IMPROVED SAMPLE PREPARATION FOR THE MOLECULAR DETECTION OF
Shigella sonnei IN FOODS

By

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OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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2006
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by

Benjamin Ray Warren
To Nikki, for your undying love and support; to Zachary, for bringing so much love into our lives; to my parents, for never losing faith in me; and to all my friends along the way, for without all of you this would not have been possible.
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IMPROVED SAMPLE PREPARATION FOR THE MOLECULAR DETECTION OF Shigella sonnei IN FOODS

By

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Chair: Keith R. Schneider
Major Department: Food Science and Human Nutrition

Shigella, the causative agent of bacillary dysentery, was the third most reported foodborne bacterial pathogen in the U.S. for 2005, and most of the isolates were identified as S. sonnei. Methods for the detection of Shigella in food, however, remain problematic. In preliminary studies, a chromosomally-located genetic target for specific detection of Shigella RNA was investigated by comparing the available genomes of Shigella and E. coli. All DNA sequences identified with potential specificity for all Shigella spp. or with potential specificity for only S. sonnei using database searches tested positive for E. coli strains that had been isolated from ground beef.

Additionally, the survival of a five-strain S. sonnei cocktail on tomato surfaces, in potato salad and in ground beef was investigated using a most probable number (MPN) method. Inoculated tomatoes were stored at 13°C at 85% relative humidity, while potato salad and ground beef samples were stored at 2.5°C and 8°C. On tomato surfaces, S. sonnei populations declined to undetectable levels by day 3. In potato salad and ground
beef samples, *S. sonnei* populations detected at the end of the product shelf-life (28 days and 11 days, respectively) were not significantly different ($P > 0.05$) than initial populations. These studies suggest that *S. sonnei* survives well in foods when not desiccated.

Flow-through immunocapture (FTI) followed by analysis of recovered anti-*Shigella* beads by spread-plate using MAC (FTI-MAC), FTI followed by analysis of recovered anti-*Shigella* beads by real-time PCR (FTI-PCR) and DNA sequence capture (DSC) were compared to the *Shigella* culture method of the FDA *Bacteriological Analytical Manual* (BAM) for the detection of *S. sonnei* on tomato surfaces, in potato salad and in ground beef. FTI-MAC was significantly better ($P > 0.05$) than the BAM *Shigella* culture method for the analysis of tomatoes, but not potato salad or ground beef. FTI-PCR and DSC were significantly better ($P > 0.05$) than the BAM *Shigella* culture method for the analysis of tomatoes and potato salad, but not ground beef.
CHAPTER 1
INTRODUCTION

The ability to analyze food products for the presence of pathogenic bacteria is essential for verifying the safety of foods, identifying agents of foodborne illness and determining sources of foodborne outbreaks. Conventionally, the microbiological analysis of food involves culture enrichment followed by isolation on selective media. Confirmation of presumptive isolates is generally through biochemical characterization and/or serology. Conventional culture methods, however, are often problematic, in that many are time-consuming and require several days to complete, appropriate selective media are not currently available for all bacterial foodborne pathogens, some bacterial pathogens require specific atmospheric or other growth conditions which may be difficult to simulate in the laboratory and some bacterial pathogens may not be culturable by currently available methods. In addition, most culture enrichment procedures used for the detection of bacterial foodborne pathogens detect only the presence or absence of the target pathogen. For enumeration, a most probable number (MPN) method must be employed; however media required for MPN analysis for some bacterial foodborne pathogens is not currently available.

For bacterial foodborne pathogens whose conventional culture methods are problematic, alternative sample preparation methods may be used to improve sensitivity. Alternative sample preparation methods provide a means of separating and concentrating bacterial pathogens or components (proteins, nucleic acids, etc.) of bacterial pathogens from food matrices. If molecular-based detection is to follow, the alternative sample
preparation method must also contend with any potential inhibitors which may be present in the food. Unfortunately, there is no alternative sampling method suitable for the separation and concentration of all types bacterial pathogens from all forms of food; therefore each combination of sampling method, food matrix and bacterial pathogen must be investigated independently.

Once bacterial pathogens are separated and concentrated from food, there are many options for detection by rapid methods, such as immunoassays (enzyme linked immunosorbent assay (ELISA) and lateral flow devices), DNA hybridizations or the polymerase chain reaction (PCR). Most rapid methods require the presence of $\geq 10^3$ colony forming units (CFU)/ml of the target bacterial pathogen for consistent and dependable detection (Stevens and Jaykus, 2004); therefore efficient separation and concentration of bacterial pathogens from food are critical. Because bacterial pathogens may be present in food at low, yet potentially infectious populations, selective or non-selective enrichment is often performed prior to sample analysis to increase the bacterial population. For some sample preparation methods, very short enrichment times (4-5 hr) may be sufficient to increase the bacterial populations to detectable levels, allowing the assay to be performed in a same-day format (Yuk et al., 2006; Schneider and Warren, unpublished data).

The detection of \textit{Shigella} spp. in food is one example of where the use of alternative sample preparation methods may be used to improve analysis over conventional culture methods. The most commonly used \textit{Shigella} culture method in the U.S. is found in the U.S. Food and Drug Administration’s \textit{Bacteriological Analytical Manual} (BAM). The enrichment media recommended in the BAM is \textit{Shigella} broth (SB),
a low-carbohydrate medium used to limit the decrease in pH associated with acid production from the microbiological metabolism of sugars. SB, however, does not provide adequate specificity for *Shigella* spp. and other members of the family *Enterobacteriaceae* have been reported to out-compete and/or overgrow *Shigella* spp. during enrichment (Uyttendaele *et al*., 2001; Warren, 2003; Warren *et al*., 2005a). Other enrichment media, such as *Enterobacteriaceae* Enrichment (EE) broth (Uyttendaele *et al*., 2000) and Gram-negative (GN) broth (CMMEF), have been suggested for the enrichment of *Shigella* spp.; however EE broth has been reported to be inhibitory to *S. boydii* serotype 18 (Warren, 2003; Warren *et al*., 2005b) and GN broth contains bile salts and sodium desoxycholate, which have been shown to inhibit stressed shigellae (Tollison and Johnson, 1985; Uyttendaele *et al*., 2001).

Isolation media are also problematic for *Shigella* spp. The BAM recommends MacConkey agar (MAC), which is selective for Gram-negative bacteria and differential based on the utilization of lactose. Typically, *Shigella* spp. are lactose negative; however some serotypes of *S. boydii* have been reported as lactose positive. During the Gulf War in the early 1990’s, lactose positive *S. sonnei* were isolated from U.S. military personnel (Dr. D.J. Kopecko, FDA, personal communication). Upon further investigation, it was discovered that typical lactose negative *S. sonnei* mutate at high frequency to lactose positive phenotypes during stationary phase on lactose-containing microbiological media (Dr. D.J. Kopecko, FDA, personal communication). It has yet to be determined whether lactose positive *S. sonnei* mutants can form in contaminated food products and whether this mutation would allow *S. sonnei* a means to evade detection by conventional culture methods, such as the BAM.
Previously, a filtration-based sample preparation method using FTA filters (Whatman, Clifton, NJ) in combination with nested PCR was investigated for the detection of *S. sonnei* and *S. boydii* on tomato surfaces (Warren, 2003; Warren *et al*., 2005b). When a sample is applied to FTA filters, the moisture from the sample activates chemical denaturants, chelating agent buffers and free radical traps embedded in the filter which lyse cells, denature enzymes, inactivate pathogens and immobilize genomic DNA (Whatman, 2006). Using a tandem filter funnel system in which the first filter funnel was for size exclusion of sample material and the second filter funnel contained an FTA filter, 100 ml PBS rinses of tomatoes were analyzed and the captured *Shigella* DNA was amplified by nested PCR. This FTA filtration-nested PCR assay was able to detect *S. sonnei* and *S. boydii* on tomato surfaces at inoculation levels as low as 6.2-7.4 CFU/tomato. Unfortunately, the FTA filtration system was not as successful when applied to other types of produce, such as strawberries, cantaloupes or retail Valencia oranges. The analysis of these types of produce in the FTA filtration system resulted in clogged filters and poor detection limits as compared to those performed on tomato rinses.

Recently, a novel device for flow-through immunomagnetic separation (IMS), the Pathatrix, has been developed (Matrix MicroScience, Inc., Golden, CO). IMS methods involve the coupling of specific antibodies to paramagnetic beads, which exhibit magnetic qualities only when placed in a magnetic field. In IMS methods, the antibodies are used to capture target bacteria and then the bead-bacteria complexes are separated and concentrated from the food matrix using a magnet. Traditional IMS methods analyze small sample volumes (1.0 ml), whereas the Pathatrix allows the analysis of a much
larger sample volume (250 ml) by using a tubing system in which IMS beads are immobilized in a capture phase and the sample is continuously pumped through the capture phase using a peristaltic pump. Once recovered, the IMS beads can be analyzed by spread-plate for the isolation of viable colonies or applied directly to DNA extraction methods for subsequent analysis by PCR.

Finally, sample preparation methods based on the specific isolation of bacterial or viral DNA/RNA from various types of matrices using oligonucleotide probes immobilized on paramagnetic beads have been reported. Most commonly, biotinylated probes are attached to paramagnetic beads pre-coated with streptavidin. The bead-probes are then used to sample mixtures containing sample DNA/RNA in hybridization buffer. Following hybridization, the captured DNA/RNA can be removed from the bead-probes using heat and used as template in PCR, RT-PCR or other molecular-based detection assay.

The hypothesis of this study was that specific antibodies and/or specific oligonucleotide probes may be attached to paramagnetic beads and used for the detection of *S. sonnei* with increased sensitivity over the current conventional culture methods. The specific objectives of this study were as follows:

1. To investigate the survival of *S. sonnei* on the tomato surfaces, in potato salad and in ground beef when held under standard refrigerated conditions.

2. To investigate chromosomally-located genetic targets for the specific detection of all *Shigella* spp. or only *S. sonnei*.

3. To develop and compare flow-through immunocapture, using the Pathatrix, for the detection of *S. sonnei* on the tomato surfaces, in potato salad and in ground beef with the BAM *Shigella* culture method.

4. To develop a nucleotide sequence capture method for the rapid detection of *S. sonnei* on the tomato surfaces, in potato salad and in ground beef.
CHAPTER 2
LITERATURE REVIEW

Shigella as a Foodborne Pathogen

Background Information

*Shigella* spp. are the causative agents of shigellosis, or “bacillary dysentery,” first discovered over 100 years ago by Kiyoshi Shiga, a Japanese scientist (Anonymous, 2002). *Shigella* are members of the bacterial family *Enterobacteriaceae* and are nearly identical genetically to *Escherichia coli* and are also closely related to *Salmonella* and *Citrobacter* spp. (Lampel, 2001). *Shigella* are characterized as Gram-negative, facultatively anaerobic, non-sporeforming, non-motile rods that typically do not ferment lactose. In addition, they are lysine-decarboxylase, acetate, and mucate negative and do not produce gas from glucose, although some *S. flexneri* six serotypes have been reported to produce gas (Echeverria *et al*., 1991; International Commission on Microbiological Specifications for Foods (ICMSF), 1996). There are four serogroups of *Shigella*: *S. dysenteriae* (serogroup A; 15 serotypes), *S. flexneri* (serogroup B; eight serotypes divided into 11 subserotypes), *S. boydii* (serogroup C; 20 serotypes), and *S. sonnei* (serogroup D; one serotype) (Centers for Disease Control and Prevention (CDC), 2004).

*Shigella* has been classically characterized as a waterborne pathogen (Smith, 1987) and outbreaks have been reported from contaminated community water sources that were un- or under-chlorinated (Blostein, 1991; Fleming *et al*., 2000; CDC, 2001). Foodborne outbreaks of *Shigella* are also common, especially with foods that are subjected to processing or preparation by hand, are exposed to a limited heat treatment, or are
served/delivered raw to the consumer (Wu et al., 2000). Some examples of food products from which Shigella spp. have been isolated are potato salad, ground beef, bean dip, raw oysters, fish, and raw vegetables.

The infective dose for Shigella is very low: 10 cells of S. dysenteriae to 500 cells of S. sonnei (Kothary and Babu, 2001). At-risk populations, such as the very young, very old, or persons with decreased immune function, may be more susceptible to infection. Due to the low infective dose of Shigella, person-to-person transmission is common, especially in day-care settings (S. sonnei) where toddlers commonly practice poor personal hygiene. Typical symptoms of infection include bloody diarrhea, abdominal pain, fever, and malaise. Although the mechanism is unknown, seizures have been reported in 5.4% of shigellosis cases involving children (Galanakis et al., 2002). Chronic sequelae from S. dysenteriae serotype 1 infections can include hemolytic uremic syndrome (HUS), while S. flexneri infections are associated with later development of reactive arthritis, especially in persons with the genetic marker HLA-B27 (CDC, 2006a). Reactive arthritis is characterized by joint pain, eye irritation, and painful urination (CDC, 2006a).

**Epidemiology of Shigella**

The four serogroups of Shigella differ in epidemiology (Ingersoll et al., 2002). S. dysenteriae is primarily associated with epidemics (Ingersoll et al., 2002) with serotype 1 associated with the highest fatality rate (5-15%) (CDC, 2003). S. flexneri predominates in areas of endemic infection, while S. sonnei has been implicated in source outbreaks in developed countries (Hale, 1991). S. boydii has been associated with source outbreaks in Central and South America but is most commonly restricted to the Indian subcontinent. S.
boydii is rarely isolated in North America; however slight increases in the numbers of isolates have been observed in both 2003 and 2004 (CDC, 2004).

According to the CDC Emerging Infections Program, Foodborne Diseases Active Surveillance Network (FoodNet), Shigella was the third most reported foodborne bacterial pathogen in 2005 (CDC, 2006b). Of 16,614 laboratory-diagnosed cases, Shigella accounted for 2,078 cases (12.5% of total cases) behind only Salmonella (6,471 cases) and Campylobacter (5,655 cases) (CDC, 2006b). From 1996-1998 to 2005 the estimated annual incidence of Shigella spp. in the U.S. decreased by 43% (CDC, 2006b). While S. sonnei continued to be the most isolated serogroup in the U.S. in 2004, the rate of isolation has declined while the rate of isolation of S. flexneri and S. boydii have increased slightly (Table 2-1).

Demographic Variability of Infections with Shigella spp.

FoodNet data on shigellosis in the U.S. collected from 1996 to 1999 were recently analyzed for trends in demographic variability (Shiferaw et al., 2004). The overall incidence of shigellosis was highest among the following groups: children aged 1-4 years, male patients, blacks, Hispanics and Native Americans (Shiferaw et al., 2004). There were also marked demographic differences between infection with S. sonnei and S. flexneri with respect to age, sex and race. While the incidence of both S. sonnei and S. flexneri were higher among those aged 1-4 years, there was a second peak of S. flexneri infection among those aged 30-39 years (Shiferaw et al., 2004). The incidence of S. sonnei among men and women were similar; however the incidence of S. flexneri among men was almost twice that of women (Shiferaw et al., 2004). In addition, the incidence of S. sonnei among blacks and whites was higher than that of S. flexneri, while the incidence of S. flexneri was higher among Native Americans (Shiferaw et al., 2004).
Table 2-1. Percentage of *Shigella* isolates in the United States reported by PHLIS in recent years.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. sonnei</em></td>
<td>83.5%</td>
<td>80.2%</td>
<td>68.9%</td>
</tr>
<tr>
<td><em>S. flexneri</em></td>
<td>12.2%</td>
<td>14.4%</td>
<td>17.2%</td>
</tr>
<tr>
<td><em>S. boydii</em></td>
<td>0.8%</td>
<td>1.1%</td>
<td>1.8%</td>
</tr>
<tr>
<td><em>S. dysenteriae</em></td>
<td>0.3%</td>
<td>0.4%</td>
<td>0.4%</td>
</tr>
<tr>
<td>Ungrouped</td>
<td>3.2%</td>
<td>3.9%</td>
<td>11.7%</td>
</tr>
</tbody>
</table>

Total isolates 12,992 11,552 9,343
(CDC, 2002; CDC, 2003; CDC, 2004)

**Recently Identified Serotype of *S. dysenteriae***

During 2001 to 2003, six biochemically, serologically and genetically identical *Shigella* strains were isolated in geographically distant locations in Canada. When analyzed biochemically, the suspect strains displayed reactions consistent with that of *Shigella* spp. (Melito *et al*., 2005). When analyzed serologically, the suspect strains produced weak reactions with *S. dysenteriae* serovars 4 and 16 and *E. coli* O159 and O173 antisera, however antisera prepared from one of the suspect isolates was completely absorbed by antigens from *S. dysenteriae* serotype 4 and *E. coli* O159 (Melito *et al*., 2005). In addition, all six strains tested PCR positive for the *ipaH* gene and the invasion associated locus. Molecular typing by PCR-RFLP of the *rfb* gene produced a *S. dysenteriae* serovar 2 and *E. coli* 0112ac pattern (Melito *et al*., 2005). Based on these analyses, the authors proposed the six suspect isolates represented a novel serovar of *S. dysenteriae*.

**Foodborne Outbreaks Involving Shigella***

A summary of selected recent foodborne shigellosis outbreaks is given in Table 2-2. Characteristic of foodborne shigellosis, several recent outbreaks have been associated with foods consumed raw (Martin *et al*., 1986; Fredlund *et al*., 1987; Davis *et al*., 1988;
Table 2-2. Selected foodborne outbreaks involving *Shigella*.

<table>
<thead>
<tr>
<th>Year</th>
<th>Serogroup</th>
<th>Food Product(s) Implicated</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1983</td>
<td><em>S. sonnei</em></td>
<td>Tossed salad</td>
<td>Martin et al., 1986</td>
</tr>
<tr>
<td>1986</td>
<td><em>S. sonnei</em></td>
<td>Shredded lettuce</td>
<td>Davis et al., 1988</td>
</tr>
<tr>
<td>1986</td>
<td><em>S. sonnei</em></td>
<td>Raw oysters</td>
<td>Reeve et al., 1989</td>
</tr>
<tr>
<td>1987</td>
<td><em>S. sonnei</em></td>
<td>Watermelon</td>
<td>Fredlund et al., 1987</td>
</tr>
<tr>
<td>1988</td>
<td><em>S. sonnei</em></td>
<td>Uncooked tofu salad</td>
<td>Yagupsky et al., 1991; Lee et al., 1991</td>
</tr>
<tr>
<td>1989</td>
<td><em>S. flexneri</em> 4a</td>
<td>German potato salad</td>
<td>Lew et al., 1991</td>
</tr>
<tr>
<td>1992</td>
<td><em>S. flexneri</em> 2</td>
<td>Tossed salad</td>
<td>Dunn et al., 1995</td>
</tr>
<tr>
<td>1994</td>
<td><em>S. sonnei</em></td>
<td>Iceberg lettuce</td>
<td>Long et al. 2002; Kapperud et al., 1995; Frost et al., 1995</td>
</tr>
<tr>
<td>1995-6</td>
<td><em>S. sonnei</em></td>
<td>Fresh pasteurized milk cheese</td>
<td>Garcia-Fulgueiras et al., 2001</td>
</tr>
<tr>
<td>1996</td>
<td><em>S. flexneri</em></td>
<td>Salad vegetables</td>
<td>PHLS, 1997</td>
</tr>
<tr>
<td>1998</td>
<td><em>S. sonnei</em></td>
<td>Uncooked, chopped curly parsley</td>
<td>CDC, 1999</td>
</tr>
<tr>
<td>1998</td>
<td><em>S. flexneri</em></td>
<td>Restaurant-associated, source unknown</td>
<td>Trevejo et al., 1999</td>
</tr>
<tr>
<td>1999</td>
<td><em>S. boydii</em> 18</td>
<td>Bean salad (parsley or cilantro)</td>
<td>CDPH, 1999</td>
</tr>
<tr>
<td>2000</td>
<td><em>S. sonnei</em></td>
<td>Five layer bean dip</td>
<td>CDC, 2000; Kimura et al., 2004</td>
</tr>
<tr>
<td>2001</td>
<td><em>S. sonnei</em></td>
<td>Raw oysters</td>
<td>Terajima et al., 2004</td>
</tr>
<tr>
<td>2002</td>
<td><em>Shigella spp.</em></td>
<td>Greek-style pasta salad</td>
<td>TPH, 2002</td>
</tr>
</tbody>
</table>

Cook et al., 1995; Dunn et al., 1995; Frost et al., 1995; Kapperud et al., 1995; Public Health Laboratory Service (PHLS), 1997; CDC, 1999) and processed or prepared by hand (Martin et al., 1986; Lee et al., 1991; Lew et al., 1991; Yagupsky et al., 1991; Dunn et al., 1995; Chicago Department of Public Health (CDPH), 1999; Trevejo et al., 1999; Toronto Public Health (TPH), 2002). In 2000, a multi-state outbreak of shigellosis was traced to a commercially prepared five-layer bean dip (Kimura et al., 2004). This outbreak involved 406 persons (14 hospitalizations, 0 deaths) across 10 states. After extensive epidemiological investigation, numerous problems were identified in the manufacturing process and investigators determined that the source of the outbreak was most likely an infected food-handler (Kimura et al., 2004). This outbreak demonstrates
the vulnerability of our food supply to point-source contamination with *Shigella* followed by wide distribution and subsequent infection of many consumers (Kimura *et al*., 2004). 

**Prevalence of *Shigella*: Food and Food Handlers**

Despite the high incidence of shigellosis, there is limited data on the prevalence of *Shigella* among food handlers or on food products. A study investigating the presence of enteropathogens among food handlers in Irbid, Jordan, isolated *Shigella* from the stools of four out of 283 examined food handlers (al-Lahham *et al*., 1990). Mensah *et al*. (2002) evaluated 511 food items from the streets of Accra, Ghana, from which *S. sonnei* was isolated from one sample of macaroni. It was noted that the macaroni was served using bare hands instead of clean utensils, which may have led to the *S. sonnei* contamination. Wood *et al*. (1983) examined foods from Mexican homes, commercial sources in Guadalajara, Mexico, and from restaurants in Houston, TX, for contamination with bacterial enteropathogens. While no *Shigella* was isolated from foods sampled from 12 Houston restaurants or from food commercially prepared in Guadalajara, Mexico, four isolates were obtained from meals prepared in Mexican homes. These studies demonstrate the importance of proper food handling and the role food handlers in the transmission of *Shigella*.

In response to President Clinton’s National Food Safety Initiative (January 1997) and Produce & Imported Foods Safety Initiative (October 1997), the U.S. Food and Drug Administration (FDA) has investigated the presence of foodborne pathogens, including *Shigella*, on imported and domestic produce (FDA, 2001a; FDA, 2001b; FDA, 2003). An FDA survey of imported broccoli, cantaloupe, celery, parsley, scallions, loose-leaf lettuce, and tomatoes found *Shigella* contamination in nine of 671 total samples: three of 151 cantaloupe samples, two of 84 celery samples, one of 116 lettuce samples, one of 84
parsley samples, and two of 180 scallion samples (FDA, 2001a). Another FDA survey of domestically grown fresh cantaloupe, celery, scallions, parsley and tomatoes found *Shigella* contamination in five of 665 total samples: one of 164 cantaloupe samples, three of 93 scallion samples, and one of 90 parsley samples (FDA, 2003). An additional survey of imported produce was conducted; however at the time of this publication results were not publicly available (FDA, 2001b).

**Survival of *Shigella***

**Environmental Factors on Survival of *Shigella***

*Shigella* spp. are heat sensitive, acid resistant, salt tolerant bacteria that can withstand low levels of organic acids (Zaika, 2001; Zaika, 2002a; Zaika 2002b). Zaika (2001) studied the survival of *S. flexneri* strain 5348 in brain heart infusion (BHI) broth as a function of pH (2 to 5) and temperature (4 to 37°C). When inoculated into BHI broth adjusted to pH 5, *S. flexneri* demonstrated growth when held at 19, 28, and 37°C, while counts declined over time at temperature of 12°C or lower. When inoculated into BHI broth adjusted to pH 2, 3, or 4, inoculated *S. flexneri* counts declined over time at all temperatures tested. *S. flexneri* in BHI broth adjusted to pH 2 reached undetectable levels in 1 to 3 days when held at temperature of 19°C or lower. In general, *S. flexneri* survival was greater in BHI broth incubated at lower temperatures and adjusted to higher pH. This study suggests that *S. flexneri* is acid resistant and that acidic foods may support the survival of *Shigella* over a long period of time (Zaika, 2001).

Zaika (2002a) studied the survival of *S. flexneri* strain 5348 in BHI broth (pH 4 to 6) containing 0.5 to 8% NaCl. In BHI adjusted to pH 6, *S. flexneri* grew in the presence of ≤6% NaCl when held at 19 and 37°C, and in the presence of ≤7% NaCl when held at 28°C. Growth of *S. flexneri* was also observed in BHI broth adjusted to pH 5 containing...
$\leq 2, \leq 4, \leq 4, \leq 0.5\% \text{ NaCl}$ when held at 37, 28, 19, and 12°C, respectively (Zaika, 2002a). 

*S. flexneri* populations gradually declined in BHI adjusted to pH 4 at all incubation temperatures and all levels of NaCl tested (Zaika, 2002a). Results from this study suggest that *S. flexneri* is salt tolerant and may survive in salty foods such as pickled vegetables, caviar, pickled herring, dry cured ham, and certain cheeses for extended periods of time (Zaika, 2002a).

*S. flexneri* survival was also studied in BHI broth supplemented with organic acids commonly found in fruits and vegetables (citric, malic, and tartaric acid) or fermentation acids commonly used as preservatives (acetic and lactic acid) at 0.04M and adjusted to pH 4 with HCl or NaOH (Zaika, 2002b). Fermentation acids (acetic and lactic acid) had a greater effect on survival than citric, malic, and tartaric acids (Zaika, 2002b). When incubated at 37°C, *S. flexneri* survived for 1 to 2 days in the presence of each organic acid tested. At 4°C, *S. flexneri* survived in the presence of all the organic acids tested for longer than 55 days (Zaika, 2002b). This study suggests that organic acids may aid in the inactivation of *Shigella*, however foods with low levels of acids stored at low temperatures may support the survival of the bacterium for extended periods of time (Zaika, 2002b).

Temperature is an important factor in survival of shigellae. Freezing (-20°C) and refrigeration (4°C) temperatures support survival, but not growth, of *Shigella* (International Commission on Microbiological Specifications for Foods (ICMSF), 1996). When studied in nutrient broth, the observed temperature ranges which permitted growth for *S. sonnei* and *S. flexneri* were 6.1 to 47.1°C and 7.9 to 45.2°C, respectively (cited in ICMSF, 1996); however *Shigella* can survive for extended periods of time when stored at
-20°C or at 4°C. Elevated temperatures are less permissive for *Shigella* survival, and traditional pasteurization and cooking temperatures are sufficient for inactivation. Evans *et al.* (1970) calculated the decimal reduction time (D-value) of *S. dysenteriae* in pasteurized whole milk to be 0.0008 sec at 82.2°C. When studied in nutrient broth, most strains of *S. sonnei* and *S. flexneri* were inactivated within 5 min at 63°C (cited in ICMSF, 1996). Sublethal heat exposure can sensitize *Shigella* to selective components of microbiological media. Tollison and Johnson (1985) demonstrated that *S. flexneri* sublethally heat-stressed by exposure to 50.0°C for 30 minutes in phosphate buffer became sensitive to 0.85% bile salts and 0.50% sodium desoxycholate. Since these compounds are ingredients in several enrichment and isolation media used for detection of *Shigella*, the thermal history of the food sample to be analyzed should be known.

**Survival of *Shigella* on Fomites**

Inanimate objects, or fomites, can serve as vectors for transmission of *Shigella* and there have been several reports on the survival of *Shigella* on various surfaces (Spicer, 1959; Nakamura, 1962; Islam *et al.*, 2001). Spicer (1959) studied the survival of *S. sonnei* dried on cotton threads at room temperature and under refrigeration at various levels of relative humidity. In general, survival was better at refrigerated temperatures and at high (84%) and low (0%) relative humidity (RH). *S. sonnei* remained detectable on the cotton threads after 12 days at 5-10°C (84% RH) (Spicer, 1959). Similar results were observed using 10 different strains of *S. sonnei* inoculated on cotton, glass, wood, paper, and metal at various temperatures (-20°C to 45°C) (Nakamura, 1962). When held at -20°C, most of the strains survived for more than 14 days on each surface, however surfaces held at 45°C did not support survival of most strains (Nakamura, 1962). Investigations on the survival of *S. dysenteriae* serotype 1 on cloth, wood, plastic,
aluminum, and glass objects suggest that 1.5 to 4 hours post-inoculation, *S. dysenteriae* serotype 1 enters a viable but non-culturable (VBNC) state (Islam *et al.*, 2001). While no *S. dysenteriae* serotype 1 could be recovered after 5 days by conventional culture methods, viable cells could be observed using fluorescent antibody techniques (Islam *et al.*, 2001). Whether or not *Shigella* is able to achieve a true VBNC state or if these results demonstrate the inadequacy of plating media in recovering viable *Shigella* has not been fully investigated. Nevertheless, these studies demonstrate that fomites can sustain viable *Shigella* for an extended time and serve as vehicles in transmission of the pathogen.

**Survival of Shigella in Food and Water**

*Shigella* can survive in water with little decline in population. Rafii and Lunsford (1997) inoculated distilled water with *S. flexneri* at 2.8 x 10^8 colony forming units (CFU)/ml and held the samples at 4°C. After 26 days, 9.2 x 10^7 CFU/ml of *S. flexneri* survived. This high survival rate of *S. flexneri* in water supports the historical association of shigellosis outbreaks and water sources.

Fruits and vegetables can support the growth or survival of *Shigella*. Escartin *et al.* (1989) artificially contaminated fresh cut papaya, jicama, and watermelon with *S. sonnei*, *S. flexneri*, or *S. dysenteriae* and within 6 hours at room temperature, growth was observed. Rafii and Lunsford (1997) inoculated raw cabbage, onion, and green pepper with *S. flexneri* and although counts decreased slightly at 4°C, survival was observed after 12 days on onion and green pepper, at which time sampling was terminated due to spoilage (Rafii and Lunsford, 1997). *S. flexneri* survival was observed on the cabbage for 26 days. Wu *et al.* (2000) studied survival of *S. sonnei* on whole and chopped parsley leaves. At 21°C, growth on chopped parsley was observed at a similar rate to that in
nutrient broth (Wu et al., 2000). At 4°C, populations declined on both chopped and whole parsley throughout the 14-day storage period, however S. sonnei survived regardless of initial population (Wu et al., 2000). These studies demonstrate Shigella survival on refrigerated produce for periods of time that exceed expected shelf life.

Low pH foods can support survival of Shigella when held at refrigerated temperatures. Bagamboula et al. (2002) demonstrated S. sonnei and S. flexneri survival in apple juice (pH 3.3 to 3.4) and tomato juice (pH 3.9 to 4.1) held at 7°C for 14 days. No reduction was observed in the tomato juice, while a 1.2 to 3.1 log_{10} CFU reduction was observed in apple juice over the 14 day study. Rafii and Lunsford (1997) observed S. flexneri survival in carrot salad (pH 2.7 to 2.9), potato salad (pH 3.3 to 4.4), coleslaw (pH 4.1 to 4.2), and crab salad (pH 4.4 to 4.5) held at 4°C. Sampling was terminated at day 11 for the carrot and the potato salads, at which time S. flexneri counts decreased from 4.3 x 10^6 to 4.2 x 10^2 CFU/g and from 1.32 x 10^6 to 8.5 x 10^2 CFU/g, respectively. Sampling of the coleslaw and the crab salads ceased due to product spoilage on days 13 and 20, respectively, however S. flexneri survived at levels of 2.16 x 10^4 and 2.4 x 10^5 CFU/g, respectively. These studies indicate that Shigella survived at refrigerated temperatures despite the presence of background microflora and low pH.

Prepared foods can also support the survival of Shigella. Islam et al. (1993b) investigated the growth and survival of S. flexneri in boiled rice, lentil soup, milk, cooked beef, cooked fish, mashed potato, mashed brinjal, and raw cucumber. All food samples, except raw cucumber, were autoclaved prior to inoculation. Ten gram or 10 ml samples of each food were inoculated with 10^5 cells of S. flexneri, incubated at 5, 25, or 37°C and sampled over 72 hr. All of the foods tested supported growth up to 10^8 to 10^{10} cells per g
or ml within 6 to 18 hr after inoculation at 25°C and 37°C (Islam et al., 1993b). Initial inoculum levels were maintained throughout the 72 hr holding period for all foods, except rice and milk. *S. flexneri* counts in rice decreased by approximately 1 log after 72 hr at 5°C, whereas counts in milk dropped after 48 hr but then returned to the initial inoculum level by 72 hr. These results demonstrate the ability of *Shigella* to grow and survive in a variety of prepared foods that may be contaminated by an infected food handler.

*Shigella* is able to survive on produce packaged under vacuum or modified atmosphere. Satchell et al. (1990) investigated the survival of *S. sonnei* in shredded cabbage packaged under vacuum or in a modified atmosphere of nitrogen and carbon dioxide when stored at room temperature or under refrigeration. When test samples were stored at room temperature, counts of inoculated *S. sonnei* remained at level for up to three days, at which time populations began to drop. When test samples were stored under refrigeration, *S. sonnei* counts did not drop after seven days. In the latter study, refrigerated samples maintained a constant pH throughout the study, while samples stored at room temperature had a drop in pH that may have contributed to the decline in *S. sonnei* cell numbers.

**Current Understanding of Shigella Pathogenesis**

This discussion will be limited to an overview of epithelial cell invasion by *Shigella*, potential roles of the IpaH effector proteins and blockage of autophagy by IcsB. The reader is directed to the following reference for a more complete review of *Shigella* pathogenesis and the toxins produced by *Shigella* spp.: Warren et al., 2006.
Shigella Invasion of Epithelial Cells

The invasion of the local epithelium of the colon (large intestine) is presented in Figure 2-1. 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 under side of the M cells, Shigella infect macrophages and induce cell apoptosis (Suzuki and Sasakawa, 2001). Once released from the macrophage, Shigella enters neighboring epithelial cells. Shigella first forms a membrane bound protrusion into the 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 escape from the double-membrane vacuole, releasing it into the cytosol of the recipient cell. In response to invasion, epithelial cells produce pro-inflammatory cytokines that contribute to inflammation of the colon (Suzuki and Sasakawa, 2001).

Potential Roles of the IpaH Effector Proteins

IpaH7.8 facilitates escape from endocytic vacuoles

Fernandez-Prada et al. (2000) reported that IpaH7.8 of S. flexneri was required for efficient escape from endocytic vacuoles. Human monocyte-derived macrophages (HMDM) and the J744 mouse macrophage cell line were infected with S. flexneri 2457T and pWR700, an ipaH7.8 deletion mutant of S. flexneri 2457T. After the infected HMDM and J744 cells were incubated in the presence of gentamicin and chloroquine, results showed that more pWR700 than 2457T was present within endocytic vacuoles, suggesting that IpaH7.8 is required for escape from the vacuole. The contrast between
Figure 2-1. Invasion of epithelial cells by *Shigella* spp. In the large intestine, *Shigella* enters through M cells that overlay the solitary lymphoid nodules, infect the resident macrophage and induce cell apoptosis. Once released from the macrophage, *Shigella* enters the neighboring enterocytes and escape from the double membrane vacuole that encompasses them. *Shigella* multiply in the cytoplasm of the host cell and polymerize actin for motility. IcsB is required to evade autophagic recognition; therefore icsB mutants are degraded once they escape from the vacuole. Figure reproduced from Ogawa and Sasakawa, 2006.

pWR700 and 2457T localization within endocytic vacuoles was more pronounced in the J744 cell line. One explanation for this was that the HMDM in tissue culture represented a heterogenous population of cells, at various stages of differentiation. The authors further suggested that the *ipaH* genes may play a bigger role in monocytes than macrophages (Fernandez-Prada *et al.*, 2000). It is noteworthy to mention that *ipaH*4.5 and *ipaH*9.8 mutants behaved like the wild-type 2457T in both HMDM and J744 cells, suggesting their role in virulence differs from that of *ipaH*7.8.

**Subversion of host cell signaling by IpaH9.8**

Toyotome *et al.* (2002) investigated the secretion of IpaH proteins from *S. flexneri* in broth cultures and determined that IpaH proteins are exported by type III secretion...
after entry into the host cell. Further investigation showed that once secreted, IpaH9.8 accumulates in the nucleus, while small amounts are present in the cytoplasm. IpaH9.8 has similar structure to the *Salmonella* Typhimurium protein SspH1, which belongs to the bacterial LPX repeat protein family. Upon infection of the local epithelium, intracellular pathogens, such as *Shigella* and *Salmonella*, elicit the secretion of proinflammatory cytokines, such as interleukin 8 (IL-8) (Haraga and Miller, 2003). Production of IL-8 and other cytokines in response to bacterial invasion are dependant, in part, on activation of transcription factor NF-kappa B. Haraga and Miller (2003) demonstrated that SspH1 and IpaH9.8 both localize to the mammalian nucleus and inhibit nuclear factor kappa B (NF-kappa B)-dependent gene expression (Haraga and Miller, 2003). In this way, IpaH9.8 serves to subvert host cell signaling events involved in the immune response to epithelial invasion.

**Blockage of Autophagy by IcsB**

Autophagy is a critical process in eukaryotic cells in which undesirable cellular components or organelles, including invading microbes, are degraded. Recently, Ogawa *et al.* (2005) identified IcsB as critical in the camouflage against autophagic recognition. IcsB is an effector protein exported by type III secretion and is located on the cell surface. IcsB mutants are fully invasive and capable of escaping from the vacuole, but defective in its ability to multiply within the host cell (Ogawa *et al.*, 2003). In the absence of IcsB, the autophagy protein Atg5 recognizes and binds to IcsA (VirG), thus initiating autophagosome formation. Ogawa *et al.* (2005) demonstrated the IcsA (VirG) binding region for both Atg5 and IcsB is the same, and Atg5 binding to IcsA (VirG) is inhibited by IcsB in a dose-dependent manner. By blocking the binding if IcsA (VirG) by Atg5,
IcsB inhibits the autophagic recognition of *Shigella* within the host cell cytoplasm, thus contributing to intracellular survival.

**Genetic Relationship Between *Shigella* and *Escherichia coli***

While it has been generally accepted that *Shigella* are within the species *E. coli*, recent studies have indicated that *Shigella*, like the other forms of pathogenic *E. coli*, derived from different evolutionary origins, suggesting convergent evolution of the *Shigella* phenotype (Pupo et al., 2000). Rolland et al. (1998) used restriction fragment length polymorphism of rDNA (ribotyping) to group 75 strains of *Shigella*, 13 strains of enteroinvasive *E. coli* (EIEC) and 72 *E. coli* strains of the *E. coli* Reference (ECOR) Collection, which have been classified into four phylogenic groups (A, B1, B2 and D). The *S. sonnei*, *S. flexneri* and most *S. dysenteriae* ribotypes were closely related to phylogenic group D, while *S. dysenteriae* serotype 1 strains were closely related to phylogenic group B1 and *S. boydii* strains were spread between phylogenic group D and B1 (Rolland et al., 1998). In contrast, the ribotypes of EIEC strains were widely distributed among phylogenic groups A, B1 and B2. This evidence suggests that *Shigella* and EIEC derived from different origins.

Pupo et al. (2000) sequenced eight housekeeping genes from four regions of the chromosome for 46 strains of *Shigella* representing all four serotypes. Three distinct clusters of *Shigella* were identified and although *S. sonnei* and *S. dysenteriae* serotype 1, 8 and 10 did not group in the main three clusters, they fell well within the species *E. coli* (Pupo et al, 2000). As with the study by Rolland et al. (1998), *S. boydii* serotype 13 was distantly related to the other *Shigella* strains. Cluster 1 contained most of the *S. boydii* and *S. dysenteriae* strains along with *S. flexneri* serotypes 6 and 6A. Cluster 2 contained seven *S. boydii* strains and *S. dysenteriae* serotype 2. Cluster 3 contained *S. flexneri*
serotypes 1-5 and S. boydii serotype 12. Unlike the results from ribotyping, the use of multiple genes for phylogenetic analysis revealed greater genetic diversity among the strains of *Shigella*, further suggesting that *Shigella* derived from different evolutionary origins.

Fukiya *et al.* (2004) used comparative genomic hybridization microarray analysis to compare the gene content of *E. coli* K-12 with that of 22 pathogenic *E. coli* and *Shigella* strains. When compared to the *E. coli* K-12 genome, the genomes of *S. sonnei*, *S. boydii* and *S. flexneri* 2a were missing only 613, 533 and 716 open reading frames (ORFs). The genomes of the other pathogenic *E. coli* strains were missing similar numbers of ORFs. Subsequent phylogenetic analysis revealed a close relationship between three of four EIEC strains and the three strains of *Shigella*, which suggests EIEC and *Shigella* form a single *E. coli* pathovar (Fukiya *et al.* 2004; Yang *et al.* 2005).

Providing further to the body of evidence that *Shigella* and EIEC derived from different origins, the complete genomes of *S. boydii* serotype 4 (strain 227), *S. dysenteriae* serotype 1 (strain 197) and *S. sonnei* (strain 046) have recently been sequenced (Yang *et al.*, 2005). Comparative genomics among the newly sequenced *Shigella* genomes and the previously sequenced genomes of *S. flexneri* 2a (strain 301) and *E. coli* K-12 (strain MG1655) supported previous work by Fukiya *et al.* (2004). While the genomes of *Shigella* share most of their genes with that of *E. coli* K-12, the *Shigella* phenotype is a result of the gain and loss of functions through bacteriophage-mediated gene acquisition, insertion sequence (IS)-mediated DNA rearrangements and formation of pseudogenes (Yang *et al.*, 2005). For example, the chromosome and virulence plasmid of *S. sonnei* strain 046 contained 327 and 28 intact IS elements and 67
and 68 partial IS elements, respectively. In contrast, the *E. coli* K-12 genome contained only 37 intact IS elements and seven partial IS elements. These studies taken together, demonstrate that the *Shigella* have evolved from distinct *E. coli* ancestors through convergent evolution.

**Detection Methods for *Shigella* in Foods**

**Conventional Culture Methods for *Shigella***

**Traditional microbiological media for enrichment and isolation of *Shigella***

Traditional microbiological techniques make use of selective and differential media for the enrichment and isolation of *Shigella*. Many variants of enrichment and plating media have been investigated for optimal recovery, often with conflicting results. Although *Shigella* is readily isolated from clinical samples, food samples are more problematic. Isolation of *Shigella* from food samples can be inhibited by indigenous microflora, especially the coliform bacteria and *Proteus* spp. (ICMSF, 1996). The addition of the antibiotic novobiocin to liquid and solid media has been shown to improve the isolation of *S. flexneri* and *S. sonnei* from investigated foods (cited in ICMSF, 1996). Contamination of food products with *Shigella* results primarily through a food handler with poor personal hygiene; therefore the concentration of *Shigella* may be very low compared to that of the indigenous microflora (Lampel and Maurelli, 2001). Currently, selective media are not available that adequately suppress the growth of background microflora, therefore *Shigella* is often overgrown by competing microorganisms (Lampel and Maurelli, 2001). More research is needed to determine more appropriate selective media and enrichment conditions for the isolation of *Shigella* from food samples.

Two enrichment broths initially used for the isolation of *Shigella* were Selenite-F (SF) and 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 (and most enterics); therefore it is no longer used in enrichment procedures for Shigella. TT is a peptone-based broth with bile salts and sodium thiosulfate that inhibits growth of most Gram-positives and Enterobacteriaceae. While TT is routinely used for the enrichment of Salmonella, it is rarely used for Shigella. 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. Both TT and GN broths contain bile salts, which can be inhibitory to stressed cultures (Tollison and Johnson, 1985). Furthermore, GN broth contains sodium desoxycholate, which has been shown to inhibit heat-stressed shigellae (Uyttendaele et al., 2001). Regardless, GN broth is listed as an alternate enrichment medium for the detection of Shigella from food by some standard methods (Lampel, 2001).

Current enrichment procedures (FDA, 1998; Lampel, 2001, Health Canada, 2004) use a low carbohydrate medium, Shigella broth (SB) with addition of novobiocin, for the detection/isolation of Shigella. One study reported that acids produced by other Enterobacteriaceae during the fermentation of carbohydrates were toxic to shigellae (Mehlman et al., 1985); however, other studies have shown that Shigella can grow at pH 4.5 to 4.75 (Bagamboula et al., 2002) and survive at pH 4.0 (Zaika, 2002). Nevertheless, the use of SB limits the production of acids, and thereby limits the introduction of low pH, during enrichment. SB is also less stringent than TT broth and GN broth for the enrichment of Shigella since it contains neither bile salts nor sodium desoxycholate. A
recent study investigated SB, GN broth, tryptic soy broth, and Enterobacteriaceae Enrichment (EE) broth with the addition of novobiocin for enrichment/detection of shigellae (Uyttendaele et al., 2001). When incubated in GN broth, S. sonnei was unable to grow to comparable levels as observed in SB and EE broths. EE broth, however, has been reported inhibitory to S. boydii (Warren, 2003; Warren et al., 2005b).

Multiple plating media with differing selectivity can be used to increase the chances of Shigella isolation. The most common low selectivity medium used for plating Shigella is MacConkey Agar (MAC). Eosin methylene blue (EMB) or Tergitol-7 (T7) agars can also be used. Since differentiation on MAC is solely based on lactose fermentation, Shigella colonies look similar to those of many lactose negative competitors (Uyttendaele et al., 2001). On MAC, Shigella colonies are translucent or slightly pink, with or without rough edges. Shigella produce colorless colonies on EMB and bluish colonies on the yellowish-green T7 agar (Lampel, 2001).

Intermediate selectivity media useful in isolating Shigella 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 since DCA required a 48 hr incubation to show clear colony morphology as opposed to overnight incubation for XLD. Unfortunately, D-xylose, which serves as a differentiating agent on XLD agar, is fermented by some strains of S. boydii while most Shigella cannot ferment xylose (Bhat and Rajan, 1975). Thus some strains of Shigella will be missed if XLD is used as the sole plating medium.

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*, especially if the culture is stressed (Lampel, 2001; Uyttendaele *et al.*, 2001). *Shigella* spp. produce colorless, translucent colonies on SSA and green colonies on HEA.

A newly developed plating medium, Chromogenic *Shigella* spp. Plating Medium (CSPM; R&F Laboratories, West Chicago, IL), offers medium selectivity (bile salts, antibiotic supplementation) with an alternative to differentiation based on lactose fermentation. Instead, differentiation on CSPM is based on proprietary components consisting of select carbohydrates, pH indicators, and chromogens (Dr. Larry Restaino, personal communication). *Shigella* spp. produce white to clear colonies on CSPM while competitors produce colored colonies. CSPM has been compared to MAC and SSA for the recovery of *S. boydii* and *S. sonnei* from tomato surfaces with no significant differences in recovery observed (Warren, 2003; Warren *et al.*, 2005a). Further evaluation of CSPM against other strains of *S. boydii* and *S. sonnei* as well as the other serogroups of *Shigella* is needed.

Recent studies at the FDA, Laboratory for Enteric and Sexually Transmitted Diseases have demonstrated stable lactose-positive mutations in stationary phase *S. sonnei* (Dr. D.J. Kopecko, FDA, personal communication). DNA sequencing experiments have revealed slip-strand mutations within the lac repressor (*lacI*) that are responsible for the lactose-positive phenotype. These mutations are significant for detection of *S. sonnei* in food since typical colonies on plating media (MAC) are differentiated based on the utilization of lactose, the typical *S. sonnei* phenotype being lactose-negative.
The FDA *Bacteriological Analytical Manual* culture method for detection of *Shigella* in foods

The FDA *Bacteriological Analytical Manual* (BAM) culture method for the isolation and detection of *Shigella* spp. from food utilizes a combination of low carbohydrate enrichment, anaerobic conditions, and elevated temperature (FDA, 1998). Briefly, a 25 g sample of the food product 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 min 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 hr (44ºC for *S. sonnei*; 42ºC for all other *Shigella* spp.) and the enrichments are streaked on MAC. Confirmation of suspicious colonies involves tests for motility, H₂S, gas formation, lysine decarboxylase, and fermentation of sucrose or lactose. Isolates negative for all confirmatory tests are further tested using 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.

*June et al.* (1993) evaluated the effectiveness of the BAM culture method for *Shigella*. 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. Using either unstressed or chilled stressed cells, acceptable recovery was achieved for both strains from the potato salad, chicken salad, cooked shrimp salad and lettuce samples, but not from the ground beef and raw oyster samples. An approximate 4-log unit difference in recovery from ground beef samples was observed between the two strains,
suggesting high strain variability. Given the low infective dose of *Shigella* (as low as 10 cells), the BAM was considered ineffective for the evaluation of raw ground beef and raw oysters.

In 2002, the BAM culture method for *Shigella* was evaluated using two strains of unstressed, chill-stressed, or freeze-stressed *S. sonnei* (strains 357 and 20143) on selected types of produce (Jacobson *et al*., 2002). Acceptable recovery of unstressed cells (<1.0 x 10^1 CFU/25g) and chill-stressed or freeze-stressed cells (<5.2 x 10^1 CFU/25g) were observed for all produce types tested (Jacobson *et al*., 2002). More recently, similar tests of a modified BAM protocol showed unacceptable recovery of unstressed *S. sonnei* (patient isolate from an outbreak involving an unknown source) and *S. boydii* (patient isolate from an outbreak involving bean salad) on tomatoes at 1.9 x 10^2 CFU/tomato and >5.3 x 10^5 CFU/tomato, respectively (Warren, 2003). These results support the observation that significant variation exists among strains of *S. sonnei* and demonstrate the importance of including the other serogroups of *Shigella* when evaluating culture methods for detection in food.

**Other culture methods for the detection of *Shigella* in foods**

Alternate culture methods for the detection of *Shigella* in foods can be found in Health Canada’s Compendium of Analytical Methods (Health Canada, 2004) and the American Public Health Association’s Compendium of Methods for the Microbiological Examination of Foods (CMMEF) (Lampel, 2001). The Health Canada method is based on the BAM culture method with a few modifications; most notably enrichments for all *Shigella* spp. (including *S. sonnei*) are supplemented with 0.5 µg/ml novobiocin and incubated anaerobically at 42°C. The CMMEF culture method is also similar to the BAM culture method, however the level of novobiocin in *S. sonnei* enrichment media is lower
(0.3 µg/ml) and enrichment conditions are aerobic at 37°C. The CMMEF further suggests that two to three 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. Confirmation of suspicious colonies is similar to methods described above for the BAM culture method.

Recently, the CMMEF culture method and an enrichment procedure involving EE broth have been compared to the BAM culture method for the detection of *S. boydii* and *S. sonnei* on tomato surfaces (Warren, 2003). Natural tomato microflora was found to have a great impact on recovery of *S. sonnei* and completely inhibited recovery of *S. boydii* in all three culture methods. No significant differences \((P > 0.05)\) were observed among the culture methods for detection of *S. sonnei*, or between the BAM and CMMEF culture methods for the detection of *S. boydii*. EE broth was found to be inhibitory to *S. boydii*. These results suggest the need for more selective enrichment protocols for the evaluation of *Shigella* spp. in food.

**Immunological Methods for *Shigella* Detection**

**Lipopolysaccharide (LPS) of *Shigella***

Gram-negative bacterial LPS consists of three distinct regions: lipid A, core oligosaccharide, and a serotype-specific O-polysaccharide chain (O-antigen) (Neidhardt, 2004). The lipid A portion anchors the LPS molecule to the outer membrane. The core oligosaccharide is composed of two regions: the inner core and the outer core. The inner core is common to many enterobacterial species and is composed of heptose and 2 keto-3 deoxyoctulosonate (KDO), while the outer core is rich in hexose and tends to be more species-specific (Tsang *et al.*, 1987). Studies of the core structure of *S. flexneri* have indicated that serotypes 1 to 5 and variants X and Y share the *E. coli* R3 core (Carlin and
Figure 2-2. Structural differences between hexose regions of *Shigella sonnei* and *Shigella flexneri* lipopolysaccharides. A) *S. sonnei* and *S. flexneri* serotype 6 share the hexose region of the *E. coli* R1 core. B) *S. flexneri* serotypes 1-5 and X and Y variants have a hexose region identical to the *E. coli* R3 core. (Structures compiled from Jansson *et al.*, 1981). Abbr.: Gal = galactose, Glc = glucose, GlcNAc = 2-acetamido-N-deoxyglucose.

Lindberg, 1986), while *S. flexneri* serotype 6 and *S. sonnei* share the *E. coli* R1 core (Gamian and Romanowska, 1982; Carlin and Lindberg, 1986; Viret *et al.*, 1992) (Figure 2-2). Antibodies have been developed with specificity to the inner core of *Shigella* spp. (Rahman and Stimson, 2001). The O-antigen is a polymer of repeating saccharides that is highly variable among species. Strains sharing identical O-antigen repeating units are of the same serotype. The O-antigens of all *S. flexneri* serotypes are a repeating tetrasaccharide (Carlin and Lindberg, 1986), while the O-antigen of *S. sonnei* is a repeating disaccharide (Kenne *et al.*, 1977) (Figure 2-3).

**Differences in *S. sonnei* form I and form II lipopolysaccharide**

Virulent *S. sonnei* produce smooth colonies, termed form I, which result from expression of the O-antigen. Unlike other *Shigella* species, the LPS genes (*rfc* and *rfb*) of *S. sonnei* are located on a large virulence plasmid, which can be spontaneously lost at
Figure 2-3. O-antigen repeating subunits of *S. sonnei* and *S. flexneri* lipopolysaccharide. (A) The disaccharide repeating subunit of the *S. sonnei* O-antigen, (B) the tetrasaccharide repeating subunit of *S. flexneri* serotype 6, and (C) the tetrasaccharide repeating subunit of *S. flexneri* serotypes 1-5 and X and Y variants. (Structures compiled from Carlin and Lindberg, 1986; Dmitriev *et al.*, 1979; and Gamian and Romanowska, 1982). Abbr: l-AltNAcA = N-acetyl-l-altrosaminuronic acid, 4-N-DFucNac = 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose, GaLA = galacturonic acid, GaLNAC = N-acetylglactosamine, Ac3Rha = 3-O-acetylrhamnose, Rha = rhamnose, GlcNAC = 2-acetamido-N-deoxyglucose.

high frequency. Sansonetti *et al.* (1981) investigated the stability of form I plasmids and observed 1 to 45% plasmid loss from re-streaking form I colonies onto MAC and incubating 24 hr at 37°C. When the large virulence plasmid is lost, rough (avirulent) colonies, termed form II, are produced that express the *Enterobacteriaceae* R1 lipopolysaccharide core (Sansonetti *et al.*, 1981; Gamian and Romanowska 1982). A defective mutant of form II *S. sonnei* LPS, termed R-form, is characterized by an incomplete core region (Gamian and Romanowska, 1982) (Figure 2-4). As antibodies specific for *S. sonnei* O-antigen will bind form I, but not form II or R-form LPS, immunological detection methods with specificity for the O-antigen of *Shigella* are compromised when the virulence plasmid is lost.

**Immunological Detection Methods for Bacteria**

Immunological methods, such as latex agglutination (LA), enzyme immunoassay (EIA), or immunomagnetic separation (IMS), have been utilized for the detection of
Figure 2-4. Lipopolysaccharides of *S. sonnei*. A) Form I, B) form II, and C) R-form. (Structures compiled from Gamien and Romanowska, 1982).

many foodborne bacterial pathogens. LA assays involve latex particles coated with antibodies specific for target bacteria. Binding causes a visible clumping of the latex particle-bacteria complexes that can be seen with the naked eye. EIA is a term used to describe many assay formats in which enzyme-labeled antibodies are used to bind antigens with detection via a colorimetric reaction using the enzyme label. Microplate
readers are generally used for detection of the colorimetric signal and these assays can be performed in high-throughput format. IMS involves small paramagnetic beads coated with antibodies against the surface antigens of bacterial cells. Paramagnetic beads exhibit magnetic properties only when placed within a magnetic field and show no residual magnetism when removed from this field. After target bacteria are bound by the antibody-coated beads, a magnetic field is used to separate the bead-bacteria complexes. Once the magnetic field is removed, the bead-bacteria complexes return to suspension (Olsvik et al., 1994). Detection of bead-bacteria complexes can be performed via direct plating on microbiological media, direct microscopy, or nucleic acid amplification. LA, EIA and IMS methods require the use of monoclonal or polyclonal antibodies to bind specific antigens present on the surface of bacterial cells. For this reason, development of antibodies with sufficient specificity is critical for the performance of immunological methods. A summary of these and other rapid methods for *Shigella* detection is presented in Table 2-3.

**Latex agglutination methods for *Shigella***

LA requires the prior isolation of a suspect colony on solid media. For this reason LA methods cannot be used for detection of *Shigella* directly from a food sample, but rather can confirm or aid in characterization of suspected *Shigella* colonies. Several latex agglutination serotyping kits for *Shigella* are commercially available, however only one representative kit will be discussed in this review.

The Wellcolex Colour *Shigella* Test (WCT-*Shigella*, Remel Inc., Lenexa, KS) allows the identification of isolates to the species level using only two reagents, each consisting of a mixture of red and blue latex particles coated with antibodies specific for
Table 2-3. Selected rapid methods for detection of *Shigella*.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Target</th>
<th>Matrix</th>
<th>Sample Preparation</th>
<th>Detection</th>
<th>Detection Limit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. sonnei</td>
<td><em>ipaH</em></td>
<td>Tomatoes</td>
<td>FTA filtration</td>
<td>Nested PCR</td>
<td>&lt; 10³ CFU/tomato</td>
<td>Warren, 2003</td>
</tr>
<tr>
<td>S. boydii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. flexneri</td>
<td><em>ipaH</em></td>
<td>Water</td>
<td>SE, boiled extract</td>
<td>Semi-nested PCR</td>
<td>≥ 1.1 x 10⁴ CFU/ml</td>
<td>Theron et al., 2001</td>
</tr>
<tr>
<td>S. flexneri</td>
<td><em>virA</em></td>
<td>Mayonnaise</td>
<td>CEP</td>
<td>PCR</td>
<td>10⁻³⁻¹⁰⁻³ CFU/ml</td>
<td>Theron et al., 2001</td>
</tr>
<tr>
<td>S. dysenteriae 1</td>
<td><em>virA</em></td>
<td>Mussels</td>
<td>PE, CEP</td>
<td>PCR</td>
<td>10⁻¹⁻¹⁰⁻² CFU/ml</td>
<td>Vantarakis et al., 2000</td>
</tr>
<tr>
<td>S. flexneri</td>
<td>plasmid DNA</td>
<td>Lettuce</td>
<td>SE, BDC</td>
<td>PCR</td>
<td>1.0 x 10⁴ CFU/g</td>
<td>Lampel et al., 1990</td>
</tr>
<tr>
<td>S. flexneri LPS</td>
<td><em>ial</em></td>
<td>Various foods</td>
<td>Alkaline denaturation</td>
<td>Nested PCR</td>
<td>1.0 x 10¹ CFU/g</td>
<td>Lindqvist, 1999</td>
</tr>
<tr>
<td>S. sonnei form I</td>
<td>LPS, <em>ial</em></td>
<td>Feces</td>
<td>IMS</td>
<td>PCR</td>
<td>1.0-1.5 x 10¹ CFU/ml</td>
<td>Achi-Berglund and Lindberg, 1996</td>
</tr>
<tr>
<td>S. dysenteriae</td>
<td>LPS</td>
<td>Rectal swabs</td>
<td>PE, DPSM</td>
<td>EIA</td>
<td>ND</td>
<td>Sonjai et al., 2001</td>
</tr>
<tr>
<td>S. dysenteriae</td>
<td>LPS</td>
<td>Various foods</td>
<td>None</td>
<td>Biosensor</td>
<td>≥ 4.9 x 10⁴ CFU/ml</td>
<td>Sapsford et al., 2004</td>
</tr>
<tr>
<td>S. dysenteriae 1</td>
<td>LPS, <em>ial</em></td>
<td>Feces</td>
<td>IMS</td>
<td>PCR</td>
<td>ND</td>
<td>Achi et al., 1996</td>
</tr>
<tr>
<td>S. flexneri</td>
<td>LPS, <em>ial</em></td>
<td>Feces</td>
<td>IMS</td>
<td>PCR</td>
<td>1.0 x 10¹ CFU/g</td>
<td>Islam and Lindberg, 1992</td>
</tr>
<tr>
<td>S. flexneri</td>
<td>LPS, 16S rRNA</td>
<td>Sewage</td>
<td>Immunocapture</td>
<td>UPPCR</td>
<td>5.0 x 10¹ CFU/ml</td>
<td>Peng et al., 2002</td>
</tr>
<tr>
<td>S. dysenteriae 1</td>
<td>LPS</td>
<td>Feces</td>
<td>IMS</td>
<td>EIA</td>
<td>1.0 x 10³ CFU</td>
<td>Islam et al., 1993a</td>
</tr>
</tbody>
</table>

one of the four different *Shigella* serogroups. Each reagent is added to one of two identical sample spots of the suspect isolate. The presence of homologous antigen results in the agglutination of one color coupled with a change in background color. Color change combinations due to any of the four *Shigella* serogroups are easily distinguishable as is the negative reaction in which the particles remain in smooth purple suspension. Non-specific agglutination results in a purple agglutination with a clear background.

Bouvet and Jeanjean (1992) tested the WCT-*Shigella* against 100 *Shigella* isolates from human stools and observed specificity and sensitivity at 100% and 98%, respectively. The two *Shigella* strains that did not give visible aggregates were *S. dysenteriae* type 2 and *S. flexneri* type 4. These results were supported by Kocka et al. (1992). Using the WCT-*Shigella*, 42 of 42 clinical *Shigella* isolates and seven of eight stock *Shigella* cultures were correctly grouped (Kocka et al., 1992). The stock *Shigella* culture that was not correctly grouped had been repeatedly passed in culture that may have resulted in the loss of some antigenicity. Lefebvre et al. (1995) evaluated WCT-*Shigella* and six commercial slide agglutination *Shigella* serogrouping kits for accuracy. The WCT-*Shigella* was easily performed and interpretation of results was less subjective than the other tests. WCT-*Shigella* met a performance standard of 90% accuracy in these evaluations.

**Enzyme immunoassay methods for *Shigella***

Although EIA methods for the detection of foodborne pathogens are common, the only commercially available kits for *Shigella* spp are the Shigel-Dot A (for *S. dysenteriae*), B (for *S. flexneri*), C (for *S. boydii*), and D (for *S. sonnei*) test kits (Science Development and Management. Ltd., Bangkok, Thailand). The Shigel-Dot kits are membrane dot-blot enzyme-linked immunosorbent assays (ELISA). These test kits have
been validated using 500 rectal swabs and have been compared to conventional culture isolation and Western blot analysis. The diagnostic accuracy of the Shigel-Dot A, B, C, and D was 99.2%, 95.0%, 94.0%, and 96.4%, respectively, when compared to conventional culture isolation, and all were 100% when compared to the conventional culture isolation and Western blot combined (Sonjai et al., 2001). Monoclonal antibodies included in the Shigel-Dot D kit are reported to detect both \textit{S. sonnei} form I and form II LPS.

**Immunomagnetic separation methods for \textit{Shigella} detection**

IMS has been investigated for the concentration and purification of bacterial pathogens from food samples (Cudjoe and Krona, 1997; Hsih and Tsen, 2001; Drysdale \textit{et al.}, 2004; Lynch, \textit{et al.}, 2004) and are reportedly more sensitive than comparable conventional culture methods (Cudjoe and Krona, 1997; Hsih and Tsen, 2001). Anti-\textit{Salmonella}, anti-\textit{E. coli} O157:H7, anti-\textit{Campylobacter}, and anti-\textit{Listeria} IMS beads are commercially available (Matrix MicroScience, Cambridgeshire, UK; Dynal Biotech, Oslo, Norway) however, anti-\textit{Shigella} IMS beads are not yet commercially available.

IMS has been used as a technique to concentrate \textit{Shigella} from clinical samples for downstream detection processes such as EIA (Islam \textit{et al.}, 1993a) or PCR (Islam and Lindberg, 1992; Achi \textit{et al.}, 1996; Achi-Berglund and Lindberg, 1996). By using IMS, PCR inhibitors inherent to fecal samples were successfully eliminated. Briefly, IMS was used to concentrate \textit{S. sonnei} (Achi \textit{et al.}, 1996; Achi-Berglund and Lindberg, 1996), \textit{S. flexneri} (Islam and Lindberg, 1992; Achi \textit{et al.}, 1996), and \textit{S. dysenteriae} serotype 1 (Islam and Lindberg, 1992; Achi \textit{et al.}, 1996) from feces prior to PCR. The detection limit for the IMS-EIA and IMS-PCR methods were $1.0 \times 10^3 \text{ CFU/ml}$ (Islam \textit{et al.}, 1993a) and $1.0-1.5 \times 10^1 \text{ CFU/ml}$ (Islam and Lindberg, 1992; Achi-Berglund and
Lindberg, 1996), respectively. In addition, the IMS-PCR method was more than two times as effective than the conventional culture method for the diagnosis of shigellosis in children with severe diarrhea (Achi et al., 1996). These studies have significance with respect to *Shigella* detection in foods, however further investigations are needed to evaluate the suitability of the IMS techniques for use with food samples.

**Immunomagnetic separation using the Pathatrix**

Recently, a novel device allowing flow-through IMS, the Pathatrix, has been developed (Figure 2-5). The Pathatrix allows a 250 ml sample to be continuously pumped through a tubing system in which IMS beads are immobilized in a capture phase, thereby allowing the analysis of a complete 25 g sample + 225 ml buffer/enrichment broth homogenate with or without prior incubation. Each Pathatrix unit can analyze up to five samples at one time. The incubation pots in which the sample (stomacher bag) is placed are temperature controlled from room temperature up to 45°C. After analysis, the tubing system is disconnected from the sample and connected to a vessel containing wash buffer which is then pumped over the capture phase to wash away any food particles or unbound microorganisms. Once washing is complete the beads are recovered in buffer and the captured microorganisms may be detected using selective plating, colorimetric assays or molecular assays.

Arthur *et al.* (2005) compared the Pathatrix method in combination with selective plating and PCR using the Lightcycler (Roche Applied Science, Indianapolis, IN) with the BAX system (DuPont Qualicon, Wilmington, DE) and the Assurance GDS (BioControl Systems, Inc., Seattle, WA) for detection of *E. coli* O157:H7 in ground beef. When samples were inoculated at 17 CFU/65 g ground beef, all of the investigated methods detected *E. coli* O157:H7 in 57 of 57 samples. However, when samples were
inoculated at 1.7 CFU/65 g ground beef, the Pathatrix in combination with selective
plating or PCR (4 hr pre-enrichment) detected *E. coli* O157:H7 in 98% of samples
whereas the BAX system (8 hr pre-enrichment) and the Assurance GDS system (8 hr pre-
enrichment) detected *E. coli* O157:H7 in 66% and 73% of samples, respectively (Arthur
*et al.*, 2005).

**Additional technologies for immunological detection of *Shigella***

In a recent report, Sapsford *et al.* (2004) describe detection of *S. dysenteriae* in
buffer and on chicken carcasses using an array biosensor developed at the Naval
Research Laboratory. The array biosensor measures total internal reflection fluorescence
using a 25-min sandwich immunoassay for antigen detection. An advantage of the array
biosensor is that little or no sample preparation is required prior to analysis. The detection
limit of the array biosensor for *S. dysenteriae* in ground turkey, chicken carcass wash,
buffered milk, and a lettuce leaf wash was observed at $7.8 \times 10^5$ CFU/g, $4.9 \times 10^4$
CFU/ml, $7.8 \times 10^5$ CFU/ml, and $2.0 \times 10^5$ CFU/ml, respectively. When tested in buffer,
the array biosensor did not respond as efficiently to the other serogroups of *Shigella* and
cross-reacted with *E. coli*, suggesting the specificity of the polyclonal antibodies was a limiting factor. The use of a monoclonal antibody with higher specificity may improve the diagnostic ability of this testing format.

**Molecular Microbiological Methods for *Shigella* Detection in Foods**

**Polymerase chain reaction detection of *Shigella* in foods**

Polymerase chain reaction (PCR) methods for detection of *Shigella* in food have previously demonstrated higher sensitivity than comparable culture methods (Warren, 2003). PCR assays for *Shigella* spp. have targeted the invasion associated locus (*ial*) (Islam and Lindberg, 1992; Lindqvist, 1999), the *virA* gene (Villalobo and Torres, 1998; Vantarakis et al., 2000), or the *ipaH* gene (Sethabutr et al., 1993; Sethabutr et al., 2000; Theron et al., 2001; Lampel and Orlandi, 2002; Warren, 2003) for amplification. The same PCR primers are used to detect each of the serogroups of *Shigella* and enteroinvasive *E. coli*. The *ial* and *virA* genes are located on the large virulence plasmid (sometimes referred to as the invasion plasmid), however sequencing of the *S. flexneri* genome (Jin et al., 2002) revealed the *ipaH* gene to be encoded multiple times on both the chromosome and the large virulence plasmid. Thus, detection of the *ipaH* gene is possible in the event the large virulence plasmid has been lost, which has been shown to occur when food samples are stored for a prolonged periods of time prior to analysis (Lampel and Orlandi, 2002). The discussion below will be limited to PCR detection of *Shigella* in food samples, however several reports of detection in fecal samples appear in the literature. These methods are summarized along with other rapid methods in Table 2-3.

Vantarakis *et al.* (2000) developed a multiplex PCR method to detect both *Shigella* spp. and *Salmonella* spp. in mussels. Multiplex PCRs involve the use of two or more sets
of primers in the same PCR such that multiple targets can be amplified in the same reaction. Artificially inoculated *S. dysenteriae* and *S. Typhimurium* 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. The *virA* gene of *Shigella* spp. and the *invA* genes of *Salmonella* spp. were amplified. Sample homogenates were inoculated at various concentrations to establish the lowest detection limit for the method. When samples were not pre-enriched prior to analysis, the multiplex PCR method was able to detect *S. dysenteriae* at 1.0 x 10^3 CFU/ml in the homogenate. Following 22 hour incubation in buffered peptone water the multiplex PCR methods was able to *S. dysenteriae* detect levels as low as 1.0 x 10^1 to 1.0 x 10^2 CFU/ml in the homogenate. Similar results were observed for *S. Typhimurium*.

Villalobo and Torres (1998) investigated PCR for the detection of *S. dysenteriae* serotype 1 in mayonnaise. Samples were homogenized in buffered peptone water and artificially contaminated with various concentrations of *S. dysenteriae* serotype 1. Bacterial cells were lysed with detergent, the DNA extracted with phenol-chloroform and precipitated with ethanol. A multiplex PCR was then used to amplify regions from the *virA* gene and the 16S rRNA gene. The lowest level of inoculation detected by this method was 1.0 x 10^2 to 1.0 x 10^3 CFU/ml in the homogenate.

In a study by Lindqvist (1999), nested PCR was investigated for the detection of the *ial* locus of *Shigella* spp. from spiked lettuce, shrimp, milk, and blue cheese samples. Nested PCR involves the use of two sequential PCRs in which the target sequence in the second PCR lies within the amplified sequence in the first PCR. Nested PCRs can be used to achieve extreme low sensitivities. Food samples inoculated were homogenized in
physiological saline and bacteria were isolated by buoyant density centrifugation
(separation of components based on density). Single PCR, using only the external primer
sets, was able to detect *S. flexneri* in aqueous solution at 0.5-1.0 x 10^5 CFU/ml. Nested
PCR was more sensitive and 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 for the detection of the *ipaH*
gene of *S. flexneri* in spiked environmental water samples. The detection limits in the
various environmental water samples were 2.0 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 that inhibited PCR. Humic
substances are transformed products from soil organic matter that do not belong to the
known classes of biochemistry. These include humic acids, fulvic acids, and humins. Pre-
enrichment in GN broth served to dilute humic substances while allowing the *S. flexneri*
to multiply, thereby increasing the concentration of target DNA.

**Improved PCR detection of Shigella by FTA filtration**

FTA filters (Whatman, Clifton, NJ) have been used in template preparation for
PCR detection of pathogenic microorganisms (Lampel *et al.*, 2000; Orlandi and Lampel,
2000; Lampel and Orlandi, 2002; Warren, 2003; Warren *et al.*, 2005b). Moisture in the
sample activates chemicals in the FTA filter that lyse cells, denature enzymes, inactivate
pathogens, and immobilize genomic DNA (Whatman website, 2004). Chemicals in the
FTA filters also protect DNA and RNA from light, free radicals, enzymes, and pathogens
during dry, room-temperature storage. FTA filtration as sample preparation for PCR has
been developed for detection of *Cyclospora* and *Cryptosporidium* in water (FDA, 1998). Recently, this sample preparation technique has been investigated for detection of *S. boydii* and *S. sonnei* from tomato rinses by nested PCR (Warren, 2003; Warren *et al.*, 2005b). Briefly, tomato rinses were passed through a two-stage filter where the first stage contained filter paper for size exclusion and the second stage contained FTA filter paper. After purification and washing, 6-mm punches are taken from the FTA filters and used directly as template in the first step of the nested PCR. The FTA filtration/nested PCR (FTA-PCR) assay detected *S. boydii* and *S. sonnei* at $6.2 \times 10^0$ CFU/tomato and $7.4 \times 10^0$ CFU/tomato, respectively (Warren, 2003; Warren *et al.*, 2005b).

Although FTA-PCR had excellent sensitivity when used to analyze tomato rinses, which are relatively clean, similar results were not observed when recovery of inoculated *S. boydii* and *S. sonnei* was attempted from cantaloupes, strawberries, or retail Valencia oranges (Schneider and Warren, unpublished data). FTA-PCR was unable to detect inoculated *Shigella* from strawberries, suggesting that the purification and washing protocol used on the FTA filters may not be adequate to remove PCR inhibitors (Warren, 2003). Despite several size exclusion filtering techniques applied prior to FTA filtration, the FTA filters were routinely clogged by cantaloupe fibers or the wax coating from retail oranges, which entered the buffer rinse during recovery procedures (Warren, 2003). The advantage of the FTA-PCR technique is that no enrichment was necessary to obtain low detection limits due to the large volume of sample analyzed by the filters. The technique warrants further investigation for the analysis of foods for *Shigella* contamination as improvements in pre-filtration techniques could improve the sensitivity.
Additional molecular microbiological techniques for detection of *Shigella*

An interesting new method, immunocapture universal primer PCR (iUPPCR) has been recently reported (Peng *et al*., 2002). Universal primer PCR employs primers designed against highly conserved regions, such as 16S rRNA genes, thus the resulting PCR can be used for amplification of almost any bacteria. Typically, the sequence of the resulting amplicon would be further analyzed for identification of the bacteria. In iUPPCR however, immunocapture is employed for specificity and universal primer PCR is used for detection of captured bacteria. Monoclonal antibodies specific for individual serotypes of *S. dysenteriae*, *S. flexneri*, *S. boydii*, or *S. sonnei* were coated into the wells of a 96-well polystyrene plate. Cross-reactivity tests demonstrated the specificity of the monoclonal antibodies to the strain level. Wells were challenged with bacteria and captured bacteria were detected using universal primer PCR, i.e. PCR with primers specific for 16S rRNA sequences conserved among closely related bacteria. The detection limit for shigellae in broth was $5.0 \times 10^2$ CFU/ml (Peng *et al*., 2002). Presumably, detection of up to 96 pathogens sharing the conserved 16S rRNA sequence could be accomplished in the same plate using specific monoclonal antibodies for bacterial capture (differentiation of cell types) and the universal primer PCR for detection. Further investigation of this method with respect to food samples as opposed to bacteria in broth is required.

More recently, Ji *et al.* (2006) reported iUPPCR in combination with denaturing gradient gel electrophoresis (DGGE) for the detection and identification of *Shigella* spp. In contrast to the iUPPCR method described above, genus-specific polyvalent monoclonal antibodies were used to coat the wells of a 96-well plate, such that all *Shigella* spp. may be captured in each well. Following iUPPCR to amplify 16S rRNA
gene fragments, DGGE was used to identify the specific serotype. Unfortunately, the iUPPCR-DGGE method was only tested against pure cultures in laboratory conditions. Although the authors speculate that it would be useful for the detection and identification of Shigella in food and environmental samples, further testing in the presence of food/environmental matrices and indigenous microflora are required.

Advances in oligonucleotide microarrays have further enabled the simultaneous detection and identification of bacterial foodborne pathogens. Oligonucleotide microarrays involve the ordered immobilization of specific probes to a solid surface followed by hybridization with labeled sample DNA. After hybridization, development of the label can allow identification/enumeration of complementary sample DNA sequences. In a recent report, Kakinuma et al. (2003) describes an oligonucleotide microarray using probes specific for the gyrB gene to detect and differentiate E. coli, Shigella, and Salmonella. PCR amplified regions of the gyrB gene were fluorescently labeled and hybridized to detection probes immobilized on glass slides. Based on reaction patterns, three species of Shigella, seven serovars of Salmonella, and one strain of E. coli were correctly identified at the species level. Identification at the subspecies level of Salmonella was problematic when multiple serovars were present in the same sample due to the overlap of microarray patterns. Assay formats such as this could be expanded to potentially detect and identify a wide range of enteric pathogens.

Song et al. (2005) reported an alternative DNA amplification method, loop-mediated isothermal amplification (LAMP), for the detection of Shigella spp. in clinical samples. In the LAMP assay, four specialized primers designed to specifically recognize six distinct regions on the target gene are used in combination with a DNA polymerase
with strand displacement activity. The amplification reaction occurs at 65°C, therefore no
thermocycler is necessary as with PCR. For a more complete description of the LAMP
assay, the reader is directed to the Eiken Chemical Co., Ltd. (Tokyo, Japan) website:
homogenized in sterile water were inoculated with various titers of *S. flexneri* YSH6000
and the DNA was extracted using a boiling method. The sensitivity of the LAMP assay
for *S. flexneri* in the inoculated stool samples was eight CFU per reaction whereas the
sensitivity of a PCR reaction was determined to be 800 CFU per reaction. A distinct
advantage of the LAMP assay is that amplified DNA may be visualized by the naked eye
as turbidity in the sample, eliminating the need for post-amplification processes, such as
gel electrophoresis.

Finally, repetitive sequence-based PCR (rep-PCR) has been demonstrated for the
identification and molecular typing of members of the family *Enterobacteriaceae*,
including *Shigella* (Raza *et al*., 2003; Lising *et al*., 2004). In rep-PCR, primers are
designed complementary to bacterial interspersed repetitive sequences and the regions
lying outward of the primers are amplified resulting in DNA fragments of varying
lengths. These fragments can then be separated by electrophoresis to form a bacterial
fingerprint unique to individual bacterial strains. The DiversiLab System (Spectral
Genomics, Inc., Houston, TX) generates such fragments by rep-PCR that are then
analyzed using microfluidics lab-on-a-chip and the Agilent 2100 Bioanalyzer (Agilent
Technologies, Inc. Palo Alto, CA). Results can be visualized in several formats including
dendograms, electropherograms, gel-like image, or scatter plots (Lising *et al*., 2004).
Rep-PCR technology has been shown to be reproducible and can allow the differentiation
of species, subspecies and strains in as little as 4 hours. Some *Shigella* serogroups show >95% similarity with the DiversiLab Enteric Kit Beta version, however the DiversiLab *Shigella* kit offers greater differentiation among serogroups and strains (Lising *et al*., 2004). Strains of *E. coli* (non-pathogenic, enterohemorragic, and enterotoxigenic) were effectively differentiated from *Shigella* spp. using rep-PCR (Raza *et al*., 2003; Lising *et al*., 2004), however no enteroinvasive *E. coli* strains were included in the studies.

**Detection of Pathogens by Sequence Capture**

Sequence capture methods have been investigated for the detection of a wide variety of bacterial, viral, and fungal pathogens. Typically, a specific oligonucleotide probe is immobilized on a solid surface and DNA/RNA prepared from the target microorganism is hybridized to the probe. Zammatteo *et al.* (1997) compared the sequence capture of human cytomegalovirus using DNA probes immobilized on 96-well microtiter plates and paramagnetic beads and found that faster hybridization kinetics were obtained with the use of beads as the solid support. Although Zammatteo *et al.* (1997) used DNA probes covalently bound to the solid supports, it is more common to use oligonucleotide probes labeled with biotin on their 5′ end with paramagnetic beads or microtiter plates with wells pre-coated with streptavidin. As with more traditional nucleic acid hybridization, variables such as salt concentration, temperature, contact time and GC content of the probe sequence all have influence on hybridization efficiency. Del Gallo *et al.* (2005) investigated steric factors affecting the hybridization of PCR amplified sequences to DNA probes immobilized on the screen-printed gold surface of disposable electrodes. The amount of probe coverage on the surface as well as the relative position of the probe on the target sequence was found to partially control hybridization efficiency. When the probe coverage was approx. $2.9 \times 10^{12}$ molecules/cm$^2$ and when the
probe sequence was located at one of the termini of the target sequence the efficiency of hybridization was increased (Del Gallo et al., 2005). In addition, the use of spacer molecules between the solid support and the probe sequence has been shown to drastically improve hybridization efficiency (Shchepinov et al., 1997; Amagliani et al., 2006). The method used to purify the probe during synthesis may also affect hybridization efficiency, with HPLC purification preferred over desalting (Amagliani et al., 2006). Finally, sequence capture methods may be used to prepare DNA/RNA from samples that contain PCR inhibitors, since these inhibitors are removed during post-hybridization washing (Maher et al., 2001; Tsai et al., 2003).

While there are numerous reports of sequence capture for the analysis of clinical samples for bacterial and viral pathogens, this discussion is limited to the few reports of sequence capture used for the analysis of foods or environmental samples. In a study by Chen et al. (1998), sequence capture in combination with PCR was investigated for the detection of verotoxigenic E. coli (VTEC) in brain heart infusion (BHI) broth cultures and in artificially contaminated ground beef. Biotin-labeled probes were used to form hybrids with specific DNA segments and then the hybrids were bound using streptavidin-coated paramagnetic beads. In BHI broth, detection of initial VTEC concentrations of $10^3$, $10^2$ and $10^0$ CFU/ml was achieved after 5, 7 and 10 hr enrichment at 37°C, respectively. In ground beef samples, the sequence capture method was able to detect VTEC at levels of $10^0$ CFU/g after 15 hr incubation in BHI broth. For both BHI broth and ground beef samples, DNA was extracted from one ml aliquots of the enrichments by the boiling method and the prepared DNA was used as template in the sequence capture method. In a subsequent study, Chen and Griffiths (2001) modified this sequence capture
method for the simultaneous detection of *Salmonella* and Shiga-like toxin-producing *E. coli*. Again using cultures in BHI broth, the detection limit was determined to be $10^0$ CFU/ml after 10 hr incubation at 37°C (Chen and Griffiths, 2001). This study demonstrates that multiple probes, each specific for a different pathogen, may be used in combination on solid supports in order to test for different pathogens at the same time.

Tsai *et al.* (2003) investigated sequence capture in combination with PCR for the detection of enterotoxigenic *E. coli* (ETEC) associated with cattle in environmental water samples. Biotin-labeled probes with specificity for the enterotoxin gene LTIIa were attached to streptavidin-coated paramagnetic beads and used to form hybrids with prepared DNA samples. The detection limit of the assay was determined to be 2.5 attogram/µl DNA. In addition, some of the extracted DNA samples were spiked with humic acids, known inhibitors of PCR, to determine if the sequence capture method was effective at removing these inhibitors prior to PCR. In the presence of humic acids, the sequence capture method increased sensitivity 10,000-fold over conventional PCR (Tsai *et al.*, 2003).

Amagliani *et al.* (2006) developed sequence capture in combination with PCR for the detection of *Listeria monocytogenes* in milk. Using NH$_2$-labeled DNA probes specific for the *hlyA* gene coupled to paramagnetic beads, the detection limit of the assay was $10^1$ CFU/ml. The sensitivity was achieved using (CH$_2$)$_{12}$ spacers between the NH$_2$-label and the DNA probe sequence. In contrast to many previously reported sequence capture methods, the method of Amagliani *et al.* (2006) required no DNA extraction/purification as capture was performed directly in the milk sample. For this reason a two-step sequence capture in which biotin-labeled probes formed hybrids with target sequences followed by
isolation of the hybrids using streptavidin-coated paramagnetic beads was not successful. It was hypothesized that indigenous biotin in the milk samples interfered with the biotin-labeled hybrids binding to the streptavidin-coated beads (Amagliani et al., 2006).
CHAPTER 3
MATERIALS AND METHODS

Preliminary Studies

Acquisition and Maintenance of \textit{Shigella sonnei} Cultures

The following cultures were purchased from the American Type Culture Collection (ATCC; Manassas, VA): \textit{S. sonnei} ATCC 9290, \textit{S. sonnei} ATCC 29031, \textit{S. sonnei} ATCC 29030, \textit{S. sonnei} ATCC 25931 and \textit{S. sonnei} ATCC 29930. Each strain was resuscitated as instructed in the package inserts and the resulting growth was streaked for isolation on MAC plates. One typical \textit{S. sonnei} colony from each plate was transferred to a TSA slant and stored at 4°C. A second typical \textit{S. sonnei} colony was transferred per product instructions to Protect™ Bacterial Preservers (Scientific Device Laboratory, Inc., Des Plaines, IL) and stored at -70°C.

Adaptation of Cultures to Rifampicin

Subcultures of each of the five \textit{S. sonnei} strains were adapted to the bactericidal agent rifampicin by spontaneous mutation. A 10,000 µg/ml (1%) stock solution of rifampicin was prepared by dissolving 2.0 g rifampicin (Fisherbrand, Fisher Scientific, Pittsburg, PA) in 200 ml methanol. The stock solution was then filter sterilized and stored in the dark at 4°C.

Stock cultures were grown overnight in 10 ml TSB (37°C, 30 rpm). Overnight cultures were transferred to 10 ml TSB supplemented with 2.5 µg/ml rifampicin and grown overnight (37°C, 30 rpm). With each transfer, the concentration of rifampicin increased until the final concentration was 200 µg/ml rifampicin. Once the cultures were
adapted to 200 µg/ml rifampicin, cultures were grown overnight (37°C, 30 rpm) three consecutive times in TSB supplemented with 200 µg/ml rifampicin to ensure well adapted populations.

Once adaptation was complete, the final overnight culture was streaked for isolation on MAC and incubated overnight at 37°C. One typical colony from the overnight MAC plate was transferred to a TSA slant supplemented with 200 µg/ml rifampicin and stored at 4°C. Another typical colony was transferred per manufacturer’s instructions to a Protect™ Bacterial Preserver and stored at -76°C.

Preparation of Microbiological Media

Wild-type cultures were grown in Tryptic Soy Broth (TSB; BD Diagnostics, Franklin Lakes, NJ) containing 10 µM Congo red (TSCR) and maintained on Tryptic Soy Agar (TSA; BD Diagnostics) slants containing 10 µM Congo red. For survival studies only, bacterial strains were adapted to 200 µg/ml of the bactericidal agent rifampicin and experiments were conducted in TSB supplemented with 100 µg/ml rifampicin (TSB rif+). All dilutions, unless otherwise specified, were performed using 0.1% Peptone (BD Diagnostics) water. Phosphate Buffered Saline (PBS; pH 7.4) was prepared using the following formula per liter: 1.2 g NaHPO4, 8.2 g Na2PO4 and 5.0 g NaCl.

*Shigella* Broth (SB) was prepared according to the U.S. Food and Drug Administration’s (1998) *Bacteriological Analytical Manual* (BAM) and supplemented with novobiocin (ICN Biomedicals Inc., Aurora, OH) at 3.0 µg/ml. MacConkey Agar (MAC; BD Diagnostics), Triple Sugar Iron Agar (TSI; BD Diagnostics), Lysine Iron Agar (LIA; BD Diagnostics) and Motility Medium (MM; BD Diagnostics) were all prepared according to manufacturers instructions. TSI and LIA were prepared as slants in
screw cap tubes. MM was supplemented with 0.005% triphenyltetrazolium chloride (TTC; BD Diagnostics). When necessary, media pH was adjusted using filter sterilized 1N NaOH.

**Acquisition/Preparation of Food Matrices**

Mature green, unwashed, unwaxed tomatoes (Florida 47 cultivar) were obtained from a nearby packinghouse. Tomatoes were held at 4°C prior to use. ‘Picnic Potato and Egg Salad’ (hereafter referred to as potato salad) was prepared the night before each experiment using a publicly available recipe (http://southernfood.about.com/od/potatosalads/r/bl00624c.htm?terms=picnic+potato+and+egg+salad ). Briefly, 6.0 lbs potatoes were skinned, cubed, boiled for 15 min, and then cooled. Eight large eggs were hard boiled for 15 min, cooled, and chopped. After straining the water from the cooked potatoes, the chopped eggs, ½ cup chopped red onion, 1 cup chopped fresh celery, 1 and ½ cup mayonnaise (Hellman’s Lite, Unilever, Englewood Cliffs, NJ), 3 tbs yellow mustard (Publix Supermarket brand, Lakeland, FL), 1 tsp salt, and 1 tsp black pepper were added and mixed well with a large serving spoon. The potato salad was stored at 4°C until use. Ground sirloin (90% lean/10% fat; hereafter referred to as ground beef) was purchased at a local grocer the morning of each experiment.

**Acquisition and Maintenance of Anti-Shigella Antibodies**

All polyclonal and monoclonal antibodies used in the following experiments are commercially available and are listed in Table 3-1. Anti-*Shigella* polyclonal antibodies AB01 and AB04 were purchased from Virostat, Inc. (catalog number 0901; Portland, ME) and AbCam, Inc. (catalog number Ab19988; Cambridge, MA), respectively. Goat anti-rabbit IgG (H+L chain specific) antibodies (AB03) were purchased from Southern Biotech Associates, Inc. (catalog number 4050-01; Birmingham, AL). Anti-*S. sonnei*
monoclonal antibodies (AB02) were purchased from Novus Biologicals (catalog number BM1316; Littleton, CO). Goat anti-mouse IgG (H+L chain specific) antibodies (AB05) were purchased from Southern Biotech Associates, Inc. (catalog number 1031-01). Upon receipt, all antibodies were stored at 4.0°C if they were to be used within two months otherwise aliquots of 100 µl were stored frozen at -20°C.

**Binding of Antibodies to Paramagnetic Beads**

The binding of antibodies to MagaCell beads was performed according to Cortex Biochem, Protocol 503 (available at [http://www.cortex-biochem.com/commerce/ccc1010-protocols.htm](http://www.cortex-biochem.com/commerce/ccc1010-protocols.htm)) except that all volumes were reduced proportionately for a starting volume of 500 µl beads. The MagaCell beads were mixed by inverting the bottle by hand until no beads were visible as a pellet on the bottom of the bottle. A 500-µl aliquot of the beads were transferred to a clean, sterile 1.5 ml microcentrifuge tube. All washing steps were performed using a magnetic particle concentrator (MPC-S; Dynal Biotech) or a magnetic separator (CD3002; Cortex BioChem) to draw the beads to the side of the tube allowing the supernatant to be removed using a pipette. The beads were washed two times in 1000 µl de-ionized water, followed by four times in 1000 µl acetone. The beads were then resuspended in 250 µl acetone containing 0.12 g 1,1 carbonyldiimidazole per 10 ml. The microcentrifuge tubes were placed on a rugged rotator and mixed by gentle end-over-end rotation for 1 hr. The beads were then washed four times in 1000 µl acetone, four times in 1000 µl de-ionized water and four times in 1000 µl 0.1M sodium bicarbonate buffer, pH 8.6. The beads were then resuspended in 400 µl 0.1M sodium bicarbonate buffer, pH 8.6, an appropriate amount of antibody solution (Table 3-1) was added and the volume was adjusted to 500 µl using 0.1M sodium bicarbonate buffer, pH
Table 3-1. Antibodies investigated for immunocapture of *S. sonnei*.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Clonality</th>
<th>Immunogen</th>
<th>Volume added per 500 µl MagaCell beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB01</td>
<td>Polyclonal</td>
<td>Mixture of <em>S. boydii</em>, <em>S. flexneri</em>, <em>S. dysenteriae</em></td>
<td>50</td>
</tr>
<tr>
<td>AB02</td>
<td>Monoclonal</td>
<td><em>S. sonnei</em>, NCTC 9774 (Wheeler phase I)</td>
<td>100</td>
</tr>
<tr>
<td>AB03</td>
<td>Polyclonal</td>
<td>Pooled antisera from goats hyperimmunized with normal rabbit IgG</td>
<td>100</td>
</tr>
<tr>
<td>AB04</td>
<td>Polyclonal</td>
<td>Membrane extract of <em>S. sonnei</em> and <em>S. flexneri</em></td>
<td>50</td>
</tr>
<tr>
<td>AB05</td>
<td>Polyclonal</td>
<td>Pooled antisera from goats hyperimmunized with mouse IgG paraproteins</td>
<td>100</td>
</tr>
</tbody>
</table>

8.6, when necessary. The beads were again placed on a rugged rotator and mixed with gentle end-over-end rotation for 18-24 hr at room temperature. The next day, the beads were washed two times in 500 µl 0.1M sodium bicarbonate buffer, pH 8.6. The beads were then resuspended in 500 µl 0.1M sodium bicarbonate buffer, pH 8.6, containing ethanolamine (3 ml/liter) and mixed by gentle end-over-end rotation for 1 hr. The beads were then washed once in 500 µl 0.1M sodium acetate buffer, pH 4.0, resuspended in 500 µl 0.1M sodium acetate buffer, pH 4.0, and mixed by gentle end-over-end rotation for 1 hr. Finally the beads were washed three times in 500 µl PBS containing 0.1% sodium azide and stored at 4°C until used.

**Evaluation and Optimization of Immunocapture Using Anti-Shigella Beads**

Preliminary challenges of anti-*Shigella* beads for immunocapture of *S. sonnei* strains were performed in PBS. A 100-µl aliquot of an overnight *S. sonnei* culture in TSCR was used to inoculate sterile stomacher bags containing 250 ml PBS such that the final cell titer was approximately $2.0 \times 10^5$ CFU/ml. The stomacher bags were then placed into Pathatrix incubation pots and the samples were circulated for 30 min at 37°C.
using 25 µl aliquots of anti-\textit{Shigella} beads for immunocapture. Anti-\textit{Shigella} beads prepared using various anti-\textit{Shigella} antibodies and various concentrations of anti-\textit{Shigella} beads were investigated for FTI. After circulation, each sample was washed in PBS and the beads were recovered in approximately 250 µl PBS. A 100-µl aliquot of the recovered beads was analyzed by spread plate using MAC. MAC plates were incubated at 37°C for 24 hr and the resulting colonies were counted. Antibodies AB01, AB02 and AB04 were bound directly to MagaCell beads and tested for primary capture in FTI. In addition, AB01 and AB02 were tested for immunocapture of \textit{S. sonnei} in broth culture followed by secondary capture using MagaCell beads coated with antibodies AB03 and AB05, respectively.

\textbf{Crude DNA Extraction from Bacteria by Boiling}

Stock cultures frozen on Protect™ Bacterial Preservers were retrieved from frozen (-70°C) storage and allowed to thaw. One bead was aseptically transferred from the Protect™ Bacterial Preserver into 10 ml TSB and grown overnight (37°C). 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).

A 1.0-ml aliquot of the overnight culture was transferred to a clean, sterile 1.5 ml microcentrifuge tube and the bacterial cells were harvested by centrifugation (3,220 x g for 10 min). The resulting supernatant was discarded and the pellet was re-suspended in 200 µl de-ionized, sterilized water. Samples were then boiled for 10 min in a dry bath incubator (Fisher Scientific, IsoTemp 125D). The supernatant (DNA template) was
aseptically transferred to a clean, sterile 1.5 ml microcentrifuge tube and stored at -20°C.
The pellet was discarded.

**DNA Extraction from Anti-Shigella Beads using the DNeasy Kit**

Extraction of DNA from bacterial cells captured on anti-Shigella immunomagnetic beads was performed using a modified protocol with DNeasy spin columns (Qiagen, Valencia, CA). A 100-µl aliquot of concentrated anti-Shigella beads was transferred to a clean, sterile 1.5 ml microcentrifuge tube and heated on a dry bath incubator at 95°C for 10 min to lyse bacterial cells. After incubation, 100 µl sterile water, 200 µl buffer AL (Qiagen) and 200 µl absolute ethanol was added to the sample. The resulting mixture was vortexed for 5 sec and placed in a MPC-S for at least 1 min to draw the magnetic particles to the side of the tube. Without disturbing the magnetic particles, the mixture was transferred to a DNeasy spin column and centrifuged at 6,000 x g for 1 min. The DNeasy spin column was transferred to a clean collection tube and 500 µl buffer AW1 (Qiagen) was passed through the column by centrifugation at 6,000 x g for 1 min. The DNeasy spin column was transferred to a clean collection tube and 500 µl buffer AW2 (Qiagen) was passed through the column by centrifugation at 16,000 x g for 3 min. The DNeasy spin column was transferred to a clean, sterile 1.5 ml microcentrifuge tube and 100 µl buffer AE (Qiagen) was added to elute bacterial DNA by centrifugation at 6,000 x g for 1 min. The DNeasy spin column was discarded and the eluted DNA was placed on ice or stored at -20°C prior to analysis by real-time PCR.

**Preparation of HeLa Cell Extracts**

Fresh HeLa cells were obtained from the laboratory of Dr. F. Southwich, University of Florida, resuspended in 1.0% Triton X-100 supplemented with Complete Mini Protease Inhibitor cocktail tablets (Roche Applied Sciences, Indianapolis, IN).
HeLa cells were lysed using three cycles of freeze-thaw. One hundred µl aliquots of the lysed cells were transferred to clean, sterile 1.5 ml microcentrifuge tubes and proteins were extracted using a modified method of Wessel and Flügge (1984). Briefly, the samples were vortexed and centrifuged at 5,000 × g for 2 min and the sample supernatant was transferred to a clean, sterile 1.5 ml centrifuge tube and saved as ‘HeLa cell extract 1’. The remaining pellet was resuspended in 400 µl methanol (Fisherbrand), vortexed and centrifuged at 9,000 × g for 10 sec. To the solution, 200 µl chloroform (Fisherbrand) was added and the resulting solution was vortexed and centrifuged at 9,000 × g for 10 sec. To the solution, 300 µl water was added and the resulting solution was vortexed vigorously and centrifuged at 9,000 × g for 1 min. The upper phase in the resulting supernatant was transferred to a clean, sterile 1.5 ml centrifuge tube and saved as ‘HeLa cell extract 2’. To the lower phase, 300 µl methanol was added and the solution was vortexed and centrifuged at 9,000 × g for 2 min. The resulting supernatant was transferred to a clean, sterile 1.5 ml centrifuge tube and saved as ‘HeLa cell extract 3’. The pellet was dried using forced air (generated using a transfer pipette) and saved as ‘HeLa cell extract 4’.

RNA Extraction Using the RNeasy Kit

Extraction of RNA from bacterial cells was performed using RNeasy spin columns (Qiagen). A 250-µl aliquot of bacterial cells were transferred to clean, sterile 1.5 ml microcentrifuge tubes containing 500 µl RNAbacterial Protect Reagent (Qiagen), incubated at room temperature for 5 min and centrifuged at 5,000 × g for 10 min. The resulting supernatant was discarded and pellet was resuspended in 100 µl TE buffer, pH 8.0, containing 1 mg/ml lysozyme (Sigma, St. Louis, MO) and incubated at room temperature for 5 min. To the mixtures, 350 µl buffer RLP (Qiagen), containing 10 µl/ml 2-mercaptoethanol (Sigma), were added and the solutions was vortexed vigorously for 5
sec. To the mixture, 200 µl absolute ethanol was added and the solution was mixed by
gentle action of the pipette. The resulting mixture (approx. 700 µl) was transferred to
clean, sterile RNeasy spin columns and centrifuged at 8,000 x g for 15 sec. In a fume
hood, the flow-through was discarded, 700 µl buffer RW1 (Qiagen) was added to the
RNeasy spin column and the sample was centrifuged at 8,000 x g for 15 sec. In the fume
hood, the collection vessel and flow-through were discarded and the RNeasy spin column
was transferred to a clean, sterile collection vessel. A 500 µl aliquot of buffer RPE
(Qiagen) was added to the RNeasy spin column, the sample was centrifuged at 8,000 x g
for 15 sec and the flow-through was discarded. A second 500 µl aliquot of buffer RPE
(Qiagen) was added to the RNeasy spin column, the sample was centrifuged at 8,000 x g
for 2 min and the collection vessel and flow-through were discarded. The RNeasy spin
column was transferred to a clean, sterile 1.5 ml microcentrifuge tube and 50 µl water
was added directly onto the RNeasy silica-gel membrane to elute RNA. The RNeasy spin
column inside of the 1.5 ml microcentrifuge tube was centrifuged at 8,000 x g for 2 min
and the RNeasy spin column was discarded. The collected RNA sample was placed on
ice or stored at -70°C prior to further analysis.

**DNase Treatment of RNA Extracts Prior to RT-PCR**

DNase treatment of collected RNA samples was performed using the DNase I
Amplification Grade Kit (Invitrogen, Carlsbad, CA). To a clean, sterile 0.5 ml
microcentrifuge tube, 1 µl 10X DNase I Reaction Buffer (Invitrogen), 2 µl DNase I Amp
Grade (1 U/µl; Invitrogen) and 2 µl DEPC-treated water (ICN Biomedicals Inc.) was
added. A 5 µl aliquot of RNA was added and the solution was mixed using the action of
the pipette and incubated for 15 min at room temperature. After incubation the reaction
was stopped by addition of 1 µl 25 mM EDTA Solution (Invitrogen) and the mixture was
heated at 65°C for 10 min. DNase I-treated RNA samples were placed on ice prior to further analysis by RT-PCR.

**Induction and Expression of ipaH RNA in S. sonnei**

A five-strain *S. sonnei* cocktail was prepared as described below in the section ‘Inoculum Preparation’. The following test solutions were prepared in clean, sterile 1.5 ml microcentrifuge tubes: 900 µl SB + 100 µl HeLa cell extract 1, 900 µl SB + 100 µl HeLa cell extract 2, 900 µl SB + 100 µl HeLa cell extract 3, 900 µl SB + 100 µl HeLa cell extract 4, 900 µl SB + 100 µl Congo red solution (see Appendix A) and 1000 µl SB (control). A 10-µl aliquot of the *S. sonnei* cocktail was added to each of the test solutions and the solutions were vortexed. Total RNA was extracted from a 250-µl aliquot of each test solution and analyzed by RT-PCR using *ipaH* gene-specific primers. The remaining portions of the test solutions were incubated at 37°C for 4 hr, after which the total RNA was extracted from a second 250-µl aliquot of each test solution and analyzed by RT-PCR using *ipaH* gene-specific primers.

**Identification of Shigella-Specific Genetic Loci**

Homologous gene cluster tables were created using the Microbial Genome Database for Comparative Analysis (MBGD; National Institute for Basic Biology, National Institutes of Natural Sciences, Japan; available at: http://mbgd.genome.ad.jp/) using the available genomes (as of December 2005) for *Escherichia coli* and *Shigella* spp. (*E. coli* O157:H7, *E. coli* CFT073, *E. coli* K-12 W3110, *E. coli* K-12 MG1655, *E. coli* EDL933, *S. flexneri* 2a 2457T, *S. flexneri* 2a 301 and *S. sonnei* Ss046). Homologous genes were analyzed using ClustalW (protein-protein alignment function included in the MBGD) to identify potential genes for the detection of *Shigella* spp. or *S. sonnei*. Specifically, those genes that contained sequences conserved among *Shigella* spp. or
sequences unique to \textit{S. sonnei} were identified as potential genetic targets. The nucleotide sequences were obtained for the identified potential genetic targets and analyzed using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn; available at the National Center for Biotechnology Information website: \url{http://www.ncbi.nlm.nih.gov/}) to further identify sequences specific to \textit{Shigella} spp. or \textit{S. sonnei}. The identified sequences were then used for primer/probe development as described below.

**Development of Primers/Probes for the Detection of \textit{Shigella}**

The primers/probes investigated in this study are listed in Table 3-2. All primers/probes were developed using the Beacon Designer 5 software (PREMIER Biosoft International, Palo Alto, CA). For design of the 01-023 primers and probe, the conserved sequence from the chromosomally-located \textit{ipaH} genes of \textit{S. sonnei} Ss046 was identified using ClustalW. Default primer settings were used with one exception; the 3' maximum Δ\textit{G} was adjusted from 10 –kcal/mol to 4.0 –kcal/mol. For primers designed for the detection of all \textit{Shigella} spp., regions with cross homology to the \textit{E. coli} K-12 genome were avoided. For primers designed for the specific detection of \textit{S. sonnei}, regions with cross homology to the \textit{S. flexneri} 2a 2457T genome were avoided. All hydrolysis probes were designed with the reporter dye FAM on the 5' end and the quenching dye TAMRA on the 3' end. All primers/probes were purchased from Sigma-Genosys (The Woodlands, TX).

**Evaluation of Primer/Probe Specificity**

All primers and probes were evaluated for specificity \textit{in silico} using BLASTn in addition to in-house testing against DNA from stock bacterial cultures (Table 3-3). Primer specificity was initially tested using real-time PCR followed by melt curve
Table 3-2. Primers designed for the detection of *Shigella*. All primers were designed using Beacon Designer 5 software with gene sequences obtained from the genome of *S. sonnei* Ss046 (accession number CP000038).

<table>
<thead>
<tr>
<th>Designation</th>
<th>Gene ID</th>
<th>Sequence (5' → 3')</th>
<th>Product length (bp)</th>
</tr>
</thead>
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<td>01-023F</td>
<td><em>ipaH</em></td>
<td>GTGAAGGAAATGCGTTTCTATG</td>
<td>106</td>
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<tr>
<td>01-023R</td>
<td></td>
<td>ACCAGTCCGTAATTCATTCTC</td>
<td></td>
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<td>01-023P</td>
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<td>AGTGACAGCAGATGACCTC</td>
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<td>01-024F</td>
<td>SSO_0670</td>
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<td>01-024R</td>
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<td>01-024P</td>
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<td>AGCTCAGCAGACCCAGCCG</td>
<td></td>
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<tr>
<td>01-025F</td>
<td>SSO_2067</td>
<td>GCCCCGCTACGCATGTC</td>
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<tr>
<td>01-025R</td>
<td></td>
<td>GTGATCTCCAGTTCCGAATTG</td>
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<td>01-026F</td>
<td>SSO_2071</td>
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<td>01-026R</td>
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<td></td>
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<td>01-027F</td>
<td>SSO_1019</td>
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<td>01-028F</td>
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<td>01-029F</td>
<td>SSO_2863</td>
<td>CGGCTGCTTGGCGCCGCTTAAG</td>
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<td></td>
<td>ATACTCTTTCTTGAGGATGTTG</td>
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Analysis in 20 µl reaction mixtures consisting of: 10 µl IQ™ Supermix with SYBR green (Bio-Rad, Hercules, CA), 200 nM (final concentration) each of forward and reverse primer, 2.0 µl DNA sample and purified water. The PCR cycling conditions were 95°C
Table 3-3. *Shigella* and non-*Shigella* strains tested for specificity.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Culture</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRS101</td>
<td><em>Shigella boydii</em> serotype 18</td>
<td>ATCC 35966</td>
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<tr>
<td>KRS102</td>
<td><em>Shigella boydii</em> serotype 18</td>
<td>Outbreak isolate</td>
</tr>
<tr>
<td>KRS103</td>
<td><em>Shigella sonnei</em></td>
<td>Patient isolate</td>
</tr>
<tr>
<td>KRS104</td>
<td><em>Shigella sonnei</em></td>
<td>Patient isolate</td>
</tr>
<tr>
<td>KRS105</td>
<td><em>Shigella sonnei</em></td>
<td>Outbreak isolate</td>
</tr>
<tr>
<td>KRS107</td>
<td><em>Shigella flexneri</em></td>
<td>Dr. K.A. Lampel, FDA</td>
</tr>
<tr>
<td>KRS108</td>
<td><em>Shigella dysenteriae</em> serotype 1</td>
<td>ATCC 9361</td>
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<td><em>Shigella sonnei</em></td>
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<td><em>Shigella sonnei</em></td>
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<td><em>Salmonella</em> Agona</td>
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<td>KRS203</td>
<td><em>Salmonella</em> Gaminara</td>
<td>ATCC BAA-711</td>
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<td><em>Salmonella</em> Poona</td>
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<td><em>Salmonella</em> Montevideo</td>
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<td>KRS206</td>
<td><em>Salmonella</em> Enteritidis</td>
<td>Dr. G.E. Rodrick, University of Florida</td>
</tr>
<tr>
<td>KRS207</td>
<td><em>Salmonella</em> Agona LJH617</td>
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<td>KRS208</td>
<td><em>Salmonella</em> Gaminara LJH618</td>
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<td><em>Salmonella</em> Michigan LJH621</td>
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<td><em>Salmonella</em> Poona LJH630</td>
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<td>KRS306</td>
<td><em>Escherichia coli</em> O157:H7 GFP-85</td>
<td>Deibel Laboratories, Gainesville, FL</td>
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Table 3-3. Continued.

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<td>KRS316</td>
<td><em>Escherichia coli</em></td>
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<td>KRS402</td>
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<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>Environmental isolate</td>
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</tr>
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</tr>
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</tr>
<tr>
<td>KRS427</td>
<td><em>Enterobacter cloacae</em></td>
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</table>

for 1 min followed by 45 cycles of 95.0°C for 10 sec then 60.0°C for 30 sec. Following PCR amplification a melt curve was performed from 60.0°C to 95°C with the temperature increasing at a rate of 0.5°C/10 sec. The annealing temperature was
optimized for primer pairs showing initial specificity and the specificity testing was repeated using reaction mixtures similar to that described above except that the hydrolysis probe was added at 200 nM (final concentration) and IQ™ Supermix was used in place of IQ™ Supermix with SYBR green. All real-time PCR was performed using the iCycler (BioRad).

**Binding of Biotinylated Capture Probes to Streptavidin-Coated Paramagnetic Beads**

The capture probe was designed using the conserved sequence of the chromosomally-located *ipaH* genes of *S. sonnei* Ss046 and was purchased from Sigma-Genosys (The Woodlands, TX). The sequence of the capture probe was tested for specificity *in silico* using BLASTn. To reduce steric hindrance during hybridization, the capture probe was constructed with a 12-carbon spacer between the 5' nucleotide and the biotin molecule as shown in Figure 3-1.

A 200-µl aliquot of Dynabeads M-280 Streptavidin (Dynal Biotech; Oslo, Norway) was transferred to a clean, sterile 1.5 ml microcentrifuge tube. The beads were washed as follows: two times in 200 µl 2X Binding/Washing buffer (B/W buffer; see Appendix A), two times (minimum of 1-3 min each) in 200 µl Dynabeads Solution A (see Appendix A), two times in Dynabeads Solution B (see Appendix A), one time in 200 µl B/W buffer and finally resuspended in 400 µl B/W buffer. A 40-µl aliquot of a 10 µM capture probe solution and 360 µl sterile water were added to the beads and the resulting mixture was incubated at room temperature with gentle end-over-end rotation for 10 min. The capture probe-bead complexes (hereafter referred to as CP-*Shigella* beads) were then washed three times in 400 µl 1X B/W buffer and resuspended in 400 µl 1X B/W buffer containing 1.5 µl ethanolamine. The CP-*Shigella* beads were incubated at room
Biotin-(CH₂)₁₂-CTCCAGCATCTCATATTTCTGC

Figure 3-1. Design of the capture probe and CP-Shigella beads. Biotinylated DNA probes were used to label streptavidin (SA)-coated paramagnetic beads for use in DNA sequence capture experiments. A 12-carbon spacer was inserted between the biotin molecule and the DNA probe sequence to alleviate steric hindrance during hybridization.

temperature with gentle end-over-end rotation for 1 hr, washed 3 times in 400µl low-salt wash buffer (see Appendix A) and finally resuspended in 400 µl low-salt wash buffer. CP-Shigella beads were stored at 4°C prior to use.

Inoculum Preparation

Three days prior to each experiment, S. sonnei cultures were individually cultivated (37°C, static incubation) in 10 ml tubes of TSCR and overnight transfers were performed using 10 µl sterile, disposable loops (BD Diagnostics) each day. On the day of the experiment, a five-strain S. sonnei cocktail was compiled by transferring 2.0 ml from each of the five 18-hr S. sonnei cultures (late stationary phase) to a clean, sterile 15 ml centrifuge tube. The cocktail was centrifuged (3,220 x g for 10 min at 4°C) and the resulting pellet was washed twice in 10 ml of 0.1% peptone. The final cell titer of the cocktail inoculum was determined by pour plate using TSA.

Calculation of Generation Time of S. sonnei in Shigella Broth

Each S. sonnei strain was cultured and transferred in TSB (37°C) overnight for three days. On the third day, 10 µl of an 18-hr culture was used to inoculate 100 ml sterile SB in a 250 ml Erlenmeyer flask. The SB flask was then swirled to disperse the
inoculum and a 1.0 ml aliquot was used to prepare dilutions in 0.1% peptone water. Appropriate dilutions were analyzed by pour plate using TSB to estimate the initial bacterial population. The SB flask was incubated aerobically at 44°C without shaking and 1.0 ml samples were analyzed as described above every 30 min. Using a Microsoft Excel spreadsheet, the log bacterial population vs. time was plotted to determine the length of lag phase and the generation time during exponential growth.

**Preliminary Experiments with Anti-Shigella Beads**

Two whole tomatoes and two 50 g ground beef samples were inoculated with the five-strain *S. sonnei* cocktail at approximately $4.3 \times 10^5$ CFU/tomato and $2.2 \times 10^5$ CFU/25 g, respectively. Two 25-g aliquots of ground beef were transferred to clean, sterile filtered stomacher bags each containing 225 ml SB and homogenized for 30 sec. The two tomato samples were transferred to clean, sterile stomacher bags each containing 250 ml SB and subjected to a 30 sec vigorous shake followed a 1 min hand manipulation. The filtered SB from the ground beef samples and the SB rinse from the tomato samples were transferred to clean, sterile stomacher bags and incubated at 44°C for 18 hr. After enrichment, the samples were analyzed by FTI using anti-Shigella beads prepared using either AB02 or AB04. The recovered anti-Shigella beads were analyzed by spread plate using MAC (44°C for 24 hr). The resulting colonies were identified based on biochemical reactions using the BBL® Enterotube™ II (Becton Dickinson, Sparks, MD).

**Separation of *S. sonnei* from Food Matrices Using Low-Speed Centrifugation**

A five-strain cocktail of the rifampicin resistant *S. sonnei* was prepared as described above and diluted in 0.1% peptone water to a final concentration of $3.3 \times 10^5$ CFU/ml. To test low-speed centrifugation for the separation of *S. sonnei* from food matrices, five replicate 25-g samples of potato salad and ground beef were homogenized.
with 25 ml SB and transferred to clean, sterile 50 ml centrifuge tubes. The SB homogenates were then spiked with 1.0 ml of the diluted *S. sonnei* cocktail and the centrifuge tubes were mixed by vigorous shaking for 30 sec. Prior to centrifugation, 1.0 ml aliquots of the homogenate were serially diluted using 9.0 ml 0.1% peptone tubes and 1.0 ml aliquots from appropriate dilutions were analyzed by pour-plate using TSA rif+. The remaining SB homogenates were subjected to low-speed centrifugation (LSC; 100 x g for 5 min). After centrifugation, 1.0 ml aliquots from the supernatant were serially diluted using 9.0 ml 0.1% peptone tubes and 1.0 ml aliquots from appropriate dilutions were analyzed by pour-plate using TSA rif+.

**Survival Studies**

**Sample Inoculation and Subsequent Recovery**

Tomatoes were placed in sterile fiberglass trays with the blossom scar faced up. Smooth surfaces around the blossom scar of tomatoes were spot inoculated at 10 sites per fruit with 10 µl per site using appropriate dilutions to obtain final inoculation levels of approximately 5.0 x 10⁵ CFU/tomato with five replicate tomatoes at each level. The inoculated tomatoes were dried for at least 1 hr in a laminar flow hood at room temperature. After drying, tomatoes were stored in humidity chamber at 13ºC with 85% relative humidity (RH) and five inoculated tomatoes were transferred to sterile Stomacher bags (Seward, Norfolk, UK) containing 100 ml of sterile PBS at each observation day. For recovery of inocula, tomatoes were shaken vigorously for 30 sec, then massaged by hand for 1 min in the stomach bag similar to that described by Zhuang *et al.* (1995).

For trials involving potato salad and ground beef, 50-g samples were aseptically weighed into sterile 4-oz specimen cups and inoculated with 1.0 ml of an appropriate dilution to obtain final inoculation levels of ca 1.0 x 10⁶ CFU/g. Using sterile tongue
depressors, the inoculum was homogenized in each sample for a minimum of 30 sec, after which the samples were allowed to sit at room temperature for 1 hr for bacterial attachment. After attachment, each set of specimen cups with samples were stored at 2.5°C and 8°C, respectively. For recovery of inocula, sterile tongue depressors were used to weigh 25 g aliquots from each sample to sterile, clean Stomacher bags after which 225 ml of PBS was added and the samples were placed in the stomacher (Tekmar Company, Cincinnati, OH) for 30 sec.

Three-Tube Most Probable Number Estimation of Survivors

Recovered inocula in 100 ml PBS rinses of tomatoes represented a 0.01 dilution of the tomatoes surface population. Recovered inocula in 225 ml PBS homogenates of potato salad or ground beef samples represented a 0.1/g dilution of the surviving population. Recovered inocula were serially diluted using 9.0 ml 0.1% peptone tubes and 1.0 ml aliquots from appropriate dilutions were used to inoculate each of three TSB rif+ tubes. To enumerate survivors at the 0.1/tomato level, three 10 ml aliquots from the 100 ml PBS tomato rinse were used to inoculate 10 ml double-strength TSB rif+ tubes. All TSB rif+ tubes were incubated overnight (37°C; static incubation) and scored as either “positive” or “negative” for Salmonella or S. sonnei based on the presence/absence of visible growth. Surviving populations were estimated using the three-tube most probable number (MPN) table located in Appendix 2 of the FDA BAM (2003). Non-inoculated samples of each food were analyzed to confirm the absence of indigenous microflora with resistance to rifampicin at 100 ppm.

Evaluation of Detection Methods

A schematic representation of the experimental design is presented in Figure 3-2. Tomato smooth surfaces, potato salad and ground beef samples were inoculated with a
Figure 3-2. Flow diagram of the experiments involving inoculated food samples. Each food samples was analyzed by the Shigella culture method of the FDA Bacteriological Analytical Manual (BAM), by flow-through immunocapture followed by analysis of recovered beads by spread plate using MacConkey agar (MAC) or using real-time PCR and by DNA sequence capture. Suspected isolates on MAC were further analyzed using Triple Sugar Iron (TSI) agar slants, Lysine Iron Agar (LIA) slants and Motility Medium (MM).

five-strain S. sonnei cocktail and recovery of the inocula was investigated using the BAM Shigella culture method, FTI-MAC, FTI-PCR and DSC.
Inoculation of Samples and Subsequent Recovery

For tomato samples, tomatoes were placed in sterile fiberglass trays with the blossom scar faced up. Smooth surfaces around the blossom scar of tomatoes were spot inoculated at 10 sites per fruit with 10 µl per site using appropriate dilutions of the *S. sonnei* cocktail to obtain final inoculation levels from $10^4$ to $10^0$ CFU/tomato with ten replicate tomatoes at each level. The inocula were allowed to dry completely at room temperature. After drying, inoculated tomatoes were transferred to sterile Stomacher bags (Seward) containing 250 ml of sterile SB pre-warmed to 44°C. For recovery of *S. sonnei*, the stomacher bags were sealed using stomacher bag clips and the tomatoes were shaken vigorously for 30 sec then massaged by hand for 1 min, similar to the method described by Zhuang *et al.* (1995).

For potato salad and ground beef samples, 50 g of sample was weighed into a sterile 4-oz specimen cup and inoculated with appropriate dilutions of the *S. sonnei* cocktail to obtain final inoculation levels from $10^2$ to $10^0$ CFU/25 g with ten replicate samples at each level. Sterile wooden tongue depressors were used to homogenize the inoculum in each specimen cup. The inoculated potato salad and ground beef samples were allowed to sit for 1 hr at room temperature for bacterial attachment. A 25-g aliquot from each sample was weighed into a sterile dual-chambered stomacher bag containing 225 ml of SB and homogenized for 30 sec.

Modified BAM Culture Method for *S. sonnei*

For tomato samples, the recovered inocula in SB was transferred to sterile 18 oz Whirl-Pak® bags (Nasco, Modesto, CA) and the top of the bag was folded over twice and secured. For potato salad and ground beef samples, the internal filter of the dual-chambered stomacher bag was used to transfer the SB supernatant only to sterile 18 oz
Whirl-Pak® bags and the top of the bag was folded over twice and secured. Samples were incubated at 44°C for 18-24 hr under anaerobic conditions using 7.0 liter rectangular jars (Mitsubishi Gas Chemical Company, Inc., Japan) with the Pack-Anaero anaerobic gas generating system (Mitsubishi Gas Chemical Company). SB enrichments were homogenized by hand and streaked for isolation on MAC and incubated at 37°C for 18-24 hr.

For confirmation, three isolates colonies demonstrating typical *Shigella* morphology were selected from each MAC plate. When three typical isolates were not present, atypical isolates were selected for confirmation. Each selected isolate was used to inoculate a TSI slant, a LIA slant and MM and incubated overnight (37°C). From each isolate that resulted in typical reactions for *Shigella* on TSI slants, LIA slants and MM, growth from the TSI slant was cultivated overnight (37°C) in TSB. Growth from the TSB tubes was then streaked for isolation on MAC and incubated overnight (37°C). Biochemical reactions were tested using the BBL® Enterotube™ II (Becton Dickinson). Enterotubes were incubated overnight (37°C) and positive reactions were read according to manufacturer’s instructions.

**Flow-Through Immunocapture (FTI) Using the Pathatrix**

All samples were inoculated and enriched as described above for the modified BAM method with the following exceptions. SB tomato rinses and the filtered SB supernatant of homogenized potato salad and ground beef samples were transferred to clean, sterile stomacher bags prior to overnight enrichment. Anaerobic conditions were not generated for any samples analyzed by FTI. Instead Stomacher clips were used to seal the Stomacher bags and samples were incubated at 44°C (static).
All FTI experiments were performed using the Pathatrix system (Matrix MicroScience, Golden, CO). The assembly/operation of the Pathatrix and the recovery of anti-\textit{Shigella} beads were performed as per manufacturer instructions. Briefly, Stomacher bags containing sample enrichments were placed into a Pathatrix incubation pot. After the tubing was properly placed, a 50-µl aliquot of anti-\textit{Shigella} beads was injected to the system per manufacturer’s instructions. After 20 min circulation at 37°C, the tubing assembly was disconnected from the sample and the anti-\textit{Shigella} beads were washed with 100 ml of PBS and recovered in the collection vessel suspended in 5 to 10 ml PBS. Using a magnet, the anti-\textit{Shigella} beads were drawn to the side of the collection vessel and the volume of PBS was reduced to approximately 250 µl using a sterile transfer pipet. A 50-µl aliquot of the resuspended anti-\textit{Shigella} beads was analyzed by spread plate using MAC. MAC plates were incubated at 37°C for 24 hr. In addition, DNA was extracted from a 100-µl aliquot of the resuspended anti-\textit{Shigella} beads as described above and analyzed by real-time PCR.

\textbf{Sequence Capture of \textit{Shigella} DNA}

A schematic representation of the DSC method is presented in Figure 3-3. Preliminary experiments were performed to evaluate hybridization buffers, hybridization temperatures, type of streptavidin-coated paramagnetic beads and sensitivity.

All procedures for inoculation and recovery of food samples was followed as described above for FTI samples. After 18-24 hr incubation, the samples were shaken briefly to mix contents. For potato salad and ground beef samples, a 10 ml aliquot was aseptically transferred to a clean, sterile 15 ml centrifuge tube and solid food material was sedimented using low-speed centrifugation (100 x g for 5 min). For tomato samples, no solid food material was present in the overnight enrichments, therefore no low-speed
Figure 3-3. Flow diagram of the DNA sequence capture method. CP-Shigella beads were prepared using Dynabeads M-280 Streptavidin coated with a 5' biotin-labeled DNA probe with specificity for the *ipaH* gene of *Shigella* and enteroinvasive *E. coli*.
centrifugation was performed. A 1.0-ml aliquot of the resulting supernatant (potato salad and ground beef samples) or a 1.0-ml aliquot of the overnight enrichment (tomato samples) was transferred to a clean, sterile 1.5 ml microcentrifuge tube. The bacterial cells were then sedimented using centrifugation (6,000 x g for 5 min) and the supernatant was discarded. The pellet was resuspended in 530 µl hybridization buffer 1 (see Appendix A), heated at 100°C for 10 min and finally cooled on ice for 10 min. Cellular and solid material were sedimented by centrifugation (6,000 x g for 5 min) and the supernatant was transferred to a clean, sterile 1.5 ml microcentrifuge tube containing 200 µl 3.75 M NaCl and 20 µl CP-Shigella beads. The contents were mixed using the action of the pipette and heated at 40°C for 5 min on a dry-bath incubator followed by end-over-end rotation (hybridization) at 40°C for 1 hr. Heated end-over-end rotation was achieved using a Rugged Rotator (Glas-Col, Terre Haute, IN) inside of an environmental chamber (Lab-Line, E2 series, Barnstead International, Melrose Park, IL). Following hybridization, the beads were recovered using a magnetic rack and washed 2 times in 200 µl wash buffer (see Appendix A), 2 times in 200 µl low-salt wash buffer (see Appendix A) and resuspended in 50 µl TE buffer. The resuspended beads were then heated at 75°C for 10 min to release the captured DNA from the probe and, using the magnetic rack, the DNA-containing supernatant was transferred to a clean, sterile 1.5 ml microcentrifuge tube. The DNA samples were placed on ice or frozen (-20°C) prior to analysis by PCR.

**Real-Time PCR and Reverse Transcriptase (RT) PCR**

All real-time PCR and real-time RT-PCR analyses were performed using the iCycler® (Bio-Rad, Hercules, CA). For the analysis of FTI samples, real-time PCR was performed using 50 µl reaction mixtures consisting of: 25 µl IQ™ Supermix (Bio-Rad), 200 nM (final concentration) each of forward and reverse primer, 200 nm (final
concentration) hydrolysis probe, 20 µl DNA sample and purified water. The PCR cycling conditions were 95°C for 1 min followed by 40 cycles of 95°C for 10 sec then 60°C for 30 sec. For the analysis for DNA sequence capture samples, real-time PCR was performed using 20 µl reaction mixtures consisting of: 10 µl IQ™ Supermix (Bio-Rad), 200 nM (final concentration) each of forward and reverse primer, 200 nm (final concentration) hydrolysis probe, 5 µl DNA sample and purified water. The PCR cycling conditions were the same as for FTI samples.

Real-time RT-PCR was performed using 20 µl reaction mixtures consisting of: 10 µl 2X RT-PCR Reaction Mix for Probes (Bio-Rad), 200 nM (final concentration) each of forward and reverse primer, 200 nM (final concentration) hydrolysis probe, 0.4 µl reverse transcriptase (Bio-Rad), 0.3 µl RNasin (Promega, Madison, WI), 5 µl RNA sample and purified water. The PCR cycling conditions were 50°C for 10 min, 95°C for 5 min followed by 40 cycles of 95°C for 10 sec then 60°C for 30 sec. For all RNA samples analyzed by RT-PCR, parallel reaction mixtures without the addition of reverse transcriptase were prepared to verify complete digestion of DNA.

All PCR and RT-PCR reaction mixtures were prepared in a Labconco Purifier Class II safety cabinet (Labconco Corporation, Kansas City, MO).

Recording of Data and Statistical Analysis

All statistical analyses of survival studies were performed using the Statview statistical software package (SAS) version 9.1 (SAS Institute Inc., Cary, NC) using a mixed model. Sample replications were treated as random variables within time. Statistical analysis of population means in experiments involving LSC were performed by hand using a two sample t-test. $P$ values < 0.05 were considered significant.
All results from the evaluation of detection methods were recorded as “positive” or “negative” for the detection of *S. sonnei*. Positive isolation on plating media resulted from typical reactions for *S. sonnei* in all confirmation steps and positive biochemical BBL® Enterotube™ II identification. Bias-reduced logistic regression (BRLR) models were constructed using the R software (The R Foundation for Statistical Computing, Version 2.2.0, [http://cran.us.r-project.org/](http://cran.us.r-project.org/)) to identify significant differences ($P < 0.05$) among the detection methods tested.
CHAPTER 4
RESULTS

This study consisted of three phases of research. The first phase consisted of preliminary trials involving the preparation of growth curves to calculate the generation time of \textit{S. sonnei} in SB, the development of primers/probes for the detection of \textit{S. sonnei} and testing the specificity of each set of primers/probes against a DNA library of positive and negative controls. The second phase consisted of experiments designed to test the ability of \textit{S. sonnei} to survive in/on selected foods. The third phase of this study involved the evaluation of newly developed sampling methods for the detection of artificially inoculated \textit{S. sonnei} on smooth tomato surfaces and in potato salad and ground beef. Recovery/detection of the inocula was tested using the BAM \textit{Shigella} culture method and the newly developed methods: flow-through immunocapture (FTI) followed by analysis of recovered anti-\textit{Shigella} beads by spread-plate using MAC (FTI-MAC), FTI followed by analysis of recovered anti-\textit{Shigella} beads by real-time PCR (FTI-PCR) and DNA sequence capture (DSC).

Preliminary Studies

Calculation of Generation Time of \textit{S. sonnei} in \textit{Shigella} Broth

Growth curves were prepared for \textit{S. sonnei} ATCC 9290, \textit{S. sonnei} ATCC 29031, \textit{S. sonnei} ATCC 29030, \textit{S. sonnei} ATCC 25931 and \textit{S. sonnei} ATCC 29930 (Figures 4-1, 4-2, 4-3, 4-4 and 4-5, respectively) in \textit{Shigella} broth (SB) incubated aerobically without shaking at 44°C. The length of the lag phase was identified and the exponential phase was used to calculate the generation time for each strain. The average growth kinetics
among the five *S. sonnei* strains was a lag phase of approximately 2 hr and a generation
time of 18.8 ± 0.6 min.

**Growth curve of *S. sonnei* ATCC 9290**

When the growth of *S. sonnei* ATCC 9290 was investigated in SB (44ºC), an initial
lag phase of 2 hr was observed prior to exponential growth (Figure 4-1). Logarithmic
regression used to analyze the exponential phase of growth showed linearity (*R^2* = 0.997)
and the equation of the line was used to calculate a generation time of 19.0 min. The
initial population of *S. sonnei* ATCC 9290 was 4.3 x 10^4 CFU/ml and the final population
was 3.8 x 10^8 CFU/ml.

**Growth curve of *S. sonnei* ATCC 29031**

When the growth of *S. sonnei* ATCC 29031 was investigated in SB (44ºC), an
initial lag phase of 2 hr was observed prior to exponential growth (Figure 4-2).
Logarithmic regression used to analyze the exponential phase of growth showed linearity
(*R^2* = 0.994) and the equation of the line was used to calculate a generation time of 19.5
min. The initial population of *S. sonnei* ATCC 29031 was 3.8 x 10^4 CFU/ml and the final
population was 2.4 x 10^8 CFU/ml.

**Growth curve of *S. sonnei* ATCC 29030**

When the growth of *S. sonnei* ATCC 29030 was investigated in SB (44ºC), an
initial lag phase of 2 hr was observed prior to exponential growth (Figure 4-3).
Logarithmic regression used to analyze the exponential phase of growth showed linearity
(*R^2* = 0.996) and the equation of the line was used to calculate a generation time of 18.6
min. The initial population of *S. sonnei* ATCC 29030 was 4.0 x 10^4 CFU/ml and the final
population was 5.0 x 10^8 CFU/ml.
Figure 4-1. Growth curve: *S. sonnei* ATCC 9290 in *Shigella* broth. A 100 ml microcosm was inoculated with a 10-µl aliquot of an 18-hr *S. sonnei* ATCC 9290 culture and incubated (44°C, static). At appropriate time intervals, a 1.0 ml aliquot was serially diluted in 0.1% peptone and the population was estimated by pour plate using tryptic soy agar. (□) lag/stationary phase growth; (■) exponential phase growth. Error bars represent one standard deviation.

Figure 4-2. Growth curve: *S. sonnei* ATCC 29031 in *Shigella* broth. A 100 ml microcosm was inoculated with a 10-µl aliquot of an 18-hr *S. sonnei* ATCC 29031 culture and incubated (44°C, static). At appropriate time intervals, a 1.0 ml aliquot was serially diluted in 0.1% peptone and the population was estimated by pour plate in tryptic soy agar. (□) lag/stationary phase growth; (■) exponential phase growth. Error bars represent one standard deviation.
y = 0.9734x + 2.5896
R² = 0.996

Figure 4-3. Growth curve: *S. sonnei* ATCC 29030 in *Shigella* broth. A 100 ml microcosm was inoculated with a 10-µl aliquot of an 18-hr *S. sonnei* ATCC 29030 culture and incubated (44°C, static). At appropriate time intervals, a 1.0 ml aliquot was used to make serial dilutions in 0.1% peptone and the population was estimated by pour plate using tryptic soy agar. (□) lag/stationary phase growth; (■) exponential phase growth. Error bars represent one standard deviation.

Growth curve of *S. sonnei* ATCC 25931

When the growth of *S. sonnei* ATCC 25931 was investigated in SB (44°C), an initial lag phase of 2 hr was observed prior to exponential growth (Figure 4-4). Logarithmic regression used to analyze the exponential phase of growth showed linearity (R² = 0.990) and the equation of the line was used to calculate a generation time of 18.0 min. The initial population of *S. sonnei* ATCC 25931 was 2.9 x 10⁴ CFU/ml and the final population was 3.7 x 10⁸ CFU/ml.

Growth curve of *S. sonnei* ATCC 29930

When the growth of *S. sonnei* ATCC 29930 was investigated in SB (44°C), an initial lag phase of 2 hr was observed prior to exponential growth (Figure 4-5). Logarithmic regression used to analyze the exponential phase of growth showed linearity
Figure 4-4. Growth curve: *S. sonnei* ATCC 25931 in *Shigella* broth. A 100 ml microcosm was inoculated with a 10-µl aliquot of an 18-hour *S. sonnei* ATCC 25931 culture and incubated (44°C, static). At appropriate time intervals, a 1.0 ml aliquot was used to make serial dilutions in 0.1% peptone and the population was estimated by pour plate using tryptic soy agar. (□) lag/stationary phase growth; (■) exponential phase growth. Error bars represent one standard deviation.

Figure 4-5. Growth curve: *S. sonnei* ATCC 29930 in *Shigella* broth. A 100 ml microcosm was inoculated with a 10-µl aliquot of an 18-hour *S. sonnei* ATCC 29930 culture and incubated (44°C, static). At appropriate time intervals, a 1.0 ml aliquot was used to make serial dilutions in 0.1% peptone and the population was estimated by pour plate using tryptic soy agar. (□) lag/stationary phase growth; (■) exponential phase growth. Error bars represent one standard deviation.
(R² = 0.998) and the equation of the line was used to calculate a generation time of 19.1 min. The initial population of S. sonnei ATCC 29930 was 1.6 x 10⁴ CFU/ml and the final population was 4.3 x 10⁸ CFU/ml.

**Evaluation Anti-Shigella Antibodies for Use with Flow-Through Immunocapture**

Two polyclonal antibodies (AB01 and AB04) and one monoclonal antibody (AB02) were evaluated for use in FTI of S. sonnei using the Pathatrix (Table 4-1). AB01 was generated using a mixture of S. boydii, S. flexneri and S. dysenteriae as the immunogens. AB04 was generated using a membrane extract mixture of S. sonnei and S. flexneri. In addition, a secondary capture in which S. sonnei pre-bound with AB01 or AB02 in solution were separated from food matrices using FTI with paramagnetic beads coated with goat anti-rabbit antibodies or rabbit anti-mouse antibodies (AB03 or AB05, respectively).

The number of S. sonnei colonies which resulted from FTI followed by analysis of recovered beads by spread plate using MAC using anti-Shigella beads prepared with the various anti-Shigella antibodies is listed in Table 4-1. When AB01 was used for the preparation of anti-Shigella beads, a population too numerous to count (TNTC) of S. sonnei ATCC 25931, two colonies of S. sonnei ATCC 29930, 78 colonies of S. sonnei ATCC 9290 and no colonies of S. sonnei ATCC 29030 or S. sonnei ATCC 29031 were observed. When AB02 was used for the preparation of anti-Shigella beads, a population TNTC of S. sonnei ATCC 25931 and S. sonnei ATCC 9290, 147 colonies of S. sonnei ATCC 29930, 112 colonies of S. sonnei ATCC 29030 and 87 colonies of S. sonnei ATCC 29031 was observed. When AB04 was used for the preparation of anti-Shigella beads, 261 colonies of S. sonnei ATCC 25931, 182 colonies of S. sonnei ATCC 29930, 15 colonies of S. sonnei ATCC 29030, 53 colonies of S. sonnei ATCC 9290 and
Table 4-1. Evaluation of anti-*Shigella* antibodies for flow-through immunocapture (FTI) of *S. sonnei*. Microcosms of individual *S. sonnei* strains were prepared at approximately 2.0 x 10^5 CFU/ml in 250 ml phosphate buffered saline (PBS). The microcosms were then analyzed by FTI for 30 min at 37°C using various anti-*Shigella* beads. After circulation, 100 µl aliquots of the recovered beads were analyzed by spread plate using MacConkey agar (MAC; 37°C for 24 hr). The numbers of resulting colonies were enumerated in order to compare immunocapture by the various antibodies. In the AB03-AB03 and AB05-AB02 experiments, AB01 and AB02 were added directly to inoculated PBS and allowed to bind for 5 min with shaking (60 rpm) followed by FTI using beads coated with AB03 and AB05, respectively.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>ATCC 25931</th>
<th>ATCC 29930</th>
<th>ATCC 29030</th>
<th>ATCC 9290</th>
<th>ATCC 29031</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB01</td>
<td>TNTC</td>
<td>2</td>
<td>NG</td>
<td>78</td>
<td>NG</td>
</tr>
<tr>
<td>AB02</td>
<td>TNTC</td>
<td>147</td>
<td>112</td>
<td>TNTC</td>
<td>87</td>
</tr>
<tr>
<td>AB03-AB01</td>
<td>148</td>
<td>263</td>
<td>TNTC</td>
<td>68</td>
<td>TNTC</td>
</tr>
<tr>
<td>AB04</td>
<td>261</td>
<td>182</td>
<td>15</td>
<td>53</td>
<td>47</td>
</tr>
<tr>
<td>AB05-AB02</td>
<td>TNTC</td>
<td>NG</td>
<td>NG</td>
<td>10</td>
<td>NG</td>
</tr>
</tbody>
</table>

^a^ too numerous to count
^b^ no growth

47 colonies of *S. sonnei* ATCC 29031 was observed. These data suggest that anti-*Shigella* beads prepared with AB02 or AB04, but not AB01, may be used for the consistent immunocapture of *S. sonnei* by FTI.

When AB01 was used to bind *S. sonnei* prior to analysis by FTI using paramagnetic beads coated with AB03 (secondary capture), populations TNTC of *S. sonnei* ATCC 29030 and *S. sonnei* ATCC 29031, 148 colonies of *S. sonnei* ATCC 25931, 263 colonies of *S. sonnei* ATCC 29930 and 68 colonies of *S. sonnei* ATCC 9290 were observed. When AB02 was used to bind *S. sonnei* prior to analysis by FTI using paramagnetic beads coated with AB05 (secondary capture), populations TNTC of *S. sonnei* ATCC 25931, 10 colonies of ATCC 9290 and no colonies of *S. sonnei* ATCC 29930, ATCC 29031 or ATCC 29030 were observed. These data suggest that secondary capture by FTI using
AB03 of *S. sonnei* which have been prior labeled with AB01 may be used for the consistent immunocapture. Secondary capture by FTI using AB05 of *S. sonnei* that had been prior labeled with AB02 did not provide consistent immunocapture under the conditions investigated. Using the Pathatrix for secondary capture of *S. sonnei* that had been prior labeled with anti-*Shigella* antibodies required additional steps and time over using the Pathatrix for primary capture.

**Preliminary Experiments with Anti-*Shigella* Beads**

Tomato and ground beef samples were inoculated with a five-strain *S. sonnei* cocktail and analyzed by FTI followed by analysis of the recovered beads by spread plate using MAC. When anti-*Shigella* beads were prepared with AB02, analysis of tomato and ground beef samples resulted in MAC plates with only colonies exhibiting morphologies typical for *S. sonnei*, and each colony selected (three colonies from each plate) for confirmation was identified as *S. sonnei*. When anti-*Shigella* beads were prepared with AB04, analysis of tomato and ground beef samples resulted in MAC plates with colony morphologies both typical and atypical for *S. sonnei*. Atypical colonies from tomato samples were identified as *Citrobacter freundii*, *Enterobacter* spp. and *Klebsiella pneumoniae*. Atypical colonies from ground beef samples were identified as *Enterobacter cloacae* and *E. coli*. Typical colonies from tomato samples were identified as *S. sonnei* or *E. coli*. These data suggest that anti-*Shigella* beads prepared with AB02, but not those prepared with AB04, may be used for the specific detection of *S. sonnei* in tomato and ground beef samples. Anti-*Shigella* beads prepared with AB02 were used for all subsequent experiments (hereafter referred to as anti-*Shigella* beads).

The manufacturer of AB02 reported no reactivity with *S. boydii*, *S. flexneri* or *S. dysenteriae*, however when anti-*Shigella* beads were tested against solutions of these
serogroups in PBS using the FTI-MAC method (as described for *S. sonnei* strains) the anti-*Shigella* beads reacted strongly with the *S. flexneri* strain (KRS106) and the *S. dysenteriae* type 1 strain (KRS108). When tested against two stock strains of *S. boydii* type 18, anti-*Shigella* beads reacted weakly with one strain (KRS101) but did not react with the other strain (KRS102).

**Optimization of Anti-*Shigella* Bead Concentration for Flow-Through Immunocapture of *S. sonnei***

To optimize the concentration of anti-*Shigella* beads required for detection of *S. sonnei* by FTI, anti-*Shigella* beads were diluted in PBS containing 0.1% sodium azide and tested against various concentrations of *S. sonnei* ATCC 25931 or *S. sonnei* 29930 (Table 4-2). Twenty-five-µl aliquots of the initial concentration of anti-*Shigella* beads (50 mg/ml) and the following diluted concentrations were tested for FTI: 25 mg/ml (1:1 dilution), 16.7 mg/ml (1:2 dilution), 12.5 mg/ml (1:3 dilution) and 10 mg/ml (1:4 dilution). The number of colonies on MAC plates which resulted from the analysis of various concentrations of *S. sonnei* ATCC 25931 and *S. sonnei* ATCC 29930 by FTI using the various concentrations of anti-*Shigella* beads are given in Table 4-2.

When *S. sonnei* ATCC 25931 microcosms of $2.8 \times 10^3$ CFU/ml and $3.7 \times 10^5$ CFU/ml were analyzed by FTI, all tested dilutions of anti-*Shigella* beads resulted in MAC plates with populations TNTC. For *S. sonnei* ATCC 25931 microcosms of $3.2 \times 10^1$ CFU/ml analyzed by FTI, undiluted anti-*Shigella* beads and dilutions of 1:1, 1:2, 1:3 and 1:4 anti-*Shigella* beads resulted in MAC plates with 27, 18, 13, 6 and 12 colonies, respectively. These data suggest the sensitivity of FTI for *S. sonnei* ATCC 25931 is approximately $3.0 \times 10^1$ CFU/ml and that any of the tested concentration of anti-*Shigella* beads may be used for detection.
Table 4-2. Optimization of anti-

Shigella bead concentration for flow-through 

immunocapture (FTI) of S. sonnei. Microcosms of S. sonnei ATCC 25931 or 

ATCC 29930 at various concentrations were prepared in phosphate buffered 

saline (PBS). The microcosms were then analyzed by FTI for 30 min at 37°C 

using 25-µl aliquots of various dilutions of anti-Shigella beads. After 

circulation, 100-µl aliquots of the recovered beads were analyzed by spread 

plate using MacConkey agar (MAC; 37°C for 24 hr). The numbers of 

resulting colonies were enumerated in order to determine the sensitivity of 

FTI using various concentrations of anti-Shigella beads.

<table>
<thead>
<tr>
<th>S. sonnei titer (CFU/ml)</th>
<th>Plate counts from dilution of anti-Shigella beads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>ATCC 25931</td>
<td></td>
</tr>
<tr>
<td>3.2 x 10^1</td>
<td>27</td>
</tr>
<tr>
<td>2.8 x 10^3</td>
<td>TNTC a</td>
</tr>
<tr>
<td>3.7 x 10^5</td>
<td>TNTC</td>
</tr>
<tr>
<td>ATCC 29930</td>
<td></td>
</tr>
<tr>
<td>2.1 x 10^1</td>
<td>NG b</td>
</tr>
<tr>
<td>2.7 x 10^3</td>
<td>NG</td>
</tr>
<tr>
<td>2.8 x 10^5</td>
<td>11</td>
</tr>
</tbody>
</table>

a too numerous to count
b no growth

When S. sonnei ATCC 29930 microcosms of 2.1 x 10^3 CFU/ml and 2.7 x 10^3 

CFU/ml were analyzed by FTI, all tested dilutions of anti-Shigella beads resulted in 

MAC plates with no colonies. For S. sonnei ATCC 29930 microcosms of 2.8 x 10^5 

CFU/ml analyzed by FTI, undiluted anti-Shigella beads and dilutions of 1:1, 1:2, 1:3 and 

1:4 anti-Shigella beads resulted in MAC plates with 11, 7, 13, 12 and 12 colonies, 

respectively. These data suggest the sensitivity of FTI for S. sonnei ATCC 29930 is 

approximately 3.0 x 10^5 CFU/ml and that any of the tested concentration of anti-Shigella 

beads may be used for detection.

Although the tested concentrations of anti-Shigella beads did not affect detection of 

S. sonnei ATCC 29531 or ATCC 29930 in PBS, the addition of 25 µl anti-Shigella bead 

aliquots per FTI analysis of ground beef samples resulted in poor visual bead recovery.
To improve visual bead recovery, 50-µl aliquots of the 10 mg/ml anti-Shigella bead dilution were used for FTI in all inoculated trials.

Identification of Potentially Shigella-Specific Genetic Loci

The MBGD and BLASTn were used to identify genetic loci potentially specific for Shigella spp. or for S. sonnei alone. The genes identified using the MBGD are listed in Table 4-3. Using BLASTn, none of the genes were identified with specificity for all five of the Shigella genomes within the database (Table 4-4), however the genes SSO_0670, SSO_2685, SSO_3247 and SSO_0721 were identified as potentially specific for some species of Shigella. When analyzed using BLASTn, the genes SSO_2067, SSO_2071, SSO_1019, SSO_2059 and SSO_2863 were identified as potentially specific for S. sonnei (Table 4-4). The nucleotide sequences of the identified genes were used to develop PCR primers (Table 3-2).

Specificity of Primers Developed for Potentially Shigella-Specific Genetic Loci

The developed primers were evaluated by real-time PCR against DNA extracted from stock Shigella spp. (Table 4-4) and closely-related microorganisms (data not shown). Primer sets 01-025, 01-026, 01-028 and 01-029 amplified DNA from eight strains of S. sonnei and one strain each of S. flexneri and S. dysenteriae, however DNA from two strains of S. boydii were not amplified. The observed amplification of DNA from the stock strains of S. flexneri and S. dysenteriae were not in agreement with the in silico analysis using BLASTn. Primer set 01-027 amplified DNA from only three of five S. sonnei strains tested; therefore it was not investigated against DNA from other Shigella
Table 4-3. Genetic targets identified with potential specificity for *Shigella* spp. or for *S. sonnei* alone. Genes were identified using the Microbial Genome Database for Comparative Analysis (MBGD) to create orthologous gene tables from the available sequences of *S. flexneri*, *S. sonnei* and *E. coli*. All Gene IDs listed below are from the genome of *S. sonnei* Ss046 (accession number CP000038).

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene Name</th>
<th>Gene Description</th>
<th>Primer Set</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSO_0670</td>
<td><em>ybgD</em></td>
<td>Putative fimbrial-like protein</td>
<td>01-024</td>
</tr>
<tr>
<td>SSO_2067</td>
<td><em>pduL</em></td>
<td>Putative propanediol utilization protein</td>
<td>01-025</td>
</tr>
<tr>
<td>SSO_2071</td>
<td><em>pduP</em></td>
<td>Putative propanediol utilization protein: CoA-dependent propionaldehyde dehydrogenase</td>
<td>01-026</td>
</tr>
<tr>
<td>SSO_1019</td>
<td>n/a</td>
<td>Putative membrane protein</td>
<td>01-027</td>
</tr>
<tr>
<td>SSO_2059</td>
<td><em>pduB</em></td>
<td>Putative propanediol utilization protein: polyhedral bodies</td>
<td>01-028</td>
</tr>
<tr>
<td>SSO_2863</td>
<td>n/a</td>
<td>Putative periplasmic or exported protein</td>
<td>01-029</td>
</tr>
<tr>
<td>SSO_2685</td>
<td>n/a</td>
<td>Conserved hypothetical protein</td>
<td>01-030</td>
</tr>
<tr>
<td>SSO_3247</td>
<td>n/a</td>
<td>Putative minor pilin and initiator</td>
<td>01-031</td>
</tr>
<tr>
<td>SSO_0721</td>
<td>n/a</td>
<td>Conserved hypothetical protein</td>
<td>01-032</td>
</tr>
</tbody>
</table>

These data demonstrate that primer sets 01-025, 01-026, 01-028 and 01-029 are not specific for *S. sonnei*.

Primer sets 01-030, 01-031 and 01-032 were tested DNA extracted from each of the *Shigella* spp. and *E. coli* isolates listed in Table 3-3. Primer set 01-030 performed in agreement with the *in silico* analysis with respect to the *Shigella* spp. tested. Primer sets 01-031 and 01-032, although not specific for *S. dysenteriae* by *in silico* analysis, resulted in positive amplification when tested against extracted DNA from the stock *S. dysenteriae* type 1 culture (Table 4-4). When tested against *E. coli* DNA however, primer sets 01-030 and 01-031 amplified DNA from all of the *E. coli*, while primer set 01-032 amplified DNA from only two strains of *E. coli* (KRS312 and KRS316). These data suggest that primer sets 01-030, 01-031 and 01-032 may not be used for the specific detection of *Shigella* spp.
Table 4-4. Evaluation of primer specificity among stock *Shigella* cultures and by comparative analysis against previously sequenced *Shigella* genomes. Extracted DNA from stock *Shigella* cultures were analyzed by real-time PCR. BLAST searches for significant alignments were performed using the National Center for Biotechnology Information (NCBI) website.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain identification/ Accession number</th>
<th>Primer set</th>
<th>01-024</th>
<th>01-025</th>
<th>01-026</th>
<th>01-027</th>
<th>01-028</th>
<th>01-029</th>
<th>01-030</th>
<th>01-031</th>
<th>01-032</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity by PCR against extracted DNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. boydii</em></td>
<td>KRS101</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>S. boydii</em></td>
<td>KRS102</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td>KRS103</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td><em>S. sonnei</em></td>
<td>KRS104</td>
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<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td>KRS105</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>S. sonnei</em></td>
<td>KRS109</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>S. sonnei</em></td>
<td>KRS110</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>S. sonnei</em></td>
<td>KRS111</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td></td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td>KRS112</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>S. sonnei</em></td>
<td>KRS113</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>S. flexneri</em></td>
<td>KRS106</td>
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<td>+</td>
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<td>ND</td>
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<tr>
<td><em>S. dysenteriae</em></td>
<td>KRS108</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
</tr>
</tbody>
</table>

Specificity by *in silico* analysis (BLAST)

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain identification/ Accession number</th>
<th>Primer set</th>
<th>01-024</th>
<th>01-025</th>
<th>01-026</th>
<th>01-027</th>
<th>01-028</th>
<th>01-029</th>
<th>01-030</th>
<th>01-031</th>
<th>01-032</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. boydii</em></td>
<td>NC_007613</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td><em>S. sonnei</em></td>
<td>NC_007384</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>-</td>
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<td><em>S. flexneri</em></td>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<td>+</td>
<td></td>
</tr>
<tr>
<td><em>S. dysenteriae</em></td>
<td>NC_007606</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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</table>

ND = not determined
Primer set 01-024 was initially tested against the complete DNA library for microorganisms listed in Table 3-3, except for \textit{E. coli} strains KRS308 through KRS316. Amplification was observed only from DNA from eight strains of \textit{S. sonnei} and one strain each of \textit{S. flexneri} and \textit{S. dysenteriae}, however DNA from two strains of \textit{S. boydii} were not amplified. The observed amplification of DNA from the stock strain of \textit{S. dysenteriae} was not in agreement with the \textit{in silico} analysis using BLASTn. The \textit{E. coli} strains KRS308 through KRS316 were isolates obtained during the BAM \textit{Shigella} culture trials involving ground beef. Despite previous testing, in which primer set 01-024 tested negative against DNA from four strains of \textit{E. coli} O157:H7 and three strains of non-pathogenic \textit{E. coli}, primer set 01-024 resulted in amplification of DNA from eight out of nine of the ground beef \textit{E. coli} isolates. These data demonstrate that primer set 01-024 is not specific for \textit{Shigella} spp. Further investigations of primer set 01-024 were terminated.

Taken together, these data demonstrate the limitations of DNA databases and the importance of verifying primer specificity against DNA extracts from numerous strains of target and non-target microorganisms.

\textbf{Separation of \textit{S. sonnei} from Food Matrices by Low-Speed Centrifugation}

Since low-speed centrifugation (LSC; 100 X g for 5 min) was to be used as a sample preparation step for potato salad and ground beef sample, the concentration of \textit{S. sonnei} before and after LSC was investigated (Table 4-5). The \textit{S. sonnei} population in potato salad was $1.1 \times 10^4$ CFU/ml in the homogenized sample before LSC and $1.9 \times 10^4$ CFU/ml in the supernatant after LSC. The \textit{S. sonnei} population in ground beef was $1.1 \times 10^5$ CFU/ml in the homogenized sample before LSC and $1.6 \times 10^5$ CFU/ml in the supernatant after LSC. In both the potato salad and ground beef samples, the \textit{S. sonnei}
Table 4-5. Effects of low-speed centrifugation on *S. sonnei* populations in sample supernatant. A 25 g sample of potato salad or ground beef was transferred to a clean, sterile 50 ml microcentrifuge tube containing 25 ml *Shigella* broth. The samples were then inoculated with 100 µl of a five-strain *S. sonnei* cocktail that was resistant to the antibiotic rifampicin and the tube was capped tightly. The mixture was homogenized by shaking the tube vigorously for 30 sec. A 1.0 ml aliquot of the supernatant was serially diluted and appropriate dilutions analyzed by pour-plate using tryptic soy agar (TSA) supplemented with rifampicin at 80 ppm (rif+). The remaining sample was centrifuged (100 x g for 5 min) and again a 1.0 ml aliquot of the supernatant was serially diluted and appropriate dilutions analyzed by pour-plate using TSA rif+.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Potato salad Before</th>
<th>Potato salad After</th>
<th>Ground beef Before</th>
<th>Ground beef After</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.98</td>
<td>4.26</td>
<td>5.12</td>
<td>5.16</td>
</tr>
<tr>
<td>2</td>
<td>4.07</td>
<td>4.31</td>
<td>5.05</td>
<td>5.26</td>
</tr>
<tr>
<td>3</td>
<td>4.12</td>
<td>4.29</td>
<td>4.92</td>
<td>5.19</td>
</tr>
<tr>
<td>4</td>
<td>3.99</td>
<td>4.24</td>
<td>4.93</td>
<td>5.18</td>
</tr>
<tr>
<td>5</td>
<td>4.02</td>
<td>4.24</td>
<td>5.07</td>
<td>5.20</td>
</tr>
<tr>
<td>Avg</td>
<td>4.04</td>
<td>4.27</td>
<td>5.03</td>
<td>5.20</td>
</tr>
<tr>
<td>Stdev</td>
<td>0.06</td>
<td>0.03</td>
<td>0.09</td>
<td>0.04</td>
</tr>
</tbody>
</table>

populations in the supernatant following LSC were significantly higher (*P* < 0.05) than those in the homogenized samples prior to LSC. In addition, some solid food particulates were successfully sedimented by LSC. Taken together, these data suggest LSC, as performed in this study, may be used for the separation of some solid food particulates from potato salad and ground beef samples without sedimentation of *S. sonnei* from sample homogenates.

**Development of DNA Sequence Capture (DSC) for the Detection of *S. sonnei***

Two hybridization buffers (HB1 and HB2) were evaluated for use in DSC for the detection of *S. sonnei* in foods (Table 4-6). When a five-strain *S. sonnei* cocktail was analyzed by DSC followed by real-time PCR using HB1 (40°C hybridization temperature) with CP-*Shigella* beads or beads prepared without the addition of a specific probe (unlabeled beads), the cycle number at which the fluorescence exceeded the
Table 4-6. Evaluation of hybridization buffers for DSC for the detection of S. sonnei. One-ml aliquots of a five-strain S. sonnei cocktail or a five-serovar Salmonella cocktail were analyzed by the DSC method using two hybridization buffers (HB1 and HB2) and two beads preparations (CP-Shigella beads and unlabeled beads). In addition, HB1 was investigated with hybridization temperatures of 40°C and 55°C, while HB2 was only investigated at 40°C. The recovered DNA was analyzed by real-time PCR. Each reported Ct value is the average of three replicates.

<table>
<thead>
<tr>
<th>Test</th>
<th>Ct value</th>
<th>HB1 (40°C)</th>
<th>HB1 (55°C)</th>
<th>HB2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg</td>
<td>Stdev</td>
<td>Avg</td>
<td>Stdev</td>
</tr>
<tr>
<td>S. sonnei</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP-Shigella beads</td>
<td>20.4</td>
<td>0.5</td>
<td>19.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Unlabeled beads</td>
<td>25.8</td>
<td>3.2</td>
<td>25.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP-Shigella beads</td>
<td>28.4</td>
<td>1.0</td>
<td>25.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Unlabeled beads</td>
<td>26.8</td>
<td>2.1</td>
<td>28.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

threshold value (Ct value) was 20.4 and 25.8, respectively. The difference in Ct values (5.4 cycles) represented an approx. 100-fold increase in ipaH DNA capture when the CP-Shigella beads were used versus the unlabeled beads. When a five-serovar Salmonella cocktail was analyzed by the DSC method using HB1 with CP-Shigella beads or unlabeled beads, Ct values of 28.4 and 26.8 were observed, respectively. These data suggest that when HB1 is used with the DSC method, S. sonnei and Salmonella DNA bind in a non-specific manner to the unlabeled beads; however the CP-Shigella beads permit specific capture of ipaH DNA.

A higher hybridization temperature (55°C) was also investigated for DSC using HB1. When a five-strain S. sonnei cocktail was analyzed by the DSC method using HB1 (55°C) with CP-Shigella beads or unlabeled beads, Ct values of 19.3 and 25.1 were observed, respectively. The difference in Ct values (5.8 cycles) represented an approx. 100-fold increase in ipaH DNA capture when the CP-Shigella beads were used versus the
unlabeled beads. When a five-serovar *Salmonella* cocktail was analyzed by the DSC method using HB1 (55°C) with CP-*Shigella* beads or unlabeled beads, Ct values of 25.9 and 28.3 were observed, respectively. These data suggest that the higher temperature for hybridization did not prevent non-specific adsorption of *Salmonella* DNA to CP-*Shigella* beads.

When a five-strain *Shigella* cocktail was analyzed by the DSC method using HB2 with CP-*Shigella* beads or unlabeled beads, Ct values of 18.7 and 18.2 were observed, respectively. When a five-serovar *Salmonella* cocktail was analyzed by the DSC method using HB2 with CP-*Shigella* beads or unlabeled beads, Ct values of 21.1 and 21.7 were observed, respectively. These data suggest that when HB2 is used with the DSC method only non-specific capture of *S. sonnei* and *Salmonella* DNA occurs. HB2 was not investigated further in this study.

Streptavidin-coated paramagnetic beads from two manufacturers were investigated for DSC of *S. sonnei* DNA (Table 4-7). A five-strain *S. sonnei* cocktail was analyzed with CP-*Shigella* beads prepared with either Dynabeads M-280 Streptavidin or MagaBeads Streptavidin. As with prior experiments, unlabeled beads of each type were tested as controls. When Dynabeads M-280 Streptavidin were used for DSC, the CP-*Shigella* beads and unlabeled beads resulted in Ct values of 20.5 and 25.7, respectively. When MagaBeads Streptavidin were used for DSC, CP-*Shigella* beads and unlabeled beads resulted in Ct values of 21.5 and 24.0, respectively. Based on differences between Ct values of CP-*Shigella* and unlabeled beads, CP-*Shigella* beads prepared with Dynabeads M-280 Streptavidin performed slightly better for the specific capture of *ipaH* DNA.
Table 4-7. Comparison of paramagnetic beads for use with CP-Shigella beads. One-ml aliquots of a five-strain *S. sonnei* cocktail was analyzed by the DSC method using CP-Shigella and unlabeled beads prepared with streptavidin-coated paramagnetic beads from two manufacturers. The recovered DNA was analyzed by real-time PCR. Each reported Ct value is the average of three replicates.

<table>
<thead>
<tr>
<th>Beads</th>
<th>Ct value</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MagaBeads</td>
<td>Dynabeads</td>
<td></td>
</tr>
<tr>
<td>CP-Shigella beads</td>
<td>21.5</td>
<td>20.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Un-labeled beads</td>
<td>24.0</td>
<td>25.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>

The specific capture of the *ipaH* DNA by CP-Shigella beads in the presence of non-target DNA was also investigated (Table 4-8). A 1.0-ml aliquot from a five-strain *S. sonnei* cocktail (2.3 x 10^8 CFU/ml) and a 1.0-ml aliquot from a five-serovar *Salmonella* cocktail (5.5 x 10^8 CFU/ml) were mixed in a sterile test tube containing 8.0 ml PBS and the resulting solution was analyzed using the DSC method using CP-Shigella beads or unlabeled beads (Table 4-8). Captured *ipaH* DNA analyzed by real-time PCR resulted in amplification at a Ct value of 23.6 when CP-Shigella beads were used and at a Ct value of 29.8 when unlabeled beads were used. This difference in Ct values (6.2 cycles) represented an approx. 100-fold increase in DNA capture when the CP-Shigella beads were used versus the unlabeled beads. Captured *Salmonella* DNA analyzed by real-time PCR resulted in amplification at a Ct value of 30.7 when CP-Shigella beads were used and at a Ct value of 33.3 when unlabeled beads were used. This difference (2.6 cycles) represented a less than 10-fold increase in capture when the CP-Shigella beads were used versus the unlabeled beads. Based on differences of Ct values (7.1 cycles), the CP-Shigella beads captured *ipaH* DNA at an approximate 100-fold increase over *Salmonella* DNA. These data suggest that the CP-Shigella beads bind *ipaH* DNA in a specific
Table 4-8. Specific capture of *ipaH* DNA in the presence of non-target DNA by CP-*Shigella* beads. One-ml aliquots of a five-strain *S. sonnei* and five-serovar *Salmonella* cocktail were analyzed by the DSC method using CP-*Shigella* and unlabeled beads. The recovered DNA was analyzed by real-time PCR. Each reported Ct value is the average of three replicates.

<table>
<thead>
<tr>
<th>Test</th>
<th>Ct value</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg</td>
<td>Stdev</td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP-<em>Shigella</em> beads</td>
<td>23.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Un-labeled beads</td>
<td>30.9</td>
<td>1.2</td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP-<em>Shigella</em> beads</td>
<td>30.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Un-labeled beads</td>
<td>33.3</td>
<td>0.5</td>
</tr>
</tbody>
</table>

manner, however non-specific binding of DNA occurs with reduced efficiency.

In order to determine the sensitivity of the DSC assay, serial dilutions of a five-strain *S. sonnei* cocktail were prepared in PBS and analyzed using the DSC method (Table 4-9). After analysis of captured DNA by real-time PCR, the sensitivity was determined to be $2.3 \times 10^2$ CFU/ml. No amplification was observed from samples diluted to $2.3 \times 10^1$ or $2.3 \times 10^0$ CFU/ml. These data suggest that *S. sonnei* populations at levels as low as $10^2$ CFU/ml may be detected by the DSC method.

**Expression of *ipaH* RNA in Log and Stationary Phase *S. sonnei***

Using a five-strain *S. sonnei* cocktail in 1.0 ml SB microcosms supplemented with HeLa cell extracts, the dye Congo red or neither the extract nor the dye, *ipaH* RNA expression was investigated in stationary and exponential phase cells (Table 4-10). When RNA was extracted from stationary phase cells and analyzed by RT-PCR, no *ipaH* RNA was detected whether the inducers were present or not. In contrast, when RNA was extracted from exponential phase cells and analyzed by RT-PCR, *ipaH* RNA was detected in all of the samples except when HeLa cell extract 3 was used. Visible growth
Table 4-9. Sensitivity of DNA sequence capture method. One-ml aliquots of a five-strain *S. sonnei* were serially diluted in PBS and analyzed by the DSC method using CP-*Shigella* beads. The recovered DNA was analyzed by real-time PCR. Each reported Ct value is the average of three replicates.

<table>
<thead>
<tr>
<th>Cell titer (CFU/ml)</th>
<th>Ct value</th>
<th>Avg</th>
<th>Stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3 x 10^6</td>
<td></td>
<td>26.2</td>
<td>0.7</td>
</tr>
<tr>
<td>2.3 x 10^5</td>
<td></td>
<td>29.8</td>
<td>0.6</td>
</tr>
<tr>
<td>2.3 x 10^4</td>
<td></td>
<td>32.4</td>
<td>0.3</td>
</tr>
<tr>
<td>2.3 x 10^3</td>
<td></td>
<td>37.6</td>
<td>0.8</td>
</tr>
<tr>
<td>2.3 x 10^2</td>
<td></td>
<td>40.6</td>
<td>1.4</td>
</tr>
<tr>
<td>2.3 x 10^1</td>
<td>n/a</td>
<td></td>
<td>n/a</td>
</tr>
<tr>
<td>2.3 x 10^0</td>
<td>n/a</td>
<td></td>
<td>n/a</td>
</tr>
</tbody>
</table>

was achieved after 4 hr incubation in all of the microcosms except those with HeLa cell extract 3, which indicated growth inhibition. Ct values from all microcosms were between 26.5 (HeLa cell extract 4) and 29.0 (HeLa cell extract 1), which did not indicate an increase in *ipaH* expression over the control culture in SB (Ct value of 28.2). These data suggest that for RT-PCR or other RNA amplification procedure to be effective for the detection of *ipaH* RNA, *S. sonnei* must be in exponential phase. Further, these data suggest that none of the HeLa cell extracts or the dye Congo red resulted in up-regulation of the *ipaH* gene to permit detection of very low populations of *S. sonnei*.

**Survival Studies**

**Survival of *S. sonnei* on Smooth Tomato Surfaces**

To evaluate the effect of drying each inoculum cocktail on the surface of the tomatoes, populations were enumerated immediately after inoculation and at 90 min after inoculation (time for inocula to be completely dry on all tomatoes). Compared with initial populations (5.68 log_{10} MPN/tomato), the level of *S. sonnei* (3.23 log_{10} MPN/tomato) was significantly reduced (*P* < 0.05) after the inoculum on tomato surfaces was allowed to dry completely. These data suggest that *S. sonnei* is not resistant to drying on the smooth
Table 4-10. Transcriptional induction of the *ipaH* gene using HeLa cell extracts and the dye Congo red. Protein extracts from HeLa cells (HeLa Extract 1-4) and Congo red were added to *Shigella* broth and the resulting solutions were inoculated with a five-strain *S. sonnei* cocktail. Total RNA was extracted from a 250 µl aliquot of each test solution and analyzed by RT-PCR using *ipaH* gene-specific primers. The remaining portions of the test solutions were incubated at 37°C for 4 hr, after which the total RNA was extracted from a second 250 µl aliquot of each test solution and analyzed by RT-PCR using *ipaH* gene-specific primers. The *S. sonnei* cocktail in SB alone was tested as a control. The Ct values given represent the average of duplicate samples.

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Ct values</th>
<th>Stationary phase</th>
<th>Exponential phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg</td>
<td>Stdev</td>
<td>Avg</td>
</tr>
<tr>
<td>HeLa Extract 1</td>
<td>n/a</td>
<td>n/a</td>
<td>29.0</td>
</tr>
<tr>
<td>HeLa Extract 2</td>
<td>n/a</td>
<td>n/a</td>
<td>27.0</td>
</tr>
<tr>
<td>HeLa Extract 3</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>HeLa Extract 4</td>
<td>n/a</td>
<td>n/a</td>
<td>26.5</td>
</tr>
<tr>
<td>Congo Red</td>
<td>n/a</td>
<td>n/a</td>
<td>28.0</td>
</tr>
<tr>
<td>Control</td>
<td>n/a</td>
<td>n/a</td>
<td>28.2</td>
</tr>
</tbody>
</table>

a no amplification

surfaces of tomatoes.

Significant decreases (*P* < 0.05) in *S. sonnei* populations were observed on days 1 and 2, indicating that *S. sonnei* populations continued to decrease rapidly after drying. No survivors (detection limit of the assay was < 3 MPN/tomato) were detected on days 3 or 5 (Figure 4-6), therefore sampling was terminated at this point. No mold growth was observed on any tomato surface in studies involving *S. sonnei* during five-day observation. These data suggest that tomato surfaces do not support the survival of *S. sonnei* when held at the recommended temperature and relative humidity combination.

Survival of *S. sonnei* in Potato Salad

For studies involving *S. sonnei* survival in potato salad, there was no significant difference (*P* > 0.05) between initial populations inoculated in samples to be stored at
Figure 4-6. Survival of a five-strain *S. sonnei* cocktail on the smooth surfaces of tomatoes. Each individual *S. sonnei* strain was resistant to the bactericidal agent rifampicin by spontaneous adaptation. Inoculated tomatoes were stored at 13°C and 85% relative humidity. Inocula were recovered by placing each tomato into a sterile stomacher bag containing 100 ml phosphate buffered saline and shaking vigorously for 30 sec followed by a 1 min hand massage. Five replicate tomatoes were sampled at each time point. Survivors were enumerated using a three-tube most probable number method in tryptic soy broth supplemented with 100 µg/ml rifampicin. Error bars represent one standard deviation.

2.5°C or 8.0°C (6.03 log10 MPN/g and 5.94 log10 MPN/g, respectively) (Figure 4-7). The potato salad had an initial pH of 5.2, however after 28 days storage at both 2.5°C and 8.0°C, a pH of 4.5 was observed. When samples were stored at 2.5°C, *S. sonnei* populations were significantly lower ($P < 0.05$) than the initial population on days 5, 14, and 28 (5.49 log$_{10}$ MPN/g, 5.52 log$_{10}$ MPN/g, and 5.54 log$_{10}$ MPN/g, respectively). When samples were stored at 8.0°C, *S. sonnei* populations were significantly lower ($P < 0.05$) than the initial population on days 7, 14, and 21 (5.51 log$_{10}$ MPN/g, 4.82 log$_{10}$ MPN/g, and 5.04 log$_{10}$ MPN/g, respectively). *S. sonnei* populations observed on days 3, 5, 7, 14, and 21 were significantly lower ($P < 0.05$) in samples stored at 8.0°C than in samples held at 2.5°C (Figure 4-7). At day 28, however, there was no significant difference ($P > 0.05$) in *S. sonnei* populations in samples stored at 2.5°C or 8.0°C (5.54 log$_{10}$ MPN/g and
Figure 4-7. Survival of five-strain *S. sonnei* cocktail in potato salad. Each individual *S. sonnei* strain was resistance to 100 µg/ml rifampicin by spontaneous adaptation. Inoculated potato salad was stored at either 2.5°C or 8.0°C. Survivors were recovered by transferring 25 g of potato salad into a sterile stomacher bag containing 100 ml phosphate buffered saline and stomaching for 30 sec. Five replicate samples were analyzed at each time point. Survivors were enumerated using a three-tube most probable number method in tryptic soy broth supplemented with 100 µg/ml rifampicin. Error bars represent one standard deviation.

5.67 log_{10} MPN/g, respectively). The increase in surviving *S. sonnei* observed on day 28 in samples stored at 8.0°C may be due to variation in potato salad ingredients among samples (i.e., onions, celery, egg, etc.). These data demonstrate the ability of potato salad to support long-term survival of *S. sonnei* at refrigerated temperatures.

**Survival of *S. sonnei* in Ground Beef**

For studies involving the survival of *S. sonnei* in ground beef, there was no significant difference (*P > 0.05*) between initial populations inoculated in samples to be stored at 2.5°C or 8.0°C (6.06 log_{10} MPN/g and 6.06 log_{10} MPN/g, respectively) (Figure 4-8). The ground beef had an initial pH of 5.6, however after nine days storage at 2.5°C or 8.0°C, a pH of 5.8 and 6.1, respectively, was observed. When samples were stored at
Figure 4-8. Survival of five-strain *S. sonnei* cocktail in ground beef. Each individual *S. sonnei* strain was resistance to 100 µg/ml rifampicin by spontaneous adaptation. Inoculated ground beef was stored at either 2.5°C or 8.0°C. Survivors were recovered by transferring 25 g of ground beef into a sterile stomacher bag containing 100 ml phosphate buffered saline and stomaching for 30 sec. Five replicate samples were analyzed at each time point. Survivors were enumerated using a three-tube most probable number method in tryptic soy broth supplemented with 100 µg/ml rifampicin. Error bars represent one standard deviation.

2.5°C, *S. sonnei* populations were significantly lower (*P* < 0.05) than the initial population on day 1 (5.68 log$_{10}$ MPN/g). When samples were stored at 8.0°C, *S. sonnei* populations were significantly lower (*P* < 0.05) than the initial population on days 1, 7, and 9 (5.65 log$_{10}$ MPN/g, 5.66 log$_{10}$ MPN/g, and 5.49 log$_{10}$ MPN/g, respectively). *S. sonnei* populations observed on days 3 and 9 were significantly lower (*P* < 0.05) in samples held at 8.0°C than in samples held at 2.5°C (Figure 4-8). Sampling was terminated on day 9 due to product spoilage, determined by color and odor. These data demonstrate that ground beef supports survival of *S. sonnei* at refrigerated temperatures beyond the shelf-life of the product.
Evaluation of Detection Methods

Detection of *S. sonnei* in Selected Foods by a Modified FDA *Bacteriological Analytical Manual* (BAM) *Shigella* Culture Method

A modified BAM *Shigella* culture method was investigated for the detection of *S. sonnei* on tomato surfaces and in potato salad and ground beef samples (Table 4-11). For tomato samples inoculated at the $10^4$ CFU/tomato, $10^3$ CFU/tomato, $10^2$ CFU/tomato, $10^1$ CFU/tomato and $10^0$ CFU/tomato levels, *S. sonnei* was detected in 9, 8, 6, 1 and 0 out of 10 samples, respectively. Typical colonies not identified as *S. sonnei* were identified as *Klebsiella pneumoniae* or *Enterobacter* spp. Atypical colonies tested for biochemical reactions were identified as *Enterobacter* spp. or *Citrobacter freundii*.

When potato salad samples inoculated at the $10^2$ CFU/25 g, $10^1$ CFU/25 g and $10^0$ CFU/25 g levels were analyzed by the BAM, *S. sonnei* was detected in 10, 10 and 9 out of 10 samples, respectively. All of the typical colonies selected for confirmation were identified as *S. sonnei* and there were no MAC plates from potato salad samples that contained atypical colonies.

When ground beef samples inoculated at the $10^2$ CFU/25 g, $10^1$ CFU/25 g and $10^0$ CFU/25 g levels were analyzed by the BAM, *S. sonnei* was detected in 7, 2 and 2 out of 10 samples, respectively. The MAC plates from ground beef samples contained extensive growth of atypical colonies. Atypical colonies tested for biochemical reactions were identified as *Enterobacter cloacae*, *Escherichia coli* or *Hafnia alvei*. Typical colonies not identified as *S. sonnei* were identified as *E. cloacae* or *E. coli*.

Taken together, these data suggest that more selective enrichment and isolation media are needed for the conventional culture analysis of foods for *S. sonnei*. 
Table 4-11. Number of samples positive for *S. sonnei* by various detection methods. Smooth tomato surfaces, potato salad, and ground beef were analyzed by the FDA, *Bacteriological Analytical Manual* (BAM) *Shigella* culture method, flow-through immunocapture (FTI) followed by direct plating on MacConkey (MAC) agar (FTI-MAC), FTI followed by real-time PCR (FTI-PCR) and DNA sequence capture (DSC).

<table>
<thead>
<tr>
<th>Food sample</th>
<th>Inoculation level</th>
<th>Detection method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAM, FTI-MAC, FTI-PCR, DSC</td>
<td></td>
</tr>
<tr>
<td>Tomato</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁴ CFU/tomato</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Potato salad</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁴ CFU/tomato</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Ground beef</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁴ CFU/25 g</td>
<td>10</td>
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Detection of *S. sonnei* in Selected Foods by Flow-Through Immunocapture (FTI)

FTI-MAC was investigated for the detection of *S. sonnei* on tomato surfaces and in potato salad and ground beef samples (Table 4-11). For tomato samples inoculated at the 10⁴ CFU/tomato, 10³ CFU/tomato, 10² CFU/tomato, 10¹ CFU/tomato and 10⁰ CFU/tomato levels, *S. sonnei* was detected in 10, 8, 10, 7 and 0 out of 10 samples, respectively. All of the colonies selected for confirmation were identified as *S. sonnei*. Only one MAC plate from a tomato inoculated at the 10³ CFU/tomato level contained atypical colonies identified as *Enterobacter* spp. through biochemical characterization. BRLR models determined the FTI-MAC method achieved greater detection (*P* < 0.05) of inoculated *S. sonnei* on tomato surfaces over the BAM *Shigella* culture method. These data suggest the FTI-MAC method may be used for the analysis of tomato surfaces for the presence of *S. sonnei*. 
When potato salad samples inoculated at the $10^2$ CFU/25 g, $10^1$ CFU/25 g and $10^0$ CFU/25 g levels were analyzed by FTI-MAC, *S. sonnei* was detected in 10, 10 and 8 out of 10 samples, respectively. All of the colonies selected for confirmation were identified as *S. sonnei* and there were no MAC plates containing atypical colonies. BRLR models revealed there was no significant difference ($P > 0.05$) between the FTI-MAC method and the BAM *Shigella* culture method. These data suggest the FTI-MAC method may be used for the analysis of potato salad for the presence of *S. sonnei*.

When ground beef samples inoculated at the $10^2$ CFU/25 g, $10^1$ CFU/25 g and $10^0$ CFU/25 g levels were analyzed by FTI-MAC, *S. sonnei* was detected in 4, 4 and 0 out of 10 samples, respectively. The MAC plates from ground beef samples contained extensive growth of atypical colonies. Atypical colonies tested for biochemical reactions were identified as *E. cloacae*, *E. coli* or *H. alvei*. Typical colonies not identified as *S. sonnei* were identified as *C. freundii*, *E. cloacae* or *E. coli*. BRLR models revealed there was no significant difference ($P > 0.05$) between the FTI-MAC method and the BAM *Shigella* culture method for the detection of *S. sonnei* in ground beef. These data suggest the FTI-MAC method may be used for the analysis of ground beef for the presence of *S. sonnei*, however more selective enrichment/isolation media and anti-*S. sonnei* antibodies are required to effectively eliminate co-isolation of closely-related *Enterobacteriaceae*, such as *E. coli*.

FTI-PCR was investigated for the detection of *S. sonnei* on tomato surfaces and in potato salad and ground beef samples (Table 4-11). For tomato samples inoculated at the $10^4$ CFU/tomato, $10^3$ CFU/tomato, $10^2$ CFU/tomato, $10^1$ CFU/tomato and $10^0$ CFU/tomato levels, *S. sonnei* was detected in 10, 10, 10, 9 and 0 out of 10 samples,
respectively. Bias-reduced logistic regression (BRLR) models determined the FTI-PCR method achieved greater detection \((P < 0.05)\) of inoculated \(S. \text{sonnei}\) on tomatoes over the BAM \(Shigella\) culture method. All FTI-PCR analyses were completed within 24 hr. These data suggest the FTI-PCR method may be used for the rapid detection of \(S. \text{sonnei}\) on tomato surfaces.

When potato salad samples inoculated at the \(10^2\) CFU/25 g, \(10^1\) CFU/25 g and \(10^0\) CFU/25 g levels were analyzed by FTI-PCR, \(S. \text{sonnei}\) was detected in 10, 10 and 8 out of 10 samples, respectively. BRLR models revealed there was no significant difference \((P > 0.05)\) between the FTI-PCR method and the BAM \(Shigella\) culture method. All FTI-PCR analyses were completed within 24 hr. These data suggest the FTI-PCR method may be used for the rapid detection of \(S. \text{sonnei}\) in potato salad.

When ground beef samples inoculated at the \(10^2\) CFU/25 g, \(10^1\) CFU/25 g and \(10^0\) CFU/25 g levels were analyzed by FTI-PCR, \(S. \text{sonnei}\) was detected in 10, 9 and 2 out of 10 samples, respectively. BRLR models determined the FTI-PCR method achieved greater detection \((P < 0.05)\) of inoculated \(S. \text{sonnei}\) in ground beef samples over the BAM \(Shigella\) culture method. All FTI-PCR analyses were completed within 24 hr. These data suggest the FTI-PCR method may be used for the rapid detection of \(S. \text{sonnei}\) in ground beef.

**Detection of \(S. \text{sonnei}\) in Selected Foods by DNA Sequence Capture (DSC)**

DSC was investigated for the detection of \(S. \text{sonnei}\) on tomato surfaces and in potato salad and ground beef samples (Table 4-11). For tomato samples inoculated at the \(10^4\) CFU/tomato, \(10^3\) CFU/tomato, \(10^2\) CFU/tomato, \(10^1\) CFU/tomato and \(10^0\) CFU/tomato levels, \(S. \text{sonnei}\) was detected in 10, 10, 10, 9 and 0 out of 10 samples, respectively. BRLR models determined the DSC method achieved greater detection \((P < \)
0.05) of inoculated *S. sonnei* on tomatoes over the BAM *Shigella* culture method. All DSC analyses were completed within 24 hr. These data suggest the DSC method may be used for the rapid detection of *S. sonnei* on tomato surfaces.

When potato salad samples inoculated at the $10^2$ CFU/25 g, $10^1$ CFU/25 g and $10^0$ CFU/25 g levels were analyzed by DSC, *S. sonnei* was detected in 10, 10 and 10 out of 10 samples, respectively. BRLR models revealed there was no significant difference ($P > 0.05$) between the DSC method and the BAM *Shigella* culture method. All DSC analyses were completed within 24 hr. These data suggest the DSC method may be used for the rapid detection of *S. sonnei* in potato salad.

When ground beef samples inoculated at the $10^2$ CFU/25 g, $10^1$ CFU/25 g and $10^0$ CFU/25 g levels were analyzed by DSC, *S. sonnei* was detected in 10, 10 and 6 out of 10 samples, respectively. BRLR models determined the DSC method achieved greater detection ($P < 0.05$) of inoculated *S. sonnei* in ground beef samples over the BAM *Shigella* culture method. All DSC analyses were completed within 24 hr. These data suggest the DSC method may be used for the rapid detection of *S. sonnei* in ground beef.

BRLR models were also used to compare the DSC method to the FTI-PCR methods for the detection of *S. sonnei* on tomato surfaces and in potato salad and ground beef samples. There was no significant difference ($P > 0.05$) between the DSC method and the FTI-PCR method for the analysis of tomato or potato salad samples; however the DSC method achieved greater detection ($P < 0.05$) of inoculated *S. sonnei* in ground beef samples over the FTI-PCR method. These data suggest that the DSC method may provide superior detection of *S. sonnei* over the FTI-PCR method when closely related

*Enterobacteriaceae*, such as *E. coli*, are present in food.
CHAPTER 5
DISCUSSION AND CONCLUSIONS

Conventional culture methods for the detection of *Shigella* in foods are problematic, in that appropriate selective media are not currently available and *Shigella* spp. are often overgrown by competitive microorganisms during enrichment. This study investigated two alternative sample preparation methods for the detection of inoculated *Shigella sonnei* from tomato surfaces and in potato salad and ground beef. Flow-through immunocapture (FTI) followed by analysis of recovered beads by spread-plate using MacConkey agar (MAC) and by real-time PCR and DNA sequence capture (DSC) were developed. Food samples were inoculated at decreasing levels to determine the lowest detection level for each assay. The FDA *Bacteriological Analytical Manual* (BAM) *Shigella* culture method was performed for comparison to the newly developed methods.

**Preliminary Studies**

Five *S. sonnei* strains were investigated in this study to account for variation among strains. All *S. sonnei* strains were purchased from the American Type Culture Collection (ATCC 9290, ATCC 29031, ATCC 29030, ATCC 25931 and ATCC 29930). *S. sonnei* represents the serogroup of *Shigella* most commonly associated with foodborne outbreaks in North America. According to the Centers for Disease Control and Prevention’s (CDC) Public Health Laboratory Information System (PHLIS), *S. sonnei* accounts for >80% of the *Shigella* isolates in the U.S. in recent years. The most current PHLIS data, however reports that *S. sonnei* isolates in 2004 accounted for only 68.9% of the *Shigella* isolates in
the U.S. It remains to be determined if this decreased incidence of *S. sonnei* will continue in future years.

Three distinctly different food matrices were chosen for analysis by the developed methods: tomatoes, potato salad and ground beef. These foods were chosen due to their association with previous shigellosis outbreaks and/or their chemical/microbiological composition. The tomato can be used as a representative for produce and is the only food matrix chosen in which the inoculum is dried completely prior to analysis. In addition, previous research on detection methods for *Shigella* spp. on tomato surfaces provides a means for direct method comparison (Warren, 2003; Warren *et al*., 2005b). Potato salad is representative of the food category ‘prepared salads’ that have been implicated in previous shigellosis outbreaks (Lew *et al*., 1991; TPH, 2002). Potato salad, composed of carbohydrates (potatoes), proteins (eggs) and lipids (mayonnaise), contains low levels of microorganisms if prepared under sanitary conditions and not subjected to temperature abuse. Ground beef, while high in protein and fat, typically contains higher microbial loads than potato salad and is often contaminated with *E. coli*, a microorganism closely-related to *Shigella*.

**Growth Characteristics of *S. sonnei* in *Shigella* Broth (SB)**

Growth characteristics were determined for each of the five *S. sonnei* strains by performing growth curves in SB incubated at 44°C without shaking. All five strains experienced an initial lag phase of 2 hr prior to entering the exponential phase of growth. The average doubling time of the five *S. sonnei* strains during exponential growth was 18.8 min. The growth data was used to estimate cell populations after *S. sonnei* on inoculated tomatoes, potato salad and ground beef samples were recovered in SB and incubated for short (4-5 hr) periods of time. From these estimates, it was determined
whether *S. sonnei* populations could meet or exceed the detection limits of the newly developed assays in order to facilitate same-day (within 8 hr) detection. Such growth estimates make the assumptions that all of the *S. sonnei* inoculated to the food matrices would be recovered into the SB enrichment and that growth of *S. sonnei* in the presence of food components and indigenous microflora would be similar to that in pure culture. In practice, it is more likely that only a portion of the inoculated *S. sonnei* would be recovered in the analysis of food samples and that these *S. sonnei* would not achieve optimal growth rates in the presence of competitive microorganisms.

The best-case estimates of *S. sonnei* populations in SB enrichments after short incubation times concluded that cell titers required for reliable detection by FTI or DSC in same-day formats could not be achieved with low initial inoculation levels. Instead of determining the minimum enrichment time necessary for reliable detection, an 18-hr enrichment was used to simulate the way the developed methods would be used by commercial diagnostic laboratories. In practice, food samples to be analyzed would be received in the morning or early afternoon and set-up in the late afternoon. The following morning, the detection assay would be performed on the enriched samples.

**Expression and Induction of the *ipaH* Gene of *S. sonnei***

In preliminary experiments, *ipaH* RNA was extracted from stationary and exponential phase *S. sonnei* in SB and analyzed by RT-PCR. The addition of HeLa cell extracts and Congo red dye was tested for their ability to induce *ipaH* expression. Results demonstrated no *ipaH* RNA was detected in stationary phase cells, however *ipaH* RNA was detected in exponential phase cells. The addition of HeLa cell extracts and the dye Congo red was found not to induce *ipaH* expression above that observed in control cultures, with the exception of HeLa cell extract 3, which resulted in no *ipaH* RNA
expression. The SB supplemented with HeLa cell extract 3 showed no visible growth after 4 hr incubation, indicating the extract inhibited the growth of the *S. sonnei* cocktail. Initial *S. sonnei* populations were approx. $2.0 \times 10^6$ CFU/ml and based on growth curve data presented in this study, *S. sonnei* populations after 4 hr incubation were $>10^7$ CFU/ml. From these results it was determined that detection of *S. sonnei* by RT-PCR or other RNA amplification methods, such as nucleic acid sequence-based amplification (NASBA) would require a genetic target other than the *ipaH* gene unless significant pre-enrichment was performed prior to RNA extraction.

**Identification of Potentially *Shigella*-Specific Genetic Loci**

Attempts were made to identify a *Shigella*-specific chromosomally-located gene whose RNA might be useful for detection by RT-PCR or NASBA. In total, nine genetic targets were identified for further analysis using the MBGD and BLASTn software (Table 4-3). Of these, the *ybgD* gene was the only identified target for which primers were developed with specificity for *S. sonnei*, *S. flexneri* and *S. dysenteriae* when tested against the DNA library available at the time (all of those listed in Table 3-3 except KRS308 through KRS316). The *ybgD* gene has been described as a putative fimbrial-like protein, therefore the expression of YbgD is questionable given that none of the sequenced *Shigella* genomes contain intact loci for fimbrial biogenesis (Yang *et al.*, 2005). The *ybgD* gene was found to be expressed in actively growing cells (in SB) when tested by RT-PCR (data not shown). Unfortunately, the addition of nine *E. coli* strains isolated from ground beef during evaluation of the BAM *Shigella* culture method to the DNA library resulted in eight of the nine testing positive for the *ybgD* sequence. Since the *ybgD* sequence was no longer specific to species within the *Shigella*, all further testing of the *ybgD* for use in detection assays was terminated.
Using the BLASTn software for testing the specificity of a given DNA sequence is limited by the number of DNA sequences available in the database. Results from this study are reflective of this; many of the molecular targets when analyzed using BLASTn returned significant hits only with the sequences of *Shigella* spp., however when tested against DNA extracted from the microorganisms in the DNA library these targets were not specific. Furthermore, the BLASTn results often did not match the in house testing for species of *Shigella*. For example, the primers developed for the *ybgD* gene (primer set 01-024) were not homologous to sequences within the *S. dysenteriae* or *S. boydii* genomes when tested using BLASTn, however primer set 01-024 amplified DNA from *S. dysenteriae* ATCC 9361 when tested in house. This is significant as *S. dysenteriae* ATCC 9361 and the *S. dysenteriae* whose genome has been sequenced are both serotype 1 strains.

*E. coli* DNA sequences recently made publicly available (April 2006) would have eliminated most of the identified genetic targets as potentially specific for *Shigella*. At onset of this project, the only *Shigella* genomes available were that of *S. flexneri* 2a strain 301 and *S. flexneri* 2a strain 2457T. Over the course of this work, the genomes of *S. boydii* serotype 4 (strain 227), *S. dysenteriae* serotype 1 (strain 197) and *S. sonnei* (strain 046) have been made publicly available (Yang *et al*., 2005). As genome sequences from additional serotypes/strains of *Shigella* and *E. coli* become available, a more complete and representative *in silico* analysis of gene distribution among the *E. coli/Shigella* species can be performed.

**Survival Studies**

In previous studies where the survival of *Shigella* was investigated on smooth tomato surfaces, samples were inoculated with rifampicin-resistant *Shigella* and rinsed
with 100 ml phosphate buffered saline (PBS) and subjected to the same shake/rub method as employed in the present study (Schneider and Warren, unpublished data). A 1.0 ml aliquot of the PBS rinse was then analyzed by pour-plate using TSA rif+ to determine the number of survivors. The detection limit for this type of method is $1.0 \times 10^2$ CFU/ml tomato rinse, therefore as *Shigella* populations declined below this level it could not be determined if a small, but viable, population had survived. To overcome this problem, the MPN method described in the present study was developed and used to investigate *S. sonnei* survival on tomatoes, in potato salad and in ground beef. The detection limit of the MPN method was $\geq 3$ MPN/ml tomato rinse.

**Rapid *S. sonnei* Inactivation on Tomato Surfaces**

The waxy surface of the tomato is very smooth, unlike the corky surfaces of cantaloupes or potatoes, and without invaginations (as found on oranges) or epidermal and peridermal pores, such as stoma or lenticels, respectively. Survival experiments revealed that *S. sonnei* inactivate rapidly when dried on the waxy surface of a tomato. The inocula took approx. 90 min to dry completely on the tomato surface, during which a 2.45 log$_{10}$ CFU/tomato decrease in *S. sonnei* was observed. After drying, *S. sonnei* continued to die rapidly and no survivors were observed after three days of storage (Figure 4-6). These results were consistent with Islam *et al.* (2001), who reported that no *S. dysenteriae* serotype 1 inoculated on cloth, wood, plastic, aluminum, and glass objects could be recovered after five days by conventional culture methods. In contrast, Spicer (1959) reported *S. sonnei* survival for up to 12 days on cotton threads held at 5-10°C and Nakamura (1962) reported *S. sonnei* survival for up to 14 days on cotton, glass, wood, paper, and metal at various temperatures. Strain variation, holding conditions
(temperature/relative humidity) and surface pH may partially explain the differences in survival observed among these reports.

**S. sonnei Survives in Potato Salad and Ground Beef**

Survival experiments revealed that the potato salad (as prepared in this study) and ground beef support survival of *S. sonnei* over the shelf-life of these products. These observations were in agreement with other reports of *Shigella* spp. survival in similar foods. Rafii and Lunsford (1997) observed *S. flexneri* survival in carrot salad, potato salad, coleslaw and crab salad held at 4°C. While *S. flexneri* populations declined in the carrot and potato salads, significant populations remained at day 11. The pH of the carrot and potato salads used by Rafii and Lunsford (1997) were pH 2.7 to 2.9 and pH 3.3 to 4.4, respectively, whereas the potato salad used in the present study was pH 5.2 to 5.9. Rafii and Lunsford (1997) reported *S. flexneri* survival at higher levels in the coleslaw and crab salads, pH 4.1 to 4.2 and pH 4.4 to 4.5, respectively. No previous reports were found documenting the survival of *Shigella* in raw ground beef, however Islam *et al.* (1993b) reported that cooked beef supported the growth of *S. flexneri* under temperature abuse conditions.

**Evaluation of Detection Methods**

**Recovery of *S. sonnei* by the BAM *Shigella* Culture Method**

The BAM *Shigella* culture method was used to analyze tomatoes, potato salad and ground beef inoculated with decreasing concentrations of *S. sonnei*. Closely related members of the Family *Enterobacteriaceae*, such as *E. coli*, *Enterobacter* spp., *Citrobacter* spp. and *Klebsiella* spp., were observed to grow in SB enriched aerobically at 44°C and resulted in non-specific colonies when SB enrichments were streaked to MAC. This is in agreement with previous studies in which the enrichment media of the BAM
Shigella culture method were investigated for the isolation of S. sonnei from foods (Uyttendaele et al., 2000; Warren et al., 2005a). Excessive presence of background microorganisms on MAC plates from tomato and ground beef samples made identification of S. sonnei colonies difficult and contributed to decreased detection. This highlights the need for more selective media for the isolation of S. sonnei from food.

Analysis of lowest detection levels of the BAM Shigella culture method

The lowest detection level (LDL) and the lowest detection level in which S. sonnei was detected in all 10 of the replicates (LDL100) were determined for the BAM Shigella culture method (Table 5-1). The LDL and LDL100 when inoculated tomatoes were analyzed by the BAM Shigella culture method were 4.9 x 10¹ CFU/tomato and >4.9 x 10⁴ CFU/tomato, respectively. The lack of detection of S. sonnei from all 10 replicate tomatoes at any of the inoculation levels was likely influenced by the effect of drying the inoculum on the tomato surface and/or the presence of competitive microorganisms. In support of this possibility, Flessa et al. (2003) reported the loss of 1.0 log₁₀ CFU when S. sonnei populations in 0.1% peptone were inoculated on glass cover slips and allowed to dry completely (approx. 40 min). The LDL and LDL₁₀₀ when inoculated potato salad was analyzed by the BAM Shigella culture method were 2.6 x 10⁰ CFU/25 g and 2.6 x 10¹ CFU/25 g, respectively. When potato salad samples were analyzed for total aerobic plate count using TSA (data not shown), it was determined that very low levels (<10³ CFU/g) of background microorganisms were present prior to inoculation. It was considered that this contributed to the increased recovery of S. sonnei from potato salad by the BAM Shigella culture method over that observed with ground beef samples. The LDL and LDL₁₀₀ when inoculated ground beef samples were analyzed by the BAM Shigella culture method were 1.1 x 10⁰ CFU/25 g and >1.1 x 10² CFU/25 g, respectively. The
Table 5-1. Lowest detection levels of the BAM *Shigella* culture method. Tomatoes, potato salad and ground beef samples were inoculated with a five-strain *S. sonnei* cocktail at various levels and analyzed using BAM *Shigella* culture method. The lowest detection level (LDL) and lowest detection level in which *S. sonnei* was detected in all 10 of the replicates (LDL<sub>100</sub>) were determined based on initial inoculation levels.

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<th>Food matrix</th>
<th>LDL</th>
<th>LDL&lt;sub&gt;100&lt;/sub&gt;</th>
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<tr>
<td>Tomato (CFU/tomato)</td>
<td>4.9 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>&gt;4.9 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Potato salad (CFU/25 g)</td>
<td>2.6 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>2.6 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ground beef (CFU/25 g)</td>
<td>1.1 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>&gt;1.1 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
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</table>

Presence of high competitive background microorganisms likely contributed to the lack of detection of *S. sonnei* in all 10 replicates at any of the inoculation levels tested in this study. In order to determine the true LDL<sub>100</sub> when the BAM *Shigella* culture method is used to analyze tomatoes or ground beef, this experiment would have to be repeated using higher inoculation levels than were investigated in this study.

In a similar study, Jacobson *et al.* (2002) evaluated the BAM *Shigella* culture method using two strains of *S. sonnei* (strains 357 and 20143) 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 x 10<sup>1</sup> CFU/25 g for all produce types, while LDLs with chill-stressed and freeze-stressed cells were less than 5.2 x 10<sup>1</sup> CFU/25 g for all produce types tested (Jacobson *et al.*, 2002). In another similar study, Warren (2003) reported the LDL of inoculated *S. sonnei* on tomato surfaces when analyzed by the BAM *Shigella* culture method to be 1.9 x 10<sup>2</sup> CFU/tomato. Variation among strains of *S. sonnei* could explain the difference in reported LDLs with the BAM *Shigella* culture method. Taken together, these and the current study demonstrate the importance of using more than one strain when evaluating detection methods for foodborne pathogens.
Evaluation of Flow-Through Immunocapture for the Detection of S. sonnei in Food

Initial attempts were to develop the FTI methods into a same-day format, as was previously reported for Salmonella (Yuk et al., 2006). For same-day enrichments, previous studies used a combination of enrichment media supplemented with yeast extract and shaking incubation to decrease lag phase and increase the growth rate of Salmonella prior to FTI in order to detect very low (10^0 CFU/25 g) inoculation levels (Schneider and Warren, unpublished data). The minimum concentration of S. sonnei ATCC 29930 required for detection by FTI-MAC (2.8 x 10^5 CFU/ml), however was too high to achieve using the short enrichment times available for same-day testing. In contrast, S. sonnei ATCC 25931 was only required at 3.1 x 10^1 CFU/ml for detection by FTI-MAC, therefore short enrichment times may have generated a sufficient cell titer for a same-day test. This demonstrates the importance of using several different strains when evaluating detection methods for foodborne bacteria.

Operational issues with anti-Shigella beads in flow-through immunocapture

Un-coated paramagnetic beads are not available from Matrix MicroScience, therefore other commercially available beads were investigated for use with the Pathatrix system. The beads from Matrix MicroScience, although of proprietary composition, are 0.8 microns in diameter with high (>50%) iron content. The Dynabeads M-270 Epoxy (Invitrogen), 2.8 microns in diameter with ~15% iron content, were investigated for use with the Pathatrix system with poor results. When un-labeled beads were added to the Pathatrix system and circulated (30 min at 37°C) with 250 ml PBS, no visible recovery of beads in the capture phase was observed. It was considered that the increased size and/or lower iron content as compared to the Matrix MicroScience beads prevented the magnetic draw of the Dynabeads M-270 out of the circulating sample. Since both the Matrix
MicroScience and the Dynabeads M-270 beads are polystyrene, it was not considered that the Dynabeads were sticking to components of the tubing system and not returning to the capture phase. Lowering the flow rate may have increased recovery, however the Pathatrix has only three possible flow rates: 280 ml/min (speed 1), 400 ml/min (speed 2) or 500 ml/min (speed 3). The normal flow rate used for sample circulation is speed 2, while washing of recovered beads is performed at speed 1. None of the currently reported Pathatrix protocols utilized speed 3. The use of speed 1 for sample circulation would limit the number of times the sample is passed through the capture phase in a 30 min run time, therefore paramagnetic beads with higher iron content were investigated. The MagaCell beads, approx. 3.0 microns in diameter with 50% iron content, were tested in the Pathatrix system using the normal circulation flow rate. Although the MagaCell beads were similar in diameter to the Dynabeads M-270 beads, the increased iron content facilitated recovery in the Pathatrix system.

Recovery of the anti-\textit{Shigella} beads during FTI experiments was observed to be variable among replicates of the same type of food. For example, when tomato samples were analyzed on the Pathatrix, some samples resulted in visible beads however a few samples did not. A portion of the beads may be immobilized within the capture phase without being visible to the naked eye (Dr. J.P. Coombs, Matrix MicroScience, personal communication). Tomato samples without visibly recovered beads tested positive for \textit{S. sonnei} by the FTI-MAC and FTI-PCR methods, which verified the presence of beads in the capture phase without being visible to the naked eye.

In some of the food samples, anti-\textit{Shigella} beads were observed to be immobilized in an unconventional manner within the capture phase. Normally, the anti-\textit{Shigella} beads
are immobilized within the capture phase at the surface bordering the top of the magnet (Figure 5-1, arrow 1). In some samples, however, some or all of the recovered anti-
*Shigella* beads were immobilized within the capture phase at the surface bordering the side of the magnet (Figure 5-1, arrow 2). It was not determined how this unconventional placement of anti-*Shigella* beads affected immunocapture. The design of the capture phase creates a vortex in the area immediately beyond the magnet and the second baffle, therefore it is likely that target microorganisms may contact anti-*Shigella* beads immobilized at the side of the magnet. Detection of *S. sonnei* was observed in samples, which contained recovered beads only at the side of the magnet, however it could not be determined if the *S. sonnei* cells were bound by the anti-*Shigella* beads before or after they were pushed to the side of the magnet by the sample flow. It should be noted that some immunocapture of *S. sonnei* may have occurred as anti-*Shigella* beads circulated through the Pathatrix system prior to immobilization in the capture phase. This could be tested by immobilizing the anti-*Shigella* beads in the capture phase before adding the sample, however this was not performed in this study.

**Non-specific immunocapture of Enterobacter cloacae and Escherichia coli**

In FTI experiments involving ground beef, non-specific binding of *E. coli* and *E. cloacae* was observed and most likely affected detection of *S. sonnei* at all of the inoculation levels. This non-specific immunocapture was not observed when ground beef was analyzed by FTI-MAC in preliminary experiments. Initially, degradation of the blocking agent (ethanolamine) used during the preparation of anti-*Shigella* beads was suspected of contributing to non-specific binding since preliminary experiments involving FTI with ground beef resulted in specific isolation of *S. sonnei*. The blocking solution (sodium bicarbonate buffer, pH 8.6, containing 0.3% ethanolamine) had been
Figure 5-1. Location of anti-Shigella beads in the capture phase of the Pathatrix during FTI. The direction of flow is indicated by the white dashed arrow. The Pathatrix is designed to immobilize IMS beads at the location shown by arrow 1, however in FTI experiments, beads were often observed to accumulate at the location shown by arrow 2.

prepared in a 50 ml centrifuge tube and held at room temperature unprotected from light.

The anti-Shigella beads were prepared again with freshly made blocking solution and 10 replicates of 25 g ground beef samples were inoculated at $10^2$ CFU/25 g and re-analyzed by FTI-MAC and FTI-PCR. *S. sonnei* was detected in all 10 samples by both methods; however MAC plates continued to contain colonies of *E. coli* and/or *E. cloacae*. Based on these findings, the non-specific immunocapture was most likely due to a shared or very similar epitope among *S. sonnei* and at least some strains of *E. coli* and *E. cloacae*.

Competitive inhibition may have also contributed to poor recovery of *S. sonnei* in ground beef by FTI-MAC. If growth of *S. sonnei* was inhibited during enrichment in SB, the ratio of *S. sonnei* to the competitive microflora after 18 hr would be low. Previous studies involving FTI of *Salmonella* in ground beef determined that when *Salmonella* populations were present in a low ratio to that of competitive background, increased non-
specific capture was observed (Schneider and Warren, unpublished data). However, when the ratio of *Salmonella* to competitive background was higher, non-specific capture was decreased or absent completely (Schneider and Warren, unpublished data). *S. sonnei* was detected in ground beef samples inoculated at the $10^0$ CFU/25 g level by FTI-PCR and DSC, which provided evidence that the growth of *S. sonnei* was not completely inhibited, since some growth would be necessary to reach the detection limit of both assays ($10^5$ CFU/ml and $10^2$ CFU/ml, respectively). Another possibility is that *E. coli* and *E. cloacae* may have achieved higher cell titers than *S. sonnei* when enriched in SB, which would have naturally created the low target-to-non-target ratio that resulted in non-specific immunocapture in previous studies. Competitive inhibition of *S. sonnei* by ground beef microflora could be investigated further using the rifampicin resistant *S. sonnei* subcultures used in the survival studies. Ground beef samples would be inoculated with a cocktail of the rifampicin resistant *S. sonnei*, enriched in SB and then dilutions of the SB enrichment analyzed by pour-plate using TSA and TSA rif+. By comparing the counts from TSA (total plate count in ground beef enrichment) with TSA rif+ (total *S. sonnei* count in ground beef enrichment) it could be determined if growth of *S. sonnei* was affected by the competing microorganisms.

**Analysis of lowest detection levels of the FTI-MAC and FTI-PCR methods**

The LDL and LDL$_{100}$ were determined for the FTI-MAC and FTI-PCR methods (Table 5-2). For tomato samples, the LDL of the FTI-MAC and FTI-PCR methods was $4.9 \times 10^1$ CFU/tomato and the LDL$_{100}$ was $4.9 \times 10^2$ CFU/tomato. Notably, *S. sonnei* was only detected in eight of ten replications inoculated at $4.9 \times 10^3$ CFU/tomato (Table 4-11). This failure to detect *S. sonnei* in all replications could have been due to variation of indigenous microflora among tomatoes. For potato salad samples, the LDL of the FTI-
Table 5-2. Lowest detection levels of the FTI-MAC and FTI-PCR methods. Tomatoes, potato salad and ground beef samples were inoculated with a five-strain *S. sonnei* cocktail at various levels and analyzed using FTI-MAC or FTI-PCR. The lowest detection level (LDL) and lowest detection level in which *S. sonnei* was detected in all 10 of the replicates (LDL100) were determined based on initial inoculation levels.

<table>
<thead>
<tr>
<th>Food matrix</th>
<th>FTI-MAC</th>
<th></th>
<th>FTI-PCR</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDL</td>
<td>LDL100</td>
<td>LDL</td>
<td>LDL100</td>
</tr>
<tr>
<td>Tomato (CFU/tomato)</td>
<td>4.9 x 10^1</td>
<td>4.9 x 10^2</td>
<td>4.9 x 10^1</td>
<td>4.9 x 10^2</td>
</tr>
<tr>
<td>Potato salad (CFU/25 g)</td>
<td>1.5 x 10^0</td>
<td>1.5 x 10^1</td>
<td>1.5 x 10^0</td>
<td>1.5 x 10^1</td>
</tr>
<tr>
<td>Ground beef (CFU/25 g)</td>
<td>1.9 x 10^1</td>
<td>&gt;1.9 x 10^2</td>
<td>1.9 x 10^0</td>
<td>1.9 x 10^2</td>
</tr>
</tbody>
</table>

MAC and FTI-PCR methods was 2.6 x 10^0 CFU/25 g and the LDL100 was 2.6 x 10^1 CFU/25 g. Low indigenous microbial levels could have contributed to the low LDL and LDL100 values observed in potato salad samples. It should be noted that in practical application, potato salad-type products may have been subjected to temperature abuse and therefore may contain far higher microbial counts, which may affect the sensitivity of the FTI-MAC and/or FTI-PCR methods. For ground beef samples, the LDL of the FTI-MAC and FTI-PCR methods were 1.5 x 10^1 CFU/25 g and 1.5 x 10^0 CFU/25 g, respectively. Since *S. sonnei* was not detected in all of the ground beef replications at any of the inoculation levels by FTI-MAC, the LDL100 was reported as >1.5 x 10^2 CFU/25 g. In order to determine the true LDL100 when FTI-MAC is used to analyze ground beef, this experiment would have to be repeated using higher inoculation levels than were investigated in this study. The LDL100 of the FTI-PCR method was 1.5 x 10^2 CFU/25 g. Higher LDL100 values for ground beef samples likely reflect the type and population of indigenous microflora as compared to that of tomatoes and potato salad.
The LDLs of the developed FTI-PCR method meet or exceed many previous reports of PCR detection of *Shigella* spp. in food. Vantarakis *et al.* (2000) reported the detection of *S. dysenteriae* serotype 1 in mussels at $1.0 \times 10^3$ CFU/ml with no pre-enrichment and $1.0 \times 10^{1-2}$ CFU/ml following 22 hr incubation in buffered peptone water using a chemical DNA extraction/ethanol precipitation method. Villalobo and Torres (1998) reported the detection of *S. dysenteriae* serotype 1 in mayonnaise at $1.0 \times 10^{2-3}$ CFU/ml following phenol-chloroform DNA extraction/ethanol precipitation. In a study by Lindqvist (1999), nested PCR in combination with buoyant density centrifugation was able to detect *S. flexneri* at $1.0 \times 10^1$ CFU/g in lettuce, shrimp, milk, and blue cheese samples. Without the use of buoyant density centrifugation prior to nested PCR, the detection limit was $1.0 \times 10^3$ CFU/ml in aqueous solution. Theron *et al.* (2001) investigated a semi-nested PCR for the detection of *S. flexneri* in spiked environmental water samples with detection limits of $2.0 \times 10^3$ CFU/ml for well water, $1.4 \times 10^1$ CFU/ml for lake water, $5.8 \times 10^2$ CFU/ml for river water, $6.1 \times 10^2$ CFU/ml for treated sewage water, and $1.1 \times 10^1$ CFU/ml for tap water following a six hr pre-enrichment in GN broth. Variability in results among the water samples was attributed to the presence of humic substances that inhibited PCR.

The FTI-MAC and FTI-PCR methods did not meet the LDL of a previously reported FTA filtration-nested PCR (FTA-PCR) method for the detection of *S. sonnei* on tomatoes ($7.4 \times 10^0$ CFU/tomato) (Warren, 2003; Warren *et al.*, 2005b). For a description of the FTA-PCR method, the reader is directed to Chapter 2, Literature Review. Survival studies determined that significant numbers of *S. sonnei* die or become sublethally injured when dried completely on the tomato surface. Therefore it is likely that all of the
*S. sonnei* inoculated at the $10^0$ CFU/tomato level in the previous and present studies may have been inactivated or sublethally injured prior to analysis. Due to the nature of FTA filters, naked DNA or sublethally injured *S. sonnei* could still be detected and the sensitivity of the FTA-PCR method did not depend on pre-enrichment of the sample. In contrast, the FTI-MAC and FTI-PCR methods required a lengthy pre-enrichment in which sublethally injured *S. sonnei* may not have recovered. Unlike the FTA-PCR method, however the FTI-MAC method allows for the isolation of viable colonies, a feature that is desirable if further characterization of the detected *Shigella* is required.

**Future research involving flow-through immunocapture for the detection of *S. sonnei***

There are several areas that warrant future research for FTI detection of *S. sonnei* in food. As more specific anti-*Shigella* antibodies become available, the non-specific binding of *E. coli* and *E. cloacae* by the anti-*Shigella* beads observed in the present study may be reduced or eliminated. Only commercially available antibodies were investigated in this report, however there are several reports in the literature that describe specific anti-*Shigella* antibodies (Rahman and Stimson, 2001). Unsuccessful attempts were made to obtain antibodies reported to be specific for *S. sonnei* from other researchers. Another area of future research that could improve the detection of *S. sonnei* by FTI methods is the development of more selective media for the enrichment or isolation of *Shigella* spp. Previously, a chromogenic media for the isolation of *Shigella* (CSPM) was compared to MAC and *Salmonella-Shigella* agar (SSA) for the isolation of *S. sonnei* and *S. boydii* from tomatoes with no significant difference noted among these isolation media (Warren, 2003; Warren et al., 2005a). In the present study, a preliminary experiment compared CSPM and MAC for the isolation of *S. sonnei* from potato salad and ground beef (data not shown). The use of MAC over CSPM resulted in greater isolation rates of *S. sonnei*
from potato salad and ground beef; therefore CSPM was not further evaluated in this study. Finally, the addition of nutrients and/or additives to SB, such as yeast extract, may be investigated for improved recovery of sublethally injured *S. sonnei* from tomato surfaces. In a preliminary study, SB was supplemented with 0.5% yeast extract (SBYE) in an attempt to shorten lag phase and boost the growth rate of *S. sonnei* to generate enough cells to allow detection in a same-day format. It was observed that one tomato inoculated at the $10^0$ CFU/tomato level tested positive by FTI-MAC and FTI-PCR. Further research is necessary to determine if this observation is repeatable and if SBYE may improve the detection of *S. sonnei* from tomato surfaces.

**Evaluation of DNA Sequence Capture (DSC) for the Detection of *S. sonnei* in Food**

**Evaluation of hybridization buffers for DSC**

Two hybridization buffers were evaluated for use with DSC. Hybridization buffer 1 (HB1), a Tris-HCl based buffer, was described by Mangiapan *et al.* (1996) for the detection of mycobacterial DNA from clinical samples. HB1 was chosen for investigation in this study since, unlike other reports of DSC, DNA preparations were crude tissue and cell lysates were prepared directly in HB1, therefore the entire sample was analyzed.

Hybridization buffer 2 (HB2), a sodium-sodium citrate (SSC) based buffer was similar to that described by Tsai *et al.* (2003). In most reports where variations of HB2 were used, DNA was extracted from samples and an aliquot of the extracted DNA was used in DSC methods. In preliminary studies, both HB1 and HB2 resulted in the capture and PCR amplification of *S. sonnei* DNA from SB cultures, potato salad and ground beef samples (data not shown).
Non-specific adsorption of DNA to CP-Shigella beads

When HB1 was used in DSC methods, a consistent and reproducible increase (approx. 100-fold) in detection of *S. sonnei* DNA was observed when CP-Shigella beads were used instead of unlabeled beads. There was, however, non-specific adsorption of DNA to the unlabeled beads. Several variations to the DSC method were investigated for the reduction of non-specific DNA adsorption, including the temperature of hybridization, the type of paramagnetic beads and the bacterial cell titer in the sample to be analyzed. Hybridizations should normally be performed at a temperature 20-25°C below the melting temperature of the probe-target hybrid (Castora and Greene, 1998). The melting temperature of the CP-Shigella probe hybrid was calculated to be 62.6°C (Sigma-Genosys website, 2006); therefore hybridization in the DSC method was performed at 40°C. To reduce non-specific binding, a higher hybridization temperature of 55°C was investigated, however non-specific binding was not reduced (Table 4-8). It was also considered that the type of paramagnetic beads used in the preparation of CP-Shigella beads may affect non-specific binding. Two types of streptavidin-coated paramagnetic beads were investigated, the Dynabeads M-280 Streptavidin and the MagaBeads Streptavidin, however neither resulted in the elimination of non-specific DNA binding (Table 4-7). Based on Ct values from real-time PCR, the Dynabeads M-280 Streptavidin resulted in the largest difference between DNA capture by the CP-Shigella beads and the unlabeled beads, indicating more specific capture of *S. sonnei* DNA. For this reason the Dynabeads M-280 Streptavidin were used for the preparation of CP-Shigella beads in all inoculated trials.

Unexpectedly, the use of HB2 in DSC methods gave poor results with respect to specific capture. Based on Ct values from PCR amplification, there was no difference
between capture of *S. sonnei* DNA by CP-*Shigella* beads and unlabeled beads when HB2 was used in the DSC method (Table 4-6). It was considered that components of HB2, such as sodium dodecyl sulfate (SDS) and/or Sarkosyl, may have disrupted bonds between the streptavidin molecules and the bead surface, the streptavidin and biotin-labeled DNA probes, or both, thereby eliminating the specificity of the CP-*Shigella* beads over that of the unlabeled beads. In contrast, Tsai *et al.* (2003) reported sequence capture of enterotoxigenic *E. coli* using a hybridization buffer similar to HB2, except that 1X SSC was used rather than 5X SSC to avoid stringency problems in subsequent PCR. Non-specific adsorption of DNA to magnetic beads was not observed in this study, however, unlike the present study, hybridization was performed at 85°C. In preliminary studies it was observed that when HB2 was used for DSC, the cell membrane/food material pellet was solubilized during the heating step and in many cases no visible pellet remained after subsequent centrifugation. It was not determined how the additional solutes may or may not have affected hybridization of *S. sonnei* DNA to the CP-*Shigella* beads in food samples.

Several previous studies also report the specific capture of verotoxigenic *E. coli* (Chen *et al.*, 1998) and *Salmonella* and Shiga-like toxin producing *E. coli* (Chen and Griffiths, 2001) using sequence capture followed by PCR. In the first study, specific capture was determined using slot hybridizations, where target and non-target bacterial DNA were cross-linked to positively-charged nylon membranes and hybridized to digoxigenin-labeled capture probes (Chen *et al.*, 1998). In the second study, specificity was determined through PCR amplification of DNA sequences, not by capture of target sequences to the capture probe/bead complexes (Chen and Griffiths, 2001). Neither study
investigated non-specific adsorption of DNA to streptavidin-coated beads with or without the presence of the capture probe.

Mangiapan et al. (1996) reported the use of a two-step capture procedure where biotinylated capture probes were hybridized to target sequences in solution in the first step, then separated from the mixture in a second step by the addition of streptavidin-coated paramagnetic beads to bind the biotin-labeled hybrids. The two-step approach increased the sensitivity of the assay >10-fold over the direct capture method (Mangiapan et al., 1996). There was no mention that non-specific adsorption of DNA was investigated, therefore it is unclear how the two-step capture compares to the non-specific DNA adsorption observed in the present study.

Amagliani et al. (2006) reported specific detection of the hlyA gene of Listeria monocytogenes using NH₂-labeled probes immobilized on amino modified nanoparticles. Specificity was tested using fluorescein-labeled oligonucleotides with complementary and non-complementary sequences to that of the capture probe. Following hybridization, the nanoparticles were washed in buffer and then heated to 80°C for 4 min to dissociate the annealed sequences for spectrophotometric analysis (Amagliani et al., 2006). Supernatants from only hybridizations with complementary fluorescein-labeled oligonucleotides resulted in significant fluorescence; therefore non-complementary fluorescein-labeled oligonucleotides were not hybridized to the probe-labeled nanoparticles.

Analysis of lowest detection levels of the DSC method

The LDL and LDL₁₀₀ were determined for the DSC method (Table 5-3). For tomato samples, the LDL and LDL₁₀₀ were 2.0 x 10¹ CFU/tomato and 2.0 x 10² CFU/tomato, respectively. For potato salad samples, the LDL and LDL₁₀₀ were both 5.6
Table 5-3. Lowest detection levels of the DSC method. Tomatoes, potato salad and ground beef samples were inoculated with a five-strain *S. sonnei* cocktail at various levels and analyzed using DSC. The lowest detection level (LDL) and lowest detection level in which *S. sonnei* was detected in all 10 of the replicates (LDL$_{100}$) were determined based on initial inoculation levels.

<table>
<thead>
<tr>
<th>Food matrix</th>
<th>LDL</th>
<th>LDL$_{100}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato (CFU/tomato)</td>
<td>$2.0 \times 10^1$</td>
<td>$2.0 \times 10^2$</td>
</tr>
<tr>
<td>Potato salad (CFU/25 g)</td>
<td>$5.6 \times 10^0$</td>
<td>$5.6 \times 10^0$</td>
</tr>
<tr>
<td>Ground beef (CFU/25 g)</td>
<td>$4.6 \times 10^0$</td>
<td>$4.6 \times 10^1$</td>
</tr>
</tbody>
</table>

$x \times 10^0$ CFU/25 g. As discussed above for the FTI methods, low indigenous microbial levels likely contributed to positive detection of *S. sonnei* in potato salad samples. For ground beef samples, the LDL and LDL$_{100}$ were $1.5 \times 10^0$ CFU/25 g and $1.5 \times 10^1$ CFU/25 g, respectively. Based on the LDL and LDL$_{100}$ values for ground beef, the DSC method was not affected by the presence of competitive background microorganisms as observed with the FTI methods. For this reason, it is likely that the analysis of temperature abused potato salad containing high levels of background microorganisms by the DSC method would give similar results as seen in the present study.

The LDL values reported in the present study are in agreement with those previously reported for DNA sequence capture of foodborne pathogens (Chen *et al.*, 1998; Chen and Griffiths, 2001; Tsai *et al.*, 2003; Amagliani *et al.*, 2006). Sequence capture followed by PCR was able to detect verotoxigenic *E. coli* at $10^0$ CFU/g ground beef after 15 hr nonselective enrichment (Chen *et al.*, 1998) and *Salmonella* and Shiga-like toxin producing *E. coli* at $10^0$ CFU/ml after 10 hr nonselective enrichment (Chen and Griffiths, 2001). Tsai *et al.*, (2003) reported a detection limit of 1.0 CFU/liter environmental water after filtering the water sample through a 0.45 micron filter,
incubating the filters face-down for approximately 24 hr on selective agar plates and extracting the DNA from suspect colonies prior to sequence capture-PCR. Finally, Amagliani et al. (2006) reported an LDL for *L. monocytogenes* in fluid milk of $1.0 \times 10^1$ CFU/ml after recovering bacterial cells from 10 ml samples by centrifugation and extracting DNA from the resulting pellet. Centrifugation of a 10-ml sample inoculated at $1.0 \times 10^1$ CFU/ml resulted in approx $1.0 \times 10^2$ CFU/sequence capture hybridization, which is in agreement with the sensitivity of the DSC method observed in the present study.

**Future research to improve DSC for the detection of *S. sonnei* in food**

Several areas in which future research may improve DSC for the detection of *S. sonnei* in food include automation of bead handling steps and elimination of non-specific binding. Excessive time and labor was necessary to perform the beads washing steps as described for the DSC method, especially for the number of samples analyzed in one day. The Dynal BeadRetriever (Invitrogen) is an instrument designed for automated IMS enrichment, using magnetic rods to transfer IMS beads from tube to tube (Invitrogen, 2006). Using the BeadRetriever, samples may be analyzed in a little as 20 min, which would greatly improve the number of analyses possible on one day. In addition to automation, the elimination of non-specific adsorption of DNA to CP-*Shigella* beads may improve sensitivity of the DSC method. The presence of large amounts of non-target DNA have been demonstrated to reduce the sensitivity of PCR (Kramer and Coen, 1994), therefore reduction of non-target DNA binding during DSC would likely improve the sensitivity of the overall method. Finally, a longer capture probe (>100 bases) for use with the CP-*Shigella* beads would allow hybridization at temperatures higher than 55°C, which may impact non-specific DNA binding to the beads.
Sources of Variation Among Inoculated Studies

The BAM *Shigella* culture method, FTI and DSC experiments were not performed on the same day; therefore several preparations of potato salad and several different lots of ground beef were used in this study. For this reason, total populations of background microflora and the types of microflora present varied among trials. The effects of this could be seen with the preliminary evaluations of anti-*Shigella* beads in ground beef versus the inoculated trials as described above. Nevertheless, *E. coli* and *E. cloacae* were isolated from all inoculated ground beef trials. For potato salad samples, variations in sugar content among potatoes may have resulted in variable amounts of simple sugars among potato salad samples. Increased sugar content in potato salad samples may result in increased sugar concentrations in SB enrichments. It has previously been reported that the acids produced by microbial fermentation of sugars may inhibit *Shigella* in broth cultures, thus the enrichment media recommended in the BAM contain very little carbohydrate. These sources of variation may be eliminated by performing all of the methods in parallel using potato salad or ground beef samples from the same preparation/purchase.

In addition, potato salad and ground beef samples were inoculated in 50-g aliquots, however only 25 g of the sample was transferred to enrichment after the attachment period. Homogenation of the inocula was by hand using a sterile tongue depressor. For ground beef samples, it was difficult to ensure complete homogenation due to the tendency of ground beef to form together. Furthermore, it was possible that for some of the samples at the low inoculation levels, none of the inoculum was in the 25-g aliquot transferred for enrichment but was rather discarded with the remaining sample. Initially, 50-g samples were inoculated so that each sample could be divided and used for two
methods (FTI and DSC). As the experiments were performed, only one method was investigated on a given day, however the inoculation method was maintained for consistency among trials. Variation due to this type of inoculation method could easily be eliminated by the inoculation of 25 g samples and then using the entire sample for each method.

As with previous studies, tomatoes used in this study were directly out of the field and variations in filth, dirt or sand present on the tomato fruits was observed among shipments (Warren, 2003). The effects of filth, dirt or sand on tomato surfaces on the attachment, recovery and survival of *S. sonnei* has yet to be determined. As previously suggested, an experiment in which recovered *S. sonnei* from both clean and filthy tomatoes are enumerated could provide insight as to the effect of filth, dirt or sand on attachment, recovery and survival (Warren, 2003).

**Practical Applications of Flow-Through Immunocapture and DNA Sequence Capture**

Recently proposed approaches for the detection of *Shigella* in food have combined conventional culture methods with PCR methods to facilitate specific and rapid detection while still providing the isolation of viable colonies. The FTI methods developed in this study fit this type of approach, in that once the anti-*Shigella* beads are recovered, rapid and specific PCR analysis may be performed while at the same time an aliquot of the beads may be analyzed using culture techniques (spread-plate) which would provide isolated colonies. The FTI methods are somewhat limited by the number of samples that could be processed in one day using a single Pathatrix unit, therefore the FTI methods would be most useful where the analysis of a small number of samples (<30) each day is required. In contrast, the DSC method can be automated; therefore rapid and specific analysis of a large number of samples (>50) in a single day is possible. For this reason,
the DSC method would be most useful in laboratories screening large numbers of food samples for the presence of *S. sonnei*. Since the DSC method does not isolate viable colonies, it would not be useful when biochemical or serological characterizations of detected strains are necessary.

**Conclusions**

The results of this study did not support the hypothesis that specific antibodies and/or specific DNA probes may be attached to paramagnetic beads and used for the analysis of foods for the presence of *S. sonnei* with increased sensitivity over the BAM *Shigella* culture method. Potato salad and ground beef support the survival of *S. sonnei* beyond their shelf-life when held under normal refrigerated conditions, while *S. sonnei* declines rapidly on the smooth surfaces of tomatoes held under normal storage conditions. All of the methods developed in this study (FTI-MAC, FTI-PCR and DSC) performed as well or better than the BAM *Shigella* culture method for the detection of *S. sonnei* on tomato surfaces and in potato salad and in ground beef. The FTI-PCR and DSC methods may be used as rapid methods for the detection of *S. sonnei* since final results were obtained within 24 hr. The FTI-MAC method resulted in the isolation of viable *S. sonnei* colonies, however the lack of specificity of commercially available anti-*Shigella* antibodies resulted in the co-isolation of competitive microorganisms, such as *E. coli* and *E. cloacae*. The DSC method was most successful for the detection of *S. sonnei* amid high levels of competitive microorganisms.
APPENDIX A
PREPARATION OF BUFFERS AND SOLUTIONS

Binding/Washing (B/W) Buffer (2X)

Formula: 10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 2.0 M NaCl

1. Calculate amount of stock 1.0 M Tris-HCl, pH 7.5 needed.

\[(1.0 \text{ M}) \times (x \text{ ml}) = (0.01 \text{ M}) \times (200 \text{ ml}) = 2.0 \text{ ml of 1.0 M Tris-HCl, pH 7.5}\]

2. Calculate amount of stock 0.25 M EDTA needed.

\[(0.25 \text{ M}) \times (x \text{ ml}) = (0.001 \text{ M}) \times (200 \text{ ml}) = 0.8 \text{ ml of 0.25 M EDTA}\]

3. Calculate the amount of NaCl needed to make 200 ml.

\[2.0 \text{ mol/L} \times 58.44 \text{ g/mol} \times 0.2 \text{ L} = 23.4 \text{ g NaCl}\]

3. Dissolve 23.4 g NaCl in 197.2 ml DI water.

4. Add 2.0 ml of stock 1.0 M Tris-HCl, pH 7.5, and 0.8 ml of stock 0.25 M EDTA.

5. Add 0.2 ml diethyl pyrocarbonate (DEPC) and shake vigorously.

6. Incubate the solution at room temperature for 1 h.

7. Autoclave at 121°C for 15 min.

Congo Red Solution

Formula: 1 mM Congo red

1. Calculate the amount of Congo Red needed.

\[0.001 \text{ mol/L} \times 696.65 \text{ g/mol} \times 0.2 \text{ L} = 0.14 \text{ g Congo Red}\]

2. Dissolve 3.4 g Congo Red in 200 ml DI water.

3. Filter sterilize the solution by passage through a 0.2 micron filter.

4. Store at room temperature.
Dynabeads Solution A

Formula: 0.1 M NaOH, 0.05 M NaCl

1. Calculate the amount NaOH needed to make 200 ml.
   
   \[
   0.1 \text{ mol/L} \times 40.00 \text{ g/mol} \times 0.2 \text{ L} = 0.8 \text{ g NaOH}
   \]

2. Calculate the amount of NaCl needed to make 200 ml.

   \[
   0.05 \text{ mol/L} \times 58.44 \text{ g/mol} \times 0.2 \text{ L} = 0.58 \text{ g NaCl}
   \]

3. Dissolve 0.8 g NaOH and 0.58 g NaCl in 200 ml DI water.

4. Add 0.2 ml DEPC and shake vigorously.

5. Incubate the solution at room temperature for 1 h.

6. Autoclave at 121°C for 15 min.

Dynabeads Solution B

Formula: 0.1 M NaOH

1. Calculate the amount NaOH needed to make 200 ml.

   \[
   0.1 \text{ mol/L} \times 40.00 \text{ g/mol} \times 0.2 \text{ L} = 0.8 \text{ g NaOH}
   \]

2. Dissolve 0.8 g NaOH in 200 ml DI water.

3. Add 0.2 ml DEPC and shake vigorously.

4. Incubate the solution at room temperature for 1 h.

5. Autoclave at 121°C for 15 min.

Hybridization Buffer 1

Formula: 100 mM Tris-HCl, pH 7.5, 50 mM EDTA, 150 mM NaCl

1. Calculate amount of stock 1.0 M Tris-HCl, pH 7.5 is needed.

   \[
   (1.0 \text{ M}) \times (x \text{ ml}) = (0.1 \text{ M}) \times (100 \text{ ml}) = 10.0 \text{ ml of } 1.0 \text{ M Tris-HCl, pH 7.5}
   \]

2. Calculate amount of stock 0.25 M EDTA is needed.

   \[
   (0.25 \text{ M}) \times (x \text{ ml}) = (0.05 \text{ M}) \times (100 \text{ ml}) = 20 \text{ ml of } 0.25 \text{ M EDTA}
   \]
3. Calculate the amount of stock 3.75 M NaCl needed.

\[(3.75 \text{ M}) \times (x \text{ ml}) = (0.15 \text{ M}) \times (100 \text{ ml}) = 4.0 \text{ ml of 3.75 M NaCl}\]

4. Combine 10.0 ml of stock 1.0 M Tris-HCl, pH 7.5, 20 ml of stock 0.25 M EDTA and 4.0 ml of 3.75 M NaCl with 66.0 ml water.

6. Add 0.1 ml DEPC and shake vigorously.

7. Incubate the solution at room temperature for 1 h.

8. Autoclave at 121°C for 15 min.

**Hybridization Buffer 2**

Formula: 5X Saline-Sodium Citrate (SSC), 0.1% Sarkosyl, 0.02% SDS, 1X Denhardt’s Blocking Reagent

1. Calculate amount of stock 20X SSC needed.

\[(20X) \times (x \text{ ml}) = (5X) \times (100 \text{ ml}) = 25.0 \text{ ml of 20X SSC}\]

2. Calculate amount of stock 1.0% Sarkosyl needed.

\[(1.0\%) \times (x \text{ ml}) = (0.1\%) \times (100 \text{ ml}) = 10.0 \text{ ml of 1.0\% Sarkosyl}\]

3. Calculate the amount of stock 1.0% SDS needed.

\[(1.0\%) \times (x \text{ ml}) = (0.02\%) \times (100 \text{ ml}) = 2.0 \text{ ml of 1.0\% SDS}\]

4. Calculate the amount of stock 50X Denhardt’s Blocking Reagent needed.

\[(50X) \times (x \text{ ml}) = (1X) \times (100 \text{ ml}) = 2.0 \text{ ml of 50X Denhardt’s Blocking Reagent}\]

5. Combine 25.0 ml 20X SSC, 10.0 ml 1.0% Sarkosyl, 2.0 ml 1.0% SDS and 2.0 ml 50X Denhardt’s Bocking Reagent with 61.0 ml water.

6. Add 0.1 ml DEPC and shake vigorously.

7. Incubate the solution at room temperature for 1 h.

8. Autoclave at 121°C for 15 min.
Low-Salt Wash Buffer

Formula: 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA

1. Calculate amount of stock 1.0 M Tris-HCl, pH 7.5 is needed.
   \[(1.0 \text{ M}) \times (x \text{ ml}) = (0.02 \text{ M}) \times (200 \text{ ml}) = 4.0 \text{ ml} \text{ of 1.0 M Tris-HCl, pH 7.5}\]

2. Calculate amount of stock 0.25 M EDTA is needed.
   \[(0.25 \text{ M}) \times (x \text{ ml}) = (0.001 \text{ M}) \times (200 \text{ ml}) = 0.8 \text{ ml} \text{ 0.25 M EDTA}\]

3. Calculate the amount of NaCl needed.
   \[0.15 \text{ mol/L} \times 58.44 \text{ g/mol} \times 0.2 \text{ L} = 1.8 \text{ g NaCl}\]

4. Dissolve 1.8 g NaCl in 195.2 ml DI water.

5. Add 4.0 ml of stock 1.0 M Tris-HCl, pH 7.5, and 0.8 ml of stock 0.25 M EDTA.

6. Add 0.2 ml DEPC and shake vigorously.

7. Incubate the solution at room temperature for 1 h.

8. Autoclave at 121°C for 15 min.

Phosphate Buffered Saline, pH 7.4

Formula: 0.058 M Na₂HPO₄, 0.01 M NaH₂PO₄, 0.085 M NaCl

1. Calculate the amount of Na₂HPO₄ needed to make 1 L.
   \[0.058 \text{ mol/L} \times 141.08 \text{ g/mol} = 8.2 \text{ g Na₂HPO₄}\]

2. Calculate the amount of NaH₂PO₄ needed to make 1 L.
   \[0.01 \text{ mol/L} \times 119.96 \text{ g/mol} = 1.2 \text{ g NaH₂PO₄}\]

3. Calculate the amount of NaCl needed to make 1 L.
   \[0.085 \text{ mol/L} \times 58.44 \text{ g/mol} = 5.0 \text{ g NaCl}\]

4. Dissolve 8.2 g Na₂HPO₄, 1.2 g NaH₂PO₄, and 5.0 g NaCl in 1 L DI water.

5. Adjust pH if necessary using 1.0 N NaOH or 1.0 N HCl.

6. Autoclave at 121°C for 15 min.
**Sodium Acetate Buffer, pH 4.0**

Formula: 0.1 M NaC\textsubscript{2}H\textsubscript{3}O\textsubscript{2} glacial acetic acid to pH 4.0

1. Calculate the amount of NaC\textsubscript{2}H\textsubscript{3}O\textsubscript{2}*3H\textsubscript{2}O needed to make 250 ml.

\[0.1 \text{ mol/L} \times 136.08 \text{ g/mol} \times 0.25 \text{ L} = 3.4 \text{ g NaC}_2\text{H}_3\text{O}_2*3\text{H}_2\text{O}\]

2. Dissolve 3.4 g NaC\textsubscript{2}H\textsubscript{3}O\textsubscript{2}*3H\textsubscript{2}O in 200 ml DI water.

3. Titrate to pH 4.0 with glacial acetic acid.

4. Adjust final volume to 250 ml using DI water.

5. Filter sterilize the solution by passage through a 0.2 micron filter.

**Sodium Bicarbonate Buffer, pH 8.6**

Formula: 0.1 M Na\textsubscript{2}CO\textsubscript{3}, 0.1M NaHCO\textsubscript{3}

1. Make stock solution of 0.1M Na\textsubscript{2}CO\textsubscript{3} (pH 8.07).

\[0.1 \text{ mol/L} \times 105.99 \text{ g/mol} = 10.6 \text{ g Na}_2\text{CO}_3/\text{L}\]

2. Make stock solution of 0.1 M NaHCO\textsubscript{3} (pH 11.0).

\[0.1 \text{ mol/L} \times 84.01 \text{ g/mol} = 8.4 \text{ g NaHCO}_3/\text{L}\]

3. Transfer 100 ml 0.1 M NaHCO\textsubscript{3} and titrate to pH 8.6 (about 4.0 ml).

4. Filter sterilize the solution by passage through a 0.2 micron filter.

**Wash Buffer**

Formula: 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA

1. Calculate amount of stock 1.0 M Tris-HCl, pH 7.5 is needed.

\[(1.0 \text{ M}) \times (x \text{ ml}) = (0.02 \text{ M}) \times (200 \text{ ml}) = 4.0 \text{ ml of 1.0 M Tris-HCl, pH 7.5}\]

2. Calculate amount of stock 0.25 M EDTA is needed.

\[(0.25 \text{ M}) \times (x \text{ ml}) = (0.001 \text{ M}) \times (200 \text{ ml}) = 0.8 \text{ ml 0.25 M EDTA}\]

3. Calculate the amount of NaCl needed.

\[0.5 \text{ mol/L} \times 58.44 \text{ g/mol} \times 0.2 \text{ L} = 5.9 \text{ g NaCl}\]
4. Dissolve 5.9 g NaCl in 195.2 ml DI water.

5. Add 4.0 ml of stock 1.0 M Tris-HCl, pH 7.5, and 0.8 ml of stock 0.25 M EDTA.

6. Add 0.2 ml DEPC and shake vigorously.

7. Incubate the solution at room temperature for 1 h.

8. Autoclave at 121°C for 15 min.
APPENDIX B
ALIGNMENT OF CHROMOSOMALLY-LOCATED *ipaH* GENES OF *Shigella sonnei*

ClustalW was used to align the sequences of the five chromosomally-located *ipaH* genes of *S. sonnei* Ss046. The ClustalW output is given below with the conserved bases designated with asterisks (*).

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ipaH1  TGACGAGGTACTGGCCCTGCGATTGTCTGAAAACGGCTCACAACTGCACCATTCATAA 1764

*******************************************
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Benjamin Ray Warren was born in Brandon, FL, on June 29, 1975. In 1998, he received his Bachelor of Science from the University of Florida in food science. After graduation he took the position of Food Safety and Product Development Manager with Blood’s Hammock Groves, Inc., a grower/shipper/processor of fresh Florida citrus. In 2003, he received his Master of Science from the University of Florida in food science where his thesis won the IFAS Award of Excellence for Graduate Research. The author worked part-time at Deibel Laboratories of Gainesville, Inc. during his master’s and doctoral research.

On May 23, 2004, the author married Nicole Leigh Sanson at the Baughman Center on the University of Florida campus. On May 2, 2006, the author and his wife celebrated the birth of their first son, Zachary Ray Warren. Upon completion of his doctoral degree, the author plans to pursue a career in research and academics in food microbiology.