A Survey of *Salmonella* Serovars and Most Probable Numbers in Rendered-Animal-Protein Meals: Inferences for Animal and Human Health

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Abstract

Salmonellae are resourceful and adaptive organisms that infect a broad range of diverse hosts. Serovars of the genus were first isolated in a poultry mash in 1948, thus establishing a need to assess the pertinence of *Salmonella* organisms in animal protein feed ingredients. In the study reported here, a random-sampling scheme was established to evaluate the *Salmonella* population level by three-tube most-probable-number (MPN) analysis and serovar identity over a period of one year. The results provide evidence of the limited relatedness of animal protein meals in the complex cycle of feed contamination and likely subsequent transmission of disease to animals or humans through the feed chain.

Introduction and Background

Species of the genus *Salmonella* have historically been associated with foodborne diseases and remain a constant challenge to the feed and food industry throughout the world. The voluminous literature and databases clearly affirm the importance of this group of organisms as disease agents of both animals and man (Ziprin, 1994). Especially relevant is that most countries of the world use *Salmonella* as an indicator of sanitation, hygiene, or contamination of products. In the United States, the U.S. Department of Agriculture's (USDA's) Food Safety and Inspection Service has introduced the genus into its regulatory mandate, establishing pathogen reduction performance standards for *Salmonella*, because raw meat and poultry products frequently harbor the organism. Salmonellae are also among the most prevalent of zoonotic infectious agents throughout the world (Werner, 1992).

In 1884, T. Smith and D.E. Salmon, two eminent research microbiologists in USDA's Bureau of Animal Industry, first reported on "the hog cholera group of bacteria" and on "swine plague," work that culminated in the naming of the genus *Salmonella* in honor of Daniel E. Salmon (Franco, 1997; Ziprin, 1994). The work of these researchers clearly demonstrates the fallacies of early research endeavors: Swine fecal specimens routinely contain salmonellae, and it is obvious that Smith and Salmon associated their isolation of these organisms with the cause of early poorly defined and challenging diseases like hog cholera. Today hog cholera is known to be a viral disease. Nonetheless, even though they did not discover the definitive cause of the disease, Smith and Salmon contributed to the annals of bacteriology and infectious diseases by discovering this ubiquitous foodborne pathogen.

In 1880, prior to the findings of Smith and Salmon, Eberth had discovered the etiological agent of typhoid fever, an acute bacterial disease of humans (Levine & Blake, 1992). It became clear that the causative organism of typhoid fever belonged to the same general group as Salmon and Smith's bacterium. Thus, by 1888, *Salmonella* had emerged as an important infectious agent in both animals and man that caused pathologic changes and other disease complications not limited to diarrhea (Franco, 1997).

The early history of *Salmonella* circumvented the rendering and feed industries until 1948, when the organism was first isolated in animal feed (poultry mash) at the University of Kentucky's Experimental Station in Lexington (Franco, 1997). The federal Food, Drug, and Cosmetic Act, the country's basic food and drug law, defines food as "articles used for food or drink for man or other animals ... and articles used for components of any such article" [Section 201(f)]. Therefore, according to the act, *Salmonella* contamination of animal feeds, which could produce infection and disease in animals, is regarded as an adulterant (Levine & Blake, 1992). The regulatory implications are firmly established, and the results of different studies throughout the world have indicated a need to assess the relevance and contextual significance of the prevalence of the organism in major ingredients used in feed. This need is especially important since the medical or veterinary literature emphasizes a causal relationship between the inclusion of animal protein meals in feed rations and the incidence of disease in livestock and poultry—and subsequently humans. To date, inferences have always been anecdotal, devoid of conclusive validated studies, and predominantly based on the assumption of "likely

cause" because of the occasional isolation of the organism in feeds.

The most widely referenced peer-reviewed publication on the public health pertinence of feed-associated salmonellosis in humans is limited to a hypothesis. Albeit interesting, it lacks scientific validation for a proven link between the feed, confirmed contamination of the poultry, and the clinical manifestations of the patients. In 1973, researchers and epidemiologists of the Bacterial Diseases Branch of the Centers for Disease Control (CDC) reported in the prestigious British medical journal the *Lancet* on the epidemiology of an international outbreak of *Salmonella* Agona (Clark, Kaufmann, Gangarosa, & Thompson, 1973). The authors reported that a foodborne-disease outbreak had occurred in Paragould, Arkansas, affecting 17 residents of the town, and that the source of the outbreak had been traced to a local restaurant and to a Mississippi poultry farm that had used imported Peruvian fishmeal as a protein supplement in its feed at a rate of 8 percent (Clark et al., 1973; Franco, 1997).

The epidemiological data implied that the fishmeal was the probable vehicle of the S. Agona contamination of the poultry and, by inference, the associated cause of the foodborne-disease outbreak. This instance was also the first inferred relationship of a rendered animal by-product in a livestock- or poultry-feed formula with the possible transmission of disease in humans (Franco, 1997). The epidemiology remained circumstantial because S. Agona was not isolated from the feed samples collected (Clark et al., 1973). Specimens for laboratory analysis were taken at all stages of production at the poultry-processing and rendering plants, and isolations of many *Salmonella* serovars were recovered, but not of S. Agona (Clark et al., 1973). The serovar (Agona) was recovered, however, from environmental swabs taken at the slaughterhouse and from offal to be rendered (Clark et al., 1973).

The authors theorized that "contaminated chicken apparently introduced S. Agona into the Paragould restaurant, but it was probably not the vehicle of infection, since cooking temperatures were sufficient to kill salmonellae" (Clark et al., 1973). This reported outbreak heightens the complexity of the chain of transmission of salmonellosis, whether in animals or humans, and illustrates inherent obstacles to making finite conclusions (Franco, 1997). The report was a well-documented effort to examine the continual challenges associated with the nuances of *Salmonella* epidemiology, and it generated questions that must be addressed. In fact, this publication has become the most important reference source for the likelihood of the feed-animal-human transmission linkages culminating in disease.

The Center for Veterinary Medicine (CVM) of the Food and Drug Administration (FDA) has expressed concern about *Salmonella* contamination in feed ingredients and finished feed, and its representatives have been collaborating with involved industries to examine and consider options for prevention and control. Conceptually, the agency recommends that preventive controls be realized by applying hazard analysis and critical control point (HACCP) principles to the manufacturing processes.

Characteristics of the Organism

Salmonella is the name associated with a genus of bacteria that is commonly related to foodborne diseases and is a member of the family Enterobacteriaceae (Blackman et al., 1992; Kaye, 1996). Salmonellae are rod-shaped, motile, Gram-negative, non-spore-forming bacilli that ferment glucose, maltose, and mannitol; almost all produce acid and gas with fermentation (Franco, 1997; Kaye). Salmonellae can be differentiated by their somatic (O) antigens, composed of lipopolysaccharides, and their flagellar (H) antigens (Kaye).

Traditionally, the serovars most commonly isolated in any country tend to be characteristic of that locale and not subject to extreme variations of isolation frequency over short periods (Clark et al., 1973). The genus is made up of more than 2,300 serovars whose simple requirements for growth enable them to multiply over a wide variety of conditions (Franco, 1997). The organisms are not highly resistant to either physical or chemical agents. They can be killed at 55°C in 1 hour, or at 60°C in 15 to 20 minutes. They are ubiquitous, and moist conditions favor growth (Franco, 1997). The optimal temperature for growth is 37°C, the normal human body temperature. Standard cooking temperatures, pasteurization, and commonly used disinfectants readily destroy the organism. Freezing decreases *Salmonella* numbers, but does not kill them. The organism survives desiccation well; under optimum conditions with no limits on food and space, a cell can divide every 20 minutes (Franco, 1997).

Study Objective

The Animal Protein Producers Industry (APPI) comprises companies in the rendering industry that produce protein meals of animal origin. Each member plant collects a sample of rendered or blended animal protein meal weekly, or at least 52 samples annually. Participating plants mail in their samples either weekly or monthly to a laboratory for *Salmonella* analysis. Currently, approximately 25 percent of the submitted samples test positive for *Salmonella*.

The major objectives of the study reported here were to determine through data collection the pertinence of *Salmonella* population numbers and serovar identity in submitted samples, and subsequently to assess the relevance of the findings in the context of food safety, including the public health inferences. The goals were compatible with the main objectives of the U.S. food safety system, which embraces the farm-to-table concept of controls, and the perceived role of every sector in the food chain in precluding hazards and ensuring a safe finished product.

Method

Experimental Protocol

The study analyzed a cross-section of approximately 200 animal protein meal samples that tested positive for *Salmonella* over a 12-month period and determined the identity of the *Salmonella* serovar and the level of *Salmonella* by most-probable-number (MPN) analysis.

Random-Sampling Scheme

A statistically generated sampling scheme used a cross-section of the APPI *Salmonella*-positive samples, on the assumption of 175 *Salmonella*-positive samples per month. Each month, 16 to 17 *Salmonella*-positive samples were selected for MPN determination and *Salmonella* serotyping. The numbers given in Table 1 for each month refer to the sequential number of each of the positive samples used that month. For example, in the first month, the first sample used for serotyping and enumeration was the third positive sample found. The 17th sample used for serotyping and enumeration was the 165th positive sample found.

Selection of Samples

Samples from participating member plants were mailed to Ralston Analytical Laboratories in St. Louis, Missouri, for *Salmonella* analysis. The presence of *Salmonella* was determined in a 25-gram (25-g) sample with a *Salmonella* DNA probe assay (Rose, B.E., Llabres, C.M., & Bennett, B., 1991). Standard cultural procedures and biochemical and serological testing confirmed samples with a Gene-Trak assay

presumptive-positive result. A tally was kept in the laboratory of the total number of *Salmonella*-positive samples for each month. Gene-Trak presumptive-positive samples were added to the tally, and the appropriate samples were selected according to the random-selection table. Presumptive-positive results were used to select samples for MPN analysis and serotyping for two reasons. First, the Gene-Trak *Salmonella* assay has shown that APPI samples that were presumed positive for *Salmonella* are almost always confirmed to be so. Second, the *Salmonella* MPN assay must be set up as soon as possible after the Gene-Trak *Salmonella* detection assay so that the MPN result will realistically reflect the *Salmonella* population in the sample in the selected time frame.

Enumeration of *Salmonella* by MPN Procedure

Salmonella enumeration was conducted on the selected *Salmonella*-positive samples using a three-tube most-probable-number (MPN) technique (CDC, 1981). Testing was performed on 40-gram samples. The minimum sensitivity of this assay, based on the dilutions, was <0.03 *Salmonella* MPN per gram.

Determination of *Salmonella* Serovar

Salmonella isolates from the Gene-Trak detection assay were used for serotyping. Identification of O antigens was performed according to a method described by Kauffmann (1996), and the identification of H antigens was performed according to the procedure described in the 11th edition of the *Difco Manual* (Difco, 1998). The author determined the identity of each *Salmonella* serovar by compiling the results of O and H serology and consulting the 1994 revision of the "Kauffmann-White *Salmonella* Antigenic Scheme" in the 11th edition of the *Difco Manual*.

TABLE 1

Random Selection of Positive *Salmonella* Samples for Serotyping, by Month

	Month of Sampling											
Sample	1	2	3	4	5	6	7	8	9	10	11	12
1	3	8	3	9	19	12	5	1	12	4	13	1
2	4	17	4	23	47	20	6	26	19	9	19	5
3	17	30	10	25	48	25	25	30	23	12	21	43
4	18	31	25	28	55	41	36	32	63	45	45	44
5	23	36	34	36	79	43	41	42	66	58	50	45
6	53	46	36	53	82	82	42	65	70	73	56	68
7	61	49	44	55	89	95	50	71	81	87	92	81
8	63	68	47	62	103	96	53	73	82	91	98	95
9	67	83	50	73	112	109	58	75	104	107	101	100
10	71	85	51	82	120	111	62	110	109	109	109	105
11	74	98	58	94	126	119	80	112	131	127	123	110
12	78	118	94	118	127	134	86	117	132	129	129	116
13	95	132	124	137	128	146	108	121	140	139	136	124
14	104	134	138	138	129	151	109	134	156	144	139	129
15	117	138	158	148	132	159	142	153	157	157	146	146
16	126	139	169	149	139	162	147	163	172	159	148	152
17	165	166	174	165	164	175	170	169				

The numbers given for each month refer to the sequential number of each of the positive samples used in that month. For example, in the first month, the first sample used for serotyping and enumeration was the third positive sample found. The 17th sample used for serotyping and enumeration was the 165th positive sample found.

Results and Discussion

Average monthly *Salmonella* MPN/g counts ranged from 0.2 (April 1999) to 78.0 (July 1998). The five highest counts were in consecutive months: June 1998 (12.3), July 1998 (78.0), August 1998 (4.0), September 1998 (74.8), and October 1998 (10.2). The average *Salmonella* MPN/g value for the entire 12 months and all 197 samples was 16.3, while the median MPN/g value was 0.09.

Table 2 reflects the frequency of the *Salmonella* MPN/g values. Almost 75 percent of the samples (148 of 197) had *Salmonella* MPN/g values of <1.0. Almost 91 percent of the samples (180 of 197) had *Salmonella* MPN/g values of <10. Only 4.5 percent of the samples had *Salmonella* MPN/g values at the 10^2 to 10^3 level.

Table 3 gives the frequency of the top 10 *Salmonella* serovars isolated during the 12-month survey. Approximately 48 percent of the *Salmonella* serovars were among these 10 serovars. From the 197 samples used in this study, 56 unique serovars were identified. The top three serovars isolated from samples in this survey—*Salmonella* Senftenberg, *Salmonella* Livingstone, and *Salmonella* Mbandaka—were found in 23 percent of the samples. Of particular interest are the serovars that have previously been associated with foodborne illness in humans. Two of these serovars, *Salmonella* Agona (3.5 percent) and *Salmonella* Infantis (3 percent), were among the top 10 serovars isolated. Although *Salmonella* Typhimurium and *Salmonella* Enteritidis are the most common serovars in the United States (Centers for Disease Control and Prevention [CDC], 2003), each was isolated in only 0.5 percent of the *Salmonella*-positive samples in this study.

Conclusions

For the 197 *Salmonella*-positive samples tested during this 12-month study, the *Salmonella* MPN/g values ranged from <0.03 to 1,100, with a mean MPN/g value of 16.3 and a median MPN/g value of 0.09. The 10 most common serovars, in order of occurrence, were as follows: *Salmonella* Senftenberg, *Salmonella* Livingstone, *Salmonella* Mbandaka, C2 Group *Salmonella*, *Salmonella* Havana, *Salmonella* Lexington, *Salmonella* Agona, *Salmonella* Arkansas, *Salmonella* Infantis, and *Salmonella* Johannesburg. These top 10 serovars accounted for 48 percent of the serovars isolated. Four serovars associated with foodborne illness—*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Infantis, and *Salmonella* Agona—accounted for only 7.5 percent of the *Salmonella* isolated.

Animal and Human Health Inferences

Clinical findings indicate that the majority of *Salmonella* infections in farm animals are transmitted from an animal of the same spe-

TABLE 2

Frequency of *Salmonella* MPN/g Values, June 1998 to May 1999

Salmonella MPN/g Value	Frequency
<0.03	52
0.03	1
0.04	21
0.07	5
0.09	23
0.14	1
0.15	5
0.23	20
0.36	1
0.43	11
0.93	7
1.2	1
1.5	2
2.1	3
2.3	10
2.9	1
4.3	7
4.4	1
4.6	4
9.3	2
11	1
12	1
15	3
23	1
24	1
39	1
46	2
93	1
110	5
150	1
930	1
1,100	1

TABLE 3

Top 10 *Salmonella* Serovars, June 1998 to May 1999

Salmonella Serovar	Number of Occurrences	Percentage
Salmonella Senftenberg	16	8%
Salmonella Livingstone	15	7.6%
Salmonella Mbandaka	15	7.6%
C2 Group *Salmonella*	8	4%
Salmonella Havana	8	4%
Salmonella Lexington	8	4%
Salmonella Agona	7	3.5%
Salmonella Arkansas	6	3%
Salmonella Infantis	6	3%
Salmonella Johannesburg	6	3%
Number of isolates serotyped	197	
Serovars of interest:		
Salmonella Typhimurium	1	0.5%
Salmonella Enteriditis	1	0.5%
Salmonella Infantis	6	3%
Salmonella Agona	7	3.5%

cies, a trend especially exemplified by the host-adapted serovars. In cattle, the two most common serovars consistently isolated are S. Typhimurium and S. Dublin (Wray, 1994). In swine, S. Choleraesuis remains the most predominant serovar, associated with acute, subacute, and chronic syndromes of infection (Wray). Poultry isolations of different *Salmonella* serovars tend to be more diverse than cattle and swine isolates and also appear to be more variable from year to year, at least in the United States. Serovars Heidelberg and Enteritidis have historically been the most consistent of the isolates (Franco, 1997; Snoeyenbos, 1994). Basically, however, the majority of the more than 2,300 *Salmonella* serovars are not host-adapted and can infect a broad range of susceptible hosts, most likely all species of mammals, birds, and reptiles, for varying periods, with or without clinical manifestations of disease (Snoeyenbos).

In the review of laboratory findings and the annals of infectious diseases, the postulate that any *Salmonella* serovar has the potential to cause disease should not be disputed. The record, however, indicates that of the various serovars, only 30 to 40 are routinely reported to have clinical pertinence in animals and man (Franco, 1997). The isolates of rendered-animal-protein meals, in general, have traditionally not been linked to the customary cause of clinical syndromes in animals and man. An evaluation of the 10 most frequently isolated serovars in this study affirms this inference. Both in animals and man, three clinically significant isolates that were serotyped are S. Enteritidis, 0.5 percent; S. Typhimurium, 0.5 percent; and S. Infantis—1 percent of the total samples serotyped.

The findings of this study are compatible with those of previous serotyping isolates done by other researchers and government institutes—for instance, Sato (1977–1983), Nagaraja (1978–1989), and the Ministry of Agriculture, Fisheries, and Food of the United Kingdom (1993) (Franco, 1997). The independent findings of other laboratories, including some in other countries (Japan and the United Kingdom), showing a marked degree of relative consistency of isolates, offer encouragement about the pertinence of this work.

The safety of rendered-animal-protein meals must be put in context. The time-and-temperature processes of rendering far exceed the range that destroys *Salmonella* and, for that matter, other genera of bacteria of importance to the feed and food cycle. The inclusion rate of animal protein meals in feed rations varies from 2.5 percent to 5 percent depending on animal species and nutritional objectives. The further processing and pelleting at the feed mill, using a conditioner meal temperature of at least 180°F and monitoring the moisture level, serve as effective additional insurance of product safety.

FDA's ultimate regulatory statute and mission is the protection of public health. The agency's compliance oversight initiatives encompass a broad range of industries' manufacturing and production practices, including aspects of feed and food safety, and are integrated to ensure that the mandated objective is accomplished. In essence, FDA remains a public health agency, and the examination of the relevance of *Salmonella* contamination of

rendered animal proteins and its health safety pertinence is directly related to the public health significance.

The findings of this study profile both the prevalent serovars and the MPN of a broad range of rendered proteins that are used in livestock/poultry rations. Most of the isolated serovars were not compatible with the usual isolates found in a clinical setting in animals and humans in the United States. Of equal significance is the mean population average of 16.3 organisms per gram. For disease to occur, it is usually necessary to ingest large numbers of organisms (on the order of five or more logs). There is, however, considerable variation in the inoculum size necessary to cause disease. This variation is dependent on the serovar, the vehicle of infection, and the host. Certain serovars cause disease in relatively small numbers (e.g., S. Typhimurium, S. Newport, and S. Heidelberg). Nonetheless, the MPNs of this study present compelling evidence of the limited risk of transmitting disease to animals or humans through rendered animal proteins in the feed rations of livestock or poultry.

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