REAL-TIME PCR DETECTION OF *PAENIBACILLUS* TO PREDICT THE SHELF-LIFE OF FLUID MILK AND DEVELOPMENT OF A PCR AND SEQUENCE BASED METHOD TO SEROTYPE *SALMONELLA*

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Matthew Louis Ranieri

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REAL-TIME PCR DETECTION OF *PAENIBACILLUS* TO PREDICT THE SHELF-LIFE OF FLUID MILK AND DEVELOPMENT OF A PCR AND SEQUENCE BASED METHOD TO SEROTYPE *SALMONELLA*

Matthew Louis Ranieri, Ph.D.

Cornell University 2013

To improve the quality of commercial dairy ingredients and consumer products, including cheese, fluid milk, milk powders and others, it is important to identify and then control factors that contribute to their degradation. Tracking and eliminating sporeforming bacteria is a particular concern, as these organisms can resist many processing hurdles. Psychrotolerant sporeformers, specifically *Paenibacillus* spp., are important spoilage bacteria for pasteurized, refrigerated foods such as fluid milk. A real-time PCR assay targeting 16S rDNA was designed to detect *Paenibacillus* spp. in fluid milk and to discriminate between *Paenibacillus* and other closely related sporeforming bacteria. Specificity was confirmed using 16 *Paenibacillus* and 17 *Bacillus* isolates. All 16 *Paenibacillus* isolates were detected with a mean cycle threshold (Ct) of 19.14 ± 0.54. While 14/17 *Bacillus* isolates showed no signal (Ct > 40), 3 *Bacillus* isolates showed very weak positive signals (Ct = 38.66 ± 0.65). The assay provided a detection limit of approximately 3.25 ! 10^{1} CFU/ml using total genomic DNA extracted from raw milk samples inoculated with *Paenibacillus*. Application of the TaqMan PCR to colony lysates obtained from heat-treated and enriched raw milk provided fast and accurate detection of *Paenibacillus.* Heat-treated milk samples where *Paenibacillus* (" 1 CFU/ml) were detected by this

colony TaqMan PCR showed high bacterial counts (> 4.30 log CFU/ml) after refrigerated storage (6°C) for 21 days. We thus developed a tool for rapid detection of *Paenibacillus* that has the potential to identify raw milk with microbial spoilage potential as a pasteurized product.

Replacement of traditional serotyping methods with molecular approaches is particularly important for *Salmonella,* which includes >2,500 different serotypes. We evaluated the ability of PFGE, rep-PCR, ribotyping, and MLST to predict serotypes for a set of 46 isolates, which were identified to represent the top 40 reported *Salmonella* from human and non-human sources reported by the Centers for Disease Control and World Health Organization. MLST was most reliable and able to accurately predict serotypes for 42/46 isolates representing the top 40 serotypes. PFGE, ribotyping, and rep-PCR were able to accurately predict 35/46, 34/46 and 30/46 serotypes, respectively. We also integrated a number of available data sources to develop and validate a PCR-based O-antigen screen with sequencing of internal *fliC* (H1 antigen) and *fljB* (H2 antigen) fragments to characterize *Salmonella* isolates to the serotype level. PCR and sequence based serotyping correctly identified 42/46 common serotypes. We continued to test our method against a selection of 70 less common *Salmonella* serotypes and were able to accurately predict 62/70 *Salmonella* serotypes. This study provides an initial comparison of the ability to identify *Salmonella* serotypes using (i) different molecular methods that predict serotypes based on banding patterns or phylogenetic relationships and (ii) a combined PCR and sequencing based approach that directly targets O and H antigen encoding genes.

BIOGRAPHICAL SKETCH

Matthew L. Ranieri was born in Syracuse, NY and grew up nearby in Elbridge, NY. He later attended Jordan-Elbridge High School, where he was captain of the soccer, basketball, and golf teams. Matthew graduated from Cornell University in Ithaca, NY in 2006 with a Bachelor of Science degree in Food Science. After graduating, he returned to Cornell University for graduate research in Food Science, and completed his Masters degree in 2009. He currently resides in Tigoni, Kenya with his spouse, Christina Crawford.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

IMPROVING THE QUALITY AND SAFETY OF FOOD

Despite advances in food preservation techniques, bacterial spoilage remains a leading cause of global food loss (Gram et al., 2002). Nearly one-third of all food produced worldwide is estimated to be lost post-harvest, much of which can be attributed to microbial spoilage (Gustavsson et al., 2011). Dairy products constitute one of the leading sectors impacted by food loss in the US (Kantor et al., 2007). With increasing demand for fluid milk, cheese, and dairy powders, processors must understand the role that spoilage bacteria play in product quality, as undesirable microbial growth can result in direct economic losses from spoiled products as well as a loss of sales to customers who may choose alternative products. Our review on tracking and eliminating dairy associated organisms highlights the main challenges that spoilage bacteria present to the dairy industry, with an emphasis on sporeforming bacteria. Developing an understanding of dairy spoilage bacteria, including their growth characteristics and transmission, is essential for implementing practical control methods that are necessary for extending the shelf lives of dairy products.

The US dairy industry has a particular interest in fluid milk spoilage, as nearly 20% of conventionally pasteurized (high temperature short time; HTST) fluid milk is discarded prior to consumption each year (Kantor et al., 1997). In the US, the shelf-life of fluid milk ranges from approximately 1-3 weeks. Most consumer complaints result from the growth of psychrotolerant bacteria, typically either non-sporeforming Gram-negative rods or Gram-positive sporeforming bacteria (Fromm and Boor, 2004; Hayes et

al., 2002; Huck et al., 2008; Ranieri and Boor 2009). In the absence of non-sporeforming Gram-negative rods (e.g., *Pseudomonas* spp.), Gram-positive psychrotolerant sporeformers can survive pasteurization as spores, germinate, and then grow during refrigerated storage to numbers capable of causing off-flavors or curdling of milk (De Jonge et al., 2010(Huck et al., 2007; Ranieri et al., 2009; Ranieri and Boor, 2009; De Jonghe et al., 2010).

The predominant Gram-positive sporeforming bacteria isolated from milk are *Bacillus* spp. and *Paenibacillus* spp. During refrigerated storage of pasteurized milk, *Paenibacillus* spp. become the predominant spoilage organisms, typically representing over 95% of the bacterial population identified late in shelf-life (> 10 days) (Ranieri and Boor, 2009). *Paenibacillus* spp. are generally present in very low numbers in raw milk and early in pasteurized milk shelf-life, yet can reproduce to high numbers during cold-storage. Numerous microbiological tests have been applied to raw milk with the goal of predicting shelf-life performance of the milk, but none are adequately predictive of HTST pasteurized fluid milk shelf-life (Martin et al., 2011). The aim of our second study was to develop a novel PCR assay targeting 16S rDNA so that specific identification of *Paenibacillus* spp. could be performed rapidly. The objectives of this study were to: (i) design primers and a probe for detection of *Paenibacillus* spp. while limiting non-specific detection of closely related *Bacillus* spp. (ii) validate primers and probe using a real-time PCR assay on select *Paenibacillus* and *Bacillus* isolates from a collection of over 1200 isolates from fluid milk and dairy environments, and (iii) develop a systematic approach to aid in identification of *Paenibacillus* spp. from raw milk. The results of this study provide the food industry with an assay to monitor the quality of raw milk.

In addition to food spoilage concerns, food safety continues to affect all aspects of the farm to fork continuum. In the US alone, it was recently estimated that the economic burden from health losses due to foodborne illness totals over $77 billion annually (Scharff, 2012). The identification of *Salmonella* serotypes remains an important public health concern as non-typhoidal *Salmonella* causes an estimated 93.8 million cases of gastroenteritis globally each year (Majowicz et al., 2010). In the US, the CDC estimates that non-typhoidal *Salmonella* accounts for 1.03 million cases of gastoenteritis, 19,000 hospitalizations, and 378 deaths annually, making it a leading cause of foodborne illness (Scallan et al., 2011). The financial burden of foodborne illnesses is also substantial, as the annual economic cost of non-typhoidal *Salmonella* infections, not including costs to the government or food industry, totals over $4.4 billion dollars in the U.S. (Scharff, 2012). To better understand its transmission throughout the food chain and to aid in epidemiological investigations, accurate discrimination of *Salmonella* spp. is critical.

The genus *Salmonella* is divided into two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is divided into 6 subspecies, including subspecies I (*enterica*), II (*salamae*), IIIa (*arizonae*), IIIb (*diarizonae*), IV (*houtenae*), and VI (*indica*) (Grimont and Weill, 2007). The traditional method of subtyping *Salmonella* below the subspecies level has been serotyping, which has been applied for over 70 years (Grimont and Weill, 2007; Guibourdenche et al., 2010). Currently, there are over 2,500 known serotypes, with the majority (over 1,500) belonging to S. *enterica* subsp. *enterica*, which is also the group having clinical relevance due to its common isolation from humans and warm-blooded animals (CDC, 2011).

Classical serotyping is performed according to the White-Kauffmann-Le Minor scheme, which identifies the somatic (O) and flagellar (H) antigens based on agglutination of bacteria with specific sera. Despite its widespread use, traditional serotyping has a number of drawbacks. Serotyping of *Salmonella* takes at least 3 days to complete, is labor intensive, requires maintenance of over 250 typing sera as well as 350 different antigens, and is unable to type rough or mucoid strains. Furthermore, serotyping is often not sensitive enough to provide the level of discrimination needed for foodborne illness outbreak investigations, and cannot be used to infer phylogenetic relationships. As a result of many traditional serotyping pitfalls, researchers have investigated a number of alternative methods to subtype *Salmonella*.

The purpose of our study was to develop and test a PCR and sequence based approach for predicting *Salmonella* serotypes that was at least as reliable at predicting serotypes as existing subtyping methods. We first evaluated PFGE, rep-PCR, ribotyping, and MLST for their ability to predict serotypes for 46 isolates representing clinically relevant *Salmonella* serotypes. Then, we characterized the same set of 46 isolates using PCR and sequencing, plus an additional set of 70 isolates that represented less common *Salmonella* serotypes. Our PCR and sequencing based approach allows for continuity with traditional serotyping data, reduces the need for expensive or proprietary equipment, and could be integrated into an open-source web-based database permitting review of sequence data for enhanced accuracy.

CHAPTER TWO

TRACKING AND ELIMINATING SPOREFORMERS IN DAIRY SYSTEMS[1]

INTRODUCTION

One challenge encountered with the production of dairy foods is the potential for product spoilage from microbial growth. Undesirable microbial growth can cause immediate, direct economic losses from spoiled products as well as long-term loss of sales to consumers who may choose to avoid consumption of food products associated with an unpleasant experience. Due to the highly perishable nature of dairy products, maintenance of product quality requires considerable attention to detail (Pasteurized Milk Ordinance, 2009). Many factors directly influence shelf-life, including raw milk quality, processing and handling parameters at the plant, equipment cleaning and maintenance, and temperature control throughout the entire dairy continuum. A lapse in control at any point can result in loss of product through bacterial spoilage. Development of an understanding of dairy spoilage bacteria, including their growth characteristics and transmission, is essential for implementing practical control methods necessary for extending the shelf lives of dairy products.

Of spoilage microorganisms of importance to the dairy industry, sporeforming bacteria represent a group that may be the most diverse and difficult to combat. These microbes can cause spoilage across the full spectrum of dairy products. To illustrate, the presence of sporeforming bacteria is associated with late blowing gas defects in some cheese products (Klijn et al., 1995; Quiberoni et al., 2008) reduced shelf-life in ultra-high

[1] Published in the Australian Journal of Dairy Technology (Ranieri and Boor, 2010).

temperature (UHT) fluid milk (Hammer et al., 1995; Scheldeman et al., 2006) and increased spoilage of valued-added products when milk powders contaminated with spores are used as food ingredients (Scott et al., 2007). Some sporeforming bacteria can survive high-temperature-short-time (HTST) pasteurization conditions, and subsequently grow in milk stored at refrigeration temperatures, thus limiting pasteurized fluid milk shelf-life. So, how does the dairy industry battle ubiquitously present microbes that can exist in a dormant spore state that enables bacterial survival in the presence of multiple stresses, such as heat, drying, and acid exposure, but then promotes germination and subsequent multiplication to large numbers when present in a favorable environment (our food)?

One challenge for the dairy industry is to identify route(s) of entry for spoilage bacteria, including sporeformers, into food products. When spoilage microbes are typically present at very low initial levels in the food products, then development of effective control measures also requires an understanding of the number of microbes necessary to cause spoilage. Further, not all members of a bacterial genus are equally likely to be present in a dairy processing system or to cause product loss through spoilage. Therefore, analytical approaches that enable accurate differentiation among closely related bacterial strains (i.e., subtyping) as well as quantification are essential for development and implementation of effective intervention strategies. Emerging molecular tools have allowed researchers to track transmission of various microorganisms in food production and processing systems, from farms to processing plants (Nightingale et al., 2004; Thimothe et al., 2004) and beyond. In the case of dairy product spoilage, molecular tools also have been used to identify some key sporeforming

bacteria (e.g., Fromm and Boor 2004; Huck et al., 2007a). Ultimately, the ability to control spoilage bacteria – and particularly sporeforming bacteria – that have the potential to grow in dairy products under typical storage conditions, will reduce product loss and improve customer satisfaction by ensuring provision of nutritious, safe, high-quality consumer foods.

QUALITY AND SHELF-LIFE OF FLUID MILK

While many tests have been developed to assess raw milk quality (somatic cell count, standard plate count, coliform count, preliminary incubation count, psychrotrophic bacteria count, etc.; Frank and Yousef 2004), an important practical question is whether or not the results from these tests predict raw milk performance post-pasteurization. Some processors have chosen to use the preliminary incubation (PI) test as a predictor of pasteurized product shelf-life. The PI test involves holding raw milk at 55°F (12.8°C) for 18 hours prior to performing a standard plate count. The theory behind the test is that preliminary incubation will enable detection of bacterial contaminants in milk (from dirty equipment, soil, etc.) that may be able to multiply during storage of raw milk, prior to pasteurization. To test the predictive ability of the PI test for the performance of the pasteurized product, commingled raw milk from dairy plant silos and corresponding commercially pasteurized milk samples were collected from four NYS fluid milk processing plants (Woodcock and Boor, unpublished). Raw milk samples were subjected to a number of microbiological tests, including: somatic cell count, coliform count, lab pasteurization count, psychrotrophic bacteria count, spore count and preliminary incubation count. All pasteurized milk samples were held at 6°C and tested for coliform

and standard plate counts at 1, 10, 14, and 21 days post-pasteurization. Additionally, all pasteurized milk samples were evaluated for flavor characteristics by a trained sensory panel on days 1, 10, 14 and 21 post-pasteurization. None of the raw milk tests, including the PI test, were effective in predicting the post-pasteurization performance of the raw milk as reflected by the shelf-life characteristics of the pasteurized products. In the absence of post-pasteurization contamination, psychrotolerant gram-positive sporeforming microbes were responsible for limiting the shelf-lives of the commercially pasteurized products. However, no currently existing raw milk assay will rapidly and accurately quantify the presence of this group of microbes. Therefore, a new raw milk test, capable of quantifying very low levels of psychrotolerant sporeforming microbes, would be an invaluable tool for the dairy industry.

Sporeforming bacteria are responsible for multiple concerns regarding the safety and quality of dairy products. For example, sporeforming bacteria can produce enzymes that degrade milk components to yield objectionable off-flavors, generate gas that can cause structural defects in cheese and produce toxins that can cause human illness (De Jonghe et al., 2010). As fluid milk handling and processing are central to dairy foods manufacturing, the following discussion is focused on understanding the role of sporeforming bacteria from farm to finished product in HTST pasteurized fluid milk products.

In the United States, the shelf-life of conventionally processed HTST pasteurized fluid milk is approximately 2-3 weeks (Fromm and Boor 2004; Carey et al., 2005; Gandy et al., 2008; He et al., 2009; Ranieri and Boor 2009). The perishable nature of fluid milk products contributes to significant product loss throughout the food processing and

handling continuum, representing approximately 20% of all foods lost by US processors, retailers and consumers (Kantor et al., 1997). Many factors have been identified that affect pasteurized milk quality and shelf life, including the microflora of the raw milk supply, design and handling parameters at the processing plant, cleaning, sanitation and maintenance programs, and control of the finished product through the retail distribution chain (Carey et al., 2005). Stringent emphasis on cleaning and sanitation measures in processing plants is essential for control of post-pasteurization bacterial contamination, a frequent cause of reduced product quality that can result in dramatic limitations in fluid milk shelf-life (Hayes et al., 2002; He et al., 2009; Ranieri and Boor 2009). When post-pasteurization contaminants are successfully eliminated from fluid milk processing systems, the next biological barrier to further shelf-life extension of HTST-processed fluid milk products beyond approximately 21 days becomes evident. This barrier is the presence of psychrotolerant, sporeforming spoilage bacteria, particularly *Bacillus* and *Paenibacillus* spp. (Ralyea et al., 1998; Fromm and Boor 2004; Huck et al., 2007b; Ranieri and Boor, 2009).

Bacillus spp. represent a diverse group of aerobic or facultatively anaerobic, rod shaped, gram-positive, sporeforming bacteria (Logan and De Vos 2009). They exhibit a wide range of physiological abilities, with some strains able to tolerate extreme temperature, pH and salt conditions. *Bacillus* spp. can be isolated from soil or from environments contaminated with soil, which explains their presence on dairy farms and in processing plants. The spores of *Bacillus* are particularly troublesome, as they can exhibit extreme resistance to heat, radiation, disinfectants and dessication (Logan and De Vos 2009).

Paenibacillus spp. are also aerobic or facultatively anaerobic, rod shaped sporeforming bacteria. *Paenibacillus* spp. have been only recently recognized as a genera distinct from *Bacillus* spp**.** (Ash et al., 1993). Traditional microbiological methods do not differentiate *Bacillus* spp. and *Paenibacillus* spp., which may at least partially explain the relative absence of dairy industry literature on *Paenibacillus* spp. As a further complication, while *Paenibacillus* spp. are considered gram-positive based on their cell wall structure, in a gram stain, they frequently appear to be gram-variable (both purple and pink) or even gram-negative (pink) (Huck et al., 2007b). The natural habitat of *Paenibacillus* spp. is soil, and they are considered to have an important role in composting plant material through the excretion of extracellular enzymes. Typical isolation practices for *Paenibacillus* and *Bacillus* spp. from environmental samples exploit their sporeforming characteristics. In general, samples are heated to destroy non-sporeforming microbes and to stimulate spore germination, thus encouraging multiplication of vegetative *Bacillus* and *Paenibacillus* spp. in the sample (Priest 2009). Spore recovery from raw milk is reported to be optimal following heat treatment at 80°C for 12 mins (Frank and Yousef 2004).

PASTEURIZATION PARAMETERS INFLUENCING BACTERIAL GROWTH IN FLUID MILK PRODUCTS

Intuitively, one would predict that the higher the temperature treatment of raw fluid milk during pasteurization (within the temperature limits commonly applied for HTST processing), the lower the resulting bacterial numbers would be throughout product shelf-life. However, this relationship does not necessarily hold. The US Grade

A Pasteurized Milk Ordinance (Pasteurized Milk Ordinance, 2009) specifies minimum processing conditions of 72°C for at least 15 seconds for HTST pasteurized milk products, but, for a number of reasons, many US milk processors exceed these minimum requirements. US plants frequently pasteurize milk at temperatures as high as 80°C with holding times of up to 30 seconds. While the initial outcome of the 80°C temperature treatment may appear beneficial, i.e., by an immediate reduced recovery of bacteria from this milk relative to recovery from milk processed at lower temperatures, many milk processors have reported shorter fluid milk shelf-lives (i.e., higher bacterial numbers in pasteurized products after fewer days) after shifting to a higher HTST processing temperature.

To investigate the effect of HTST processing temperatures on post-pasteurization bacterial numbers, 2% fat raw milk was heated to 60°C, homogenized, and treated for 25 seconds at 1 of 4 different temperatures (72.9, 77.2, 79.9, or 85.2°C) and then held at 6°C for up to 21 days (Ranieri et al., 2009). Aerobic bacterial plate counts were measured in pasteurized milk samples at days 1, 7, 14, and 21 post-processing to compare the relative numbers of bacteria growing in milk that had been treated at different temperatures. Counter-intuitively, higher bacterial numbers were consistently found in milk that had been processed at higher temperatures relative to milk that had been pasteurized at 72.9^{o}C. The increased rate of growth among bacteria present in milk pasteurized at 85.2^{o}C relative to that among those in milk pasteurized at 72.9^{o}C suggests that factors intrinsic to the milk, spores, or both are thermosensitive. Potential factors that could affect bacterial growth rates could include an increased availability of milk-based nutrients in milk heated at higher temperatures, or greater destruction of heat sensitive antibacterial factors

that are indigenous to the milk. For example, the lactoperoxidase system is a heat sensitive antimicrobial system that is naturally present in milk (Barrett et al., 1999). It is also possible that interactions among sporeformers are influenced by heat (McGuiggan et al., 2002).

To probe the microbial ecology of fluid milk pasteurized at different temperatures (i.e., to determine if the differences in bacterial numbers in milk pasteurized at different temperatures were due to outgrowth of different microbial populations), 490 psychrotolerant sporeforming bacteria were isolated from the milk samples during 21 days of refrigerated storage. All isolates were identified using a DNA sequence-based subtyping method, described in detail below, that differentiates strains on the basis of partial DNA sequences obtained for the *rpoB* gene (Durak et al., 2005; Huck et al., 2007a). Regardless of processing temperature, >85% of the isolates characterized at 0, 1, and 7 days post-processing were of the genus *Bacillus*, whereas more than 92% of the isolates characterized at 14 and 21 days post-processing were of the genus *Paenibacillus*. Furthermore, although typically present at low numbers in raw milk (<1 spore/mL), *Paenibacillus* spp. were capable of multiplying to numbers higher than 10^6 CFU/mL in pasteurized milk. The presence of *Paenibacillus* spp. at low numbers early in pasteurized milk shelf-life (1-10 days post-pasteurization), and the capacity of these microbes to predominate at the end of shelf-life (days 14-21 post-pasteurization) have been highlighted in a number of studies, some of which are summarized in Figure 2.1. The ability of *Paenibacillus* spp. to limit the shelf-life of pasteurized fluid milk, despite being present at very low levels in raw milk, indicates the need to identify and eliminate niches where *Paenibacillus* spp. spores may contaminate or re-contaminate fluid milk products.

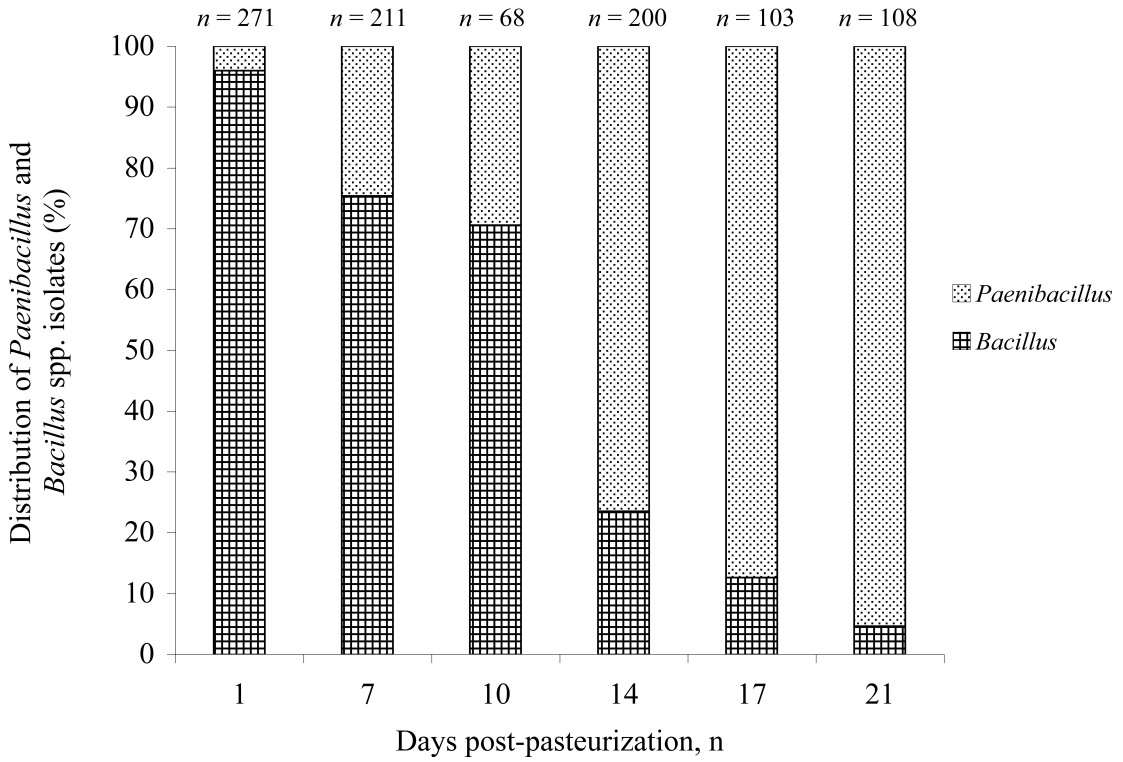

Figure 2.1: Percent of isolates characterized at days 1, 7, 10, 14, 17 and 21 following pasteurization and subsequent storage at 6°C. *Bacillus* spp. represent the predominant bacteria isolated directly after pasteurization (days 1-10), however, the predominant species by day 14 is *Paenibacillus*, reflecting the potential for microbes in this genus to spoil pasteurized fluid milk. Figure adapted from Fromm and Boor (2004), Ranieri et al., (2009), and Ranieri and Boor (2009).

TRACKING SPOILAGE ORGANISMS FROM FARM TO FINISHED PRODUCT

Tracking organisms throughout dairy systems requires the implementation of a subtyping method that is reproducible, cost-effective and discriminatory. One strategy for distinguishing among closely related bacterial strains is to develop and employ a DNA sequence-based subtyping method (Sukhnanand et al., 2005). The premise of sequence-based subtyping strategies is to obtain and compare a specific DNA sequence(s) that is universally present within a specific group of bacteria. Using polymerase chain reaction (PCR), a targeted segment of DNA is amplified from any bacterium that shares a specific genetic target. Following amplification, the DNA fragment is sequenced to determine each base pair (A, T, C or G) within the targeted region. By comparing sequences from different isolates, investigators can infer genetic relationships. Specifically, DNA sequences are compared to determine if they are identical or different by at least one base pair. Gene sequences targeted for DNA sequence-based subtyping typically encode proteins that are integral to a bacterium's function (known as 'housekeeping' genes), to provide an increased likelihood that the target will be present in all bacteria of interest.

Fromm and Boor (2004) utilized a 16S rDNA subtyping method to investigate the diversity of bacterial isolates collected throughout product shelf-life from fluid milk samples stored at 6^{o}C. Analysis of the bacterial isolate distribution indicated that *Paenibacillus* spp. and *Bacillus* spp. were the predominant bacterial isolates found in commercial New York State fluid milk. Additionally, a clear trend was identified in the microbial ecology of the pasteurized milk samples during storage at 6^{o}C, as the number of *Paenibacillus* isolates increased from 3 (~6%) on initial day to 30 (~60%) on day 17 of

shelf-life (From and Boor 2004). This study identified the roles of *Paenibacillus* spp. and *Bacillus* spp. as the biological barriers to shelf-life extension when post-pasteurization contamination, typically by gram-negative bacteria (primarily *Pseudomonas* spp.), is adequately controlled.

While the 16S rDNA subtyping method is presently broadly applied for bacterial identification in microbiological research, it did not allow sensitive discrimination among unique and distinct strains of psychrotolerant, sporeforming gram-positive bacteria (i.e., *Paenibacillus* and *Bacillus* spp. could not be clearly differentiated into the appropriate genera). Our goal, therefore, was to develop an assay that would enable classification of closely related *Bacillus* and *Paenibacillus* spp. To that end, an *rpoB* DNA sequence-based assay was developed that provides an enhanced ability, relative to 16S rDNA typing, to discriminate among gram-positive sporeforming bacteria (Durak et al., 2006). The enhanced discrimination of the *rpoB* DNA method relative to the 16S rDNA method reflects the fact that the targeted portion of the *rpoB* gene is less conserved than the targeted portion of the 16S rDNA gene (Durak et al., 2006). The *rpoB* gene encodes for the beta subunit of RNA polymerase in all bacteria.

Pure bacterial cultures must be used to generate *rpoB* subtyping data. Following enumeration of bacteria from a given sample, representative bacterial colonies are selected to represent visually distinct morphologies. PCR is performed to generate a 740 bp product from the *rpoB* gene (Drancourt et al., 2004; Durak et al., 2005). DNA sequences are aligned, then trimmed to a 632-nucleotide fragment in MegAlign (DNAstar®, Lasergene, Wis., USA), corresponding to nt 2455 to 3086 of the 3,534 *rpoB* open reading frame of *Bacillus cereus* ATCC 10987 (GeneBank AEO17194, locus tag

BCE_0102; Huck et al., 2007a). The sequences are compared to one another using a sequence comparison tool [e.g., BioEdit (Hall 1999)]. If one sequence differs from the other(s) by 1 or more base pairs, it is considered to represent a different allelic type (AT). If two sequences are identical (the base pairs are exact matches), the bacteria are considered to be the same allelic type.

Allelic types are useful for isolate characterization and analysis of contamination patterns. One of the main benefits of a DNA sequence-based subtyping method such as *rpoB* subtyping is the production of unambiguous sequence data, which are highly reproducible between laboratories (Aires-de-Sousa et al., 2006). Additionally, if strains are curated, the resulting culture collections are invaluable for further characterization of isolates for specific phenotypic or genetic characteristics of interest. Furthermore, DNA sequence-based typing strategies are less expensive than other commonly applied subtyping methods, including pulsed field gel electrophoresis (PFGE) and ribotyping. One drawback is the need for DNA sequencing equipment or access to a lab with sequencing capabilities, which may not be present in a traditional microbiology laboratory. Also, DNA sequence subtyping is a culture-based method that is only capable of identifying bacterial colonies that can grow on bacteriological media. Overall, however, sequence based subtyping methods provide a reliable, cost-effective strategy for identifying and characterizing bacteria in food production and processing systems.

In a recent study, the *rpoB* subtyping method was applied to bacterial isolates that had been collected from all segments of the dairy processing continuum (Huck et al., 2008). Specifically, samples were collected from dairy farms, raw milk tank trucks, dairy plant storage silos, and pasteurized milk. The bacterial isolates obtained from these

samples were then subtyped. The resulting data indicated that some bacterial allelic types (i.e., strains) were isolated throughout the dairy system continuum, from the farm to the packaged product, suggesting that: potential entry points for sporeforming bacteria occur throughout the entire system (e.g., in raw milk bulk tanks, tanker trucks, etc.; Figure 2.2); or sporeforming bacteria present in raw milk can be transmitted throughout the entire system; or both. While other studies have examined the presence of *Bacillus* spp. and closely related microbes in milk from the farm (Crielly et al., 1994; Sutherland and Murdoch 1994; Lukasova et al., 2001; Scheldeman et al., 2004; Bartoszewicz et al., 2008), processing plant (Lin et al., 1998; Huck et al., 2007b) and pasteurized packaged products (Huck *et al.,* 2007b; Ranieri and Boor 2009), the study reported by Huck *et al.,* (2008) used a discriminatory subtyping method to characterize spoilage bacteria, which enabled identification of potential contamination points from the dairy farm environment to packaged HTST-pasteurized fluid milk products. Clearly, sporeforming bacteria, including those able to grow under refrigeration temperatures, exist in the dairy farm environment (i.e. cow bedding materials, cow feed, manure, wash water, and soil). Thus, the farm represents a potential source of contamination with sporeforming bacteria that can survive pasteurization conditions used for HTST-pasteurized fluid milk products. Importantly, a number of characterized isolates were found only in the commercially packaged products, suggesting the potential for contamination or re-contamination of fluid milk at the processing plant.

While initial bacterial subtyping studies focused on isolates collected from dairy farms, processing plants and fluid milk in the Northeastern US, a further examination of fluid milk processed in 5 different regions across the US was completed to investigate the

presence of gram-positive psychrotolerant sporeforming bacteria in other regions. To determine the microbial ecology of milk from 5 geographical regions, 2% HTST pasteurized fluid milk samples were obtained from 18 different plants representing the Northeast, Southeast, South, Midwest, and West (Ranieri and Boor 2009). To examine the bacterial ecology of the milk during refrigerated storage, isolates were collected from milk stored at 6°C on days 1, 7, 10, 14 and 17 post-pasteurization. Of 589 bacterial isolates identified from milk samples, 346 were identified as gram-positive sporeforming bacteria, and of those, 240 were identical to those previously identified from samples that had been obtained previously in NYS, indicating the widespread presence of sporeforming bacteria in fluid milk production and processing systems within the US. Further, the bacterial ecology of the products during refrigerated storage mirrored work reported by Fromm and Boor (2004) and Huck et al., (2007b). On days 1, 7 and 10, *Bacillus* spp. comprised over 84% of the gram-positive sporeforming isolates collected, whereas at day 17 *Paenibacillus* spp. totaled more than 92% of the isolates characterized. These results indicate a clear shift in gram-positive spoilage genera from *Bacillus* spp. to *Paenibacillus* spp. during refrigerated storage of pasteurized milk. Due to their predominance at the end of shelf-life, and low numbers in raw and initial days post-pasteurization, *Paenibacillus* spp. pose a considerable challenge to dairy processors that desire to further extend HTST pasteurized milk shelf-life.

The ability to identify and track transmission of sporeforming gram-positive bacteria was enabled by development of the *rpoB* subtyping method, which has been applied to psychrotolerant sporeforming bacteria isolated from milk production and processing systems (Durak et al., 2006; Huck et al., 2007a,b; Huck et al., 2008; Ranieri et

al., 2009; Ranieri and Boor 2009). Currently, over 1,100 gram-positive sporeforming isolates from New York State farms, dairy processing environments, raw milk and pasteurized milk samples have been isolated and subtyped. An additional 346 isolates have been isolated from across the U.S., including from fluid milk processing plants in Pennsylvania, Florida, Georgia, Tennessee, Texas, Michigan, Wisconsin, Minnesota, California, New Mexico and Idaho, as described above. Based on *rpoB* subtyping analysis, from the > 1,400 isolates characterized to date, over 260 unique subtypes have been classified, illustrating the rich diversity of sporeforming microbes present in fluid milk production and processing systems (Huck et al., 2008; Ranieri et al., 2009; Ranieri and Boor, 2009). Figure 2.2 illustrates the ability to trace bacterial contaminants throughout a food production, processing and distribution system using data generated by DNA subtyping. Further, it is clear that sporeforming bacteria capable of limiting HTST fluid milk shelf-life are present in products manufactured across the US. Therefore, psychrotolerant sporeforming bacteria represent important target organisms for development of tests designed to assess the quality of raw milk relative to its post-processing functionality. Specifically, we hypothesize that implementation of effective strategies for controlling the presence of allelic types 1, 15, and 27, which were found frequently and in all sample types (farm, tanker truck, plant silos, and pasteurized products; Figure 2.2) will reduce the overall presence of sporeforming bacteria in milk processing systems.

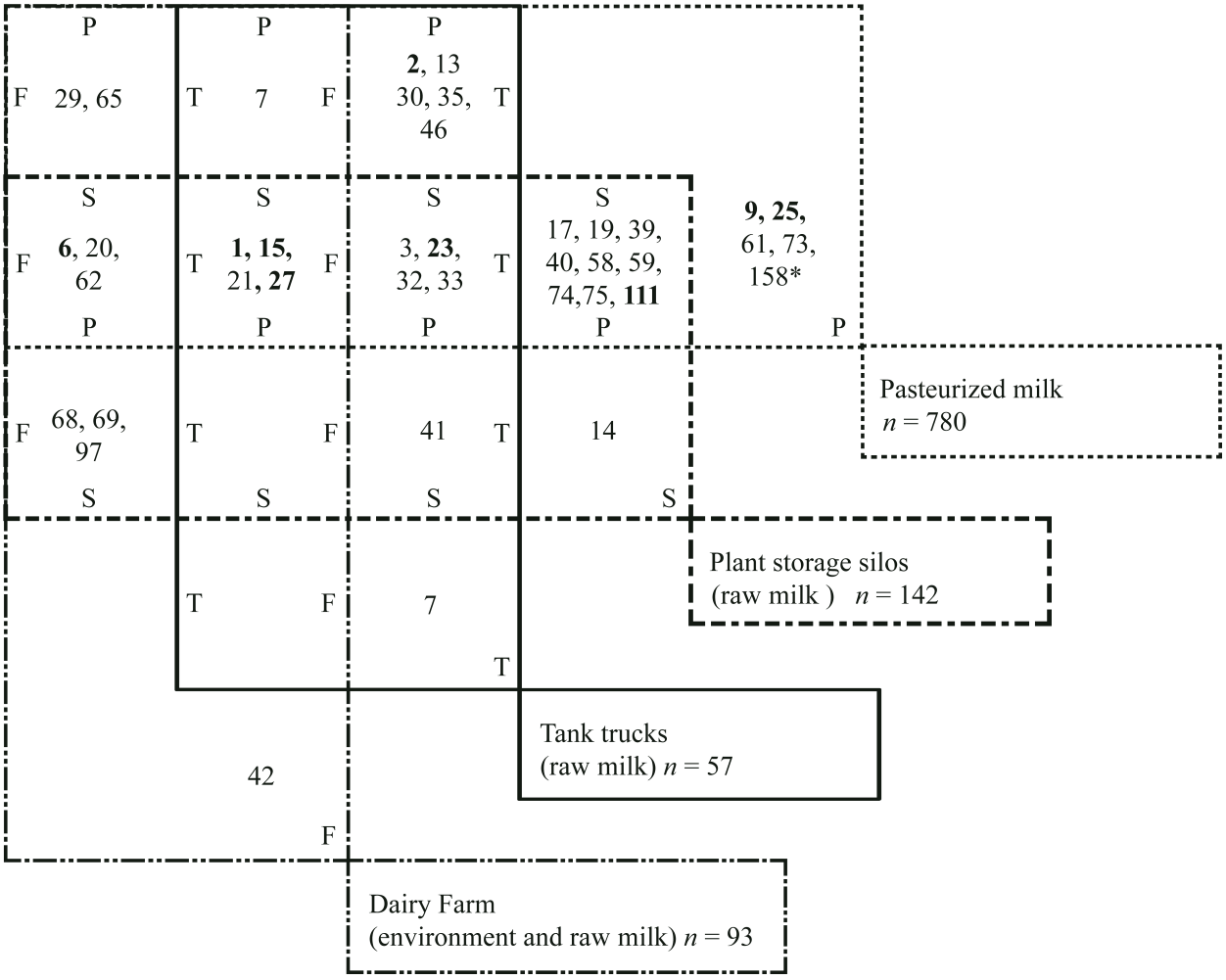

Figure 2.2: Venn diagram (non-proportional) indicating the distribution of common *rpoB* allelic types (AT) isolated from dairy farm (F), raw milk tank truck (T), raw milk storage silo (S), and pasteurized milk (P) samples. Letters in each square indicate sample types in which these *rpoB* AT were identified. AT in bold represent those found in pasteurized milk from across the United States, including plants representing the Northeast, Southeast, South, Midwest and West (Ranieri and Boor 2009). Common *Bacillus* spp. includes AT1, AT6, and AT9 (*B. licheniformis*), AT17 and AT73 (*B. arenosi*), AT20 (*B. pumilus*), and AT158 (*B.cereus*). Common *Paenibacillus* spp. includes AT2, AT13, AT15, AT25, and AT27 (*P. odorifer*) and AT23 and AT111 (*P. amylolyticus*). Figure adapted from Huck et al., (2008).

**rpoB* AT158 was found solely in pasteurized milk samples from a single processing plant (n=149), highlighting the existence of processing plant-specific contamination.

Consequences of Spores in Dairy Products Other than Fluid Milk

The presence of sporeforming bacteria can be detrimental to a wide spectrum of dairy products. It is well documented that clostridia can cause late blowing defects in Dutch and Italian hard cheeses (Ingham et al., 1998). More recently, *Bacillus polymyxa* and *Bacillus macerans* were associated with the spoilage of Argentinean cheeses (Quiberoni et al., 2008). Spoilage of these products was attributed to the presence of *Bacillus* spoilage organisms in raw milk and the subsequent ability of these microbes to resist heat treatments, or to the entry of these organisms via post-pasteurization contamination.

In the manufacture of whole milk powder, the predominant sites identified as harboring large numbers of thermotolerant spores were the pre-heater plate heat exchanger and the evaporator (Scott et al., 2007). While not proven, initial contamination of milk powder plants is thought to arise from the presence of small numbers of thermophilic microbes present in raw milk that can survive pasteurization and multiply in accommodating niches. The sporeforming isolates identified in the plant survey were *Anoxybacillus flavithermus* and *Geobacillus* spp. While Scott et al., (2007) classified thermophilic organisms based on partial 16S rDNA sequences, as described by Flint et al., (2001b), other methods such as randomly amplified polymorphic DNA (RAPD) have been employed to identify thermophilic bacterial isolates recovered from milk powder products (Ronimus et al., 2003). Real-time methods have also been reported, allowing the rapid detection and enumeration of thermophilic bacilli in milk powder. Rueckert et al., (2005) designed a TaqMan-based real-time PCR assay targeting the 16S rRNA gene for selective and quantitative detection of thermophilic bacilli, and a

SYBR Green-based real-time PCR assay targeting the *spo0A* sporulation gene (Rueckert et al., 2006). UHT milk products are also recognized to spoil due to the presence of thermotolerant sporeforming bacteria. Specifically, highly heat-resistant spores of *Bacillus sporothermodurans* have been isolated from UHT milk (Montanari et al., 2004).

Highly heat resistant spores were also recently isolated from dairy farm samples following sample treatment at 100°C for 30 minutes (Scheldeman et al., 2005; Scheldeman et al., 2006). As the dairy industry wishes to develop novel UHT and extended shelf-life products, the presence of such spores in milk production systems will become increasingly important. Typing methods, such as *rpoB* subtyping, will provide for rapid identification of such organisms from farm to finished product, thus enabling development of effective intervention strategies. Additionally, characterization of the diversity of sporeforming dairy spoilage organisms will help prepare the industry to address issues related to the presence of highly heat resistant sporeforming bacteria.

While we have concentrated on the presence of spoilage organisms in dairy products, it is important to describe the potential of some dairy-associated sporeforming bacteria to cause human illness. In particular, *Bacillus cereus* represents a common aerobic sporeforming bacterium associated with raw and pasteurized milk. *B. cereus* can produce heat stable enzymes capable of causing foodborne illness (Granum 2002). The toxigenic potential of closely related aerobic sporeformers is also under investigation (From et al., 2005; De Jonge et al., 2009). In the past year a product recall has been associated with the presence of *B. cereus*; the recall involved a commercial ready-to-drink dairy product (Larsen 2010). Such incidences highlight the need for effective methods for tracking and screening potentially toxic *Bacillus* spp. in dairy products.

CONCLUSIONS

Sporeforming spoilage bacteria play an important role in the quality of dairy products. As dairy processors strive to meet consumer demand by developing new products with extended or novel shelf-life characteristics, the need to understand characteristics, ecology, and spoilage potential of sporeforming bacteria will become increasingly important. Spores are ubiquitous in nature, and are capable of enduring many of the processing hurdles developed and implemented to date. With reliable tracking and characterization methods, we will be able to mitigate problems associated with sporeforming spoilage organisms by using a systematic approach for controlling points of entry and multiplication for these microbes in dairy systems. The combined efforts of farmers, dairy processors, retailers and researchers will be needed to provide consumers with the highest quality dairy products possible. To that end, it is essential that all segments of the dairy industry work together to integrate practical measures for control of spoilage organisms in dairy processing systems.

CHAPTER THREE

REAL-TIME PCR DETECTION OF *PAENIBACILLUS* SPP. IN RAW MILK TO PREDICT SHELF-LIFE PERFORMANCE OF PASTEURIZED FLUID MILK PRODUCTS[2]

INTRODUCTION

Despite advances in food preservation techniques, bacterial spoilage remains a leading cause of global food loss (Gram et al., 2002). Nearly one-third of all food produced worldwide is estimated to be lost post-harvest, much of which can be attributed to microbial spoilage (Gustavsson et al., 2011). Dairy products constitute one of the leading sectors impacted by food loss in the US, as nearly 20% of conventionally pasteurized (high temperature short time; HTST) fluid milk is discarded prior to consumption each year (Kantor et al., 1997). In the US, the shelf-life of fluid milk ranges from approximately 1-3 weeks. Most consumer complaints result from the growth of psychrotolerant bacteria, typically either non-sporeforming Gram-negative rods or Gram-positive sporeforming bacteria (Mayr et al., 1999; Hayes et al., 2002; Fromm and Boor, 2004; Huck et al., 2008; Ranieri and Boor, 2009; Schmidt et al., 2011). The presence of psychrotolerant, non-sporeforming bacteria (e.g., *Pseudomonas*) in pasteurized milk indicates either inadequate heating of the milk or, more commonly, post-pasteurization contamination (Eneroth et al., 2000). Therefore, pasteurized milk contamination with *Pseudomonas* and other non-sporeforming bacteria can be controlled or eliminated by adhering to pasteurization specifications for minimum time and temperature

[2] Published in Applied and Environmental Microbiology (Ranieri et. al., 2012).

combinations (FDA, 2011) and by adhering to proper sanitation and equipment maintenance protocols, particularly with respect to milk filler sites (Ralyea and Wiedmann, 1998). Conversely, Gram-positive psychrotolerant sporeformers can survive pasteurization as spores, germinate, and then grow during refrigerated storage to numbers capable of causing off-flavors or curdling of milk (Huck et al., 2007; Ranieri et al., 2009; Ranieri and Boor, 2009; De Jonghe et al., 2010).

The predominant Gram-positive sporeforming bacteria isolated from milk are *Bacillus* spp. and *Paenibacillus* spp. Both *Bacillus* spp. and *Paenibacillus* spp. have been isolated from farm environments (e.g., soil, water, and feed), raw milk, dairy processing plants, and pasteurized milk (De Jonghe et al., 2010; Huck et al., 2008; Giffel et al., 2002; Scheldeman and Goossens, 2004). In HTST pasteurized milk, when post-pasteurization contamination is excluded, *Bacillus* spp. represent the predominant bacteria found early in shelf-life (< 7 days). However, during refrigerated storage of pasteurized milk, *Paenibacillus* spp. become the predominant spoilage organisms, typically representing over 95% of the bacterial population identified late in shelf-life (> 10 days) (Ranieri and Boor, 2010). *Paenibacillus* spp. are generally present in very low numbers in raw milk and early in pasteurized milk shelf-life, yet can reproduce to high numbers during cold-storage. Numerous microbiological tests have been applied to raw milk with the goal of predicting shelf-life performance of the milk, but none are adequately predictive of HTST pasteurized fluid milk shelf-life (Martin et al., 2011). This, in part, is likely due to the inability of traditional microbiological tests to identify or quantify low levels (< 10 spores/ml) of *Paenibacillus* spp. Currently, only limited phenotypic methods are available to differentiate between *Bacillus* spp. and closely

related *Paenibacillus* spp., including cold-growth, which requires 7 to 10 days of incubation, and lactose utilization, which can be difficult to interpret and is not a consistent indicator of sporeformer genus (Ivy et al., 2012).

The aim of this study was to develop a novel PCR assay targeting 16S rDNA so that specific identification of *Paenibacillus* spp. could be performed rapidly. The objectives of this study were to: (i) design primers and a probe for detection of *Paenibacillus* spp. while limiting non-specific detection of closely related *Bacillus* spp. (ii) validate primers and probe using a real-time PCR assay on select *Paenibacillus* and *Bacillus* isolates from a collection of over 1200 isolates from fluid milk and dairy environments, and (iii) develop a systematic approach to aid in identification of *Paenibacillus* spp. from raw milk. The results of this study will provide the food industry with an assay to monitor the quality of raw milk. This assay may even be adapted to aid in the development of strategies to limit spoilage of other pasteurized, refrigerated foods like vegetable purees (Carlin et al., 2000; Guinebretiere et al., 2001) and fermented beverages (Haakensen and Ziola, 2008) . Finally, our assay has potential for use as a screening tool to isolate novel enzyme producing *Paenibacillus* spp. from other foods (Piuri et al., 1998) and the natural environment (Naghmouchi et al., 2011; Sakai et al., 2005), as previous identification of *Paenibacillus* strains has led to the discovery of many compounds with promising applications in agriculture and medicine (30).

MATERIALS AND METHODS

TaqMan Probe and Primer Design. *rpoB* and 16S rDNA alignments were performed in MegAlign (DNASTAR, Inc. Madison, WI). *rpoB* sequences (632 bp) from a total of

1,288 isolates representing *Paenibacillus* (n = 737), *Bacillus* (n = 467), and genera formerly classified as *Bacillus* (n = 84) (e.g., *Viridibacillus*), collected from farm environments, raw milk, fluid milk processing plants, and HTST fluid milk products were analyzed to identify unique subtypes (Ivy et al., 2012). *rpoB* sequences lacked sufficient conservation for design of TaqMan primers and probes that could detect all 737 *Paenibacillus* sequences represented in this collection. Therefore, alignments of partial (> 600 bp) 16S rDNA sequences representing each of the 283 *rpoB* subtypes identified among these *Bacillus* and *Paenibacillus* spp. were used to create consensus sequences for (i) all *Paenibacillus* rDNA sequences and (ii) all non-*Paenibacillus* rDNA sequences (which includes sequences for *Bacillus*, *Lysinibacillus*, *Oceanobacillus, Psychrobacillus, Solibacillus*, and *Viridibacillus*). The consensus sequences were exported to Primer Express (Version 2.0.0 Applied Biosystems, Foster City, CA) for primer-probe design. Primers were designed to detect a conserved region within the *Paenibacillus* genus, while excluding *Bacillus* spp. and other closely related genera. The designed amplicon was 158 bp, and included a 24 bp probe located 34 bp downstream from the 5' end of the forward primer (see Table 4.1 for primers and probe). The probe was labeled on the 5' end with 6-carboxyfluorescein (FAM) and the 3' end with tetramethylrhodamine (TAMRA). Detailed information on all isolates used in this study, including 16S and *rpoB* sequences, can be accessed at www.pathogentracker.net.

TaqMan Conditions. Real-time PCR was conducted in a 12.5 µl reaction containing 6.25 µl of 2X TaqMan Universal Master Mix (Applied Biosystems), 900 nM of each forward and reverse primer (MR-18_16S F, MR-19_16S R), 250 nM TaqMan probe (MR-21_16S Probe), and 1.375 µl water (Table 4.1). Each reaction also contained 1.25

µl of 10X Exogenous Internal Positive Control (IPC) Mix and 0.25 µl of 50X Exogenous IPC DNA (PE Applied Biosystems). Finally, 1.0 µl of DNA template was added to each reaction.

Real-time PCR was performed as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s followed by extension and annealing at 60°C for 1 min. Threshold cycle (C_t) values represent the fractional PCR cycle in which fluorescence first passed a defined threshold for each sample amplification plot.

Bacterial Isolate Selection and Assay Validation. To validate the primers and probe, *Paenibacillus* and closely related *Bacillus* strains (n = 9 for each genus) were selected to represent the most frequently isolated *rpoB* allelic types (AT; i.e., those isolated ≥ 10 times) from a collection of over 1200 isolates collected from dairy farms, processing plants, raw milk and pasteurized fluid milk (Ivy et al., 2012). An additional 8 *Bacillus* (or closely related genera of *Lysinibacillus*, *Oceanobacillus*, and *Viridibacillus*) and 7 *Paenibacillus* strains were included to represent genetic diversity (Table 3.2).

Pure bacterial cultures, stored in 15% glycerol at -80°C, were streaked onto brain-heart infusion (BHI) agar (Difco, BD Diagnostics, Franklin Lakes, NJ) and grown for 18-24 h at 32°C. A single colony from plates that confirmed a pure culture was inoculated into 5 ml of BHI broth (Difco) and grown for 18-24 h at 32°C. Total genomic DNA was extracted from 1 ml of overnight culture according to QIAamp DNeasy kit instructions (Qiagen Inc., Valencia, CA). Purified DNA concentrations were determined using Hoechst Dye Assay (Thermo Fisher Scientific, Wilmington, DE) and standardized to 10^5 genomes/µl.

To determine amplification efficiency, genomic DNA from *Paenibacillus odorifer* isolate FSL H7-592, representing the predominant spoilage allelic type (AT15), was serially diluted (10^7 to 10^1 genomes/ml) to produce a standard curve. Amplification efficiency was calculated using the following equation: $E = [10^{(-1/slope)}]-1$.

Detection Limit and Raw Milk Sample Testing. To determine the detection limit for *Paenibacillus* in the presence of other bacteria in a complex matrix, raw milk was obtained from the Cornell Teaching and Research Center (Dryden, NY). An overnight culture of *Paenibacillus odorifer* (FSL H7-592; AT15) was grown in BHI broth (Difco), then centrifuged at 10,000 × *g* (Eppendorf 5417C, Hamburg, Germany) and re-suspended in phosphate buffered saline solution (Weber Scientific, Hamilton, NJ) before serial dilution into the raw milk; final *Paenibacillus* concentrations of 10^5, 10^4, 10^3, 10^2, and 10^1 CFU/ml of milk were achieved. A negative control containing no added *Paenibacillus* DNA was also included. To test the sensitivity of the PCR assay with a high background flora of mesophilic sporeforming bacteria typically found in milk, 100 ml of raw milk was heated to 80°C and held for 12 min, cooled, and then incubated at 32°C for 18 h before inoculation with *Paenibacillus odorifer* to achieve final *Paenibacillus* DNA concentrations of 10^5, 10^4, 10^3, 10^2, and 10^1 CFU/ml of enriched milk. The Norgen Milk Bacteria DNA Isolation Kit (Norgen Biotek Corp., Ontario Canada) was used according to manufacturer's instructions to extract DNA from 1 ml of all milk samples, and a final elution volume of 100 µl was obtained.

***Paenibacillus* Assay Testing of Raw Milk Samples**. Approximately 400 ml of raw milk was collected from ten different farms across upstate NY from March to May of 2011. Bulk tank raw milk samples (n = 24) were shipped on ice to the Cornell University Milk

Quality Improvement Laboratory (Ithaca, NY). Upon receipt, raw milk was spore-shocked (80°C for 12 min) to eliminate vegetative cells and activate spores (Franks and Yousef, 2004). Approximately 150 ml of milk was aliquoted to 4 sterile 250 ml screw-capped Pyrex containers for aerobic plate count (APC) determination on the initial day of heat treatment and at days 7, 14 and 21 of storage at 6°C; APCs were performed according to Standard Methods for the Examination of Dairy Products (Franks and Yousef, 2004). An additional 25 ml of spore-shocked milk was aliquoted into a sterile vial; this sample was incubated at 13°C for 48 h to encourage growth of *Paenibacillus* spp. while limiting *Bacillus* spp. growth. Bacterial counts in the 13°C enrichment were monitored immediately following the spore-shock, at 24 h post spore-shock, and 48 h post spore-shock; bacterial counts were determined by plating 1 ml of milk over 5 BHI plates (200 µl per plate) supplemented with bromo-chloro-indolyl-galactopyranoside (X-gal; 100mg/L; Gold Biotechnology, St. Louis, MO). Plates were incubated at 32°C for 24 h before enumeration. Plating onto BHI agar supplemented with X-Gal allowed for simultaneous APC determination and identification of β-galactosidase positive sporeforming bacteria, which, in milk, generally have been found to be *Paenibacillus* spp. (Ivy et al., 2012). From APC plates, both X-gal positive and negative colony counts were recorded. Up to 5 isolates, representing colonies with unique morphologies and including both β-gal positive and negative activity, were selected from each plate for *Paenibacillus* TaqMan PCR; crude lysates were prepared by touching a single colony with a sterile toothpick, transferring the cells into 100 µl of sterile water in a 1.5 ml Eppendorf tube (Eppendorf, Hamburg, Germany), vortexing briefly, then microwaving on high for 4 minutes. TaqMan PCR results from colony lysates were interpreted as positive for

Paenibacillus if the C_t value was < 36.71; this cutoff value was the mean C_t for the non-*Paenibacillus* isolates (38.66 ± 0.65) that were used to evaluate assay specificity (Table 3.2) minus 3 standard deviations (to limit false-positive detection). An isolate representing each colony was also characterized to the genus and species level by 16S rDNA or *rpoB* sequence based subtyping, as previously described (Huck et al., 2007).

In addition to direct testing of colonies, total genomic DNA was isolated from milk, after incubation of the spore-shocked milk at 13°C for 48 h, using the Norgen Milk Bacteria DNA Isolation Kit. Final elution volumes of 100 µl were collected and used in the TaqMan PCR reported here to test for the presence of *Paenibacillus*.

To test for an association between the detection of *Paenibacillus* colonies in raw milk samples (after heat-shock of milk, 48 h incubation at 13°C and plating onto BHI agar supplemented with X-gal) and final bacterial count in heat treated milk samples stored for 21 d at 6°C, Fisher's Exact tests were performed (JMP Version 8.0; SAS Institute Inc., Cary, NC). *Paenibacillus* assay results were coded as presence (≥ 1 *Paenibacillus* colony confirmed by TaqMan PCR) or absence (no detectable *Paenibacillus* colonies) depending on TaqMan colony PCR results. For statistical analysis, final bacterial counts at day 21 were used to assign milk samples into one of two groups ($\leq 2 \times 10^4$ or $> 2 \times 10^4$ CFU/ml) based on the Pasteurized Milk Ordinance (PMO, 2009) bacterial count limit of 2×10^4 CFU/ml for Grade A pasteurized fluid milk. For descriptive analysis, milk samples with day 21 bacterial counts $> 2 \times 10^4$ CFU/ml were separated into 'intermediate' ($> 2 \times 10^4$ and $\leq 1 \times 10^6$ CFU/ml) and 'high' ($> 1 \times 10^6$ CFU/ml) categories, while day 21 bacterial counts $\leq 2 \times 10^4$ remained designated at 'low.' P-values less than 0.05 were considered significant.

RESULTS

TaqMan allows for specific detection of *Paenibacillus* spp. The TaqMan primers and probes designed here (Table 3.1) were first used to generate a standard curve based on mean C_t values from assays performed in duplicate with *Paenibacillus* DNA representing 10^7 to 10^1 log genome copy numbers (Figure 3.1). The linear regression line relating log genome copy number to C_t values was: $y = -3.58x + 37.98$ and the R^2 value for the linear equation was 0.98. The amplification efficiency for real-time PCR amplification was determined to be 90.11%.

TABLE 3.1: TaqMan primers and probe designed for the detection of *Paenibacillus* spp. 16S rDNA.

Primer or probe	Sequence (5'-3')	Denaturation temp (°C)[a]
MR-18_16S F	AAA TCA TCA TGC CCC TTA TG	61.1
MR-19_16S R	CGA TTA CTA GCA ATT CCG ACT	59.8
MR-21_16S Probe	CGT ACT ACA ATG GCC GGT ACA ACG	69.6

[a]Denaturation temperatures were calculated using the Sigma-Aldrich DNA calculator (Sigma-Aldrich, St. Louis, MO)

The specificity of primers and probe for detection of *Paenibacillus* spp. was evaluated using 10^5 copies of genomic DNA isolated from 16 *Paenibacillus* isolates. All 16 *Paenibacillus* isolates were detected with the assay, and the mean C_t value was 19.14 ± 0.54 (Table 3.2). The 16 isolates tested represented 16 *rpoB* allelic types (ATs). These *rpoB* ATs represent over 56% (414/737) of *Paenibacillus* isolates previously collected from each of the four fundamental steps in dairy processing (i.e., from dairy farms [feed, bedding materials, manure, soil and milking parlor wash water], tank trucks, plant storage silos, and pasteurized milk) that were classified into these 16 ATs. These ATs also represent five of the predominant *rpoB* ATs identified among sporeformer isolates

obtained from HTST pasteurized milk processed in different geographical regions throughout the US (AT2, AT15, AT23 and AT27; isolated from milk processed in the Northeastern, Midwest, West, South and Southeastern US) (Ranieri and Boor, 2009).

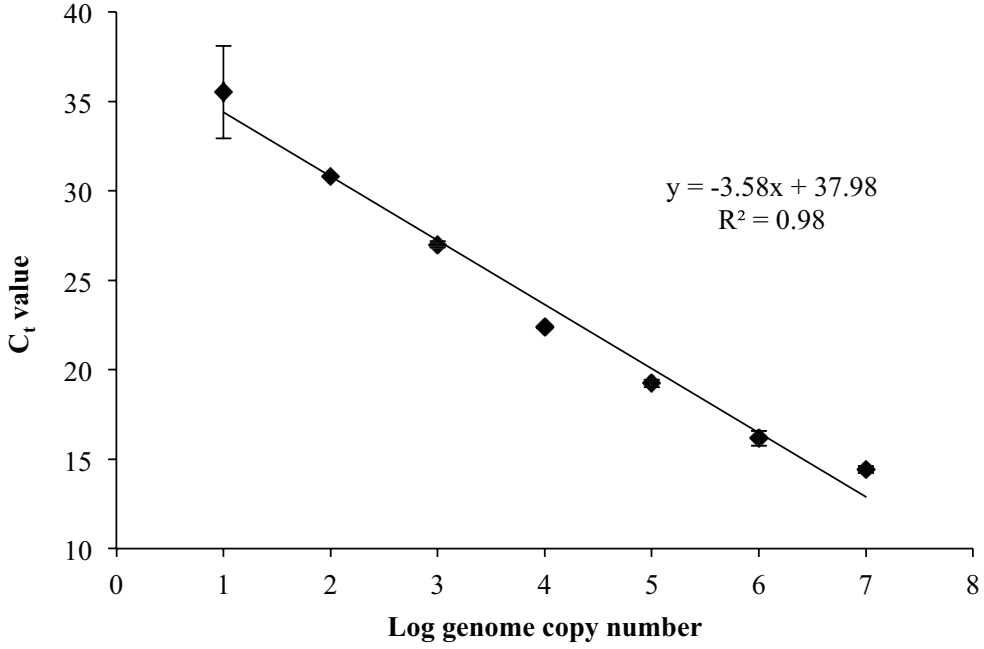

Figure 3.1: Standard curve for determination of amplification efficiency. Error bars indicate ±1 standard deviation for duplicate tests of each genome copy number. The average efficiency for real-time amplification was 90.11%.

A total of 17 isolates representing *Bacillus* and other genera closely related to *Bacillus* (i.e., *Viridibacillus*, *Lysinibacillus*, and *Oceanobacillus*) were also tested with the TaqMan PCR. These isolates represented 17 unique *rpoB* ATs, including 9 common ATs (i.e., ATs that represented ≥ 10 isolates among a total of 551 non-*Paenibacillus* isolates). In total, > 85% (470/551) of non-*Paenibacillus* isolates collected and

Table 3.2: Bacterial isolates used to evaluate the specificity of a real-time PCR assay for detection of *Paenibacillus* spp.

Isolate	AT[a]	Group ID[b]	No. isolates in AT[c]	Mean C_t[d]
FSL R5-510	1	*Bacillus licheniformis* s.l. 1	134	>40
FSL H7-687	3	*Bacillus weihenstephanensis*	19	>40
FSL R5-450	6	*Bacillus licheniformis* s.l. 1	35	39.53
FSL R5-213	17	*Viridibacillus* spp.	24	>40
FSL H7-346	20	*Bacillus pumilus*	24	>40
FSL H7-608	59	*Bacillus cereus* s.l.	26	>40
FSL R5-280	73	*Viridibacillus* spp.	18	>40
FSL H8-103	75	*Bacillus weihenstephanensis*	23	>40
FSL R5-860	158	*Bacillus cereus* s.l.	137	>40
FSL H3-288	34	*Lysinibacillus* spp.	3	>40
FSL H7-305	55	*Bacillus clausii*	2	>40
FSL H7-431	64	*Bacillus* sp. 2	2	>40
FSL H7-432	65	*Bacillus subtilis* s.l. 1	6	>40
FSL H7-719	84	*Oceanobacillus chironomi*	1	>40
FSL H7-729	85	*Bacillus* cf. *flexus*	1	38.65
FSL H8-493	135	*Bacillus aerophilus* s.l.	9	>40
FSL R5-231	140	*Bacillus safensis*	6	38.22 ± 0.34
FSL F4-077	2	*Paenibacillus odorifer* 1	52	19.06 ± 0.06
FSL F4-126	13	*Paenibacillus odorifer* 1	21	18.31 ± 0.25
FSL H7-592	15	*Paenibacillus odorifer* 1	112	19.09 ± 0.11
FSL F4-190	21	*Paenibacillus odorifer* 3	28	18.62 ± 0.18
FSL H7-689	23	*Paenibacillus amylolyticus* s.l.	35	18.88 ± 0.04
FSL F4-242	25	*Paenibacillus odorifer* 1	19	18.40 ± 0.08
FSL F4-248	27	*Paenibacillus odorifer* 1	79	19.01 ± 0.10
FSL R5-925	30	*Paenibacillus odorifer* 3	12	20.28 ± 0.22
FSL H3-442	32	*Paenibacillus odorifer* 1	16	19.43 ± 0.10
FSL F4-100	8	*Paenibacillus lautus*	3	18.92 ± 0.21
FSL H3-318	41	*Paenibacillus* sp. 1	3	19.87 ± 0.28
FSL R7-277	45	*Paenibacillus graminis* 1	3	19.02 ± 0.13
FSL H7-331	58	*Paenibacillus* sp. 10	5	19.95 ± 0.06
FSL H8-287	100	*Paenibacillus* cf. *xylanilyticus*	9	18.94 ± 0.04
FSL H8-551	157	*Paenibacillus* cf. *peoriae*	8	19.38 ± 0.23
FSL R5-978	163	*Paenibacillus graminis* 2	9	19.01 ± 0.14

[a]*Bacillus* AT1, AT6, AT17 and *Paenibacillus* AT2, AT15, AT23 and AT27 represent AT commonly isolated from HTST milk produced in plants throughout the US (35). AT1, AT15, AT21 and AT27 also represent ATs commonly isolated throughout the dairy processing continuum (i.e., dairy farm environment, tank trucks, plant storage silos, and pasteurized milk) in New York State (20).

[b]Group ID based on phylogenetic comparison previously described (22).

[c]Numbers are based on a total of 737 *Paenibacillus* and 551 non-*Paenibacillus* (i.e., *Bacillus*, *Lysinibacillus*, *Oceanobacillus*, *Viridibacillus*) dairy associated isolates characterized by *rpoB* sequence based subtyping (22). AT isolated > 10 times considered predominant and used to test assay specificity; all other isolates included to represent unique phylogenetic clades based on partial *rpoB* sequence comparison.

[d]Samples not detected in 40 cycles assigned ">40". Samples without SD only detected in one of two replicates.

characterized from the fluid milk-processing continuum, including dairy farm environments, tank trucks, plant storage silos, raw and pasteurized milk were classified into the 17 ATs tested here. Overall, 14 isolates were negative in the TaqMan PCR (C_t > 40), including 8/9 predominant *Bacillus* AT found in fluid milk or dairy processing environments. The remaining three isolates (FSL R5-450, FSL H7-729, and FSL R5-231) yielded weakly positive results in the TaqMan PCR (i.e., C_t values ≥ 38.22). Isolate FSL R5-450, which represents a common AT (i.e., AT6, see Table 3.2) was negative in one and weakly positive in the other replicate (C_t = 39.53). FSL H7-729 (AT85, an isolate included for genetic diversity; Table 3.2) was also negative in one and weakly positive in the other replicate (C_t = 38.65). *Bacillus* strain FSL R5-231 (AT140, an AT isolated only 6 times) was the only non-*Paenibacillus* strain that yielded a positive result in both TaqMan replicates (C_t = 38.22 ± 0.34).

Detection limit for vegetative *Paenibacillus* cells in raw milk is 3.25×10^1 CFU/ml. The ability of the assay to detect vegetative *Paenibacillus* cells in whole raw milk, with and without spore enrichment, was tested. Detection of *Paenibacillus* in raw milk (no enrichment) inoculated with *Paenibacillus* isolate FSL H7-592 (AT15) was possible at concentrations ranging from $3.25 \times 10^5 \pm 0.21 \times 10^5$ CFU/ml (C_t = 26.14 ± 0.78) to as few as $3.25 \times 10^1 \pm 0.21 \times 10^1$ *Paenibacillus* CFU/ml (C_t = 39.15; only one of two replicates had a C_t < 40) (Table 3); background flora in the raw milk was present at $3.85 \times 10^3 \pm 1.91 \times 10^3$ CFU/ml (Table 3.3). The negative control was not detected in two biological replicates (C_t > 40).

The detection of *Paenibacillus* cells inoculated into spore activated and enriched raw milk ranged from $3.25 \times 10^5 \pm 0.21 \times 10^5$ *Paenibacillus* CFU/ml (C_t = 26.73 ± 0.09)

to $3.25 \times 10^2 \pm 0.21 \times 10^5$ *Paenibacillus* CFU/ml (C_t = 39.46; only one of two replicates detected). *Paenibacillus* was not detected ($C_t > 40$) in the enriched milk sample containing $3.25 \times 10^5 \pm 0.21 \times 10^5$ CFU/ml or the negative control. While the C_t values at higher *Paenibacillus* concentrations (3.25×10^4 and 3.25×10^5 CFU/ml) were similar for both raw milk and spore enriched raw milk, at lower *Paenibacillus* concentrations (3.25×10^1, 3.25×10^2, and 3.25×10^3 CFU/ml) the C_t values were higher for heat shocked and enriched samples. In enriched milk samples, the sensitivity of detection for *Paenibacillus* was approximately 10-fold lower when *Paenibacillus* was inoculated in the non-enriched raw milk (with a mean background flora of $3.85 \times 10^3 \pm 1.91 \times 10^3$ CFU/ml) as compared to when *Paenibacillus* was inoculated in the enriched milk samples, which showed a background flora of $4.65 \times 10^7 \pm 0.21$ CFU/ml. A high concentration of mesophilic sporeforming (i.e., *Bacillus*) bacterial 16S rDNA may have contributed to the decreased *Paenibacillus* sensitivity observed in the enriched milk samples.

Table 3.3: Sensitivity of *Paenibacillus* detection using real-time PCR.

Paenibacillus (CFU/ml)	C_t after	
	Paenibacillus inoculated into raw milk[a]	*Paenibacillus* inoculated into heat-shocked and enriched (32°C for 18 h) raw milk[b]
$3.25 \times 10^5 \pm 0.21 \times 10^5$	26.14 ± 0.78	26.73 ± 0.09
$3.25 \times 10^4 \pm 0.21 \times 10^4$	29.47 ± 0.40	30.80 ± 0.50
$3.25 \times 10^3 \pm 0.21 \times 10^3$	31.76 ±1.20	38.22 ± 0.06
$3.25 \times 10^2 \pm 0.21 \times 10^2$	35.61 ± 0.95	39.46[c]
$3.25 \times 10^1 \pm 0.21 \times 10^1$	39.15[c]	>40[d]
Negative Control	>40[d]	>40[d]

[a]Mean aerobic plate count of raw milk: $3.85 \times 10^3 \pm 1.91 \times 10^3$ CFU/ml.
[b]Milk was incubated at 32°C for 18 h to achieve high levels of competitive microflora. The mean aerobic plate count post enrichment was: $4.65 \times 10^7 \pm 0.21 \times 10^7$ CFU/ml.
[c]Only one of two sample replicates detected in 40 cycles.
[d]Samples not detected in 40 cycles assigned C_t ">40."

Assay detects low levels of *Paenibacillus* spores capable of germination and outgrowth to spoilage levels in milk. In order to evaluate the utility of the *Paenibacillus* TaqMan colony PCR, we also compared results from *Paenibacillus* detection in raw milk by TaqMan colony PCR to bacterial counts of milk stored at 6°C post heat-treatment. Briefly, 24 raw milk samples collected from farm bulk tanks were (i) evaluated by the TaqMan colony PCR and (ii) subjected to simulated HTST pasteurization, followed by monitoring of bacterial numbers in the HTST treated milk over a simulated shelf life of 21 days (i.e., incubation at 6°C) (Figure 3.2). While initial day counts for all 24 milk samples were below 2×10^2 spores/ml, and ranged from < 1 spore/ml to 117 spores/ml (mean of 11 spores/ml), subsequent bacterial outgrowth varied. At day 21 post spore-shock treatment, bacterial numbers in the milk samples ranged from < 10 CFU/ml (8 samples) to 4.37×10^7 CFU/ml (Sample D-3, Table 3.4). Bacterial numbers after storage at 6°C for 21 d were categorized: 5 samples had bacterial counts > 1×10^6 CFU/ml (high); 16 samples remained < 2×10^4 CFU/ml (low); and 3 had numbers between 2×10^4 and 1×10^6 CFU/ml (intermediate). In 4/5 milk samples that reached bacterial numbers over 1×10^6 CFU/ml by day 21, *Paenibacillus* was detected by applying the *Paenibacillus* TaqMan to β-gal positive colonies recovered from raw milk after a 48 h enrichment at 13°C (t = 48 h assay result [+]; Figure 3.2). For sample D-4, total bacterial counts were 4, 15, and 153 CFU/ml after enrichment at 13°C for 0, 24 and 48 h, respectively. Of these counts, 0 β-gal positive CFU/ml were identified at t = 0, 5 β-gal positive CFU/ml were identified at t = 24, and 114 β-gal positive CFU/ml at t = 48 h.

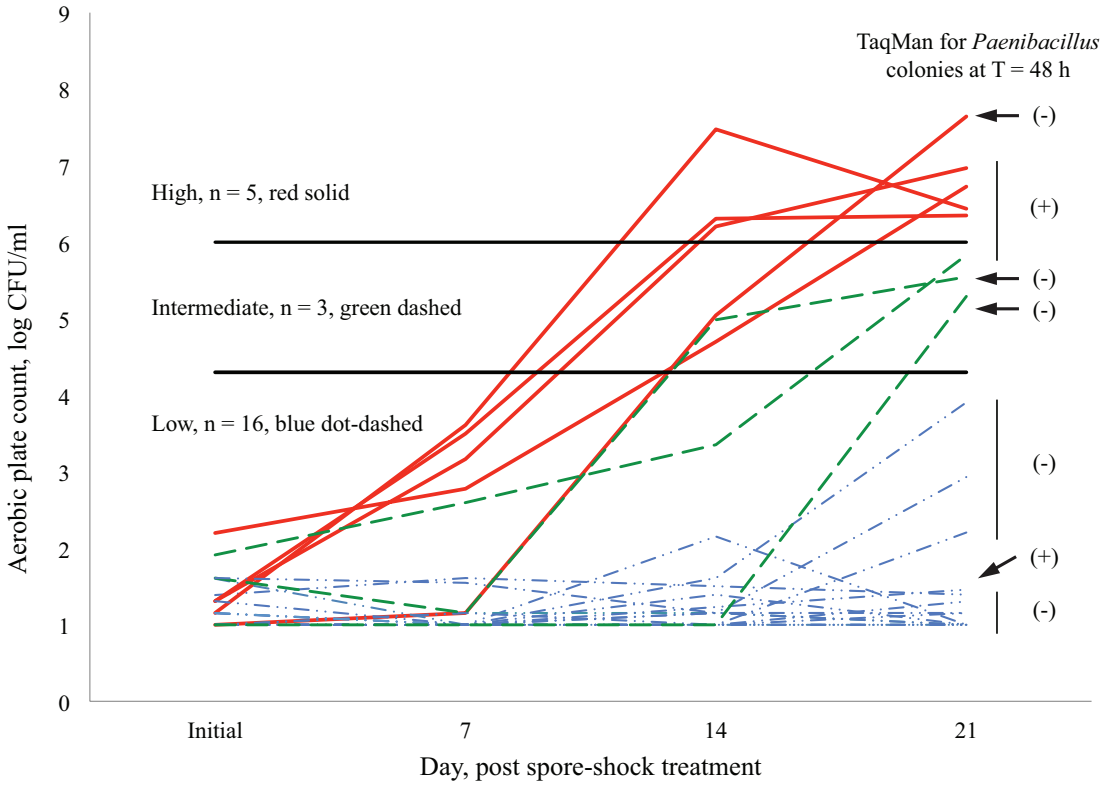

Figure 3.2: Aerobic plate counts of spore shocked milk stored at 6°C for 21 days. For each milk sample (n = 24), the *Paenibacillus* TaqMan assay was applied to individual colonies following heat treatment (80°C for 12 min), enrichment (13°C for 48 h), and plating of raw milk samples. Assay results indicate presence (+) or absence (-) of one or more *Paenibacillus* colonies. The horizontal line at 4.3 log CFU/ml indicates the maximum permissible bacterial count in high temperature short time pasteurized milk in the US. The horizontal line at 6 log CFU/ml indicates the maximum bacterial count typically associated with sensory scores of 8 and above ("good" flavor) on a 10 point scale.

Representative blue colonies selected from t = 24 (FSL R7-693) and t = 48 h (FSL R7-708) were identified as *Paenibacillus* by the TaqMan colony PCR reported here (C_t = 22.56 and 21.69, respectively). Confirmation of genus and species was performed by *rpoB* or 16S rDNA sequence-based characterization, and isolates FSL R7-693 and FSL R7-708 were determined to be *Paenibacillus peoriae* and *Paenibacillus polymyxa*, respectively. By day 21, milk sample D-4 reached a bacterial count of 9.33×10^6

CFU/ml. The predominant spoilage bacteria identified in the heat-treated milk stored at 6°C for 21 d was also determined to be *Paenibacillus*.

Only one sample reached the "high" bacterial count category (> 1×10^6 CFU/ml) after storage for 21 d at 6°C and did not contain detectable *Paenibacillus* after enrichment (D-3; Table 3.4). For the raw milk corresponding to this sample, the aerobic plate counts were 2, 2, and < 1 CFU/ml following 0, 24, and 48 h of enrichment. The only colonies obtained at t = 0 and t = 24 h were determined to be *Bacillus* (Table 3.4). After 21 days of storage at 6°C, the bacterial count of sample D-3 reached 4.37×10^7 CFU/ml; the predominant organisms detected at this time were *Paenibacillus*, suggesting that very low levels (< 1 spore/ml) of *Paenibacillus* are still capable of reaching high numbers in pasteurized products stored at refrigeration temperatures.

Among the 3 milk samples reaching intermediate bacterial counts by day 21 of cold storage (samples C-5, D-5, and J-5), only sample C-5 contained detectable *Paenibacillus* colonies during the 13°C enrichment and plating on BHI supplemented with X-gal. Aerobic plate counts during enrichment of sample C-5 were 117 (6 β-gal positive colonies), 87, and 550 (10 β-gal weakly positive [partial or light blue] colonies) at 0, 24 and 48 h enrichment times, respectively. Two isolates, FSL R7-726 and FSL R7-727, from the t = 0 plating were β-gal positive and were determined to be *Paenibacillus* by the TaqMan colony PCR (C_t = 21.5 and 23.9, respectively).

Table 3.4: Summary of assay results for raw milk samples that showed evidence for bacterial spoilage after heat-treatment and subsequent incubation at 6°C.

Spoilage growth category[b]	Milk sample	CFU/mL after 13°C milk enrichment time (β-gal positive CFU/ml)			Colony screening results after 13°C milk enrichment time												Milk assessment following 21 d at 6°C	
					T = 0 h				T = 24 h				T = 48 h					
		T = 0 h	T = 24 h	T = 48 h	Isolate ID	β-gal (+/-)[c]	TaqMan (C_t)[d]	*rpoB* based genus	Isolate ID	β-gal (+/-)[c]	TaqMan (C_t)[d]	*rpoB* based genus	Isolate ID	β-gal (+/-)[c]	TaqMan (C_t)[d]	*rpoB* based genus	Bacterial count (CFU/ml)	Predominant spoilage bacteria (*rpoB* or 16S based identification)
High	D-3	2	2	<1	FSL R7-644	-	>40	*Bacillus*	FSL R7-645	-	>40	*Bacillus*	NA				4.37×10^7	*Paenibacillus*
	D-4	4	15 (5)	153 (114)	FSL R7-677	-	>40	*Bacillus*	FSL R7-692	-	>40	*Bacillus*	FSL R7-708	+	21.69	*Paenibacillus*	9.33×10^6	*Paenibacillus*
					FSL R7-678	-	>40	*Bacillus*	FSL R7-693	+	22.56	*Paenibacillus*	FSL R7-709	-	>40	*Bacillus*		
													FSL R7-710	-	>40	*Bacillus*		
	C-4	30	29	313 (13)	FSL R7-674	-	>40	*Bacillus*	FSL R7-690	-	>40	*Bacillus*	FSL R7-704	-	>40	*Bacillus*	5.37×10^6	*Paenibacillus*
					FSL R7-675	-	>40	*Bacillus*	FSL R7-691	-	>40	*Bacillus*	FSL R7-705	-	>40	*Bacillus*		
					FSL R7-676	-	>40	*Bacillus*					FSL R7-706	+	25.18	*Paenibacillus*		
													FSL R7-707	+	20.73	*Paenibacillus*		
	C-3	27	23	197 (40)	FSL R7-646	-	>40	*Bacillus*	FSL R7-650	-	>40	*Bacillus*	FSL R7-652	+	18.9	*Paenibacillus*	2.75×10^6	*Paenibacillus*
									FSL R7-651	-	>40	*Bacillus*	FSL R7-653	-	>40	*Bacillus*		
													FSL R7-654	-	>40	*Bacillus*		
													FSL R7-655	-	>40	*Bacillus*		
													FSL R7-656	+	17.9	*Paenibacillus*		
	G-4	14	42 (1)	530 (190)	FSL R7-679	-	18.37	*Paenibacillus*	FSL R7-695	-	>40	*Bacillus*	FSL R7-711	+	21.8	*Paenibacillus*	2.24×10^6	*Paenibacillus*
					FSL R7-680	-	>40	*Bacillus*	FSL R7-696	-	>40	*Bacillus*	FSL R7-712	+/-	>40	*Bacillus*		
					FSL R7-681	-	>40	*Bacillus*	FSL R7-697	-	>40	*Bacillus*	FSL R7-713	-	>40	*Bacillus*		
					FSL R7-682	-	>40	*Bacillus*					FSL R7-714	-	>40	*Bacillus*		
Intermediate	C-5	117 (6)	87	550 (10)	FSL R7-722	-	>40	*Bacillus*	FSL R7-733	-	>40	*Bacillus*	FSL R7-736	-	>40	*Bacillus*	6.76×10^5	*Paenibacillus*
					FSL R7-723	-	>40	*Bacillus*	FSL R7-734	-	>40	*Bacillus*	FSL R7-737	-	>40	*Bacillus*		
					FSL R7-724	-	>40	*Bacillus*	FSL R7-735	-	>40	*Bacillus*	FSL R7-738	-	>40	*Bacillus*		
					FSL R7-725	-	>40	*Bacillus*					FSL R7-739	+/-	18.98	*Paenibacillus*		
					FSL R7-726	+	21.5	*Paenibacillus*										
					FSL R7-727	+	23.9	*Paenibacillus*										
	D-5	<1	3	<1	NA				FSL R7-740	-	>40	*Bacillus*	NA				3.55×10^5	*B. weihenstephanensis*
									FSL R7-741	-	>40	*Bacillus*						
									FSL R7-742	-	>40	*Bacillus*						
	J-5	<1	12	686	NA				FSL R7-744	-	>40	*Bacillus*	FSL R7-745	-	>40	*Bacillus*	1.95×10^5	*B. weihenstephanensis*

[a]Bacterial counts for the remaining 16 milk samples remained below 2×10^4 CFU/ml after heat shock and storage at 6°C for 21 d. See Supplemental Table 1 for complete summary of all 24 samples.

[b]Growth category assigned based on APCs following heat shock and storage of milk at 6°C for 21 d. "High" category indicates APC > 1×10^6 CFU/ml; "Intermediate" category indicates APC ≤ 1×10^6 CFU/ml and > 2×10^4 CFU/ml; "Low" category indicates APC ≤ 2×10^4 CFU/ml.

[c]β-gal positive (blue) colonies represented by "+"; β-gal negative colonies represented by "-"; and β-gal weakly positive colonies (partial blue or light blue colony) indicated by "+/-."

[d]TaqMan C_t values < 36.71 intepreted as a positive *Paenibacillus* colony.

Characterization by *rpoB* sequence analysis confirmed both isolates as *Paenibacillus*. Plating at 24 h of enrichment yielded only *Bacillus* colonies (n = 3), however, one *Paenibacillus* colony was identified after 48 h of enrichment (FSL R7-739; C_t = 18.98). After storage at 6°C for 21 d, the bacterial count for milk sample C-5 reached 6.76×10^5 CFU/ml, and the predominant bacteria identified was *Paenibacillus*. The other two milk samples (D-5 and J-5) in the "intermediate" count category contained no detectable *Paenibacillus*. Plating at 0, 24 and 48 h during sample enrichments yielded no β-gal positive colonies. Analysis of colonies using the *Paenibacillus* TaqMan determined colonies to be genera other than *Paenibacillus* ($C_t > 40$). *rpoB* sequence-based characterization identified all 5 isolates collected from enrichment samples as *Bacillus pumilus* or *licheniformis* (FSL R7-740 to FSL R7-745). The predominant spoilage organism identified after storage of milk samples at 6°C for 21 d was determined to be cold-tolerant *Bacillus weihenstephanensis*. Final bacterial counts were 3.55 and 1.95×10^5 CFU/ml for samples D-5 and J-5, respectively.

A total of 16 raw milk samples had bacterial counts below 2×10^4 (4.30 log) CFU/ml after storage at 6°C for 21 d (Table S1). During enrichment of those samples, 54 isolates were collected and only one sample (H-5) contained detectable *Paenibacillus*. After 48 h of enrichment, plating of sample H-5 resulted in 12 CFU/ml, 5 of which were weakly β-gal positive. β-gal weakly positive isolate FSL R7-747 was tested with the assay and determined to be *Paenibacillus* (C_t = 20.37). *rpoB* based characterization confirmed FSL H7-747 identification of *Paenibacillus*. Following storage of milk sample H-5 for 21 d at 6°C, the bacterial count was 2.88×10^1 CFU/ml.

Results for the 24 milk samples were tested for a statistical association between detection of *Paenibacillus* (Table S1; *Paenibacillus* colonies detected in 6 of 24 samples at T = 48 h) and final APC after heat treatment and storage of raw milk samples for 21 d at 6°C. In raw milk samples where *Paenibacillus* was detected, there was a significant association with higher bacterial counts at d 21 ($> 2 \times 10^4$ CFU/ml; P = 0.0069).

Overall, a total of 109 bacterial isolates were collected during screening for *Paenibacillus* colonies by 13°C enrichment for 48 h and plating onto BHI supplemented with X-gal. Of these, 97 isolates were β-gal negative; 96/97 β-gal negative isolates were also negative in the TaqMan colony PCR ($C_t > 40$; Table S1). The only β-gal negative colony that yielded a positive signal with the TaqMan colony PCR (FSL R7-679, C_t = 18.37) was confirmed as *Paenibacillus* by *rpoB* sequence-based characterization. *rpoB* sequence-based identification identified the remaining 96 isolates as: *Bacillus* (n = 92), *Brevibacillus* (n = 2), *Oceanobacillus* (n = 1), and *Staphylococcus* (n = 1). All 9 β-gal positive colonies were positive in the TaqMan colony PCR (mean $C_t = 21.57 \pm 2.26$). There were also 3 weakly β-gal positive (+/-; Table S1) colonies. Based on *rpoB* characterization, 2/3 of these colonies were identified as *Paenibacillus* and were detected with the TaqMan PCR (FSL R7-739 and FSL R7-747; C_t = 18.98 and 20.37, respectively). The remaining weakly β-gal positive colony (FSL R7-712) was determined to be *Bacillus*, and was not detected by the TaqMan colony PCR ($C_t > 40$).

In addition to testing individual colonies, total genomic DNA was collected from each of the 24 raw milk samples after 48 h of incubation at 13°C. Among these samples, only one milk sample was positive for *Paenibacillus* with the TaqMan PCR (G-4; 190 β-gal positive CFU/ml; $C_t = 34.49 \pm 0.81$). This suggests that *Paenibacillus* contamination

in the raw milk is typically at levels below the detection limit of the TaqMan PCR when used on DNA directly extracted from milk (i.e., $< 3.25 \times 10^1 \pm 0.21$ spores/ml).

DISCUSSION

Our real-time PCR based approach represents an improved tool for identifying the predominant psychrotolerant sporeforming spoilage bacteria associated with pasteurized fluid milk stored at refrigerated temperatures. Based on a diverse collection of aerobic sporeforming bacteria, which included over 1200 isolates collected from different segments of the dairy production continuum (Ivy et al., 2012), we targeted *Paenibacillus* spp., the microbes that present the current biological limit to extension of pasteurized fluid milk shelf-life. Our detection method requires heat-treating raw milk at 80°C for 12 min to activate spores and eliminate vegetative bacterial cells, followed by a 48 h enrichment at 13°C to enrich for psychrotolerant bacteria. After enrichment, milk samples are plated onto BHI supplemented with X-gal to allow direct colony screening of colonies, including β-gal positive colonies, which, in milk, generally represent *Paenibacillus* spp. Next, crude colony lysates are prepared for immediate testing of individual colonies using our TaqMan PCR, and final testing results (i.e., *Paenibacillus* or non-*Paenibacillus* spp.) can be obtained within a few hours. Overall, this colony screening strategy combined with a TaqMan PCR presents a novel approach for detecting *Paenibacillus* in raw milk, and for predicting psychrotolerant bacterial outgrowth in milk held at 6°C.

A *Paenibacillus* real-time PCR assay has potential applications for detection of psychrotolerant sporeforming bacteria in a variety of foods. Few rapid, molecular

based detection methods targeting sporeforming bacteria responsible for food spoilage have been developed (Luo et al., 2004; Fernández-No et al., 2011; Jang et al., 2011), and of these, none have focused on *Paenibacillus,* the psychrotolerant sporeforming genera associated with dairy spoilage. The absence of appropriate tools may reflect, in part, the fact that bacterial ecology present in pasteurized fluid milk has only recently been characterized at the molecular level, which led to identification of *Paenibacillus* as the predominant fluid milk sporeforming spoilage genera (Fromm and Boor, 2004; Huck et al., 2008; Ranieri and Boor, 2009; Ivy et al., 2012). Rapid methods to detect sporeforming bacteria have primarily focused on foodborne pathogens, e.g., *Bacillus cereus* (Martínez-Blanch et al., 2009; Wehrle et al., 2010; Gracias and McKillip, 2011), that pose a significant health threat. However, the presence of sporeforming bacteria that can resist multiple processing hurdles and affect food product quality represents considerable economic and food security concerns. One commercial assay has been developed by Pall GeneSystems for the detection of spore-forming bacteria in food (Postollec et al., 2010). However, when testing 34 food matrices, the authors reported the detection system was unable to identify any *Paenibacillus*. Conversely, when applying standard methods to the same 34 food matrices, researchers were able to identify *Paenibacillus* in sliced nuts and chocolate (Postollec et al., 2010), which illustrates the difficulty of reliably identifying low levels of *Paenibacillus* in food. Other than this method, development of assays for *Paenibacillus* spp. to date has focused on *P. larvae* (Martínez et al., 2010; Chagas et al., 2010), an important honeybee pathogen. Thus, an assay targeting psychrotolerant *Paenibacillus* associated with milk spoilage represents a new and important tool for the dairy industry to identify high quality raw milk, as well as

potential contamination sites at the farm and processing facility level. Sporeformers, including *Paenibacillus,* have the potential to form biofilms (Yegorenkova et al., 2011), reside within processing facilities (Huck et al., 2007), and have been isolated from paperboard packaging (Pirttijärvi et al., 1996). Thus, it is important to develop sensitive tools for detection of spoilage organisms and to apply them throughout the processing chain to identify entry points to enable development of control strategies to reduce spoilage and improve the quality of our foods. In the future, our assay could be extended to other refrigerated and pasteurized foods, including processed vegetables (Carlin et al., 2000; Guinebretiere et al., 2001; Fangio et al., 2010) where psychrotolerant *Paenibacillus* are a potential spoilage concern.

Direct PCR based detection of *Paenibacillus* in raw milk to predict shelf-life is challenging due to the high sensitivity required. Previous studies have demonstrated that low spore levels are typically found in raw milk. For example, sampling of raw milk from 43 processing plant silos in New York State yielded a mean aerobic spore count of 52 spores/ml (Martin et al., 2011). Additional studies in Europe reported similar findings, as mean counts of 131 mesophilic aerobic spores/ml (Stulova et al., 2010) and < 100 spores/ml of raw milk (Giffel et al., 2002) were detected. Of these aerobic spores, only a small percentage are likely to be *Paenibacillus*, as a number of studies have found *Bacillus* spp. comprise the majority of spores identified in raw, and in recently heat-treated milk (Fromm and Boor, 2004; Huck et al., 2008; Coorevits et al., 2008; Ranieri et al., 2009). Consistent with this, only 12/109 (11%) isolates collected during our study represented *Paenibacillus* spp., and 9/12 of those *Paenibacillus* isolates were detected only after enrichment for 24 or 48 hours at 13°C. Thus, due to the low levels of spores

naturally present in raw milk, particularly of psychrotolerant *Paenibacillus* spp., an enrichment or concentration step is needed to improve assay sensitivity.

In addition to low levels of *Paenibacillus* spp., high levels of closely related *Bacillus* spp. further complicate detection, particularly for assays targeting 16S rDNA. The detection limit for our assay increased nearly 10-fold when *Paenibacillus* were inoculated into heat-shocked and enriched (32°C for 18 h) raw milk. This reduction in sensitivity is likely due to high levels of closely related *Bacillus* spp. competing for primers and probe. Postollec and colleagues (Postollec et al., 2010) encountered cross reactivity when testing a commercial assay based on 16S rDNA primers and probes, and reported *Paenibacillus* detection with *Bacillus* primers and vice versa. Many *Bacillus* and *Paenibacillus* spp. share over 99% identity based on partial (632 bp) 16S rDNA analysis (Ivy et al., 2012). Therefore, continued development of new assays, particularly through leveraging full genome sequencing technologies and concentrating on defining characteristics of sporeforming bacteria, such as the differential presence of cold growth genes (Francis et al., 1998), will be critical to further improve detection capabilities.

PCR based detection of individual colonies after enrichment and plating allows for sensitive and specific detection of *Paenibacillus* spp. Results from TaqMan detection, performed on DNA extracted from milk samples, were predominantly negative due to low levels of *Paenibacillus* and competition from closely related *Bacillus* spp. Our observed detection limit for *Paenibacillus* inoculated into raw milk was 3.25×10^1 CFU/ml, which explains why only 1/24 raw milk samples tested positive for *Paenibacillus*. However, plating the same spore-shocked and enriched milk samples onto BHI supplemented with X-gal allowed for detection of *Paenibacillus* in 6/24 raw milk

samples. Therefore, use of a TaqMan colony PCR following a short enrichment and plating on BHI supplemented with X-gal greatly improves the reliability of the assay. Direct colony screening allowed us to lower the detection limit for *Paenibacillus* from 3.25×10^1 CFU/ml to 1 CFU/ml when a 1 ml sample was plated. In addition to improved sensitivity, the colony screening method avoids the time and costs associated with genomic DNA purification steps.

The colony screening method employs two important phenotypes that aid in distinguishing *Paenibacillus* from other sporeformers: cold-growth and β-galactosidase activity. In general, *Paenibacillus* spp. are capable of growth at 6°C, whereas most *Bacillus* spp. are not; the most notable exception is *Bacillus weihenstephanesis* (Ivy et al., 2012). By applying a 48 h incubation step for heat shocked milk at 13°C, we were able to enrich for psychrotolerant *Paenibacillus* without promoting growth of mesophilic *Bacillus* spp., which typically represent a higher proportion of spores in raw milk. However, two samples (C-5 and J-5) reached counts above the Pasteurized Milk Ordinance (FDA, 2011) limit for pasteurized milk (> 20,000 CFU/ml) after storage at 6°C for 21 d, and were not detected by our assay. The predominant spoilage organism in the two milk samples was determined to be *B. weihenstephanensis*. This outcome demonstrates the need for a detection system that utilizes genetic targets, such as cold growth genes, shared by the psychrotolerant spoilage organisms of concern (i.e., *Paenibacillus* spp. and *B. weihenstephanensis*).

In addition to cold-growth, β-galactosidase activity proved useful in identification of *Paenibacillus*. Previous work has shown that the majority of dairy-associated *Paenibacillus* subtypes are β-galactosidase positive, whereas the majority of *Bacillus*

subtypes are not (De Jonghe et al., 2010; Ivy et al., 2012). However, as some dairy-associated *Bacillus* isolates have expressed positive or weakly positive β-gal activity, this phenotypic test cannot be completely relied upon to distinguish *Paenibacillus* from other sporeformers (Ivy et al., 2012). In fact, we identified two β-gal weakly positive isolates and one β-gal negative isolate as *Paenibacillus* by TaqMan colony PCR and *rpoB* sequence based characterization. Thus, the combination of β-gal screening and a 16S rDNA TaqMan assay proved necessary for accurate and sensitive detection of *Paenibacillus* spp.. Application of this culture dependent assay to screen for *Paenibacillus* spp. in non-dairy environments could facilitate identification of strains with important metabolic capabilities (e.g., production of polymyxin, bio-remediation, or nitrogen fixing ability) of importance to agriculture, food processing, and medicine (Sakai et al., 2005; Naghmouchi et al., 2011; Shaheen et al., 2011).

Conclusion. We developed a sensitive and specific TaqMan assay that can detect psychrotolerant sporeforming *Paenibacillus* spp. associated with dairy spoilage. While the low levels of spores initially present in raw milk prevented direct detection of *Paenibacillus* in DNA extracted from raw milk or from enriched milk samples, an alternative colony screening method proved feasible. A 16S rDNA-based TaqMan assay on crude colony lysates obtained from heat-shocked milk that had been enriched at 13°C for 48 h and plated on BHI supplemented with X-gal provided fast and accurate identification of *Paenibacillus*. Overall, the assay provides an improved tool for the dairy industry to differentiate raw milk with the potential for lower post-pasteurization bacterial outgrowth. Further development of rapid and effective detection methods for

psychrotolerant sporeformers within a comprehensive farm to fork framework are needed for improved control of these important spoilage organisms in the food supply.

CHAPTER FOUR

PREDICTION OF *SALMONELLA* SEROVARS BY DNA-BASED SUBTYPING METHODS AND A PCR AND SEQUENCE-BASED SEROTYPING METHOD FOR IDENTIFICATION OF O, H1 AND H2 ANTIGENS[5]

INTRODUCTION

Salmonellosis is a considerable public health concern as non-typhoidal *Salmonella* serovars cause an estimated 93.8 million cases of gastroenteritis globally each year (Majowicz et al., 2010). The genus *Salmonella* is divided into two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into 6 subspecies, including subspecies I (*enterica*), II (*salamae*), IIIa (*arizonae*), IIIb (*diarizonae*), IV (*houtenae*), and VI (*indica*) (Grimont and Weill, 2007). The traditional method of subtyping *Salmonella*, below the subspecies level, has been serotyping, which has been applied for over 70 years (Grimont and Weill, 2007; Guibourdenche et al., 2010). Serotyping can provide valuable information regarding likely pathogen sources (as certain serovars are associated with specific hosts or geographical regions), potential disease severity, and potential antimicrobial resistance of *Salmonella* isolates. Identification of *Salmonella* serovars thus remains an important public health diagnostic need. There are over 2,600 currently recognized serovars, with the majority (over 1,500) belonging to S. *enterica* subsp. *enterica*, which is also the group of greatest clinical relevance due to its common association with humans and warm-blooded animals (CDC, 2011).

Traditional serotyping is performed according to the White-Kauffmann-Le Minor scheme, which identifies the somatic (O) and flagellar (H) antigens based on

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agglutination of bacteria with specific sera (Grimont and Weill, 2007). Despite its widespread use, traditional serotyping does have a number of drawbacks. Serotyping of *Salmonella* takes at least 3 days to complete, is labor intensive, requires maintenance of over 250 typing sera as well as 350 different antigens, and is unable to type rough or mucoid strains. Furthermore, traditional serotyping is often not sensitive enough to provide the level of discrimination needed for foodborne illness outbreak investigations, and cannot be used to infer phylogenetic relationships. Currently, 46 somatic (O) and 114 flagellar (H) variants for *Salmonella* have been identified (Grimont and Weill, 2007). The O antigen is a component of the lipopolysaccharide that is exposed on the bacterial cell surface, and multiple O antigens may be expressed together at the same time (Samuel and Reeves, 2003; Reeves et al., 1996). Genes responsible for O antigen expression (e.g., sugar transferases, O antigen flippase [*wzx*], and polymerase [*wzy*]) are located within a large regulon called the *rfb* cluster (Samuel and Reeves, 2003). Comparison of *wzx* and *wzy* genes from common serogroups has shown that these genes have little similarity even at the amino acid sequence level, making *wzx* and *wzy* appropriate candidates for serogroup-specific primer design (Fitzgerald et al., 2003; Herrera-León et al., 2007). Additional work has shown that sugar synthase genes within the *rfb* cluster can be targeted to distinguish between common serogroups (Luk et al., 1993). The genes responsible for the flagellin structure are *fliC* (phase 1 flagellin) and *fljB* (phase 2 flagellin). Both *fliC* and *fljB* are generally conserved at the terminal ends, but highly variable in the central region that encodes antigens (Joys, 1985; McQuiston et al., 2004). A number of studies have utilized variability in the *rfb* region, *fliC* and *fljB* to identify serovars, typically using probe based assays or PCR strategies (Franklin et al., 2011; Yoshida et al., 2007; McQuiston et al., 2011). While these approaches have been reported to show good concordance with traditional serotyping, limitations of

these methods include problems with characterization of new or unusual serovars or allelic variants that do not react with existing primers or probes (Franklin et al., 2011; Yoshida et al., 2007; McQuiston et al., 2011).

In addition to serotype identification through use of genetic targets that are directly responsible for O and H antigen expression, molecular subtyping methods (e.g., pulsed-field gel electrophoresis [PFGE]) can be used to predict the serovars of *Salmonella* isolates. In addition to PFGE (Zou et al., 2012; Kérouanton et al., 2007), ribotyping (Esteban et al., 1993; Bailey et al., 2002), repetitive extragenic palindromic sequence-based PCR (rep-PCR) (Wise et al., 2009; Chenu et al., 2011), multi-locus sequence typing (MLST) (Kotetishvili et al., 2002; Achtman et al., 2012), and molecular typing based on genomic markers (Wattiau et al., 2008b; Kim et al., 2006) have been investigated for their ability to replace or complement traditional serotyping. While many of these methods have been able to reliably predict a limited set of serovars, they still lack widespread adoption, likely due to requirements for specialized equipment as well as a lack of proven reliability for predicting *Salmonella* serovars. Furthermore, these methods are based on genomic targets that are not directly responsible for antigen expression, which may lead to serovar misidentification, particularly for newly emergent serovars (e.g., 4,5,12:i:-), which may be misidentified as the serovar of the evolutionary ancestor (Soyer et al., 2009; Moreno Switt et al., 2009). To facilitate further development and implementation of DNA-based approaches for serovar identification of *Salmonella* isolates, we compared the ability to predict serovars between different molecular subtyping methods (i.e., PFGE, rep-PCR, ribotyping, and MLST) and a newly implemented combined PCR and sequencing based approach that directly targets O and H antigen encoding genes.

MATERIALS AND METHODS

Bacterial Isolates. *Salmonella* isolates were selected to include representation of (i) the top 20 serovars among US human sources, the top 20 serovars among US non-human sources, and the top 20 serovars among non-clinical nonhuman sources (all as reported to CDC) (CDC, 2006) and (ii) the top 20 serovars among human sources worldwide (as reported to the WHO) (Galanis et al., 2006); this strategy identified a total of 40 serovars (Table S1). Two isolates were chosen to represent the 5 most commonly reported serovars (i.e., Typhimurium, Enteritidis, Newport, Heidelberg, and Javiana), and a single isolate of Typhimurium var. 5- (formerly *Salmonella* var. Copenhagen) was included, for a total of 46 isolates. In addition, we assembled a set of 70 isolates that included all additional 63 serovars present on our laboratory strain collection; these isolates represent less common (rare) serovars not represented in the top 40 set (Table S1). Finally, seven isolates that included incomplete serovar information (e.g., IIIb 35:Rough) or that were identified as "Untypable" by traditional serotyping were included in the less common isolate set. Detailed isolate information can be found at www.foodmicrobetracker.com under the isolate ID (e.g., FSL R8-1987).

PFGE. PFGE with XbaI (Roche Molecular Diagnostics, Pleasanton, CA) was performed according to the CDC PulseNet protocol using a CHEF-Mapper (Bio-Rad Laboratories, Hercules, CA) (RIBOT et al., 2006). The CDC *Salmonella* Braenderup strain H9812 was used as the reference (Hunter et al., 2005). PFGE gel images were captured with the Gel ChemiDoc system (Bio-Rad Laboratories). BioNumerics version 5.1 (Applied Maths, Austin, TX) was used to analyze the PFGE patterns. Similarity analysis was performed using the Dice coefficient and clustering was performed using the unweighted pair group method by arithmetic mean. PFGE patterns for test isolates were compared against a custom PFGE database available in

the Cornell Food Safety Laboratory (FSL); this database included, at the time of analysis, 5,935 isolates representing 170 serovars (this database is available upon request). A serovar was assigned to a given test isolate based on the serovar associated with the isolate that provided the top match in the PFGE pattern comparison; only PFGE patterns that showed ≤ 3 band differences to the pattern of the test isolate were considered; if a test isolate did not match any isolate in the database by ≤ 3 band differences, the serovar for the isolate was considered "Unidentified."

rep-PCR. *Salmonella* isolates were cultured on BHI agar for 18 h at 37°C, and the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA) was used to extract DNA according to the manufacturer's instructions. All DNA samples were amplified using the DiversiLab *Salmonella* Kit for DNA fingerprinting (bioMerieux, Inc., Durham, NC) according to the manufacturer's instructions. Analysis of rep-PCR patterns was conducted as previously described (Wise et al., 2009), using DiversiLab software version 3.4. The 'Top Match' feature of the software was utilized; a query sample that matched a serovar library entry at > 85% was considered to represent a positive identification. At the time of analysis the rep-PCR database included 313 isolates (309 *S*. subsp. *enterica* and 4 *S*. subsp. *arizonae* isolates) representing 55 serovars.

Ribotyping. Automated ribotyping with the restriction enzyme PvuII was performed using the RiboPrinter microbial characterization system, and reagents from the DuPont Qualicon ribotyping kit according to the manufacturer's instructions (DuPont Qualicon, Wilmington, DE). PvuII patterns were compared, using the RiboPrinter software, against the DuPont *Salmonella* PvuII database, which at the time of analysis included 592 isolates representing 227 serovars. The top match was used to predict the serovar of a tested isolate; if no pattern in the DuPont database matched with >70% similarity, the isolate serovar was reported as "Unidentified."

MLST. Partial sequencing of seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) was performed as previously described (Kidgell et al., 2002) at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY). Sequences were assembled and analyzed using Lasergene 7.2.1 software (DNAstar). Allelic type (AT) and sequence type (ST) numbers were assigned by submitting the sequences and strain information to the *Salmonella* MLST website (http://mlst.ucc.ie/mlst/dbs/Senterica). When a sequence from a *Salmonella* isolate matched an existing ST in the database, the serovar information for the existing ST was assigned to our query. For new STs, the nearest ST (matching 6/7 ATs) was used to assign a serovar; all new ATs (including corresponding electropherograms) and STs were submitted to the MLST database. All sequences for the 7-gene MLST are available at www.foodmicrobetracker.com.

DNA Preparation for PCR. For PCR amplification of O serogroups, *fliC* and *fljB*, total genomic DNA was extracted from 1 mL of overnight culture in BHI according to QIAamp DNeasy kit instructions (Qiagen Inc., Valencia, CA). DNA concentrations were determined using Nanodrop 1000 (Thermo Scientific, Wilmington, DE) and standardized to 25 ng/ml.

PCR detection of O serogroups. PCR detection of serogroups was performed using (i) a multiplex PCR that identifies serogroups O:4, O:7, O:8, O:9, and O:3,10 (19) and (ii) two separate single PCRs that identify O:13 (12) and O:18 (13); PCRs were performed using previously published primers (Table 4.1) and optimized PCR conditions (Table S2). PCR products were separated by agarose gel electrophoresis using Tris-acetate-EDTA buffer and visualized by staining with 0.005% ethidium bromide. PCR products obtained from select O antigen PCRs were also sequenced, using standard methods as detailed below.

PCR amplification and sequencing of genes encoding H1 and H2 antigens. Amplification of *fliC* and *fljB* was performed using primers (Table 4.1) and optimized PCR conditions (Table S2) previously described (Mortimer et al., 2004; Imre et al., 2005). We also designed an alternative set of *fljB* PCR primers (*fljB* set 2; Table 4.1) that was used for amplification of an approximately 1600 nt fragment (see Table S2 for PCR conditions); this set was designed as the previously described set of *fljB* primers (*fljB* Set 1, Table 4.1) did not allow for reliable amplification of *fljB*, predominately among isolates representing rare serovars (Table S1 details primers that were used for each isolate). Prior to sequencing, all PCR products were purified using Exonuclease I and shrimp alkaline phosphatase according to the manufacturer's instructions (Affymetrix, Cleveland, Ohio). As sequencing with previously published *fliC* or *fljB* primers only provided single coverage of the PCR product, newly designed primers MR-1_forward and MR-2_reverse (Sequencing Set 1) were used to obtain double coverage of the variable internal regions in *fliC* or *fljB* (Table 4.1). Sequencing was carried out on the Applied Biosystems Automated 3730 DNA Analyzer using Big Dye Terminator Chemistry at the Cornell University Life Sciences Core Laboratories Center. Sequences were assembled and analyzed using Lasergene 7.2.1 software (DNAstar, Madison, WI). BLASTN search analysis was used to compare *fliC* and *fljB* sequences with those in GenBank (Altschul et al., 1990), and to infer *fliC* or *fljB* antigens. Alignment of *fliC* and *fljB* sequences was performed using MAAFT (Katoh and Toh, 2008), and cluster analysis was performed using the maximum-likelihood (ML) algorithm in RAxML (Stamatakis, 2006) with rapid bootstrapping (100 bootstrap replicates). Amino acid sequence distances (p-distances) were calculated using MegA (Version 5.05) (Tamura et al., 2011).

TABLE 4.1: Summary of primers used to determine *Salmonella* serovars

Gene target (serogroup)	Designation	Amplicon	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Reference
wzx (O:4 [B])	Multiplex PCR 1	230	GGC ATA TAT TTC TGT ATT CGC G	GCC TTA ATT AAG TAA GTT AGT GGA AGC	Herrera-Leon et al., 2007
wzx (O:7 [C1])	Multiplex PCR 1	483	CAG TAG TCC GTA AAA TAC AGG GTG G	CAA TGC TAT AAA TAC TGT GTT AAA TTG C	Herrera-Leon et al., 2007
wzx (O:8 [C2-C3])	Multiplex PCR 1	154	ACT GAA GGT GGT ATT TCA TGG G	AAG ACA TCC CTA ACT GCC CTG C	Herrera-Leon et al., 2007
tyv (O:9 [D])	Multiplex PCR 1	615	GAG GAA GGG AAA TGA AGC TTT T	TAG CAA ACT GTC TCC CAC CAT AC	Herrera-Leon et al., 2007
wzx (O:3,10 [E1])	Multiplex PCR 1	345	TAA AGT ATA TGG TGC TGA TTT AAC C	GTT AAA ATG ACA GAT TGA GCA GAG	Herrera-Leon et al., 2007
wzy (O:13 [G])	Serogroup Set 1	90	CTC TTG ATG AAT GTT ATT A	GTT AAC CCC TCC TAA TA	Fitzgerald et al., 2007
wzx (O:18 [K])	Serogroup Set 2	360	CTC TAG GAT CAA CTG AAG GTG GTC	CAA CCC AGC AAT AAA GCA GAA	Fitzgerald et al., 2006
fliC	*fliC* Set 1[b]	~1520	ATG GCA CAA GTC ATT AAT AC	TTA ACG CAG TAA AGA GAG GAC	Mortimer et al., 2004
fliC	*fliC* Set 2[b]	~1520	CCG AAT TCA TGG CAC AAG TCA TTA ATA CAA AC	CCG GAT CCT TAA CGC AGT AAA GAG AGG ACG T	Imre et al., 2005
fljB	*fljB* Set 1[b]	~1520	CCG AAT TCA TGG CAC AAG TAA TCA ACA CTA A	CGG GAT CCT TAA CGT AAC AGA GAC AGC ACG	Imre et al., 2005
fljB	*fljB* Set 2[b]	~1600	GGC ACA AGT AAT CAA CAC TAA CA	CAT TTA CAG CCA TAC ATT CCA TA	Current study
fliC or *fljB*[a]	Sequencing Set 1	~887	AAC AAC AAC CTG CAG CGT GTG	GTC GGA ATC TTC GAT ACG GCT AC	Current study

[a]Primer MR-1 Forward and MR-2 Reverse used exclusively for sequencing of *fliC* and *fljB* PCR products. These primers provided double of the internal variable region of *fliC* and *fljB*.

[b]When used for sequencing these primers did not provide full double coverage of the internal variable region of *fliC* or *fljB*.

Traditional Serotyping. Immunological serotyping was completed by either the New York State Department of Health or the National Veterinary Services Laboratory (Ames, IA).

RESULTS

PFGE. PFGE patterns were generated for all 46 isolates tested, and then compared to a custom database that included PFGE patterns for isolates representing 170 serovars, including all 40 serovars evaluated here. Using the methods detailed above, serovars were predicted correctly for 35/46 (75%) isolates (Table 4.2). Among the 11 isolates that were not accurately predicted, 3 isolates were predicted to be serovars that were not congruent with traditional serotyping; one serovar Typhimurium isolate matched serovar 4,5,12:i:- (0 band difference), one serovar Saintpaul isolate matched serovar Typhimurium (2 band difference), and one serovar Typhimurium var. 5- isolate matched serovars Typhimurium (0 band difference) and Typhimurium var. 5- (0 band difference) (Table S3). No serovar could be assigned for 8/46 isolates as PFGE patterns differed by > 3 bands from all isolates in the database; these isolates represented serovars Choleraesuis, Give, Mississippi, Orion var. 15+,34+, Reading, Virchow, Weltevreden, and Worthington (Table 4.2).

rep-PCR. rep-PCR patterns were generated, on the DiversiLab system, for all 46 isolates tested. Overall, the DiversiLab rep-PCR system accurately predicted 30/46 (65%) serovars tested when applying an 85% similarity cutoff (Table 4.2). Of the remaining 16 isolates, 11/16 had rep-PCR patterns that matched an existing pattern in the rep-PCR library at > 85% identity, but the assigned serovar was not congruent with traditional serotyping (Table 4.2). Among the 5 isolates that had rep-PCR patterns with < 85% identity to patterns in the DiversiLab library, four represented serovars were not included in the library (Give, Orion var. 15+, 34+, Typhimurium var.

TABLE 4.2: Comparison of DNA based subtyping methods used to predict the 'top 40' *Salmonella* serovars evaluated in this study

DNA based subtyping method	No. of isolates for which the serovar was identified correctly (n = 46)	Serovar was incorrectly identified (no.)	Serovar was not identified (no.)
MLST	42 (91%)	4,5,12:i:- (1); Typhimurium var. 5- (1)	Orion var. 15+,34+ (1)[a], Reading (1)[a]
Molecular Serotyping	42 (91%)	Choleraesuis (1); Senftenberg (1); Typhimurium (1); Typhimurium var. 5- (1)	(0)
PFGE	35 (76%)	Saintpaul (1); Typhimurium (1); Typhimurium var. 5-	Choleraesuis (1)[b]; Give (1)[b]; Mississippi (1)[b]; Orion var. 15+,34+ (1)[b]; Reading (1)[b]; Virchow (1)[b]; Weltevreden (1)[b]; Worthington (1)[b]
Rep-PCR	30 (65%)	Derby (1); Infantis (1); Kentucky (2); Muenster (1); Paratyphi B. var. Java (1); Reading (1); Senftenberg (1); Stanley (1); Typhimurium (1); Virchow (1);	Give (1)[d]; Javiana (1)[c]; Orion var. 15+,34+ (1)[d]; Typhimurium var. 5- (1)[d]; Weltevreden (1)[d]
Ribotyping	34 (74%)	4,5,12:i:- (1); Braenderup (1); Give (1); Javiana (1); Muenster (1); Orion 15+,34+; (1); Uganda (1)	Blockley (1)[e]; Dublin (1)[e]; Montevideo (1)[f]; Typhi (1)[f]; Typhimurium var. 5- (1)[e]

[a]For unidentified serovars traditional serotyping information was not available for the most similar isolate(s) in the MLST database.
[b]PFGE patterns for the most similar patterns differed by more than 3 bands, thus a serovar could not determined.
[c]DiversiLab percent identity to library strains was less than 85%.
[d]Serovar not in DiversiLab library at time of analysis.
[e]Serovar was not in ribotype database at time of analysis.
[f]Serovar could not be assigned as ribotype pattern did not match existing pattern in database at > 70%.

5- and Weltevreden; Table 4.2). While rep-PCR patterns for 5 serovar Javiana isolates were in the DiversiLab library, one serovar Javiana isolate tested (FSL S5-406) did not match an existing pattern at > 85% identity (top match was Mississippi at 72.3% identity) (Table S3).

Ribotyping. Automated ribotyping produced ribotype patterns for all 46 isolates. A total of 34/46 (74%) serovars predicted by ribotyping were congruent with traditional *Salmonella* serotyping results. Of the 12 serovars that were not accurately predicted, 7 isolates had ribotype patterns that matched database patterns with > 70% identity, but the assigned serovars were not congruent with traditional serotyping results (Table 4.2). Ribotype patterns for serovars Montevideo (FSL S5-630) and Typhi (FSL R6-540) did not match any existing patterns in the database at > 70% similarity and thus could not be assigned a serovar; both Montevideo and Typhi ribotype patterns were available in the database (Table S2). An additional 3 isolates did not match any existing patterns at > 70% and the database did not contain those serovars (i.e., serovars Blockley [FSL S5-648], Dublin [FSL S5-439], and Typhimurium var. 5- [FSL S5-786]) (Table 4.2).

MLST. The Max Planck 7-gene MLST scheme was able to accurately predict serovars for 42/46 (91%) isolates (Table 4.2). Two isolates, representing serovars 4,5,12;i;- and Typhimurium var. 5- (FSL S5-580 and FSL S5-786, respectively), were identified as serovar Typhimurium. An additional 2 isolates representing serovars Orion var. 15+,34+ (FSL R8-3408) and Reading (FSL R8-1987) could not be identified; isolates representing the corresponding STs in the MLST database lacked serovar information. Among the 322 partial housekeeping gene sequences submitted, new ATs were identified for serovars Javiana (FSL S5-406; *hisD* AT520), Oranienburg (FSL S5-642; *hemD* AT315), and Give (FSL S5-487; *sucA* AT397). A total of 6 new STs were identified for isolates representing serovars Javiana (ST1674),

Montevideo (ST1677), Oranienburg (ST1675), Dublin (ST1673), Uganda (ST1676), and Give (ST1678) (Table S4).

PCRs targeting O antigen genes allowed for reliable identification of clinically important *Salmonella* serogroups, but specific primers for less common O antigens need to be developed. PCRs targeting O antigen genes were used to determine serogroups in 46 isolates representing clinically important *S. enterica* subsp. *enterica* serovars and 70 less common *S. enterica* serovars (Table S1). Based on traditional serotyping data, these PCRs were expected to allow for identification of the O-groups for 44/46 isolates representing common serovars and 40/64 isolates representing less common serovars for a total of 84/110 isolates (Table 4.3). PCR based serogroup results were congruent with immunological serotyping data for all 84 of these isolates, including 44 isolates representing common serovars. Correctly identified serogroups included O:4 (n = 21), O:7 (n = 15), O:8 (n = 16), O:9 (n = 11), O:3,10 (n = 9), O:13 (n = 11) and O:18 (n = 1) (Table 4.3). Sequencing of selected O-group PCR products revealed limited diversity within a given O-group; for example, a 532 nt partial *tyvD* sequence obtained from six O:9 isolates showed only 4 polymorphic nucleotides, all present in the same isolate (Figure S1). Also, sequencing of a 402 nt *wzx* fragment in one E4 and seven E1 isolates revealed limited diversity and no polymorphisms that could differentiate E4 from E1 (Figure S2).

Twenty-six isolates represented, by traditional serotyping, O-groups that were not targeted by the O-group PCR assays used. Among these 26 isolates, 18 did not yield PCR products with any of the O-group PCRs evaluated (Table 4.3). However, 8 isolates each yielded a positive PCR result with one primer set; for these isolates PCR-based serogroups were not congruent with traditional typing, these isolates included O-groups O:11 (n = 5), O:9,46 (n = 1), O:1,3,19 (n = 1), O:54 (n = 1). All five O:11 isolates were positive with O:7 primers (Table 4.3); we subsequently found that the

TABLE 4.3 Results of O-group determination using serogroup specific PCRs

Serogroup	No. isolates within serogroup[a]	No. isolates with a positive PCR result for O-group							No. isolates with neg. PCR result
		O:4 (B)	O:7 (C1)	O:8 (C2-C3)	O:9 (D1)	O:3,10 (E1)	O:13 (G)	O:18 (K)	
O-groups with primers for detection									
O:4 (B)	21	21							0
O:7 (C1)	15		15						0
O:8 (C2-C3)	16			16					0
O:9 (D1)	11				11				0
O:3,10 (E1)	9					9			0
O:13 (G)	11						11		0
O:18 (K)	1							1	0
O-groups lacking primers for detection									
O:2 (A)	1								1
O:9,46 (D2)	1				1[d]				0
O:1,3,19 (E4)	1					1[e]			0
O:11 (F)	5		5[b]						0
O:6,14 (H)	1								1
O:16 (I)	4								4
O:28 (M)	2								2
O:30 (N)	1								1
O:35 (O)	4								4
O:38 (P)	1								1
O:39 (Q)	1								1
O:40 (R)	2								2
O:51	1								1
O:54	1		1[c]						0
Untypable	6				1			2	3

[a]Serogroups for 116 isolates determined by immunological serotyping. Among the 116 isolates, 95 yielded positive PCR results with our primers (Table 1). The remaining 21 isolates did not show amplification with any of the 7 O-group primer sets tested.

[b]Analysis of serogroup O:7 primers revealed non-specific primer match to serovar Rubislaw (O:11).

[c]Factor O:54 is plasmid-controlled and may mask factors O:6,7,14 (C1) for serovar Montevideo.

[d]Analysis of serogroup O:9 primers revealed primer match to serovar Baildon (O:9,46).

[e]Primer design was based on *Salmonella* sequences representing serogroups O:3,10 and O:1,3,19.

serogroup O:7 forward (22/22) and reverse (23/23) primers matched *tyv* (an O antigen gene present in the *rfb* region) in *Salmonella* Rubislaw (O:11), with a predicted amplicon size (615 nt) that matched the size expected for O:7. The only isolate representing serogroup O:9,46 was positive with the O:9 primers; the serogroup O:9 forward (24/25 nt) and reverse (28/29 nt) primers matched *tyv* in *Salmonella* Baildon (O:9,46). Sequencing and alignment of *tyvD* in serogroup O:9 revealed that this gene is highly conserved (Figure S1). The one serogroup O:1,3,19 isolate was positive with the O:3,10 primers; sequencing and alignment revealed that *wzx* was highly conserved between the two serogroups (Figure S2) and primers had been designed to detect both O:3,10 and O:1,3,19 (Herrera-León et al., 2007). Serovar Montevideo (serogroup O:54) was detected by O:7 primers; this exception was not completely unexpected as Montevideo serogroup expression is plasmid controlled and may mask factor O:7 (Popoff and Le Minor, 1985).

Among the 7 isolates that could not be classified by immunological serotyping, three isolates yielded positive results with one of the O-group primer sets used here; these isolates were classified as serogroups O:3,10 (FSL R8-2289) and O:18 (FSL R6-592 and R8-904) (Table 4.4). The remaining 4 untypable isolates (FSL R8-3567, FSL A4-524, FSL R8-143, and FSL R8-756) did not yield PCR products with any of the O-group primer sets used.

***fliC* and *fljB* sequencing allows for prediction of H1 and H2 antigens, that is also congruent with serological typing.** Among the 109 tested isolates with serovar information, 28 H1 antigens and 15 unique H2 antigens were represented. Flagellar antigens for these isolates were identified through a molecular approach that includes amplification of *fliC* and *fljB,* encoding for H1 and H2, respectively, and sequencing to obtain coverage of the internal, variable region. Results for PCR and sequence based determination of H1 antigens were congruent with traditional serotyping for all

TABLE 4.4: Molecular serotyping results for serologically untypable isolates[a]

Isolate	Immunological serotyping results				Molecular serotyping results			
	Serogroup	H1 antigens	H2 antigens	Serovar	Serogroup	H1 antigens	H2 antigens	Serovar[b]
FSL R8-3567	O:35 (O)	NA	NA	IIIb 35:Rough	ND[c]	l,v	1,5	requires O-antigen identification
FSL R6-592	NA	NA	NA	Untypable	O:18 (K)	z4,z23	–	*S.* I, II or IIIa 18:z4,z23:-
FSL R8-904	NA	NA	NA	Untypable	O:18 (K)	z4,z23	–	*S.* I, II or IIIa 18:z4,z23:-
FSL R8-2289	NA	NA	NA	Untypable	O:3,10 (E1)[d]	g,[s],t	–	*S.* II 3,10:g,[s],t:-
FSL A4-524	NA	NA	NA	Untypable	ND[c]	y	1,7	requires O-antigen identification
FSL R8-143	NA	NA	NA	Untypable	ND[c]	z52	1,7	requires O-antigen identification
FSL R8-756	NA	NA	NA	Untypable	ND[c]	k	1,5,7	requires O-antigen identification

[a]Represents all isolates where immunological determination of antigens was inhibited by strain phenotype (e.g., rough, mucoid, or non-motile).

[b]Species other than *S. enterica* subsp. *enterica* are designated by the following symbols: II for serovars of *S. enterica* subsp. *salamae*; IIIa for serovars of *S. enterica* subsp. *arizonae*

[c]ND indicates serogroup was not detected with primer sets tested in this study (i.e., primer sets for detection of O:4, O:7, O:8, O:9, O:3,10, O:13, and O:18)

[d]Serogroup primers for O:3,10 (E1) were also found to detect serogroup O:1,3,19 (E4).

109 isolates (Table S1), while H2 antigen determination was congruent with traditional serotyping for 104/109 isolates. Isolates where molecular and traditional H2 antigen determinations did not match included 2 isolates from the isolate set representing the 40 most common serovars as well as three isolates from the set representing less common serovars (Table 4.5). Specifically, for one serovar Typhimurium isolate (FSL S5-433), we obtained a PCR product, but were unable to sequence the product and for one serovar Choleraesuis isolates no PCR product was obtained with the *fljB* primers. In addition, for a serovar Corvallis isolate, sequencing determined the H2 antigens to be 1,5 while immunological serotyping indicated [z6] and for a serovar Wandsworth isolate sequencing determined the H2 antigens to be 1,7 while traditional serotyping indicated a 1,2 H2 antigen (Table 4.5). Finally, for one serovar Wangata isolate no *fljB* PCR product could be obtained (Table 4.5). While H1 antigens could be determined, by molecular serotyping, for all seven untypable isolates tested here, the H2 antigen encoding gene was only amplified for four isolates, which were identified as 1,5 (n=1); 1,7 (n=2), and 1,5,7 (n=1) (Table 4.4).

Cluster analysis performed on the 116 partial *fliC* aa sequences obtained here (Figure 4.1) showed three distinct clades that represented (i) the g-complex with "g" or "m,t" antigenic factors; (ii) the "z4,z23" antigenic group; and (iii) a large cluster with predominately single antigens (e.g., "a" or "b"; previously described as the "non g-complex") (Mortimer et al., 2004). The tree also included a large number of well supported nodes (bootstrap values > 90) within these clades, typically supporting branches that included sequences for a given H1 antigen (a total of 26 unique antigenic factors were represented in this tree). Most *fliC* antigenic groups represented highly homologous sequences; for example, sequence similarities within antigenic group r were > 99%. However, not all *fliC* antigen groups were as homologous; for

TABLE 4.5: Discrepancies between traditional and molecular serotyping of O, H1 and H2 antigens

Isolate set	Isolate	Discrepancy	Immunological serotyping results				Molecular serotyping results				Conclusion
			Serogroup	H1 antigens	H2 antigens	Serotype	Serogroup	H1 antigens	H2 antigens	Serovar	
Top 40	FSL S5-433[a]	H2 antigen	O:4 (B)	i	1,2	Typhimurium	O:4 (B)	i	–[b]	4,5,12:i:-	*fljB* sequencing failure[b]
	FSL R8-3632[c]	H2 antigen	O:7 (C1)	c	1,5	Choleraesuis	O:7(C1)	c	–	6,7:c:-	*fljB* primer exception
	FSL S5-658	Serogroup	O:1,3,19 (E4)	g,[s],t	–	Senftenberg	O:3,10 (E1)[d]	g,[s],t	–	Westhampton	Non-specific serogroup primer
Rare 70	FSL R8-092	H2 antigen	O:8 (C2-C3)	z4, z23	[z6]	Corvallis	O:8 (C2-C3)	z4, z23	1,5	6,8:z4,z23:1,5	H2 identification error
	FSL R8-1542	H2 antigen	O:9 (D1)	z4, z23	1,7	Wangata	O:9 (D1)	z4, z23	–	9,12:z4,z23:-	*fljB* primer exception
	FSL R6-199	Serogroup	O:9,46 (D2)	a	e,n,x	Baildon	O:9 (D1)[e]	a	e,n,x	Lomalinda	Non-specific serogroup primer[e]
	FSL R6-526	H2 antigen	O:39 (Q)	b	1,2	Wandsworth	NA[f]	b	1,7	Incomplete[f]	H2 identification error
	FSL A4-595	Serogroup	O:11 (F)	k	e,n,x,[z15]	Kisarawe	O:7(C1)	k	e,n,x	Singapore	Non-specific serogroup primer[g]
	FSL R8-3524	Serogroup	O:11 (F)	i	1,2	Aberdeen	O:7(C1)	i	1,2	Augustenborg	Non-specific serogroup primer[g]
	FSL R8-3555[h]	Serogroup	O:11 (F)	a	e,n,z15	Luciana	O:7(C1)	a	e,n,z15	6,7:a:e,n,z15	Non-specific serogroup primer[g]
	FSL S5-477	Serogroup	O:11 (F)	r	e,n,x	Rubislaw	O:7(C1)	r	e,n,x	6,7:r:e,n,x	Non-specific serogroup primer[g]
	FSL S5-654	Serogroup	O:11 (F)	z	z6	Nyanza	O:7(C1)	z	z,6	S. enterica subsp. II	Non-specific serogroup primer[g]

[a]Repeated immunological serotyping confirmed isolate was *S.* Typhimurium.

[b]Primers amplified PCR product of expected size; sequencing quality was noisy and deteriorated and could not be interpreted.

[c]Repeated immunological serotyping confirmed isolate was *S.* Choleraesuis.

[d]Primers targeting serogroup O:3,10 also detected serogroup O:1,3,19.

[e]Primers targeting serogroup O:9 also detected serogroup O:9,46.

[f]NA indicates primers were not available for detection of serogroup O:39, which led to incomplete molecular based serovar result.

[g]Primers targeting serogroup O:7 also detected serogroup O:11.

[h]Isolate chosen to represent five serogroup O:11 (F) isolates with O-group discrepancy. Repeated immunological serotyping confirmed isolate was *S.* Luciana (serogroup O:11).

example sequence similarities for antigenic group k ranged from 74.1% - 100%. Despite this, the k antigenic group represented a clearly defined clade.

Cluster analysis of 90 *fljB* partial aa sequences (Figure 4.2) also showed that the majority of the 11 unique antigenic factors (represented by 32 isolates representing common serovars, 54 isolates representing rare serovars, and 4 untypable isolates), grouped into well-defined clades, with many antigenic groups displaying a high level of aa homology. For example, partial aa sequence similarities for antigenic group e,n,x ranged from 99.5 - 100%. Antigenic group 1,5 showed the lowest level of homology, sequence similarities ranged from 88.4 - 100%; even though this group is paraphyletic with aa sequences for antigenic factors 1,6, *fljB* sequencing still allowed for antigen determination that was congruent with traditional serotyping. Overall, phylogenetic trees based on partial aa sequence for *fliC* and *fljB* display clearly defined clusters that allow for identification of antigenic groups, indicating their potential for sequence based identification of H1 and H2 antigens, respectively.

Comparison of DNA based subtyping methods and their ability to predict serovars. Based on the 46 isolates representing the 40 most common *Salmonella* serovars, the predictive ability of DNA based subtyping methods evaluated in this study ranged from 30/46 (65%; rep-PCR) to 42/46 (91%; MLST and molecular serotyping) (Table 4.2). Serovars 4,5,12:i:-, Typhimurium, and Typhimurium var. 5- represented the 3 serovars for which molecular methods were most frequently unable to predict a serovar that was congruent with traditional serotyping.

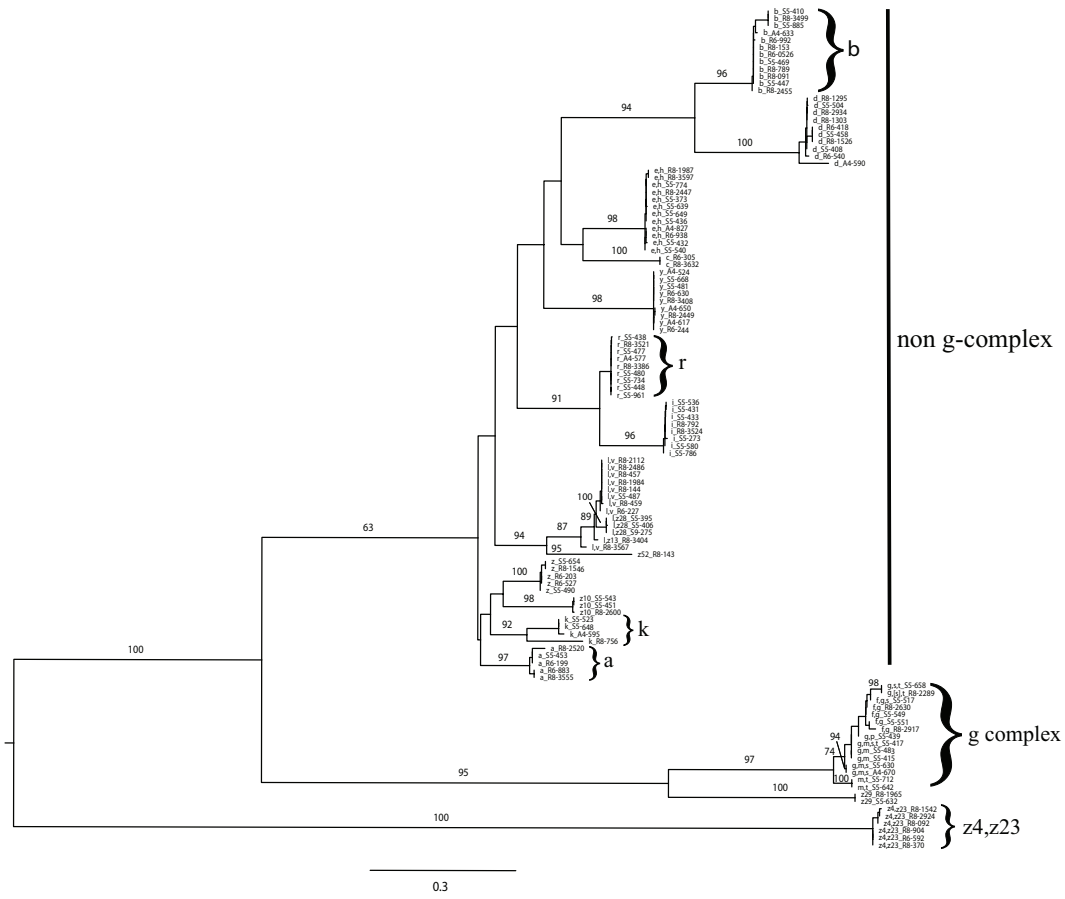

Figure 4.1: Midpoint-rooted maximum-likelihood phylogenetic tree of partial *fliC* amino acid sequences from 116 *Salmonella* isolates representing 46 common, 63 uncommon, and 7 untypable serovars. The scale represents the estimated number of amino acid substitutions per site. Numerical values represent the percentage of bootstrap replications that support the respective node. Bootstrap values greater than 60 are shown for major clades. Label shows the H1 antigen, followed by Food Safety Laboratory (FSL) number; e.g., b_S5-410 indicates H1 antigen b, isolate FSL S5-410.

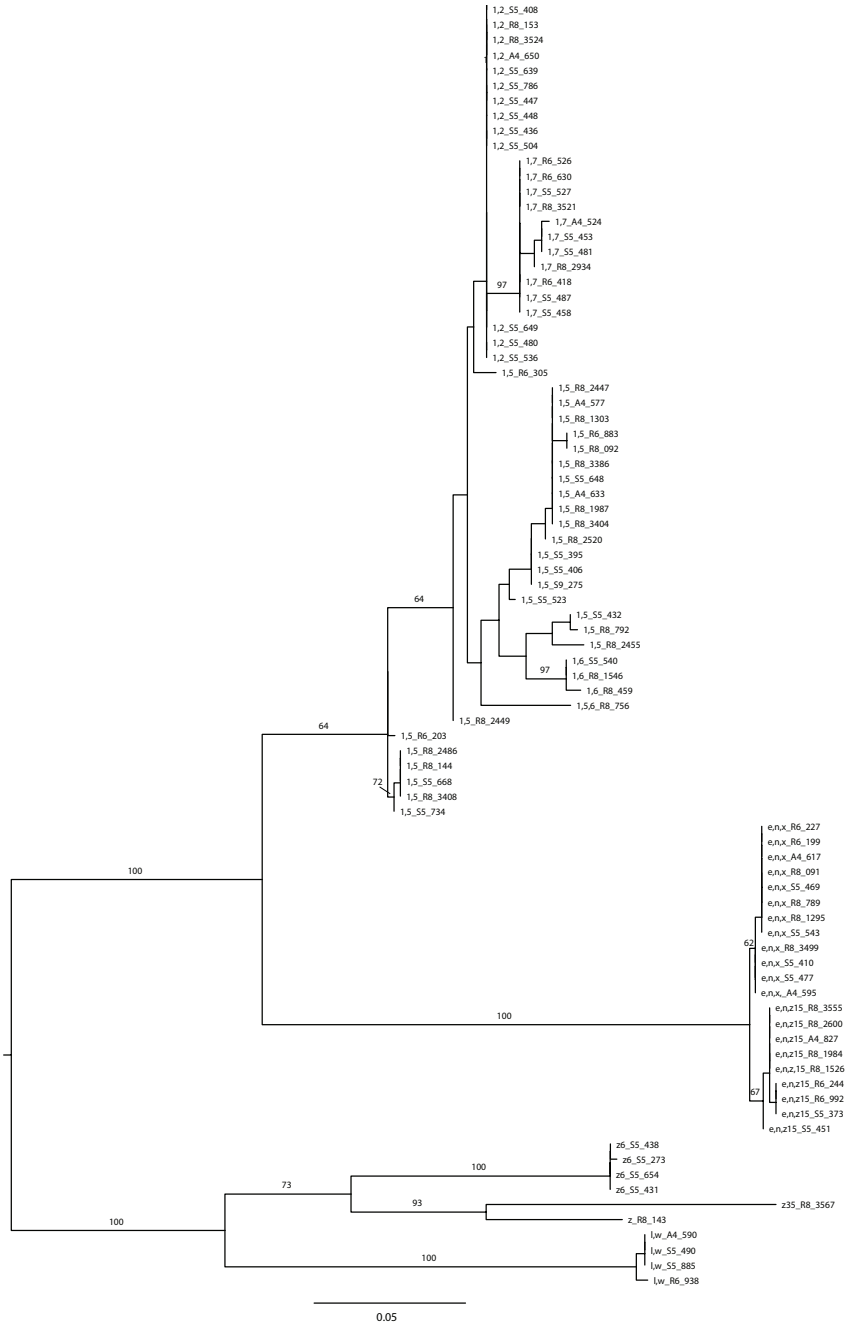

Figure 4.2: Midpoint-rooted maximum-likelihood phylogenetic tree of 90 partial *fljB* amino acid sequences from *Salmonella* isolates representing 32 common and 58 rare serotypes. The scale represents the estimated number of amino acid substitutions per site. Numerical values represent the percentage of bootstrap replications that support the respective node. Bootstrap values greater than 60 are shown for major clades.

DISCUSSION

Salmonella serotyping remains a critical component of *Salmonella* surveillance efforts as it facilitates rapid identification and source tracking of salmonellosis outbreaks, particularly if rapid access to molecular subtyping, such as PFGE, is not available. Traditional serotyping not only provides subtyping data that allow for worldwide comparison, which has facilitated detection of a number of salmonellosis outbreaks with international scope (Werber et al., 2005; Elviss et al., 2009; Nicolay et al., 2011), but also facilitates comparison with historical datasets since serotyping has been in use for about 70 years. As new methods for serotyping and subtyping of *Salmonella* are developed, it is thus important that these methods can be referenced and correlated to serovars according to the existing White-Kauffmann-Le Minor scheme, to maintain continuity of information based on serovar data, as well as to facilitate communication with laboratories that use traditional serotyping. Conceptually, molecular approaches to serotyping of *Salmonella* may use either (i) characterization of genetic targets that are directly responsible for O and H antigen expression or (ii) genetic characterization of *Salmonella* through banding or sequence-based subtyping methods (targeting genes unrelated to O and H antigen expression), followed by serovar prediction through comparison with databases that contain references patterns for isolates with traditional serovar information. Our study indicates that (i) serovar prediction based on banding pattern-based methods (i.e., PFGE, rep-PCR, and ribotyping) and DNA sequence typing schemes (i.e., MLST) is feasible for most serovars, but requires large and comprehensive databases and that (ii) sequence based serotyping provides an alternative method to SNP or microarray based O and H antigen determination or subtyping-based serovar prediction.

Serovar prediction based on banding pattern-based methods and DNA sequence typing schemes is feasible for most serovars, but requires large and

comprehensive databases. For banding pattern-based subtyping methods, the ability to correctly predict serovars ranged from 65% to 76% correct prediction of serovars among isolates representing the 40 most common *Salmonella* serovars; by comparison MLST correctly predicted the serovars of 91% of these isolates. Previous studies typically only tested the ability of one or a few subtyping methods to predict serovars in isolates representing limited diversity and a few serovars (Weigel et al., 2004; Gaul et al., 2007; Bailey et al., 2002; Wise et al., 2009; Zou et al., 2010; Chenu et al., 2011). For example, Gaul *et al*. (Gaul et al., 2007) compared one banding pattern method, PFGE, to traditional serotyping on a collection of 674 swine *Salmonella* isolates. In general, if subtyping data are to be used for serovar prediction, they will require establishment of a large and comprehensive libraries of subtype patterns, which should represent the diversity of at least a majority of the 2,500 *Salmonella* serovars. We specifically observed that in some cases common serovars could not be identified due to database limitations, i.e., the serovar was not available in the database. In contrast to most databases for banding pattern methods, which are typically proprietary (e.g., for automated ribotyping, rep-PCR) or restricted access (e.g., PulseNet), MLST is characterized by the availability of open source databases (http://pubmlst.org/databases.shtml) with continuous community addition of subtype data. Among the subtype methods evaluated, PFGE and MLST have the largest databases, even though the PulseNet PFGE database could not be used for the study reported here as it not publicly available. While the *Salmonella* MLST database is large (it included > 5,700 *Salmonella* isolates and > 600 serovars as of October 15, 2012), a recent study suggested that reliable MLST-based prediction of *Salmonella* serovars may remain challenging (Achtman et al., 2012). In particular, this study showed that a number of phylogenetic groups (e-Burst groups) contained multiple serovars and that many serovars are distributed across distinct e-Burst groups,

suggesting polyphyletic origins of these serovars. In our study, rather than using phylogenetic groupings to predict serovars, we used perfect ST matches to isolates in the MLST database to predict serovars; only in cases where no prefect ST match was available used the serovars for closely related isolates (matches in 6 of 7 ATs) to predict the serovar for a query isolate. While this approach is more pragmatic and may be more likely to not yield a "match" that allows for serovar prediction, it, based on our data, shows a good ability to predict serovars. Importantly, traditional serotyping of *Salmonella* has been estimated to allow for correct serovar identification with about 92 to 95% of isolates (Wattiau et al., 2008b), suggesting that at least for the isolate set used here, the accuracy of MLST for prediction of serovars is in the same range as expected for traditional serotyping. For example, Wattiau *et al*. reported that 90.8% of 754 *S. enterica* subsp. *enterica* isolates were correctly serotyped by classical methods with 9.1% of isolates showing no results with classical serotyping due to strain autoagglutination or lack of antigen expression (Wattiau et al., 2008a).

While development of larger databases for subtyping methods may allow for some improvements with regard to the ability of these methods to correctly predict *Salmonella* serovars, there are inherent limitations to serovar prediction by subtyping methods, as, for example, detailed by Achtman *et al*. (Achtman et al., 2012) for MLST-based prediction of serovars. Our data specifically support that many subtyping methods are not likely be able to correctly identify and differentiate the closely related *Salmonella* serovars Typhimurium (4,5,12:i:1,2), 4,5,12:i:-, and Typhimurium var. 5-. This is consistent with recent studies (Gaul et al., 2007; Zou et al., 2012) that also showed that the majority of isolates for which serovars were not correctly predicted by PFGE belonged to 4,5,12:i:-; in one study 135 misclassified 4,5,12:i:- isolates were predicted to either be serovar Typhimurium (95 isolates) or Typhimurium var. 5- (40 isolates) (Zou et al., 2012). Similar limitations with closely related *Salmonella*

serovars have been reported when evaluating ribotyping; in one study 20 serovar 4,5,12:i:- isolates were predicted to be serovar Typhimurium (Bailey et al., 2002). While rep-PCR reported to predict were serovar 4,5,12:i:- in one study with three 4,5,12:i:- isolates (Wise et al., 2009), our study reported problems with correct prediction of serovar 4,5,12:i:- across banding pattern-based subtyping methods, consistent with the observation that strain with this serovar appears to represent multiple independent emergence events from serovar Typhimurium ancestors. In addition, previous studies have also shown that subtyping methods can, in some instances, not correctly predict serovars differing by one or two antigens, such as with (i) serovars Newport (I 6,8,20:e,h:1,2) and Bardo (I 8:e,h:1,2) (Soyer et al., 2010) and (ii) serovars Hadar (I 6,8:z10:e,n,x) and Istanbul (I 8:z10:e,n,x) (Wise et al., 2009).

Sequence based serotyping provides an alternative method to SNP or microarray based O and H antigen determination. Methods that directly characterize genetic targets that are responsible for O and H antigens conceptually represent an attractive opportunity for "molecular serotyping," which should address a number of the drawbacks of serovar prediction based on molecular subtyping methods. To date, some methods have been developed are available that use primers and probes in various assay formats to detect specific O, H1 and H2 antigen markers (within the *rfb* cluster, *fliC* and *fljB*), including a Luminex based system (Fitzgerald et al., 2007; McQuiston et al., 2011) and ArrayTube genoserotyping tool (Franklin et al., 2011). In initial evaluations, these methods have demonstrated good congruency with traditional serotyping. For example, the Luminex-based system developed by the CDC allowed accurate O-group prediction for 362/384 isolates (94.3%) representing 6 common O-groups (Fitzgerald et al., 2007) and accurate H antigen prediction for 461/500 isolates (92.2%) (McQuiston et al., 2011). In a smaller study, the ArrayTube genoserotyping tool allowed for correct serovar prediction for 76/100 (76%) isolates (Franklin et al.,

2011). While these methods offer the potential for rapid, ease-of-use and high throughput molecular serovar prediction, including for both rough and mucoid strains, these methods can currently only identify a portion of the over 1,500 *Salmonella* subsp. I serovars. For example, the most recently described Luminex assay was not able to determine H antigens for 46/500 isolates due to a limited number of probes (McQuiston et al., 2011) and the ArrayTube genoserotyping tool is currently only able to detect 41/114 flagellar antigens (Franklin et al., 2011). While both of these approaches appear to work reasonably well for serovar identification of common serovars where sufficient genetic information (e.g., full genome sequence data) is available for design of appropriate reagents (i.e. primers and probes), difficulties are likely encountered when these systems are challenged with isolates representing rare serovars that were not used for the design of the primers or probes. Examples of specific concerns include (i) no reaction with primer and probes as genes encoding for O or H antigens are not targeted by primer and probes, and (ii) false positive results for a given O or H antigen if primers and probes target a region that is conserved between common and rare antigens that were not considered in the assay design.

In contrast to molecular serotyping systems that rely on primers and probes to identify genes that determine the antigenic formula for *Salmonella* isolates, we implemented an approach that combines (i) PCR-based detection of genes that are specific for a given O antigen based on previous studies that used PCR to identify major O antigen groups (Fitzgerald et al., 2006; Herrera-León et al., 2007) and (ii) PCR amplification of *fliC* and *fljB*, followed by sequencing of the internal variable region of these genes to allow for H1 and H2 antigen determination. Overall, this approach allowed for correct identification of 91% of the isolates representing the common 40 serovars and 85.7% of the isolates representing less common serovars. While sequencing of *fliC* and *fljB* has previously been used to discover target

sequences for development of probe-based molecular serotyping approaches, we are not aware of any comprehensive studies that used sequencing of these two genes as the primary approach for molecular serotyping. While our data suggest that PCR-based O antigen typing along with *fliC* and *fljB* sequencing presents a viable approach for molecular serotyping, some challenges remain to be overcome to develop this method so that it can be used broadly and allows for serotyping of a wide range of *Salmonella* serovars. For one, our current method only detects 7 common O antigens, with some primers showing positive reaction with two antigens, therefore causing some false positive results, including one primer set that yields positive results with both serogroup O:3,10 (E1) and O:1,3,19 (E4) isolates (Herrera-León et al., 2007), and a set that yields positive results with both serogroup O:7 (C1) and O:11 (F). Design of better PCR primers and approaches that use PCR and subsequent sequencing of target genes that contribute to O antigen expression should, in the future, be able to address this issue. Specifically, as full genome sequences for isolates representing additional O-groups become available (Bakker et al., 2011; Allard et al., 2012), design primers capable of detecting all 46 *Salmonella* serogroups should be feasible. With regard to identification of H1 and H2 antigens, the design of primer sets that allow reliable amplification of *fliC* and *fljB* remains a challenge; these genes include internal variable and external conserved regions, which represents a challenge in the design of primers that only amplify the target gene (i.e., either *fliC* and *fljB*) and allow for reliable amplification across diverse serovars. We also found that previously reported *fljB* primers failed to amplify *fljB* in a number of isolates representing less common serovars. Even though the majority of isolates evaluated here allowed for successful *fliC* and *fljB* amplificaton, with the new set of *fljB* primers designed here we found a few exceptions, including an inability to amplify *fljB* in one serovar Choleraesuis isolate, supporting the need to develop additional or improved primers. Again,

availability of full genome sequences for additional serovars should help in the design of improved primers for *fliC* and *fljB* amplification, even though use of more than one primer set may be necessary to allow for amplification in isolates representing diverse serovars. Genome sequences should also facilitate development of PCR-based approaches for the detection of rare flagellar antigens encoded by other genes (Mehta and Arya, 2002). Finally, development of robust and large *fliC* and *fljB* sequence databases will be necessary to allow for broad use of the sequencing based molecular serotyping approaches described here; to this end we have deposited the *fliC* and *fljB* sequence data reported here in the public Food Microbe Tracker database (www.foodmicrobetracker.com).

Conclusions. As a variety of efforts are under way to replace or supplement traditional serotyping of *Salmonella* with molecular methods, many laboratories around are faced with decisions as to which technology or approaches to implement. Current approaches use either serovar prediction based on molecular subtyping data or direct characterization of genes affecting O or H antigen expression. Among the methods evaluated here, sequencing based approaches including (i) MLST and (ii) a combination of a PCR-based O antigen screen and sequencing of internal *fliC* (H1 antigen) and *fljB* (H2 antigen) fragments provided for the best serovar prediction. Both of these methods also use equipment that can be used for a variety of applications, as compared to the more specialized equipment used for many banding pattern based subtyping (e.g., ribotyping, Rep-PCR) or other molecular serotyping methods that were not evaluated here (e.g., PremiTest (Wattiau et al., 2008a), Luminex (Fitzgerald et al., 2007; McQuiston et al., 2011), ArrayTube genoserotyping (Franklin et al., 2011)); this may favor implementation of PCR and sequencing-based methods in some laboratories, particularly as advances in sequencing technology could make these methods more attractive. Our data also indicate that banding pattern-based

subtyping methods may have the potential to allow for serovar prediction that may be adequate under some conditions, particularly for users that have or can develop larger databases that contain subtype patterns for isolates representing diverse serovars or at least the serovars typically encountered by a given laboratory. In addition, combination of multiple molecular and possibly traditional serotyping approaches will facilitate improved serovar classification of *Salmonella.*

Importantly, the combination of a PCR-based O antigen screen and sequencing of internal *fliC* and *fljB* fragments reported here allows for continuity with traditional serotyping data. While some authors have proposed that MLST-based approaches should fully replace serotyping (Achtman et al., 2012), we believe that compatibility with traditional serovar data is critical for *Salmonella* characterization, at least in the medium term future. In addition, this approach will be highly compatible with full genome sequencing-based strategies for *Salmonella* characterization as serovar specific sequence data can easily be extracted from full genome sequences and be used to predict serovars, using the information created through PCR-based O antigen screen and sequencing of internal *fliC* and *fljB* fragments.

CHAPTER FIVE

CONCLUSIONS

Continued improvement of food quality and food safety remains important to create a sustainable and adequate food supply. Our research presents a general overview of the microbiological hurdles facing dairy processors, and provides a rapid assay to identify the current hurdle to fluid milk shelf-life extension– *Paenibacillus* spp. In addition, we demonstrated the ability to utilize basic lab equipment in order to facilitate rapid, economical serotype determination for *Salmonella enterica*, which is responsible for the largest number of known foodborne illnesses, caused by bacteria, in the US. Accurate serotyping of *Salmonella* is important to continue surveillance and intervene during outbreaks of salmonellosis.

Sporeforming spoilage bacteria play an important role in the quality of dairy products. As dairy processors strive to meet consumer demand by developing new products with extended or novel shelf-life characteristics, the need to understand characteristics, ecology, and spoilage potential of sporeforming bacteria will become increasingly important. Spores are ubiquitous in nature, and are capable of enduring many of the processing hurdles developed and implemented to date. With reliable tracking and characterization methods, we will be able to mitigate problems associated with sporeforming spoilage organisms by using a systematic approach for controlling points of entry and multiplication for these microbes in dairy systems. The combined efforts of farmers, dairy processors, retailers and researchers will be needed to provide consumers with the highest quality dairy products possible. To that end, it is essential

that all segments of the dairy industry work together to integrate practical measures for control of spoilage organisms in dairy processing systems.

We developed a sensitive and specific TaqMan assay that can detect psychrotolerant sporeforming *Paenibacillus* spp. associated with dairy spoilage. While the low levels of spores initially present in raw milk prevented direct detection of *Paenibacillus* in DNA extracted from raw milk or from enriched milk samples, an alternative colony screening method proved feasible. A 16S rDNA-based TaqMan assay on crude colony lysates obtained from heat-shocked milk that had been enriched at 13°C for 48 h and plated on BHI supplemented with X-gal provided fast and accurate identification of *Paenibacillus*. Overall, the assay provides an improved tool for the dairy industry to differentiate raw milk with the potential for lower post-pasteurization bacterial outgrowth. Further development of rapid and effective detection methods for psychrotolerant sporeformers within a comprehensive farm to fork framework are needed for improved control of these important spoilage organisms in the food supply.

The results of our *Paenibacillus* detection system will provide the food industry with an assay to monitor the quality of raw milk. This assay may even be adapted to aid in the development of strategies to limit spoilage of other pasteurized, refrigerated foods like vegetable purees (Carlin et al., 2000; Guinebretiere et al., 2001) and fermented beverages (Haakensen and Ziola, 2008). Finally, our assay has potential for use as a screening tool to isolate novel enzyme producing *Paenibacillus* spp. from other foods (Piuri et al., 1998) and the natural environment (Naghmouchi et al., 2011; Sakai et al., 2005), as previous identification of *Paenibacillus* strains has led

to the discovery of many important compounds (e.g., polymyxin) with promising applications in agriculture, food processing, and medicine (Naghmouchi et al., 2011).

In addition to the development of a dairy spoilage detection tool, we also developed a rapid, economical PCR and sequencing based *Salmonella* serotyping method. This method utilizes basic lab equipment to serotype *Salmonella* spp., which are responsible for the largest number of known foodborne illnesses caused by bacteria in the US. Our method provides an important alternative to traditional serotyping, which is labor intensive, time-consuming (3-5 days) and requires maintenance of over 250 antisera. Accurate serotyping of *Salmonella* is important to detect and rapidly intervene during outbreaks of salmonellosis.

We used both known and novel primers to develop a simple, cost-effective PCR and sequence-based scheme to determine O, H1 and H2 antigens. Overall, we were able to predict 42/46 common serotypes and 62/70 rare serotypes. Additional work to expand O-antigen detection primers, and to include more robust primer sets, will further improve molecular serotyping.

As full-genome sequencing has become more accessible, a number of studies have generated full-genome *Salmonella* sequences that can be leveraged to design new, primers to enhance molecular detection of serotypes (Bakker et al., 2011; Allard et al., 2012). Specifically, these *Salmonella* genomes can be utilized to design primers capable of detecting all 46 *Salmonella* serogroups, including those that we could not differentiate between (e.g., serogroup O:3,10 vs. O:1,3,19). Genome sequences for *fliC* and *fljB* could be extracted to expand available *fliC* and *fljB* databases, plus for development of primers to detect rare flagellar antigens encoded by other genes.

Overall, our PCR and sequencing based strategy allows for continuity with traditional serotyping data, reduces the need for expensive or proprietary equipment, and could be integrated into an open-source web-based database permitting review of sequence data for enhanced accuracy.

APPENDIX

Table S1 (Chapter 3): Complete results of TaqMan assay used to screen 24 raw milk samples for Paenibacillus spoilage potential.

Spoilage growth category[a]	Milk sample	CFU/ml after 13°C enrichment for (β-gal positive count)			Colony screening results after 13°C milk enrichment for												Milk assessment following 21 d at 6°C	
					T = 0 h				T = 24 h				T = 48 h					
		T = 0 h	T = 24 h	T = 48 h	Isolate ID	β-gal (+/-)[b]	TaqMan (C_t)[d]	*rpoB* based species ID	Isolate ID	β-gal (+/-)[b]	TaqMan (C_t)[d]	*rpoB* based species ID	Isolate ID	β-gal (+/-)[b]	TaqMan (C_t)[d]	*rpoB* based species ID	Bacterial count (CFU/ml)	Predominant spoilage bacteria (*rpoB* or 16S based identification)[c]
High	D-3	2	2	<1	FSL R7-644	-	>40	*Bacillus*	FSL R7-645	-	>40	*Bacillus*	NA				4.37×10^7	*Paenibacillus*
	D-4	4	15 (5)	153 (114)	FSL R7-677	-	>40	*Bacillus*	FSL R7-692	-	>40	*Bacillus*	FSL R7-708	+	21.69	*Paenibacillus*	9.33×10^6	*Paenibacillus*
					FSL R7-678	-	>40	*Bacillus*	FSL R7-693	+	22.56	*Paenibacillus*	FSL R7-709	-	>40	*Bacillus*		
													FSL R7-710	-	>40	*Bacillus*		
	C-4	30	29	313 (13)	FSL R7-674	-	>40	*Bacillus*	FSL R7-690	-	>40	*Bacillus*	FSL R7-704	-	>40	*Bacillus*	5.37×10^6	*Paenibacillus*
					FSL R7-675	-	>40	*Bacillus*	FSL R7-691	-	>40	*Bacillus*	FSL R7-705	-	>40	*Bacillus*		
					FSL R7-676	-	>40	*Bacillus*					FSL R7-706	+	25.18	*Paenibacillus*		
													FSL R7-707	+	20.73	*Paenibacillus*		
	C-3	27	23	197 (40)	FSL R7-646	-	>40	*Bacillus*	FSL R7-650	-	>40	*Bacillus*	FSL R7-652	+	18.9	*Paenibacillus*	2.75×10^6	*Paenibacillus*
									FSL R7-651	-	>40	*Bacillus*	FSL R7-653	-	>40	*Bacillus*		
													FSL R7-654	-	>40	*Bacillus*		
													FSL R7-655	-	>40	*Bacillus*		
													FSL R7-656	+	17.9	*Paenibacillus*		
	G-4	14	42 (1)	530 (190)	FSL R7-679	-	18.37	*Paenibacillus*	FSL R7-695	-	>40	*Bacillus*	FSL R7-711	+	21.8	*Paenibacillus*	2.24×10^6	*Paenibacillus*
					FSL R7-680	-	>40	*Bacillus*	FSL R7-696	-	>40	*Bacillus*	FSL R7-712	+/-	>40	*Bacillus*		
					FSL R7-681	-	>40	*Bacillus*	FSL R7-697	-	>40	*Bacillus*	FSL R7-713	-	>40	*Bacillus*		
					FSL R7-682	-	>40	*Bacillus*					FSL R7-714	-	>40	*Bacillus*		
Intermediate	C-5	117 (6)	87	550 (10)	FSL R7-722	-	>40	*Bacillus*	FSL R7-733	-	>40	*Bacillus*	FSL R7-736	-	>40	*Bacillus*	6.76×10^5	*Paenibacillus*
					FSL R7-723	-	>40	*Bacillus*	FSL R7-734	-	>40	*Bacillus*	FSL R7-737	-	>40	*Bacillus*		
					FSL R7-724	-	>40	*Bacillus*	FSL R7-735	-	>40	*Bacillus*	FSL R7-738	-	>40	*Bacillus*		
					FSL R7-725	-	>40	*Bacillus*					FSL R7-739	+/-	18.98	*Paenibacillus*		
					FSL R7-726	+	21.5	*Paenibacillus*										
					FSL R7-727	+	23.9	*Paenibacillus*										
	D-5	<1	3	<1	NA				FSL R7-740	-	>40	*Bacillus*	NA				3.55×10^5	*B. weihenstephanensis*
									FSL R7-741	-	>40	*Bacillus*						
									FSL R7-742	-	>40	*Bacillus*						
	J-5	<1	12	686	NA				FSL R7-744	-	>40	*Bacillus*	FSL R7-745	-	>40	*Bacillus*	1.95×10^5	*B. weihenstephanensis*
Low	J-4	10	15	9	FSL R7-685	-	>40	*Bacillus*	FSL R7-699	-	>40	*Bacillus*	FSL R7-716	-	>40	*Bacillus*	8.13×10^3	NA
					FSL R7-686	-	>40	*Bacillus*	FSL R7-700	-	>40	*Bacillus*	FSL R7-717	-	>40	*Bacillus*		
					FSL R7-687	-	>40	*Bacillus*	FSL R7-701	-	>40	*Bacillus*	FSL R7-718	-	>40	*Bacillus*		
	B-4	30	44	2410	FSL R7-671	-	>40	*Bacillus*	FSL R7-688	-	>40	*Bacillus*	FSL R7-702	-	>40	*Bacillus*	8.51×10^2	NA
					FSL R7-672	-	>40	*Bacillus*	FSL R7-689	-	>40	*Bacillus*	FSL R7-703	-	>40	*Bacillus*		
					FSL R7-673	-	>40	*Bacillus*										
	H-4	3	1	<1	FSL R7-683	-	>40	*Bacillus*	NA				NA				1.62×10^2	NA
					FSL R7-684	-	>40	*Bacillus*										
	H-5	6	4	12 (5)	NA				FSL R7-746	-	>40	*Bacillus*	FSL R7-747	+/-	20.37	*Paenibacillus*	2.88×10^1	NA
													FSL R7-748	-	>40	*Bacillus*		
													FSL R7-749	-	>40	*Bacillus*		
	J-3	6	5	937	FSL R7- 637	-	>40	*Bacillus*	FSL R7-639	-	>40	*Bacillus*	FSL R7-642	-	>40	*Bacillus*	2.45×10^1	NA
					FSL R7-638	-	>40	*Bacillus*	FSL R7-640	-	>40	*Bacillus*	FSL R7-643	-	>40	*Bacillus*		
									FSL R7-641		>40	*Bacillus*						
	A-4	3	2	3	FSL R7-662	-	>40	*Bacillus*	FSL R7-664	-	>40	*Bacillus*	FSL R7-666	-	>40	*Bacillus*	2.04×10^1	NA
					FSL R7-663	-	>40	*Bacillus*	FSL R7-665	-	>40	*Bacillus*	FSL R7-667	-	>40	*Bacillus*		
	E-5	1	<1	<1	FSL R7-743	-	>40	*Bacillus*	NA				NA				1.41×10^1	NA
	F-5	<1	29	1017	FSL R7-719	-	>40	*Brevibacillus*	FSL R7-720	-	>40	*Brevibacillus*	FSL R7-732	-	>40	*Bacillus*	1.41×10^1	NA
									FSL R7-721	-	>40	*Bacillus*						
	E-3	1	1	<1	FSL R7-635	-	>40	*Oceanobacillus*	FSL R7-636	-	>40	*Bacillus*	NA				$<1.00 \times 10^1$	NA
	F-3	<1	<1	577	NA				NA				FSL R7-657	-	>40	*Bacillus*	$<1.00 \times 10^1$	NA
													FSL R7-658	-	>41	*Bacillus*		
	H-3	2	<1	<1	FSL R7-659	-	>40	*Bacillus*	NA				NA				$<1.00 \times 10^1$	NA
					FSL R7-660	-	>40	*Bacillus*										
	I-3	1	<1	<1	FSL R7-661	-	>40	*Bacillus*	NA				NA				$<1.00 \times 10^1$	NA
	E-4	<1	2	<1	NA				FSL R7-694	-	>40	*Bacillus*	NA				$<1.00 \times 10^1$	NA
	F-4	<1	1	16	FSL R7-668	-	>40	*Bacillus*	FSL R7-669	-	>40	*Bacillus*	FSL R7-670	-	>40	*Bacillus*	$<1.00 \times 10^1$	NA
	I-4	<1	8	5700	NA				FSL R7-698	-	>40	*Bacillus*	FSL R7-715	-	>40	*Bacillus*	$<1.00 \times 10^1$	NA
	I-5	4	<1	<1	FSL R7-750	-	>40	*Staphylococcus*	NA				NA				$<1.00 \times 10^1$	NA

[a]Growth category assigned based on APCs following heat shock and storage of milk at 6°C for 21 d. "High" category indicates APC > 1×10^5 CFU/ml; "Intermediate" category indicates APC ≤ 1×10^5 CFU/ml and > 2×10^4 CFU/ml; "Low" category indicates APC ≤ 2×10^4 CFU/ml.

[b]β-gal positive (blue) colonies represented by "+"; β-gal negative colonies represented by "-"; and β-gal weakly positive colonies (partial blue or light blue) indicated by "+/-."

[c]Predominant spoilage bacteria not identified in milk samples where the bacterial count remained ≤ 2×10^4 CFU/ml or when no bacterial colonies could be isolated; indicated by NA.

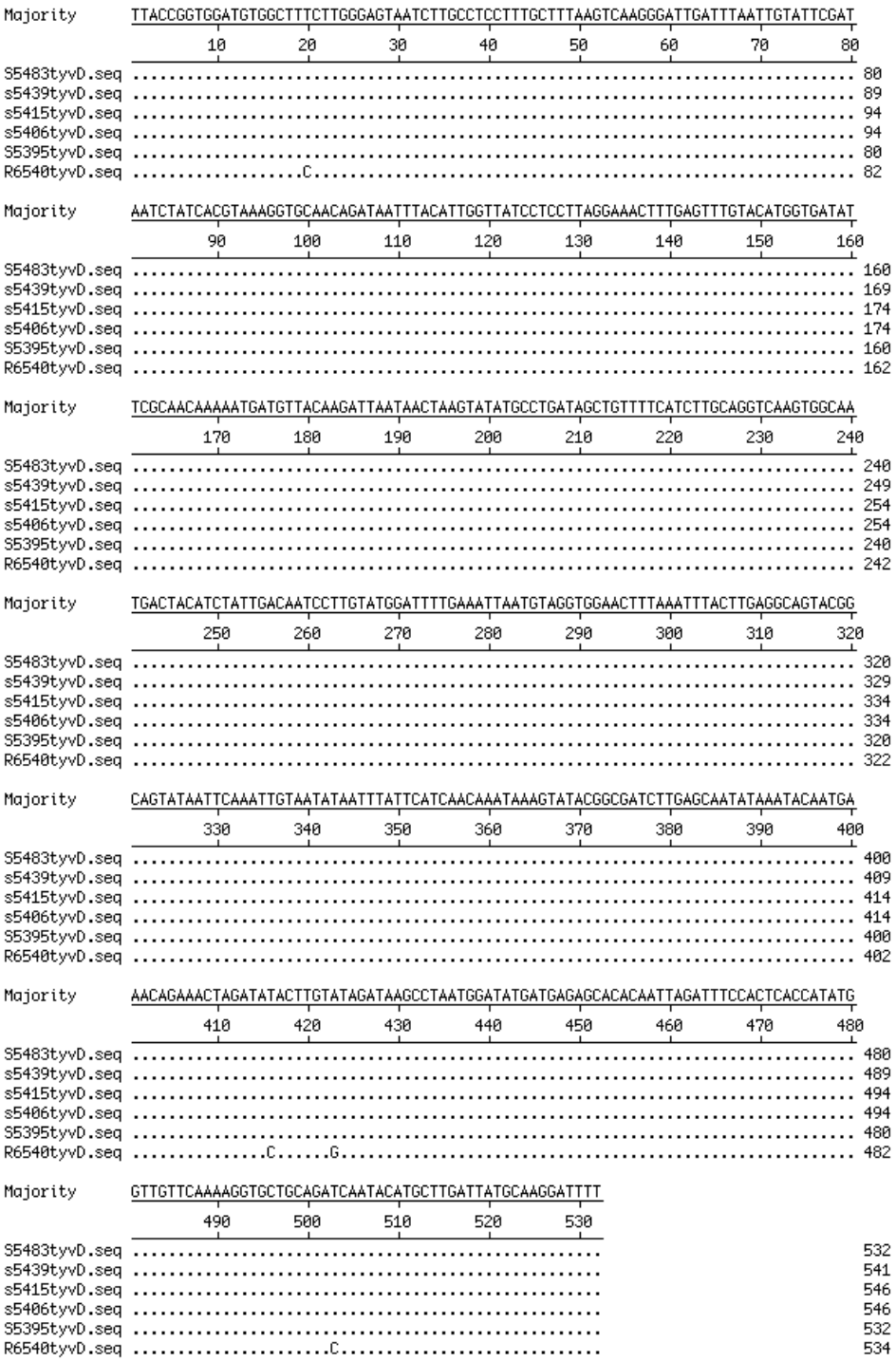

Figure S1 (Chapter 4): Alignment of 532 nt from *tyvD* for 6 serogroup O:9 (D1) isolates representing common serovars. Left hand label includes isolate identification information, e.g., s5483tyvD is isolate FSL S5-483.

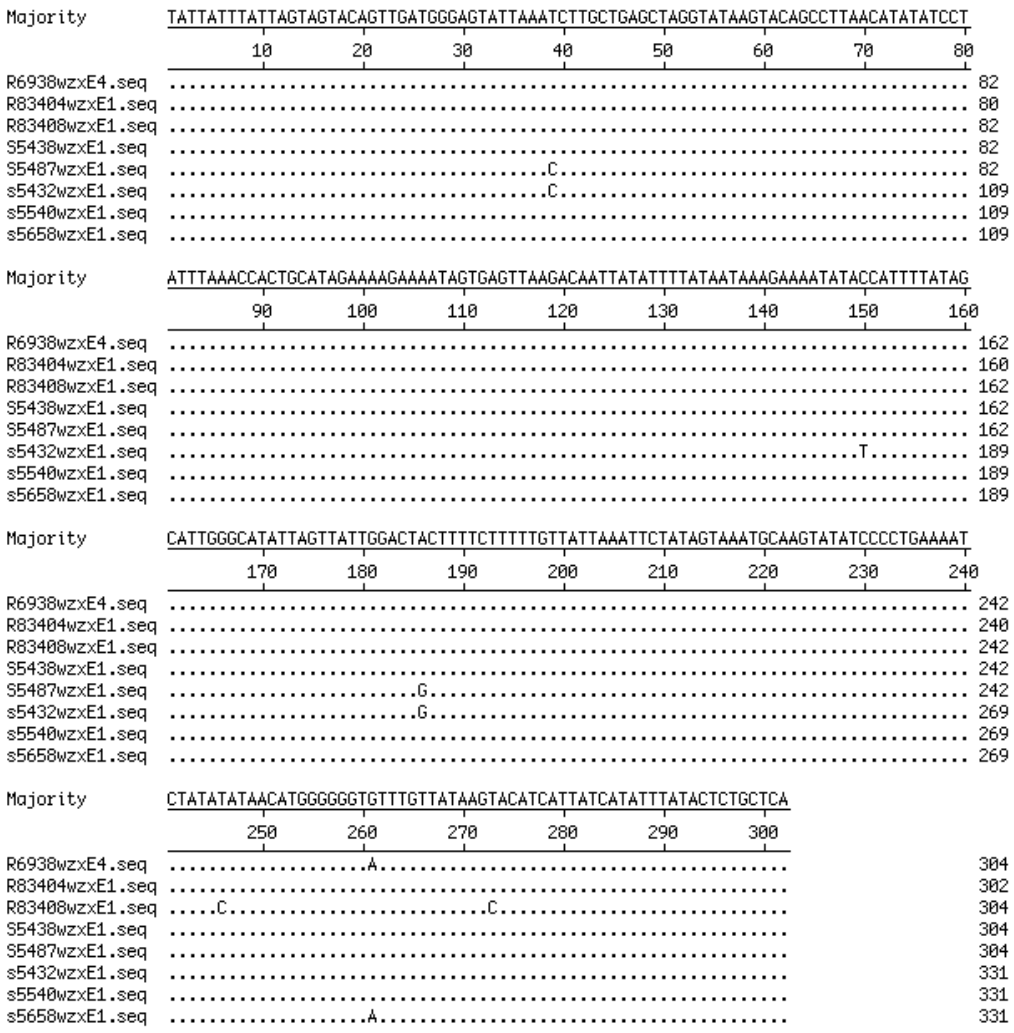

Figure S2 (Chapter 4): Alignment of 302 nt fragment from *wzx* for 7 serogroup O:3,10 (E1) isolates and 1 serogroup O:1,3,19 (E4) isolate. Left hand label includes isolate identification information, e.g., R6938wzxE4 is isolate FSL R6-938, representing serogroup E4.

Table S1 (Chapter 4): Summary of molecular serotyping results for all top 40 and rare 70 Salmonella isolates[a].

		Results by traditional serotyping			O-antigen PCR results[b]							H1-antigen PCR and sequencing results[c]						H2-antigen PCR and sequencing results[c]					
Serotype	Isolate	Serogroup	H1 antigens	H2 antigens	B	C1	C2	D	E	G	K	PCR primers	Sequencing primers	Sequence length (No. nt)[d]	Sequencing coverage	*fliC* Blastn	*fliC* antigens	PCR primers	Sequencing Primers	Sequence length (No. nt)[d]	Sequencing Coverage	*fljB* Blastn	*fljB* antigens
Top 40																							
Reading	FSL R8-1987	O:4 (B)	e,h	1,5	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1372*	single	Anatum	e,h	*fljB* Set 1	*fljB* Set 1	1408*	single	Bareilly	1,5
Derby	FSL R8-2630	O:4 (B)	f,g	[1,2]	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	794	double	Derby	f,g	*fljB* Set 1	no pcr product	–	–	–	–
Stanley	FSL S5-408	O:4 (B)	d	1,2	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1375*	single	Muenchen	d	*fljB* Set 1	Sequencing Set 1	867	single	Newport	1,2
Typhimurium	FSL S5-433	O:4 (B)	i	1,2	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1321*	single	Typhimurium	i	*fljB* Set 1	pcr product	–	NA	–	–
Paratyphi B var. Java	FSL S5-447	O:4 (B)	b	1,2	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1314*	single	Paratyphi B	b	*fljB* Set 1	*fljB* Set 1	1412*	single	Hissar	1,2
Heidelberg	FSL S5-448	O:4 (B)	r	1,2	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1345*	single	Heidelberg	r	*fljB* Set 1	*fljB* Set 1	1412*	single	Heidelberg	1,2
Schwarzengrund	FSL S5-458	O:4 (B)	d	1,7	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1378*	single	Schwarzengrund	d	*fljB* Set 1	*fljB* Set 1	1407*	single	Schwarzengrund	1,7
Heidelberg	FSL S5-480	O:4 (B)	r	1,2	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1388*	single	Heidelberg	r	*fljB* Set 1	*fljB* Set 1	1414*	single	Heidelberg	1,2
Agona	FSL S5-517	O:4 (B)	f,g,s	[1,2]	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	673	double	Agona	f,g,s	*fljB* Set 1	no pcr product	–	NA	–	–
Typhimurium	FSL S5-536	O:4 (B)	i	1,2	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1382*	single	Typhimurium	i	*fljB* Set 1	*fljB* Set 1	1414*	single	Typhimurium	1,2
4,5,12:i:-	FSL S5-580	O:4 (B)	i	–	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1383*	single	Typhimurium	i	*fljB* Set 1	no pcr product	–	NA	–	–
Saintpaul	FSL S5-649	O:4 (B)	e,h	1,2	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1391*	single	Anatum	e,h	*fljB* Set 1	*fljB* Set 1	1420*	single	Saintpaul	1,2
Typhimurium. var. 5-	FSL S5-786	O:4 (B)	i	1,2	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1353*	single	Typhimurium	i	*fljB* Set 1	*fljB* Set 1	1412*	single	Typhimurium	1,2
Tennessee	FSL R8-1965	O:7 (C1)	z29	[1,2,7]	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	737	double	Tennessee	z29	*fljB* Set 1	no pcr product	–	NA	–	–
Choleraesuis	FSL R8-3632	O:7 (C1)	c	1,5	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1401*	single	Choleraesuis	c	*fljB* Set 1	no pcr product	–	NA	–	–
Braenderup	FSL S5-373	O:7 (C1)	e,h	e,n,z15	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1334*	single	Saintpaul	e,h	*fljB* Set 1	*fljB* Set 1	1399*	single	Braenderup	e,n,z15
Mbandaka	FSL S5-451	O:7 (C1)	z10	e,n,z15	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1383*	single	Hadar	z10	*fljB* Set 1	*fljB* Set 1	1399*	single	Mbandaka	e,n,z15
Thompson	FSL S5-523	O:7 (C1)	k	1,5	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1383*	single	Thompson	k	*fljB* Set 1	*fljB* Set 1	1398*	single	Thompson	1,5
Oranienburg	FSL S5-642	O:7 (C1)	m,t	[z57]	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1353*	single	Oranienburg	m,t	*fljB* Set 1	no pcr product	–	NA	–	–
Infantis	FSL S5-734	O:7 (C1)	r	1,5	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1371*	single	Heidelberg	r	*fljB* Set 1	*fljB* Set 1	1410*	single	Infantis	1,5
Virchow	FSL S5-961	O:7 (C1)	r	1,2	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1350*	single	Heidelberg	r	*fljB* Set 1	*fljB* Set 1	1197*	single	Hissar	1,2
Kentucky	FSL S5-273	O:8 (C2-C3)	i	z6	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1372*	single	Kentucky	i	*fljB* Set 1	*fljB* Set 1	1349*	single	Kentucky	z6
Kentucky	FSL S5-431	O:8 (C2-C3)	i	z6	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1381*	single	Typhimurium	i	*fljB* Set 1	*fljB* Set 1	1394*	single	Kentucky	z6
Newport	FSL S5-436	O:8 (C2-C3)	e,h	1,2,	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1402*	single	Newport	e,h	*fljB* Set 1	*fljB* Set 1	1416*	single	Newport	1,2
Muenchen	FSL S5-504	O:8 (C2-C3)	d	1,2	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1378*	single	Muenchen	d	*fljB* Set 1	*fljB* Set 1	1410*	single	Muenchen	1,2
Hadar	FSL S5-543	O:8 (C2-C3)	z10	e,n,x	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1394*	single	Hadar	z10	*fljB* Set 1	*fljB* Set 1	1398*	single	Bonn	e,n,x
Newport	FSL S5-639	O:8 (C2-C3)	e,h	1,2,	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1388*	single	Newport	e,h	*fljB* Set 1	*fljB* Set 1	1401*	single	Newport	1,2
Blockley	FSL S5-648	O:8 (C2-C3)	k	1,5	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1386*	single	Thompson	k	*fljB* Set 1	*fljB* Set 1	1348*	single	Blockley	1,5
Typhi	FSL R6-540	O:9 (D1)	d	–	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1417*	single	Typhi	d	*fljB* Set 1	no pcr product	–	NA	–	–
Javiana	FSL S5-395	O:9 (D1)	l,z28	1,5	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1395*	single	Javiana	l,z28	*fljB* Set 1	*fljB* Set 1	1409*	single	Thompson	1,5
Javiana	FSL S5-406	O:9 (D1)	l,z28	1,5	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1049*	single	Javiana	l,z28	*fljB* Set 1	*fljB* Set 1	1399*	single	Thompson	1,5
Enteritidis	FSL S5-415	O:9 (D1)	g,m	–	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1282*	single	Enteritidis	g,m	*fljB* Set 1	no pcr product	–	–	–	–
Dublin	FSL S5-439	O:9 (D1)	g,p	–	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	774	double	Dublin	g,p	*fljB* Set 1	no pcr product	–	–	–	–
Enteritidis	FSL S5-483	O:9 (D1)	g,m	–	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1286*	single	Enteritidis	g,m	*fljB* Set 1	no pcr product	–	–	–	–
Meleagridis	FSL R6-938	O:3,10 (E1)	e,h	l,w	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1383*	single	Anatum	e,h	*fljB* Set 1	*fljB* Set 1	1385*	single	Meleagridis	l,w
Uganda	FSL R8-3404	O:3,10 (E1)	l,z13	1,5	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1372*	single	Kinshasa	l,z13	*fljB* Set 1	*fljB* Set 1	1408*	single	Uganda	1,5
Orion va. 15+,34+	FSL R8-3408	O:3,10 (E1)	y	1,5	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1382*	single	Orion	y	*fljB* Set 1	*fljB* Set 1	1414*	single	Thompson	1,5
Muenster	FSL S5-432	O:3,10 (E1)	e,h	1,5	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1391*	single	Anatum	e,h	*fljB* Set 1	*fljB* Set 1	1402*	single	Vanier	1,5
Weltevreden	FSL S5-438	O:3,10 (E1)	r	z6	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1351*	single	Weltevreden	r	*fljB* Set 1	*fljB* Set 1	1305*	single	Weltevreden	z6
Give	FSL S5-487	O:3,10 (E1)	l,v	1,7	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1352*	single	Panama	l,v	*fljB* Set 1	Sequencing Set 1	928	double	Give	1,7
Anatum	FSL S5-540	O:3,10 (E1)	e,h	1,6	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1362*	single	Anatum	e,h	*fljB* Set 1	*fljB* Set 1	1409*	single	Anatum	1,6
Senftenberg	FSL S5-658	O:1,3,19 (E4)	g,[s],t	–	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	723	double	Senftenberg	g,[s],t	*fljB* Set 1	no pcr product	–	–	–	–
Mississippi	FSL A4-633	O:13 (G)	b	1,5	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 1	*fliC* Set 1	1360*	single	Paratyphi B	b	*fljB* Set 1	*fljB* Set 1	1407*	single	Mississippi	1,5
Worthington	FSL S5-490	O:13 (G)	z	l,w	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 1	*fliC* Set 1	1362*	single	Indiana	z	*fljB* Set 1	*fljB* Set 1	1392*	single	Cloucester	l,w
Cerro	FSL R8-370	O:18 (K)	z4,z23	[1,5]	(-)	(-)	(-)	(-)	(-)	(-)	(+)	*fliC* Set 1	*fliC* Set 1	1093*	single	Stanleyville	z4,z23	*fljB* Set 1	no pcr product	–	NA	–	–
Montevideo	FSL S5-630	O:54[f]	g,m,s	–	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	*fliC* Set 1	1379*	single	Montevideo	g,m,s	*fljB* Set 1	no pcr product	–	NA	–	–
Rare 70																							
Paratyphi A	FSL R6-883	O:2 (A)	a	1,5	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	773	double	Paratyphi A	a	*fljB* Set 2	Sequencing Set 1	874	double	Paratyphi A	1,5
Abony	FSL S5-469	O:4 (B)	b	e,n,x	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	643	double	Paratyphi B	b	*fljB* Set 2	Sequencing Set 1	921	double	Hadar	e,n,x
Arechavaleta	FSL S5-453	O:4 (B)	a	1,7	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	692	double	Miami	a	*fljB* Set 2	Sequencing Set 1	769	double	Schwarzengrund	1,7
Brandenburg	FSL R8-1984	O:4 (B)	l,v	e,n,z15	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	635	double	Give	l,v	*fljB* Set 2	Sequencing Set 1	928	double	Brandenburg	e,n,z15
Indiana	FSL R6-527	O:4 (B)	z	1,7	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	635	double	Indiana	z	*fljB* Set 2	Sequencing Set 1	809	double	Indiana	1,7
Kiambu	FSL R6-203	O:4 (B)	z	1,5	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	603	double	Indiana	z	*fljB* Set 2	Sequencing Set 1	847	double	Nima	1,5
Paratyphi B[e]	FSL R8-153	O:4 (B)	z4, z23	z64	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	682	double	Paratyphi B	b	*fljB* Set 2	Sequencing Set 1	901	double	Newport	1,2
Remo	FSL R8-3521	O:4 (B)	r	1,7	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	727	double	Weltevreden	r	*fljB* Set 2	Sequencing Set 1	808	double	Schwarzengrund	1,7
Sandiego	FSL A4-827	O:4 (B)	e,h	e,n,z15	(+)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	617	double	Chester	e,h	*fljB* Set 2	Sequencing Set 1	904	double	Brandenburg	e,n,z15
Bareilly	FSL R8-2449	O:7 (C1)	y	1,5	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	722	double	Coeln	y	*fljB* Set 2	Sequencing Set 1	634	double	Cholerasuis	1,5
Concord	FSL R8-457	O:7 (C1)	l,v	1,2	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	730	double	London	l,v	*fljB* Set 2	Sequencing Set 1	739	double	Typhimurium	1,2
Georgia	FSL R6-992	O:7 (C1)	b	e,n,z15	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	700	double	Paratyphi B	b	*fljB* Set 2	Sequencing Set 1	860	double	Brandenburg	e,n,z15
Hartford	FSL A4-617	O:7 (C1)	y	e,n,x	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	525	double	Coeln	y	*fljB* Set 2	Sequencing Set 1	805	double	Hadar	e,n,x
Mikawasima	FSL R6-244	O:7 (C1)	y	e,n,z15	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	480	double	Coeln	y	*fljB* Set 2	Sequencing Set 1	709	double	Brandenburg	e,n,z15
Ohio	FSL S5-885	O:7 (C1)	b	l,w	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	821	double	Paratyphi B	b	*fljB* Set 2	Sequencing Set 1	654	double	Ohio	l,w
Paratyphi C	FSL R6-305	O:7 (C1)	c	1,5	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	650	double	Choleraesuis	c	*fljB* Set 2	Sequencing Set 1	808	double	Michigan	1,5
Bardo	FSL S5-774	O:8 (C2-C3)	e,h	1,2	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	616	double	Newport	e,h	*fljB* Set 2	Sequencing Set 1	805	double	Typhimurium	1,2
Bovismorbificans	FSL A4-577	O:8 (C2-C3)	r,[i]	1,5	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	626	double	Heidelberg	r	*fljB* Set 2	Sequencing Set 1	666	double	Paratyphi A	1,5
Corvallis	FSL R8-092	O:8 (C2-C3)	z4, z23	[z6]	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	592	double	Cerro	z4,z23	*fljB* Set 2	Sequencing Set 1	805	double	Paratyphi A	1,5
Glostrup	FSL R8-2600	O:8 (C2-C3)	z10	e,n,z15	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	761	double	Hadar	z10	*fljB* Set 2	Sequencing Set 1	831	double	Brandenburg	e,n,z15
Hindmarsh	FSL R8-3386	O:8 (C2-C3)	r	1,5	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	726	double	Bovismorbificans	r,[i]	*fljB* Set 2	Sequencing Set 1	911	double	Paratyphi A	1,5
Holcomb	FSL R6-227	O:8 (C2-C3)	l,v	e,n,x	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	681	double	Give	l,v	*fljB* Set 2	Sequencing Set 1	678	double	Hadar	e,n,x
Kottbus	FSL R8-2447	O:8 (C2-C3)	e,h	1,5	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	771	double	Newport	e,h	*fljB* Set 2	Sequencing Set 1	738	double	Paratyphi A	1,5
Litchfield	FSL R8-2112	O:8 (C2-C3)	l,v	1,2	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	712	double	London	l,v	*fljB* Set 2	Sequencing Set 1	769	double	Typhimurium	1,2
Manhattan	FSL R8-1303	O:8 (C2-C3)	d	1,5	(-)	(-)	(+)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	711	double	Muenchen	d	*fljB* Set 2	Sequencing Set 1	827	double	Paratyphi A	1,5
Berta	FSL R8-2917	O:9 (D1)	[f],g,[t]	–	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	773	double	Rissen	f,g	*fljB* Set 2	Sequencing Set 1	–	–	–	–
Javiana[e]	FSL S9-275	O:9 (D1)	z38	1,2	(-)	(-)	(-)	(+)	(-)	(-)	(-)	fliC Set 1	Sequencing Set 1	719	double	Javiana	l,z28	*fljB* Set 2	Sequencing Set 1	794	double	Paratyphi A	1,5
Miami	FSL R8-2520	O:9 (D1)	a	1,5	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	742	double	Miami	a	*fljB* Set 2	Sequencing Set 1	589	double	Paratyphi A	1,5
Panama	FSL R8-2486	O:9 (D1)	l,v	1,5	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	817	double	Panama	l,v	*fljB* Set 2	Sequencing Set 1	637	double	Nima	1,5
Wangata	FSL R8-1542	O:9 (D1)	z4, z23	1,7	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	659	double	Cerro	z4,z23	*fljB* Set 2	Sequencing Set 1	–	NA	–	–
Baildon	FSL R6-199	O:9,46 (D2)	a	e,n,x	(-)	(-)	(-)	(+)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	728	double	Paratyphi A	a	*fljB* Set 2	Sequencing Set 1	682	double	Hadar	e,n,x
Amager	FSL A4-650	O:3,10 (E1)	y	1,2	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	684	double	Coeln	y	*fljB* Set 2	Sequencing Set 1	944	double	Newport	1,2
London	FSL R8-459	O:3,10 (E1)	l,v	1,6	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	785	double	London	l,v	*fljB* Set 2	Sequencing Set 1	959	double	Agama	1,6
Aberdeen	FSL R8-3524	O:11 (F)	i	1,2	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	659	double	Typhimurium	i	*fljB* Set 2	Sequencing Set 1	928	double	Newport	1,2
Kisarawe	FSL A4-595	O:11 (F)	k	e,n,x,[z15]	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	858	double	Zanzibar	k	*fljB* Set 2	Sequencing Set 1	955	double	Abortusequi	e,n,x
Luciana	FSL R8-3555	O:11 (F)	a	e,n,z15	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	613	double	50:a:-	a	*fljB* Set 2	Sequencing Set 1	885	double	Sanktgeorg	e,n,z15
Nyanza	FSL S5-654	O:11 (F)	z	z6	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	819	double	Indiana	z	*fljB* Set 2	Sequencing Set 1	595	double	Weltevreden	z,6
Rubislaw	FSL S5-477	O:11 (F)	r	e,n,x	(-)	(+)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	715	double	Weltevreden	r	*fljB* Set 2	Sequencing Set 1	932	double	Abortusequi	e,n,x
Agbeni	FSL S5-417	O:13 (G)	g ,m,[s],[t]	–	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 1	Sequencing Set 1	707	double	Enteritidis	g,m	*fljB* Set 2	no pcr product	–	–	–	–
Cubana	FSL S5-632	O:13 (G)	z29	–	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 2	Sequencing Set 1	655	double	Tennessee	z29	*fljB* Set 2	no pcr product	–	–	–	–
Grumpensis	FSL R6-418	O:13 (G)	d	1,7	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 1	Sequencing Set 1	709	double	Schwarzengrund	d	*fljB* Set 2	Sequencing Set 1	929	double	Schwarzengrund	1,7
Havana	FSL S5-549	O:13 (G)	f , g,[s]	–	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 1	Sequencing Set 1	746	double	Derby	f,g	*fljB* Set 2	no pcr product	–	–	–	–
Kintambo	FSL S5-712	O:13 (G)	m,t	–	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 2	Sequencing Set 1	665	double	Oranienburg	m,t	*fljB* Set 2	no pcr product	–	–	–	–
Mississippi	FSL R8-2455	O:13 (G)	b	1,5	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 1	Sequencing Set 1	831	double	Paratyphi B	b	*fljB* Set 2	Sequencing Set 1	887	double	Paratyphi A	1,5
Poona	FSL R8-1546	O:13 (G)	z	1,6	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 2	Sequencing Set 1	702	double	Indiana	z	*fljB* Set 2	Sequencing Set 1	916	double	Agama	1,6
Putten	FSL A4-590	O:13 (G)	d	l,w	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 1	Sequencing Set 1	680	double	Putten	d	*fljB* Set 2	Sequencing Set 1	834	double	Ohio	l,w
Telelkebir	FSL R8-1526	O:13 (G)	d	e,n,z15	(-)	(-)	(-)	(-)	(-)	(+)	(-)	*fliC* Set 1	Sequencing Set 1	660	double	Schwarzengrund	d	*fljB* Set 2	Sequencing Set 1	943	double	Brandenburg	e,n,z15
Madelia	FSL R6-630	O:6,14 (H)	y	1,7	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	725	double	Coeln	y	*fljB* Set 2	Sequencing Set 1	638	double	Nola	1,7
Barranquilla	FSL R8-1295	O:16 (I)	d	e,n,x	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	651	double	Muenchen	d	*fljB* Set 2	Sequencing Set 1	978	double	Hadar	e,n,x
Gaminara	FSL R8-2934	O:16 (I)	d	1,7	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	696	double	Muenchen	d	*fljB* Set 2	Sequencing Set 1	850	double	Indiana	1,7
Hvittingfoss	FSL R8-789	O:16 (I)	b	e,n,x	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	780	double	Paratyphi B	b	*fljB* Set 2	Sequencing Set 1	967	double	Hadar	e,n,x
Hvittingfoss[e]	FSL R8-091	O:16 (I)	i	1,5	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	783	double	Paratyphi B	b	*fljB* Set 2	Sequencing Set 1	914	double	Hadar	e,n,x
Cotham	FSL R8-792	O:28 (M)	i	1,5	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	715	double	Typhimurium	i	*fljB* Set 2	Sequencing Set 1	945	double	Agama	1,6
Pomona	FSL S5-481	O:28 (M)	y	1,7	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	663	double	Coeln	y	*fljB* Set 2	Sequencing Set 1	885	double	Indiana	1,7
Urbana	FSL S5-410	O:30 (N)	b	e,n,x	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	676	double	Paratyphi B	b	*fljB* Set 2	Sequencing Set 1	649	double	Hadar	e,n,x
Adelaide	FSL S5-551	O:35 (O)	f,g	–	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	786	double	Rissen	f,g	*fljB* Set 2	no pcr product	–	–	–	–
Alachua	FSL R8-2924	O:35 (O)	z4, z23	–	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	791	double	Cerro	z4,z23	*fljB* Set 2	no pcr product	–	–	–	–
Ealing	FSL A4-670	O:35 (O)	g,m,s	–	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	654	double	Amsterdam	g,m,s	*fljB* Set 2	no pcr product	–	–	–	–
Freetown	FSL S5-668	O:38 (P)	y	1,5	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	626	double	Coeln	y	*fljB* Set 2	Sequencing Set 1	821	double	Nima	1,5
Wandsworth	FSL R6-526	O:39 (Q)	b	1,2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	529	double	Paratyphi B	b	*fljB* Set 2	Sequencing Set 1	680	double	Indiana	1,7
Johannesburg	FSL R8-3499	O:40 (R)	b	e,n,x	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	760	double	Paratyphi B	b	*fljB* Set 2	Sequencing Set 1	818	double	Hadar	e,n,x
Tilene	FSL R8-3597	O:40 (R)	e,h	1,2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	621	double	Saintpaul	e,h	*fljB* Set 2	Sequencing Set 1	851	double	Typhimurium	1,2
Overschie	FSL R8-144	O:51	l,v	1,5	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	601	double	Give	l,v	*fljB* Set 2	Sequencing Set 1	653	double	Nima	1,5
IIIb 35:Rough	FSL R8-3567	O:35 (O)	NA	NA	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	609	double	Give	l,v	*fljB* Set 2	Sequencing Set 1	734	double	IIIb 38:(k):z35	z35
Untypable	FSL R8-143	NA	NA	NA	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	699	double	Arizonae	z52	*fljB* Set 2	Sequencing Set 1	604	double	IIIb 61:i:z	z
Untypable	FSL R8-756	NA	NA	NA	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 2	Sequencing Set 1	501	double	Blockley	k	*fljB* Set 2	Sequencing Set 1	653	double	42:l,v:1,5,7	1,5,7
Untypable	FSL R8-2289	NA	NA	NA	(-)	(-)	(-)	(-)	(+)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	767	double	Senftenberg	g,[s],t	*fljB* Set 2	no pcr product	–	–	–	–
Untypable	FSL R6-592	NA	NA	NA	(-)	(-)	(-)	(-)	(-)	(-)	(+)	*fliC* Set 2	Sequencing Set 1	730	double	Cerro	z4,z23	*fljB* Set 2	no pcr product	–	–	–	–
Untypable	FSL R8-904	NA	NA	NA	(-)	(-)	(-)	(-)	(-)	(-)	(+)	*fliC* Set 1	Sequencing Set 1	792	double	Cerro	z4,z23	*fljB* Set 2	Sequencing Set 1	NA	NA	NA	NA
Untypable	FSL A4-524	NA	NA	NA	(-)	(-)	(-)	(-)	(-)	(-)	(-)	*fliC* Set 1	Sequencing Set 1	618	double	Coeln	y	*fljB* Set 2	Sequencing Set 1	1297*	single	Schwarzengrund	1,7

[a]Antigenic formulae reported according to White-Kauffmann-Le Minor scheme.
[b]Presence of expected PCR amplicon indicated by (+) and lack of amplicon indicated by (-).
[c]For specific primer details refer to Table 1.
[d]Sequencing primers resulting in only single coverage for majority of targeted gene marked with an asterisk.
[e]Following molecular serotyping and repeated immunological serotyping, the original serotype designation was amended. *S.* Hvittingfoss was previously identified as *S.* Idikan; *S.* Paratyphi B was previously identified as *S.* Aaruhus; *S.* Javiana was previously identified as *S.* Lille.

TABLE S2 (Chapter 4): PCR conditions used for serogroup, *fliC* and *fljB* amplification[a].

Primer Set	Primer Conc. (µM)	Start (°C, min)	30 cycles of[b]		
			Denaturation (°C, s)	Annealing (°C, s)	Extension (°C, s)
Multiplex PCR 1	0.2	95, 10	95, 30	58, 30	72, 45
Serogroup Set 1	0.5	95, 10	94, 30	48, 60	72, 90
Serogroup Set 2	0.5	95, 10	94, 30	58, 30	72, 45
fliC Set 1	0.5	95, 10	95, 30	59, 30[c]	72, 90
fliC Set 2	0.5	95, 10	95, 30	70, 60[d]	72, 90
fljB Set 1	0.5	95, 10	95, 60	65, 30[e]	72, 90
fljB Set 2	0.4	95, 10	95, 30	58, 30	72, 90

[a]Each 25 µL PCR reaction also contained: 1X PCR buffer, 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.625 units of AmpliTaq Gold 360 DNA Polymerase, and 25 ng of purified *Salmonella* DNA. Refer to Table 1 for additional primer information.

[b]All PCRs ended with a final extension at 72°C for 7 min and were then stored at 4°C.

[c]Touchdown at -0.5°C per cycle for 20 cycles, followed by 20 cycles at 49°C.

[d]Touchdown at -0.5°C per cycle for 20 cycles, followed by 20 cycles at 60°C.

[e]Touchdown at -0.5°C per cycle for 20 cycles, followed by 20 cycles at 55°C.

TABLE S3 (Chapter 4): Results for subtyping methods evaluated for their ability to predict *Salmonella* serovars in 46 isolates representing 40 common serovars.

Serovar	Isolate	Serovar predicted by				
		PFGE (band difference from most similar	Rep-PCR (Diversilab % identity to top match)	Ribotyping (DuPont ID % identity to top match)	MLST	Molecular Serotyping
Typhimurium	FSL S5-536	Typhimurium (0)	Typhimurium (94.4)	Typhimurium (94)	Typhimurium	Typhimurium
Typhimurium	FSL S5-433	4,5,12:i:- (0)	4,5,12:i:- (96.9)	Typhimurium (97)	Typhimurium	4,5,12:i:-
Enteritidis	FSL S5-415	Enteritidis (0)	Enteritidis (97.1)	Enteritidis (95)	Enteritidis	Enteritidis
Enteritidis	FSL S5-483	Enteritidis (0)	Enteritidis (98.5)	Enteritidis (96)	Enteritidis	Enteritidis
Newport	FSL S5-639	Newport (0)	Newport (97.4)	Newport (94), Bardo (94)	Newport	Newport
Newport	FSL S5-436	Newport (0)	Newport (94.6)	Newport (93)	Newport	Newport
Heidelberg	FSL S5-448	Heidelberg (0)	Heidelberg (96.3)	Hiedelberg (98)	Heidelberg	Heidelberg
Heidelberg	FSL S5-480	Heidelberg (1)	Heidelberg (97)	Heidelberg (96)	Heidelberg	Heidelberg
Javiana	FSL S5-395	Javiana (0)	Javiana (86.5)	Binza (93), Orion (93), Tomasville (93)	Javiana	Javiana
Javiana	FSL S5-406	Javiana (0)	Mississippi (72.3)	Javiana (96)	Javiana	Javiana
4,5,12:i:-	FSL S5-580	4,5,12:i:- (0)	4,5,12:i:- (96.9)	Typhimurium (93)	Typhimurium	Typhimurium
Montevideo	FSL S5-630	Montevideo (1)	Montevideo (98.2)	Unidentified (< 70)	Montevideo	Montevideo
Muenchen	FSL S5-504	Muenchen (1)	Muenchen (94.9)	Muenchen (97)	Muenchen	Muenchen
Oranienburg	FSL S5-642	Oranienburg (0)	Oranienburg (96.9)	Oranienburg (95)	Oranienburg	Oranienburg
Mississippi	FSL A4-633	Unidentified (> 3)	Mississippi (92.9)	Mississippi (88), Minnesota (88)	Mississippi	Mississippi
Saintpaul	FSL S5-649	Typhimurium (2)	Saintpaul (94.6)	Saintpaul (95)	Saintpaul	Saintpaul
Braenderup	FSL S5-373	Braenderup (0)	Braenderup (97.3)	Bareilly (96)	Braenderup	Braenderup
Agona	FSL S5-517	Agona (1)	Agona (98.4)	Agona (88)	Agona	Agona
Infantis	FSL S5-734	Infantis (0)	Typhimurium (95.3)	Infantis (93)	Infantis	Infantis
Thompson	FSL S5-523	Thompson (0)	Thompson (96.3)	Thompson (95)	Thompson	Thompson
Paratyphi B var. Java	FSL S5-447	Paratyphi B var. Java (0)	4,5,12:i:- (96.5)	Paratyphi B (71)	Paratyphi B var Java	Paratyphi B var Java
Typhi	FSL R6-540	Typhi (0)	Typhi (94.5)	Unidentified (< 70)	Typhi	Typhi
Stanley	FSL S5-408	Stanley (1)	Senftenberg (89.6)	Stanley (96)	Stanley	Stanley
Tennessee	FSL R8-1965	Tenneessee (0)	Tennessee (95)	Tennessee (81)	Tennesee	Tennesee
Hadar	FSL S5-543	Hadar (0)	Hadar (97.2)	Hadar (91)	Hadar	Hadar
Virchow	FSL S5-961	Unidentified (> 3)	Bareilly (94.2)	Virchow (96)	Virchow	Virchow
Blockley	FSL S5-648	Blockley (2)	Blockley (97.2)	Haardt (92)	Blockley	Blockley
Anatum	FSL S5-540	Anatum (0)	Anatum (92.3)	Anatum (97), Newington (97)	Anatum	Anatum
Weltevreden	FSL S5-438	Unidentified (> 3)	Berta (82.8)	Weltevreden (90)	Weltevreden	Weltevreden
Orion var. 15+,34+	FSL R8-3408	Unidentified (> 3)	Thompson (96.4)	Paratyphi B (93)	Serotype not identified[a]	Orion var. 15+,34+
Dublin	FSL S5-439	Dublin (0)	Dublin (87.1)	Enteritidis (97), San Diego (97)	Dublin	Dublin
Derby	FSL R8-2630	Derby (0)	Hadar (93)	Derby (98)	Derby	Derby
Senftenberg	FSL S5-658	Senftenberg (1)	Schwarzengrund (96.2)	Senftenberg (97)	Senftenberg	Senftenberg
Kentucky	FSL S5-273	Kentucky (0)	Blockley (91.6)	Kentucky (95)	Kentucky	Kentucky
Kentucky	FSL S5-431	Kentucky (0)	I8,20:-:z6 (96.9)	Kentucky (92)	Kentucky	Kentucky
Muenster	FSL S5-432	Muenster (0)	Javiana (96.2)	Lomita (91)	Muenster	Muenster
Mbandaka	FSL S5-451	Mbandaka (1)	Mbandaka (93.2)	Mbandaka (93)	Mbandaka	Mbandaka
Cerro	FSL R8-370	Cerro (0)	Cerro (95.4)	Cerro (88)	Cerro	Cerro
Choleraesuis	FSL R8-3632	Unidentified (> 3)	Litchfield (95.2); Choleraesuis (95.0)[c]	Choleraesuis (88)	Choleraesuis var. Kunzendorf	6,7;c;-
Reading	FSL R8-1987	Unidentified (> 3)	Bareilly (94.6)	Reading (93)	Serotype not identified[a]	Reading
Meleagridis	FSL R6-938	Meleagridis (0)	Meleagridis (96.5)	Meleagridis (94)	Meleagridis	Meleagridis
Uganda	FSL R8-3404	Uganda (1)	Uganda (95.6)	Enteritidis (90)	Uganda	Uganda
Schwarzengrund	FSL S5-458	Schwarzengrund (0)	Schwarzengrund (97.7)	Schwarzengrund (95), Bredeney (95)	Schwarzengrund	Schwarzengrund
Give	FSL S5-487	Unidentified (> 3)	Oranienburg (96.6)	Abaetetuba (88)	Give	Give
Worthington	FSL S5-490	Unidentified (> 3)	Worthington (87.5)	Worthington (96)	Worthington	Worthington
Typhimurium var. 5-[b]	FSL S5-786	Typhimurium (0); T. Copenhagen (0)	Typhimurium (96.4)	Typhimurium (76)	Typhimurium	Typhimurium

[a]MLST identified an exitisting sequence type, but isolates for that sequence type available in the database lacked serotype information.

[b]*S.* Typhimurium var. 5- was formerly *S.* Typhimurium var. Copenhagen.

[c]Examination of rep-PCR patterns indicated *S.* Choleraesuis was a better match to isolate FSL R8-3632

TABLE S4 (Chapter 4): Summary of the Max Planck 7-gene MLST for the 'top 40' *Salmonella* serovars.

Serovar	FSL number	*aroC* AT	*dnaN* AT	*hemD* AT	*hisD* AT	*purE* AT	*sucA* AT	*thrA* AT	ST	Serovar predicted by MLST
Typhimurium	FSL S5-433	10	7	12	9	5	9	2	19	Typhimurium
Typhimurium	FSL S5-536	10	7	12	9	5	9	2	19	Typhimurium
Enteritidis	FSL S5-415	5	2	3	7	6	6	11	11	Enteritidis
Enteritidis	FSL S5-483	5	2	3	7	6	6	11	11	Enteritidis
Newport	FSL S5-436	10	7	21	14	15	12	12	45	Newport
Newport	FSL S5-639	16	43	45	43	36	39	42	5	Newport
Heidelberg	FSL S5-448	2	7	9	9	5	9	12	15	Heidelberg
Heidelberg	FSL S5-480	2	7	9	9	5	9	12	15	Heidelberg
Javiana	FSL S5-395	13	12	17	16	13	16	4	24	Javiana
Javiana	FSL S5-406	13	12	17	520[a]	13	16	4	1674[a]	Javiana
4,5,12:i:-	FSL S5-580	10	7	12	9	5	9	2	19	Typhimurium
Montevideo	FSL S5-630	11	41	55	42	34	58	12	1677[a]	Montevideo
Muenchen	FSL S5-504	41	9	21	12	8	37	17	83	Muenchen
Oranienburg	FSL S5-642	13	11	315[a]	15	12	15	4	1675[a]	Oranienburg
Mississippi	FSL A4-633	48	128	96	119	116	119	118	448	Mississippi
Saintpaul	FSL S5-649	5	21	18	9	6	12	17	50	Saintpaul
Braenderup	FSL S5-373	12	2	15	14	11	14	16	22	Braenderup
Agona	FSL S5-517	3	3	7	4	3	3	7	13	Agona
Infantis	FSL S5-734	17	18	22	17	5	21	19	32	Infantis
Thompson	FSL S5-523	14	13	18	12	14	18	1	26	Thompson
Paratyphi B var. Java	FSL S5-447	46	44	46	46	38	18	34	88	Paratyphi B var Java
Typhi	FSL R6-540	1	1	1	1	1	1	5	1	Typhi
Stanley	FSL S5-408	16	16	20	18	8	12	18	29	Stanley
Tennesee	FSL R8-1965	118	107	8	51	2	117	16	319	Tennesee
Hadar	FSL S5-543	2	5	6	7	5	7	12	33	Hadar
Virchow	FSL S5-961	6	7	10	10	8	10	14	16	Virchow
Blockley	FSL S5-648	23	9	15	12	17	20	12	52	Blockley
Anatum	FSL S5-540	10	14	15	31	25	20	33	64	Anatum
Weltevreden	FSL S5-438	130	97	25	125	84	9	101	365	Weltevreden
Orion va. 15+,34+	FSL R8-3408	99	175	58	11	111	9	2	639[b]	639 serovar not in database
Dublin	FSL S5-439	5	2	3	6	5	5	2	1673[a]	Dublin
Derby	FSL R8-2630	39	35	8	36	29	9	36	71	Derby
Senftenberg	FSL S5-658	7	6	8	8	7	8	13	14	Senftenberg
Kentucky	FSL S5-273	76	14	3	77	64	64	67	198	Kentucky
Kentucky	FSL S5-431	62	53	54	60	5	53	54	152	Kentucky
Muenster	FSL S5-432	119	10	17	42	12	13	4	321	Muenster
Mbandaka	FSL S5-451	15	70	93	78	113	6	68	413	Mbandaka
Cerro	FSL R8-370	14	112	43	123	118	115	120	367	Cerro
Choleraesuis	FSL R8-3632	34	31	35	14	26	6	8	66	Choleraesuis var. Kunzendorf
Reading	FSL R8-1987	46	60	10	9	6	12	17	1628[b]	1628 serovar not in database
Meleagridis	FSL R8-938	92	125	78	128	138	9	141	463	Meleagridis
Uganda	FSL R8-3404	147	13	15	123	15	9	17	1676[a]	Uganda
Schwarzengrund	FSL S5-458	43	47	49	49	41	15	114	322	Schwarzengrund
Give	FSL S5-487	84	11	16	42	40	398[a]	4	1678[a]	Give
Worthington	FSL S5-490	189	70	68	132	175	9	172	592	Worthington
T. var. 5- (Copenhagen)	FSL S5-786	10	7	12	9	5	9	2	19	Typhimurium

[a]Represents a new allelic type or sequence type that was submitted to the MLST database

[b]An exisiting sequence type was found in the MLST database, however, no serovar information was available for the isolate(s).

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