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I am submitting herewith a thesis written by Andres Rodriguez Lozano entitled "Multistate Study to Determine the Presence of *Salmonella spp*. in Farm Animals and their Environment." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

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Multistate Study to Determine the Presence of *Salmonella spp*. in Farm Animals and their Environment

A Thesis Presented for the Master of Science Degree

The University of Tennessee, Knoxville

Andres Rodriguez Lozano

August 2004

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DEDICATION

I would like to dedicate this thesis to the following people:

My parents, Andres and Ana Maria, for all their support, friendship and encouragement during all my life that made the person that I am. You are the best parents ever.

My brother and best friend, Jose Maria, who has always been a source of encouragement and inspiration to me.

To Sylvia, whose love, friendship and patience became an essential part of my life.

"*Gutta cavat lapidem, non vi, saepe cadendo"*

Bei Ovid, *Epistulae ex Ponto* IV: 10-15.

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ABSTRACT

Salmonella is the second most common cause of foodborne infection in the Unites States (US). It is estimated that about 1.4 million people suffer from salmonellosis a year in the US with an estimated annual cost of $2.9 billion. Human cases of salmonellosis are often related to the consumption of raw meat and unpasteurized milk and milk products. Whereas the fecal-oral contamination is well established at farm level, less in known about the role of the environment on the *Salmonella* survival. The objective of this study was to compare isolation of *Salmonella* in farm animals and their environment based on spatial, temporal, and environmental factors to provide population-based epidemiological information that can be used in assessing risk and development risk management strategies. Samples were collected from 12 different locations in 5 states (Tennessee, Alabama, North Carolina, California and Washington). Samples originated from dairy cows, beef cattle and swine herds. Environmental samples (n=360) and rectal swabs (n=1200) were analyzed using BAM modified protocols. *Salmonella* positives were characterized with Riboprinter® and Pulsed-Field Gel Electrophoresis using *Pvu*II and *Xba*I restriction enzymes. *Salmonella* was most frequently isolated from swine, the animals being the major reservoir, with an isolation of fecal materials of 11.9%, followed by rectal swabs (8.8%) and feed (7.7%). For dairy cows and beef cattle, the major reservoir of *Salmonella* was the environment, especially the feed (3.1%), followed by soil samples (2.0%). The most common serotypes isolated from swine were *S*. Anatum, *S*. Javiana, *S*. Newington, and *S*. Worthington. The most common

serotypes found in dairy cows were *S*. Anatum, *S*. Newington and *S*. Javiana, whereas only two serotypes were isolated from beef cattle, *S*. Anatum and *S*. Newington. The Simpson's diversity index was calculated for Riboprinter (0.86) and PFGE (0.98). This data indicates significant diversity among the *Salmonella* isolated, but we were able to find regional and spatial differences among the *Salmonella* isolates. Breaking the contamination cycle between the animals, their environment, and management practices to control swine fecal materials will be essential to reduce the isolation of *Salmonella* in farm animals.

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Part I: Literature Review

Salmonella general characteristics

Salmonella is a rod-shape motile non-sporeforming Gram-negative bacterium (the exceptions for non-motile species are *S*. Gallinarum and *S*. Pullorum) (FDA 2004). Currently there are 2541 *Salmonella* serovars (Popoff et al. 2004). These are classified in two species, *S*. *enterica* and *S. bongori*. *S. enterica* is divided into six subspecies, *Salmonella enterica* subsp. *arizonae*, *Salmonella enterica* subsp. *diarizonae*, *Salmonella enterica* subsp. *enterica*, *Salmonella enterica* subsp. *houtenae*, *Salmonella enterica* subsp. *indica*, and *Salmonella enterica* subsp. *salamae* (Popoff et al. 2004). Although this new nomenclature has not been yet authorized by the Bacteriological Code, it is widely used by the scientific community, including the World Health Organization (WHO) and the Institute Pasteur in Paris, France (Euzeby 2000; Yan et al. 2003, Popoff et al. 2004). The Center for Disease Control and Prevention (CDC) recently adopted a new system to name *Salmonella* as oppose to the Kauffman-White scheme (CDC 2003).

Salmonella is the second most important cause of foodborne infection in the United States (US). In the US there are about 1.4 million cases of human salmonellosis every year (CDC 2003) with an estimated annual cost of $ 2.9 billion (ERS USDA 2004). Salmonellosis can occur through consumption of raw meat and poultry products as well as through unpasteurized milk (MMWR 1995; MMWR 2003). *Salmonella* can be shed in milk from asymptomatic cows (Smith et al. 1994). Meat can be contaminated with *Salmonella* at slaughter through carcasses (Smith et al 1994) or during further processing. S*almonella* can

produce a variety of symptoms, from non-clinical to diarrhea, septicemia, abortion in animals and death. The infectivity of *Salmonella* depends on the host immune system, strain and amount of *Salmonella* ingested as well as environmental factors (Hirsh and Zee 1999).

The largest outbreak of salmonellosis in the US involved 224,000 people in 1994. The outbreak was caused by ice cream produced from milk that was transported by trucks that previously carried raw eggs (Jay 2000). The second biggest outbreak of salmonellosis reported in the US was related to milk from a dairy plant and involved 200,000 people (Jay 2000).

Prevalence of *Salmonella*

Prevalence in humans

Major serotypes of *Salmonella* associated with human salmonellosis reported from the period 1995-2002 are shown in Table 1. *S.* Typhimurium and *S*. Enteriditis are the two most common serotypes associated with human salmonellosis since 1995. *S.* Heidelberg and S Newport have been routinely listed in the top five *Salmonella* causing human salmonellosis between 1995 and 2002 (Table 1). Frequency of isolation of other serotypes has been outbreak related.

In 2003, five serotypes accounted for about 60% of the human isolates, and these were *S*. Typhimurium, *S*. Enteriditis, *S*. Newport, *S*. Heidelberg, and *S*. Javiana (MMRW 2004). Whereas the isolation of *S*. Typhimurium has decreased within the last 7 seven years, the incidence of *S*. Javiana has increased 227% for

Table 1. Top ten *Salmonella* serotypes isolated from human cases of salmonellosis reported to CDC during 2002, 2000, 1997 and 1995.

	2002	**2000**	**1997**	**1995**
1	*S*. Typhimurium	*S*. Typhimurium	*S*. Typhimurium	*S*. Enteriditis
2	*S*. Enteriditis	*S*. Enteriditis	*S*. Enteriditis	*S*. Typhimurium
3	*S*. Newport	*S*. Newport	*S*. Heidelberg	*S*. Newport
4	*S*. Heidelberg	*S*. Heidelberg	*S*. Newport	*S*. Heidelberg
5	*S*. Javiana	*S*. Javiana	*S*. Agona	*S*. Hadar
6	*S*. Montevideo	*S*. Montevideo	*S*. Montevideo	*S*. Javiana
7	*S*. Muenchen	*S*. Muenchen	*S*. Thomson	*S*. Muenchen
8	*S*. Oranienburg	*S*. Infantis	*S*. Javiana	*S*. Montevideo
9	*S*. Saintpaul	*S*. Thomson	*S*. Infantis	*S*. Agona
10	*S*. Infantis	*S*. Oranienburg	*S*. Hadar	*S*. Thomsom

(Adapted from CDC selected annual reports during the years 1995, 1997, 2000 and 2002).

the same period (MMWR 2004). Other common human serotypes have remained fairly constant within the last seven years.

Prevalence in animals

A comparison of clinical *Salmonella* serotypes (non-human) and non-clinical isolates (healthy animals) reported to the CDC during 2002 is shown in Tables 2 and 3. Clinical cases reported during 1995 to CDC that were associated with specific animals are provided in table 4. Non-clinical *Salmonella* were not reported by CDC until 1999.

Not all of the *Salmonella* serotypes isolated from animals match the ones isolated from human salmonellosis cases. There were also differences between serotypes from clinical and non-clinical animals. The clinical serotypes reported in 2002 were related to most of the human isolates, but several of the non-clinical isolates were not listed in the top ten serotypes isolated from human cases (CDC 2002). However, *Salmonella* serotypes from poultry were quite similar regardless of the species. This shows the importance of the carrier state, where the animal can be shedding *Salmonella* without sign of sickness. The shedding of *Salmonella* through feces may contaminate the environment, making the farm animals and their environment an important reservoir for *Salmonella*.

Several surveys have been done in the US to determine the prevalence of *Salmonella* in farm animals and farm environments. Smith et al. (1994) did a survey to evaluate the prevalence of *Salmonella* in California cattle. They analyzed fecal and environmental samples, as well as blood samples to determine the exposure to *Salmonella.* They found that 75% of cattle had recent

Table 2. Top five *Salmonella* serotypes isolated from clinical cases of salmonellosis in animals during 2002 reported to CDC.

	Bovine	**Porcine**	**Chicken**	**Turkey**
1	*S*. Newport	*S*. Typhimurium	*S*. Heidelberg	*S*. Senftemberg
2	*S*. Typhimurium	*S*. Derby	*S*. Kentucky	*S*. Heidelberg
3	*S*. Dublin	*S*. Cholerasuis	*S*. Typhimurium	*S*. Typhimurium
4	*S*. Agona	*S*. Heidelberg	*S*. Enteriditis	*S*. Bredeney
5	*S*. Montevideo	*S*. Agona	*S*. Montevideo	*S*. Montevideo

(Adapted from CDC 2003 annual report).

Table 3. Top five *Salmonella* serotypes isolated from non-clinical cases in animals during 2002 reported to CDC.

	Bovine	**Porcine**	**Chicken**	**Turkey**
1	*S*. Montevideo	*S*. Derby	*S*. Heidelberg	*S*. Senftemberg
2	*S*. Senftemberg	*S*. Senftemberg	*S*. Kentucky	*S*. Heidelberg
3	*S*. Dublin	*S*. Agona	*S*. Typhimurium	*S*. Hadar
4	*S*. Kentucky	*S*. Uganda	*S*. Enteriditis	*S*. Muenster
5	*S*. Anatum	*S*. Mbandaka	*S*. Mbandaka	*S*. Saintpaul

(Adapted from CDC 2003 annual report).

Table 4. Top five *Salmonella* serotypes isolated from animal clinical cases of salmonellosis during 1995 reported to CDC.

	Bovine	**Porcine**	**Chicken**	**Turkey**
1	*S*. Typhimurium	*S*. Derby	*S*. Enteriditis	*S*.Brandenburg
2	*S*. Typhimurium Var Copenhagen.	*S*. Typhimurium var Copenhagen.	*S*. Heidelberg	*S*. Muenster
3	*S*. Kentucky	*S*. Typhimurium	*S*. Kentucky	*S*. Montevideo
4	*S*. Dublin	*S*. Heidelberg	*S*. Hadar	*S*. Bredeney
5	*S*. Montevideo	*S*. Agona	*S*. Typhimurium	*S*. Heidelberg

(Adapted from CDC annual report 1996).

exposure to *Salmonella.* This percentage of exposure was lower when they analyzed the fecal and environmental samples, with the serotypes isolated including *S*. Typhimurium, *S*. Dublin, and *S*. Oranienburg.

In 1995 The USDA National Animal Health Monitoring System (NAHMS) published a national study (Fedorka-Cray et al. 1998) to determine the prevalence of *Salmonella* in feedlot pens (Table 5). A total of 13 states were sampled and *Salmonella spp* was recovered in 38% of the feedlots. Overall, 5% of all the samples were positive for *Salmonella*. With the exception of *S*. Newington, the other four serotypes were on the top ten list for clinical *Salmonella* isolates from bovine according to the CDC Annual Report in 1997(Fedorka-Cray et al. 1998). The authors reported that the five most common isolates related to sick cattle in 1991 were *S*. Typhimurium, *S*. Dublin, *S*. Typhimurium var. Copenhagen, *S*. Cerro, and *S*. Newport. The top five clinical isolates in that time period were S. Typhimurium, *S*. Typhimurium var Copenhagen, *S*. Dublin, *S*. Anatum and *S*. Montevideo (Fedorka-Cray et al. 1998).

In 1999, the USDA NAHMS conducted another national survey to estimate the prevalence of *Salmonella* among feedlot cattle by sampling 12 states (USDA 2001). *Salmonella* isolation in the fecal samples was 6.3%. *S*. Newport was the only isolate from feedlots related to human illnesses that year. The most common serotypes isolated in feedlot cattle during 1999 are shown in Table 5.

Table 5. Comparison of the different surveys for *Salmonella spp.* isolation in US from non-clinically ill cattle.

USDA 1995	**USDA 1999**	**Dargatz et al. (2000)**	**Beach et al. (2002) feedlot cattle**	**Beach et al. (2002) non feedlot cattle**
S. Anatum (27.9%)	*S*. Anatum	*S*.Orainenburg (21.8%)	*S*. Anatum (18.3%)	*S*. Kentucky (35.4%)
S. Montevideo (12.9%)	*S*. Montevideo	*S*. Cerro (21.8%)	*S*. Kentucky (17.5%)	*S*. Montevideo (21.7%)
S. Muenster (11.8%)	*S*. Reading	*S*. Anatum (10.3%)	*S*. Montevideo (9.2%)	*S*. Cerro (7.5%)
S. Kentucky (8.2%)	*S*. Newport	*S*. Bredeney (9.0%)	*S*.Senftemberg (8.3%)	*S*. Anatum (6.8%)
S. Newington (4.3%)	*S*. Kentucky	*S*. Mbandaka (5.1%)	*S*. Mbandaka (7.5%)	*S*. Mbandaka (5.0%)

A national study of health and management for the US beef cow-calf industry was conducted by Dargatz et al. (2000). They collected fecal samples from 187 beef cow-calf operations located in 22 states. The overall prevalence of *Salmonella spp* in cow feces was 1.4%. For specific serotypes found in this study see Table 5.

Another study by Beach et *al*. (2002) analyzed *Salmonella* in beef cattle during transportation to slaughter in central Texas. The prevalence of *Salmonella* recovery from fecal samples in feedlot cattle was 4.0%; whereas, for nonfeedlot cattle it was 10.9%. The serotypes most commonly isolated from feces in feedlot cattle were *S*. Anatum (25%), and *S*. Senftemberg (25%). For nonfeedlot cattle, the most common serotypes were *S*. Kentucky (35.4%) and *S*. Montevideo (21.7%). The most common serotypes isolated in all sample types in this study are shown in Table 5.

In Canada, a study conducted to determine the most common serotypes isolated from Alberta ground beef found that these were *S*. Anatum, *S*. Heidelberg, *S*. Montevideo, and *S*. Typhimurium (Sorensen et al. 2002).

In 1996, the USDA conducted Dairy' 96, a national survey to establish the prevalence of *Salmonella* in US dairy operations during the period October 1996 to September 1997. Fecal samples were analyzed from dairy operations in 19 states with a fecal shedding of 5.4% reported (Wells et al. 2001). Fecal samples were collected from animals in 91 dairies and 97 cull dairy cow markets from 19 states. The recovery of *Salmonella* from milk cows was 5.4%. *Salmonella* shedding was detected in 21.1% of dairies. The most common serotypes as well

as the top serotypes for clinically ill dairy cows are shown in Table 6 (Wells et al. 2001). In 1995, the USDA conducted a national survey to estimate the prevalence of *Salmonella* in finisher hogs. The study was conducted in 16 states. A total of 6655 fecal samples were collected from pens of late finisher hogs and analyzed. *Salmonella* was present in 38.2% of the operations. The prevalence of *Salmonella* in swine feces was as low as 6%. The majority (60.3%) of the operations had only one serotype (USDA 1997b). The most frequent serotypes recovered are shown in table 7 (USDA 1997b).

In 1997 Davies et al. evaluated the prevalence of *Salmonella* in two different swine production systems in North Carolina, a finishing site using all-in/all-out management and a farrow-to-finish system using continuous flow management of finishing pigs. The prevalence of *Salmonella* from fecal samples in swine was 24.6%. The most common serotypes are shown in Table 7 (Davies et al. 1997).

The prevalence of *Salmonella* in 25 Minnesota swine farms was determined by analyzing the ileocecal lymph nodes of slaughtered swine. In this study, 3.69% of the swine were positive. For *Salmonella*, the prevalence during shipment was 32%, varying from 0 to 33% overall (Carlson 2001). Most common serotypes isolated from lymph nodes at slaughter are shown in Table 7.

Barber et al. (2002) studied the distribution of *Salmonella* in swine production units in Illinois from farms that tested positive at slaughter. They analyzed fecal samples from swine and other domestic and wild animals, feed, water, boots, flies and mice (Table 7).

Table 6. *Salmonella* serotypes isolated from non-clinical cases in dairy cows. This study was part of a USDA national study "Dairy'96".

Wells et al. 2001
S. Montevideo (21.3%)
S. Cerro (13.4%)
S. Kentucky (8.5%)
S. Menhaden (7.7%)
S. Anatum (6.2%)
S. Meleagridis (6.2%)

Table 7. Comparison of different studies for *Salmonella* serotypes isolated from several studies from non-clinically ill swine.

USDA 1997	**Davies et al. (1997)**	**Carlson and Blaha (2001)**	**Barber et al. (2002)**
S. Derby	*S*. Derby	*S*. Agona (23.88%)	*S*. Derby
S. Agona	*S*. Typhimurium var Copenhagen.	*S*. Infantis (16.67%)	*S*. Agona
S. Typhimurium var Copenhagen.	*S*. Heidelberg	*S*. Newhaw (11.9%)	*S*. Worthington
S. Brandemburg	*S*. Schuarzenground	*S*. Typhimurium (7.14%)	*S*. Uganda
S. Mbandaka	*S*. Mbandaka	*S*. Mbandaka (7.14%)	

Bailey et al. (2001) studied the prevalence of *Salmonella* in integrated poultry operations. Different types of samples were analyzed from the hatchery to the end process. *Salmonella* was present in all types of samples and 9.1% of the samples were positive for *Salmonella*. They identified 36 different serotypes, the most prevalent being *S*. Senftemberg, *S*. Thomson, and *S*. Montevideo

A survey conducted in the Pacific Northwest analyzed 4725 samples from poultry products, poultry and their environment in 1999-2000 (Roy et al. 2002). The total prevalence of *Salmonella* was 11.99% (Table 8), though the highest prevalence came from carcass rinse (34.17%), ground broiler meat (29.49%), and fluff samples (15.04%). *S*. Heidelberg and *S*. Kentucky accounted for almost 50% of the isolates. In the Hazard Analysis Critical Control Point (HACCP) survey, these two serotypes accounted for 47.41% of the total (FSIS 1999).

A study was conducted in Canada to determine the prevalence of *Salmonella* in turkey flocks (Irwin 1994). They analyzed pooled litter, dust and feed samples. *Salmonella* was recovered in 86.5% of the environmental samples.

Hird et al. (1993) compared *Salmonella* serotypes obtained from two different sources in turkey (California Veterinary Diagnostic Laboratory System, CVDLS, and NAHMS) and compared this with the most common human isolates in California. *S*. Heidelberg and *S*. Agona were found to be a common isolates in both, humans and turkeys.

The Food Safety and Inspection Service (FSIS) initiated a pathogen reduction plan using the Hazard Analysis Critical Control System

Table 8. Comparison of *Salmonella* serotypes isolated from chicken and turkey in different studies.

Roy et al. (2002)	**Irwin et al. (1994)**	**NAHMS [1] (1988-89)**	**CVDLS [1] (1988-89)**
S. Heidelberg (25.77%)	*S*. Anatum (19.6%)	*S*. Kentucky	*S*. Kentucky
S. Kentucky (21.65%)	*S*. Hadar (18.1%)	*S*. Anatum	*S*. Anatum
S. Montevideo (11.34%)	*S*. Agona (18.1%)	*S*. Arizonae	*S*. Heidelberg
S. Enteriditis *S*. Hadar (5.15%)	*S*. Saintpaul (15.5%)	*S*. Heidelberg	*S*. Reading
S. Infantis *S*. Typhimurium S. Thomson (4.12%)	*S*. Bredeney (12.6%)	*S*. Reading S. Senftenberg	*S*. Senftenberg
S. Mbandaka *S*. Cerro (3.09%)		*S*. Agona *S*.Meleagridis	*S*. Broughton

(1) Adapted from Hird et al. 1993.

(HACCP) in order to ensure the safety of the raw ground products from meat and poultry. During the 1-year period from 1998 to 1999, FSIS conducted a *Salmonella* study to determine the effectiveness of the HACCP system. The *Salmonella* isolates were serotyped and the results are shown in Table 9.

Salmonella in the environment

The major reservoir for *Salmonella* is the gastrointestinal tract of humans and animals (Jay 2000). Several *Salmonella* serovars are host adapted including *S.* Gallinarum in poultry, *S.* Dublin in cattle, and *S.* Cholerasuis in swine (Jay 2000). *S.* Cholerasuis is the most common serotype found in sick swine, but its isolation in feed or in the environment is very low. Therefore the major reservoir of *S*. Cholerasuis is the swine and transmission occurs primarily through infected swine (Schwartz 1991). According to Ferris et al. (1991), *S*. Typhimurium and *S*. Derby are the two serotypes that cause most of the clinical cases of salmonellosis in cattle in the United States (Smith et al. 1994). *S.* Dublin is typically isolated from ill cattle (CDC 2000). Infected animals that shed *Salmonella* through feces asymptomatically are called carriers. This is important because they can serve as a reservoir for further spread. According to House et al. (1993), cattle that recover from infections from *S.* Typhimurium and from the group B, C and E *Salmonella*, stop shedding *Salmonella* in 3 to 12 weeks whereas cattle infected with *S.* Dublin and group D can become chronic carriers (Anderson et al. 2001).

Table 9. Five most common *Salmonella* serotypes isolated from plants processing under HACCP(1) plan from meat and poultry during 1998.

	Large broiler HACCP plan	**Large ground beef HACCP plan**	**Large ground turkey HACCP plan**	**Large swine HACCP plan**
1	*S*. Kentucky	*S*. Anatum	*S*. Hadar	*S*. Derby
2	*S*. Heidelberg	*S*. Hadar	*S*. Heidelberg	*S*. Typhimurium var. Copenhagen
3	S. Typhimurium var Copenhagen	*S*. Muenster	*S*. Senftenberg	*S*. Agona
4	*S*. Typhimurium	*S*. Meleagridis	*S*. Reading	*S*. Dranderburg
5	*S*. Hadar	*S*. Typhimurium var. Copenhagen	*S*. Saintpaul	*S*. Schwarzengrund

(1) Hazard Analysis Critical Control Point.
(Adapted from USDA FSIS 1999).

Most of the foodborne related *Salmonella* are non-host specific and are pathogenic for humans and animals (Jay 2000). According to Rings (1985) most of the serotypes isolated from cattle tend to be non-host adapted (Fedorka-Cray et al. 1998).

Several factors have been linked to the presence of *Salmonella* in animals including exposure to new animals without quarantine, use of lagoon wastewater, not properly monitoring feed components, presence of rodents or wild animals, rendering trucks, and inadequate handling of sick animals (Smith et al. 1994). Stress factors such as transportation, food deprivation or confinement may also increase the spread of *Salmonella*. *Salmonella* shedding in feces tends to be intermittent (Corrier et al.1990).

Salmonella **in soil**

According to Thomason et al. (1975) and Bohm (1993), *Salmonella spp.* can survive in the environment for long periods of time (Letellier et al. 1999). The survival of *Salmonella* in soil for years has been shown and this increases the probability of infecting a new host (Winfield and Groisman 2003).

Salmonella can move in soil horizontally, being able to be spread from one location to another, or vertically, contaminating wells and water reservoirs. The vertical movements of bacteria in soil will depend on the soil type, pH, soil water content, surface properties, plants and temperature (Mawdsley 1995). Chandler et al. (1980) proved that the moisture content of the soil was a limiting factor for *S.* Typhimurium survival, although they were still able to isolate them from dry

soils after 14 days. Zibilske and Weaver (1978) also that found that moisture (dry) and temperature (high) were limiting factors for *Salmonella* Typhimurium survival in soil.

Soil type has an impact on the survival and spread of *Salmonella,* since impermeable soils are more prone to runoff after heavy rains as opposed to more permeable soils where the bacterial content can be absorbed (Mawdsley et al. 1995). The movement of pathogens was reported to occur easier in coarse soils that in fine soils (Mawdsley et al. 1995).

Other factors also play a major role in survival of *Salmonella* in soil (Gudding and Krogstad 1975) such as the presence of indigenous flora (Hussong et al. 1985). Turpin et al (1993) showed the prevalence of *Salmonella* was greater in sterile soil. In non sterile soil, Turpin et al. (1993) suggested that *Salmonella* undergoes a state called viable-but-non-culturable in soil.

Salmonella **in feces/litter**

Sick animals can shed *Salmonella* in their feces and contaminate other animals through the oral-fecal route or through contamination of the farm environment. Asymptomatic carriers can shed *Salmonella* in feces at a prevalence of 3-4% (Smith et al. 1994), although according to Fedorka-Cray (2000), the prevalence of fecal shedding in asymptomatic cattle is only 1.4%. The presence of *Salmonella* in feces can be used to estimate the environmental contamination (Irwin et al. 1994), although presence in feces may underestimate

the prevalence of *S*. Dublin in cows due to the low shedding ratio of this microorganism by the carriers (Smith et al. 1899; 1991; 1994).

Salmonella has been shown to survive for long periods of time under different environmental conditions (Morse et al. 1982). Even the host adapted *S.* Cholerasuis serotype can survive in swine feces and remains detectable in dry feces for up to 2 to 4 months (Gray and Fedorka-Cray 2001).

Extensive research on poultry litter and feces has been done to reduce and control the presence of *Salmonella* and prevent further contamination (Opara et al. 1992; Carr et al. 1995; Mallison et al. 2000; Hayes et al. 2000; Eriksson et al. 2001). *Salmonella* is unequally distributed through poultry houses and high Aw is a risk factor for *Salmonella* growth (Hays et al. 2000). Eriksson et al. (2001) concluded that *Salmonella* was detected in poultry samples with high Aw (0.90) and moisture content greater than 35%. Aw values of 0.90-0.95 in dry litter have also been related to the presence of *Salmonella* (Carr et al. 1995). Aw, moisture content and airflow seem to be critical factors which must be controlled to reduce the growth of *Salmonella* in poultry litter (Mallison et al. 2001).

Although most of these studies have been done with poultry litter and feces, this principle could be applied to any farm animal manure management plan. Areas exposed to some air flow had drier litter and lower bacterial counts.

Salmonella **in water**

Water is an important source of *Salmonella* spread and contamination. *Salmonella* shed by humans can be isolated in municipal sewage (Kinde et al.

1997). The contamination by human sewage of water effluents for irrigation of plants used as cattle feed can lead to cattle infection (Anderson et al. 2001). According to Kinde et al. (2000), poultry contamination may be related to human sources through effluent that contaminates rodents, which can be an environmental source for poultry contamination (Roy et al. 2001). Rivers can spread *Salmonella*, becoming a vehicle for contamination of farm downstream contributing to the regional spread of *Salmonella* serotypes (Anderson 2001). Lamar (2003) studied the influence of a farm on the TN river and found that the farm did not contribute to a great extent to the river contamination since the samples upstream from the farm were already positive for *Salmonella*. Rivers downstream from farms can be contaminated due to heavy rains through runoff (Martinez-Urtaza et al. 2004) or through irrigation. Water runoffs will move pathogens but this will depend on the soil type, rate of rainfall and the topography (Mawdsley et al.1995). More permeable soils will transfer bacteria more quickly, possibly contaminating land drains or ground water (vertical movement of microorganisms). Jawson et al. (1982) found higher amounts of fecal contaminants from runoffs from grazed soil than from non grazed soils, even after removal of the cattle (Mawdsley et al. 1995). Flushing water to remove manure from alleys in dairy cattle facilities has been considered a risk factor to increase shedders among cattle (Kabagambe et al. 2000).

Salmonella in feed

According to Bailey et al. (2001), Erwin first discovered viable *Salmonella* in poultry feed in 1955. The presence of *Salmonella* in animal feed is considered to be one of the major factors causing contamination and spreading of the bacteria among animals. Contamination of feed can occur prior to arrival to the farm in the processing plant and during transporting, or at farm level through animals and environment. According to Lo Fo Wong et al. (2002), feed can serve as a way to introduce *Salmonella* into the farm or as a way to establish infection in animals. *Salmonella* has been isolated from animal feed but the serotypes isolated do not usually correlate with the most prevalent ones found in humans and animals (Jay 2002; Bredens et al. 1996; Fedorka-Cray et al. 1998), although Krytenburg et al. (1998) found one *S.* Typhimurium in cattle feed in the Pacific Northwest. Another study found one serotype of *Salmonella* in meat that was also found on the final processed carcass, although 10 different serotypes were identified in feed (Bailey et al. 2001). This might be due to the limited microbiological surveys on animal feeds, and different infectious dose for animals and humans (Crump et al. 2002). Crump et al. (2002) showed the relationship between contaminated animal feed and human outbreaks of salmonellosis. They described the importance of *S*. Hadar and *S.* Agona serotypes in human outbreaks and how *S*. Agona has increased in human cases since its introduction in animal feeds in the US.

Krytenburg et al. (1998) studied the prevalence of *S. enterica* in the Pacific Northwest cattle. They found several types of contaminated feed including grain, wet and dry forages, by products and protein supplements. They found an

incidence of 9.8% *Salmonella* in cattle feed whereas for swine feed the incidence was 2.8% (Harris et al. 1997). Irwin (1994) found that 9.8% of feed for turkeys was contaminated with *Salmonella*. Out of 26 flocks, 12 shared the same serovars in feed and environment (Irwin 1994). Environmental contamination at the farm is also important. In one study, six farms had the same serotype in feces and feed, whereas in five, the serotype isolated in feed was not present in feces (Davies et al. 1997). Other sources of feed contamination include rodents, wild birds (Harris et al. 1997) or irrigation water contaminated with human sewage (Anderson 2001).

Samuel et al. (1988) studied the effect of feed management in cattle before slaughter. They fed three groups of animals different diets (first group taken to the feedlot and slaughter within two days, the second group fed *ad libitum* for 18 days and the third group fattened and fed for 80 days). At time of slaughter, rumen and lymph node samples were analyzed, as well as soil samples. The major number of *Salmonella* was isolated from cattle from the second group, whereas none was isolated from the third group. High levels of Volatile Fatty Acids (VFA) and a low ruminal pH can decrease the numbers of *Salmonella* present in the rumen (Samuel et al. 1988). The study concluded that adjustments to a new diet resulted in higher incidence of isolation of *Salmonella*. According to Jones (1992) and Grau et al. (1969), *Salmonella* survival is increased by the reduction in dry matter intake by reducing production of VFA in the rumen (Anderson et al. 2001). There are some differences in the serotypes shed by feces in feedlot cattle compared to non-feedlot cattle that might be related to

differences in the ruminal pH (Beach et al. 2002). Davies (1994) stated that contamination in ruminants with *Salmonella* is primarily due to the consumption of rations rich in cottonseed meal and palm kernel (Hinton 2000). Kabagambe et al. (2000) found that feeding TMR (Total Mixed Ration) to cattle increased *Salmonella* shedding.

Glikman et al. (1981) found that the source of a *S*. Anatum outbreak in cattle was the silage that was used to feed the cattle. Contamination of the hayfield with bird droppings and improper silage conditions were the cause of the outbreak. The survival of *Salmonella* in grass undergoing silage will depend on conditions under which it is ensiled and the final pH (Anderson et al. 2001).

Salmonella can survive in dry environments for long periods on time (such as on feed) (Juven et al. 1984). Pelleting seems to reduce the contamination of animal feed but only low levels of contamination can be reduced through pelleting, whereas high levels might not be reduced (Fedorka-Cray et al. 1997). Davies et al. (1997) found that the prevalence of *Salmonella* in swine farms feeding pelleted rations (38.1%) was higher than farms feeding meal rations (5.7%) in North Carolina. Juven et al. (1984) estimated the survival of *Salmonella* in poultry feed and bone meal to be 14 weeks.

Contamination in the milling plant when preparing animal feed has been shown to occur. Whyte et al. (2003) studied the prevalence of *Salmonella* in a poultry feed mill. They found that the recovery of *Salmonella* in feed in the preheating area was 18.8% (11.8% feed ingredients and 33.3% dust), whereas post-heating recovery of *Salmonella* was 33% (dust samples 24.2%). The

environment in major animal feed operations plays a major role in controlling *Salmonella* contamination (Whyte et al. 2003). Introducing contaminated raw ingredients to the mills is common (Whyte et al. 2003), which may cause feed ingredients to be an important source of *Salmonella*. A survey conducted by the FDA (McChesney et al. 1995) showed that *Salmonella* was detected in 56.4% of animal protein and 36% of vegetable proteins used for animal feed. During an outbreak of *Salmonellosis* in California, fat added to the feed ratio seemed to be directly related to the infection in cattle attributed to *S*. Menhaden (Anderson et al. 1997). The amount of fat added to the feed had implications also on the differences in morbidity rates among herds (Anderson et al. 1997). Harris et al. (1997) found that the S*almonella* isolation rate in feed and feed ingredients was 2.8% and was isolated from 46.7% of the farms. Seven of the serotypes obtained were *S*. Worthington, Agona, Anatum, Montevideo, Heidelberg, Oranienburg and Derby. Barber et al. (2002) did not isolate any *Salmonella* from swine feed samples.

Transportation is another point where feed can get contaminated with *Salmonella*. Fedorka-Cray et al. (1997) sampled feed and feed trucks and found an isolation rate of 0.7% in feed (included meat/bone meal, fish, bonemeal, meatmeal, and soybean meal) but 22.7% in trucks. Whyte et al. (2003) found that the feed transport vehicles had a rate of contamination with *Salmonella* of 57.1%. Proper truck sanitation and purchasing *Salmonella*-free animal feed may be a key points to control the *Salmonella* contamination of food.

Other environmental sources

Several environmental sources contribute to *Salmonella* contamination in the farm and might also serve as a possible reservoir for *Salmonella*. Barber et al. (2002) sampled several environmental swine compartments and found that the environmental samples that showed higher *Salmonella* recovery were cats (12%), boots (11%), bird feces (8%), flies (6%), and mice (5%).

Birds at the farm are a possible reservoir for *Salmonella* that can contaminate the environment as well as the feed, or can contaminated themselves through ingesting contaminated feed. The presence of *Salmonella* in wild birds has been well established (Kirk et al. 2002; Craven et al. 2000).

The presence of rodents has been established as a risk factor for *Salmonella* contamination (Warnick et al. 2001). Mice present in farms are possible contaminants spreading *Salmonella* through feces (Davies and Wray 1995). Barber et al. (2002) found that the number of positive samples in bird droppings and mice were related to the positive *Salmonella* samples in cats.

Letellier et al. (1999) found that most of the environmental samples (flies, rodents, spiders, bird fecal material) were positive for *Salmonella* at clinically ill swine farms. Flies play such an important role in spreading *Salmonella* that Bailey et al. (2001) suggested flies as a cheap way to monitor the presence of *Salmonella* on farms since they found 18.6% positive *Salmonella* recovery from fly samples. Humans are another source of contamination for animals (Barber et al. 2002). Cross contamination through boots has been well established (Barber et al. 2002; Bailey et al. 2001; Letellier 1999; Radke et al. 2002).

Farm management

Stress during transport and feed deprivation of the animals can lead to rapid growth of *Salmonella* in the gastrointestinal tract, and when followed by provision of a feedyard, can increase the fecal shedding of *Salmonella* (Corrier 1990). Prevalence of shedding in cattle due to transportation was found to be 1.9% (Sorensen et al. 2001). Beach et al. (2002) studied the prevalence of *Salmonella* in beeflot cattle from transport to slaughter. They found that the shedding of *Salmonella* was increased for adult cattle from 1 to 21% during transport, whereas for feedlot cattle it remained constant. The reason might be the diet since it changes the liquor composition of the rumen (Samuel et al. 1988). Use of calves in feeder marketing, and moving from one location to another through feedyard fattening has been shown to increase the shedding of *Salmonella* in feces (Corrier et al. 1990).

Regional differences in the isolation of *Salmonella* have been shown in the US, with *Salmonella* being most prevalent in the Southern US (Kabagambe et al. 2002). *Salmonella* in swine was most prevalent in the southeastern US (65.5% of operations positive), whereas the Midwest and Northcentral states showed a lower prevalence in their operations (29.9% and 36.1% respectively) (USDA 1997a). The prevalence of *Salmonella* in feedlot cattle was higher in USDA region one (AZ, CA, ID, WA) than the other two regions (IA, IL, MN, SD and CO, KS, OK, NE, TX) (Fedorka-Cray et al. 1998). *Salmonella* was isolated with higher prevalence from the South Central, Central and Southeastern US (Dargatz et al. 2000). These differences are probably due to the climate differences but

conflicting climatic data have been reported (Fedorka-Cray et al. 1998; Dargatz et al. 2000; Sorensen et al. 2002; Barber et al. 2002). Bailey et al. (2001) found that *Salmonella* was more frequently isolated during the fall and winter than during spring and summer in vertically integrated poultry operations.

Herd size and the use of flush-water systems are reported to be important risk factors associated with shedding of *Salmonella* (Kabagambe et al. 2000). Herd size increased *Salmonella* shedding in swine (USDA 1997b). Large-size cattle operations tend to bring more cattle into the operations, increasing the risk of contact with subclinically ill cattle and the effect of other stress factors (Kabagambe et al. 2000). Stress caused by transportation of animals increased shedding of *Salmonella* (Barber et al. 2002). Letellier et al. (1999) studied *Salmonella* contamination at two swine farms using integrated production systems and found an overall *Salmonella* prevalence of 7.9%. They found higher rates of isolation of *Salmonella* in replacement gilts (15.9%) and finishing units (21.9%). This study concluded that *Salmonella* was introduced to the farm via carriers at the breeding level (Letellier et al. 1999).

Similar results were obtained by Warnick et al. (2003) who found a significant association between herd size and fecal shedding of *Salmonella* when they studied 65 dairy herds with a recent history of salmonellosis. Warnick et al. (2001) in a separate study concluded that herd size was a risk factor when risk factors associated with clinical salmonellosis in Virginia cattle were studied. Fedorka-Cray et al. (1998) reported that smaller herds have less *Salmonella* recovery than larger operations. One study in beef cattle concluded that the herd

size was not correlated with the shedding of *Salmonella* in beef cattle (Fedorka-Cray et al. 2000).

Molecular diversity

Traditional methods to identify *Salmonella* are based on phenotypic characteristics (FDA 1998). New methods based on DNA patterns are used to identify and compare genetic differences among isolates. These techniques are very useful to study the epidemiology of outbreaks and to track isolates of *Salmonella*. Some of these techniques, such as Riboprinter and Pulsed-Field Gel Electrophoresis (PFGE) are based on the use on restriction enzymes targeted at DNA that cut the DNA into pieces or fragments. The resulting fragments are separated into bands based on molecular weight (Oscar 1998).

Riboprinter®

The Riboprinter® (Qualicom, Wilmington, DE) is an automated system for analysis of ribosomal DNA. This technique is based on the use of restriction enzymes that especially cut ribosomal DNA. DNA fragments are separated by size on an agarose gel and then hybridized with a labeled probe. A computerized camera captures the band images that can be use for further analysis. The Riboprinter® compares band pattern with a library and classifies *Salmonella* isolates into ribogroups. Riboprinter® was found to have limited use for identifying *Salmonella* based on band match but was effective in characterizing *Salmonella* serotypes (Oscar 1998). Several restriction enzymes have been

used, but *Pvu*I seems to give greater band patterns differentiation (Bailey et al. 2002).

Pulsed-Field Gel Electrophoresis

PFGE has been considered the gold standard of molecular typing techniques for many years (Olive and Bean 1999). The bacterial genome is cut by the action of restriction enzymes and separated by electrophoresis. PFGE uses pulsed electrical fields in order to separate large molecular weight fragments (Olive and Bean 1999). The bands on the gel are commonly visualized with ethidium bromide. Although PFGE is superior to other typing technique (Olive and Bean 1999; Yan et al. 2003), the success in discrimination among isolates depends on the serotype (Liebana et al. 2001). Although PFGE is more sensitive in differentiating among strains, Riboprinter® is very useful when a large volume of samples are to be analyzed (Pfaller et al. 1996; Hollis et al. 1999) or when highly trained personnel is not available. PFGE has been used to study clonal *Salmonella* populations in swine and other food applications (Wonderling et al. 2003; Baloda et al. 2001; Sandvang et al. 2000). PFGE is also the method of choice used by CDC for characterizing *Salmonella* isolates under the PulseNet program.

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Part II: Multistate Study to Determine the Presence of *Salmonella spp.* in Dairy Cows, Beef Cattle and their Environment

Abstract

Salmonella is the second most important cause of foodborne infections in the United States after *Campylobacter jejuni*. Human cases of salmonellosis are often linked to the consumption of raw meat and unpasteurized milk and dairy products. Although fecal-oral contamination among animals has been well established, less is known about contamination through the environment at the farm level. The objective of this study was to compare isolation of *Salmonella* in dairy cows and beef cattle and their farm environments based on temporal, spatial, and environmental factors, including production or management practices to provide population-based epidemiological information for *Salmonella* that can be used in assessing risk and developing risk management strategies. Samples from 8 locations in four different states (Alabama, Tennessee, California and Washington) were collected over 21 months. Environmental samples (n=240) and fecal swab samples (n=800) were analyzed to determine the presence of *Salmonella* using modified BAM protocols. In beef cattle, *Salmonella* was recovered from feed (3.1%), soil (2.0%), and bedding (1.0%). In dairy cows, *Salmonella* was recovered from fecal swabs (1%), feed (4.2%), soil (3.1%), and bedding (4.1%). The most common serotypes isolated from beef cattle were *S*. Anatum (89.9%) and *S*. Newington (11.1%). For dairy cows, the most common serotypes isolated were *S*. Anatum (56%), *S*. Newington (20%), *S*. Javiana (8%), and non serotyped *Salmonella spp*. (16%). *Salmonella* was found to be more prevalent in dairy cows than beef cattle possibly due to differences in management practices and their environment. The environment appeared to be

the major reservoir for *Salmonella*. Breaking the contamination cycle between animals and their environment will be essential to reduce the isolation rate of *Salmonella* in beef cattle and dairy cows.

I. Introduction

Salmonella is the second most important cause of foodborne infection in the United States (US). There are about 1.4 million cases of human salmonellosis every year in the US (FDA 2004), with an estimated annual cost of $ 2.9 billion (ERS USDA 2004). Human salmonellosis is often related to the consumption of or exposure to raw meat, and unpasteurized milk and milk products (Jay 2000; MMRW 1995; MMRW 2003). The top ten serotypes from human cases in 2002 were *S*. Typhimurium, *S*. Enteriditis, *S*. Newport, *S*. Heidelberg, *S*. Javiana, *S*. Montevideo, *S*. Muenchen, *S*. Oranienburg, *S*. Saintpaul, and *S*. Infantis (CDC 2003). The most common serotypes isolated from clinically ill bovine during the same period were *S*. Newport, *S*. Typhimurium, *S*. Dublin, *S*. Agona, and *S*. Montevideo (CDC 2002). The most common serotypes isolated from non-clinical cases were *S*. Montevideo, *S*. Senftenberg, *S*. Dublin, *S*. Kentucky, *S*. Anatum (CDC 2002). The most common serotypes of *Salmonella* isolated from beef cattle in the US were *S*. Anatum, *S*. Montevideo, *S*. Muenster, *S*. Kentucky, and *S*. Newington (Fedorka-Cray et al. 1997). The most common serotypes of *Salmonella* in feedlot cattle (USDA 2001) were *S*. Anatum, *S*. Montevideo, *S*. Reading, *S*. Newport, and *S*. Kentucky. In 1996, the USDA conducted “Dairy’96”, a national survey to estimate the isolation rate of *Salmonella* in US dairy operations (USDA 1997). The most common serotypes obtained in dairy cows were *S*. Montevideo, *S*. Cerro, *S*. Kentucky, *S*. Menhaden, *S*. Anatum, and *S*. Meleagridis (Wells et al. 2001). Most of these studies focused on *Salmonella* isolation from fecal swabs or fresh feces, but little research has been done to

establish the significance of the environment as a possible reservoir of *Salmonella*. In 1998, the USDA FSIS established a mandatory HACCP program in slaughter plants in order to decrease the cases of human salmonellosis and diseases due to other enteric pathogens. A decrease in isolation of foodborne diseases in the US was noted for the first time since CDC began intensive monitoring in 2003 (CDC 2003).

Serotypes of *Salmonella* associated with clinical animal cases in 2002 were similar to serotypes in human cases, but most of the non-clinical cases did not account for the top ten serotypes causing foodborne illness in humans (CDC 2002). This gives importance to the carrier state, where the animal may shed *Salmonella* with no obvious sign of sickness. Shedding of *Salmonella* through feces may contaminate the farm environment, which becomes an important source for *Salmonella*. Control of *Salmonella* in animals at the farm level is important to reduce further contamination at the slaughter plant.

The objective of this study was to evaluate associations in farm animals and the surrounding environment over time to provide a better understanding of the distribution of *Salmonella* on the farm. This will help to establish better control programs and intervention strategies at the farm level to reduce *Salmonella* contamination of animals before slaughter.

II. Material and Methods

Sample collection: Samples were collected from a total of 8 farms in four different states (Tennessee, Alabama, Washington, and California) during a

period of 21 months (from August 2002 to June 2004). Farms were selected based on previous collaborative studies with the University of Tennessee and the Universities at the states sampled. Each farm was sampled every 3-4 months, obtaining a total of 6 samplings per farm. Several sample types were collected for each sampling period. A total of 1040 samples were analyzed. Rectal swabs were collected from 20 randomly selected cows by the person taking the samples (n=800). All other sample types originated from the surrounding environment (n=240). From beef cattle farms, soil samples were taken from 3 locations, the grazing area, the watering, resting area, and area inaccessible to cattle. From the dairy farms, soil was collected from areas near the holding facilities, and bedding samples were collected from inside the holding area itself. From each location, feed/foodstuff samples were collected from whatever source the animals were feeding at the time. These samples were diverse and included pasture grass, Total Mixed Ration (TMR, a mixture of silage and high energy feedstuffs), hay, and silage. A sample protocol was developed (box containing empty sample container and instructions for sample collection). Samples were collected by the farmers and Geographical Position System (GPS) coordinates were recorded at the sampling place using eTrex (Garmin, Olathe, KS). Samples were sent to the University of Tennessee overnight and kept refrigerated at 4^{o} C until completely analyzed within the next 3 to 4 days.

Farm description: Four different farms were sampled for beef cattle and dairy cows. Beef cattle farms belonged to universities, with the exception of a Washington farm that was privately owned. Dairy cow farms belonged to

universities with the exception of one located in California that was privately owned.

Salmonella isolation: Samples were analyzed for the presence of *Salmonella* using FDA-BAM modified methods (Pangloli et al. 2003). All the media used was obtained from Difco (Sparks, MD) except Rappaport-Vassiliadis (RV) that was obtained from Difco and Oxoid (Ogdensburg, NY). The rectal swabs were first transferred into a sterile tube containing 10 ml of Universal Broth in order the divide the sample to analyzed for the presence of different pathogens. Then, 1 ml was transferred into a tube containing 10 ml of RV broth or Tetrathionate broth (TT) (added Brilliant Green 20 ml/l and iodine 10ml/l), and incubated as described in Table 1. For the environmental samples, 25 grams were weighed and mixed with TT or RV (225 ml) and incubated as described in Table 1. Dairy and beef samples were weighed (25 grams) and mixed with 225 ml of Lactose Broth (LB) and incubated as described in Table 1. Pre-enrichment broth (1ml) was transferred into a sterile test tube with 10 ml of TT and incubated (Table 1). All samples were streaked for isolation onto XLT4 (xylose-lysine-Tergiol 4)/BSA (bismuth sulfite agar) plates (Table 1).Typical *Salmonella* colonies on XLT4 (black) and atypical colonies (yellow with red background) were selected for further confirmation. Typical BSA *Salmonella* colonies (metallic sheen) were also selected. Presumptive colonies were transferred onto TSI (Triple Sugar Iron) agar tubes and into Urea broth and incubated at 35.5C for 24 hours. A TSI result of K/A (red slant /yellow butt) or K/S (red slant/ black precipitate), and urea negative was considered presumptive positive for

Table 1. Protocol followed for the isolation of *Salmonella* from dairy and beef cattle fecal swabs and environment. This protocol was done as described by Pangloli et al. 2003.

Origin of sample	Pre-enrich. T [1] t Medium	Enrichment T t Medium	Plating T t Medium
Rectal swab	-	RV[3] 42C 24h	BSA[5] 35.5C 48h
Feed	LB[2] 35.5C 24h	TT[4] 35C 24 h	BSA 35.5C 48h
Soil	-	TT 42C 24h	XLT4[6] 35.5C 24h
Bedding	-	RV 42C 24h	XLT4 35.5C 24h

(1)T: time; t: temperature.
(2) LB= Lactose Broth.
(3) RV= Rappaport-Vassiliadis.
(4) TT= Tetrathionate.
(5) BSA= Bismuth Sulfate Agar.
(6) XLT4= Xylose-Lysine-Tergitol 4 Agar.

Salmonella. Presumptive colonies were kept for further serological analysis with somatic antigen O (Difco, Sparks, MD). A TSI result of K/A (red slant /yellow butt) or K/S (red slant/ black precipitate), and urea negative was considered presumptive positive for *Salmonella.* Presumptive colonies were kept for further serological analysis with somatic antigen O (Difco, Sparks, MD). Agglutination was considered positive for *Salmonella*. Positive antigen O colonies were analyzed using Analytical Profile Index (API) 20E (Biomeriux, Hazelwood, MO) to confirm the presence of *Salmonella spp* and or *S*. Arizona.

Aerobic Plate Count: Aerobic plate count (APC) was prepared according to Feldsine et al. (2003) using SimPlate (Biocontrol, Bellevue, WA). Fecal swabs were vortexed in 10 ml lactose broth. For other samples, 25 grams were weighed into sterile filtered bags with 225 ml of 0.1% peptone water (% w/v) and 10-fold dilutions were prepared. SimPlates were incubated at 35C for 24h. The total number of wells showing color change were counted. To calculate the MPN (most probable number) of organisms, the conversion Table provided by the manufacturer (Biocontrol, Bellevue, WA) was used and multiplied by its corresponding dilution.

Total Coliforms/ *Escherichia coli* :Total Coliforms and *E. coli* were enumerated using SimPlates using instructions provided by the manufacturer (Biocontrol, Bellevue, WA). SimPlates were incubated at 35C for 24 h. Total coliforms were counted based on wells with color change. *E. coli*, were counted by observing wells that fluoresced under UV light. The total MPN of coliforms/ *E. coli* was

calculated using the SimPlate conversion Table provided by manufacturer (Biocontrol, Bellevue, WA).

Fecal streptococci: Fecal streptococci were counted as described by Downes and Ito (2001). For fecal swabs, the swabs were vortexed in 10 ml lactose broth. A 1 ml aliquot was transferred into sterile tubes containing 9ml of 0.1% peptone water. For other samples, 25 grams was weighed into sterile filtered bags with 225 ml of 0.1% peptone water (%w/v). Decimal dilutions were prepared. A 1ml aliquot from each dilution was pourplated with 15-20 ml. KF Streptococcus Agar (%). Plates were incubated at 35C for 48h. Pink colonies were counted using a manual colony counter.

Riboprinter: Frozen isolates were thawed and streaked onto BHI Agar at 35.5C for 24 h. Samples were processed using a RiboPrinter (Qualicom, Wilimgton, DE) manufacturer's protocol using *Pvu*II as the restriction enzyme (Bailey et al. 2002b). The ribosomal DNA fragments were digested and processed on a nylon membrane and hybridized with an *E. coli* DNA probe. Using chemiluminescent, the image was captured by a CCD camera and taken to computer software for further analysis (Bruce 1996). The RiboPrinter identified isolates by band matching and also classified them into ribotypes (Oscar 1998).

III. Results and Discussion

Our study was part of a multistate study to determine the isolation rate of *Salmonella* in farm animals and their environment. Comparative data on swine, turkey, and chickens is provided courtesy of the University of Tennessee and the

Food Safety Center of Excellence (Draughon 2004). Data on swine, chickens and turkey are adapted from a recent presentation and are shown in Table 2 for comparison purposes (Rodriguez et al. 2004a, 2004b). *Salmonella* isolation in beef cattle was the lowest among all of the animals tested (Table 2). Overall, *Salmonella* was more frequently isolated in dairy cows (11.5%) than in beef cattle (6.2%) (Table 2).

Salmonella in fecal swabs and bedding

Salmonella was not isolated from any of the beef cattle fecal swabs tested, whereas for dairy cows, the isolation rate was 1% (Table 3). The isolation rate of *Salmonella* in bedding samples was also lower for beef cattle (1%) than for dairy cows (3.2%). Other studies have found an isolation rate from fecal swabs and/or feces as high as 5.5% (Losinger et al. 1997), 5% (Fedorka-Cray et al.1998), 6.3% (USDA 2001), and 11.2% (Dargatz et al. 2000) for beef cattle. Fecal shedding reported in dairy cows was 5.4%, (Wells et al. 2001) and 2.1% (Losinger et al. 1995), which are similar to the results, found in our study.

The background microflora from fecal swabs were very consistent, showing high values of total coliforms, fecal streptococci, and *E. coli* (Tables 4, and 5). The reason we found lower isolation rates in fecal swabs from beef cattle compared to other studies may be due to a variety of reasons. Animals in our study were not from feedlots and beef cattle density was low. Dairy cows were primarily from university experiment stations rather than commercial facilities and probably had similar herd density. Beef cattle bedding material consisted of mixtures of dry feces and soil. For dairy cows, bedding consisted of a mixture of

Table 2. Percentage of *Salmonella* isolation by state and farm animal type over a period of 21 months (August 2002 to June 2004). Five different states were sampled, Tennessee, Alabama, California, Washington and North Carolina.

	TN	AL	CA	WA	NC	AVERAGE
			% positive			
Beef	12.5	8.3	4.2	0.0	ns(1)	6.2
Dairy	8.3	16.7	4.2	16.7	ns	11.5
Swine(2)	6.3	Ns	10.3	6.3	7.3	7.8
Poultry(2)	12.5	Ns	ns	16.7	5.0	11.4
Turkey(2)	ns	Ns	ns	ns	30.0	30.0

(1) ns: not sampled.
(2) Data obtained from Rodriguez et al. 2004a, and Rodriguez et al. 2004b.

Table 3. Percentage of *Salmonella* isolated over a period of 21 months (August 2002 to June 2004) in beef cattle and dairy cows rectal swabs and their environment. Four different states were sampled for dairy cows and beef cattle: Tennessee, Alabama, California, and Washington.

BEEF	SWAB[1]	FEED[2]	SOIL[3]	BED[4]
		% Positive		
TN	0.0	4.2	4.2	4.2
AL	0.0	4.2	4.2	0.0
CA	0.0	4.2	0.0	0.0
WA	0.0	0.0	0.0	0.0
Average	0.0	3.1	2.0	1.0
DAIRY	SWAB	FEED	SOIL	BED
TN	0.0	4.2	4.2	0.0
AL	0.0	4.2	8.3	4.2
CA	0.0	4.2	0.0	0.0
WA	4.2	4.2	0.0	8.3
Average	1.0	4.2	3.1	12.5

(1) Rectal swab.
(2) Feed varied with season, animal type, and state.
(3) Soil was obtained from the grazing areas, watering resting areas and inaccessible areas for beef cattle. For dairy cows soil was collected from areas near the holding area.
(4) Bedding was composed of a mixture of dry feces, soil and/or peanuts hulls.

Table 4. Enumeration of fecal microorganisms and percentage positive *Salmonella* in rectal swabs, bedding and soil from beef cattle premises by sample type for each state.

Beef		TN	AL	CA	WA
Rectal swab	APC[(1)(2)]	10	9.9	9.8	10.1
	Total coliforms[(2)]	9.2	9.6	9.5	9.7
	E coli[(2)]	8.9	9.4	9.1	9.6
	Fecal *Streptococcus*[(2)]	7.8	8.0	7.2	7.9
	% *Salmonella*[(3)]	0.0	0.0	0.0	0.0
Holding material	APC	7.1	8.2	7.8	7.8
	Total coliforms	5.9	8.1	6.5	5.3
	E coli	4.8	8.1	6.5	5.3
	Fecal *Streptococcus*	4.7	7.0	4.5	7.5
	% *Salmonella*	4.2	0.0	0.0	0.0
Soil	APC	6.7	7.6	7.5	8.3
	Total coliforms	5.5	6.3	4.2	5.4
	E. coli	4.6	6.2	3	4.7
	Fecal *Streptococcus*	3.5	4.8	6.8	3.2
	% *Salmonella*	4.2	4.2	0.0	0.0

(1) APC= Aerobic Plate Count.
(2) The APC, Total coliforms, *E. Coli* and Fecal *Streptococcus* are given in log CFU/recal swab and logCFU/gram sample.
(3) *Salmonella* is given in percentage of isolation by state and sample type.

Table 5. Enumeration of fecal microorganisms and percentage positive *Salmonella* in rectal swabs, bedding and soil from dairy cows premises by sample type for each state.

Dairy		TN	AL	CA	WA
Fecal swab	APC[(1)(2)]	10.3	9.9	9.9	9.5
	Total Coliforms[(2)]	9.4	9.2	9.3	8.8
	E. coli[(2)]	8.8	9.6	8.8	8.6
	Fecal *Streptococcus*[(2)]	7.6	7.7	8.2	7.8
	% *Salmonella*[(3)]	0.0	0.0	0.0	4.2
Bedding material	APC	8.0	9.0	8.6	7.7
	T. Coliforms	6.3	7.2	7.9	7.4
	E. coli	5.4	6.7	6.7	6.3
	Fecal *Streptococcus*	5.2	7.0	7.3	6.5
	% *Salmonella*	0.0	4.2	0.0	8.3
Soil	APC	7.0	8.9	7.3	6.8
	Total Coliforms	4.9	6.6	6.9	3.8
	E. coli	4.1	6.4	5.1	3.6
	Fecal *Streptococcus*	3.8	4.3	5.0	6.4
	% *Salmonella*	4.2	8.3	0.0	0.0

(1) APC= Aerobic Plate Count.
(2) The APC, Total coliforms, *E. Coli* and Fecal *Streptococcus* are given in log CFU/fecal swab logCFU/gram sample.
(3) *Salmonella* is given in percentage of isolation.

dry feces, fresh feces, soil, and/or peanuts hulls. *Salmonella* was only isolated in beef cattle bedding in Tennessee (4.2%) (Table 3). In 3 states, Alabama, California, and Washington, *Salmonella* was not isolated from bedding or rectal swabs (Table 3).

Background microflora were higher for dairy bedding than for beef bedding material (Tables 4 and 5). The dairy bedding appeared to contain more feces than the beef cattle bedding. *Salmonella* was isolated in 4.2% of Alabama, and 8.3% of Washington dairy cow bedding (Table 3). *Salmonella* was isolated in both bedding material and rectal swabs from Washington dairy cows (Table 3). According to Irwin et al. (1994), feces could be used to estimate the isolation rate of *Salmonella* in farm animals. We found analysis of feed a better estimation of the *Salmonella* status on the farm. This is probably also due to the type of fecal samples we obtained.

Feces may underestimate the isolation rate of *Salmonella* because carriers only shed *Salmonella* at a rate of 3-4% (Smith et al. 1994), although *Salmonella* survival in feces has been well established (Gray and Fedrorkra-Cray 2001).

Serotypes in fecal swabs and bedding material

The serotypes that we most commonly isolated from beef cattle feces were *S*. Anatum and *S*. Newington (Table 6). From dairy cows, the most common serotypes were found in bedding samples were *S*. Anatum, *S*. Javiana, *S*. Newington, and *Salmonella spp.* (Table 7). From dairy cow fecal swabs, only *S*. Anatum and *S*. Newington were isolated (Table 7). These findings are in

Table 6. *Salmonella* serotypes isolated from beef cattle by state and sample type.

	RECTAL SWAB	FEED	SOIL	BEDDING
TN	-[1]	S. Anatum	S. Anatum	S. Anatum S. Newington
AL	-	S. Anatum	S. Anatum	-
CA	-	S. Anatum	-	-
WA	-	-	-	-
Overall serotype distribution	-	44% *S*. Anatum	33% *S*. Anatum	11.1% *S*. Anatum 11.1% *S*. Newington

(1) No *Salmonella* isolated.

Table 7. *Salmonella* serotypes isolated from dairy cows by state and sample type.

	SWAB	FEED	SOIL	BEDDING
TN	-(1)	S. Anatum S. Newington	S. Anatum	
AL	-	S. Anatum	S. Anatum *Salmonella* spp	S. Anatum
CA				
WA	S. Anatum S. Newington	*Salmonella spp*		S. Anatum S. Javiana S. Newington *Salmonella spp*
Serotypes isolated	S. Anatum 4% S. Newington 4%	*S. Anatum 20%* S. Newington 8% *Salmonella spp*. 4%	S. Anatum 12% *Salmonella spp*. 4%	*S. Anatum 20%* S. Newington 8% S. Javiana 8% *Salmonella spp*. 8%

(1) No *Salmonella* isolated.

agreement with the major national surveys done in beef cattle and dairy cows where S. Anatum was one of the most frequent serotypes isolated in dairy cows (Wells et al. 2000) beef cattle (Fedorka-Cray 1998; Losinger et al. 1997), and feedlot cattle (Beach et al. 2002).

***Salmonella* in soil samples**

Salmonella isolation from soil samples from beef cattle premises was 2.0% overall (Table 3). Only soil samples from two states, Tennessee (4.2%) and Alabama (4.2%), were positive for *Salmonella* (Table 3). Similar results were obtained from dairy cows soil samples, where the isolation rate was 3.1% (Table 3). Samples from Alabama had overall higher background microflora compared to other states. It is interesting to note that *Salmonella* was isolated in both Southern US soil samples but not from soil samples in the Western US. The clay soil type, warm humid climate and strong poultry production history of the region may have contributed to higher recovery of *Salmonella* since clay soils and high humidity have been associated with increased *Salmonella* survival (Zibilske et al. 1978; Mawdsley et al. 1995).

Soil samples were taken from different locations within the farm in each sampling period. Although we do not have enough samples to correlate soil type and the presence of *Salmonella*, our data strongly suggests that *Salmonella* survival in the soils from the Southeast is higher that the other two regions. The survival of *Salmonella* in soil has been demonstrated and this increases the risk of further infection to new host (Winfield and Groisman 2003). *Salmonella* survival in soil has been shown to depend on the soil type, pH, soil water content,

surface soil properties, and presence of plants and temperature (Mawdsley et al. 1995).

The presence of indigenous flora will have an impact on the survival of enteric pathogens in soil (Hussong et al. 1985). *Salmonella* survival in sterile soil has been proved to be higher that in soils where there is a native flora present (Turpin et al. 1993). The native flora will compete with enteric pathogens for nutrients, decreasing its survival. The background microflora did not differ much from one location to another, so probably the soil type and climate had a major impact on the survival of *Salmonella.*

Winfield and Groisman (2003) suggested that the use of *E. coli* as an indicator for *Salmonella* might not be useful in soil due to the longer survival of *Salmonella* in the environment compared to *E. coli*. Our results are in agreement with this, and lead us to suggest that specific tools to target specific pathogens need to be used in soil instead of the use of the indicator bacteria or biomass as a marker.

***Salmonella* in feed**

Feed samples had the highest isolation of *Salmonella* for beef cattle (3.1%), and dairy cows (4.2%) (Table 3). *Salmonella* was found positive in at least one sampling period in all states except Washington. In beef cattle, grass was the most commonly contaminated sample (Table 8), probably due to the presence of feces from carriers within the farm. For dairy cows, TMR was the most contaminated feed (Table 8). *Salmonella* was isolated in all states in dairy cows. The mean isolation rate for *Salmonella* in feed for all four states was 4.2%

Table 8. Isolation of *Salmonella* from different types of feed sources in beef cattle and dairy cows by state. Animal feed varied depending on the season sampled and the state.

		STATE			
Animal type	Sample type	TN	AL	CA	WA
Beef	Grass	+ (1)	+	+	- (2)
	Fresh hay	-	ns (3)	-	ns
	Bunk hay	-	ns	-	ns
	Fresh TMR(4)	ns	ns	ns	-
	Bunk TMR	ns	ns	ns	-
Dairy	Fresh TMR	-	+	+	-
	Bunk TMR	-	+	-	+
	Fresh silage	+	-	ns	-
	Bunk silage	+	-	ns	-

(1) + = positive for *Salmonella.*
(2) - = negative for *Salmonella.*
(3) ns = not sampled.
(4) TMR= Total Mixed Ration.

(Table 3). Krytenburg et al. (1998) found that 9.8% of the feed used for cattle in the Pacific Northwest samples was contaminated with *Salmonella*.

Animal feed contaminated with *Salmonella* has been widely reported (Crump et al. 2002). The FDA (1995) did a survey and concluded that 56.4% of the animal protein and 36% of vegeTable proteins used for animal feed were contaminated with *Salmonella*.

In Tennessee and Alabama, beef cattle were fed on grass during all the sampling periods (Table 8). *Salmonella* was isolated in both states. When indicator (*E. coli* and streptococci) were compared to *Salmonella* isolation rates (Table 9), no pattern was found. Grass samples were highly contaminated, probably due to the presence of fecal material in the pasture area (Table 9). An outbreak of *Salmonella* Newport in dairy herds has been associated with the presence of *Salmonella* in the pasture (Clegg et al. 1983). *Salmonella* survival in pasture was reported to occur for over 14 months, even after the removal of the cows. The use of wastewater to irrigate pasture land has been associated with salmonellosis in herds (Anderson et al. 2001).

California beef cattle were feed on grass and hay depending on the season. Although the Aerobic Plate Count (APC) was higher in bulk hay, total coliforms and fecal *Streptococcus* were found more frequently in fresh hay where *Salmonella* was isolated. Similar values were found in Washington fresh hay and no *Salmonella* was isolated (Table 9). Therefore, there was no association between fecal indicator bacteria and occurrence of *Salmonella* in samples.

Table 9. Enumeration of fecal microorganisms and percentage positive *Salmonella* in different feed types in bee cattle premises for each state

Beef	Microbial	TN	AL	CA	WA
Fresh hay	APC[(1)(2)]			5.8	6.9
	Total Coliforms[(2)]			5.3	5.9
	E. coli[(2)]			1.2	2.1
	Fecal *Streptococcus*[(2)]			4.5	5.4
	% *Salmonella*[(3)]			4.2	0.0
Bunk hay	APC			6.5	
	Total Coliforms			3.9	
	E. coli			1.2	
	Fecal *Streptococcus*			3.5	
	% *Salmonella*			0.0	
Grass	APC	8.3	9.0	8.2	7.8
	Total Coliforms	6.6	8.0	6.8	6.6
	E. coli	5.5	5.7	6.8	5.8
	Fecal *Streptococcus*	5.8	6.9	6.0	6.6
	% *Salmonella*	4.2	4.2	0.0	0.0
Fresh TMR	APC				5.6
	Total Coliforms				4.9
	E. coli				4.5
	Fecal *Streptococcus*				5
	% *Salmonella*				0.0
Bunk TMR	APC				6.3
	Total Coliforms				6
	E. coli				5.5
	Fecal *Streptococcus*				5.5
	% *Salmonella*				0.0

Washington cattle had more feed changes among seasons (Table 8). The feed consisted of hay, grass, and TMR. No *Salmonella* was isolated from Washington feed. The highest APC counts in Washington feed came from grass, and the lowest from fresh TMR (Table 9).

For dairy cows, the background microflora did not differ in fresh *vs.* bunk silage samples. High background microflora including fecal indicators were found in beef and dairy silage (Table 10). Silage fermentation is important to control the level of acidity in order to decrease the resident microflora (Glickman et al. 1981). *Salmonella* survival in dry feed not only depends on the Aw values but also moisture content (Juven et al. 1983). Although *Salmonella* was not isolated from Washington fresh hay, the background microflora values were very high (Table 10). *Salmonella* was probably not isolated because of the small amount of sample analyzed or because of competition with background microflora. Contamination could have occurred from other environmental sources such as birds or rodents. Glickman *et al.* (1981) reported an outbreak of *S.* Anatum that was related to an improper fermentation of the haylage and contaminated by wild birds.

Fecal *Streptococcus* was infrequently found in both, fresh and trough TMR samples from Tennessee (Table 10). Kabagambe et al. (2000) found that feeding TMR to cattle increased *Salmonella* shedding. Anderson et al. (1997) also associated the use of TMR with an outbreak of *S.* Menhaden in dairy cows in California. The source of feed was traced and the source of the contamination

Table 10. Enumeration of fecal microorganisms and percentage positive *Salmonella* in rectal swabs, bedding and soil from dairy cows premises by sample type for each state.

Dairy		TN	AL	CA	WA
Fecal swab	APC[(1)(2)]	10.3	9.9	9.9	9.5
	Total Coliforms[(2)]	9.4	9.2	9.3	8.8
	E. coli[(2)]	8.8	9.6	8.8	8.6
	Fecal *Streptococcus*[(2)]	7.6	7.7	8.2	7.8
	% *Salmonella*[(3)]	0.0	0.0	0.0	4.2
Bedding material	APC	8.0	9.0	8.6	7.7
	T. Coliforms	6.3	7.2	7.9	7.4
	E. coli	5.4	6.7	6.7	6.3
	Fecal *Streptococcus*	5.2	7.0	7.3	6.5
	% *Salmonella*	0.0	4.2	0.0	8.3
Soil	APC	7.0	8.9	7.3	6.8
	Total Coliforms	4.9	6.6	6.9	3.8
	E. coli	4.1	6.4	5.1	3.6
	Fecal *Streptococcus*	3.8	4.3	5.0	6.4
	% *Salmonella*	4.2	8.3	0.0	0.0

(1) APC= Aerobic Plate Count
(2) The APC, Total coliforms, *E. Coli* and Fecal *Streptococcus* are given in log CFU/fecal swab logCFU/gram sample.
(3) *Salmonella* is given in percentage of isolation

was related to the addition of beef tallow which was contaminated with *Salmonella*. The use of animal by-products have been shown to be related to the presence of *Salmonella* in animal feed. Animal feed can get contaminated at milling (Whyte *el at*. 2003), during transportation (Fedorka-Cray et al. 1997), at the farm, from animals or due to the presence of rodents (Davies and Wray 1995).

Seasonality of *Salmonella*

For beef cattle, all of the *Salmonella* positive samples were found during the period December 2002-February 2003 (Table 11). No *Salmonella* was isolated in any other season from Washington. For dairy cows, all *Salmonella* positives were isolated during the period November 2002-May 2003 and May 2004 (Table 12). Most of the *Salmonella* positive samples were obtained in the same period for beef cattle and dairy cows. In Washington dairy cows, *Salmonella* isolation was distributed among seasons, being isolated in Spring 2003 and 2004. These findings differ from the major national surveys were they found higher isolation rate of *Salmonella* isolation in the summer months (Wells et al. 2000; Losinger et al. 1997). Most of these studies were conducted during a 1 year period or less and only focused on the isolation rate of *Salmonella* in fresh feces or fecal swabs. Our study covered a longer period of time and included environmental samples in addition to animal samples. Our data agrees with other studies where they isolated *Salmonella* in the winter months (Bailey et al. 2001). Most of the national studies have suggested a national distribution of *Salmonella*, being more prevalent in the Southern states (Kabagambe et al. 2000;

Table 11. *Salmonella* isolation from beef cattle by season and state over a period of 21 months (August 2002 to June 2004).

	Summer/ Autumn02	**Winter 02/03**	**Spring 03**	**Summer 03**	**Autumn 03**	**Winter 04**	**Spring 04**
TN	-		-	-	-	-	ns
AL	-		-	-	-	-	ns
CA	-		-	-	-	-	ns
WA	-	-	-	ns	-	-	-

(+) *Salmonella* isolated; (-) no *Salmonella* isolated; ns: not sampled.

Table 12. *Salmonella* isolation from dairy cows by season and state over a period of 21 months (August 2002 to June 2004).

	Summer/ Autumn02	**Winter 02/03**	**Spring 03**	**Summer 03**	**Autumn 03**	**Winter 04**	**Spring 04**
TN			-	-	-	-	ns
AL	-			-	-	-	ns
CA	-		-	-	-	ns	ns
WA				ns	-	-	

(+) *Salmonella* isolated; (-) no *Salmonella* isolated; ns: not sampled.

Fedorka- Cray et al. 1998). Most of these studies sampled the farms in the South during the summer periods (Kabagambe et al. 2002; Fedorka-Cray et al. 2000).

However, Fedorka-Cray et al. (1998) found that *Salmonella* was more prevalent in a region which included Arizona, California, Idaho and Washington state.

A high isolation of *Salmonella* was identified during the winter of 2003 in the US. Most of the *Salmonella* isolated came from the environment. The presence of wild birds or rodents in warmer areas has been related to the increase of *Salmonella* isolation (Kirk et al. 2002; Craven et al. 2000; Davies and Wray 1995). *Salmonella* was not isolated during the summer periods, but we found it during other seasons. High temperatures in the Southern states might have impeded the survival of *Salmonella* in soil samples.

There were also differences in the *Salmonella* isolation by states. For beef cattle, *Salmonella* was more prevalent in Tennessee (12.5%), whereas no *Salmonella* was isolated from Washington beef samples (Table 2). For dairy cows, the highest isolation rate was seen in Alabama and Washington (16.7% each). California was the only state where we found the same isolation rate for dairy cows and beef cattle (4.2%).

Serotype distribution

S. Anatum was the most common serotype isolated from beef cattle and dairy cows (Tables 6 and 7). This is not one of the most common human isolates (CDC 2003). *S*. Anatum is not commonly isolated from sick ruminants (CDC 2003), but during 2002, was the 5th most common isolate from non sick bovine

(CDC 2003). We are in agreement with other authors that the most common serotypes found in animal feeds do not usually correlate with the most common serotypes isolated from human cases (CDC 2003). *S*. Anatum is not species-specific and seems not to be very infective for ruminants. Other authors have also found *S*. Anatum among the most common serotypes isolated from ruminant feed (Krytemburg et al. 1998).

The only two serotypes isolated from beef cattle samples were *S*. Anatum (89.9%) and *S*. Newington (11.1%) (Table 6). *S*. Anatum was found in every sample type and *S*. Newington only in Tennessee bedding (Table 6).

The most common serotypes isolated from dairy cows were *S*. Anatum (56%), *S*. Newington (20%), *Salmonella spp* (16%), and *S*. Javiana (8%) (Table 7). In beef cattle (Figure 1), *S*. Anatum was well distributed across all sample sites in the US with the exception of Washington, and S. Newington was only found in Tennessee. The same trend was found for dairy cows for *S*. Anatum (Figure 2). It was more frequently isolated in Washington state, and less in California. *S*. Javiana was only isolated in Washington state. *S*. Newington was isolated in Washington state and Tennessee. *Salmonella spp*. (unidentified serotype) were found in Washington state and Alabama.

IV. Conclusion

Salmonella isolation rate differed in dairy cows and beef cattle probably due to differences in animal management practices and their environment. We found

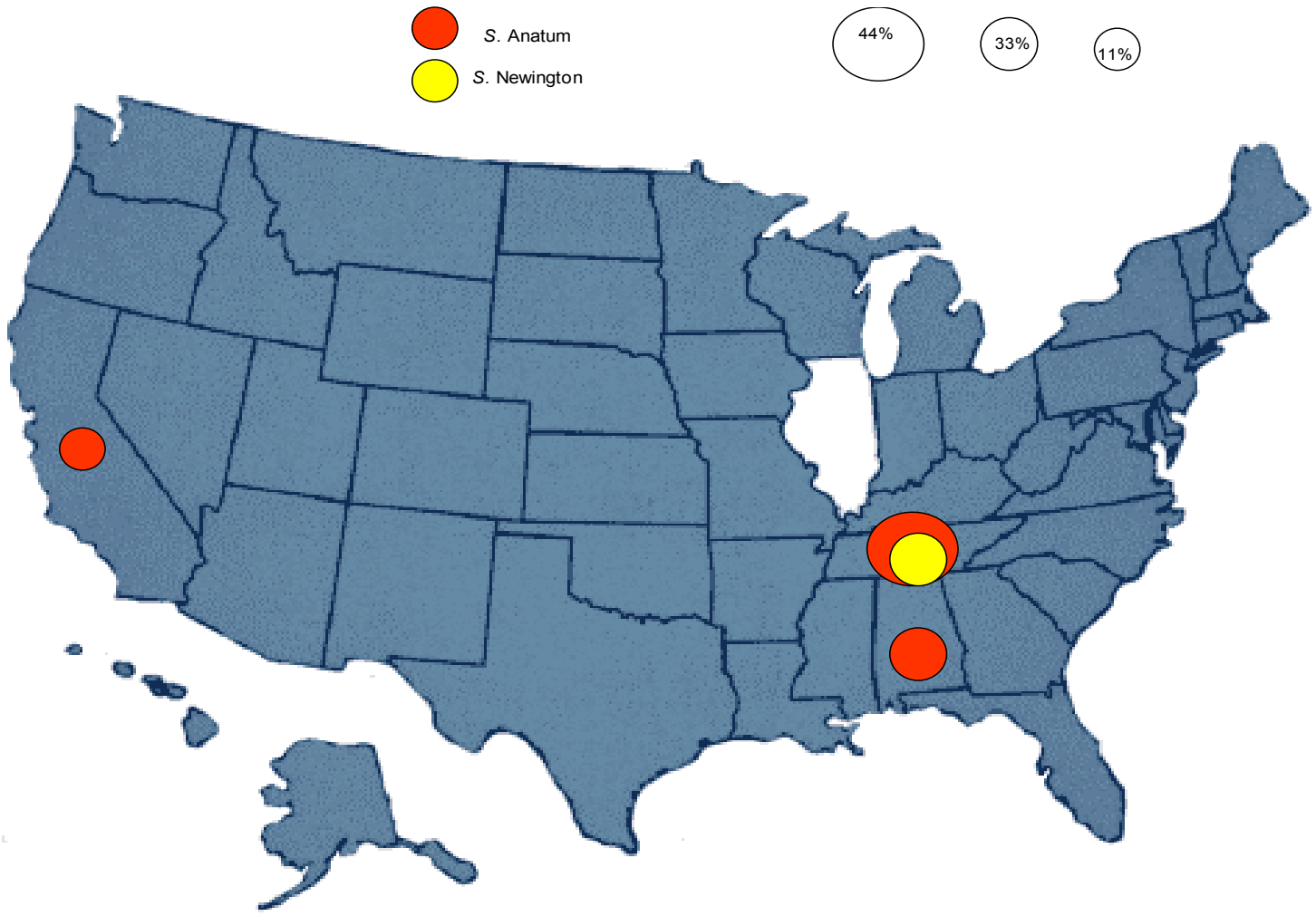


Figure 1. Percentage of *Salmonella* serotypes isolated by state in beef cattle.

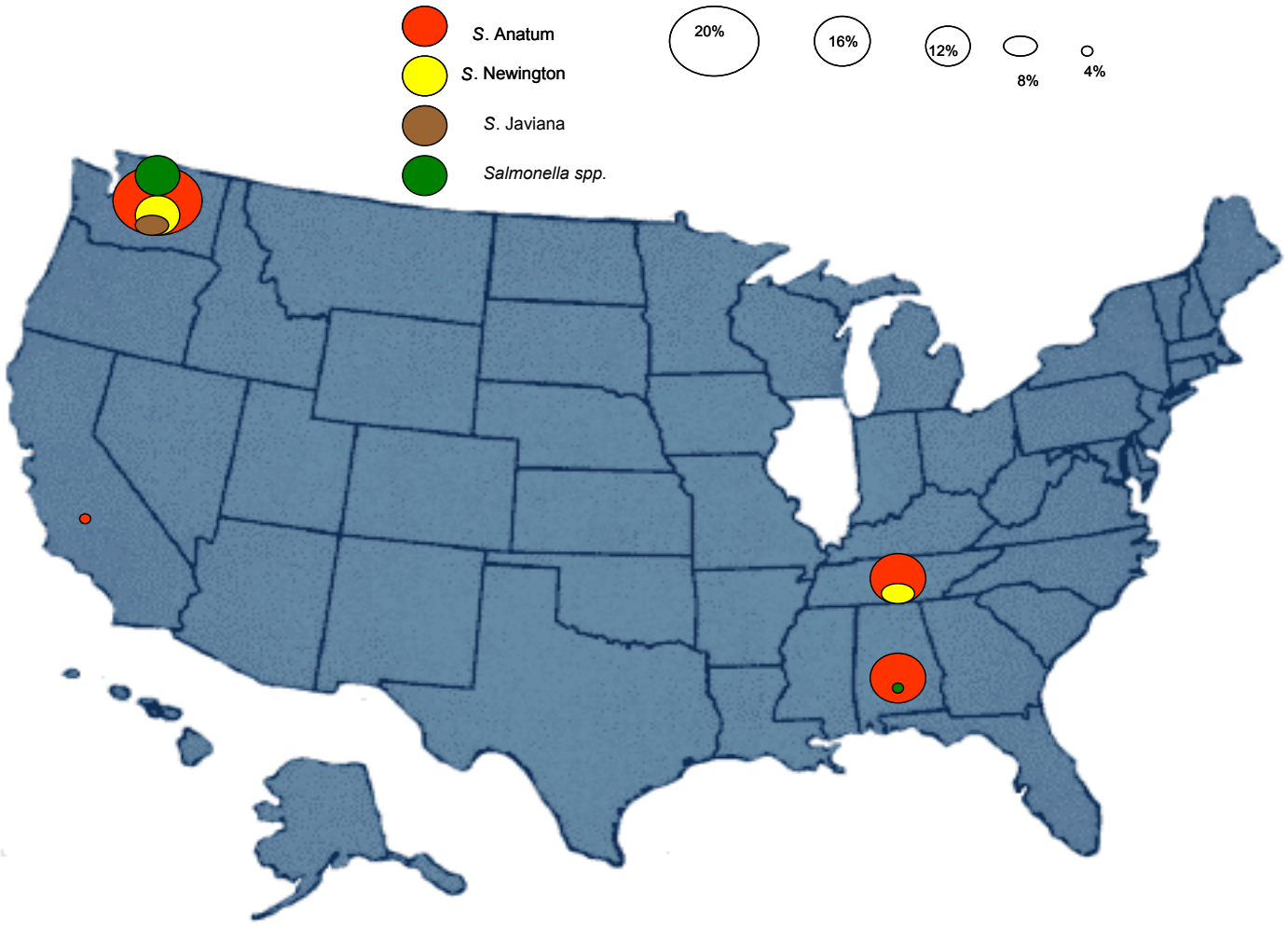


Figure 2. Percentage of *Salmonella* serotypes isolated by state in dairy cows

a national distribution of S Anatum, although isolation rate was higher in Southern beef cattle. Although S. Anatum does not represent one of the top ten serotypes most common in humans in the US every year, care must be taken to control the presence of these serotypes. S. Javiana is increasingly isolated in human cases of salmonellosis (CDC 2004) and one cause may be its presence in bovine and their environment. The serotypes found in this study are similar to those obtained by other authors in previous studies. Soil contamination with *Salmonella* was common. The survival in soil might have an impact in further contamination within the farm. *Salmonella* was more frequently isolated during colder months from environmental samples. The environment appeared to be a major reservoir for *Salmonella* at the farm from which animals can be contaminated repeatedly. Breaking the contamination cycle for *Salmonella* between animals and the environment will be essential to reduce isolation rate of *Salmonella* in beef and dairy cows.

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Part III: Multistate Study to Determine the Presence of *Salmonella spp.* in Swine Herds and their Environment

Abstract

Human cases of salmonellosis have been linked to the consumption of pork products. The most common *Salmonella* serotypes isolated from human cases of salmonellosis usually differ from the most common serotypes isolated from healthy swine. Whereas *Salmonella* contamination during transportation, or at slaughterhouses has been well established, less in known about the role of swine environments in *Salmonella* survival and further contamination of the animals. The objective of this study was to determine the *Salmonella* occurrence in animals at selected farms as well as its occurrence in the environment geographically and temporally to try to establish the major reservoirs of *Salmonella* in swine farm operations. Samples from 4 locations in four different states (Tennessee, North Carolina, California and Washington) were collected over 21 months. Environmental samples (n=120) and fecal swab samples (n=400) were analyzed to determine the presence of *Salmonella* using modified BAM protocols. *Salmonella* was more prevalent in California swine (10.3%), followed by North Carolina (7.3%), and Tennessee and Washington (6.3% each) swine. *Salmonella* was often isolated from fecal samples (11.9%), followed by fecal swabs (8.8%), feed (7.7%) and soil samples (5.4%). A total of 40 serotypes were isolated from swine fecal swabs and environment. The most common *Salmonella* serotypes isolated were *S*. Anatum, *S*. Javiana, *S*. Newington, and *S*. Worthington. We found regional differences in *Salmonella* serotypes. Management practices must be addressed to control swine fecal matter to reduce further contamination of the farm.

I. Introduction

Human cases of salmonellosis have been linked to the consumption of pork products. The most common serotypes isolated from human cases of salmonellosis during 2002 were *S*. Typhimurium, *S*. Enteriditis, *S*. Newport, *S*. Heidelberg, *S*. Javiana, *S*. Montevideo, *S*. Muenchen, *S*. Oranienburg, *S*. Saintpaul, and *S*. Infantis (CDC 2003). These usually differ from the most common serotypes isolated from healthy swine (CDC 2003). In 1995, the USDA conducted a national survey to determine the most common serotypes of *Salmonella* found in finisher pigs in the US. The serotypes most commonly found were *S*. Derby, *S*. Agona, *S*. Typhimurium var Copenhagen, *S*. Brandemburg, and *S*. Mbandaka (USDA 1997). *Salmonella* contamination of pork meat at slaughter has been well established (Wonderling et al. 2003). To reduce contamination of swine with enteric pathogens at slaughter, USDA initiated a mandatory Hazard Analysis Critical Control Point (HACCP) program in 1997 in US meat plants (FSIS 1999). Stress factors can increase the shedding of *Salmonella* by carriers and contaminate other animals (Corrier et al. 1990) during transportation and in holding areas prior to slaughter. Several studies have been done to determine the status of *Salmonella* at the farm level to investigate the level of this pathogenic organism before slaughter. Most farm level studies have sampled fecal material (USDA 1997), whereas a few studies have focused on feed samples (Harris et al. 1997), transportation (Lo Fo Wong et al. 2002) or the environment (Letellier et al. 1999; Barber et al. 2002). The objective of this study was to determine the *Salmonella* occurrence in animals at the farm as well as its

occurrence in the environment geographically and temporally to try to establish the major reservoirs of *Salmonella* in swine farm operations.

II. Material and Methods

Sample collection: Samples were collected from a total of 4 farms in 4 different states (Tennessee, California, Washington, and North Carolina) during a period of 21 months. Each farm was sampled every 3-4 months, obtaining a total of 6 samplings per farm. Several sample types were collected for each sampling period. A total of 520 samples were analyzed. Rectal swabs were collected from 20 randomly selected swine on each farm. Animals were randomly chosen by the person that was taking the samples (n=400). The other sample types were taken from the surrounding environment (n=120). These consisted of soil samples from outside the area where swine were held, and fresh feces from the swine holding area. Also, from each farm, fresh feed and feed bunk samples were collected from whatever source the animals were feeding. Samples were collected by the farmers using a detailed sampling plan, individualized pre-weighed sample containers, and Geographical Position System (GPS) coordinates were recorded at the sampling place using eTrex (Garmin, Olathe, KS). All sample containers and sampling supplies were mailed to farm collaborators approximately two weeks prior to each sampling with a return address label. Samples were sent to the University of Tennessee via overnight express and kept refrigerated at 4^{o} C until analyzed within the next 3-4 days.

Farm description: The farms from Washington, North Carolina and California were private premises. The Tennessee study site was a privately owned farm for the first 3 sampling periods. Beginning with the fourth sampling period samples were taken from a nearby University swine farm. The North Carolina swine farm was a farrowing swine facility.

Salmonella isolation: Samples were analyzed for the presence of *Salmonella* using FDA-BAM modified methods (Pangloli et al. 2003). All media used was obtained from Difco (Sparks, MD) except Rappaport-Vassiliadis (RV) that was obtained from Difco and Oxoid (Ogdensburg, NY). The rectal swabs were first transferred into a sterile tube containing 10 ml of Universal Broth in order the divide the sample to analyzed for the presence of different pathogens. Then, 1 ml was transferred into a tube containing 10 ml of RV broth or Tetrathionate broth (TT) (added Brilliant Green 20 ml/l and iodine 10ml/l), and incubated as described in Table 1. For the environmental samples, 25 grams were weighed and mixed with TT or RV (225 ml) and incubated as described in Table 1. Dairy and beef samples were weighed (25 grams) and mixed with 225 ml of Lactose Broth (LB) and incubated as described in Table 1. Pre-enrichment broth (1ml) was transferred into a sterile test tube with 10 ml of TT and incubated (Table 1).

All the samples were streaked for isolation onto XLT4 (xylose-lysine-Tergitol 4)/BSA (bismuth sulfite agar) plates (Table 1).Typical *Salmonella* colonies on XLT4 (black) and atypical colonies (yellow with red background) were selected for further analysis. Typical BSA *Salmonella* colonies (metallic sheen) were selected. Presumptive colonies were transferred onto TSI (Triple

Table 1. Protocol followed for the isolation of *Salmonella* from swine rectal swabs and environment. This protocol was done as described by Pangloli et al. 2003.

Origin of sample	Enrichment T[1] t Medium	Plating T t Medium
Rectal swab	RV[2] 42C 24h	XLT4[5] 35.5C 24h
Feed	RV 42C 24 h	BSA[4] 35.5C 48h
Soil	TT[3] 42C 24h	XLT4 35.5C 24h
Bedding/feces	TT 42C 24h	XLT4 35.5C 24h

(1)T: time; t: temperature.
(2) RV= Rappaport-Vassiliadis.
(3) TT= Tetrathionate.
(4) BSA= Bismuth Sulfate Agar.
(5) XLT4= Xylose-Lysine-Tergitol 4 Agar.

Sugar Iron) agar slants, and into Urea broth and incubated at 35.5C for 24 hours. A TSI result of K/A (red slant /yellow butt) or K/S (red slant/ black precipitate), and urea negative were considered presumptive positive for *Salmonella.* Presumptive colonies were kept for further serological analysis with somatic antigen O (Difco, Sparks, MD).

Agglutination was considered positive for *Salmonella*. Positive antigen O colonies were analyzed using Analytical Profile Index (API) 20E (Biomeriux, Hazelwood, MO) to confirm the presence of *Salmonella spp* and or *S*. Arizona.

Aerobic Plate Count : Aerobic plate count (APC) was prepared according to Feldsine et al. (2003) using SimPlate (Biocontrol, Bellevue, WA). Fecal swabs were vortexed in 10 ml lactose broth. For other samples, 25 grams were weighed into sterile filtered bags with 225 ml of 0.1% peptone water (% w/v) and 10-fold dilutions were prepared. SimPlates were incubated at 35C for 24h. The total number of wells showing color change were counted. To calculate the MPN (most probable number) of organisms, the conversion Table provided by the manufacturer (Biocontrol, Bellevue, WA) was used and multiplied by its corresponding dilution.

Total Coliforms/ *Escherichia coli* :Total Coliforms and *E. coli* were enumerated using SimPlates using instructions provided by the manufacturer (Biocontrol, Bellevue, WA). SimPlates were incubated at 35C for 24 h. Total coliforms were counted based on wells with color change. *E. coli*, were counted by observing wells that fluoresced under UV light. The total MPN of coliforms/ *E. coli* was

calculated using the SimPlate conversion Table provided by manufacturer (Biocontrol, Bellevue, WA).

Fecal streptococci: Fecal streptococci were counted as described by Downes and Ito (2001). For fecal swabs, the swabs were vortexed in 10 ml lactose broth. A 1 ml aliquot was transferred into sterile tubes containing 9ml of 0.1% peptone water. For other samples, 25 grams was weighed into sterile filtered bags with 225 ml of 0.1% peptone water (%w/v). Decimal dilutions were prepared. A 1ml aliquot from each dilution was pourplated with 15-20 ml. KF Streptococcus Agar (%). Plates were incubated at 35C for 48h. Pink colonies were counted using a manual colony counter.

Riboprinter: Frozen isolates were thawed and streaked onto BHI Agar at 35.5C for 24 h. Samples were processed using a RiboPrinter (Qualicom, Wilimgton, DE) manufacturer's protocol using *Pvu*II as the restriction enzyme (Bailey et al. 2002). The ribosomal DNA fragments were digested and processed on a nylon membrane and hybridized with an *E. coli* DNA probe. Using chemiluminescent, the image was captured by a CCD camera and taken to computer software for further analysis (Bruce 1996). The RiboPrinter identified isolates by band matching and also classified them into ribotypes (Oscar 1998).

III. Results and Discussion

Salmonella was isolated from animals and their environment in a multistate study to determine its isolation rate in beef cattle, dairy cows, swine, poultry, and turkey in the US. The overall results of this study are shown in Table 2. In this

Table 2. Percentage of *Salmonella* isolation by state and farm animal type over a period of 21 months (August 2002 to June 2004). Five different states were sampled, Tennessee, Alabama, California, Washington and North Carolina.

	TN	AL	CA	WA	NC	AVERAGE
Beef [2]	12.5	8.3	4.16	0.0	ns [1]	6.2
Dairy [2]	8.3	16.7	4.16	16.7	ns	11.5
Swine	6.3	ns	10.3	6.3	7.3	7.8
Poultry [2]	12.5	ns	ns	16.7	5.0	11.4
Turkey [2]	ns	ns	ns	Ns	30.0	30.0

(1) ns: not sampled.
(2) Data obtained from Rodriguez et al. 2004a, and Rodriguez et al. 2004b.

study we focus only on the *Salmonella* isolation rate in swine and the farm environment. Data on other animals are provided for comparison only by the University of Tennessee Food Safety Center of Excellence (Draughon 2004).

Salmonella was found in over 5% of swine samples in all states tested during this study.

***Salmonella* in fecal swabs and feces**

Salmonella was not isolated from the Washington swine feces samples (holding area) (Figure 1). However, the highest isolation rate of *Salmonella* isolation from fecal swabs was found in Washington (12.5%). The background microflora (Table 3) was similar for all fecal swabs. The swine feces samples obtained from North Carolina, California, and Tennessee were fresh feces. The Aerobic Plate Count (APC) counts of feces were similar in all states (Table 3). Tennessee fecal samples showed background counts lower than California and North Carolina, but *Salmonella* was still present (Table 3).

Swine feces not containing *Salmonella* came from a Washington farm from a mixture of bedding material, grains and dry feces. The background microflora counts for this sample type were similar to those found in Tennessee and California swine feces (Table 3). The bedding material added to the dry feces was highly contaminated and one can argue that the presence of a native microflora could have prevented the growth or survival of enteric pathogenic microorganisms. *Salmonella* survival in fecal material has been previously established (Gray and Fedrorkra-Cray 2001). Repeated exposure to swine feces is a risk factor for *Salmonella* shedding in the farm (Davies et al 1997)

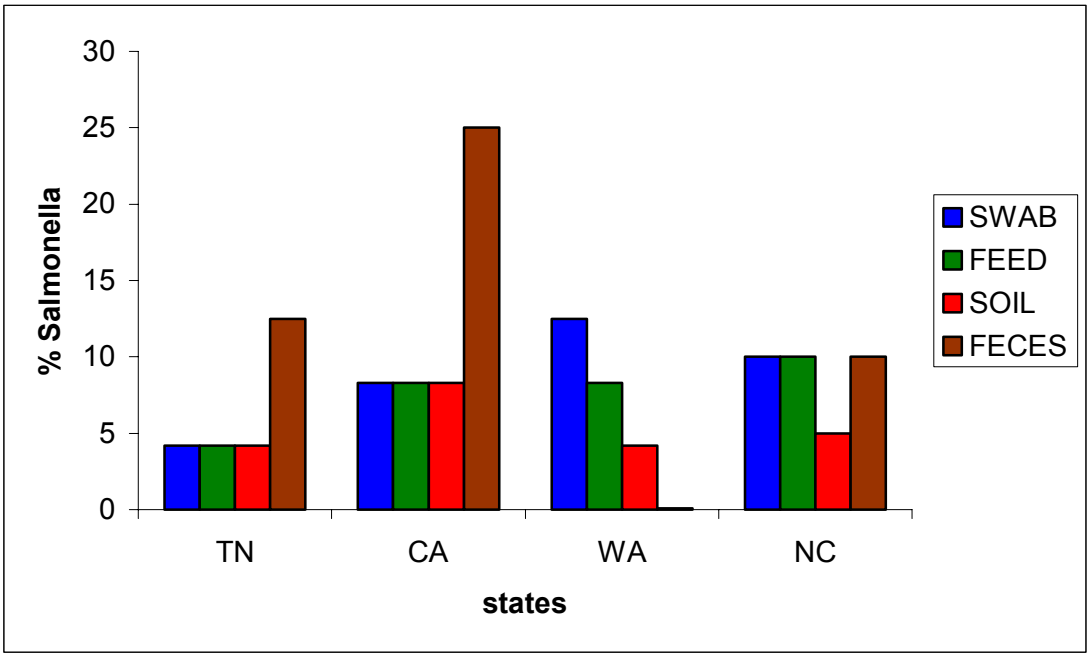


Figure 1. Percentage of *Salmonella* isolated over a period of 21 months (August 2002 to June 2004) in swine rectal swabs (swab) and their environment (feed, soil and feces). Four different states were sampled for dairy cows and beef cattle: Tennessee, Alabama, California, and North Carolina.

Table 3. Enumeration of fecal microorganisms and percentage positive *Salmonella* in rectal swabs, bedding and soil from swine premises by sample type for each state.

Swine	Microbiology	WA	TN	CA	NC
Fecal swab	APC (1)(2)	10.0	9.9	9.6	9.5
	Total Coliforms (1)	9.6	9.4	9.5	9.2
	E. Coli (1)	8.8	9.0	9.3	9.2
	Fecal *Streptococcus* (1)	6.5	7.7	7.2	7.0
	% *Salmonella* (3)	12.5	4.2	8.3	10.0
Feces	APC	8.4	8.1	8.9	9.5
	Total Coliforms	7.0	5.4	8.6	7.6
	E. Coli	6.1	5.4	8.5	7.1
	Fecal *Streptococcus*	7.0	5.9	6.9	6.9
	% *Salmonella*	0.0	12.5	25.0	10.0
Soil	APC	6.6	6.2	7.9	7.4
	Total Coliforms	4.6	4.3	6.1	5.2
	E. Coli	6.7	2.2	5.3	5.1
	Fecal *Streptococcus*	4.5	3.9	6.6	4.7
	% *Salmonella*	4.2	4.2	8.3	5.0

(1) APC= Aerobic Plate Count.
(2) The APC, Total coliforms, *E. Coli* and Fecal *Streptococcus* are given in log CFU/fecal swab logCFU/gram sample.
(3) *Salmonella* is given in percentage of isolation

Serotypes in fecal material

The most common serotypes obtained from swine feces were *S*. Javiana (7.3%), *S*. Anatum, *S*. Derby, *S*. Saintpaul, S. Arizona/Tennessee and *S*. Worthington (2.4% each) (Table 4). *S*. Javiana was found in all swine feces positive for *Salmonella*. *S.* Javiana is one of the top ten most common serotypes isolated from human salmonellosis cases, and the CDC reported an increased of 227% in human cases within the last seven years (MMRW 2004). It was significant that this serotype was found in the feces of all states sampled except Washington (Table 4). *S*. Javiana was also found in North Carolina soil (Table 4) that probably was contaminated by feces.

***Salmonella* in feed**

Swine feed samples were contaminated with *Salmonella* in all states included in our study. Percent positive salmonellae in feed were (Figure 1): North Carolina (10%), California and Washington (8.3%), and Tennessee (4.2%). The type of feed given to the animals varied depending on the location. North Carolina swine were fed pelleted feed, whereas Tennessee and California in the study used a mixture of grains and corn. Washington feed consisted of a mixture of grains. While some authors consider pelleting as a risk factor for *Salmonella* in feed (Harris et al. 1997), other researchers show different results (Lo Fo Wong et al. 2004). Our data show higher *Salmonella* isolation from pelleted feed than from non-pelleted, but these differences were small.

Table 4. *Salmonella* serotypes by state and sample type in swine.

	Swab	Feed		Soil	Feces
		Fresh	Bunk		
TN	S. Heidelberg *S*. Mbandaka *S*. Worthington	S. Anatum S. Newington	S. Anatum	*S*. Anatum	*S*. Javiana *S*. Arizona *S*. Tennessee *S*. Anatum
WA	*Salmonella spp.*	*S*. Anatum S. Newington	*Salmonella spp.*	*S*. Anatum *S*. Newington	
NC	*S*. Anatum *S*. Newington Salmonella *spp.*	*S*. Anatum	*S*. Typhimurium S. Anatum	*S*. Javiana	*S*. Javiana *S*. Worthington
CA	*Salmonella spp.*		*Salmonella spp.*	*Salmonella spp.*	*S*. Saintpaul *S*. Derby *S*. Javiana *Salmonella spp.*

Salmonella was isolated from feed bunk samples in all states (Table 5). The animals probably contaminated feed. Interestingly, *Salmonella* was isolated on fresh feed (Table 5) in all states sampled except California. Fresh feed was considered to be feed that was at the farm that had not been opened or freshly mixed. This fresh feed could have been contaminated before arrival to the farm, during transportation (Fedorka-Cray et al.1997), or in the farm facility due to environmental factors or the presence of wild birds or rodents. The background microflora when compared for fresh feed and feed bunk were higher for feed bunk (Table 5). There was no association between the presence of total coliforms, *E. coli*, or fecal *Streptococcus* with the presence of *Salmonella* in feeds.

Serotypes in feed

The most common serotypes of *Salmonella* obtained from swine feed were *S*. Anatum (12.2%), and *S*. Typhimurium and *S*. Newington (2.4% each). A group of *Salmonella* were not identifiable to the serotype level and were classified as *Salmonella spp.* (Table 4). The most common serotypes in feed in Tennessee and Washington were not the same as those isolated from fecal swabs of fecal samples. These data indicate that contamination of these feed samples probably occurred prior to arrival to the farm. An important finding was the isolation of *S*. Typhimurium from bunk feed in North Carolina (Table 4). This is not a common serotype frequently found in animal feed (Davies et al. 1997; Schneider 2002). Since it was isolated from feed bunk, we could conclude that feed was most likely contaminated by animals. This serotype is the major one isolated from human

Table 5. Enumeration of fecal microorganisms and percentage positive *Salmonella* in fresh and bunk feed samples from swine premises by sample type for each state.

Swine	Microbial	WA	TN	CA	NC
Fresh feed	APC (1)(2)	6.0	4.4	4.7	5.0
	Total Coliforms (2)	3.4	3.9	4.3	4.2
	E. Coli (2)	3.2	1.0	1.0	4.2
	Fecal *Streptococcus* (2)	3.1	3.9	4.4	2.3
	% *Salmonella* (3)	4.2	2.1	0.0	3.3
Bulk feed	APC	6.8	5.5	6.4	6.0
	Total Coliforms	4.4	4.3	4.9	3.9
	E. Coli	2.8	1.0	3.9	3.5
	Fecal *Streptococcus*	5.2	4.8	5.3	4.3
	% *Salmonella*	4.2	2,1	8.3	6.7

(1) APC= Aerobic Plate Count.
(2) The APC, Total coliforms, *E. Coli* and Fecal *Streptococcus* are given in log CFU/fecal swab logCFU/gram sample.
(3) *Salmonella* is given in percentage of isolation.

cases (CDC 2003), and is also commonly isolated from sick swine (CDC 2003). The presence of this serotype in the farm can be a risk for further contamination of other animals in the farm, or further contamination during transportation or at the slaughter plant, becoming a hazard for human health. *S*. Anatum was the most common serotypes isolated from fresh feed, followed by *S*. Newington. S. Anatum was found in feed in all the states sampled except California (Table 4). *S*. Anatum was also found in feed bunk samples from Tennessee and North Carolina, whereas the most common serotype found in California and Washington feed bunk was identified only was *Salmonella spp*. These serotypes isolated in feed are not usually associated with disease in swine (Schwartz 1991).

***Salmonella* in soil**

Soil samples around swine facilities were positive for *Salmonella* in all states (Figure 1), being slightly higher in California (8.3%), followed by North Carolina (5%), Tennessee and Washington (4.2% each). Soil samples were taken on the farm outside the area where the animals were kept. The presence of *Salmonella* in this sample type suggests that soil could have been contaminated by the presence of wild birds, rodents, and animal transfer or by the farmers through boots when moving about the swine facility. Contamination of the farmer's boot is not unusual; especially considering the amount of *Salmonella* isolated from swine feces.

Salmonella survival in soil and swine environment has been described by Morse et al. (1982), and cited by Gray and Fedorka-Cray (2001). The length of

survival will be determined by the soil characteristics (Zibilske et al. 1978). Soil contamination in the farm could serve as a reservoir for further transmission to swine on the farm.

Serotypes in soil

The most common serotypes isolated from soil were *S*. Anatum (7.3%), *S*. Javiana (4.9%), *Salmonella spp*. (4.9%), and *S*. Newington (2.4%). *S*. Anatum and *Salmonella spp*. were isolated from every sample type. *S*. Javiana was isolated from feces in all states except Washington, and was also found in North Carolina soil. *S*. Newington was also isolated from fecal swabs, and feed samples.

Our best success in isolating *Salmonella* from swine farms came from combining rectal swabs and feces samples. The use of feces in combination with feed may also be a reliable tool to determine the total presence of *Salmonella* in swine farms.

***Salmonella* and seasons**

The distribution of *Salmonella* by seasons and states is shown in Table 6. It is well established that *Salmonella* is most commonly isolated during the summer periods (Huges et al1971; Wray et al. 1987; Currier et al. 1986), although Berends et al. (1996) did not find any seasonality. However, this depends on the location sampled according to our data. *Salmonella* was isolated from California swine at least once in every season during the six sampling periods (100%). In Washington, *Salmonella* was isolated in five out of six sampling periods (83.3%), whereas in North Carolina *Salmonella* was found in four of the five sampling

Table 6. *Salmonella* isolation from swine samples by season and state over a period of 21 months (August 2002 to June 2004).

Swine	Summer/Fall 02	Winter 02/03	Spring 03	Summer 03	Fall 03	Winter 04	Spring 04
TN	-				-		-
CA							
WA						-	
NC				-			

(+) one sample type positive; (++) two sample types positive; (+++) three sample types positives.

periods (80%). *Salmonella* was found in three of the six sampling periods in Tennessee (50%).The first swine farm from Tennessee was located next to a beef farm we had sampled in a previous study. *Salmonella* was isolated from the beef farm only during winter 02/03 (data not shown). *Salmonella* was not isolated in any other season from this farm (data not shown). The majority of samples that came from the swine farm in Tennessee situated close to the beef farm were positive for *Salmonella* during the same period. The same serotype was found in both farms, and corresponded to S. Anatum. This was found in swine feed, soil and feces (Table 3), and in beef cattle grass, soil and bedding. Due to the proximity between these two farms, it appears that cross contamination may have occurred from one farm to another via contaminated water, wild birds (Craven et al. 2000), rodents (Davies and Wray 1995) or workers (Barber et al. 2002). Runoff may have been another possible mode of transmission from one farm to another. Further research is under way to evaluate the clonacity of this isolate.

***Salmonella* serotypes**

A total of 40 *Salmonella* were isolated from swine fecal swabs and environmental samples (Table 7). The most common serotypes were *S*. Anatum, *S*. Javiana, *S*. Newington, and *S*. Worthington. One isolate (2.4%) was classified as *S*. Arizona/ *S*. Tennessee. *S*. Anatum was the most prevalent isolate from feed and soil, whereas *Salmonella spp* was the most common isolate from feces followed by *S*. Javiana.

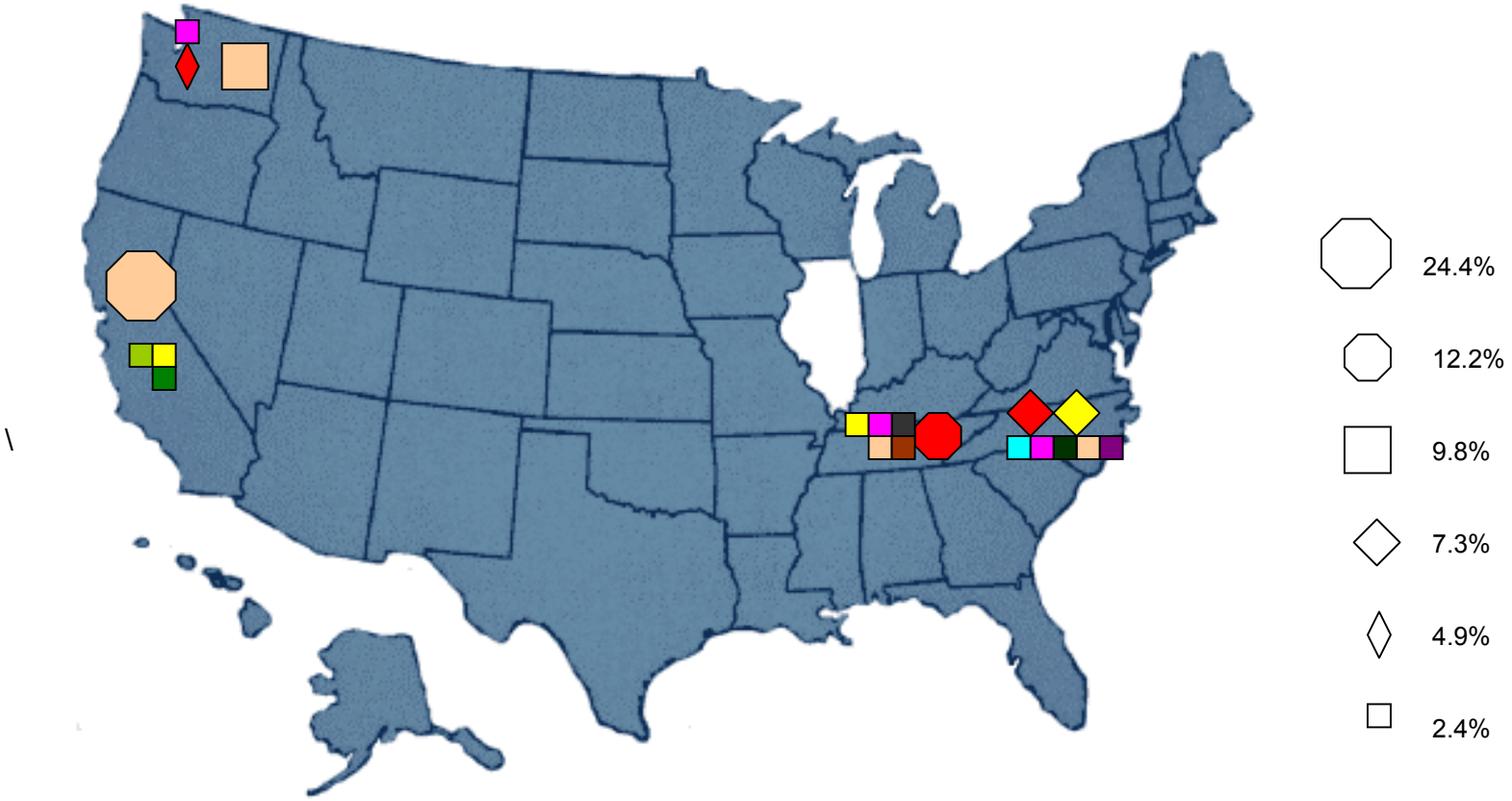
Table 7. Percentage of *Salmonella* serotypes isolation from each sample type in swine herds and their environment (n=40).

Serotype	Swab	Feed	Soil	Feces	Total
S. Anatum	2.4	12.2	7.3	2.4	6.1
S. Arizona/TN	-	-	-	2.4	0.6
S. Derby	-	-	-	2.4	0.6
S. Heidelberg	2.4	-	-	-	0.6
S. Javiana	-	-	4.9	7.3	3.0
S. Mbandaka	2.4	-	-	-	0.6
S. Newington	2.4	2.4	2.4	-	1.8
S. Saintpaul	-	-	-	2.4	0.6
Salmonella spp.	14.6	7.3	4.9	9.8	10.4
S. Typhimurium	-	2.4		-	0.6
S. Worthington	2.4	-	-	2.4	1.2

The most common serotypes isolated in “Swine ‘96” were *S*. Derby, *S*. Agona, *S*. Typhimurium var Copenhagen, *S*. Brandenburg, *S*. Mbandaka, *S*. Typhimurium, *S*. Heidelberg, *S*. Anatum, *S*. Enteriditis, and *S*. Worthington (USDA 1997). This study did not sample any farm from the Pacific Coast and was focused on the presence of *Salmonella* in finishing hogs. Davies et al. (1997) compared the isolation rate of *Salmonella* in finishing pigs raised under two different production systems. The most common serotypes they isolated were *S*. Derby, *S*. Typhimurium, *S*. Heidelberg, *S*. Worthington, and *S*. Mbandaka. Isolates in both studies were similar to ours. The major difference was in the isolation of *Salmonella* spp in our study that was not found in any of the other two studies. Most of the unserotyped *Salmonella spp.* in our study was found in California and Washington (Figure 2). Although *Salmonella* were repeatedly streaked for isolation, it is possible that the *Salmonella spp.* are two or more serotypes which resist separation. They could also be slightly different from other recognized serotypes.

S. Anatum was found among the top ten serotypes in “Swine’96” and not found in Davies’ study. Most of our farms were not finisher farms, and due to the nature of the samples, slight differences in the serotypes isolated is not unexpected.

The geographical distribution of *Salmonella* is shown in Figure 2. At a glance we can see differences in the distribution of *S.* Anatum, being more prevalent in the Southeastern US. In the Pacific coast states, most of the *Salmonella* isolated were classified as *Salmonella spp*. S. Javiana was the most.



Anatum ; Derby ; Heidelberg ; Javiana ; Mbandaka ;Newington ;Typhimurium ; Saintpaul

Worthington ; *Salmonella spp*

Figure 2. Percentage of *Salmonella* serotypes isolated from swine herds and their environment by state.

frequent isolate found in North Carolina, although it was also found in Tennessee and California *S*. Derby in the most common serotype isolated from non-clinical cases in swine and the fourth most frequent cause of clinical salmonellosis in swine in 2002 (CDC 2003). *S*. Heidelberg is the fourth most common serotype isolated from human cases of salmonellosis; it is not typically isolated from healthy swine.

S. Typhimurium is the number one cause of human and swine salmonellosis. The finding of this isolate in swine feed, though rare, is important due to its clinical implication. Further research has to be done to establish differences among serotypes isolated from swine farm environments in the US to establish levels of risk and determine if certain geographic areas are more prone to introduce specific serotypes to the food chain.

IV. Conclusion

Salmonella serotypes can vary widely depending on the geographical location. Within the farm, most of the isolates came from feces or rectal swab samples. Management practices must address the isolation rate of *Salmonella* in feces since this is a potential source for further contamination. The isolation of *Salmonella* in the soil area outside the farm was related to the amount of *Salmonella* isolated from feces. Practices to control wild birds and rodents, as well as access to the farm by personnel is essential to avoid further spread of *Salmonella* to the environment. Although the common serotypes found in feed did not correlate to the serotypes isolated from fecal material, animal feed needs

to be free of *Salmonella* to reduce transmission to animals. Identification and control of *Salmonella* positive carriers at the farm is important to stop further shedding and contamination.

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Part IV: Comparison of *Eco*RI and *Pvu*II Enzymes Using Riboprinter® for the Identification of *Salmonella spp.*

Abstract

Salmonella is a pathogenic enteric microorganism that belongs to the Enterobacteriaceae family. Traditional identification methods of pathogenic microorganisms are based on biochemical and serological analysis. However, newer techniques rely on DNA analysis. Automated Riboprinter® is one such widely used method to identify Salmonella spp. This technique is based on the enzymatic digestion of ribosomal DNA by restriction enzymes. The objective of this study was to compare the efficacy of two restriction enzymes for identification of Salmonella spp. originating from farm animals and the surrounding environment. Samples were obtained for a multisate study conducted by the University of Tennessee Food Safety Center of Excellence. Isolates were characterized using biochemical and serological tests. Two different restriction enzymes were used with the automated Riboprinter® protocol, *Eco*RI and *Pvu*II. Different results were obtained with each enzyme. *Eco*RI digested isolates were mistakenly identified as Escherichia coli, whereas, *Pvu*II digested isolates were identified as a variety of *Salmonella spp*. and *Pseudomona fluorescens*. Further biochemical analysis indicated the isolate was not *Pseudomona fluorescens*. These data suggest that care must be taken when using molecular tools such as Riboprinter® to identify *Salmonella spp.* because misinterpretation can lead to a misclassification of the organism. Understanding the limitations, choosing the correct protocol, and confirming results with appropriate biochemical and serological tests will ensure more accurate identification of *Salmonella* serotypes.

I. Introduction

Salmonella is a pathogenic enteric microorganism in the *Enterobacteriaceae* family. Traditionally, *Salmonella* has been classified based on biochemical and serological tests in the Kauffman-White scheme. New methods of identification of organisms based on DNA analysis have moved *Salmonella* nomenclature into two different species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is subdivided onto five different groups (Jay 2000) which contains the majority of the *Salmonella* serotypes. The Centers for Disease Control (CDC) have recently adopted this new nomenclature (CDC 2002). This new scheme is also used by the World Health Organization (WHO), and the Pasteur Institute in France (Euzeby 2000; Yan et al. 2003, Popoff et al. 2004).

Ribotyping is a genetic tool that classifies organisms based on the differences in the DNA that transcribes for ribosomal RNA. Automated ribotyping was developed by Qualicom (Wilimgton, DE) to analyze, compare and classify organisms using their patented Riboprinter®. Genes that codify for ribosomal RNA are one of the most conserved regions in the bacterial genome (Snyder and Champness 2003) Small differences among different organisms are used to classify them into different groups or serotypes.

Restriction enzymes cut DNA at specific nucleotide sequences. Bacteria use restriction enzymes to defend themselves against foreign DNA, such as virus, plasmids and prophages, or in recombination events (Snyder and Champness 2003). These enzymes can be grouped into three categories, type I, type II, and type III. The most common restriction enzymes used in molecular genetics

belong to the type II (Pingoud 2002). The two most common restriction enzymes used for Salmonella with the Qualicom Riboprinter® are *Eco*RI and *Pvu*II. The restriction enzymes are named after the name of the organisms from which they were isolated. *Eco*RI was isolated from *Escherichia coli* and *Pvu*II from *Proteus vulgaris.*

There are over 2300 *Salmonella* serotypes, and the Riboprinter® library does not contain representatives of all of them (Oscar 1997). Riboprinter® has many useful applications, however studies are needed to compare efficacy of different restriction enzymes and to understand how phenotypic and serological characteristics are related to ribogroups. Therefore this study was undertaken to determine the ability of the Riboprinter® to differentiate and identify *Salmonella* isolates in comparison to serological analysis, and to determine which restriction enzymes, *Eco*RI or *Pvu*II, is more useful for differentiation of *Salmonella* from animal and environmental samples.

II. Materials and Methods

The *Salmonella* isolates were obtained from a multistate study conducted by the University of Tennessee Food Safety Center of Excellence to determine the presence of *Salmonella* on swine farms. Samples were taken from four farms located in four different states (Tennessee, Washington, California and North Carolina) during a period of 21 months. Sample types included fecal material, soil outside the holding area, and swine feed. The media used was obtained from Difco (Sparks, MD) except Rappaport-Vassiliadis (RV) which was obtained from

Difco and Oxoid (Ogdensburg, NY) *Salmonella* was isolated according to BAM protocol as modified by Pangloli et *al.* (2003). Atypical colonies on selective plating were tested for serological reaction with Ag O (Difco). Triple Sugar Iron (TSI) and urea test were performed. On TSI, colonies appeared as K/S (alkaline slant black butt) or A/S (acid slant black butt). Urea test was negative. Agglutination was considered a positive results. Colony identification was confirmed using Analytical Profile Index (API) 20E (Biomerieux, Hazelwood, MO).

Ribotyping of these isolates was conducted using two different restriction enzymes, *Eco*RI and *Pvu*II (Qualicom, Millington, DE), using the automated Riboprinter® (Qualicom). Colonies were grown overnight at 35^0 C on Brain Heart Infusion (BHI) Agar (Difco), and DNA prepared for enzymatic digestion according to the manufacturer's protocol. Samples were loaded in a sample carrier. A lysing agent was added and the DNA was digested with the selected restriction enzyme (Bruce 1996). The DNA fragments were separated by electrophoresis, transferred to a membrane, and then hybridized with a labelled DNA probe (Oscar 1997). A picture of the resulting bands was taken and processed by a computer (Bruce 1996). The organism was identified by comparing the banding pattern with an existing computer library (Bruce 1996). If no match was found for the isolate a new ribogroup was assigned (Oscar 1997).

Serological analysis with Ag O (Difco) test was done to confirm a positive result. King's Agar media and OF medium were prepared according to manufacturer's formulation (Difco) for identification of possible *Pseudomonas fluorescens*. Colonies were plated and incubated 48 hours at 35C. After

incubation, the plates were examined under UV light and colony fluorescens indicated a positive result. In addition, fermentation of glucose was considered negative for *Pseudomona fluorescens* (Compendium of methods for the exam of food products).

III. Results and Discussion

The results obtained when both enzymes were compared (*Pvu*II and *Eco*RI) are shown in table 1. Both *EcoR*I and *Pvu*II are 6 bases cutters. Although *Eco*RI and *Pvu*II belong to the type II restriction enzyme class, they are structurally different and cut DNA in a different manner (Koval and Matthews 1999; Pingoud and Jeltsch 2001). As can be see in table 1, most of the *Eco*RI digested isolates were mistakenly identified as *Escherichia coli*, whereas the *Pvu*II digested isolates were classified as a variety of *Salmonella* serotypes and *P. fluorescens.* Although the isolates were positive for the agglutination test, and positive for *Salmonella spp.* according to the Analytical Profile Index (API) 20 E, they could not be classified as a specific serotype. The *Salmonella* isolates were typed with the two enzymes, the *Eco*RI protocol classified one as *Salmonella* AA, whereas *Pvu*II identified it as *S*. Anatum. Similar results were obtained with another isolate that *Eco*RI classified as *Salmonella* AA, BB (*S*. Senftenberg and S. Reading), whereas *Pvu*II classified it also as S. Anatum. This difficulty arises because of the genetic diversity of the thousands of *Salmonella* serotypes and the limited number of serotypes available for comparison in computer databases.

To verify the results obtained with *Pvu*II, we prepared King's Media and

Table 1. Comparison of *Pvu*II and *Eco*RI enzymes using Riboprinter®.

Sample code	*Eco*RI	*Pvu*II	Ag O
WASSW5- 6503 WASSW8-6903		Pseudomona fluorescens *Salmonella spp* 60%	+ +
WASSW13-22503	Escherichia coli	*S*. Paratyphi B 60% *S*. Montevideo 57% *S*. Oraienburg 57%	+
WADSW1-11402	*Salmonella* AA	*Salmonella* Anatum	+
WADSW2-11402	*Salmonella* AA AB *S*.Reading, Senftenberg	*Salmonella* Anatum	+
WAPTF-61103	Escherichia coli	*Pseudomonas fluorescens* 78% *S*.IV Houten 58% *S*. Miller 57%	+
WASSW14-6903	*Escherichia coli*	Pseudomona fluorescens	+
WASTF61103 CASSW13-82203	*Escherichia coli* *Escherichia coli*	*Pseudomona fluorescens* 79% *S*. Pullorum 60% *S*. Marina 63% *S*. Houten IV 59% *Pseudomona fluorescens* 65% *S*. Arizona III 63% *S*.Tennessee 59% *S*. Paratyphy B 56%	+ +
CASSW14-11603 CASFC1-22503	*Escherichia coli* *Escherichia coli*	Pseudomona fluorescens Pseudomona fluorescens	+ +
CASSW19-51903	*Escherichia coli* 84%	Pseudomona fluorescens	+
CABSW12-51903	Escherichia coli	*S*. Pullorum 59% *S*. Senftenberg 59% *Pseudomona fluorescens* 69% *S*. Bangkok 60%	+
CASS1-11603	Escherichia coli 80%	*Pseudomona fluorescens* 85% *S*. Pullorum 65% *S*. Marina 62% *S*. IV Houten 59%	+
CASFC1-11603	Escherichia coli	*Pseudomona fluorescens* 82% S. Marina 62% S. Pullorum 64% S. IV Houten 62%	+
CASSW17-51903	Escherichia coli	*Pseudomona flurescens* 83% *S*. Pullorum 66% *S*. Marina 62% *S*. IV Houten 62%	+
TNSFC2-62803	Escherichia coli	*S*.Arizona III 73% *S*.Tennessee 83%	+

incubated isolates to determine if fluorescence under the UV light could be observed. None of the isolates fluoresced and other phenotypic characteristics of the isolates also did not support the possibility of the isolates being Pseudomonas. Therefore, *Pvu*II misclassified the isolates as *P. fluorecens.* *Eco*RI gave very limited information and was only able to report a single species, which was *Escherichia coli*. According to Bailey et *al.* (2002), *Pvu*II seems to be a better enzyme when identifying *Salmonella* isolates. It has been reported that the bacterial DNA is resistant to digestion when using *Eco*RI (Oscar 1997). Bailey et al. (2002) found from 80 to 90% correlation between serotypes of *Salmonella* analyzed with subtyping methods and Riboprinter® using *Pvu*II enzyme. Several studies have shown that the identification of *Salmonella* by restriction enzymes depends on the type of the restriction enzyme and the *Salmonella* strain.(Chadfield et al. 2001; Esteban et al. 1993; Millemann 1995; Olsen et al. 1992).The strains used in this study are non-clinical isolates from varied environments. This might explain why significant diversity was found among the strains and why the enzymes provided different results. The use of *Eco*RI was not successful in discriminating among these *Salmonella* strains.

Salmonella and *E. coli* live in the same environment, the gastrointestinal tract of humans and animals. They have been sharing genetic information for a long time. This may explain why *E. coli* and *Salmonella* sometimes overlap phenotypically and genotypically. The colonies from this study were expressing antigens that reacted for *Salmonella* antibodies. For this reason, all of the isolates that showed some *Salmonella* grouping using *Pvu*II and were AgO

positive, were classified as *Salmonella spp*. Until further serological grouping, care must be taken when analyzing the results from ribotyping, especially when using different restriction enzymes on *Salmonella* from environment or food samples.

IV. Conclusion

There are more than 2400 serotypes of *Salmonella* known. Biochemical and serological tests have been used for years to identify and classify the members of the *Salmonella* genus. One must be careful when using molecular tools such as Riboprinter® to identify *Salmonella* isolates because misinterpretation can lead to misclassification of the organism. Understanding the principles, the limitations, and choosing the correct restriction enzyme as well as ensuring that biochemical and serological tests are not overlooked, will ensure more accurate identification of *Salmonella* serotypes.

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Part V: Comparison of Riboprinter® and Pulsed-Field Gel Electrophoresis (PFGE) to Identify *Salmonella spp.*

Abstract

Salmonella is the second most common cause of human salmonellosis in the United States (US). Whereas conventional methods have been successfully used for many years, new methods based on DNA analysis are increasingly used. The objective of this study is to compare Riboprinter® and Pulsed-Field Gel Electrophoresis (PFGE) for discriminatory power among *Salmonella* isolates from animals and farm environment as well as study their spatial and geographical relationships. *Pvu*II was the restriction enzyme used for ribotyping, and *Xba*I was used for PFGE. The discrimination index obtained for Riboprinter® was 0.86. PFGE had a discrmination index of 0.98. Ribotyping classified the isolates into 13 different ribogroups. Further differentiation was seen when a dendrogram was done. Most of the S. Anatum isolated were found to be clonal using Riboprinter®. PFGE was able to further discriminate among these isolates, suggesting even more diversity among the isolates. This study revealed significant diversity of *Salmonella* isolates from environmental samples. It was also found that related isolates grouped within geographical regions.

I. Introduction

Salmonella is the second most common cause of foodborne infections in the United States. Human cases of salmonellosis have been long linked to the consumption of raw meat and unpasteurized dairy products (CDC 1994; CDC 2003). Conventional methods based on biochemical and morphological methods for isolation and identification have been successfully used for many years. However, new technologies based on DNA analysis are increasingly used to classify and compare *Salmonella spp*. Two of theses techniques, ribotyping and Pulsed-Field Gel Electrophoresis (PFGE) use restriction enzymes to digest DNA that is then separated by electrophoresis (Olive and Bean 1999).

PFGE is considered to be “gold standard” for *Salmonella* identification and this method is currently used for the “PulseNet” program at the Centers for Disease Control and Prevention (CDC) (Swaminathan 2001). PFGE has been proven to be very useful when studying clonal populations (Sandvang et al. 2000;Baloda et al. 2001Wonderling 2003) and when investigating human salmonellosis outbreaks (Barret et al. 1994; Gruner et al. 1997). Ribotyping using the automated Riboprinter® is less discriminatory than PFGE (Hollis et al. 1999; Pfaller et al. 1996) but much faster and requires less technical training.

In this study both techniques, Riboprinter® and PFGE, are compared for discriminatory power among *Salmonella* isolated from several farm animals and their surrounding environment. Understanding the limitations of these techniques as well as the movement of *Salmonella* in the environment will help to establish better control for *Salmonella* at the farm.

II. Materials and Methods

Salmonella isolation: *Salmonella* was isolated in a multistate study to determine the prevalence of *Salmonella spp*. in animals and their environment. This study was conducted by the Food Safety Center of Excellence at the University of Tennessee.

Pulsed-field gel electrophoresis: Genomic DNA was prepared in agarose blocks. The method utilized for PFGE was described by Gautom (1997) for typing Gram-negative organisms. Isolates of *Salmonella spp* were grown overnight using Brain Heart Infusion (BHI) Agar (Difco, Sparks, MD) at 35 C and were suspended in 2 to 3 ml of TE buffer (100 m*M* Tris - 100 m*M* EDTA, pH 7.5). The cell suspension was adjusted with TE buffer to 20% transmittance using a colorimeter (bioMérieux). A 200 µl aliquot of the bacterial suspension was transferred to a 1.5 ml micro-centrifuge tube. To each tube, 10 µl proteinase K (20 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN, USA) was added and gently mixed. Added to this was 200 µl of 1.6% InCert/SDS agarose (BioWhittaker Molecular Applications, Rockland, ME, USA) maintained at 55°C. This bacterium-agarose mixture was added immediately to plug molds (Bio-Rad Laboratories, Hercules, CA, USA). Plugs were allowed to solidify for 10 min at 4°C, and then transferred to a 2 ml tube containing 1.5 ml ES buffer (0.5 *M* EDTA, pH 9.0: 1% sodium-lauroyl-sarcosine; Sigma Chemical Co.) and 40 µl of proteinase K (20 mg/ml; Roche Molecular Biochemicals). Plugs were incubated in a 55°C shaker water bath for 45 min. After incubation, ES buffer was removed and plugs were transferred to 50 ml tubes. Plugs were washed in 10 ml sterile

distilled water that was preheated to 50°C for 15 min in a shaker water bath. Water was removed and replaced with 10 ml Plug Wash TE buffer (10 m*M* Tris pH 7.5 and 1 m*M* EDTA, pH 7.5) preheated to 50°C. This was incubated at 50°C in a shaker water bath for 15 min. This wash was repeated 2 times with Plug Wash TE buffer at 50°C in a shaker water bath for 15 min. Plugs were stored at 4°C in 1 ml Plug Wash TE until used.

For restriction endonuclease digestion of genomic DNA, two 1-mm wide slices of plugs were incubated at 37°C for 1 to 1.5 h with 30 units *Xba*I (BioWhittaker Molecular Applications) restriction endonuclease enzyme in 100 µl of the appropriate restriction enzyme buffer.

The DNA fragments were separated by clamped homogeneous electric field (CHEF) electrophoresis using a CHEF-Mapper (Bio-Rad Laboratories). Plug slices were loaded and electrophoresed in 1% SeaKem gold agarose (BioWhittaker Molecular Applications) with 2 L of 0.5X TBE (0.9 *M* Tris base, 0.9 *M* Boric acid, 0.02 *M* EDTA pH 8.0) running buffer. Electrophoresis was performed with a CHEF-Mapper using the following conditions: initial switch time, 2.16 s; final switch time, 35.07 s; angle, 120°; gradient, 6.0V/cm; temperature, 14°C; ramping, linear; run time, 14 h. After electrophoresis, gels were stained in 500 ml distilled water with 50 µl ethidium bromide (10 mg/ml; Sigma Chemical Co.) for 20 min followed by two 15 min washes with distilled water. The DNA fragments were visualized by transillumination (Fotodyne Inc.) and photographed with type 55 Polaroid film (Polaroid Corp.).

Riboprinter®: The samples were processed following RiboPrinter® (Qualicom, Wilington, DE) protocol using *Pvu*II as a restriction enzyme (Bailey et al 2002). The ribosomal DNA fragments were digested and processed ion a nylon membrane and hybridized with an *E. coli* DNA probe. Fragments were visualized by chemiluminescens and the image is captured by a CCD camera and taken to computer software for further analysis (Bruce 1996). The RiboPrinter® identifies isolates by bands matching and also classifies them into ribotypes (Oscar 1998).

Strain classification: Molecular Analyst Software version 1.6 (Bio-Rad Laboratories) was used to determine strain relatedness for each of the two typing methods. The Dice binary coefficient along with the UPGMA (unweighted pair group method using arithmetic averages) was used to construct dendrograms and to determine similarities. The dendrogram is visual illustration of the hierarchic representation of linkage levels between pairs of strains. Band position tolerance of 3% was used for comparison of DNA patterns. The Dice method only considers the presence or absence of bands. Strains that exhibited 93% similarity were considered to be the same subtype. A similarity of 93% was chosen to correspond with the Riboprinter® similarity index which is used under stringent quality control. The automated RiboPrinter® System uses a similarity index of 90% as a cut-off for identical strains. The RiboPrinter® Data Analysis System (DuPont-Qualicon) was also used for strain classification of *Salmonella spp.* isolates that were analyzed using the automated RiboPrinter®. Grouping of strains by the RiboPrinter® Microbial Characterization Data Analysis System

(DuPont-Qualicon) was compared to grouping of strains by the Molecular Analyst Software version 1.6 (Bio-Rad, Laboratories).

III. Results and Discussion

The Simpson's index of diversity was calculated as described by Hunter and Gaston (1998). The Simpson's index of diversity for the Riboprinter® was 0.86, and for the PFGE was 0.98. This index was used to measure the genetic diversity in a population.

Riboprinter® classified the isolates into 13 different ribogroups (figure 1). Ribogrouping is calculated by the program depending on the restriction enzyme used. Ribogrouping with *Pvu*II is more flexible (Qualicom, personal communication) as oppose to *Eco*RI. The Riboprinter® compares the isolates to an internal library, and if it is not recognized, generates new ribogroups based on the new isolates loaded. *Salmonella* isolates used were not recognized by the *Pvu*II library and were classified under new ribogroups of *Salmonella*.

The dendrogram generated from the *Salmonella* isolates analyzed using Riborpinter® with *Pvu*II restriction enzyme is shown in figure 1. Although the riboprinter generated only 13 ribogroups (figure 1), the dendrogram generated more clusters. This is in agreement with the way Riboprinter® classifies the isolates into ribogroups and was expected since the ribogrouping is flexible.

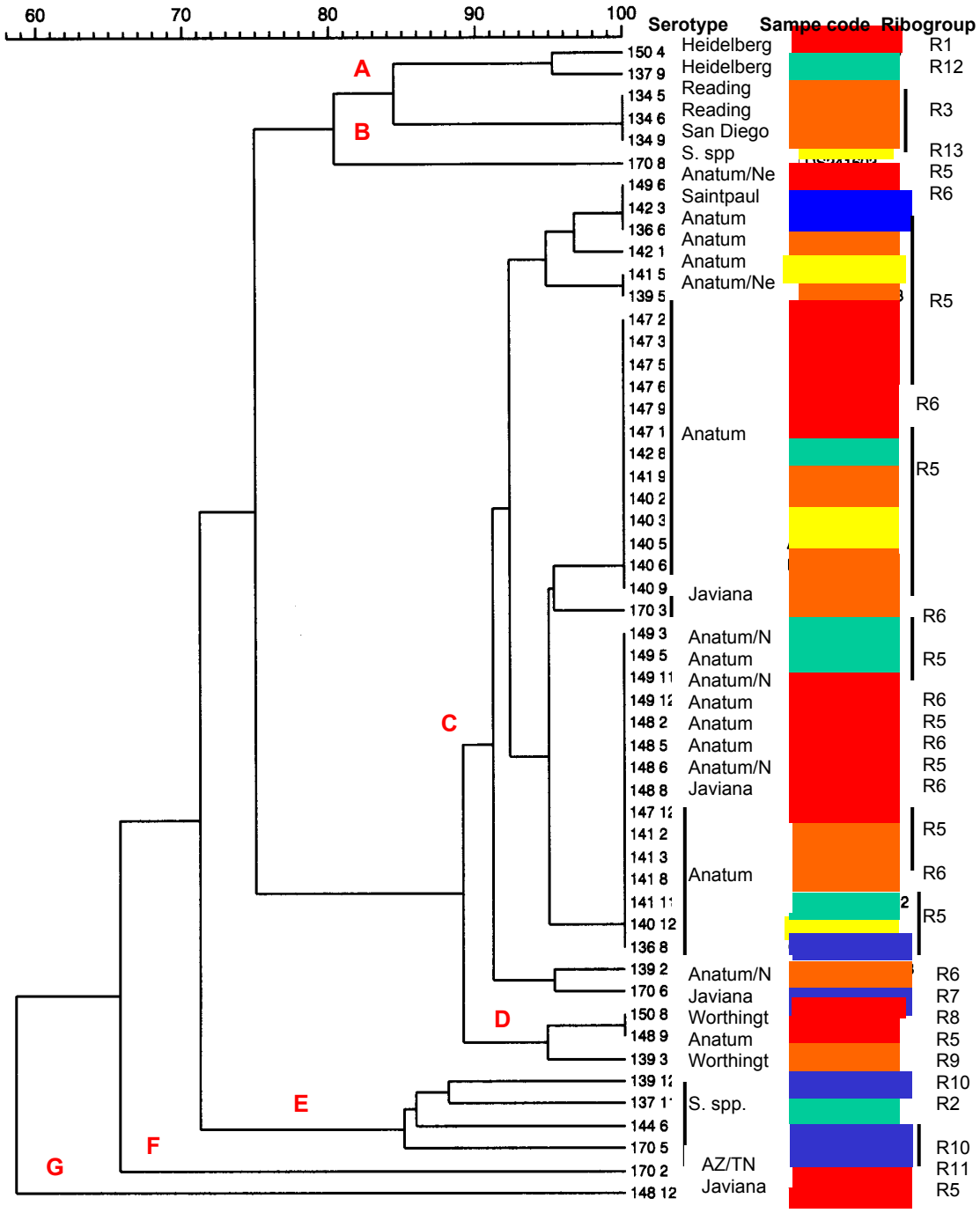


Figure1. Riboprinter Dendrogram generated using *Pvu*II restriction enzyme. Correlation: Bands, Dice (Tol. 3.0%, Opt 0.5%, Min area 0.0%). Clustering: UPGMA.

The serotypes that followed under cluster C were 91% related (Figure 1). Cluster C was divided into 3 subclusters. Isolates with a similarity above 93% was identified as the same serotype. This was true for most of the serotypes that followed under C cluster. In the subcluster C1, there were two groups of serotypes that were 100% related. The first group consisted of three serotypes that were identified by riboprinting as S. Anatum/Newington (149-6), S. Saintpaul (142-3), and S. Anatum (136-6). The other two clonal serotypes identified were S. Anatum (141-5), and S. Anatum/Newington (139-5). All of these serotypes are so closely related genetically that the Riboprinter® may classify them into different serotypes even though they are the same. This is one of the limitations of the automated Riboprinter®. Ribotyping using *Pvu*II as the restriction enzyme shows a great capability to classify the organisms as *Salmonella spp*. but sometimes can misclassify the serotypes depending on how closely related they are. The serotypes above may have arisen from the same clonal population.

The subcluster C2 was divided into three groups. The first one showed that all of the isolates were clonal and serotyped as S. Anatum. Interestingly, one serotype (147-9) was classified in a different ribogroup (R6). The isolate 140-9 was serotyped as S. Javiana. Although it was considered clonal with the other members of this cluster, it was close to 170-3 which was also a serotype as S. Javiana. The other major subcluster contained a clonal population where most of the serotypes corresponded to *S*. Anatum, with the exception of *S*. Javiana (148-8) and *S*. Anatum/Newington (149-3; 149-11; 148-6). The third subcluster represented two different serotypes *S*. Anatum (139-2) and *S*. Javiana (170-6)

that were closely related (95%). The D cluster showed two clonal serotypes that have different ribogroups (R8 and R5) and different serotypes (*S*. Anatum and *S*. Worthington).The E cluster had all of the serotypes typed as *Salmonella spp*. These unclassified serotypes were expected since Riboprinter® did not classify them into any serotype. These isolates belonged to *Salmonella spp.* isolated from Washington State and California. Three *Salmonella spp*. from California were classified under the same ribogroup. Under the F cluster, the serotype was *Salmonella* Arizona/Tennessee, but according the API20E it was only *S*. Arizona. This might not have been detected by the Riboprinter® due to limitations of the library. These serotypes were classified under a different cluster in the dendrogram due to the classification of S. Arizona. An outlier was found under the last cluster and was classified a serotype under *S*. Javiana, which was not closely related to the other *S*. Javiana found.

Under cluster A, there were two groups. The first corresponded to the serotypes S. Heidleberg, being 95% similar. The second group was a clonal population of serotypes identified as S. Reading (134-5; 134-6) and *S*. Sandiego (134-9). Since these three were clonal and were isolated from North Carolina turkey soil and swabs, probably the S. Sandiego was misclassified. Cluster B only had one sertotype that was classified under *Salmonella spp.*

By states, most of the Southeastern serotypes tended to cluster close to each other (Tennessee and North Carolina). Washington and California serotypes were more dispersed and this correlates with the fact that most of the serotypes were classified as *Salmonella spp* because of their diversity. Temporally, most of

the serotypes obtained from Tennessee, Alabama and North Carolina were clustered together, especially those obtained during the winter 2002. The other serotypes were found to be more dispersed and less related.

When analyzing the same isolates using PFGE (figure 2) a higher genetic diversity was observed. The Simpson's index of diversity is a reflection of this, with a value of 0.98. This means that few of the salmonellae isolated were clonal. The dendrogram classified the isolates in two major clusters. The cluster A had the serotype identified as S. Arizona/Tennessee and S. Javiana which were isolated from North Carolina swine.

Cluster B consisted of more different pulsotypes where most of the serotypes obtained were S. Anatum. A close relationship was found among isolates obtained in different states. The PFGE dendrogram grouped the isolates obtained from Southeastern US (Tennessee, North Carolina and Alabama), and more differentiation was found among *Salmonella* isolates found in California and Washington State. This is similar to the results found with Riboprinter®. Several clonal populations were found with PFGE. Two isolates typed as S. Anatum came from North Carolina turkey litter. S. Anatum isolates that came from Tennessee poultry and swine, and an Alabama dairy were considered clonal.

The Tennessee swine farm was located close to a beef farm. Although not clonal, the serotypes obtained during this sample period in both farms were 95% similar. In these two farms the same serotype (S. Anatum) was isolated during the same season and due to the proximity of the two farms, a clonal population was suspected. Although not clonal, there was a high degree of similarity.

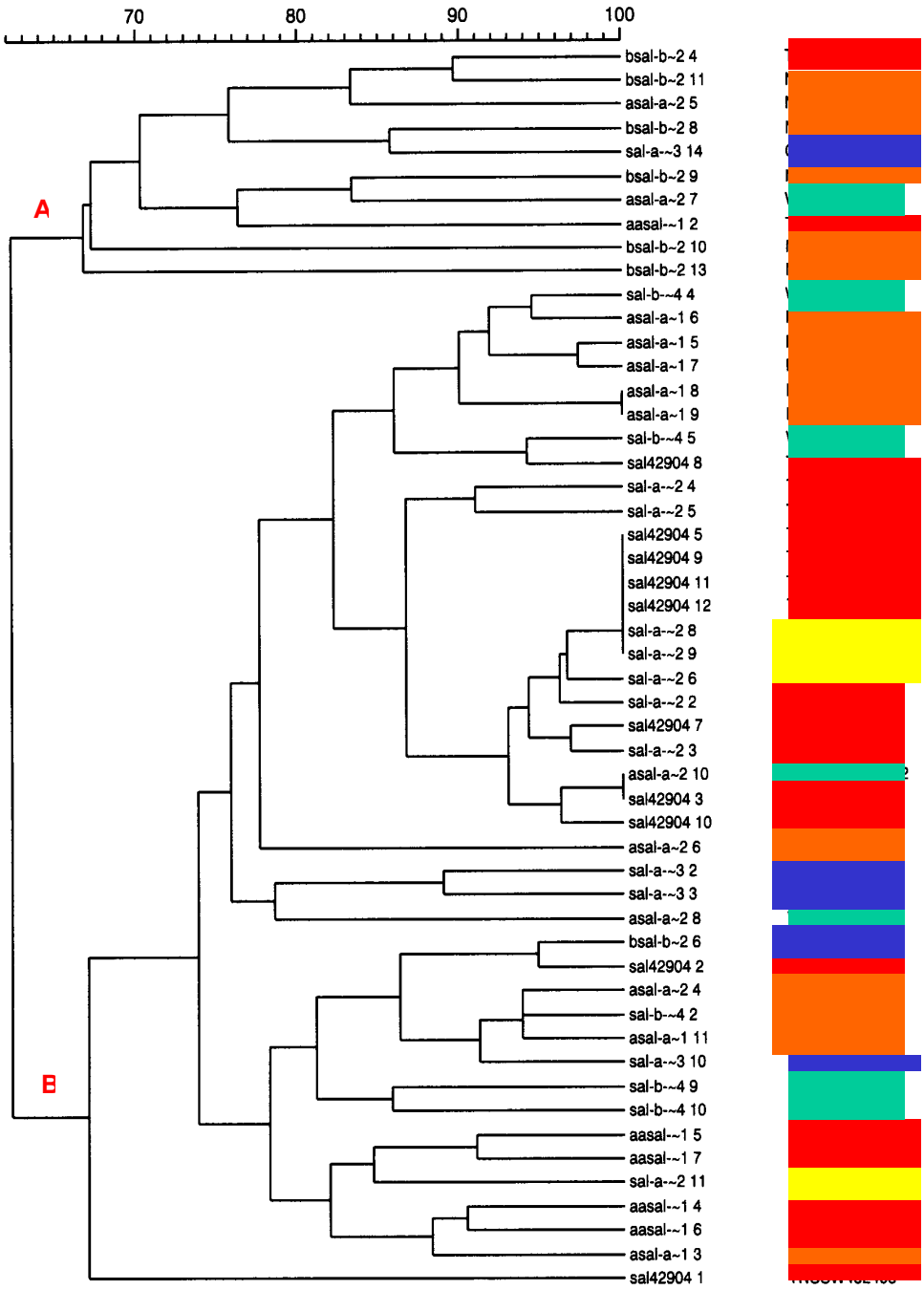


Figure2. PFGE Dendrogram generated using XbaI restriction enzyme. Correlation: Bands, Dice (Tol. 1.5%, Opt 0.5%, Min area 0.0%). Clustering: UPGMA.

One of the S. Anatum serotypes obtained from Washington State (asala2-10) that came from a dairy fecal swab was found to be clonal with a Tennessee poultry trough feed sample. Riboprinter® also classified these two serotypes in the same cluster.

PFGE showed more discriminatory power than ribotyping for S. Anatum serotypes. Similar results were found by Nayak et al. (2004). Ribotyping has shown excellent capability for determining if the bacterium isolated belongs to the *Salmonella* species. Each technique differs in the way it analyzes the DNA. While Riboprinter® only accounts for the ribosomal DNA, the PFGE takes in account the whole bacterial genome. The ribosomal DNA is a conserved region and this may explain the high degree of clonacity found with the Riboprinter®. The technique also generates fewer bands than PFGE limiting its ability to further discriminate among isolates (Olive and Bean 1999). Mutations in the genome can easily be detected by PFGE (Bennekov et al. 1996). This may explain why a higher diversity was found in isolates using PFGE compared with Riboprinter®. PFGE has been shown to be a great tool to establish clonacity among isolates, so this would be very useful in determining the source of an outbreak (Barret et al. 1993). In this study the same serotype was isolated from dairy cows and a geographically close swine farm in Tennessee (data provided by the Food safety center of Excellence, The University of Tennessee). In this case, PFGE was used to discriminate among the serotypes and concluded that the isolates did not come from the same clonal population, although were closely related (93%).

For the isolates that were found clonal on PFGE, Ribotyping also found most of them to be clonal. The exception was an isolate from Alabama dairy bedding 1 (Figure 1:141-5) that was 92% related to the others. Several outliers were found with PFGE for S. Anatum that were found clonal using Riboprinter. Weigel et al. (2004) also found that some serotypes (Agona, Anatum, Derby, Infantis and Worthington) were located across the riboprinting clusters.

In the cluster where most of the S. Anatum were isolated, there were similarities to the Riboprinter® dendrogram. S. Javiana was classified with the S. Anatum serotypes (sal42903-7; sal42903-9). Similarly, S. Sandiego and S. Reading, were classified with a 95% similarity in the dendrogram (asala2-4; salb4-2; asala1-11). These serotypes came from the same North Carolina turkey farm, but from different seasons. The genetic similarity suggests a long survival of the salmonellae in the environment.

Riboprinter® analyzes band pattern based on band position and intensity, whereas for PFGE, the way to analyze the bands is chosen by the operator. The Dice UPMGA is based on band position and not on band intensity. Genetic similarity is based on the distance between the bands analyzed. When entering PFGE bands in a computer to analyze, care must be taken to verify that all the bands are properly marked. The resolution of the computer may interfere with some of the bands and this can lead to a misclassification of the isolate.

According to several authors, the use of both techniques can be used to determine the presence of *Salmonella* serotypes and to study their relationships (Fontana et al. 2003). PFGE has been extremely useful when studying human

outbreaks (Barret et al. 1994; Bender et al. 2004). Other authors have determined that depending on the serotype (Barret et al. 1994; Thong et al. 1995; Liebana et al. 2001) and the restriction enzyme used (Gunner et al. 1997), one technique can be superior to the other. Liebana et al. (2002) found that PFGE did not provide a good differentiation for the serotypes Enteriditis and Typhimurium. However, PFGE is generally considered to have a superior discrimination compared to Ribotyping (Hollis et al. 1999; Pfaller et al. 1996) because the larger number of bands obtained for analysis.

IV. Conclusion

PFGE is considered to be the Gold Standard for molecular analysis of *Salmonella*. Our Simpon's diversity index supports this statement. PFGE demonstrated a tremendous genetic diversity among the salmonellae isolated, but this technique is more time consuming than the Riboprinter® and required more expertise.

Riboprinter® is a faster technique and has good capacity to distinguish among *Salmonella* isolates to a level where different serotypes can be studied. Depending on the requirements of the study the use of ribotyping has advantages, especially if large numbers of samples need to be analyzed. The automated Riboprinter® is a fast, reliable and repeatable technique, whereas PFGE requires a higher degree of expertise and more time.

There was a significant diversity in *Salmonella* isolates across the US in the farm samples analyzed. However, geographic and seasonal relationships were

clearly demonstrated. Survival and competition with other enteric and native bacteria at the farm may have increased the diversity of the salmonellae in the farm animals. Understanding this genetic diversity will help to track salmonellae and develop programs to reduce and control the spread of salmonellae in the farm environment to prevent further spread and contamination the animals and the food supply.

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VITA

Andres Rodriguez Lozano was born on May 31st 1976 in Murcia, Spain. He was raised in Castellon and attended the I. B. Penyagolosa High School. In 1994 he went back to Murcia to enroll at the College of Veterinary Medicine, University of Murcia, to pursue his veterinary studies. During the academic year 1999-2000 he was awarded a Socrates/Erasmus scholarship funded by the European Union to study at the Faculty of Veterinary Medicine at the University of Helsinki, Finland, as an exchange student where he also worked at the Veterinary Teaching Hospital. During the academic year 2000-2001 he was an exchange student at the College of Veterinary Medicine, The University of Tennessee, thanks to the ISEP (International Student Exchange Program) program. Andres worked also at the Veterinary Teaching Hospital Small Animal Clinic during the winter break. He also became an active member of RAM (Remote Area Medical, Veterinary Branch) and served as a translator in a trip to Guatemala (June 16th-28th 2001) led by Dr. Eric Davis. During the Fall 2001 he went back to his home University where he obtained his degree in Veterinary Medicine and Surgery.

He enrolled the University of Tennessee in the Spring 2002 to pursue his Master degree in Food Science - Food Microbiology under Dr. Draughon's supervision. He concluded his Master's degree in the summer 2004. During his master he did an internship in a dairy company "La Esmerlada", San Miguel de Allende, GA, Mexico. Andres was also was a member of the 2003 Dairy Product Evaluation Team at the University of Tennessee where he was awarded third place in yogurt evaluation for the graduate student division at the National

Collegiate Dairy Product Evaluation competition held in Illinois, Chicago. In recognition to his achievements he obtained the 2004 Jim Collins International Award at the Department of Food Science, The University of Tennessee.

Andres is going to continue his education pursuing a PhD at the University of Massachusetts in the area of Food Microbiology under Dr. McLandsborough's supervision. He will be working with *Listeria monocytogenes* and biofilms.