A Survey of *Salmonella* Serovars and Most Probable Numbers (MPN) 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. A random sampling scheme was established to evaluate the Salmonella population level by three-tube most probable numbers (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 food borne 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 Food Safety and Inspection Service, U.S. Department of Agriculture, has introduced the genus into their regulatory mandate by 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 the U. S. Department of Agriculture's Bureau of Animal Industry, first reported on "the hog cholera group of bacteria" and on "swine plague" culminating in the naming of the genus *Salmonella* in honor of Daniel E. Salmon (Franco, 1997; Ziprin, 1994). These researchers clearly demonstrate the fallacies of early research endeavors: Swine fecal specimens routinely contain salmonellae, and it was 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, we know that hog cholera is a viral disease. Nonetheless, even though they did not discover the definitive cause of the disease, they, 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, causing pathologic changes and other disease complications that were 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) of the Act). Therefore, within the meaning of 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 is especially important since the medical or veterinary literature substantiates a causal relationship between the inclusion of animal protein meals in feed rations and disease incidence 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 was limited to a hypothesis. Albeit interesting, it lacks scientific validation of 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 and Prevention (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 was traced to a local restaurant and to a Mississippi poultry farm that had used imported Peruvian fishmeal as a protein supplement at an inclusion rate in the feed of 8% (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 was also the first inferred relationship of a rendered animal by-product in a livestock- or poultry-feed formula being linked to 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 *S.* Agona (Clark et al., 1973). The serovar (Agona) was recovered, however, from environmental swabs taken at the slaughterhouse and 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, either 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 association of the feed-animal-human transmission linkages, likely 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 their 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 the principles of hazard analysis and critical control points (HACCP) 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). They 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, 1996). Salmonellae can be differentiated by their somatic (O) antigens, composed of lipopolysaccharides, and flagellar (H) antigens (Kaye, 1996).

Traditionally, the most commonly isolated serovars 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 2300 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% 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 to preclude hazards and assure a safe finished product.

Experimental Protocol

The study analyzes a cross-section of approximately 200 animal protein meal samples that tested positive for *Salmonella* over a 12-month period and determines 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, based on an assumption of 175 *Salmonella*-positive samples per month. Each month 16 to 17 *Salmonella*-positive samples were selected for MPN determination and *Salmonella* serotyping. For each month, the number in Table 1 refers to the sequential number for the positive samples for the month. For example, in the first month, the first sample for serotyping and enumeration is the third positive sample found.

Selection of Samples

Samples from participating member plants were mailed to Ralston Analytical Laboratories, St. Louis, Missouri, for *Salmonella* analysis. The presence of *Salmonella* is determined in a 25-g sample using 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 using the random selection table. For two reasons presumptive positive results were used to select samples for MPN analysis and serotyping. First, the Gene-Trak *Salmonella* assay has always 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 (Centers for Disease Control and Prevention [CDC], 1983). 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 using a method described by Kauffmann (1996) and the identification of H antigens was performed per the procedure described in the 11th edition of the *Difco Manual* (Difco, 1998). The identity of each *Salmonella* serovar was determined 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*.

Results and Discussion

The *Salmonella* MPN/g values and the *Salmonella* serovar identities are listed monthly, the average for the month appearing at the bottom of each table, and a summary of the average *Salmonella* MPN/g counts per month shown in another table. 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% of the samples (148 of 197) had *Salmonella* MPN/g values of <1.0. Almost 91% of the samples (180 of 197) had *Salmonella* MPN/g values of <10. Only 4.5% 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% of the *Salmonella* serovars were among these 10 serovars. From the 197 samples 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% of the samples. Of particular interest are those serovars that have been previously associated with foodborne illness in humans. Two of these serovars, *Salmonella* Agona (3.5%) and *Salmonella* Infantis (3%), were among the top 10 serovars isolated. Although *Salmonella* Typhimurium and *Salmonella* Enteritidis are the most common serovars in the United States (CDC, 2003), each was isolated in only 0.5% 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 1100, 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: *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% of the serovars isolated. Four serovars associated with foodborne illness—*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Infantis, and *Salmonella* Agona—accounted for only 7.5% 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 species, 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, sub-acute, and chronic syndromes of infection (Wray, 1994). 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 2300 *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, 1994).

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%; *S.* Typhimurium, 0.5%; and *S.* Infantis—1% of the total samples serotyped.

The findings of this study are compatible to those of previous serotyping isolates done by other researchers and government institutes, e.g., 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 comparisons with other countries (Japan and the United Kingdom), with a marked degree of relative consistency of isolates, offers encouragement to 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% to 5% 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 entail 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 the health safety pertinence are 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 man in the United States. Of equal significance is the mean population average of 16.3 organisms per gram. In general, 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 is dependent on the serovar, the vehicle of infection, and the host. Certain serovars cause disease in relatively small number, e.g., *S.* Typhimurium, *S.* Newport, and *S.* Heidelberg. Nonetheless, the MPN of this study present compelling evidence of the limited risk of transmitting disease to animals or humans through rendered animal proteins in feed rations of livestock or poultry.

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