Detection of Salmonella in wheat grains

by

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Abstract

Dry breakfast cereals, breads, pasta, dough, dry mixes for cakes, cookies, batters and coatings contain the cereal grain, wheat. As a raw agricultural product, several studies have shown that wheat grains can be potentially contaminated with foodborne pathogens, such as *Escherichia coli, Salmonella, Clostridium, Bacillus,* etc., as well as nonpathogenic organisms. *Salmonella* is of particular interest as a foodborne pathogen because of the involvement in numerous local and national outbreaks. Although the awareness of *Salmonella* detection in meat, fruits, vegetables and ready to eat foods is well known, our knowledge of the prevalence of *Salmonella* in cereal grains, such as wheat, is limited. The purpose of this study was to detect and isolate *Salmonella* from wheat grains that were harvested, transported and stored in different regions of the country. A total of 1,016 wheat grain samples were transported to the laboratory and stored at -80°C until analyzed. Over a thirteen-week period, 625 samples were randomly selected, thawed at 4°C, and tested. Three methods were used for detection and isolation of *Salmonella*. Method A consisted of 50 g of wheat grains suspended in 450 ml of modified buffered peptone water with pyruvate (mBPWp) and incubated at 37°C for 30 minutes. An aliquot of 10 ml of sample was pipetted into 90 ml of mBPWp with novobiocin (22 μg/ml) and incubated for a total of 24 hours. Ten milliliters of the suspension was added to 90 ml of Rappaport Vassiliadis (RV) broth and incubated for 24 hours at 42°C. Method B was a modified version of method A in which the sample size was decreased to 25 g of wheat grains and suspended in 225 ml of mBPWp. The suspension was incubated at 37°C for 30 minutes and blended in a stomacher before incubation. Method C consisted of the wheat grains incubated in RV broth with novobiocin (22 μg/ml) for 48 hours. In all three methods, DNA was extracted from the RV broth and subjected to quantitative real-time PCR (qPCR) for the detection of *invA* and *pagC* genes. Samples positive for both genes were streaked onto Hektoen-Enteric (HE) agar and incubated at 37°C overnight. Presumptive *Salmonella* colonies were tested for agglutination with *Salmonella* O antiserum Poly A- I & Vi and retested by qPCR for both genes to confirm the species. *Salmonella* confirmed isolates were submitted to the National Veterinary Services Laboratory (NVSL) in Ames, IA for serotyping. The isolates were also tested for antimicrobial susceptibility with the National Antimicrobial Resistance Monitoring Systems (NARMS)

Sensititre ™ CMV3AGNF Gram negative panel. None of the samples were qPCR positive for *invA* and *pagC* genes by method A; however, methods B and C identified *Salmonella enterica* positive samples. Overall, eight samples with a prevalence of 1.3% (8/625) were positive for *Salmonella* by qPCR and culture methods. Six samples were positive for *Salmonella* by method B, five samples were positive by method C, and three of the eight samples were identified as positive by both methods. Out of the eight isolates identified five belonged to subsp. *enterica* and three belonged to subsp. *diarizonae*. The isolates of subsp. *enterica* belonged to serotypes Anatum, Hartford, Infantis, Norwich and Oranienburg. The three *diarizonae* were identified as serotype 61:1,v:1,5(7). Antimicrobial susceptibility testing revealed that five of eight strains were pan-susceptible; however, *S*. Infantis and one strain of *S. diarizonae* showed resistance to cefoxitin. Two *S. diarizonae* strains were resistant to tetracycline and amoxicillin-clavulanic acid. These results showed that harvested wheat grains carry *Salmonella* and supported other studies showing similar results. Further investigation is needed to determine the source of contamination and pathogenic potential of the isolated strains. In order to assess the virulence potential of the *Salmonella* strains isolated from these wheat grains, whole genome sequencing of the eight strains was performed and analyzed for the presence of important virulence genes. *In silico* analysis of the genomic sequences of the eight strains confirmed the serotyping by NVSL. Multilocus sequence typing (MLST) analysis revealed that all the *diarizonae* strains belonged to same sequence type (ST-243); however, *enterica* strains belonged to multiple sequence types. The strains carried virulence genes, including fimbrial genes, typhoid toxin genes, *Salmonella* pathogenicity island (SPI)-1 and 2 encoded type three secretory system (TTSS) genes and a repertoire of effectors of TTSS. All of the strains carried the aminoglycoside resistance gene, *aac(6")-laa*. *Salmonella* phage SEN22 was the only intact phage sequence found in *Salmonella* strains belonging to subspecies *diarizonae*, while *enterica* strains carried diverse phage populations. Based on these studies, analyses of *Salmonella* strains isolated from wheat grains reveal their virulence potential, suggesting the possibility that these strains could cause foodborne illness in humans.

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Dedication

For GS and DB who convince me to go back to school, this is all yours fault.

Chapter 1 - Introduction

Members of the genus *Salmonella* are persistent, surviving in a variety of environments for long periods of time. Contamination from these environments and other sources have played a role in the entry of the bacteria into our food system, causing a biological hazard for food manufacturers which has consequently led to numerous local and multistate outbreaks. Epidemiological findings have linked several of these outbreaks to low moisture foods. This review takes a brief look at potential sources of *Salmonella* contamination, outbreaks associated with cereal grains, and possible mechanisms of survival in low moisture foods.

Salmonella is a Gram-negative, motile, facultative anaerobic bacterium that belongs to the family *Enterobacteriaceae.* It is also a zoonotic pathogen whose natural hosts, depending on serotype, include cold- and warm-blooded animals. *Salmonella* consists of two species: *S. bongori* and *S. enterica*. *Salmonella enterica* is further divided into six subspecies: *S. enterica* subsp. *enterica* (1), *S. enterica* subsp. *salamae* (ll), *S. enterica* subsp. *arizonae* (llla), *S. enterica* subsp. *diarizonae* (lllb), *S. enterica* subsp. *houtenae* (lV) and *S. enterica* subsp. *indica* (VI) (Lehman et al, 2015). Over 2600 serotypes have been identified within *S. enterica* subsp. *enterica,* all of which are capable of causing illness.

History

As far back as the middle ages, *Salmonella* has been the cause of enteric disease leading to outbreaks and death. A recent study theorized that Spanish conquistadors might have introduced *Salmonella* to the Aztec people when they arrived in Mexico, which may have been a contributing factor to the collapse of the Aztec empire (Callaway, 2017). To test this theory, the Megan Alignment Tool (MALT) was applied to non-enriched DNA sequence data from the pulp chamber of teeth from the indigenous people of Teposcolula-Yucundaa, and from this data researchers were able to identify ancient *Salmonella enterica* DNA using ancient pathogen screening (Vågene et al., 2018).

During the1880s, Georg Gaffky in referencing the work of Karl Eberth and Robert Koch (his mentor) on typhoid fever, theorized that the inability to isolate the causative agent of typhoid fever was due to the culture methods being used. He refined those techniques and used them to isolate and grow pure cultures of the bacillus. He termed this bacteria Bacillus Typhus abdominalis (now called *S.* Typhi). In 1884, he published the results of his work in the Mittheilungen aus dem Kaiserlichen Gesundheitsamte (Announcements from the imperial health department) ("Georg Theodar August Gaffky," n.d.). Around the same time, Theobald Smith under the supervision of Daniel Salmon, was able to isolate the bacteria he believed was responsible for causing hog cholera. He named it Bacillus *cholerae suis* (now called *S.* Choleraesuis). Eventually, a virus [classical swine fever virus (CSFV)] was determined to be the primary cause of hog cholera, with *S. choleraesuis* as a secondary invader. In humans, *S. choleraesuis* can cause severe typhoidal illness (Dolman, 1982).

Foodborne Pathogen

The Centers for Disease Control and Prevention (CDC) estimates that there are at least 31 pathogens that cause foodborne diseases in the United States. These pathogens consist of bacteria, viruses, and parasites causing an estimated total of 37.2 million cases of illnesses in the US annually. One of the major and most important of these pathogens is *Salmonella* (Burden of Foodborne Illness, Centers for Disease Control and Prevention, 2011).

The CDC estimates that there are 1.2 million reported cases of salmonellosis annually, with one million due to food sources. Approximately 23,000 cases are hospitalized due to severe symptoms, and 450 individuals die from this foodborne illness. The members of *Salmonella* mainly cause gastroenteritis: abdominal pain, nausea, vomiting and watery diarrhea. For most people the infection is self-limiting, lasting anywhere from four to seven days. However, there are several serotypes such as *S.* Typhi and Paratyphi A-C that cause typhoid fever (paratyphoid fever). Typhoid fever is the most severe and deadly form of enteric fever (CDC, 2018a).

In the US, a national surveillance system for *Salmonella* illnesses was established in 1962 by the Communicable Diseases Center (later to be known as the Centers for Disease Control and Prevention) Epidemiology Branch. This small surveillance unit was originally established within

the Investigation and Veterinary Public Health Sections. The unit served many functions including obtaining investigation reports on *Salmonella* outbreaks among human and animals, providing states laboratory field or epidemiologic assistance and laboratory investigations into the characteristic of the *Salmonella* serotype, its mode of introduction and survival in foodstuffs, animal and poultry feeds (Sanders et al., 1963).

The data collected by the national surveillance for *Salmonella* shows that the most common *Salmonella* serotype detected since 1962 has been *S.* Typhimurium. By 1997, the top three consistent *Salmonella* serotypes were Typhimurium followed by Enteritidis and Newport, respectively. However, in 2008, Typhimurium was supplanted by Enteritidis, a serotype frequently isolated from poultry and their eggs. The increase of this serotype has most likely been due to the growing interest in backyard poultry (CDC, 2008a). By 2016, detection of the Newport serotype surged to become the second most common serotype (National *Salmonella* Surveillance, 2018). Although these three serotypes are the major causes of clinical cases, it is the other serotypes of *Salmonella* that are responsible for most multistate outbreaks. In 2018, there were 18 multistate outbreaks, of which, 16 were food related. Among those, three were due to *S*. Enteritidis, while two were due to *S.* Typhimurium and *S*. Newport (CDC, 2018c).

Salmonella **in Soil**

Research has documented the persistence of *Salmonella* and its ability to disseminate in the outdoor environment, which has been associated with bacterial contamination of wildlife and crops. Studies have shown that depending on the strain, depth level, temperature, moisture and nature of the environment (water/soil), *Salmonella* can survive from a few weeks to several years. A study by Davies and Wray (1996) demonstrated the survival of *S.* Typhimurium in a burial pit for almost two years. The ability to detect the organism in the soil appeared to be associated with the variations in the seasons. In addition, the researchers found that the bacteria had infected a large population of *Lucilia sericata* (common green bottle/blow fly) larvae found in the pit. *S.* Typhimurium was also discovered in wild-bird droppings in a drainage ditch that was 13 feet away from an alternative decomposition pit. They believe that the wild birds became

infected from contaminated rain water from the pit or ingestion of the infected larvae (Davies & Wray, 1996).

During the winter of 2000, an outbreak of *S*. Enteritidis PT30 was attributed to raw almonds. The positive results from environmental swabs led investigators to almond orchards on three different farms. According to the growers, no manure or biosolids had been used in the previous five years, which was consistent with what investigators had observed. There were no livestock or poultry farms nearby and the system that was used for irrigation, including the water, tested negative for *S*. Enteritidis PT30 (Isaacs et al., 2005). One of the orchards that had tested positive was studied for a period of five years. Environmental swabs were obtained during different times of each year. From 2002 to 2006, 53 swabs tested positive for *S*. Enteritidis PT30. These results in addition to the evidence of contamination from 2000, suggest that *Salmonella* could have persisted in this orchard for at least seven years (Uesugi et al., 2007).

Wang et al. (2018) found that *Salmonella* in an agricultural environment could persist for a few days to well over 130 days. In the same manner, the survival of the bacteria in contaminated manure used in field plots depended on the type of soil, the time of year (moisture and temperature), depth in the soil and manure type.

Plants As Hosts?

Since 1990, there have been numerous produce-related outbreaks, such ascraw tomatoes, precut melon and alfalfa sprouts (Hedberg et al., 1999; Mohle-Boetani et al., 2001; CDC, 2018d). The majority of epidemiological investigations led to the grower or packing plant but rarely was the original source of the contaminant known (Waldner et al., 2012). Although *Salmonella* can infect humans and a variety of animals (cold- and warm-blooded), is it possible for the bacteria to use plants as hosts? Asplund and Nurmi (1991) reported that *S*. Enteritidis, *S*. Infantis and *S*. Typhimurium were able to grow at 22ºC and 30ºC in cut up pieces of ripe tomatoes. The pH in the tomatoes ranged from 3.99 to 4.37, and yet, this acidic pH did not inhibit *Salmonella* growth. Guo et al. (2001) inoculated open flowers and the stem surface tissue of tomato plants with *Salmonella*. The fruit was allowed to develop and ripen on the plant, which was then harvested and tested. Salmonella was detected in 37% of the tomatoes harvested

from the inoculated plants. These results suggest that *Salmonella* is capable of surviving in the tomato or on the plant from flowering to harvest. Kutter et al. (2006) found that *Salmonella* inoculated into quartz sand (monoxenic hydroponic model system) was able to colonize barley plants. Bacteria were found on the main and side roots including the root hairs. *Salmonella* formed microcolonies in the rhizodermis cell layers and in the inner root cortex of the central cylinder. Moreover, the bacteria spread systemically through the whole barley plant. The researchers noted that since the study was done with hydroponically grown barley, the results might differ in soil. Cooley et al. (2003) demonstrated *Salmonella* survival in *Arabidosis thaliana* (Thale cress) in a gnotobiotic system as well as in autoclaved and un-autoclaved potting soil, in addition to a potting soil mixture and Yolo fine sandy loam. *Salmonella* was able to survive longer in the autoclaved soil and the hydroponic medium. On the other hand, the bacteria did not survive as long in the un-autoclaved potting soil, potting soil mixture, or Yolo fine sandy loam. They theorize that there might be bacteria in the soils competing with *Salmonella* for colonization of the plant.

Contamination in Cereal Grains

The microflora of milled cereal grains includes *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium botulinum*, *Salmonella*, molds and yeasts (Bullerman & Bianchini, 2008). Yet there have been few reports of microbiological content of milled cereal grains in the US. *Escherichia coli* counts, yeast, mold, coliform and aerobic plate counts (APC) are the most commonly required quantitative indicator tests, while the presence of *Salmonella* is the most commonly required qualitative pathogen test (Sperber, 2007).

In 1989, researchers examined 4796 samples of wheat flour derived from hard red winter (HRW), soft red winter (SRW), spring (SPG) and durum (DUR) wheat species. They found that the durum flour had the highest APC, but the coliform counts were consistent among the wheat flour types. A total of 3,040 wheat flour samples were tested for *Salmonella* and 1.32% of the samples were positive. SRW had the highest percentage of positive wheat flour samples. The winter months had the highest occurrence of *Salmonella* (Richter et al., 1993). In Sperber's review, he obtained contemporary microbiology data from 2003 to 2005 for five types of milled

cereal grains (corn, oats, whole wheat, durum and wheat). He examined the test results of 13,484 milled cereal grain samples and found that *Salmonella* was not detected in whole wheat, corn, oat or durum, but the bacteria was found in wheat at 0.14% (Sperber, 2007).

From 2012 to 2014, 3891 wheat samples comprised from hard red winter, hard red spring, soft red winter (early), and soft red winter (late) were compiled and tested for *Salmonella* along with several other pathogens. *Salmonella* was detected in 48 of the 3891 wheat samples, a prevalence of 1.23% (Myoda et al., 2019). As with the Richter et al. study (1993), the highest occurrence of *Salmonella* was during the winter season.

Flour from cereal grains is a raw agricultural commodity, and milling of these grains has little effect on the microbiology of the flour. Steps to promote a decrease in microbial count in flour would destroy some of its functional properties (Richter et al., 1993). In addition, Sperber stated that with improved sanitation in grain handling and dry milling along with the hazard analysis critical control point (HACCP) system of food safety management most microbiological specifications and required tests were not necessary for milled cereal grains (Sperber, 2007).

Although *Salmonella* contamination in wheat flour has not been common, it can occur. In 2015, the Navajo Pride located in Farmington, NM voluntarily recalled its bleached all-purpose flour after routine screening detected *Salmonella* in a 5 pounds sample (US Food & Drug Administration Safety Recalls, 2015). The recall covered the states of Arizona, New Mexico, Texas and Utah, where the flour was sold (Food Safety News, 2015). A few weeks later, the Navajo Pride flour mill shut down for a year to replace equipment and to have additional training (Baking Busines.com, 2015).

In January 2019, General Mills voluntarily recalled Gold Medal unbleached flour in the 5 pound bags due to *Salmonella* contamination which was detected during routine testing (US Food & Drug Administration Safety Recalls, 2019a). Then in March 2019, Hometown Food Company voluntarily recalled its 5 pound bags of Pillsbury unbleached all-purpose flour due to possible *Salmonella* contamination (US Food & Drug Administration Safety Recalls, 2019b).

Outbreaks Associate with Milled Cereal Grains

Over the last three decades *Salmonella* outbreaks associated with milled cereal grains have been detected in products ranging from dry cereal to dry cake mix (Table 1.1). The earliest cereal grain related outbreak occurred during April and May of 1998, where the CDC reported that *S.* Agona had been found in Toasted Oats cereal manufactured by Malt-O-Meal. The recall encompassed eleven states, and by June, there were 209 reported clinical cases with 47 of these patients being hospitalized (CDC, 1998). In the investigation, the CDC was unable to determine how the *S.* Agona contamination occurred. *S.* Agona was detected in samples taken from the floor, exhaust system and the production line equipment (Breuer, 1999). As a result, the company had all the surfaces in the Toasted Oats production area stripped to the bare concrete, decontaminated, and refinished with epoxy (Russo et al., 2013).

In July of 2005, a recall was issued for Cold Stone Creamery cake batter ice cream. During the months of May and June 2005, 26 people in nine states had acquired *Salmonella* Typhimurium and five were hospitalized (Zhang et al., 2007; Minnesota Dept. of Health, 2005). When samples of the cake batter ice cream were originally tested, *S.* Typhimurium was not detected. So, in July 2006, the samples were retested using several different growth media and pulsed-field gel electrophoresis (PFGE). The results showed that *Salmonella* Typhimurium was the same bacteria that had caused the outbreak (Zhang et al., 2007). They found that *S.* Typhimurium was in the cake mix, and not the ice cream; however, it was not known whether the spray-dried eggs whites or the flour of the cake mix contained the bacteria (Minnesota Dept. of Health, 2005).

From June to September of 2007, people were being infected with *Salmonella* serotype I 4,5,12:i; however, the CDC at the time was unable to establish the source. B October 3rd, the investigation had found that Banquet frozen pot pies was the source of the infection, and on October 11th, ConAgra issued a recall on all nine brands of the frozen pot pies. By December 2007, there were a total of 401 cases in 41 states, of which 108 were hospitalized and three individuals died (CDC, 2007). Environmental testing was performed by the United States Department of Agriculture's Food Safety and Inspection Service (FSIS). The plant plus the five facilities that supplied the poultry and precooked meat were tested and the results were negative. Since several different types of pot pies were made at the plant, testing was done on 93 pot pies which consisted of chicken (50), turkey (35), beef (2) and two unknown types of pies. *Salmonella* was detected in 14 of the turkey pot pies. The original source of the contamination

was not found; however, it was theorized that the raw flour crust may have contained the bacteria, or that the raw poultry was not properly cooked. Another possibility was there might have been cross-contamination with raw poultry (Mody et al., 2013).

In April of 2008, there was a multistate outbreak of *S.* Agona in dry cereal. Once again, the same strain of *S.* Agona was found in Malt-O-Meal manufactured cereal, but this time it was unsweetened Puffed Rice and Puffed Wheat cereals. Fifteen states were involved; 28 people had salmonellosis with twelve individuals being hospitalized (CDC, 2008b). However, further research by Russo et al. (2013) found that 33 had actually become ill. In the following investigation, it was noted that in early 2008, a wall in the manufacturing facility adjacent to where the 1998 contamination had taken place, was opened for maintenance work. It was postulated that the dust from the wall contained the same *S.* Agona strain. This dust could have combined with the wet cleaning area around the breached wall and somehow contaminated the puffed rice and wheat cereals before packaging. After the investigation, the cereals were discontinued, and that area of the plant was no longer used for food production (Russo et al., 2013).

In June of 2018, Kellogg Co. issued a recall for Honey Smacks cereal due to an outbreak of *S.* Mbandaka. At the time, it had extended to over 31 states with 71 cases and 24 hospitalizations. Then in July, the CDC issued a warning as people were still eating the cereal and getting sick. Twenty-four additional cases brought the total to 100 in 33 states. By September, the CDC had to issue yet another warning for Kellogg Honey Smacks cereal after 30 more people became ill. The CDC closed the investigation on September 26, 2018 with a final tally of 135 cases, including 34 hospitalizations, in 36 states (CDC, 2018e). Unlike the 1998 and 2008 Malt-O-Meal contamination events, the 2018 investigation found that *Salmonella* had been an ongoing issue at the Kerry (Kellogg) production plant. Records indicate that the plant had repeatedly found *Salmonella* (not only *S.* Mbandaka) throughout the facility since 2016. Company documents showed that they had 113 positive lab results, 81 from environmental samples, 32 vector samples, as well as other positive results for *Salmonella* (Food Safety News, 2018).

On November 5, 2018, ConAgra Brands recalled four types of its Duncan Hines cake mix because *Salmonella* Agbeni had been found in the Duncan Hines Classic White cake mix. There were seven clinical cases in five states and none were hospitalized. Despite the documentation

of illnesses, there was not enough epidemiological data collected during the outbreak to link the salmonellosis to the contaminated Duncan Hines cake mix (CDC, 2019) (Table 1.1).

Table 1.1 *Salmonella* Outbreaks associated with wheat grains

Outbreaks (Year)	**Serotype**	**Product**	**Location**	**Reference**
1998	*Salmonella* Agona	Malt-O-Meal Toasted Oats cereal	USA	CDC, 1998; Breuer, 1999; Russo et al., 2013
2005	*Salmonella* Typhimurium	Cold Stone Creamery cake batter ice cream	USA	Zhang et al., 2007; Minnesota Dept. of Health, 2005
2007	*Salmonella* serotype I 4,5, 12:i	ConAgra Banquet frozen pot pies	USA	CDC, 2007; Mody et al., 2013
2008	*Salmonella* Agona	Malt-O-Meal Puffed Rice and Puffed Wheat cereal2018	USA	CDC, 2008; Russo et al., 2013
2018	*Salmonella* Mbandaka	Kellogg Honey Smacks cereal	USA	CDC, 2018
2018	*Salmonella* Agbeni	ConAgra Duncan Hines cake mix	USA	CDC, 2019
Recall (Year)	**Bacteria**	**Product**	**Location**	**Reference**
2015	*Salmonella*	Navajo Pride bleached all-purpose flour	USA	Food Safety News, 2015 Baking Business.com, 2015
2019	*Salmonella*	General Mills 5 lbs Gold Medal unbleached flour	USA	US Food & Drug Administration Safety Recalls, 2019
2019	*Salmonella*	Hometown Food Company Pillsbury 5 lbs unbleached all purpose flour	USA	US Food & Drug Administration Safety Recalls, 2019

Salmonella in Dry Pet Food

Salmonella presence in animal feed for livestock and poultry is well documented (Erwin, 1954; Boyer et al., 1958; Gray et al., 1958; Newell et al., 1959; Glickman et al.,1981; Jones et al., 1982; Molla et al., 2010). In addition, most households feed their pets dry foods that contain ingredients from animal origins as well as grains which can be at risk for *Salmonella* contamination (Imanishi et al., 2014). According to the American Pet Products Association (APPA), 2017-2018 National Pet Owners Survey, 48% U.S. of households own dogs, while 38% own cats (American Pet Products Association, 2018). *Salmonella* is a zoonotic pathogen affecting pets, and in turn, their owners. Salmonellosis is not common in cats and dogs, but when exposed to the bacteria, many can be asymptomatic. Fecal shedding has been observed for three to six weeks, and in a few cases, even longer after infection. As with humans, they too can develop gastroenteritis with symptoms ranging from fever, vomiting, diarrhea, abdominal cramps to sepsis (Food and Drug Administration, 2017; Imanishi et al., 2014). The FDA and/or the Association of American Feed Control Officials (AAFCO) define what current ingredients are allowed in pet foods. This was made possible when Congress mandated in 2007 that the federalization of animal feed ingredients standards and definitions include input from the AAFCO, as well as other relevant parties such as veterinarians (Thompson, 2008).

The ingredients in pet foods can be simple or complicated, as long as the nutritional content keeps pets happy and healthy. Protein, carbohydrates and fat are the three largest nutrient components of pet food. For example, protein sources can include meat and bone meal, soybean meal, along with some contribution of protein from whole wheat, whole corn, barley, animal digest (common ingredient/additive) and amino acids. Carbohydrates supply the glucose as well as provide the fiber in pet food. Examples of carbohydrates include whole grain wheat, brewers rice, grain flour and sorghum (Thompson, 2008).

Dry Pet Food Outbreaks Due to *Salmonella*

Just as food safety is important to humans, pet owners want the same thing for their animals. *Salmonella* has been linked to numerous recalls and outbreaks in dry pet food and dry

treats in the last ten years. Salmonellosis in pets and humans has been caused by direct contact with contaminated pet food, exposure to infected pets or possible cross-contamination (Lambertini et al, 2016). An outbreak of *Salmonella* Schwarzengrund from January 2006 to October 2008, caused 79 cases of infection in 21 states. Samples from exposed pets were collected, but no illnesses were reported. The investigation found that two brands of the dry pet food produced by Mars Petcare US were probably the source of the infections. The manufacturing plant associated with the contaminant was shut down for nearly four months, cleaned, disinfected, and renovated. However, after six months in operation, another outbreak of *S*. Schwarzengrund occurred. By September 2008, production at the plant had ceased and by that October, Mars Petcare US closed the facility permanently (CDC, 2008c; Behravesh et al., 2010).

This was the first of two outbreaks linked to dry pet foods to result in salmonellosis for both human and pets. The second outbreak occurred in April 2012, when during a routine surveillance of dry dog food, *Salmonella* Infantis was detected. A few days later, Diamond Pet Foods issued a voluntary recall for the dog food product. The following week the plant was shut down to be cleaned and disinfected. At the same time, investigators were looking into a possible multistate outbreak of S. Infantis. Further testing found the bacteria in another brand of dry dog food made by Diamond Pet Foods. Two additional recalls were issued, which led to 16 other dry foods for pets being removed from retail shelves. By July 2012, there were 53 cases in 21 states with 2 infected individuals from Canada. Thirty-seven of those who had contracted salmonellosis were hospitalized and none died. In addition, 31 dogs that became ill during the outbreak were associated with the recalled pet foods. In the initial investigation of the production plant, *S*. Infantis was only found on the finished product. However, during a follow-up visit to the plant in September 2012, investigators found multiple strains of *Salmonella*, implying that the bacteria was still being introduced into the environment (Imanishi et al., 2014).

Research has shown that *Salmonella* can survive in low moisture foods. Furthermore, *Salmonella* serotypes have been found in dry dog foods and dry pet treats like pig ears. To better understand the "kinetics" of bacteria survival in dry pet foods, 12 different strains of *Salmonella* were inoculated onto pieces of dry dog food kibbles. The kibbles were stored at ambient temperature (23.2^{o}C), and while the detection of *Salmonella* declined, the researchers were still able to isolate and quantify the bacteria 19 months later (Lambertini et al., 2016).

Certain pet foods allow for moistening of the dry food and some owners mix it with wet food before serving it to their pet. This rehydrated pet food may remain at room temperature for some time before it is consumed or discarded. What if and in what manner would *Salmonella* grow in rehydrated dry dog food? A study by Oni et al. (2016) applied a bacteria cocktail using two different strains of *Salmonella* on eight commercial brands of dry dog food. The dog food was rehydrated at three moisture levels (20, 35, & 50%) and stored at 18, 22, and 28ºC for 72 hours. At temperatures of 22 and 28ºC, bacterial growth was detected on 5 of the 8 brands tested that had been rehydrated at 35 and 50%. They concluded that *Salmonella* growth on the dry dog food depended on the brand, moisture content and storage temperature.

Contamination & Survival in Low Moisture Foods

Although *Salmonella* detection focuses mainly on poultry, eggs, food products containing poultry, ready to eat foods, fruits, and vegetables, there has been an increasing trend of contamination of low moisture foods like dry cereal, peanut butter, nuts, spices, and grains. Moreover, its presence in cereal grains has been an ongoing issue for food manufacturers as grains are the basic ingredients in many products. Contamination of cereal grains such as wheat can occur during harvest, transport or storage especially if they are in contact with wildlife (Erwin, 1955; Beuchat & Ryu, 1997; Daniels et al., 2003). Furthermore, the contaminated grain may be unevenly distributed when feces from rodents is deposited, which when sampled may give false negative results (Binter et al., 2011; Davies & Wales, 2013). Insects may also play a role in cross contamination. (Podolak et al., 2010). Crumrine et al. (1971) showed how several species of common grain insects were able to transfer *Salmonella* Montevideo from contaminated wheat to clean wheat grains. In addition, adult muscoid flies collected from the manure area of commercial dairies and poultry ranches were shown to be carriers of several strains of *Salmonella* (Mian et al., 2002). Moreover, the flies were able to transfer *Salmonella* to food (fruit peels) as well as other surfaces up to 20 days after being infected (Ostrolenk & Welch, 1942).

In the past, there has been a misunderstood belief that low-moisture foods do not support bacterial survival. However, *Salmonella* is capable of surviving in dry food for a long duration

of time and can survive in low moisture foods, like wheat, for weeks or even years (Podolak et al., 2010; Finn et al., 2013). Notably, a study examined 18 strains of Gram-positive and Gram-negative bacteria, which included *Salmonella.* They discovered that these microbes had the ability to survive for long periods of time after being suspended in anhydrous silica gel at 22ºC (Janning et al., 1994). Another study mixed 5 strains of *Salmonella* into concentrated cell suspensions that were inoculated into 15 grams of flour. The sealed bags of flour were then stored at room temperature (23 $\pm$ 1ºC) or at 35ºC. The *Salmonella* inoculated into wheat flour stored at room temperature was still detected after a year, whereas the ones stored at 35ºC were detected up to 98 days (Forghani et al., 2019). Previous studies have found that the storage temperature, level of contamination, and product formulation may likewise affect bacterial survival (Beuchat & Heaton, 1975; Burnett, Gehm, Weissinger & Beuchat, 2000; Cavallaro et al., 2011).

Mechanisms of Survival

Research has shown that *Salmonella* has the ability to survive desiccation, but it is not clear is how this is achieved. A study by Janning et al. (1994) led researchers to believe that members of *Salmonella* genus have some structural or protective mechanism that allows the microbes to survive desiccation and maintain stability in low moisture foods and nutrients. One of those protective mechanisms may be a dormancy state called VBNC - viable but nonculturable state (Podolak et al., 2010). A state that many nonpathogens and pathogens including *Salmonella*, can enter into. Bacteria in the VBNC state will not grow on regular bacteriological media and their metabolic activity is very low, but when resuscitated the VBNC become culturable (Oliver, 2005). There have been several studies showing how to achieve the transition state of VBNC, such as using UV-C (used in water and wastewater treatments plants) in seawater, chloramphenicol, saline solution or glucose (Caro et al., 1999; Asakura et al., 2002; Smith et al., 2002; Morishige et al., 2014). The question surrounding VBNC is whether bacteria can be revitalized out of the dormant state to a point of dividing under favorable conditions. If so, can the pathogen infect and cause disease in the host (Winfield & Groisman, 2003)? Studies, such as the ones by Gupte et al. (2003) and Reissbrodt et al. (2000), have demonstrated the resuscitation of *Salmonella* Typhimurium that had been induced into the VBNC state. These

bacteria were tested in a dormant state from a few months to almost two years. Experiments by Reissbrodt et al. (2000 & 2002) revealed that siderophore ferrioxamine E as well as heat-stable enterobacterial autoinducer were effective in reviving the stressed bacterial cells. Yet, do these resuscitated cells have the potential to be infectious? One study using morphine-treated mice to cause systemic immunosuppression was able to infect them with resuscitated VBNC *Salmonella* Oranienburg (Asakura et al., 2002b). However, other experiments testing the pathogenicity of *Salmonella* VBNC in mice were unable to initiate any infection (Caro et al., 1999; Smith et al., 2002; Asakura et al., 2002).

Another mode of survival for *Salmonella* is the formation of biofilms. Biofilms are organized populations of bacteria in an enclosed polymeric matrix that can bind to many different types of niches (Costerton et al., 1978; Donlan & Costerton, 2002; Steenackers et al., 2012). This structure allows *Salmonella* to survive hostile environmental pressure, various temperatures, antibiotics and sanitizers (Joseph et al., 2001; Donlan & Costerton, 2002; Steenackers et al., 2012). Various components of the extracellular matrix make up the *Salmonella* biofilms. The two most significant of these components are cellulose and curli produced by the red dry and rough (rdar) morphotype (Steenackers et al., 2012). Cellulose in bacteria is an exopolysaccharide that provides mechanical and chemical protection, and cohesive as well as structural stability for cell-cell interconnections (Zogaj et al., 2001; Pontes et al., 2015). However, further research has shown that cellulose in *Salmonella* does not play a role in virulence (Solano et al., 2002; Pontes et al., 2015). The thin aggregative fimbriae or curli are nonbranching bacterial amyloid fibers associated with cell aggregation and adhesion to various surfaces (Collinson et al., 1991; Romling et al., 1998; Barnhart & Chapman, 2006). Collinson et al. (1991) found that the thin aggregative fimbriae were highly hydrophobic plus “very resistant to disaggregation, denaturation, and depolymerization”. In addition, it was discovered that when cellulose and thin aggregative fimbriae were expressed together, they formed a hydrophobic network of cells tightly embedded in a highly inert matrix (Zogaj et al., 2001).

Conclusion

Salmonella serotypes are resilient bacteria found throughout nature with structural or protective mechanisms that allow the microorganism to survive in harsh environments. *Salmonella* increased capability to survive in low moisture foods poses a challenge to established sanitary procedures. Epidemiological investigations during outbreaks have been effective in pinpointing the manufacturing location where the contamination occurred but the original source of this contamination is rarely found.

From soil to plant life to wildlife to existing food sources, *Salmonella* has created a cycle of contamination that has proven difficult to control. To improve sanitation, would it not make sense to prevent the contamination early in the cycle especially in areas where raw agricultural commodities such as cereal grains are of concern? Perhaps a policy of incentives should be implemented to aid in places where regulations and sanitation are lacking in addition to providing increased funding for testing of raw agricultural products coming into the US. With continued research in both the medical and veterinary fields, the combined efforts from food manufacturers (animal and human), and those who handle the initial raw agricultural products, we will continue to make progress in decreasing *Salmonella* contamination and reduce the number of foodborne illnesse

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Chapter 2 - From diphasic Arizona to *Salmonella* subspecies (IIIb) *diarizonae*

The detection of *S. diarizonae* in the wheat grain samples was an unexpected find. *Salmonella diarizonae* is a subspecies (IIIb) in the genus *Salmonella* and has been isolated from humans, animals, and food sources. Illnesses from *S. diarizonae* are rare, about 96 individuals are affected each year (CDC, 2007 & 2016). The current epidemiological data on *S. diarizonae* has been limited due to several different name changes and classifications. When discovered, *diarizonae* was biochemically and serologically similar to *arizonae*, and therefore, classified in the same group. *Salmonella* species display phase variation, or diphasic states[1], which cause them to display different flagellar (H) antigens, and it was assumed that *diarizonae* was a phasic state of *arizonae*. With the advancement of technology, researchers discovered that the bacterium was its own distinct group and was separated from *arizonae*.

The subject matter of this chapter has been divided into two main sections. The intent of the first section is to provide a comprehensive historical characterization of *Salmonella* subspecies *diarizonae* and its relation to the pathogenesis in different animals. The second section gives a brief summary on the complex and sometimes confusing *Salmonella* nomenclature and its history focusing on the subspecies *diarizonae*.

Arizona

In 1939, Caldwell and Ryerson were among the first to discover a *Salmonella* pathogen in reptiles. At the time there were no known reports of the bacteria using reptiles as a host. The bacteria was isolated from ailing Gila monsters then used to inoculate horned lizards (Phrynosoma solare), a chuckwalla (Sauromalus ater) and other Gila monsters (Heloderma suspectum). Rabbits, which were believed to be immune to the *Salmonella* species as well as guinea pigs were also inoculated. The microbe proved to be pathogenic to most of the reptiles as well as the rabbits and guinea pigs. The results of the biochemical tests performed showed that the new strain was similar to the *Salmonella* species Dar-es-salaam. The Dar-es-salaam strain

was discovered in 1922, first isolated from a patient in Tanzania, Africa. Like Dar-es-salaam, the bacterium liquefied gelatin but it did not ferment dulcitol. While no antigenic analysis was done, they suggested that the bacterium be known as the Dar-es-salaam type of *Salmonella* (variety from Arizona) (Caldwell & Ryerson, 1939).

Diphasic *S.* Arizona

Kauffman received the Dar-es-salaam type of *Salmonella* (variety from Arizona) culture from Caldwell, noting it as "*S.* arizona". When tested, he found that Dar-es-salaam and *S.* Arizona strains were not serologically related. Biochemical testing done by Kauffman agreed with the Caldwell and Ryerson findings, and showed the bacterium was *Salmonella* and highly pathogenic. He also noted that Caldwell and Ryerson stated that the Dar-es-salaam type did not ferment lactose (Caldwell & Ryerson, 1939). However when Kauffman tested it, he found that the bacterium could ferment lactose after a period of time which was ironic as known *Salmonella* strains did not ferment lactose. The bacteria did not have Vi antigens nor did it have the common smooth O antigen but a different antigen that he termed the antigenic structure as XXXIII**. *Salmonella* flagellar (H) antigen consisted of c and d antigens, but *S.* Arizona H antigens did not contain those, and so, the antigenic formula of Z_4, Z_{23}, Z_{26}[2] were assigned to them (Kauffman, 1941).

Peluffo et al. (1942) also obtained a Dar-es-salaam type of *Salmonella* (variety from Arizona) culture which they called "Arizona" from Caldwell. As noted by Kauffman, the Arizona culture fermented lactose quite slowly. The culture was then transferred to lactose broth where it generated acid and gas within 24 hours. In 1945, Edwards and West published a paper about Pc110, a bacterial culture that they had received from Dr. Hinshaw. It had been isolated from a garter snake and he had classified it as a paracolon bacillus. While Pc110 was culturally and morphologically similar to coliform bacteria, the microorganism also produced H_2S, liquefied gelatin and fermented lactose. They found that the H antigens were related to that of *Salmonella* and assigned the antigenic formula of XVI: z_{10} – e, n... (Edward & West, 1945). The results of biochemical tests suggested that the bacteria was similar to Arizona, however, the Pc110 flagellar antigens had a diphasic state (West et al., 1947).

Diphasic *S.* Arizona to Diphasic Arizona

The *Salmonella* bacteria discovered by Caldwell and Ryerson, called *S.* arizona by Kauffman and referred to as the Arizona group of paracolon bacteria by Edwards et al. (1947), would be shorten to the "Arizona Group". Four hundred and fifty-six cultures were collected belonging to the Arizona group. These cultures were isolated from chickens, turkeys, canaries, guinea pigs, snakes, swine, dogs, mink, capybara (a South America rodent), man, ice cream and egg powder. Although most of the cultures came from young turkeys (241) and egg powder (121), the researchers did a complete biochemical and serological workup on all the isolates. While no pathogenicity tests were done on these cultures, the researchers relied on the pathogenic history of *S.* Arizona and the epidemiological data of the 456 strains. All but five of the cultures fermented lactose, the rate varying from 24 hours to 30 days. The 456 strains were divided into 19 O (somatic) groups with one group having 3 subgroups. The H group was more complex, but these were assigned 3 groups. In total, there were 19 O groups and 55 serologic types for classification (Edwards et al., 1947). They believe that members of Arizona might be present in human illnesses, but they were being overlooked because they fermented lactose and might be indistinguishable from *Escherichia* strains (Edwards & McWhorter, 1956; Edwards et al., 1959). Around the same time, West et al. (1947) examined eight cultures they received, one from Dr. Solowey and the other seven were from Dr. Hinshaw. Six of the cultures were from reptiles, and the other two were from a young turkey and egg powder. The bacteria varied in the rate in which they fermented lactose. Although the cultures varied slightly biochemically from the members of the Arizona Group, the H antigens were found to be diphasic. As with their previous work, no pathogenicity testing was performed.

In the spring of 1951, three dead premature lambs were presented to the Wyoming State Veterinary Laboratory. A culture containing a *Salmonella*-like bacteria was sent to Dr. Edward. Upon further study, he confirmed the bacterium to a diphasic Arizona type 26: 29-30 (Ryff & Browne, 1952). As Edward's work with the Group Arizona became more known, he began to receive cultures from other counties as well. Isolated from a Puff Alder (*Bitis arietans*) was bacterium D860/51, a culture received from Belgian Congo. As with the previous cultures, this

bacterium contained the same biochemical characteristic as the Arizona group, and the H antigens were diphasic (Edwards, Kauffman & Van Oye, 1952). From Kasenyi (Uganda), a culture 466-52 containing a diphasic Arizona strain was isolated from a healthy duck. Around the same time, he received culture 557-52, from another Puff Alder (*Bitis arietans*). While some earlier diphasic bacteria fermented lactose in four days, culture 557-52 fermented lactose in eleven days having slightly different somatic and flagellar antigens (Edwards, Kauffman & Fain, 1953).

Since the discovery of the Arizona Group, many monophasic strains had come to light but only few diphasic ones were documented. Edward believed the reason for this was diphasic serotypes rapidly fermented lactose and when grown on differential media they look no different than *Escherichia,* and so, these samples were likely discarded. In the meantime, he received additional samples of diphasic cultures pertaining to the Arizona Group. Whereas the majority of the samples had been from ill or dead specimens, cultures 3834/53, 599/54, and 4601/54 were from three healthy Mexican snakes (referred to as *Constrictor constrictor*). The researchers found the same Arizona strain (29:31-33) in two of the Mexican snakes and noted that the diphasic strains tend to be less pathogenic then the monophasic ones. Another culture added to the collection was 3829/53, discovered in a healthy Greek tortoise (*Testudo graeca*) from Morocco (Edwards & Boycott, 1955). Cultures from the monophasic strains varied in source, whereas the majority of the diphasic bacteria seemed to be from snakes, like samples 5395-52 and 1377-54. Then, there were infrequent non-cold-blooded animal samples, like sample 3853-54, isolated from a chicken and sample 2758-55, from river water in Taunton, England (Edwards & McWhorter, 1956).

Occurrence and distribution of Diphasic Arizona (I)

Bacteria from the Arizona group had been isolated from humans, other mammals, fowl and reptiles. The majority, almost 75% of the Arizona cultures studied were from fowl and reptiles. Further breakdown of the Arizona cultures revealed that monophasic serotypes seem to favor warm-blooded animals, such as fowl and humans. In contrast, the diphasic serotypes seemed to favor the cold-blooded animals, such as snakes. The Arizona cultures that had been

collected were organized into different groups. They contained 32 O groups and 180 antigenic combinations from Asia, Africa, Europe, North and South America. Out of the 140 Arizona serotypes listed, 89 were diphasic and two serotypes (19:26- and 20:33-) were questionable and not included. Seventy-three of those serotypes were isolated from reptiles alone. The categories from man, duck, and eggs/egg products had one serotype each. The other 13 serotypes were isolated from multiple sources including man, monkeys, reptiles, turkeys, chickens, sheep, water sewage, eggs/egg products and a hog (Edwards et al., 1959).

Edwards's collaboration with Japanese scientists resulted in the identification of eight new diphasic Arizona serotypes from healthy snakes (Sakazaki et al., 1960). Furthermore, samples isolated from several dead snakes (an anaconda and two western diamondbacks) from the Smithsonian Zoological Gardens plus an Indian python from a zoo in England were sent to Edward. These cultures were tested for reference diagnosis and found to be diphasic (Fife et al., 1962).

Diphasic Arizona discovery in England

In Cardiff, England, crushed bones from India and Pakistan were used in the preparation of animal feed stuff. Other studies had shown that organic materials used in animal feed had tested positive for *Salmonella.* This led the researchers to obtain 57 samples of crushed bones to test them for *Salmonella*. They found 56 *Salmonella* plus eight Arizona serotypes. The eight cultures were sent to Dr. Edwards, who confirmed them as diphasic Arizona, two of which were new serotypes (Harvey & Price, 1962; Fife, McWhorter & Edwards, 1962). Due to results from the crushed bones study, researchers were prompted to examine native animal material. Four abattoirs (slaughter houses) in England and South Wales were chosen and gauze swabs (Moore's swabs) were placed in the drains. Over a nine-year period these swabs were collected and cultured for *Salmonella*. Six positive subgenus III (Arizona) samples were found, four of those were diphasic. The diphasic samples came from two slaughter houses in South Wales, one that killed cattle and sheep, while the other killed cattle, pigs and sheep. The researchers believed that the serotype 26: 29-30 was from cattle, but the reference from the Communicable Diseases Center (Edwards) showed the strain was commonly found in sheep. This serotype had also been

cultured twice from Native American sheep-herding tribes (W.H. Ewing, personal communication) (Harvey et al., 1966).

Antigenic formulas instead of names

In 1966, the members of Enterobacteriaceae Subcommittee to the Nomenclature Committee proposed antigenic formulas, instead of names, be given to new serotypes of *Salmonella* subgenus II, Arizona group (subgenus III), and subgenus IV (Carpenter, 1968). However, the names for serotypes belonging to subspecies II-IV before 1966, including VI and *S. bongori,* would remain as is by the CDC for compatibility with old data and surveillance purposes (Brenner et al., 2000).

Occurrence and distribution of Diphasic Arizona (II)

In 1955 and 1959, Edwards et al. wrote reports on the occurrence and distribution of the Arizona Group cultures received by the Communicable Diseases Center (which would later be called the Centers for Disease Control). These reports were followed up by Martin et al. in 1967, and the final one by Weiss et al. in 1986. The 1967 report noted that from July 1956 to December 1966, the Communicable Diseases Center received 1804 Arizona cultures isolates from humans, reptiles, poultry, food as well as other animals. These cultures contained 34 O antigen groups that represented 251 serotypes, in which 167 were diphasic. The cultures were tested for serotypes, and then, placed into categories with reptiles having the most serotypes at 99. The number of serotypes isolated from humans had increased to 6 and so had those from food/food products that were now at 4. The chicken and monkey column had one serotype each. Thirteen serotypes were isolated from unknown sources, and the other 81 were from a mixture of sources including man. The researchers noted serotype 26:23-30, first isolated from monkeys, had the most samples of the diphasic strains. This serotype had 88 samples from various sources including humans (31), reptiles (22), monkeys (10), turkeys (1) and food products (1) with 23 unknowns. Also mentioned was serotype 26:29-30 which is believed to be host-adapted,

frequently cultured from sheep and to a lesser extent from humans (Martin et al., 1967). During this time, research had been published identifying Arizona as either *Salmonella* (subgenus III) or as Arizona and *Salmonella* since there was no overall consensus in nomenclature.

Diphasic Arizona discovery in Australia

In Western Australia investigations were performed to look into *Salmonella* prevalence in foodstuffs, discovering a wide variety of serotypes from *Salmonella* subgenera II, III and IV. One theory was that there was an ecological relationship between the species of *Salmonella* and the animals of Australia. A study was done examining reptiles to estimate their importance as carriers. The researchers examined 70 lizards, 40 snakes, 4 tortoises and 2 crocodiles native to the Western region of the country for *Salmonella* and Arizona. Seventeen diphasic Arizona serotypes were identified along with 49 serotypes from *Salmonella* subgenera II and IV. While Arizona was not found in the tortoises or crocodiles, the bacteria was isolated in 4 lizards and 15 snakes. Only two of the diphasic Arizona serotypes were found in both lizards and snakes. Two serotypes of diphasic Arizona were isolated from six samples of rodent litter used for feeding. In addition, three diphasic Arizona serotypes were found from ten samples of eastern Australia reptilian litter. Arizona was also identified in ticks that had infested the monitor lizards (*Varanus varius*). The researchers felt that the current host-parasite relationship of Arizona and *Salmonella* in reptiles was significant but complicated due to the lack of knowledge (Iveson et al., 1969).

Continual discovery of new serotypes

Research continued into the bacteria Arizona with ongoing discovery of new serotypes. Moreover, animal cultures were now being sent to Animal and Plant Health Inspection Service for serotyping. Eight of those samples were new serotypes belonging to Kauffman and Edwards' Arizona arizonae. Two of those strains were reported as diphasic and were isolated from snakes;

a rubber boa (*Charina bottae bottae*) snake housed in a zoo, and a water snake (*Natrix spiedon*) (Blackburn, Nelson & Karns, 1973).

Diphasic Arizona discovery in zoos

Samples requiring testing for Arizona had been sent to Edwards et al. (1955, 1956 and 1962) from various zoos, but one study examined the frequency of *Salmonella* and Arizona in various turtle populations in zoos. Nine major zoos and zoological gardens from five states in the US were chosen. Samples were collected from 124 turtles and sent off for biochemical testing and serotyping. One serotype of diphasic Arizona and six serotypes of *Salmonella* were isolated. Diphasic Arizona was discovered in three (Blanding's, Barbour's map & Alligator snapping) of the 15 turtles that tested positive. The unique finding of *S*. subgenus IV (*S*. houten) in turtles was rare, and they believed that this might be due to fecal/water contamination. The researchers felt the number of positive samples was low, possibly due to the fact that they were using a mobile and not a permanent laboratory (Jackson Jr. & Jackson, 1971).

Iveson et al. noted in 1969 that the findings of *Salmonella* and Arizona in reptiles were important but complicated because of the lack of knowledge pertaining to the host-parasite relationship. Cambre et al. (1980) began questioning that relationship as the frequency in which *Salmonella* and Arizona were isolated from reptilian necropsy was increasing. Three hundred and seventeen reptiles from the National (Smithsonian) Zoological Park were sampled for these microbes. The results showed that 117 snakes and lizards tested positive for both bacteria, but no turtles or tortoises tested positive for Arizona. Twenty-four *Salmonella* serotypes plus two un-typed strains were detected, whereas 38 Arizona serotypes plus two un-typed strains were discovered. Twenty-nine of 38 Arizona serotypes were diphasic, 19 were found only in the snakes, while 5 were found only in lizards, with 5 serotypes found in both. Water samples from shipments of live amphibians as well as three sets of mice, rats and baby chickens intended as food were also tested, neither bacteria were isolated. During the eight-month study period, 29 new reptiles were admitted to the zoo. Three of the 9 snakes and 5 of the 12 lizards tested positive for *Salmonella* or Arizona, but none of the 8 turtles tested positive for either one. In addition to the new arrivals, 10 reptiles died during the study period and were examined. The

necropsy results from three of the reptiles proved to be interesting. A Green Tree Python (*Chondropython viridis*) and a Rosy Boa (Lichanura trivirgata) tested positive for a certain Arizona strain but at their death they tested positive for different Arizona serotypes. An Arizona serotype was isolated from a Green Tree Monitor (Varanus prasinus), but when the lizard died two months later, it tested negative for the strain.

Is it possible that over time reptiles and bacteria have evolved to have the ideal host-parasite relationship? When examined, the reptiles infected with *Salmonella* and Arizona showed no signs of illness nor were the researchers able to show that these microorganisms were the cause of clinical illness or death even though they were able to culture the microbes from lesions found at necropsy. The data from the pathology findings suggest that *Salmonella* and Arizona tend to be opportunistic pathogens. One of the authors noted that Arizona (salmonellae) findings in the past might not have been missed if selective enrichment media/broth had been used (Cambre et al., 1980).

Diphasic Arizona to *S.* Arizona subgenus IIIb

With the advancement of technology researchers could now examine microorganisms molecularly. Nucleic acid hybridization research by Crosa et al. (1973) revealed that the Arizona shared 70 to 80 % nucleotide sequences with *Salmonella* reference DNA. The researchers found them to be highly interrelated but separable. The data also showed considerable overlapping between *Salmonella* and Arizona similar to *E.* coli and shigella. In addition, when the monophasic and diphasic strains of Arizona were tested, the results showed that they form two separate, non-overlapping populations that based on their antigenic properties can be divided into two subgroups (Crosa et al., 1973). Likewise, Le Minor et al. (1982a) studied 88 strains of *Salmonella*, 21 of those from subgenus III (Arizona), and found that monophasic and diphasic genetic properties, formed two distinct groups. Due to these results, they were separated, keeping monophasic Arizona as subgenus III but expanding Kauffman subgenus to include diphasic Arizona as subgenus IV diarizonae (Le Minor et al., 1982/b). However, to avoid confusion, diarizonae was reclassified from subgenus IV back to subgenus IIIb (Le Minor et al., 1986).

Occurrence and distribution of (Diphasic) Arizona subgroup of *Salmonella* (III)

Le Minor et al. (1982a) extension of Kauffman subgenera classification for the genus *Salmonella* was controversial but grew in acceptance. However, CDC had not yet accepted Le Minor et al. system in the 1986 report summarizing the occurrence and distribution of Arizona (referred to as Arizona subgroup of *Salmonella*). From 1967 to 1976, the CDC received 858 Arizona cultures from various sources. There were 33 somatic groups from 143 serotypes with 84 (8 questionable ones) belong to Arizona subgroup. When compared to the data from the previous study done in 1967, there was a decrease in the number of cultures but an increase in the number of strains isolated from humans (13) alone. Of course, with fewer cultures the number of serotypes found in reptiles also declined from 99 to 23. The other 34 serotypes were from a mixture of the sources including human. Four serotypes were found in turtles, where there had been none and a slight increase in food/food products at five. One of most isolated serotypes was 61:l,v:1,5(7) (26:23-30), which not only had the most cultures for *S.* Arizona subgroup (IIIb) at 184, but for both subgroup III. This serotype had 72 cultures isolated from humans, while the other 112 were found in sheep, snakes, turtles, other reptiles and food/food products. Serotype 61:k:1,5,(7) (26:29-30) is frequently found in sheep and appeared to be more invasive, and seems to have higher mortality rates. This strain had 39 cultures, 27 isolated from humans with the cultures from a mixture of sheep, food/food products and unknown sources. They concluded that certain serotypes of the Arizona subgroup of *Salmonella* seem to have noticeable patterns of human isolation over time and an associated increase to virulence for humans (Weiss et al., 1986).

Even in the US, the number of *S. diarizonae* isolations is quite small compared to *S. enterica* (subgroup I). Researchers found that the number of cultures from *S.* Arizona subgroup (IIIb) isolated in the United Kingdom were rare. In fact, 90 *S.* Arizona subgroup (IIIb) samples were collected from 1966 to 1990 where sixty-six cultures were isolated from humans, 14 from animals and 10 from other sources. Out of forty serotypes, 31(3 questionable) were *S.* Arizona subgroup (IIIb). The two most common serotypes were 61:k:1,5,(7) (26:29-30) and serotype 61:1,v:1,5,(7) (26:23-30). Serotype 61:k:1,5,(7) had been detected in 43 cultures that were

isolated from sheep, humans, animals (dog, pig) and animal feed. Serotype 61:1,v:1,5,(7) had 31 cultures and was the most isolated serotype from humans (13). The other 14 cultures originated from terrapin (small turtles) and the terrapin tank water. *Salmonella* Arizona subgroup (IIIb) is a concern in human infections, especially in children. Serotype 61:c:z35- was isolated from a three year old child and the first recorded human case of *S*. Arizona subgroup (IIIb) in the UK.

Although snakes have been shown to be natural carriers of many *S*. Arizona subgroup (IIIb) strains only 2 serotypes were detected in snakes and 7 serotypes were isolated from the 29 cultures of terrapins (small turtles) including tortoises (Edwards & Boycott, 1955; Cambre et al., 1980; Hall & Rowe, 1992).

Salmonella colonize certain reptile species, but the frequency is uncertain. To evaluate the prevalence of Salmonella in pet snakes, fecal samples were collected from 10 rhinoceros-horned vipers (*Bitis nasicornis*) and 6 eyelash vipers (Bothriechis schlegelii). Follow-up samples were then taken over a 22 month time frame from four snakes. *S. diarizonae* was detected in all ten of the rhinoceros-horned vipers, but only three of the eyelash vipers. A total of 12 serotypes were detected, 10 in the rhinoceros-horned vipers and 2 in the eyelash vipers. Only serotype 48:i:z was isolated from both species. Three of the 4 snakes tested during the follow-up changed serotypes, something also noted by the Cambre et al. (1980) study. The researchers questioned whether *Salmonella* was acquired in utero or by ingestion of infected prey or by contaminated feces of other reptiles. The rodents fed to the snakes were regularly tested for enteric pathogens by the supplier. The rhinoceros-horned vipers are ground dwellers while the eyelash vipers inhibit trees and brushes, which might explain why only 3 tested positive (Schöter et al., 2003).

S. diarizonae **discovery in Germany and Austria**

Geue et al. (2002) further examined the epidemiological nature of *Salmonella* among reptiles in Germany and Austria. Over a ten-month period, 189 fecal samples were collected from turtles, lizards, and snakes representing 48 reptilian species. Out of the 86 samples that tested positive for *Salmonella*, 30 were from subspecies IIIb. *S. diarizonae* was found in the lizards and snakes, but not the turtles. There were 42 serotypes detected with 16 belonging to *S.*

diarizonae. The most common serotype in the lizards was 50:k:z with 5 cultures, this strain was not detected in any of the snakes. Serotype 47:k:z35 was the most isolated strain in the snakes with 6 cultures, but it was not found in any of the lizards. The reptiles belonged to 28 owners and/or breeders that were either homebred or bought from a pet store. The data showed that a high percentage of the positive samples came from reptiles that had been purchased from a pet store

S. diarizonae **today**

Kauffman had biochemically divided *Salmonella* into four subgenera, and the advancement of nucleic acid technology confirmed his work. Le Minor et al. (1982a) research added two more subspecies and divided *S. arizonae* subgenera III into IIIa for *arizonae* (monophasic) and IIIb (diphasic) for *diarizonae*. Based on the *Salmonella* annual summary that the CDC publishes, the data on the number of *S. diarizonae* serotypes and cultures can be calculated. From 1997 through 2005, there were 231 *S. diarizonae* cultures isolated from human sources, and yet, from 2006 to 2016, there were 956 cultures. The total of 1187 cultures represented 99 serotypes. Most common serotype isolated was 61:1,v:1,5(7), which constituted 14% (166) of the cultures. The next largest number of cultures was serotype 50:k:z at 7.8% (92) along with 50:r:z at 7% (82) (CDC, 2007 & 2016). Both of these serotypes have been isolated from reptiles as well as humans (Weiss et al., 1986).

Together with a snapshot of animal sourced cultures from 2007 to 2012, the data received by the CDC from United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), and National Veterinary Services Laboratory (NVSL) showed 525 nonhuman isolated cultures that tested positive for *S. diarizonae*. There was a total of 108 serotypes with the cultures divided into nine categories. Of the 525 cultures, 232 were isolated from reptiles, and 132 were isolated from the other domestic animals/environment category. In fact, 130 of the 132 cultures were from serotype 61:-:1,5,[7] which has been frequently found in sheep (Hall & Rowe, 1991; Alvseike & Skjerve, 2002; Dargatz et al., 2015). The other categories included other birds/wild birds with 49 cultures, bovine with 21 cultures, chickens with 10 cultures, porcine with 9 cultures, equine with 5, cultures and all others with 67 cultures. The

most common subgroup IIIb was serotype 61:-:1,5,[7] that was 29% (150) of the cultures. Serotype 61:1,v:1,5(7), which had the most human cultures, had only 6 cultures with three from reptiles, a culture from equine, other birds/wild birds, and all others. While serotype 61:k:1,5(7) had 55 human isolates, it had only one nonhuman culture (CDC, 2007-2012).

S. diarizonae serotype 61:k:1,5(7)

The most well known *S. diarizonae* is serotype 61:k:1,5(7). The strain was first documented in 1951 when it was isolated from three premature lambs in Wyoming (Ryff & Browne, 1952). Edwards et al. (1959) noted that this bacterium was closely associated with sheep since that is where it frequently found. Bacteria was detected in a case of suppurative epididymo-orchitis in a ram from Spain. They suggested that serotype 61:k:1,5(7) is pathogenic for sheep genitalia due to the isolate of the bacteria from the genitalia tissues, in the absence of other pathogens (Ferreras et al., 2007). In Switzerland, *S. diarizonae* 61:k:1,5(7) was found in the tonsils of slaughtered sheep (43%) and goats (2%). While fecal samples were also tested, Bonke et al. (2012) found that tonsils were a better indicator of prevalence in asymptomatic sheep. In addition, the bacteria have been indicated in chronic proliferative rhinitis in sheep in Spain and Switzerland (Lacasta et al., 2012; Stokar-Regenscheit et al., 2017).

Serotype 61:k:1,5(7) was isolated in ten sheep fetuses that were aborted or stillborn from six ewes in Canada. Results of vaginal swabs collected over a three-month period from two of the six ewes showed the bacteria were present at 30 and 50 days. The serotype 61:k:1,5(7) was still detected in one of the ewes 90 days postpartum. They theorized that the bacteria might be a more common cause of sheep abortion than current literature indicated (Long et al., 1979). In England, serotype 61:k:1,5(7) was first identified in two slaughter houses in South Wales (Harvey et al., 1966). However, the first isolation was from ewes in 1976 that were originally from Scotland. Investigations in Scotland found serotype 61:k:1,5(7) in three different areas, including oats kept in a sheep feeding trough (Hall & Rowe, 1980).

From 1975 to 1981, there were 30 incidences of serotype 61:k:1,5(7), This data indicated a slow but steady increase, but then, the incidence decreased in 1981. This serotype has been isolated in diseased as well as healthy sheep. The researchers suggested that the increase in

exotic “serotypes” maybe associated with contaminated animal feed. They also noted the appearance of serotype 61:-:1,5,[7], which had also been isolated from sheep purchased from Scotland (Sojka et al., 1983). Yet, the rate of *S. diarizonae* serotype 61:k:1,5(7) found in sheep continued to increase in England. In 1990, there were 29 isolates, and then, there was a decrease for three years. From 1994 to 1999, the number of incidences increased from 30 to 84. There were several possible reasons for the increased incidence. In December of 1997, a new standard abortion protocol was introduced requiring *Salmonella* screening of all submitted sheep abortions. The timeframe of increase also coincided with the import of breeding stock from other countries and declining number of purebreds. Furthermore, serotype 61:k:1,5(7) had been detected in the country for several decades, and the increase in infection could be that immunologically naive animal may have been exposed on endemic farms or in livestock markets. The researchers concluded that more studies were needed to examine the epidemiological characteristics of the infections and compare the genetic relationships between the past and current serotype 61:k:1,5(7) from other countries (Davies et al., 2001).

In Norway, the discovery of S. IIIb 61:k:1,5,(7) has occasionally been found in domestic animal sources. From 1997 to 1999, the bacteria was isolated from ileocecal lymph nodes of five cattle herds, though serotype 61:k:1,5,(7) appears to have had low virulence. A study was performed to describe the occurrence in adult sheep and slaughtered lambs as well as identify any major risk factors associated with serotype 61:k:1,5,(7) at the herd level. A total of 1330 fecal samples were collected from 133 herds throughout 5 regions of Norway. The serotype was found in 16 of 133 herds from the northern, central and eastern parts of Norway, but not in the western or southern, parts where 51% of the herds are located. The prevalence varied in the regions infected from about 8% to 45% and was higher in adult sheep (Alvseike & Skjerve, 2002). In addition, the researchers noted that sheep fecal samples sent to the National Institute of Public Health in Norway and National Veterinary Institute in Sweden had detected 61:-:1,5[7] (Alvseike et al., 2004).

A similar prevalence study associated *S. diarizonae* (SASd) in sheep herds was performed in Sweden. *Salmonella* presence in the country is low due to the *Salmonella* control program. Any finding of *Salmonella* in food, feed, and/or animals is notifiable, and actions are taken to eliminate the contamination. Infected herds are put under restrictions and movements are prohibited, or if chronically infected, then these animals are eliminated. Steps are taken to

improve hygiene and the stable environment, and any contaminated areas are cleaned and disinfected. The study examined 262 sheep herds throughout Sweden and found a prevalence of 17.6%. Serotype 61:k:1,5,(7) is endemic to the area as there were positive sheep herds found all over the country. Due to the results of the study, SASd in sheep has been exempt from the *Salmonella* control program (S*ö*r*è*n et al., 2015).

Due to prevalence studies performed in other countries, an investigation was performed to examine the occurrence of *Salmonella* species in sheep in Thuringia, a federal state in Germany. For one year, 270 fecal samples were collected from 90 herds throughout Thuringa. *Salmonella enterica* subspecies *diarizonae* 61:k:1,5(7) (SASd) was detected in 206 samples, and 74 out of the 90 sheep herds were positive for SASd. Prevalence was found to be at 82%, and yet, serotype 61:k:1,5(7) has not been isolated from meat, meat products or cheese derived from sheep. Due to the lack of knowledge on infection, the researchers suggested that further studies on SASd pathogenicity and transmission routes would be valuable (Methner & Moog, 2018).

In contrast, a prevalence study performed in the US isolated a different *S. diarizonae* in sheep herds. From March to June 2011, a total of 3722 fecal samples were collected from 247 sheep operations in 22 states. The study was divided into two groups. One group was composed of 152 sheep operations that collected composite samples (697). The second group was composed of 95 sheep operations that collected both composite (436) and individual animal (2,589) samples. There were 1,003 positive samples, representing eight serotypes, seven of which belonged to *Salmonella* subspecies *enterica* and one belonged to *S. diarizonae*. The most common *S. diarizonae* serotype was 61:-:1,5,[7] with 948 (353 composite and 595 individual samples). There was an overall prevalence of 26.9% with the bacteria detected in 72.1% of the sheep operations (Dargatz et al., 2015).

Caldwell and Ryersons' discovery of *Salmonella* subspecies *arizona* in 1939, was novel in the fact that it was isolated from an infected gila monster. It was previously it was thought that *Salmonella* was only found in warm-blooded animals, and also notably it was a highly pathogenic bacterium that killed most of the infected lab animals. The finding was fundamental in the groundwork performed by Kauffman, Edwards, and their colleagues. This groundwork consisted of a series of biochemical tests that would lead to the discovery of *diarizonae* six years later in 1945. Edwards' findings would result the bacterium becoming reclassified as a new genus, stating it was closely related to *Salmonella* but distinguishable from them by biochemical

methods. Kauffman's tests, on the other hand, produced similar results but designated the bacterium as a subspecies of *Salmonella*. Kauffman's classification would be confirmed by research performed in 1970s and 1980s. Edwards and his team received *diarizonae* cultures from veterinarians, and scientists from all over the world, and through his research, led others to identify the bacterium in their own respective countries. Samples that were collected were wide-ranging in sources, including reptiles, domestic animals, poultry/birds, humans, water, and food. Edwards and his team chose not to give the *diarizonae* serotypes names, but rather, to identify them by antigenic formulas, a practice adopted by the 1966 Enterobacteriaceae Subcommittee to the Nomenclature Committee and applied to all *Salmonella* subspecies except subspecies *enterica* (Carpenter, 1968).

Salmonella diarizonae has 308 serotypes. The most well known serotype is 61:k:1,5(7) also [called sheep associated *S. diarizonae* (SASd)], and it is considered to be host adapted. While prevalent in sheep herds in other countries, serotype 61:k:1,5(7) has been supplanted by 61:-:1,5[7] in the US. *S. diarizonae* is zoonotic and frequently isolated from snakes more than any other reptiles. It is interesting to note though that the number of isolations from human has been increasing since 2006. Whether this is due to a greater distribution of the bacteria in food sources or increased ownership of exotic reptiles is uncertain.

[1]Today, we know that flagellar (H) antigens in certain members of *Salmonella* have a diphasic state or phasic variation. This was brought to light through the research of Andrewes. In his work, he tried to culture a certain *Salmonella* strain as two entities, but he found that it had the ability to change into another. He wasn't sure what events led to this change but he believed that the unspecific forms were so similar that further investigation was needed. He theorized that absorption tests or monospecific (absorbed) sera might be useful in distinguishing them. His research showed that *Salmonella* had two antigens, in which specific characteristics of the group were not evenly distributed, where a member might have attributes that are different from the whole, or where only two well-defined types were present and culture conditions may transform into either type. He called the unique phenomena a "kaleidoscope", variations exhibited by the bacteria (Andrewes, 1922). The description by Andrewes of the diphasic condition was further studied by White. White studied strains of specific and nonspecific "races" for serological comparisons, as there was little data on agglutination and absorptive reciprocity between the phases. He then examined the nonspecific and specific O "races" and the H constituents of the diphasic *Salmonella*, stating that one phase of diphasic *Salmonella* always has traces of the distinctive antigens of the other phase (White, 1926).

[2] In the Kauffman-White scheme, the antigenic structure is given in capital letters. The thermo stable O antigen is written in Roman numerals (XI, XXI, etc.), the specific H antigen in small letters (z_1, z_2, etc.) and the nonspecific H antigen in Arabic numerals (5, 6, etc.) (*Salmonella* subcommittee, 1934).

Salmonella **subspecies (III)** ***diarizonae*** **nomenclature**

The construction of *Salmonella* nomenclature has been long, complex and confusing due to the lack of uniformity. This led to many issues in how to report the bacteria from those in laboratories, by medical professionals, and scientists, and so forth. It was not until 2005 that a system was officially approved a taxonomy that scientists from the late 1800s to the early 2000s played a role in shaping. The classification of subspecies *diarizonae* has in some ways paralleled the same confusion of the genus *Salmonella* itself. While there are many papers written on the taxonomy of *Salmonella* as a whole, there are few, if any, that focus on the subspecies *diarizonae*.

The genus *Salmonella* was suggested by Dr. J. Lignières in 1900. However, the name *Salmonella Lignières 1900*, was the generic name accepted for the microorganism by the members of the subcommittee with Bruce White defining the organism. In addition, the classification of *Salmonella* used by the *Salmonella* subcommittee was based on the Kauffman-White (K-W) Scheme. This scheme is based on antigenic analysis of the somatic, flagellar, and capsular antigens and was first presented to the subcommittee by Kauffman in 1931. The K-W scheme was the continuation of the pioneering work of White, Schütze, Scott, and others (*Salmonella* subcommittee, 1934).

The concept for *Salmonella* having three species began with Borman, Stuart and Wheeler, as they were the first to put into print (Borman et al., 1944; Ewing, 1963). The three species were *S*. Choleraesuis, *S*. Typhosa and *S*. Kauffmannii, which was later changed to *S*. Enteriditis (Ewing, 1963). In the "Classification and Nomenclature of *Enterobacteriaceae*" by Kauffmann and Edwards, they suggested the name for the three species of *Salmonella* as *S*.

choleraesuis, *S. typhi* and *S. enterica* (Kauffman & Edwards, 1952). However, Ewing noted that if *Salmonella* was limited to one species, then *Salmonella enterica* might be conserved for use in connection with it. If the single species concept was adopted, then serotypes could be written as *Salmonella* enterica serotype *typhi* (Ewing, 1963). Arizona was listed as a separate group/genus from *Salmonella* and its type species, Arizona arizonae (Kauffman & Edwards, 1952). Edwards wrote that Arizona and *Salmonella* were closely related but distinguishable by biochemical tests, even though the results of the reactions were very similar (Edwards et al., 1956; Edwards, Fife & Ramsey, 1959). He believed that these bacteria composed a well-defined group and so deserved to be recognized as a distinct entity. Arizona is defined as a Gram negative, motile bacterium that produces hydrogen sulfide, utilizes citrate, and liquefies gelation. In addition, it produces acid and gas from glucose, ferments lactose, is methyl-red positive, is unable to form indole, and is Voges-Proskauer negative. Most serotypes are unable to ferment sucrose, salicin, dulcitol, inositol, or adonitol (Edwards et al., 1956).

With the use of certain biochemical tests for differential diagnosis, such as β-galactosidase, Kauffman split the members of *Salmonella* into three subgenera. Subgenus I was identified as typical and subgenus II as atypical *Salmonella*. The testing also showed that Arizona could be listed as a subgenus (III) of *Salmonella* (Kauffman, 1962). Furthermore, it was possible to merge Arizona into the original K-W scheme. Yet, a special one was used in accordance to the simplified K-W scheme. This scheme, however, did not affect the one already set up by Edwards and others (Kauffman & Rhode, 1962). Later on, Kauffman did incorporate Arizona into the original K-W scheme, and so, for example, diphasic Arizona 30:23:31 would be *Salmonella* 65:1,v:z (Kauffman, 1965). Continuing his work with *Salmonella*, Kauffman found that the genus could be divided into 4 biochemical subgenera (Kauffman, 1965b). When "An Outline of Nomenclature for the Family Enterobacteriaceae" came out in 1963, listed as Tribe II was Salmonelleae and its three genera were *Salmonella Lignières*, Arizona and *Citrobacter.*

Since the introduction of Arizona arizonae, the name had appeared in multiple publications. In 1966 Ewing wrote the request of validation for the species. However, the "Kauffmann and Edwards type species; Arizona arizonae" was rejected due to the repeat of the generic name (1966 International Code of Nomenclature of Bacteria, Rule 25). So, the bacteria, formerly known as Arizona arizonae, became Arizona Hinshawii, in honor of Dr. Hinshaw "who was responsible for much of the pioneer work on the genus" (Ewing, 1966; Ewing, 1969).

Kauffman biochemically defined the four subgenera of *Salmonella*, yet only subgenus III (Arizona) had been named. This changed with Le Minor et al. (1970), they suggested that subgenus I become *S*. Kauffmanii (a name chosen to honor Dr. Kauffman's work on *Salmonella*) originally proposed by Borman, Stuart and Wheeler in 1944. Subgenus II became *S*. salamae from dar-es-salaem as it was the first serotype of this species. Subgenus IV became *S*. houtenae named after Houten, a city in Holland where it was first described.

Salmonellae of the family *Enterobacteriaceae* consisted of three genera; *Salmonella Lignières,* Arizona and *Citrobacter*. The classification was based on biochemical and serological tests. In addition, the advancement in nucleic acid technology aided in the classification by giving scientists the ability to evaluate the genetic relatedness of strains to each other or other bacteria. Strains of *Salmonella*, *Citrobacter* and other members of *Enterobacteriaceae* were compared to *E. coli* for relatedness. The results revealed that *Citrobacter* strains showed about 50% relatedness to *E. coli* while *Salmonella* showed about 45% (Brenner & Falkow, 1971).

Crosa et al. (1973) furthered that study by examining the genetic relationship of the tribe Salmonellae. He analyzed multiple strains of *Salmonella*, Arizona, and *Citrobacter*, along with strains of the other members of *Enterobacteriaceae* and *S*. t*yphimurium*. The results showed that as a group (typical and atypical) *Salmonella* nucleotide sequences was 70 to 80% in common with *S. typhimurium,* in the same fashion, Arizona shared 70 to 80 % of nucleotide sequences with the tribe Salmonellae. However, *Citrobacter* shared between 45 to 50% with the degrees of relatedness decreasing with other members of *Enterobacteriaceae*. In addition, the researchers found that DNA-DNA duplex formation between the three species (*S*. Choleraesuis, *S*. Typhi and *S*. Enteriditis) could not be differentiated. The results of their work suggested that salmonellae could only be one species. However, they agreed with Ewing's proposal on the three species concept, since the experts had never agreed on the nomenclature of *Salmonella* (Ewing, 1972; Crosa et al., 1973).

While the Crosa et al. (1973) furthered these findings based on the Edwards definition of *Salmonella* and Arizona, Stoleru et al. (1976) was based on Kauffman's system that divided the genus *Salmonella* into four subgenera. They observed that the serotypes in Crosa et al. (1973) did not include strains from subgenus IV, and therefore, tested that group's genetic relatedness to other strains of *Salmonella* and *Citrobacter*. Their results agreed with the Crosa et al. (1973) findings, including those for *Citrobacter*. This, in essence, confirmed the Kauffman system of

classification for *Salmonella* and confirmed that *Citrobacter* did not belong in that genus. On the other hand, Stoleru et al. (1976) disagreed with the three species concept due to the high degree of DNA homology between *S*. Typhi, *S*. Choleraesuis, and *S*. Enteritidis (Crosa et al., 1973; Stoleru et al., 1976).

In 1973, the Judicial Commission of International Committee on Systemic Bacteriology (ICSB) met to appoint an Ad Hoc Committee that would review the current valid names of bacteria and retain only those that describe a Type, Neotype or Reference strain available to be published in the 1980 January issue. Among the approved names for *Salmonella* was *S. lignieres 1900*, *S. choleraesuis* and *S. arizona* (Skerman et al., 1980).

More pieces of the *Salmonella* puzzle came into place when Le Minor et al. (1982a) analyzed 20 strains from *Salmonella* subgenera I & IV and 21 strains from subgenera II and III as well as six strains from the Bongor group. These were strains that didn't fit any of Kauffman's four subgenera. The first atypical strain of this group was isolated from a lizard in Chad in 1966, and five other strains that were biochemically similar were discovered and sent to the WHO International *Salmonella* Center.

DNA-DNA hybridization data showed that the strains fell into two groups, one consisting of subgenera I-IV and the other the Bongor group. They also found that monophasic and diphasic Arizona genetic properties formed two distinct groups. This information led to them being separated and expanding *Salmonella* from four subgenera to six. Subgenus I was changed from *S*. Kauffmanii to *S*. Choleraesuis, subgenus II (*S*. Salamae) and III (*S*. Arizonae) stayed the same. Subgenus IV became *S*. Diarizonae, while *S*. Houtenae moved to subgenus V, and *S*. Bongor became subgenus VI. In addition, the results from the tests confirmed *Salmonella* as a single species. Based on the DNA-DNA hybridization results, the Bongor group could be considered another species (Le Minor et al., 1982a/b). These findings led to a majority of laboratories, like the CDC, to switch to the single species concept (Farmer et al., 1984).

In 1985, the Le Minor et al. (1982a) proposal for the *Salmonella* single species *S. choleraesuis* subsp. six names combination were validated by the International Journal of Systemic Bacteriology (International Journal of Systemic Bacteriology, 1985). Le Minor et al. (1986) continued work with *Salmonella* that led to adding Indica as a new subgenus named after the first serotype that was isolated from Vrindaban, India. Furthermore, to clear up any confusion, they returned to Kauffman subgenera. Subgenus III was divided into IIIa (*S.*

arizonae) and IIIb (*S. diarizonae*), *S. houtenae* moved back to subgenus IV, *S. bongori* became subgenus V, and *S.* Indica subgenus VI (Le Minor et al., 1986). The Le Minor et al. (1985) proposal for the combination *S. choleraesuis* subsp. *indica* was validated in 1987 (International Journal of Systemic Bacteriology, 1987).

At the meeting of the International Committee on Systemic Bacteriology Taxonomic Subcommittee on Enterobacteriaceae in 1986, the issue of *S. choleraesuis* being both a type species and serotype name presented to be confusing. Le Minor suggested *Salmonella enterica*, a name that originated with Kauffman & Edwards in 1952. The members of the subcommittee unanimously supported the name and asked for a request of opinion to be written to conserve *S.* enterica over *S. choleraesuis* (Penner, 1988). In 1987, Le Minor and Popoff wrote the request of opinion to recognize *S.* enterica as the type and only species of the genus *Salmonella*. It was noted that the DNA relatedness between the subgroups was consistent within the single species *Salmonella,* and yet, the Bongor group showed to be less related, possibly being a second species. However, they also stated that this was not a proposal for the rejection of the name *S.* choleraesuis, since there were those who did not accept the single species concept (Le Minor and Popoff, 1987).

Interestingly, while working on *S. typhi* and 48 isolates representing subgenus I though VI on gel electrophoresis, Reeves et al. (1989) found several genetic results similar to Le Minor et al. (1982a). The *Salmonella* strains had formed two clusters, one containing the subgroup V and the second containing the other six subgroups. Subgenus *bongori* biochemically, serologically and genetically belongs to the genus *Salmonella*. However, the findings suggest that the subgroup is actually a separate species. The researchers proposed that *S. choleraesuis* subsp. *bongori* be elevated to species *Salmonella* bongori (Reeves et al., 1989). The name *Salmonella bongori* (basonym: *Salmonella choleraesuis* subsp. *bongori*) was validated as the new combination in 1989 (International Journal of Systemic Bacteriology, 1989).

The Le Minor and Popoff request was denied by the Judicial Commission of the ICSB in 1990. They agreed on phylogenetic unity of the species in the genus, but it was rejected for safety concerns. The members did not want *S. typhi* lowered to a serotype (*Salmonella enterica* subsp. *enterica serovar typhi*). No alternative proposal was forthcoming, and so, the request of opinion was reopened. They wanted a request that could solve the problem of using a long and

complicated name for a bacterium with epidemic potential in medical laboratories paperwork (Judicial Commission of the ICSB, 1991).

In 1999, Euzèby wrote the Request for Opinion, stating that the nomenclature should reflect advances in science when it came to taxonomy and not the indifference of a majority of bacteriologists. He requested the rejection of *Salmonella choleraesuis* corrig. as an ambiguous name (confusion factor) and the recognition of the species *Salmonella enterica*, which had been divided into six subspecies. He also requested the conservation of the name *Salmonella typhi* and the emendation of the genus *Salmonella Lignières* with the establishment of neotype species, *Salmonella enterica* (Euzèby, 1999). His request was met with some resistance. Yabuuchi and Ezaki wrote that the original proposal made by Le Minor and Popoff and reaffirmed by Euzèby in 1999 were an infringement of Code 20a of the Bacteriological code. They requested the rejection of '*Salmonella enterica*' and the use of 'neotype species'. In addition, they requested the conservation of *Salmonella choleraesuis* as the type species of the genus Salmonella (Yabuuchi & Ezaki, 2000). Furthermore, they wanted the Judicial Commission to recognize *S. typhi* as a legitimate name, and according to Rule 56b, confirm the name as nomen conservandum. By the same token, they made the exact same request for *S. typhimurium* and *S. enteritidis*. To avoid confusion, they recommended changing the serotype name from Choleraesuis to Hogcholera (Ezaki, Kawamura & Yabuuchi, 2000). However, Popoff and Le Minor had written that these names (*S. typhimurium*, *S. typhi*, *S. enteritidis* & others) were mistakenly considered as species and had been italicized, but in fact, were devoid of taxonomic status (Popoff & Le Minor, 1997).

In July 2002, the members of Judicial Commission agreed to make *S. enterica* the type species of the genus *Salmonella*. They also agreed not to reject the name *S. choleraesuis*, but it would be considered heterotypic synonyms with *S. enterica*. The "old system" would be incorporated into the "new system", and over time, the name *S. choleraesuis* would eventually disappear from literature. The nomenclatural changes would be printed in the Opinion 80. The requests made by Ezaki et al. (2000) were considered inappropriate, and the committee could not agree with the formulated proposals (Judicial Commission, 2005). In the Opinion 80, the members decided that *Salmonella enterica* is the type species for the genus *Salmonella Lignières 1900,* which replaces *S. choleraesuis*. The epithet enterica is conserved over all earlier names that applied to the *Salmonella* species. Pertaining to Ezaki et al. (2000) arguments, the Judicial

Commission stated that the term "neotype species" has no standing in nomenclature. In addition, they did not grant the requests to conserve a number of epithets in the genus *Salmonella.* Due to the importance of the nomenclature and taxonomy, experts in these fields were asked to write a commentary (Judicial Commission, 2005b; Tindall et al., 2005). In 2007, it was proposed that the Kauffmann-White scheme change to the White-Kauffmann-Le Minor scheme, considering Le Minor was responsible for most of the serovars descriptions known at that time (Grimont & Weill, 2007).

To properly organize and characterize *Salmonella* into a unified taxonomy, it took the combination of biochemical, serological, and molecular diagnostics. The final product was over a hundred years in the making and took the research of many scientists from different countries, some of whom were the principal investigators responsible for the work on *S. diarizonae*. For this subspecies' transition from Kauffman's *S*. arizona, Edwards' Arizona arizonae (diphasic), Ewings' Arizona hinshawii (diphasic), to finally the current standard of Le Minor's *S. diarizonae* (subspecies IIIb), a systematic name has been agreed upon to describe this particular bacteria. As a genus, *Salmonella* has over 2600 serotypes with new ones constantly being discovered and classified. This classification is based on the Kauffman-White (Le Minor) scheme that uses antigenic analysis to determine the serotype and the subgroup. Currently *Salmonella* is defined as having two species: *S. bongori* and *S. enterica. Salmonella. enterica* is further divided into six subspecies *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI) (Table 2.1).

Table 2.1 Biochemical reactions for *Salmonella* species (Bergey's Manual of Systematic Bacteriology, 2004)

Biochemical reactions	***Salmonella enterica* subspecies**						***Salmonella bongori***
	enterica	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
Lactose	-	-	- (75%)	+ (75%)	-	d	-
Gelatinase	-	+	+	+	+	+	-
Culture with KCN	-	-	-	-	+	-	+
ONPG[1] (2 h)	-	-	+	+	-	d	+
Dulcitol	+	+	-	-	-	d	+
Salicin	-	-	-	-	+	-	-
Malonate	-	+	+	+	-	-	-
γ-Glutamyltransferase	+*	+	-	+	+	+	+
Mucate	+	+	+	- (70%)	-	+	+
L(+)-tartrate**	+	-	-	-	-	-	-
b-Glucuronidase	d	d	-	+	-	d	-
Sorbitol	+	+	+	+	+	-	+
Galacturonate	-	+	-	+	+	+	+

[1] *o*-nitrophenyl-β-D-galactopyranoside

* Typhimurium d, Dublin -
** d-tartrate
+ Positive for 90% or more
- Negative for 90% or more
d Varied results by different strains (positive for 11-89%)

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Chapter 3 - Detection, Isolation, and Antimicrobial Susceptibility Testing of *Salmonella enterica* from Wheat Grain Samples

Abstract

Dry breakfast cereals, breads, pasta, dough, dry mixes for cakes, cookies, batters and coatings contain the cereal grain, wheat. As a raw agricultural product, several studies have shown that wheat grains can be potentially contaminated with foodborne pathogens, such as *Escherichia coli, Salmonella, Clostridium, Bacillus,* etc., as well as nonpathogenic organisms. *Salmonella* is of particular interest as a foodborne pathogen because of the involvement in numerous local and national outbreaks. Although the awareness of *Salmonella* detection in meat, fruits, vegetables and ready to eat foods is well known, our knowledge of the prevalence of *Salmonella* in cereal grains, such as wheat is limited. The purpose of this study was to detect and isolate *Salmonella* from wheat grains that were harvested, transported and stored in different regions of the country. A total of 1,016 wheat grain samples were transported to the laboratory and stored at -80°C until analyzed. Over a thirteen-week period, 625 samples were randomly selected, thawed at 4°C, and tested. Three methods were used for detection and isolation of *Salmonella*. Method A consisted of 50 g of wheat grains suspended in 450 ml of modified buffered peptone water with pyruvate (mBPWp) and incubated at 37°C for 30 minutes. An aliquot of 10 ml of sample was pipetted into 90 ml of mBPWp with novobiocin (22 μg/ml) and incubated for a total of 24 hours. Ten milliliters of the suspension was added to 90 ml of Rappaport Vassiliadis (RV) broth and incubated for 24 hours at 42°C. Method B was a modified version of Method A in which the sample size was decreased to 25 g of wheat grains suspended in 225 ml of mBPWp. The suspension was incubated at 37°C for 30 minutes and was blended in a stomacher before incubation. Method C consisted of the wheat grains incubated in RV broth with novobiocin (22 μg/ml) for 48 hours. In all three methods, DNA was extracted from the RV broth and subjected to quantitative real-time PCR (qPCR) for the detection of *invA* and *pagC* genes. Samples positive for both genes were streaked onto Hektoen-Enteric (HE) agar and

incubated at 37°C overnight. Presumptive *Salmonella* colonies were tested for agglutination with *Salmonella* O antiserum Poly A- I & Vi and retested by qPCR for both genes to confirm the species. *Salmonella* confirmed isolates were submitted to the National Veterinary Services Laboratory (NVSL) in Ames, IA for serotyping. The isolates were tested for antimicrobial susceptibility with National Antimicrobial Resistance Monitoring Systems (NARMS) Sensititre ™ CMV3AGNF Gram negative panel. None of the samples were qPCR positive for *invA* and *pagC* genes by method A, however methods B and C identified *Salmonella enterica* positive samples. Overall, eight samples with a prevalence of 1.3% (8/625) were positive by qPCR for *Salmonella* and culture methods. Six samples were positive for *Salmonella* by method B, five samples were positive by method C, and three of the eight samples were identified as positive by both methods. Out of the eight isolates identified, five belonged to subsp. *enterica* and three belonged to subsp. *diarizonae*. The isolates of subsp. *enterica* belonged to serotypes Anatum, Hartford, Infantis, Norwich and Oranienburg. The three *diarizonae* were identified as serotype 61:1,v:1,5(7). Antimicrobial susceptibility testing revealed that five of the eight strains were pan-susceptible; however, *S*. Infantis and one strain of *S. diarizonae* showed resistance to cefoxitin. Two *S. diarizonae* strains were resistant to tetracycline and amoxicillin-clavulanic acid. These results showed that harvested wheat grains carry *Salmonella* and support other studies showing similar results. Further investigation is needed to determine the source of contamination and pathogenic potential of the isolated strains.

Introduction

The *Salmonella* species are a major foodborne pathogen, and according to the Centers for Disease Control (CDC) cause an estimated 1.2 million cases of salmonellosis each year with a million of these cases resulting from exposure to food sources (CDC, 2018). Moreover, the United States Department of Agriculture (USDA) estimates that $3.7 billion dollars is lost to foodborne illnesses caused by *Salmonella* annually (United States Department of Agriculture, 2017).

The presence of *Salmonella* in cereal grains, such as whole wheat, has been an ongoing problem as these grains are some of the basic ingredients in many food products. *Salmonella* has

the ability to survive in dry (low moisture) foods. The mechanisms employed by the bacteria that allow it to survive in these environments are not clearly understood (Podolak et al., 2010; Finn et al., 2013). Furthermore, studies have shown that depending on the bacterial strain, depth level, temperature, moisture and nature of the environment (water/soil), *Salmonella* can survive for vast periods of time, ranging from several days to years (Davies and Wray, 1996; Himathongkham et al., 1999; Wang et al., 2018).

The presence of *Salmonella* in feeds for livestock and poultry is well documented (Erwin, 1955; Boyer et al., 1958; Gray et al., 1958; Newell et al., 1959; Glickman et al., 1981; Jones et al., 1982; Molla et al., 2010). Cereal grains (such as wheat) used in these feeds may become contaminated with *Salmonella* during harvest, transport, or storage, especially if the feed comes in contact with wild and domestic animals including fowl (Erwin, 1955; Beuchat & Ryu,1997; Daniels et al., 2003). In addition, detection of the bacteria in the contaminated grain may be challenging due to uneven distribution of rodent and wildlife feces in the feed, which may result in false negative results (Binter et al., 2011; Davies & Wales, 2013). Insects may also play a role in cross-contamination (Podolak et al., 2010). A study by Crumrine et al. (1971) demonstrated how several species of common grain insects transferred *Salmonella* Montevideo from contaminated wheat to clean wheat grains. Adult muscoid flies have also been shown to be carriers of several strains of *Salmonella* (Mian et al., 2002).

The bacterial flora of cereal grains includes *Salmonella*, *Escherichia coli*, *Bacillus cereus* as well as other microorganisms, though there are few reports on the microbiological content of milled cereal grains in the US (Richter et al., 1993; Sperber, 2006; Bullerman & Bianchini, 2011; Forghani et al., 2019; Myoda et al., 2019). Therefore, the purpose of this study was to detect and isolate *Salmonella* from wheat grain that had been harvested, stored and transported from different locations in the US.

Materials and Methods

Samples

Between August and September 2017, Kansas State University College of Veterinary Medicine Pre-Harvest Food Safety laboratory received 1016 bags of wheat grain samples that were harvested and transported from 21 states in the US. The wheat grain samples were mixed well, and approximately 0.5 kg of sample was placed into Whirl-Pak bags (Nasco, Fort Atkinson, WI), assigned accession numbers, and stored at -80ºC until analyzed. Two days before sample processing, samples were removed from the -80ºC freezer and placed in a refrigerator to thaw. The day before processing, samples were removed from the refrigerator and 50 g of each wheat grain sample were weighed, labeled and placed into an individual Whirl-Pak filter bag (Nasco, Fort Atkinson, WI). The weighed samples were kept at room temperature overnight During the course of the study, the samples were subjected to two methods of pre-enrichment/enrichment and one method of enrichment only for detection and isolation of *Salmonella*.

Novobiocin stock solution

A stock solution containing 220 mg of Novobiocin in 10 ml of sterile distilled water was prepared (22 μg/ml). An aliquot of the stock solution was then added to the mBPWp or RV broth to obtain a final concentration of 22 μg/ml.

Method A: Pre-Enrichment in modified buffered peptone water with pyruvate

Fifty grams of wheat grain were placed into Whirl-Pak filter bags and suspended in 450 ml modified Buffered Peptone Water with Pyruvate (mBPWp; Neogen Corp, Lansing, MI). The filter bag samples were incubated at 37ºC for 30 minutes and then mixed by hand. A 10 ml aliquot of this sample was pipetted into 90 ml of mBPWp in T-75 flasks (TTP, Trasadingen, Switzerland) and incubated at 37ºC. After 5 hours, 100 μl of novobiocin (22 μg/ml) (Sigma, St Louis, MO) stock solution was pipetted into each T-75 flask (containing the 100 ml of diluted sample) and incubated at 37ºC for additional 19 hours. On the next day, 10 ml of sample was

transferred into T-75 flasks containing 90 ml of Rappaport-Vassiliadis R10 broth (RV) (BD, Sparks, MD) and incubated at 42ºC for 24 hours.

Method B: Pre-Enrichment in modified buffered peptone water with pyruvate, revised

Method A was modified to include a wheat sample size which was reduced from 50 g to 25 g and suspended in 225 ml of mBPWp to bring total volume to 250 ml. After 30 minutes of incubation at 37ºC, the wheat grain suspensions were homogenized in a laboratory blender stomacher Seward 400 (Seward, UK) and Bagmix 400 (Interscience, France) for 60 seconds. The wheat grain samples in the filter bags were incubated at 37ºC for 5 hours after which 250 µl of novobiocin (22 µg/ml) stock solution was added. The filter bags with wheat grain samples were returned to the 37ºC incubator for an additional 19 hours. On the next day, an aliquot of 10 ml was pipetted into T-75 flasks containing 90 ml RV broth and incubated at 42ºC for 24 hours.

Method C: Enrichment in Rappaport-Vassiliadis

Method C consisted of 25 g of wheat grain samples suspended in 225 ml of RV broth. After 30 minutes of incubation at 42ºC, 250 µl of novobiocin (22µg/ml) stock solution was added and homogenized in a laboratory blender stomacher for 60 seconds. The filter bag was then incubated at 42ºC for 48 hours.

Detection of *Salmonella*

To detect the presence of the *Salmonella*, 1 ml of enrichment RV selective broth was boiled for 10 minutes and centrifuged (Labnet Spectrafuge 24D, Melville, NY) at 9,400 x g for 5 minutes. The supernatant was subjected to Geneclean Turbo Kit (MP Biomedical, Solon, OH) and analyzed by quantitative real-time PCR (qPCR) using *inv*A (Rahn et al., 1992; Chen et al., 1997; Fach et al., 1999; Bai et al., 2018) and *pag*C (Nolan et al., 1995; Skyberg et al., 2006;

Mezal et al., 2013) genes. The primers and probes used to detect *invA* and *pagC* genes were obtained from Integrated DNA Technologies, Coralville, IA. Samples positive for both genes were streaked onto Hektoen-Enteric (HE) agar and incubated at 37°C overnight. A single presumptive *Salmonella* colony was then streaked onto Sheep Blood Agar (SBA) and incubated overnight at 37°C. *Salmonella* O antiserum Poly A- I & Vi was used for agglutination on a suspected colony which was further tested by qPCR for *invA* and *pagC* genes to confirm the species. The isolates positive for agglutination and *invA* and *pagC* genes were then stored in CryoCare beads (Key Scientific Products, Stamford, TX) at -80°C degrees.

Salmonella serotyping

The confirmed *Salmonella* isolates were sent to the National Veterinary Services Laboratories (NVSL) in Ames, Iowa for serotyping.

Antimicrobial susceptibility testing

The *Salmonella* strains were tested for antimicrobial susceptibility using the National Antimicrobial Resistance Monitoring Systems (NARMS) Sensititre CMV3AGNF Gram negative panel (Thermo Scientific, West Sussex, UK). Antimicrobial susceptibility was determined by the minimal inhibitory concentration (MIC) in the micro-broth dilution method as outlined in the Clinical and Laboratory Standard Institute (CLSI) guidelines. The Gram negative 96-well plate contained 14 different antibiotics and *E. coli* ATCC 25922 strain was used for quality control.

Detection of _Salmonella_ in spiked wheat grain samples

Three strains, *S*. Typhimurium ATCC 700408 (American Tissue Culture Collection, Manassas, VA), *S*. Oranienberg (Strain 362, KState collection) and *S*. Newport (Strain F10, KState collection) were used to inoculate wheat grain samples, confirmed to be negative for

Salmonella, to compare methods A, B, and C for detection of *Salmonella*. Frozen stocks were streaked onto SBA plates and incubated overnight at 37°C. A colony for each strain was inoculated into 10 ml of Luria-Bertani (LB) broth and incubated at 37°C for 16 hours. An aliquot of 100 µl was pipetted into 10 ml of LB and incubated at 37°C until the turbidity reached a 0.4 absorbance at 600 nm measured in a spectrophotometer (Milton Roy Thermo Fisher, Chicago, IL). The culture was then serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) with 9 ml mBPWp. Three dilutions (10^{-5}, 10^{-6}, and 10^{-7}) were chosen for *S.* Oranienberg and *S.* Newport strains and four dilutions (10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}) were chosen for *S.* Typhimurium for inoculation into the wheat grain samples. Each dilution was pipetted (2.5 ml) into 10 replicates of 25 g of wheat grain samples (10 replicates for each dilution and serotype) and then subjected pre-enrichment/enrichment and detection described for methods A, B, and C. Wheat grain samples without inoculum were used as negative controls for each method. An aliquot of 100 µl of 10^{-4}, 10^{-5} and 10^{-6} dilutions of each strain was spread plated onto SBA and incubated overnight at 37°C to determine the bacterial concentration (CFU/ml).

Results

Over the course of 13 weeks, 625 of the 1,016 wheat grain samples were tested using method A, in which no *Salmonella* was detected. The same 625 wheat grain samples selected for method A were then reexamined using methods B and C. Using these methods, a total of eight samples tested positive for *Salmonella* with a prevalence of 1.3% (8/625). Method B detected 5 of the 8 positive samples, while method C detected 6 out of 8 positive samples. Three of the 8 positive samples were detected by both methods. These qPCR positive samples were cultured on HE agar for isolation. The presumptive *Salmonella* colonies were tested for agglutination and reconfirmed as *Salmonella* by qPCR. The results from NVSL for serotyping revealed that 5 of the 8 isolates belonged to subspecies *S. enterica* (subsp. I) and the other 3 isolates belonged to subspecies to *S. diarizonae* (subsp. IIIb). The 5 *S. enterica* strains were identified as serotypes Anatum, Hartford, Infantis, Norwich and Oranienberg and the other 3 were identified as *S. diarizonae* serotype 61:1,v:1,5(7). Three of the 5 *Salmonella enterica* isolates originated from

Kansas, one from South Dakota and the other was unknown. The three *S. diarizonae* isolates originated from Indiana and two different locations in Texas (Tables 3.1 & 3.2).

Antimicrobial Susceptibility of the Isolates

Of the 8 strains tested for antimicrobial susceptibility, 5 strains were negative for resistance to the 14 antibiotics. *Salmonella* Infantis and one strain of *S. diarizonae* were resistant to cefoxitin. In addition, one *S. diarizonae* strain was intermediate for amoxicillin/clavulanic (2:1 ratio), and a different *S. diarizonae* strain was intermediate for tetracycline (Table 3.3).

Analyses of Spiked Wheat Grain Samples

Three *Salmonella* serotypes *S.* Typhimurium, *S.* Oranienberg and *S.* Newport were inoculated into wheat grain samples to compare methods A, B, and C. The concentrations of the inocula before inoculation into the wheat grain samples for *S.* Typhimurium, *S.* Oranienberg and *S.* Newport serotypes were 4.23 x 10^8 CFU/ml and 3.93 x 10^8 CFU/ml (two separate runs), 2.44 x 10^8 CFU/ml, and for 2.25 x 10^8 CFU/ml, respectively. Tables 3.4-3.7 and Figures 3.1-3.11 show the final concentrations of each serotype. All wheat grain samples spiked with three concentrations (10^{-5}, 10^{-6} & 10^{-7}) except those spiked with *S.* Typhimurium which had four concentrations (10^{-5}, 10^{-6}, 10^{-7} & 10^{-8}) of the serotypes were positive for *invA* and *pagC* genes for all three methods of isolation and detection (A, B, and C).

Discussion

Cereal grains and the products produced from them are an important food resource for both humans and animals. Wheat, a cereal grain, is widely used as an ingredient in low moisture foods, such as doughs, pastas, dry cereals, dry cake and batter mixes (Sperber, 2006; Bullerman

and Bianchini, 2009). Several studies have shown that wheat grains can be contaminated with foodborne pathogens, such as *Salmonella* (Richter et al., 1993; Sperber, 2006; Bullerman and Bianchini, 2009; Davies & Wales, 2013; Myoda et al., 2019). However, our knowledge of the prevalence of *Salmonella* in wheat grains is limited but its potential importance in human health has been emphasized recently with the detection of *Salmonella* in flour. Most recently in 2019, General Mills (Gold Medal) and Hometown Food Company (Pillsbury) voluntarily recalled their flours due to the detection of *Salmonella* in five pound unbleached flour bags (US Food and Drug Administration Safety Recalls, 2019a/b).

In this study, the Pre-Harvest Food Safety Laboratory at Kansas State University examined 625 of the 1,016 wheat grain samples of unknown wheat type. The wheat grains samples originated from 19 of the 21 states included in this experiment. Analysis of the samples showed that the prevalence of *Salmonella* in the wheat samples was low (1.3%), similar to results found by Richter et al. (1993) at 1.3%, Sperber (2006) at 0.14%, and Myoda et al. (2019) at 1.23%. However, in those studies the number of wheat grains tested consisted of several thousand samples.

The detection of *S. diarizonae* serotype 61:1,v:1,5(7) in the wheat grain samples was an unexpected finding, since the occurrence of *S. diarizonae* (subspecies IIIb) infections are rare, affecting less than a hundred people annually (CDC, 2007 & 2016). The majority of *S. diarizonae* serotypes have been isolated from reptiles, primarily snakes, but, there are a few *S. diarizonae* serotypes that have been detected in warm-blooded animals, such as serotype 61:1,v:1,5(7). This serotype was first isolated from primates and has been found to be the most common *S. diarizonae* serotype isolated from humans as well as sheep, but it can be found to a lesser extent in reptiles, food products, and poultry (Edward et al., 1959; Martin et al., 1967; Weiss et al., 1986).

In preparation of the first method (A), mBPWp was used instead of buffered peptone water (BPW) to help in the resuscitation of the bacteria that might be stressed or injured due to either heat or freezing temperatures, as adding pyruvate has been shown to increase recovery of *Salmonella* (Martin et al., 1976; Rayman et al., 1978). The antibiotic novobiocin was added to the pre-enrichment broth to inhibit the growth of certain competitive bacteria, such as *E. coli* and to enhance the recovery of *Salmonella*. Previous research has found that the number of *Salmonella* isolates detected in the pre-enrichment broth increases with the addition novobiocin

(Jensen et al., 2003). For 13 consecutive weeks, 50 wheat grain samples were tested with the exception of the last week where only 25 samples were analyzed. However, in using this method (A), no *Salmonella* was detected in the samples. Given these results, two methods were then developed to address some of the possible inadequacies of method A.

One of the potential insufficiencies associated with method A was the effectiveness of homogenization of the wheat grain once the filter bags were filled with the pre-enrichment broth. Instrumentation meant to homogenize within the filter bags did not work properly, and so, this required manual manipulation to release microbes from the wheat grain into the mBPWp. Another possible insufficiency of the method may have been the amount of time used for the initial incubation step (30 min at 37^{o}C), and then, removal of 10 ml from the mBPWp filter bags. This initial 30 min incubation time may not have been long enough for the bacteria to release from the wheat grains. The fact that no *Salmonella* was detected using method A suggests that perhaps a longer incubation time with the wheat grain may have been required. Therefore, the method was redesigned and two additional methods (B & C) were introduced. Method B was designed to address the issues of mechanical processing and incubation time, while method C was developed to see if the mBPWp pre-enrichment step was necessary.

The redesigned methods proved to be effective as *Salmonella* were detected in eight wheat samples analyzed using methods B and C. Method B, consisting of the pre-enrichment and selective enrichment broths, detected 5 of the 8 isolates, while method C, consisting of only the selective enrichment broth, detected 6 of the 8 isolates. Three strains (*S*. Hartford, *S. diarizonae*, sample no. 200, and *S. diarizonae,* sample no. 539) were detected with both methods B and C. While method A proved to be ineffective in detecting *Salmonella*, methods B and C led to the identification of bacteria, but differed somewhat in the type of serotypes that were detected. One possible reason for this is while the wheat grain samples were homogenized, the distribution of the contamination may still have been uneven, suggesting that one sampling of wheat grain might contain *Salmonella,* while the second sampling from the same bag might not (Binter et al., 2011; Davies & Wales, 2013).

In the case of the wheat grains, the use of the RV broth (method C) was as effective as mBPWp (method B) in the recovery of *Salmonella.* While the use of a pre-enrichment/enrichment media is recommended, the performance by method C demonstrated that

the mBPWp pre-enrichment step was not necessary. The advantage of eliminating this step translates to the reduction of preparation time as well as cost for materials.

The isolation of *Salmonella* from the wheat grain samples begs the question as to where the cross-contamination might have taken place. Previous studies have shown that contamination of wheat grains can come from air, soil, dust, water, insects, and fecal contamination from birds, rodents, domestic animals and wildlife (Crumrine et al., 1971; Davies & Wrays, 1996; Beuchat & Ryu, 1997; Daniels et al., 2003; Laca et al., 2006; Davies & Wales, 2013). Other factors that can lead to bacterial contamination may occur during harvest with farm equipment, during postharvest handling, processing or shipping (Beuchat & Ryu, 1997; Davies & Wales, 2013). For instance, the study by Davies and Wales (2013) isolated *Salmonella* from buildings that served as temporary grain stores, but also housed livestock at other times of the year. Bacteria were also detected in permanent grain storage areas (like silos) that were accessible to wildlife, auger systems, and farm equipment, such as combine harvesters, balers and grain trailers. For this study, the wheat grain samples that were analyzed were identified by either city or region, but all other information, such as storage conditions, was unknown. Therefore, it was not possible to ascertain where the source(s) of contamination may have originated.

To test the effectiveness of the three methods, strains of *S*. Typhimurium, *S*. Oranienburg and *S*. Newport were used to spike wheat grains. All three methods detected the lowest concentrations that were to spike the wheat grain samples, which was 4.23 CFU/g and 0.393 CFU/g (two replicates) for *S*. Typhimurium, 2.44 CFU/g for *S*. Oranienburg, and 2.25 for *S*. Newport CFU/g. In the study by Myoda et al. (2019), found the levels of detection for *Salmonella* using most probable number (MPN) was 0.110 ± 0.448 MPN/g in their evaluation of a baseline level for the contamination of raw wheat grains.

In conclusion, the presence of *Salmonella* in wheat grains, though minimal in this study, poses a serious risk to human foodstuffs and animal feeds. The ability of *Salmonella* to survive in harsh conditions in nature and for varied periods of time in low moisture foods makes controlling the bacteria difficult. Although cereals grains are a raw agricultural commodity, milling of these grains has little effect on the microbiology of the flour (Richter et al., 1993). Therefore, *Salmonella* prevention should involve controlling the bacterial contamination before it enters any type of post-harvest facility. In addition, the control measures would need to adjust

to the constant challenge of controlling *Salmonella*, since this bacteria is quite persistent and adaptable to different environments.

Table 3.1 Detection of *Salmonella* in wheat grain samples by real-time PCR and culture methods

Wheat grain Samples			Method B[a]						Method C[b]					
			Real-time PCR[c]		Presumptive colony on Hektoen-Enteric agar	Pure isolate			Real-time PCR[c]		Presumptive colony on Hektoen-Enteric agar	*Pure isolate*		
State	No. of samples	Number of samples positive	*invA*	*pagC*		Agglutination with polyvalent serum	Real-time PCR[c]		*invA*	*pagC*		Agglutination with polyvalent serum	Real-time PCR[c]	
							invA	*pagC*					*invA*	*pagC*
Alabama	5	0												
Arkansas	13	0												
Colorado	22	0												
Illinois	1	0												
Indiana	13	1	32.61	31.26	Yes	No	17.58	17.37	24.69	23.53	Yes			
Kansas	186	3							22.21	18.4	Yes	Yes	18.46	16.74
									25.62	23.5	Yes	Yes	16.99	16.56
									29.72	26.95	Yes	Yes	17.33	15.87
Maryland	19	0												

Minnesota	24	0												
Mississippi	1	0												
Missouri	36	0												
Montana	30	0												
Nebraska	30	0												
North Carolina	5	0												
North Dakota	55	0												
Ohio	1	0												
Oklahoma	47	0												
South Dakota	49	1	27.65	25.66	Yes	Yes	16.51	15.63						
Texas	34	2	32.02	29.85	Yes	No	15.36	15.41	27.16	25.05	Yes			
			31.86	31.12	Yes	No	18.43	18.12						
Virginia	4	0												
Unknown	50	1	33.09	31.46	Yes	Yes			24.91	24.17	Yes		17.7	17.18

Total	625	8												

[a] Samples were enriched in modified buffered peptone water for 24 hrs and in Rapport-Vassiliadis broth for another 24 hrs
[b] Samples were enriched in Rapport-Vassiliadis broth for 48 hrs.
[c] Cycle threshold (CT) values

Table 3.2 Serotyping of *Salmonella* isolates from wheat grains

Wheat grain sample number	Method B[a]	Method C[b]	Serotype
62	+	-	61:1,v:1,5(7)
200	+	+	61:1,v:1,5(7)
345	+	+	S. Hartford
375	-	+	S. Oranienburg
539	+	+	61:1,v:1,5(7)
595	+	-	S. Norwich
626	-	+	S. Anatum
737	-	+	S. Infantis
Total 8	5	6	

[a] Samples were enriched in modified buffered peptone water for 24 hrs and in Rapport-Vassiliadis broth for another 24 hrs

[b] Samples were enriched in Rapport-Vassiliadis broth for 48 hrs.

Table 3.3 Antimicrobial susceptibility patterns of *Salmonella* isolates from wheat grain samples

Antimicrobial agent	MIC Breakpoint (μg/ml)			*Salmonella enterica* serotypes							
	S	I	R	Diarizonae (62)	Diarizonae (200)	Diarizonae (539)	Infantis	Anatum	Oranienburg	Hartford	Norwich
Amoxicillin/ Clavulanic acid 2:1 ratio	≤ 8/4	16/8	≥ 32/16	16/8 (I)							
Ampicillin	≤ 8	16	≥ 32								
Azithromycin	N/A	N/A	N/A								
Cefoxitin	≤ 8	16	≥ 32	32 (R)			32 (R)				
Ceftiofur	≤ 2	4	≥ 8								
Ceftriaxone	≤ 1	2	≥ 4								
Chlormaphenicol	≤ 8	16	≥ 32								
Ciprofloxicin	≤ 1	2	≥ 4								
Gentamicin	≥ 4	8	≥ 16								
Nalidixic Acid	≤ 16	N/A	≥ 32								
Streptomycin	≤ 32	N/A	≥ 64								
Sulfisoxazole	≤ 256	N/A	≥ 512								
Tetracycline	≤ 4	8	≥ 16			8 (I)					

Trimethoprim/ Sulfamethoxazole	**≤ 2/38**	**N/A**	**≥ 4/76**								

S- Susceptible; I- Intermediate; R- Resistant
Breakpoints were adopted from Clinical and Laboratory Standard Institute (CLSI) guidelines (M100, 27th ed.), except for streptomycin, which has no CLSI breakpoint

Table 3.4 Detection of *Salmonella,* based on real-time PCR assays for *invA* and *pagC* genes in wheat grain samples spiked with *Salmonella* Typhimurium

Wheat grain samples	Inoculum concentration, 423 CFU/g of wheat grain						Inoculum concentration, 42.3 CFU/g of wheat grain						Inoculum concentration, 4.23 CFU/g of wheat grain					
	Method A		Method B		Method C		Method A		Method B		Method C		Method A		Method B		Method C	
	invA	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Samples that have threshold cycle (ct) value <38 are positive for *Salmonella*. Samples having a ct value of >38 are negative CFU-colony forming unit
Method A: wheat grain suspended in modified Buffered Peptone Water with Pyruvate (mBPW) at 37 degrees for 30 minutes, again in mBPWp for 24 hours and transferred to Rappaport-Vassiliadis (RV) at 42 degrees for 24 hours
Method B: Wheat grain in mBPWp at 37 degrees for 24 hours and transferred to RV at 42 degrees for 24 hours
Method C: Wheat grain in RV at 42 degrees for 48 hours

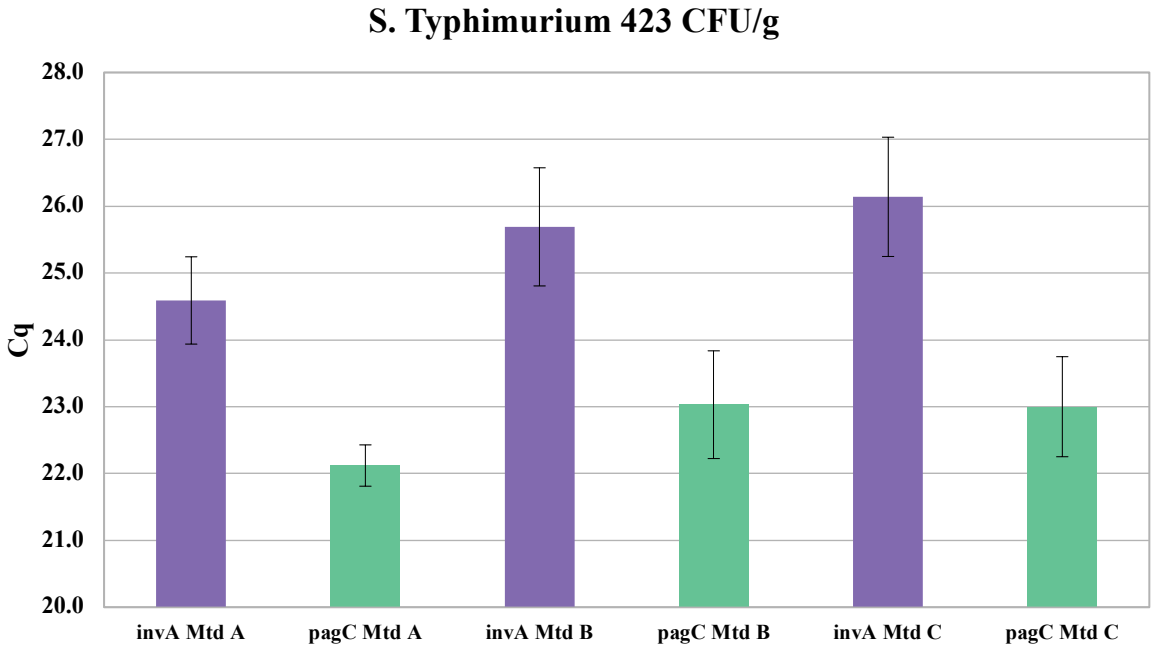

Figure 3.1 Cq values for S. Typhimurium using Methods A-C at 423 CFU/g.

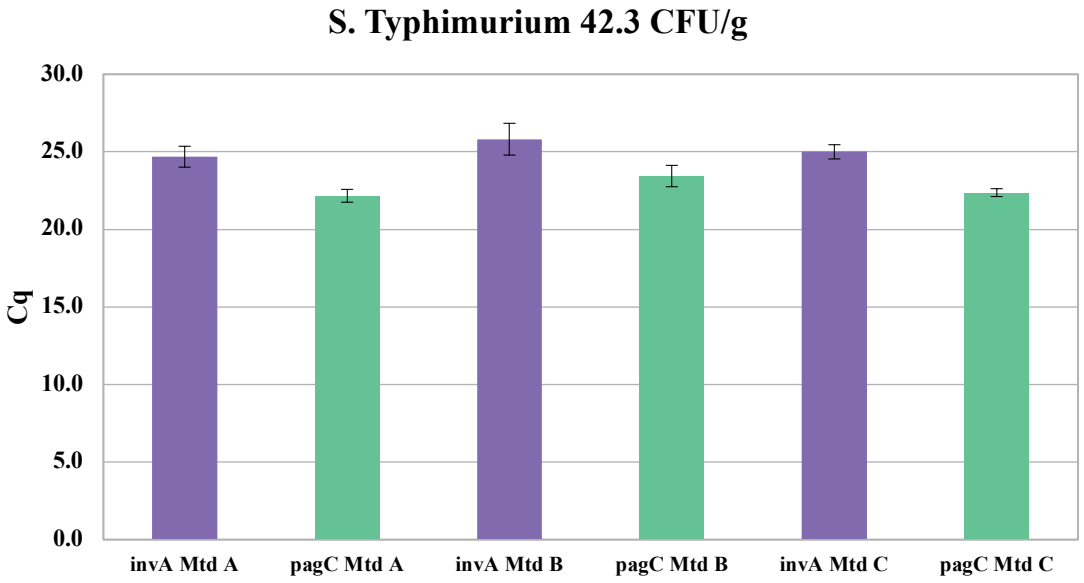

Figure 3.2 Cq values for S. Typhimurium using Methods A-C at 42.3 CFU/g.

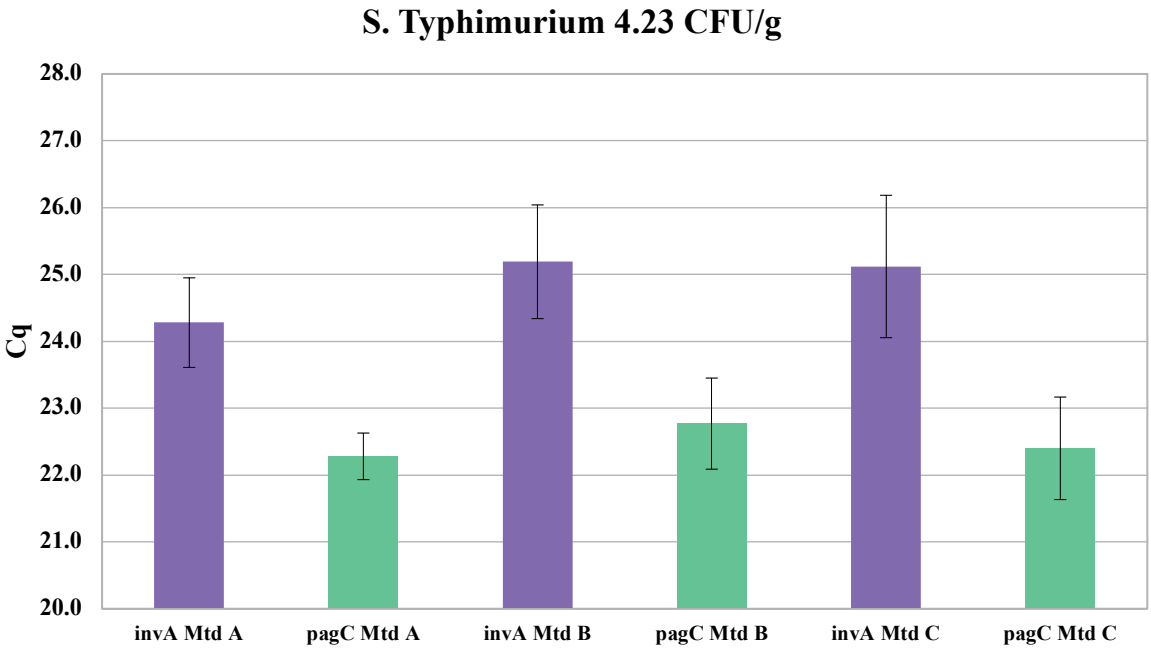

Figure 3.3 Cq values for S. Typhimurium using Methods A-C at 4.23 CFU/g.

Table 3.5 Detection of *Salmonella,* based on real-time PCR assays for *invA* and *pagC* genes in wheat grain samples spiked with *Salmonella* Typhimurium

Wheat grain samples	Inoculum concentration, 3.93 CFU/g of wheat grain						Inoculum concentration, 0.393 CFU/g of wheat grain					
	Method A		Method B		Method C		Method A		Method B		Method C	
	invA	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*
1	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+

Samples that have threshold cycle (ct) value <38 are positive for *Salmonella*. Samples having a ct value of >38 are negative
CFU-colony forming unit
Method A: wheat grain suspended in modified Buffered Peptone Water with Pyruvate (mBPW) at 37 degrees for 30 minutes, again in mBPWp for 24 hours and transferred to Rappaport-Vassiliadis (RV) at 42 degrees for 24 hours
Method B: Wheat grain in mBPWp at 37 degrees for 24 hours and transferred to RV at 42 degrees for 24 hours
Method C: Wheat grain in RV at 42 degrees for 48 hours

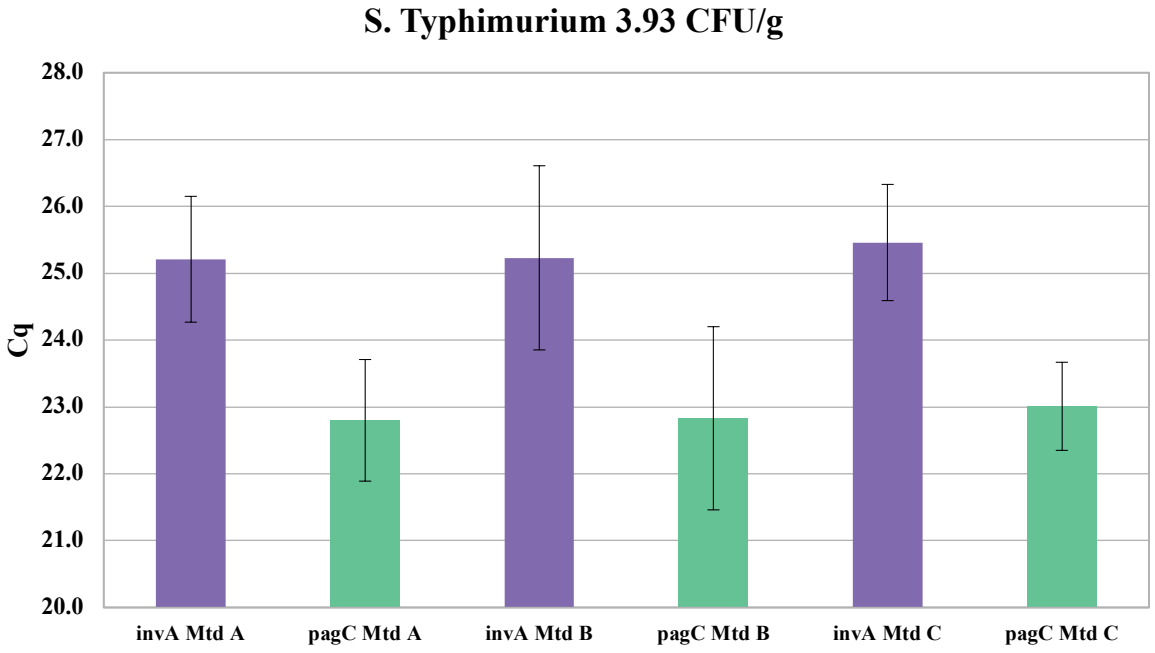

Figure 3.4 Cq values for S. Typhimurium using Methods A-C at 3.93 CFU/g.

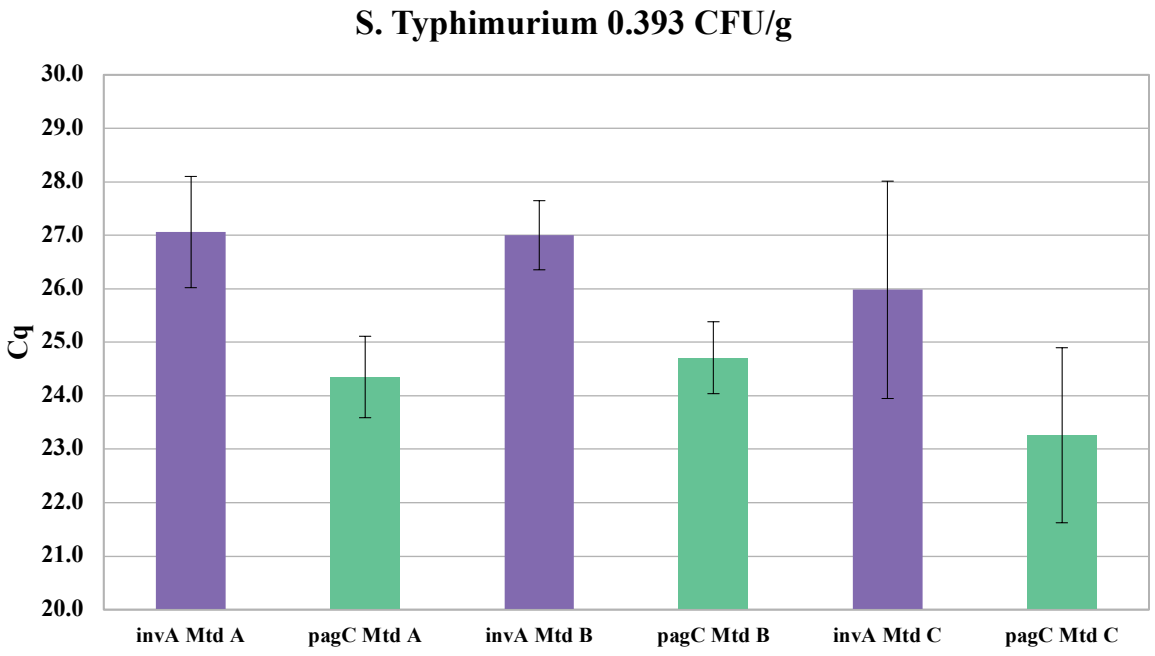

Figure 3.5 Cq values for S. Typhimurium using Methods A-C at 0.393 CFU/g.

Table 3.6 Detection of *Salmonella,* based on real-time PCR assays for *invA* and *pagC* genes in wheat grain samples spiked with *Salmonella* Oranienburg

Wheat grain samples	Inoculum concentration, 244 CFU/g of wheat grain						Inoculum concentration, 24.4 CFU/g of wheat grain						Inoculum concentration, 2.44 CFU/g of wheat grain					
	Method A		Method B		Method C		Method A		Method B		Method C		Method A		Method B		Method C	
	invA	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Samples that have threshold cycle (ct) value <38 are positive for *Salmonella*. Samples having a ct value of >38 are negative CFU-colony forming unit

Method A: wheat grain suspended in modified Buffered Peptone Water with Pyruvate (mBPW) at 37 degrees for 30 minutes, again in mBPWp for 24 hours and transferred to Rappaport-Vassiliadis (RV) at 42 degrees for 24 hours
Method B: Wheat grain in mBPWp at 37 degrees for 24 hours and transferred to RV at 42 degrees for 24 hours
Method C: Wheat grain in RV at 42 degrees for 48 hours

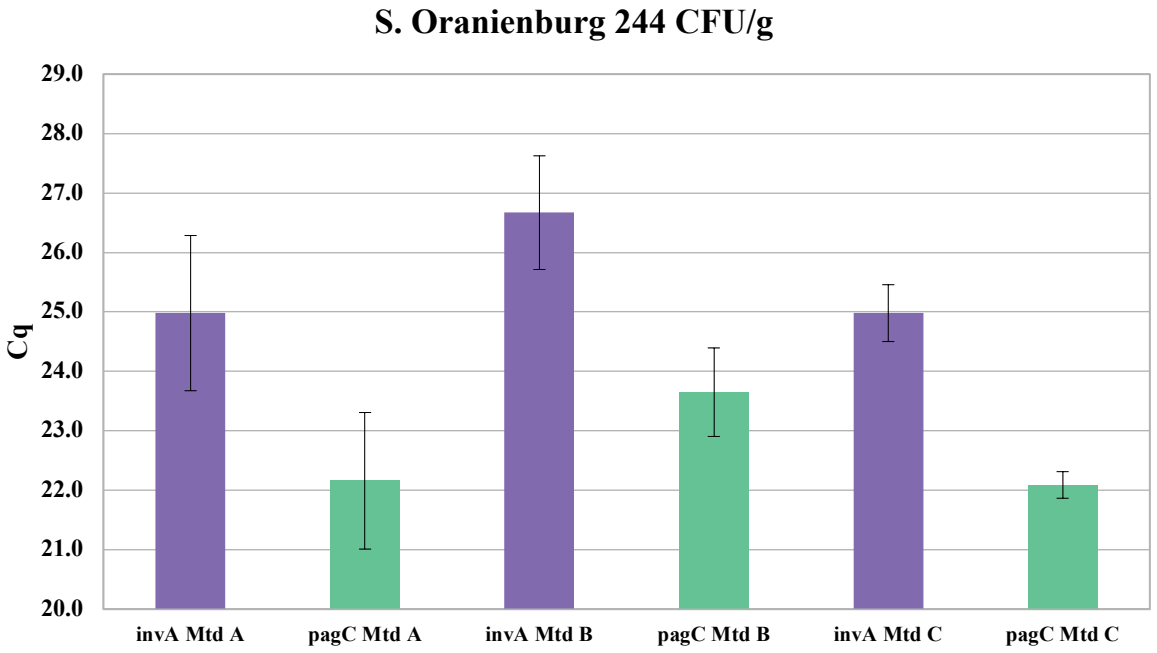

Figure 3.6 Cq values for S. Oranienburg using Methods A-C at 244 CFU/g.

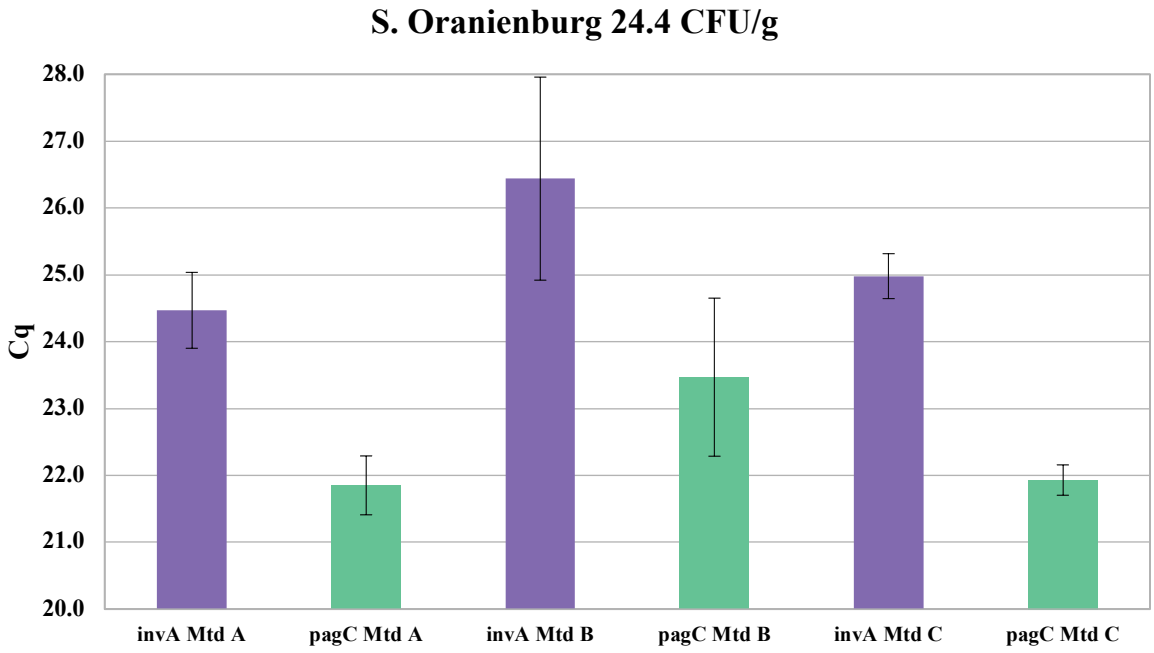

Figure 3.7 Cq values for S. Oranienburg using Methods A-C at 24.4 CFU/g.

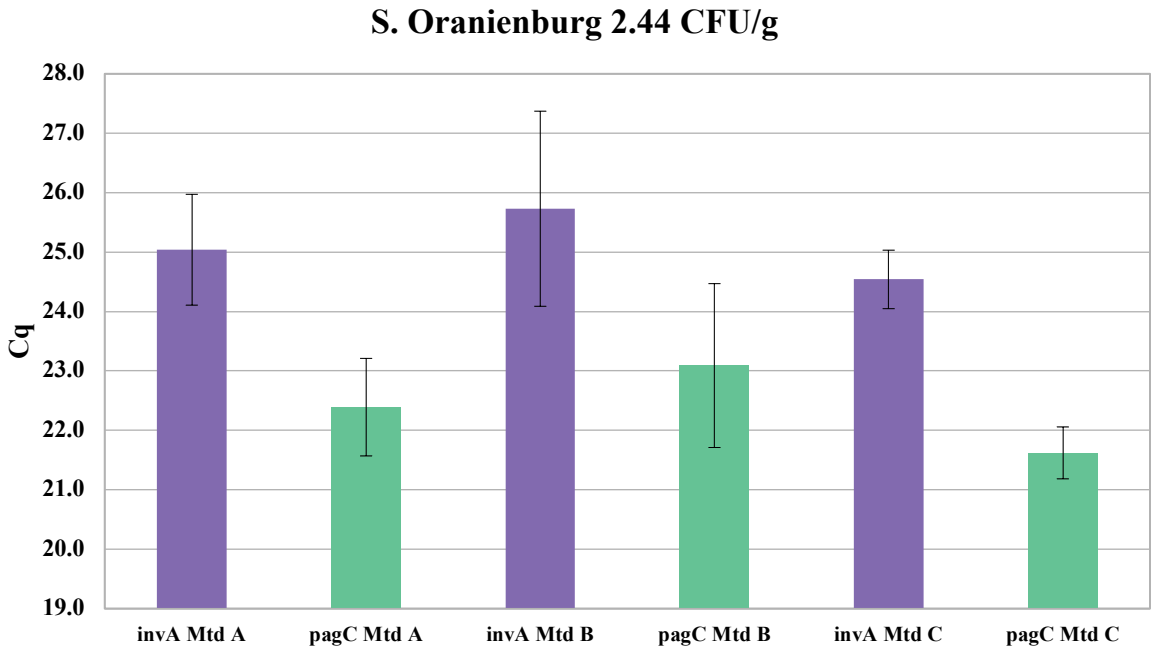

Figure 3.8 Cq values for S. Oranienburg using Methods A-C at 2.44 CFU/g.

Table 3.7 Detection of *Salmonella,* based on real-time PCR assays for *invA* and *pagC* genes in wheat grain samples spiked with *Salmonella* Newport

Wheat grain samples	Inoculum concentration, 225 CFU/g of wheat grain						Inoculum concentration, 22.5 CFU/g of wheat grain						Inoculum concentration, 2.25 CFU/g of wheat grain					
	Method A		Method B		Method C		Method A		Method B		Method C		Method A		Method B		Method C	
	invA	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*	*invA*	*pagC*
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Samples that have threshold cycle (ct) value <38 are positive for *Salmonella*. Samples having a ct value of >38 are negative CFU-colony forming unit
Method A: wheat grain suspended in modified Buffered Peptone Water with Pyruvate (mBPW) at 37 degrees for 30 minutes, again in mBPWp for 24 hours and transferred to Rappaport-Vassiliadis (RV) at 42 degrees for 24 hours
Method B: Wheat grain in mBPWp at 37 degrees for 24 hours and transferred to RV at 42 degrees for 24 hours
Method C: Wheat grain in RV at 42 degrees for 48 hours

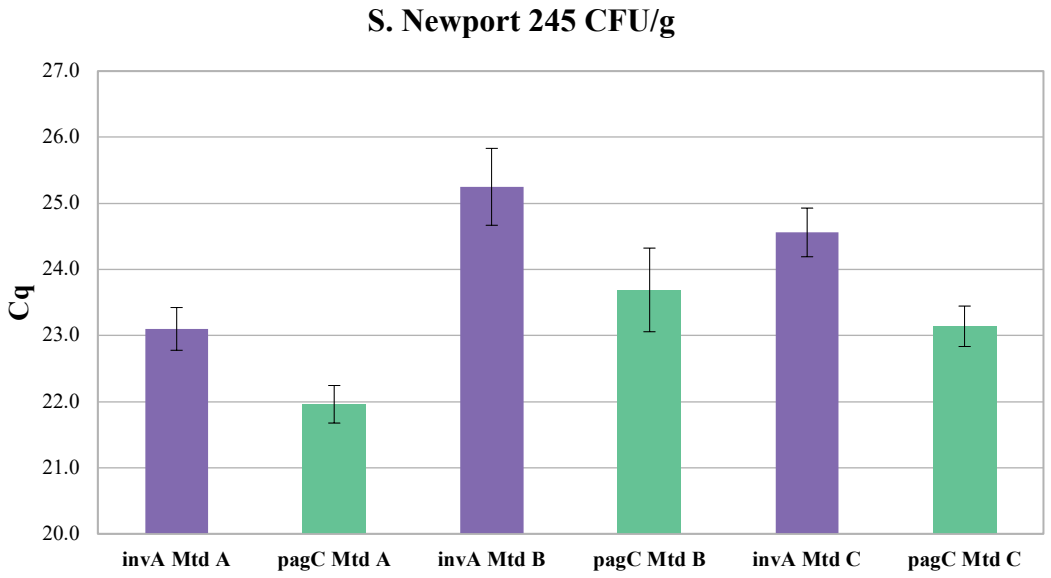

Figure 3.9 Cq values for S. Newport using Methods A-C at 245 CFU/g.

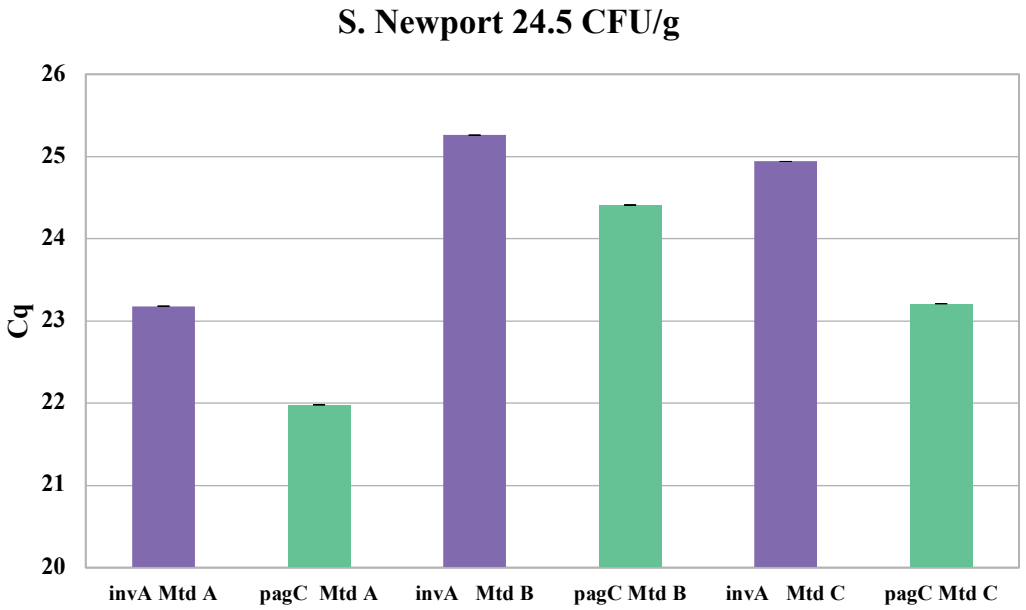

Figure 3.10 Cq values for S. Newport using Methods A-C at 24.5 CFU/g.

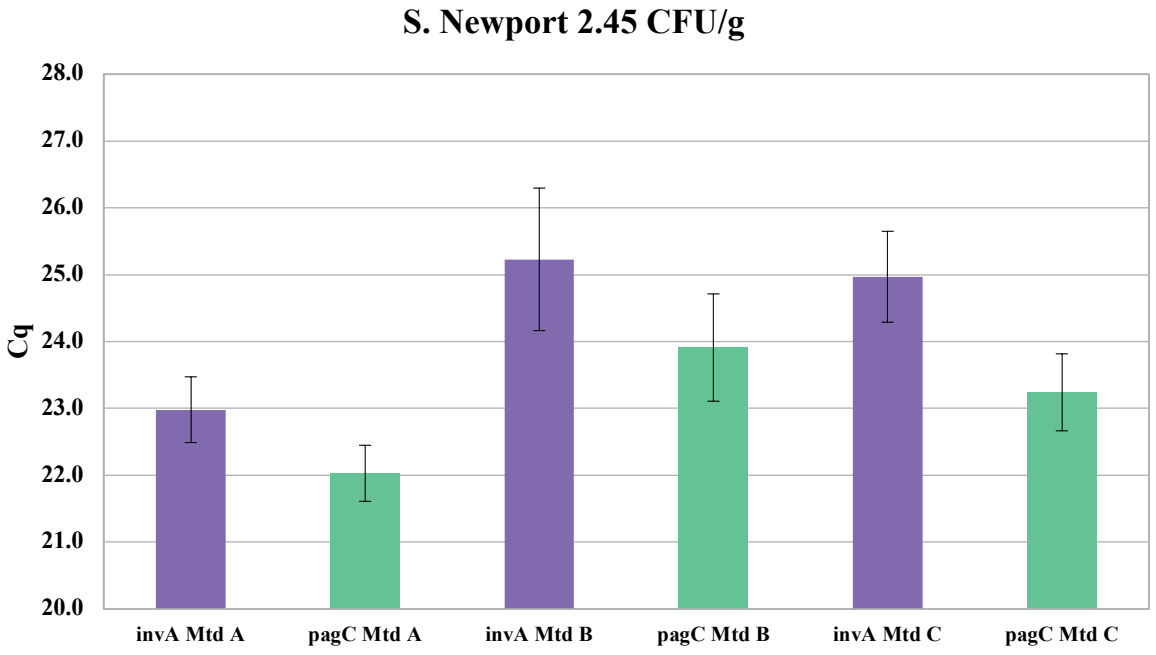

Figure 3.11 Cq values for S. Newport using Methods A-C at 2.45 CFU/g.

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Chapter 4 - Analysis of Virulence Potential of Salmonella enterica and Salmonella diarizonae Strains Isolated from Wheat Grains

Abstract

Salmonella is one of the major foodborne pathogens responsible for human illness outbreaks. It is caused by the consumption of contaminated food products. Several food products, including fresh produce, meat, egg, raw wheat flour, cake mix, toasted oats cereal, puffed rice and wheat cereal, have been implicated in human foodborne illness. The objective of this study was to determine the virulence potential of *Salmonella* strains isolated from wheat grains. Of the eight strains recovered from the screening of 625 wheat samples (Chapter 3), five belonged to subspecies *enterica*, and three to *diarizonae*. The three *diarizonae* strains belonged to serotype 61:I,v:1,5,7 while the strains of subspecies *enterica* belonged to serotypes Anatum, Hartford, Infantis, Norwich and Oranienburg. *In silico* MLST analysis revealed that all the *diarizonae* strains belonged to same sequence type (ST-243); however, *enterica* strains belonged to multiple sequence types. The strains carried virulence genes, including fimbrial genes, typhoid toxin genes, *Salmonella* pathogenicity island (SPI)-1 and -2 encoded type three secretory system (TTSS) genes, and a repertoire of effectors of TTSS. All of the strains carried aminoglycoside resistance gene, *aac(6")-laa*. IncFII was the only plasmid type found in one of the strains. *Salmonella* phage SEN22 was the only intact phage sequence found in *Salmonella* strains belonging to subspecies *diarizonae*, while *enterica* strains carried diverse phage populations. The whole genome sequence-based analysis of *Salmonella* strains isolated from wheat grains revealed their virulence potential, suggesting the possibility of these strains to cause foodborne illness in humans.

Introduction

Salmonella consists of two species*: S. bongori* and *S. enterica. Salmonella enterica* is further divided into six subspecies*: S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica subsp. diarizonae* (IIIb), *S. enterica* subsp.

houtenae (IV), and *S. enterica* subsp. *indica* (VI) (Lehman et al., 2015). They are zoonotic bacteria that exist in nearly any natural or man-made environments. They are pathogenic agents to humans and some animals, although a majority of the animals are asymptomatic. Reptiles, especially snakes, have been found to be reservoirs to certain *Salmonella* serotypes (Jackson Jr. & Jackson, 1971; Cambre et al., 1980; Schöter et al., 2003). In addition, the Centers for Disease and Prevention (CDC) has noted an increase in the number of children under the age of 5 who have become ill due to exposure to these "exotic" pets. Similarly, immunocompromised adults and the elderly also have a higher chance of illness when coming in contact with reptilian pets (CDC, 2019a).

Salmonella has been detected in the products of milled cereal grains such as wheat flour, cake mixes, wheat-based dry cereals, pie crusts, as well as animal feeds. Their presence in grains, which are raw ingredients in many foods products, are a potential health risk (Jones et al., 1982; Sperber, 2007; Zhang 2007; CDC, 2007; US Food & Drug Administration Safety Recalls, 2019). In a recent study, we determined that *Salmonella* prevalence was 1.3% (8/625) in wheat grain samples obtained from different regions of the United States (see previous chapter). Of the eight isolates confirmed as *Salmonella,* five isolates belonged to subsp. *enterica* and three isolates belonged to the subsp. *diarizonae*. The isolates of subsp. *enterica* belonged to Anatum, Hartford, Infantis, Norwich and Oranienburg. The three strains of *diarizonae* were identified as belonging to the serotype 61:I,v:1,5,7. In order to assess the virulence potential of the *Salmonella* strains isolated from these wheat grains, we performed whole genome sequencing of the eight strains and analyzed the sequence data for the presence of important virulence genes.

Materials and Methods

Salmonella strains (n=8) isolated from wheat grains and serotyped by the National Veterinary Services Laboratories (NVSL) were subjected to whole genome sequencing using the Thermo Fisher Ion S5 200bp sequencing platform. *De novo* genome assembly was performed using SPAdes (Bankevich et al., 2012). The draft genomes of *Salmonella* strains were initially annotated using the RAST (Rapid Annotation using Subsystem Technology) server (http://rast.nmpdr.org/rast.cgi) (Aziz et al., 2008). The RAST server also provides data on the distribution of genes in various categories. *In silico* MLST (multilocus sequence typing) analysis and serovar prediction was performed using SISTR (*Salmonella in silico* typing resource)

(https://lfz.corefacility.ca/sistr-app/) (Yoshida et al., 2016). The virulence gene profile was determined using the Virulence Factor Database (VFDB; (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi) (Liu et al., 2018). Different types of plasmid sequences and antimicrobial resistance genes were identified by PlasmidFinder 1.3 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) (Carattoli et al., 2014) and ResFinder 3.1 (https://cge.cbs.dtu.dk/services/ResFinder/) (Zankari et al., 2012), web-based tools developed by the Center for Genomic Epidemiology (CGE) at the Danish Technical University (DTU), Lyngby, Denmark (http://www.genomicepidemiology.org/). The total number of prophage sequences were determined using Phage Search Tool Enhanced Release (PHASTER; http://phaster.ca/). The tool identifies intact, questionable, and incomplete prophage sequences by scores of >90, 70–90, and < 70, respectively (Arndt et al., 2016; Zhou et al., 2011). The Harvest Suite, a software package, which includes tools such as Parsnp and Gingr, was used to determine the phylogenetic relationship among the *Salmonella* strains (Treangen et al., 2014). Parsnp v1.2 (http://harvest.readthedocs.io/en/latest/content/parsnp.html) was used to align the core genomes of *Salmonella* strains, followed by the construction of a maximum likelihood tree. Whole genome sequences of various *Salmonella* serotypes downloaded from NCBI were used as reference sequences for the phylogenetic analysis (Figure 4.1).

Results

In silico serotype prediction and MLST analysis

Of the eight strains used in this study, three of them belonged to subspecies *diarizonae*, and the remaining strains belonged to subspecies *enterica*. Serotypes of all the strains determined by classical serotyping method was confirmed by *in silico* serotyping (Table 4.1). *In silico* MLST analysis revealed that all the *diarizonae* strains belonged to same sequence type (ST-243); however, *enterica* strains belonged to multiple other sequence types (Table 4.1).

RAST subsystem summary

Based on the RAST subsystem annotation, the average genome size of *Salmonella* strains was 4.73 Mb (range 4.59-4.86), and the average number of genes associated with virulence, disease and defense was 58 (56-60). The highest average number of genes belonged to the membrane transport category, while the least average number of genes belonged to mobile

genetic elements. The average number of genes associated with the major subsystem categories in all of the strains is provided in Table 4.2.

Virulence gene profile

Adherence genes

A total of 17 fimbrial operons were identified in *Salmonella* strains, of which only five were present in all the strains (*csg*, *bcf*, *fim*, *stb*, *std*). The remaining fimbrial operons were absent in some of the strains. For example, *sta* was present only in two strains (2018-3-375 and 2018-3-626), *stf* was present in only four strains (2018-3-345, 2018-3-595, 2018-3-626, 2018-3-737). Nonfimbrial adherence genes including *misL*, *ratB*, *shdA*, and *sinH,* were also present, of which *misL* was present in all strains.

Type three secretory system genes (TTSS)

A total of 30 *Salmonella* pathogenicity island (SPI)-1 encoded TTSS genes were present in all of the strains. However, a few strains lacked some of the genes; i*nvB* was absent in the diarizonae strains (2018-3-62, 2018-3-200 and 2018-3-539); and *iagB* was absent in 2018-3-375; *iagB* and *orgA* were absent in 2018-3-595; *iagB* and *invC* were absent in 2018-3-626.

A total of 28 SPI-2 encoded TTSS genes were present in all of the strains. However, a few strains lacked some of the genes (*ssaM*, *ssaS*, and *sseD* were absent in 2018-3-62; *ssaM*, *ssaR*, *ssaS*, and *sseD* were absent in 2018-3-200; *ssaR* and *sseA* were absent in 2018-3-345; *ssaR*, *ssaS*, and *sseA* were absent in 2018-3-375; *ssaM*, *ssaS*, *sseC*, and *sseD* were absent in 2018-3-539; *ssaI*, *ssaR*, *ssaS*, and *sseA* were absent in 2018-3-595; *ssaR*, *ssaS*, and *sseA* were absent in 2018-3-626; *ssaS* and *sseA* were absent in 2018-3-737). The strains also carried a repertoire of TTSS-1 and TTSS-2 translocated effector protein encoding genes.

Typhoid toxin

The typhoid toxin encoding genes (*cdtB*, *pltA*) were present in four strains (2018-3-62, 2018-3-200, 2018-3-375 and 2018-3-539); however, all strains lacked the *pltB* subunit encoding gene.

Antimicrobial resistance genes

All the strains carried the aminoglycoside resistance genes (*aac(6")-laa*).

Plasmid and prophage sequences

IncFII was the only plasmid type found in one of the strains (2018-3-345). A total of 12 intact phage sequences were present in *Salmonella* strains (Table 4.3). *Salmonella* phage SEN22 was the only intact phage sequence found in *Salmonella* strains belonging to subspecies *diarizonae*. However, *enterica* strains carried a diverse set of phages, including *Salmonella* phages Gifsy-1, Fels-1, SEN34, and 118970_sal3; and *Enterobacteria* phages Fels-2, P4, fiAA91-ss, and *stx*2-converting phage 1717 (Table 4.4).

Phylogenetic analysis

Based on phylogenetic analysis, all three 61:l,5,(7) strains belonging to subspecies *diarizonae* clustered together in the maximum likelihood tree (Figure 4.1) and were closely related to *diarizonae* reference strains belonging to different serotypes. Subspecies *enterica* strains belonging to serotypes Anatum, Hartford, Infantis, Norwich and Oranienburg clustered with corresponding reference strains belonging to the same serotype.

Discussion

Non-typhoidal *Salmonella* is the leading cause of bacterial foodborne illness in humans, responsible for the vast majority of hospitalizations and deaths (Scallan et al., 2011). The majority of human salmonellosis cases are caused by serotypes of subspecies *enterica* (CDC, 2017). Human salmonellosis occurs due to consumption of contaminated food products (Antunes et al., 2003; Foley et al., 2011; Mazengia et al., 2014). Based on whole genome sequencing analysis of *Salmonella* isolates from wheat grain samples from across the country, this study revealed the possibility of these strains as potential source of foodborne illness.

There are several methods for the identification of strains of *Salmonella* from different sources. The classical method for serotyping uses the White-Kauffman-Le Minor scheme based somatic (O) and flagellar (H) (*Salmonella* subcommittee, 1934; Grimont & Weill, 2007).

Genotyping (DNA-based serotyping) relies on genetic variations in the O and H antigenic determinants (Yoshida et al., 2016). DNA sequencing techniques, such as whole genome sequencing, can also provide information on pathogen identity that includes strain typing, toxin type, encoding adherence genes as well as antimicrobial resistance genes (Gilmour et al., 2013).

Before *Salmonella* can colonize, it must be able to attach to the host cells to invade. *Salmonella* species have fimbriae (proteinaceous pili) as well as nonfimbriae (surface adhesins) that allow them to attach in multiple environments in order to survive. Most fimbriae types are based on how they are produced, their structure, and quality of the adhesins the bacteria use (Wilson et al., 2011). *Salmonella* possess a wide variety of pili encoded by adherence genes that confer different binding features, which may be needed for maintenance and survival (Forest et al., 2007). Fimbrial operons, including *csg*, *bcf*, *fim*, *stb*, and *std,* were found in all eight strains belonging to both *enterica* and *diarizonae* subspecies. Whorley et al. (2018) reported that *csg* and *fim* operons are core to S. *enterica*, yet only *bcf* and *sth*, appear to be core to S. enterica subspecies *enterica*. Fimbrial genes carried by *Salmonella* have been reported to play an important role in host tropism and colonization (Bäumler et al., 1997). Previous mouse studies have confirmed these genes play an important role in virulence in mice (Edwards et al., 2000; Lawley et al., 2006; van der Velden et al., 1998; Weening et al., 2005). In humans, five fimbrial operons (*bcf*, *csg*, *stb*, *sth*, *sti*) were reported to be present in twelve serovars of non-typhoidal *Salmonella* associated with invasive disease in humans (Suez et al., 2013).

The strains in this study also carried genes associated with TTSS encoded on SPI-1 and SPI-2. Most specifically, genes encoded on SPI, including *iacP*, *sseC*, and *spa* genes, are involved in the pathogenesis of various *Salmonella* serotypes (Bhowmick et al., 2011; Collazo and Galan, 1996; Kim et al., 2011). Genes encoding effector proteins of TTSS were carried by the *Salmonella* strains investigated in this study. Effector proteins of TTSS, including SipA, SipC, and SopE, have been shown to be involved in membrane ruffling and internalization of bacteria by modulating host cell cytoskeleton and signal transduction pathways (Chang et al., 2005; Hardt et al., 1998; McGhie et al., 2004; Myeni and Zhou, 2010; Zhou et al., 1999).

Salmonella typhi causes typhoid fever, a serious disease that causes stomach pain, high fever, headache, weakness and occasionally, death. Typhoid fever is rare in the United States with about 300 reported illnesses annually with the majority of those cases acquiring the infection while traveling (CDC, 2018). Typhoid toxin is an A-B-type toxin that is composed of

two subunits. An enzymatic component or domain (A) has a toxic effect on the cell by activating or inactivating an intracellular target or signaling pathway. The second subunit (B), a binding component or domain, transports the A subunit into the cell cytoplasm upon recognition of specific receptors on the host cells (Wilson et al., 2011).

The typhoid toxin is different from the usual A-B-type toxins because it has two A subunits and 5 subunits of B (A_2B_5). The three genes that encode the A_2B_5 toxin are PltB (B), CdtB (A) and PltA (A) (Song et al., 2013). A loss or mutation to either PltA or PltB prevents CdtB toxicity (Spanó et al., 2008). In the present study, four of the eight strains carried CdtB and PltA subunits of typhoid toxin; however, none of the strains carried the PltB subunit of the toxin. A previous study has also reported a similar finding (Worley et al., 2018). Spanò et al. (2008) have reported the lack of virulence activity of the typhoid toxin following deletion of PltB subunit of typhoid toxin.

The most common type of plasmid replicon found in subspecies *enterica* is IncFII, and only one of the strains carried the plasmid replicon (IncFII) (Worley et al., 2018). All the strains belonging to subspecies *diarizonae* carried *Salmonella* phage SEN22; however, subspecies *enterica* carried a diverse phage population. Previous studies have revealed the presence of phage-encoded virulence factors in *Salmonella* that contribute to their pathogenesis (Coombes et al., 2005; Lopez et al., 2012; Mirold et al., 1999). Coombes et al. (2005) have reported that *gogB* gene, encoded within the Gifsy-1 phage, acts as a modular and promiscuous TTSS secreted substrate of the infection process. One of the strains investigated in our study carried phage Gifsy-1, and it was also the only strain to carry the *gogB* gene.

Phylogenetic analysis revealed close clustering of the isolated strains to the reference strains belonging to the same serotype, which is in agreement with previous studies (Pornsukarom et al., 2018).

Aminoglycosides are trisaccharides with amino groups that kill bacteria by binding to the ribosomes and disrupting protein synthesis (Wilson et al., 2011). Antimicrobial resistance occurs when *Salmonella* uses the AAC(6') family of enzymes to inactivate the aminoglycosides (Poole, 2005). Aminoglycoside 6' acetyltransferases (AAC(6')-Iaa) are chromosomal-encoded broad-spectrum enzymes in *Salmonella enterica* that catalyze the acetyl CoA-dependent acetylation of an amino group in tobramycin, kanamycin and amikacin (aminoglycoside-based antibiotics) but are less effective against gentamicin. In contrast, AAC(6')-Iaa does not support

resistance to the aminoglycoside, streptomycin (Barnhart et al., 2002; Vakulenko and Mobashery, 2003; Salipante and Hall, 2003; Poole, 2005). This was consistent with the results of the antimicrobial susceptibility testing (see previous chapter), as none of the strains were resistant to either gentamicin or streptomycin. Testing revealed most of the strains were pan-susceptible; however, *S*. Infantis and *S. diarizonae* showed resistance to cefoxitin. Two of the *S. diarizonae* strains were resistant to tetracycline and amoxicillin-clavulanic acid.

In conclusion, whole genome sequence-based analysis of virulence gene profiles of *Salmonella* strains isolated from wheat grains reveals the potential risk of these strains in causing foodborne illness in humans. Given the limited molecular information about subspecies *diarizonae* in the published literature, this study also provides important information regarding the genetic composition of this subspecies. However, the data presented in this chapter is the result of a single analysis and future studies would need to confirm these results.

Figure 4.1 Maximum likelihood tree of whole genome sequences of isolated (as marked) and reference *Salmonella* strains

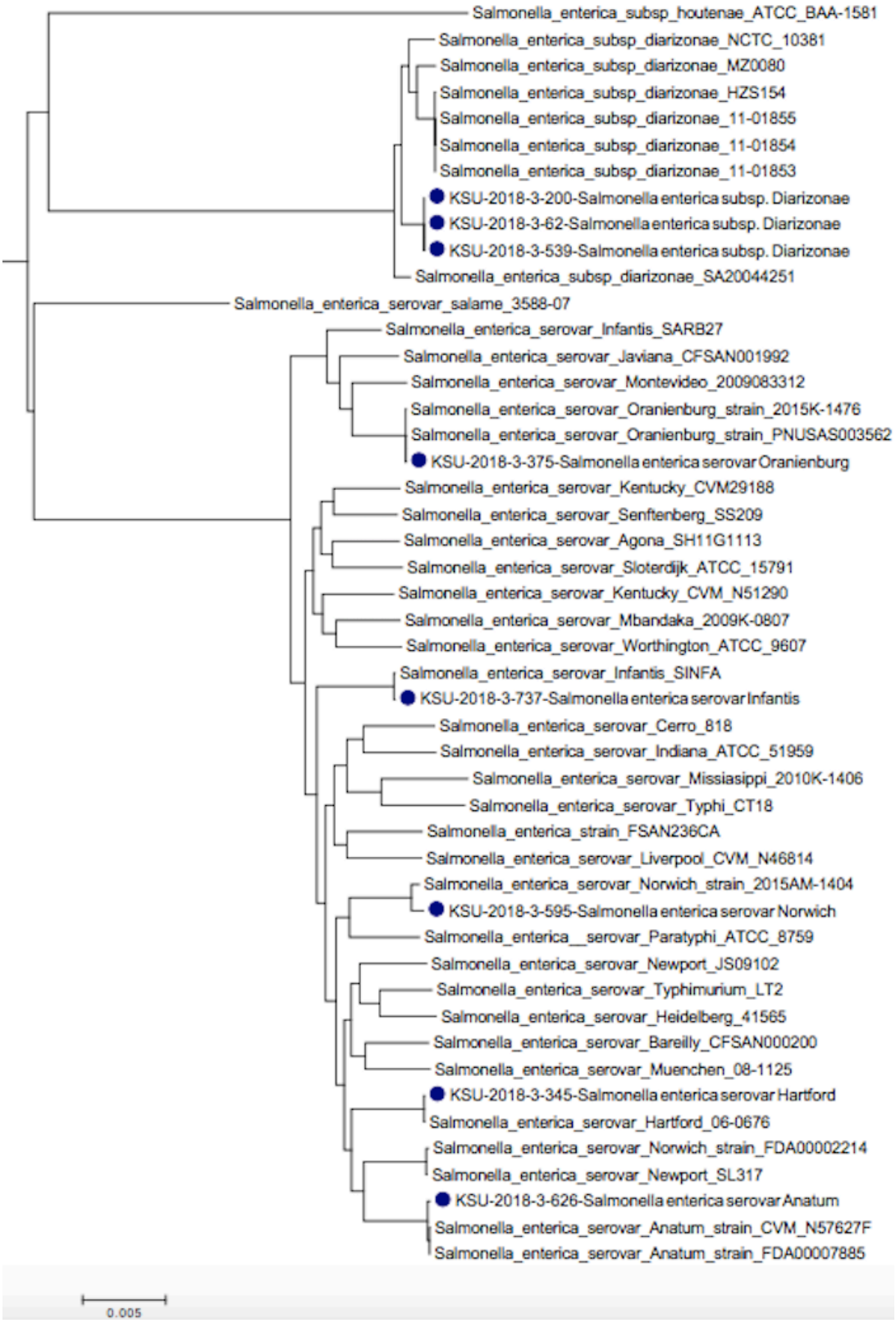

Table 4.1 Serotyping of *Salmonella* strains isolated from wheat grain samples by conventional and *in silico* analysis and sequence typing by multilocus sequence typing

***Salmonella* strains number**	**Subspecies**	**Serotype[a]**	**Serogroup**	**H1**	**H2**	***In silico* serotyping[b]**	**Multi locus sequence typing**
2018-3-62	*diarizonae*	IIIb 61:I,v:1,5,7	O61	I, v	1, 5, 7	IIIb 61:I, v:1,5,7:[z57]	243
2018-3-200	*diarizonae*	IIIb 61:I,v:1,5,7	O61	I, v	1, 5, 7	IIIb 61:I, v:1,5,7:[z57]	243
2018-3-345	*enterica*	Hartford	C1	y	e, n, x	Hartford	405
2018-3-375	*enterica*	Oranienburg	C1	m, t	[Z_{57}]	Oranienburg	23
2018-3-539	*diarizonae*	IIIb 61:I,v:1,5,7	O61	I, v	1, 5, 7	IIIb 61:I, v:1,5,7:[z57]	243
2018-3-595	*enterica*	Norwich	C1	e, h	1, 6	Norwich	2711
2018-3-626	*enterica*	Anatum	E1	e, h	1, 6	Anatum	64
2018-3-737	*enterica*	Infantis	C1	r	1, 5	Infantis	32

[a] Serotyping was performed by National Veterinary Services Laboratory, Ames, IA
[b]Yoshida, *et al*., 2016.

Table 4.2 Average genome size and average number of different categories of genes in *Salmonella* strains (n=8) based on RAST subsystem annotation (http://rast.nmpdr.org/rast.cgi)*

***Salmonella* strains number**	**Subspecies**	**Genome size (Mb)**	**Virulence, disease, and defense**	**Phages, prophages, transposable elements and plasmids**	**Membrane transport**	**Iron acquisition and metabolism**	**Stress response**
2018-3-62	*diarizonae*	4.7	60	19	169	65	93
2018-3-200	*diarizonae*	4.7	56	18	170	65	94
2018-3-345	*enterica*	4.79	56	11	182	28	91
2018-3-375	*enterica*	4.82	60	39	169	28	92
2018-3-539	*diarizonae*	4.7	56	19	171	64	94
2018-3-595	*enterica*	4.86	59	19	172	30	92
2018-3-626	*enterica*	4.59	56	5	177	28	91
2018-3-737	*enterica*	4.65	57	31	172	28	90

***Aziz et al., 2008.**

Table 4.3 Total number of prophage sequences in *Salmonella* strains (n=8) identified using PHASTER (http://phaster.ca/)*

Salmonella strains number	Subspecies	Completeness of prophage sequences		
		Intact	Questionable	Incomplete
2018-3-62	*diarizonae*	1	0	1
2018-3-200	*diarizonae*	1	0	1
2018-3-345	*enterica*	3	1	1
2018-3-375	*enterica*	2	0	3
2018-3-539	*diarizonae*	1	0	1
2018-3-595	*enterica*	1	3	4
2018-3-626	*enterica*	1	0	2
2018-3-737	*enterica*	2	3	1

#Prophage sequences were classified as intact, questionable and incomplete based on the PHASTER scores >90, 70–90, and < 70, respectively
***Zhou et al., 2011.**
***Arndt et al., 2016.**

Table 4.4 Intact prophage sequences present in *Salmonella* strains (n=8)

***Salmonella* strains number**	**Subspecies**	**Prophage**
2018-3-62	*diarizonae*	*Salmonella* phage SEN22
2018-3-200	*diarizonae*	*Salmonella* phage SEN22
2018-3-345	*enterica*	*Salmonella* phage Fels-1, Phage Gifsy-1, *Salmonella* phage SEN34
2018-3-375	*enterica*	*Enterobacteria* phage Fels-2, *stx*2-converting phage 1717
2018-3-539	*diarizonae*	*Salmonella* phage SEN22
2018-3-595	*enterica*	*Salmonella* phage 118970_sal3
2018-3-626	*enterica*	*Salmonella* phage Fels-1
2018-3-737	*enterica*	*Enterobacteria* phage P4, *Enterobacteria* phage fiAA91-ss

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Chapter 5 - Final conclusion and Discussion

Over 2500 different serotypes make up the genus *Salmonella*, a bacterium that shows little to no host specificity with the exception of few serotypes. It honors no international or local boundaries and is capable of infecting most host species that crosses its path. Detection of this bacterium goes back to the 1800s, although traces of *Salmonella*'s ability to infect humans may have led to the Aztec collapse during the middle ages. When first discovered, it was believed to only infect warm-blooded species until the discovery of this bacterium in reptiles in the early 1900s. The persistence of *Salmonella* in soil, water, dust and in fecal matter has allowed the bacteria to survive in almost any environmental niche for days, weeks and years. Its ability to disseminate throughout nature has caused the colonization of flora, and infection of livestock and poultry. This, in turn, has caused the contamination of animal by-products used for pets, livestock and poultry feeds, resulting in outbreaks and recalls. In addition, the decrease in the number of farming communities along with the increase of imported food products have added to the problem. The contamination of raw agricultural products, such as fruits, vegetables, seeds and nuts, has been responsible for multiple outbreaks, both local and nationally.

Despite implementation of a variety of control measures *Salmonella* prevalence has increased over the last couple of decades. *Salmonella* has been detected in raw food materials, such as wheat, a basic ingredient in many low moisture foods. The low moisture foods do not support the growth of the bacteria and yet, in several studies, outbreaks and recalls have revealed that not only was this bacterium able to survive in low moisture foods, but it survived for long periods of time. Mechanisms, like the viable but nonculturable (VBNC) state or the formation of biofilm, may be the means by which *Salmonella* survives hostile conditions.

Salmonella's consist adaptability and ever growing numbers of serotypes have made reducing the bacterial distribution in the food chain a challenge. Consequently, over a million people become infected with *Salmonella* yearly, costing the US several billions dollars in revenue. Unfortunately, in the last twenty years, the number of recalls and outbreaks due to *Salmonella* has increased. Some of the products involved were from the cereal grain wheat and wheat-based products in which there are few reports on *Salmonella* detection. A study was conducted examining 625 wheat grains samples from various states. The objectives of the study were to detect, isolate and characterize virulence potential and antimicrobial susceptibility.

While the original method failed to detect the *Salmonella*, the redesigned methods prove to be more effective. Overall, a total of eight isolates were detected, representing six serotypes that belonged to two *Salmonella* subspecies, *enterica* and *diarizonae*. The findings of the experiment showed that mechanical homogenization rather than manual manipulation was needed to help release the bacteria. In addition, the results of the two methods were interesting as both detected *Salmonella* but the serotypes that were isolated from each method were somewhat different which might be due to the uneven distribution of contamination, even though each sample of wheat grain was homogenized. Since the wheat grain samples were frozen upon arrival, the modified buffered peptone water with pyruvate (mBPWp) was used instead of buffered peptone water (BPW) to increase recovery of *Salmonella*. Yet by using only the Rappaport Vassiliadis (RV) enrichment broth the results were just as effective as the mBPWp pre-enrichment broth in the detection and isolation of the bacteria. Which seems to suggest that the pre-enrichment broth (though recommended) may not be needed for some food sample testing. The possible elimination of this step would be advantageous in saving time and money.

The detection and isolation of *Salmonella,* subspecies *diarizonae* in the wheat grain samples was novel. Like the other members of the genus *Salmonella*, *diarizonae* is zoonotic, and though human infections are rare, illnesses by this subspecies have been steadily increasing. Most *diarizonae* serotypes have been isolated from cold-blooded animals, although a few serotypes have become host-adapted and non-host adapted to warm-blooded animals. With the increase of "exotic" pets such as lizards, turtles and snakes in more households, certain S. *diarizonae* serotypes normally found in these animals have been responsible for salmonellosis in humans. However, the S. *diarizonae* serotype 61:1,v:1,5(7) isolated from the wheat grains is the most common serotype isolated from humans. Yet, data from the National Veterinary Services Laboratories (NVSL) for the National *Salmonella* Surveillance reports shows that this serotype has seldom been isolated from nonhuman sources.

Salmonellosis is a serious concern to public health, especially if the pathogenic bacteria has antimicrobial resistance, or with some serotypes, multiple antibiotic resistance. While antibiotic resistance level in *Salmonella* has been low, there as been a slow increase from 1% to 5% in the last twenty years. The antimicrobial susceptibility testing performed on the eight isolates revealed that three of the serotypes showed resistance or were intermediate to three antibiotics. The other five isolates showed no resistance to the 14 antibiotics tested.

Antimicrobial susceptibility is just one of the tests performed to understand the virulent nature of *Salmonella*. With the use of whole genome sequencing, the eight isolates were analyzed and multiple programs were used to analyze the data for phylogenetic relationships, serotyping, and virulence genes. The analysis revealed a number of fimbrial operons as well as nonfimbrial adherence genes and multiple *Salmonella* pathogenicity islands encoding several different type three secretory systems genes. Typhoid toxin requires the encoding of three genes for toxicity, but only two of the three genes were found in the isolates.

As progression in technology advances our understanding of these pathogens, what we've discovered today would not have been possible without the basis of the classification system that was built on the work of others. White furthered that earlier knowledge by analyzing the antigenic structure of the somatic (O) and flagellar (H) antigens that would lead to the basis in which *Salmonella* would be classified by. This would later be modified by Kauffman and extended further by Le Minor. However, the continue path to *Salmonella's* official taxonomy and nomenclature was filled with complexity. Such as the three to one species concept, the division of *Salmonella* into subspecies, was Arizona its own genus or a subspecies of *Salmonella* and the use of technology to distinguish between the serotypes that biochemical tests could not do. Nonetheless, the taxonomy of the genus *Salmonella* took over 100 years to construct, representing the work of many scientists.

The members of *Salmonella* are persistent pathogens and resilient throughout nature with mechanisms that enable the bacteria to survive hostile environments. However, *Salmonella's* ability to adapt to different niches has made control of the bacteria difficult. Even with the number of current policies and procedures set in place for food safety *Salmonella* is still responsible for a large number of foodborne illnesses every year. Further studies are needed that focus on preventing the bacteria from entering the food system and a better understanding of the mechanisms that allow *Salmonella* to circumvent sanitation protocols.