmonella-Shigella* agar (SSA), MacConkey agar (MAC). Furthermore, tomato rinses were evaluated using a newly developed FTA® filtration/ nested PCR method.

Preliminary Studies

The two *Shigella* spp. isolates selected for this study were *S. boydii* UI02 and *S. sonnei* UI05. Both strains were obtained from the laboratory of Dr. Hans Blaschek, University of Illinois, and were involved in Chicago area outbreaks involving fresh

produce or products containing produce. *S. sonnei* represents the serogroup of *Shigella* most common to North America, while *S. boydii* is rarely isolated in North America.

In order to evaluate the capability of conventional culture methods given total specificity for *Shigella* spp., rifampicin resistance was generated in *S. boydii* UI02 and *S. sonnei* UI05 subcultures by spontaneous mutation. Initially, dimethyl sulfoxide (DMSO) was used to make stock solutions of rifampicin; however the use of DMSO resulted in the production of strong malodors determined to be from the metabolism of DMSO to dimethyl sulfide and methanethiol (data not shown). As the source of the malodor was being determined, cultures were adapted to nalidixic acid as an alternative to rifampicin; however in preliminary trials, natural microflora on tomatoes proved resistant of up to 400 ppm nalidixic acid. After the source of malodor was determined to be DMSO, methanol was used to make stock solutions of rifampicin, which did not result in the production of malodors. In a study on antimicrobial activities of aqueous and methanol extracts of *Juniperus oxycedrus*, Karaman et al. (2003) used the addition of methanol alone to various media as negative controls. Methanol was found to have no inhibitory effects to various strains of *Enterobacter*, *Klebsiella*, *Acinetobacter*, and *Pseudomonas* along with one strain of *Escherichia coli*. Based on these findings with closely related microorganisms, methanol was assumed to have negligible effect on cell viability of *S. boydii* UI02 and *S. sonnei* UI05.

Growth Characteristics of *S. boydii* UI02 and *S. sonnei* UI05

Growth characteristics of *S. boydii* UI02 and *S. sonnei* UI05 were determined by performing growth curves and constructing optical density (O.D.) standard curves. In a preliminary trial, a growth curve of *S. sonnei* 9290 (data not shown) was performed and the absorbance was read at two wavelengths, 400 nm and 600 nm. When O.D. standard

curves were constructed using data from both wavelengths, there was no difference in the range of linearity between absorbance and $\log_{10}$ CFU/ml. For all the remaining growth curves a wavelength of 600 nm was used.

Using growth curve and O.D. standard curve data, the exponential growth phase doubling times for each strain investigated was calculated (Table 5-1).

Table 5-1. Doubling times associated with exponential growth phase of investigated strains of *Shigella* spp.

Strain	*S. boydii* UI02	*S. sonnei* 9290	*S. sonnei* UI05
Wild type	43.8 min	22.9 min	30.0 min
Nalidixic acid adapted	47.4 min	N/A	45.0 min
Rifampicin adapted	N/A	30.5 min	N/A

It was observed that *S. sonnei* UI05 entered exponential phase growth and achieved stationary phase faster than *S. boydii* UI02. This can be explained by the faster growth rate calculated for *S. sonnei* UI05 and an extension in the lag phase associated with *S. boydii* UI02. The wild strain of *S. sonnei* UI05 doubled every 30 minutes compared to every 43.8 minutes for *S. boydii* UI02 (Table 5-1). The lag phase prior to exponential growth observed for the wild strain of *S. boydii* UI02 was approximately 4 hours (Figure 4-1) compared to only approximately 3 hours for the wild strain of *S. sonnei* UI05 (Figure 4-3), with the onset of stationary phase at approximately 8 hours and 6 hours, respectively.

Growth curve data from the wild strain *S. sonnei* 9290 was comparable to that of the wild strain *S. sonnei* UI05. The time before exponential growth observed in the wild strain *S. sonnei* 9290 was approximately 3 hours with the onset of stationary phase at approximately 6 hours (data not shown). The *S. sonnei* 9290 wild strain doubled every 22.9 minutes compared to every 30.0 minutes for the *S. sonnei* UI05 wild strain When compared with data collected for a rifampicin adapted strain of *S. sonnei* 9290 (Figure 4-

5), entry into exponential growth of rifampicin-adapted culture was delayed by approximately 2 hours. Furthermore, the doubling time in exponential growth phase increased to 30.0 minutes with the rifampicin adapted strain.

For rifampicin adapted strains of *S. boydii* UI02 and *S. sonnei* UI05, similar growth characteristics to the *S. sonnei* 9290 rifampicin adapted strain were assumed. These assumptions included a 2 hour extension of stationary phase between the wild strain and rifampicin-adapted strain and slightly slower growth rates during exponential growth phase. This assumption was supported by growth curve data collected for strains of *S. boydii* UI02 and *S. sonnei* UI05 adapted and grown in the presence of nalidixic acid (data shown in Appendix). Exponential growth rates of nalidixic acid-adapted strains grown in the presence of nalidixic acid for both *S. boydii* UI02 and *S. sonnei* UI05 were slower than the observed rates of the wild strains (Figure 5-1). Both nalidixic acid-adapted strains experienced 4 hour extensions in lag phase compared to that of the wild strain, which supports the assumption of a 2 hour extension for both strains adapted to rifampicin. Assumptions for rifampicin adapted strains of *S. boydii* UI02 and *S. sonnei* UI05 maintained entry into stationary phase at no later than 12 hours. Growth curve data concluded that 18-hour cultures used in this study were late stationary phase cultures.

Evaluation of Primers Specific for *Shigella* spp.

Several sets of primers were investigated for the detection of *Shigella* spp. by PCR. Results indicated that primer sets 01-001 and 01-005 were the only sets specific for all four serogroups of *Shigella*. None of the primers amplified DNA from the non-*Shigella* library. Primer sets 01-001 and 01-005 targeted 620 and 290 bp regions, respectively, of the *ipaH* gene of *Shigella* spp. and enteroinvasive *E. coli*. When Jin et al. (2002) sequenced the genome of *S. flexneri* 2a, the *ipaH* gene was located seven times on the

chromosome and five more times on the plasmid. Primer sets 01-002 and the nested set 01-003 and 01-004 target regions of the *virA* gene and the invasion associated locus (*ial*), respectively. Unlike the *ipaH* gene, the *virA* gene and the *ial* are only located on the large virulence plasmid of *Shigella* spp. Negative results observed with primer sets 01-002, 01-003, and 01-004 in this study could have been due to loss of plasmid during storage or repeated transfers of the *Shigella* strains not amplified. No studies were conducted to determine if plasmids were intact in these strains. The potential loss of plasmid does yield an advantage to using primers which amplify the *ipaH* gene since detection of non-pathogenic, plasmid-less strains would still be possible.

Inoculation Studies

Evaluation of Enrichment Protocols

Three enrichment protocols were investigated for the isolation of *S. boydii* UI02 and *S. sonnei* UI05 from inoculated tomatoes in the presence of natural background microflora. When the *S. sonnei* UI05 data (Figure 4-7) were analyzed using a logistic regression model, no significant difference ($P = 1.000$) among enrichment procedures was observed. *S. boydii* UI02, however, was never recovered regardless of inoculation level in any of the three investigated enrichment procedures. The inability for conventional enrichment protocols to recover inoculated *S. boydii* UI02 demonstrates the difficulty of isolating *Shigella* spp. by traditional methods and the need for more selective and sensitive media.

Conventional culture enrichment was repeated with background contamination eliminated using enrichments supplemented with 50 µg/ml rifampicin (rif+) and rifampicin-adapted inocula. In these studies, *S. boydii* UI02 was recovered by CMMEF rif+ and FDA BAM rif+ protocols but not from the EE broth rif+ enrichment (Figure 4-

8), while *S. sonnei* UI05 was recovered by all three enrichments (Figure 4-9). Logistic regression modeling revealed no significant difference ($P = 1.000$) between the CMMEF rif+ and the FDA BAM rif+ enrichment methods for the recovery of *S. boydii* UI02, and no significant differences ($P = 1.000$) among the CMMEF rif+, FDA BAM rif+, and EE broth rif+ enrichments for the recovery of *S. sonnei* UI05. It was observed that although *S. boydii* UI02 was unable to compete amid natural tomato microflora, it was recovered at higher percentages than *S. sonnei* UI05 at each inoculation level in the CMMEF rif+ and FDA BAM rif+ enrichments. In contrast, *S. boydii* UI02 was rarely recovered when enriched in EE broth rif+ (1 out of 60 samples; Figure 4-8).

EE broth contains bile salts which have been reported to be inhibitory to some *Shigella* spp. particularly stressed cells (Tollison and Johnson, 1985). The *S. boydii* strain used in this study may have been inhibited by the bile salts in EE broth. When Uyttendaele et al. (2000) investigated EE broth among other enrichment broths for the recovery of stressed and un-stressed Shigellae, only strains of *S. sonnei* and *S. flexneri* were used. These results stress the importance of including all four serogroups of *Shigella* when evaluating enrichment procedures. These results do not suggest EE broth may be used in place of *Shigella* broth for the enrichment of *Shigella* spp. since a known pathogenic strain, *S. boydii* UI02, may be missed.

Evaluation of Plating Media

Using logistic regression models, the isolation of *Shigella* spp. among isolation media was compared. In all studies involving plating media, there was no significant difference ($\alpha = 0.05$) among isolation rates on the three media. It can be noted, however, that differentiation of *S. boydii* UI02 and *S. sonnei* UI05 colonies from those of background contaminants was far easier on SPM than on MAC or SSA. The most

common contaminants observed in this study were species of *Enterobacter*, *Citrobacter*, and *Klebsiella*. These background contaminants were also identified in other studies in which *Shigella* spp. isolation was attempted by conventional culture methods (Uyttendaele et al., 2000; Schneider et al., unpublished data).

Figure 5-1 demonstrates the ability of SPM to allow easier differentiation of various contaminants as compared to MAC and SSA. Colonies of *Shigella* spp. on SPM are white while colonies of *Enterobacter*, *Citrobacter*, *Acenitobacter*, and *Klebsiella* are either bluish or greenish. On MAC, *Shigella* spp. are translucent and slightly pink, with and without rough edges. *Enterobacter*, *Citrobacter*, and *Klebsiella* spp. all make pink colonies on MAC which may or may not be easily differentiated from colonies of *Shigella* spp. Since differentiation on MAC is based on lactose fermentation, lactose negative strains of *Enterobacter*, *Citrobacter*, and *Klebsiella* will produce colonies that are more translucent, resembling those of *Shigella* spp. Furthermore, *Acinetobacter* produces tiny translucent to pink colonies on MAC which are difficult to differentiate from *Shigella* spp. On SSA, *Enterobacter* and *Klebsiella* spp. produce pink colonies however *Shigella*, *Citrobacter* and *Acinetobacter* spp. produce colonies that are translucent to light pink. Isolation of *Shigella* spp. on MAC and SSA became increasingly difficult as colony morphologies of *Shigella* spp. and/or several contaminant colony morphologies were present on the same plate. While plating on SPM allowed greater differentiation between *Shigella* spp. and contaminant colonies, the results support previous suggestions that several different media with varying selectivity should be used in order to increase the chance of isolating *Shigella* spp. If only one plating

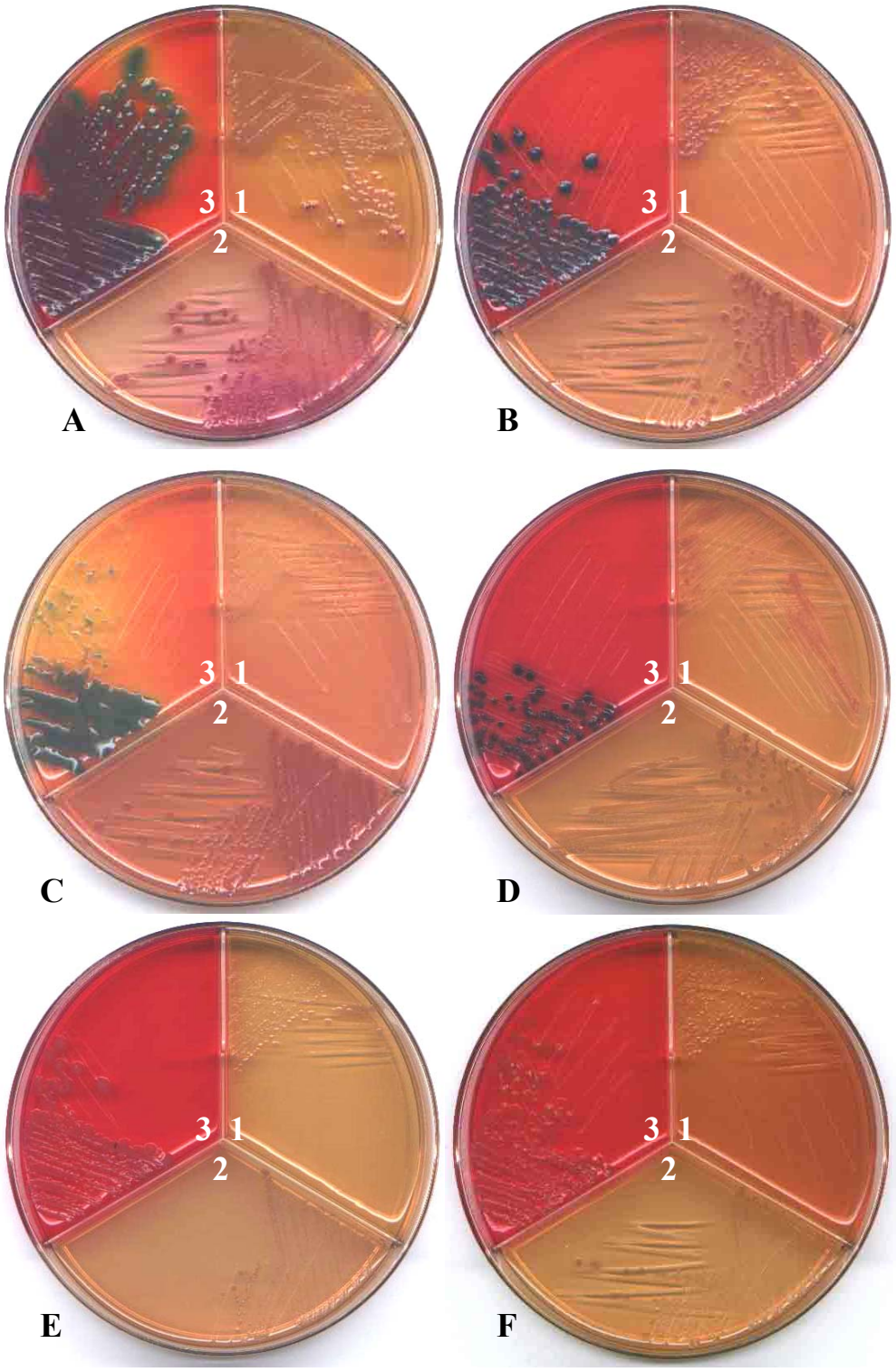

Figure 5-1. Differentiation of background microflora by isolation media. Tri-Plates contain: 1) SSA, 2) MAC, and 3) SPM, streaked with: A) *Enterobacter cloacae*, B) *Klebsiella ozanae*, C) *Citrobacter freundii*, D) *Acinetobacter anitratus*, E) *Shigella boydii* UI02, and F) *Shigella sonnei* UI05.

media is to be used, the results in this study suggest SPM can be used with equivalent isolation rates of *Shigella* spp. as compared to MAC and SSA.

Analysis of Lowest Detection Levels of Conventional Culture Methods

The lowest detection levels (LDLs) of conventional culture methods for *S. boydii* UI02 and *S. sonnei* UI05 were determined (Table 4-1). In a similar study, Jacobson et al. (2002) evaluated the FDA BAM *Shigella* method using two strains of *S. sonnei* on selected types of produce. LDLs were determined using unstressed, chill-stressed, and/or freeze-stressed cells. LDLs with unstressed cells were less than 1.0×10^1 CFU/25g for all produce types, while LDLs with chill-stressed and freeze-stressed cells were less than 5.2×10^1 CFU/25g for all produce types tested (Jacobson et al., 2002). LDLs with the FDA BAM enrichment in this study for *S. sonnei* UI05 (1.9×10^2 CFU/tomato; Table 4-1) were higher than LDLs reported by Jacobson et al. for *S. sonnei* strains 357 and 20143. Variation between strains of *S. sonnei* could explain the difference in reported LDLs with the FDA BAM method. Based upon its prevalence in outbreaks of shigellosis in the U.S., Jacobson's group chose to evaluate only strains of *S. sonnei;* however, results of this study demonstrate the importance of including other serogroups as well. While the FDA BAM appears to be quite effective based on evaluations with strains of *S. sonnei* (present study and Jacobson et al., 2002) the LDLs observed for *S. boydii* UI02, a strain responsible for a Chicago area outbreak, was $>5.3 \times 10^5$ CFU/tomato. This indicates more effective methods are needed for the detection of *Shigella* spp. in food.

The lowest detection levels in which the inoculum was recovered from 100% of the replicates (LDL100s) of the conventional culture methods were also determined for *S. boydii* UI02 and *S. sonnei* UI05 (Table 4-2). LDL100s of $>5.3 \times 10^5$ CFU/tomato and $>1.5 \times 10^5$ CFU/tomato were observed for *S. boydii* UI02 and *S. sonnei* UI05,

respectively, by conventional culture methods in the presence of natural tomato microflora. The inability of the conventional culture methods to detect *S. boydii* UI02 or *S. sonnei* UI05 at high levels of contamination demonstrates the need for a more sensitive and specific conventional culture method for the detection of *Shigella* spp. on tomato surfaces. When rif+ enrichments were used to select rifampicin-adapted inocula, LDL100s were 6.3 x 10^2 CFU/tomato (FDA BAM rif+ and CMMEF rif+) and >5.3 x 10^5 CFU/tomato (EE Broth rif+) for *S. boydii* UI02 and 1.5 x 10^5 CFU/tomato (FDA BAM rif+ and CMMEF rif+) and 1.5 x 10^4 CFU/tomato (EE Broth rif+) for *S. sonnei* UI05. The exclusion of natural tomato microflora by rif+ enrichment did not facilitate the consistent detection of inoculated *S. boydii* UI02 or *S. sonnei* UI05 at levels sufficient to cause disease, further demonstrating the need for a more sensitive and specific conventional culture method for the detection of *Shigella* spp. on tomato surfaces.

Sources of Variation Among Trials

In Figure 4-8, inconsistent recovery results were obtained from *S. boydii* UI02 data sets at the 10^4 CFU/tomato inoculation levels. Recovery by conventional culture methods with rifampicin supplemented enrichment at the 10^5 CFU/tomato and 10^3 CFU/tomato both resulted in 100% recovery, while the 10^4 CFU/tomato was less than 100% for the CMMEF enrichment. In this study, trials involving 10^6, 10^5, and 10^4 CFU/tomato inoculations were performed on one day, while the trials involving 10^3, 10^2, and 10^1 CFU/tomato inoculations were performed on another day. This gap in recovery could possibly be explained by variations in attachment and/or potential biofilm formation. In support of this theory, the 10^4 CFU/tomato set was run last on its day of sampling, while the 10^3 CFU/tomato was run first on its day of sampling. This would allow a significantly greater amount of time for the10^4 CFU/tomato inoculum to attach and/or form biofilms

while the 10^6 and 10^5 CFU/tomato data sets were run that day. Unpublished data using the *S. boydii* UI02 strain (Blaschek et al., University of Illinois) has suggested the formation of biofilms on the surfaces of produce, which resulted in difficult removal and resistance to produce washes. Another factor was that tomatoes used in different trials were harvested and shipped in different lots. On some trial days the tomatoes available were visibly cleaner than for other trials. The presence of excess filth on the surface of tomatoes might have contributed to sub-optimal attachment.

The potential for *S. boydii* UI02 and *S. sonnei* UI05 to form biofilms can be tested using several methods. To simply determine whether these strains produce biofilms, two traditional tests could be performed: the tube test (Christensen et al., 1982) or the microtiter-plate test (Christensen et al., 1982; Christensen et al., 1985). The tests involve staining the bacterial film with a cationic dye and reading the degree of film formation either visually (tube test) or by optical density (microtiter-plate assay). The major drawbacks of these tests are in their qualitative nature; the tube test is visual, while the microtiter-plate assay only measures the film formation on the bottom of the well (Stepanović et al., 2000). In order to better quantify biofilm formation, Stepanović et al. (2000) describe a modified microtiter-plate assay which involves fixing of bacterial films with methanol, staining with crystal violet, releasing the bound dye with 33% glacial acetic acid, and measuring the optical density with a plate reader. This assay could be performed with *S. boydii* UI02 and *S. sonnei* UI05 strains using several time periods for attachment/formation to determine the rate of biofilm formation, if any. Conversely, biofilm formation on tomato surfaces could be directly observed by environmental scanning electron microscopy (Blaschek et al., unpublished material).

The effect of filth on tomato surfaces on *S. boydii* UI02 and *S. sonnei* UI05 attachment and subsequent recovery could be tested as well. Tomatoes with visible filth could be obtained and a portion washed and rinsed to remove any visible filth. Using rifampicin adapted strains, dirty and clean tomatoes could be used in inoculation/recovery procedures as described in Chapter 3, Materials and Methods. Enumeration of recovered *S. boydii* UI02 and *S. sonnei* UI05 from both clean and filthy tomatoes should provide insight as to their effect on attachment/recovery characteristics.

Comparison of Conventional Culture Methods and FTA® Filtration/ Nested PCR

Logistic regression analysis was used to compare isolation rates of the conventional culture methods to the detection rates of FTA® filtration/ nested PCR method for *S. boydii* UI02 and *S. sonnei* UI05. For studies involving conventional culture methods and *S. sonnei* UI05, the FTA® filtration/ nested PCR method was significantly better than the CMMEF ($P = 0.007$), the FDA BAM ($P = 0.003$), and the EE broth enrichment ($P = 0.001$). For studies involving rifampicin supplemented enrichments and *S. boydii* UI02, the FTA® filtration/ nested PCR method was significantly better than enrichment by the CMMEF ($P = 0.010$) or enrichment in EE broth ($P < 0.001$), however it was not significantly different then enrichment by the FDA BAM ($P = 0.177$). For studies involving rifampicin supplemented enrichments and *S. sonnei* UI05, the FTA® filtration/ nested PCR method was significantly better than the CMMEF ($P = 0.007$), the FDA BAM ($P = 0.003$), and enrichment in EE broth ($P = 0.001$).

When tomatoes were analyzed by FTA® filtration/ nested PCR method, much faster results were obtained than with conventional culture methods. The time required for a confirmed result by conventional culture methods was 4 days at best. Since positive confirmation was based on a positive result in the second step PCR, the FTA® filtration/

nested PCR could produce a confirmed result in as quickly as 2 days. It should be noted that until a tandem filter funnel assembly comparable to that used in this study can be commercially purchased, the assembly process of such tandem filters requires significant time, energy, and attention to aseptic techniques. The assembly process of tandem filter funnels alone provides a risk of contamination that needs to be addressed by proper laboratory practices. Contamination problems experienced in this study were attributed to the assembly process of tandem filter funnels.

Analysis of Lowest Detection Levels of FTA® Filtration/ Nested PCR

The LDLs and LDL100s were determined for *S. boydii* UI02 and *S. sonnei* UI05 for FTA® filtration/ nested PCR (Table 4-3). Other studies which investigated the detection of *Shigella* spp. in food by PCR techniques reported LDLs of: 1.0×10^1 -1.0×10^2 CFU/ml (Vantarakis et al., 2000), 1.0×10^2 -1.0×10^3 CFU/ml (Villalobo and Torres, 1998), 1.0×10^1 CFU/ml (Lindqvist, 1999), and 1.1×10^1 CFU/ml (Theron et al., 2001). Vantarakis et al. (2000) were only able to achieve detection of *S. dysenteriae* type 1 from homogenized mussel samples at 1.0×10^1 -1.0×10^2 CFU/ml after a 22 hour pre-enrichment step in peptone water was employed. Without the pre-enrichment a LDL of 1.0×10^3 CFU/ml was observed. In a similar study, Villalobo and Torres (1998) observed LDLs of *S. dysenteriae* type 1 to be 1.0×10^2 -1.0×10^3 CFU/ml in homogenized mayonnaise samples. Lindqvist (1999) utilized buoyant density centrifugation food inoculated with *S. flexneri* to increase sensitivity of a nested PCR assay from 1.0×10^3 CFU/ml to 1.0×10^1 CFU/ml. Finally, a 6 hour pre-enrichment step in GN broth was necessary for Theron et al. (2001) to detect *S. flexneri* at 1.1×10^1 CFU/ml in environmental water samples by semi-nested PCR. While the pre-enrichment increased

sensitivity through multiplication of *S. flexneri*, it also served to dilute PCR inhibitors found in several of the environmental water samples.

In comparison to these previous studies investigating the detection of *Shigella* spp. in food with PCR techniques, the FTA® filtration/ nested PCR method detects *S. boydii* UI02 and *S. sonnei* UI05 with equivalent sensitivity. Tandem filter funnels were able to filter large volumes of tomato rinse, concentrating bacteria in the center of the FTA® filter thereby increasing the ability to detect *Shigella* spp. present at low levels. In addition, no pre-enrichment step was required to increase sensitivity in FTA® filtration/ nested PCR, therefore faster results can be obtained. Furthermore, washing protocols for FTA® punches serve to remove any potential PCR inhibitors.

Optimization of the FTA® Filtration/ Nested PCR Assay

Further development of the FTA® filtration/ nested PCR method is required to obtain the desired amplification on the first step PCR. Figure 4-10A and 4-10B show electrophoresis gels obtained with step 1 PCR amplification of sample tomatoes inoculated with *S. boydii* UI02 at 10^4 CFU/tomato and 10^2 CFU/tomato, respectively. At the 10^4 CFU/tomato inoculation level only very faint bands can be seen upon close observation, while at 10^2 CFU/tomato no bands can be observed. In step 2 PCR however, all inoculation levels were successfully amplified as shown in Figure 4-11. If negative amplification in step 1 of the FTA® filtration/ nested PCR method is to be used to determine a sample "negative" for *Shigella* spp., further optimization of this step is required as *Shigella* spp. present at levels $\leq 10^3$ CFU/tomato would be missed.

Preliminary optimization trials were performed using inoculation levels of 10^2 CFU/tomato in order to visualize bands from the step 1 PCR by adjusting the ramp rate of the annealing step from 3º/sec to 1º/sec, increasing the number of PCR cycles from 30 to

40, sectioning the FTA® punches, and adjusting the temperature control on the thermocycler from "tube" to "block." The ramp rate and temperature control settings were adjusted to account for variation between our Eppendorf Mastercycler gradient and thermocyclers used in previous studies with FTA® punches (Orlandi and Lampel, 2000; Lampel et al., 2000). Sectioning FTA® punches into two or four equal parts with a sterile scalpel was attempted to allow the punch to remain completely submerged in PCR reaction mix for the duration of the PCR (Figure 5-2). Even though high speed centrifugation was used to force the FTA® punches down into the reaction mix, the 6 mm diameter of the punch forced them back to the surface during PCR. FTA® punch sections sank to the bottom of reaction tubes and remained there for the duration of the PCR.

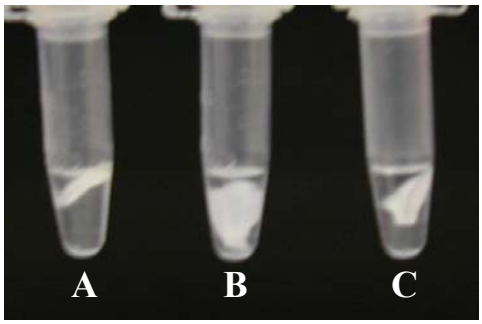

Figure 5-2. FTA® punches in 0.5 ml microcentrifuge tubes. A) Un-manipulated punches held at top of reaction mix by diameter; B) punches sectioned into halves; C) punches folded with forceps.

Both changing the ramp rate for the annealing step and adjusting the temperature control from "tube" to "block" resulted in non-specific amplification (data not shown). Increasing PCR cycles from 30 to 40 alone, and increasing the number of cycles in combination with sectioning FTA® punches into halves or quarters both resulted in observable bands from step 1 PCR (data not shown). As previously mentioned, the FTA® punches were sectioned using a sterile scalpel and forceps. Although positive results were obtained from sectioning FTA® punches, this practice is not recommended for future

studies due to the increased potential for cross-contamination. Instead, FTA® punches in future optimization studies will be folded during transfer using forceps against the inside wall of the microcentrifuge reaction tube prior to step 1 PCR. Folding allows the punches to remain completely submerged (Figure 5-2) in the PCR reaction mix to allow more optimal amplification without adding the potential chance of contamination associated with sectioning.

Predictive Value of Testing for *Shigella* spp.

It should be noted that the predictive value (PV) of any microbiological assay for the detection of *Shigella* spp. in food is affected by both the prevalence of *Shigella* spp. associated with that food product and the specificity and sensitivity of the testing procedure. In the Institute of Food Technologists (IFT) Expert Report on Emerging Microbiological Food Safety Issues, Implications for Control in the 21st Century, PV of testing is explained as a function of prevalence, specificity, and sensitivity. According to the PV model, if the pathogen is prevalent at a high frequency the PV of a test with high specificity and sensitivity will be quite high. Conversely, if a pathogen is prevalent at very low frequency, then the PV of a test with high specificity and sensitivity remains quite low. Such is the case with *Shigella* spp. whose prevalence on produce has been demonstrated at $\leq 4.1\%$ (FDA 2001a; FDA 2001b); therefore the PV of the sampling is very low. Although the conversion from conventional culture methods to nucleic-acid assays such as PCR will allow for greater test specificity and sensitivity, the PV of these tests to screen produce for potential *Shigella* spp. contamination will remain low.

Conclusions

The results of this study demonstrate the superiority of the FTA® filtration/ nested PCR method over conventional culture methods for the detection of *Shigella* spp. from

inoculated tomato surfaces. The FTA® filtration/ nested PCR method was successful in detecting as few as 6.2 *S. boydii* UI02 cells and 7.2 *S. sonnei* UI05 cells amid high background contamination. If future proposed methods for the isolation/detection of *Shigella* spp. from food are to include a conventional culture analog, the results of this study suggest that either the enrichment protocols described by the FDA BAM or the CMMEF should be considered. EE broth, although adequately effective for *S. sonnei* UI05, did not recover *S. boydii* UI02 from inoculated tomato surfaces. Previous suggestions of using several isolation media of varying selectivity were supported by results of this study. SPM is capable of isolating *S. boydii* UI02 and *S. sonnei* UI05 at rates equivalent to MAC and SSA, while providing a greater differentiation between colonies of *Shigella* spp. and closely related contaminants.

APPENDIX
GROWTH CHARACTERISTICS OF NALIDIXIC ACID ADAPTED STRAINS

Growth curves and optical density (O.D.) standard curves were prepared for *S. boydii* UI02 and *S. sonnei* UI05 strains adapted to nalidixic acid (NA).

S. boydii UI02 – NA adapted strain

The growth curve for *S. boydii* UI02 – NA adapted strain demonstrates that stationary phase was reached in approximately 13 hours (Figure B-1). A lag phase of approximately 7 hours was observed prior to exponential growth.

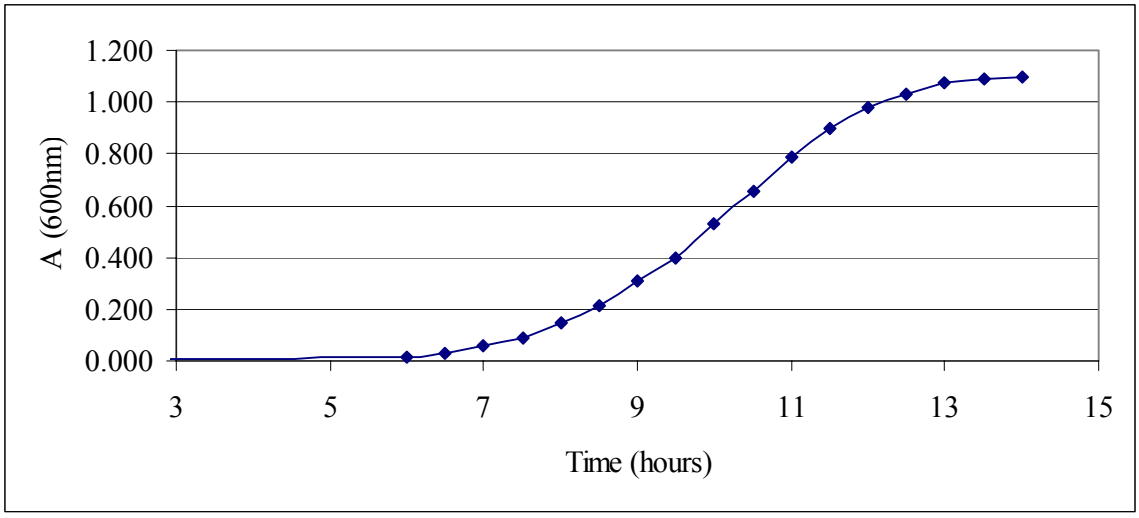

Figure A-1. Growth curve: *S. boydii* UI02 – nalidixic acid adapted strain. Each of three 100 ml TSB (200 ppm NA) microcosms were inoculated with 10 µl aliquots of an 18-hour *S. boydii* UI02 culture. The microcosms were incubated (37ºC, 30 rpm) and sampled at appropriate time intervals. At each time of sampling, the absorbance at 600 nm (A 600 nm) was measured spectrophotometrically. Shown above is the average A 600 nm of the three trials plotted against time.

An O.D. standard curve for *S. boydii* UI02 – NA adapted strain was prepared. The cell titer in which the relationship between $\log_{10}$ CFU/ml and absorbance at 600 nm (A

600 nm) showed linearity (R^2 = 0.9764; Figure B-2) was approximately 9.17 x 10^6 to 4.90 x 10^7 CFU/ml.

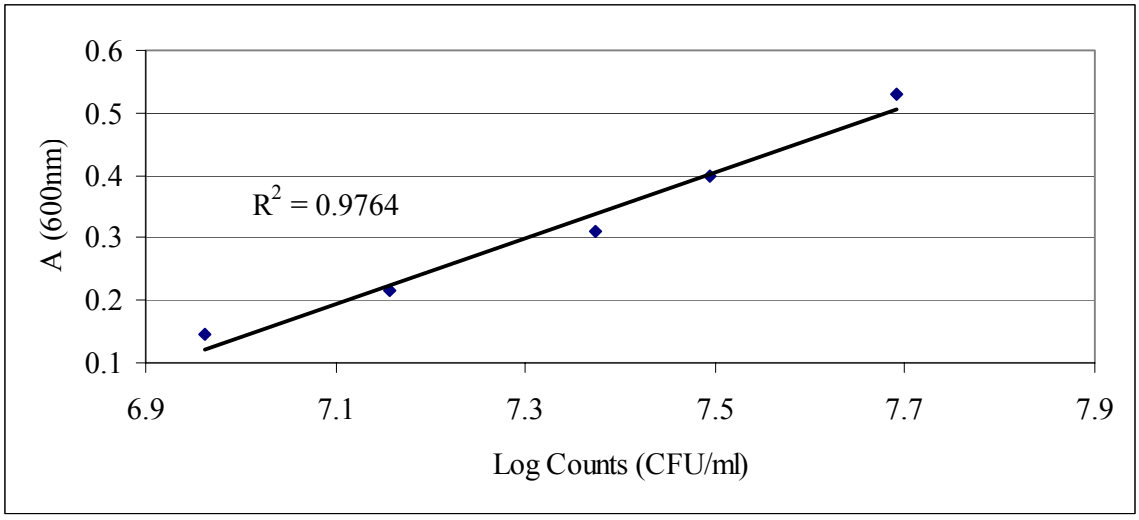

Figure A-2. Standard curve of O.D. (600 nm) of *S. boydii* UI02 – nalidixic acid adapted strain compared to log plate count. Each of three 100 ml TSB (200 ppm NA) microcosms were inoculated with 10 µl aliquots of an 18-hour *S. boydii* UI02 culture. The microcosms were incubated (37ºC, 30 rpm) and sampled at appropriate time intervals. At each time of sampling, appropriate serial dilutions in PBS were pour-plated using TSA (100 ppm NA) and incubated overnight (37ºC). Shown above is the average absorbance at 600 nm (A 600 nm) of the three trials (from Fig 4-1) plotted against the $\log_{10}$ CFU/ml for the data points which form a linear relationship.

<u>*S. sonnei* UI05 – NA adapted strain</u>

The growth curve prepared for *S. sonnei* UI05 – wild strain demonstrates stationary phase was reached in approximately 10 hours (Figure B-3), with an initial lag phase of approximately 7 hours.

An O.D. standard curve for *S. sonnei* UI05 – wild strain was prepared. The cell titer in which the relationship between $\log_{10}$ CFU/ml and absorbance at 600 nm (A 600 nm) showed linearity (R^2 = 0.9821; Figure B-4) was approximately 4.13 x 10^7 to 6.43 x 10^8 CFU/ml.

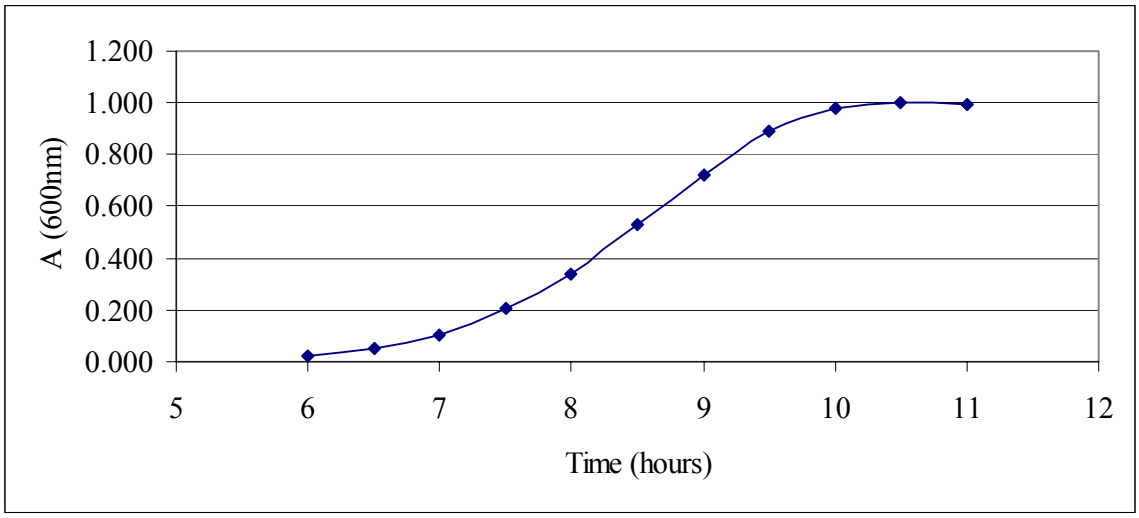

Figure A-3. Growth curve: *S. sonnei* UI05 – nalidixic acid adapted strain. Each of three 100 ml TSB (200 ppm NA) microcosms were inoculated with 10 µl aliquots of an 18-hour *S. sonnei* UI05 culture. The microcosms were incubated (37ºC, 30 rpm) and sampled at appropriate time intervals. At each time of sampling, the absorbance at 600 nm (A 600 nm) was measured spectrophotometrically. Shown above is the average A 600 nm of the three trials plotted against time.

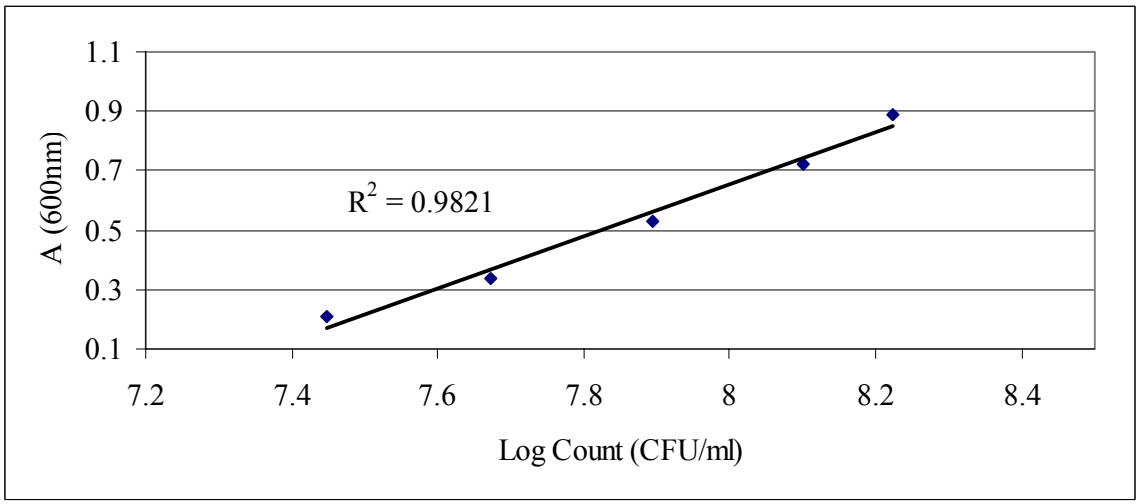

Figure A-4. Standard curve of O.D. (600 nm) of *S. sonnei* UI05 – nalidixic acid adapted strain compared to log plate count. Each of three 100 ml TSB (200 ppm NA) microcosms were inoculated with 10 µl aliquots of an 18-hour *S. sonnei* UI05 culture. The microcosms were incubated (37ºC, 30 rpm) and sampled at appropriate time intervals. At each time of sampling, appropriate serial dilutions in PBS were pour-plated using TSA (100 ppm NA) and incubated overnight (37ºC). Shown above is the average absorbance at 600 nm (A 600 nm) of the three trials (from Fig 4-1) plotted against the $\log_{10}$ CFU/ml for the data points which form a linear relationship.

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BIOGRAPHICAL SKETCH

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