DEVELOPMENT OF RECOMBINANT FLAGELLAR ANTIGENS FOR
SEROLOGICAL DETECTION OF SALMONELLA ENTERICA SEROVARs
ENTERITIDIS, HADAR, HEIDELBERG AND TYPHIMURIUM IN POULTRY

by
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(Under the Direction of John J. Maurer)

ABSTRACT

Accurate and fast serological detection of harmful Salmonella in poultry is a major concern of food safety. Common Salmonella serotypes responsible for human associated foodborne outbreaks are Enteritidis, Hadar, Heidelberg, and Typhimurium. Recombinant flagellin for phase 1 (H1) and phase 2 (H2) were cloned into Pinpoint-T® protein expression vector and purified to be used as antigens in an ELISA. An ELISA was developed for the serological detection of S. enterica Enteritidis in this study. 500 ng of purified recombinant g,m antigen and a 1:64 dilution was determined to be optimal for testing field serum samples through checkerboard titration analysis. A negative baseline cutoff was calculated to be an optical density (OD) of 0.35. This new ELISA eliminates common cross reactivity inherent in lipopolysaccharide (LPS) or whole cell antigens by focusing specifically on the H1 and H2 phase combination of flagellin found on common Salmonella serotypes present in poultry.

INDEX WORDS: ELISA, Salmonella, serology, Typhimurium, Enteritidis, Heidelberg, Hadar, recombinant DNA technology, flagella
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DEVELOPMENT OF RECOMBINANT FLAGELLAR ANTIGENS FOR
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CHAPTER 1

Literature Review

Introduction

Foodborne diseases are one of the most widespread health problems in the world (4). It is estimated that foodborne illnesses represent less than 10% of the real incidence, and surveys in a few countries indicate that foodborne diseases may be 300 – 350 times more frequent than reported cases tend to indicate (67, 94). In the United States alone there are over 38 million illnesses each year attributed to foodborne pathogens (63). It is estimated that foodborne infections account for 181,177 hospitalizations for acute gastroenteritis caused by known pathogens in the United States (63). Foodborne disease related deaths are estimated to be 2,718 (63). Most common bacterial pathogens associated with gastroenteritis are Campylobacter spp. at 2,453,926 cases per year, Salmonella spp. at 1,412,496 cases per year, and Shigella spp. at 448,240 cases per year (63). While Campylobacter is the most common cause of gastroenteritis, Salmonella has a higher death rate per year than Campylobacter (63).
Common food vehicles of many foodborne outbreaks include bakery products, beef, chicken, Chinese food, dairy products, eggs, finfish, fruits and vegetables, ice cream, Mexican food, mushrooms, nondairy beverages, pork, shellfish, turkey, and others (11, 30, 63, 72, 98, 103). Several foodborne pathogens such as *Campylobacter* spp., *Escherichia coli* O157, and *Salmonella* spp. have the ability to survive in many different foods. *Campylobacter* is often found on poultry and seafood (15), and *E. coli* O157 can be found contaminating a variety of food products (31) although it is most commonly associated with beef and dairy products (11, 63). Non-typhoidal *Salmonella* has the highest incidence of death in the United States and can be commonly found in poultry products (17, 36, 55, 63).

Food preparation and personal hygiene are important factors that can impact the risk of contracting a foodborne illness. Product preparation by food handlers plays an important role in keeping products clean. Food handlers that are ill or have poor personal hygiene have been implicated as the source of several outbreaks in United States (21, 39, 50, 77). Other risks include keeping surfaces and utensils clean during food preparation, as cross contamination can easily occur (27, 53).

*Salmonella* causes primarily gastroenteritis in adults, although it can cause septicemia in the elderly, children, or immunocompromised patients (37, 59, 68, 69). The elderly tend to have a weaker immune system, making them more susceptible to *Salmonella* infection. Children are more likely to develop systemic infection with *Salmonella* due to their developing, naive immune system (92). In the case of AIDS patients, the immune system is compromised to the point where they may have trouble
fighting off the invading bacteria; infection is easily facilitated by the *Salmonella* with little resistance from its host (37).

**Distribution of *Salmonella* in Nature**

*Salmonella* spp. colonize a variety of animal hosts, therefore its not surprising that vehicles commonly responsible for United States foodborne outbreaks are animal in origin (11). *Salmonella* are found in wildlife, food animals, companion animals (51, 87, 88), warm blooded or cold blooded vertebrate (80), and from every continent or region except for the arctic polar regions (64). In 1975 the FDA banned the sale of pet turtles because of the associated risk of *Salmonella* infections in children (20, 22). Cases were also reported involving pet iguanas and snakes as well as an outbreak in 1996 at a lizard exhibit in the Denver zoo (3, 35). *Salmonella* have been found in wild birds, including passerines (81), psittacines (38, 71), raptors (8, 79), and gallinaceous birds (73). Captive exotic birds, such as psittacines, can serve as a reservoir of multi-drug resistant, *S. enterica* Typhimurium DT104 (51). *Salmonella* is commonly isolated from a variety of domesticated, gallinaceous birds comprising of chickens, turkey, waterfowl, and quail (9, 16, 26, 41, 42, 66, 88).

Chickens are the most abundant and commercially farmed poultry product in the world (60). The *S. enterica* serotypes commonly encountered in poultry include: Enteritidis, Typhimurium, Hadar, Newport, Seftenberg, Virchow, Infantis, and Schwarzengrund (6, 12, 75, 96). In the United States, common *S. enterica* serotypes in commercial chickens, turkeys, quail and ducks include Typhimurium, Enteritidis, Heidelberg, Newport, and Hadar (69), with additional serotypes: Agona in turkeys (12, 73, 96), Paratyphi in quail (88), and Orion in ducks (7, 9). The Centers for Disease
Control have reported that the most common serotypes responsible for human salmonellosis in the United States are *S. enterica* Typhimurium, Enteritidis, Heidelberg, Newport, and Hadar (69) which are, with the exception of Newport, the same *Salmonella* serotypes present in poultry. To curtail the transmission of salmonellae to humans through the food chain, it is important to identify its source within the integrated, poultry production system.

**Sources of Salmonella Contamination in Poultry**

*Salmonella* can be isolated from many different stages of poultry production, from the hatchery to the processing plant. Approximately 12% of hatcheries are contaminated with *Salmonella* (17). The prevalence of *Salmonella* on broiler chicken farms in the United States is approximately 42% (17). A significant source of flock contamination is derived from *Salmonella* present in houses prior to the placement of chickens (58). A common problem, and source of *Salmonella* contamination, is rodent infestation in flock houses (25, 74). Commensal rodent species have a high potential to transmit pathogens to humans (74). House mice captured on poultry farms are often culture positive for *Salmonella* (46). Davies and Wray have reported *Salmonella* in 48.7% of house mice captured in poultry units (25). Mice may play an important part in introducing *Salmonella* to the poultry environment, but there are other possible sources of *Salmonella* to consider. *Salmonella enterica* Enteritidis has been found in litter-beetles and centipedes on poultry farms (23). Wild birds are another potential source for introducing *Salmonella* into the poultry environment with *S. enterica* serotypes Typhimurium, phage types DT129, DT40, DT104, and DT99; Ohio; and Kentucky having been isolated from
wild bird fecal dropping (24). Animal feed has long been considered an important source for *Salmonella* contamination on the poultry farm as well (24).

Increased *Salmonella* transmission has been observed in poultry during transport, and the transport crates themselves have been identified as an important source for contamination (83, 84). Rigby *et al* found that 86.6% of transport crates were contaminated with 15 different *Salmonella* serotypes prior to the loading of broiler chickens onto transport trucks (83). There is also evidence that poultry can become contaminated at the plant while the carcasses are being processed (48).

**Salmonella, Poultry, and On-Farm HAACP**

In 1996 the USDA Food Safety and Inspection Service (FSIS) introduced a new plan to monitor the safety and proper handling of meat and poultry (1). Each poultry processing plant must ensure its *Salmonella* contamination rate is below the current national baseline prevalence of twenty percent (90). Developed by the USDA FSIS, Hazard Analysis and Critical Control Point (HACCP) is a system that allows the production of safe meat and poultry products through the analysis of production processes. This includes identification of all hazards that are likely to occur in production, identification of critical points in the process at which these hazards may be introduced into product and therefore should be controlled, establishing critical limits for control at those points, verification of these prescribed steps, and methods of monitoring how well the plan is working (52). Since the inception of HACCP plan, *Salmonella* carcass contamination levels have been reduced in U. S. poultry processing plants to 11.5% in 2002 (http://www.fsis.usda.gov). Some consumer groups call for additional measures to further reduce the level of *Salmonella* entering processing plants through live
animals. Further reduction of *Salmonella* contamination on the final product will require pre-harvest, on-farm intervention strategies.

The National Poultry Improvement Plan (NPIP) is a cooperative federal-state-industry mechanism for controlling certain poultry diseases. The NPIP consists of many programs which target egg-transmitted and hatchery-disseminated diseases (2). Company involvement in the NPIP is voluntary, however, becoming NPIP certified allows producers to move their products interstate and classify their poultry as “U. S. Pullorum-Typhoid Clean,” or “U. S. *S. enteritidis* Monitored” (2). The benefits for voluntarily performing under the NPIP allow for customer confidence that poultry from a particular poultry farmer has tested clean of certain diseases or has been produced under disease-prevention conditions (2).

The NPIP suggests various plans to improve the cleanliness of breeder/layer poultry facilities. Common plans of the NPIP include protocols to clean and disinfect surfaces, and the frequency at which those surfaces should be cleaned in hatcheries (95). Laboratories that are able to approve a flock as “U. S. Pullorum-Typhoid Clean,” or “U. S. *S. enteritidis* Monitored” must meet a regimen of requirements to maintain the standard of the NPIP programs (2). The NPIP has many programs for poultry monitoring. Programs that help maintain NPIP standards include serological testing poultry at required stages and ages, screens for antibodies to certain known pathogens, performing sporadic tests confirming a “disease-free environment,” methods to correctly and effectively take samples from houses and birds, methods to effectively culture for known pathogens such as *Salmonella*, disinfecting protocols, fumigation, isolation, sanitation, and good management practices (2).
**Salmonella Detection Methods**

Several different culture methods have been developed over time for the isolation and detection of *Salmonella*. Tetrathionate-Brilliant Green (TBG) broth, Rappaport-Vassiliadis (RV) broth, Selenite (S) broth, and Peptone Buffered Water (PBW) are the most common enrichment media used for *Salmonella* (10, 43, 47, 70). Each enrichment medium has its advantages and disadvantages with regards to the isolation of *S. enterica* serotypes from clinical, environmental and food samples (10, 43, 47, 70). Tetrathionate-Brilliant Green suppresses the growth of most bacteria other than *Salmonella* (43). Ingredients of TBG broth include sodium desoxycholate and brilliant green for inhibiting coliforms, *Proteus*, and gram-positive bacteria (43). The two main selective ingredients of RV broth are magnesium chloride and malachite green. The malachite green concentration suppresses the growth of many bacteria including *Salmonella*. However, Vassiliadis *et al* identified the malachite green concentration which was optimal for *Salmonella* growth without impairing its selectivity (97). Harvey *et al* compared modifications to the Rappaport-Vassiliadis procedures to determine the best method for enriching *Salmonella* and found a later modification (R25) of the RV technique proved to be more sensitive (47). Osborne *et al* determined that selenite broth would not inhibit *Proteus* and *E. coli* without the addition of small amounts of sulfapyridine (70). The problem of balancing the concentration of sulfapyridine so microbial competitors were repressed without inhibiting *Salmonella* itself proved difficult and often resulted in either the growth of too many competitors or the inhibition of *Salmonella* growth altogether (70). It has also been observed that selenite broth is generally useful in the isolation of *Salmonella* from most samples, except egg products (70). A study comparing various
enrichments determined that RV and TBG broth enrichments are equally effective in isolation of *Salmonella* and PBW is also effective in recovering injured or stressed *Salmonella* (10, 44).

Delayed secondary enrichment with TBG broth increases the likelihood of isolating *Salmonella* from poultry samples (82, 99). Secondary enrichment with TBG broth involves placing 1 ml of primary, overnight enrichment into a fresh enrichment broth for an additional four to five days at room temperature or at 42°C before plating on differential agar (76,82).

Common differential agars used for isolation and identification of *Salmonella* following enrichments include: Brilliant Green (BG), Xylose-Lysine-Tergitol-4 (XLT4), *Salmonella-Shigella* (SS), and Bismuth Sulfite (BS) agar (28). Each medium has ingredients that will inhibit growth of commensal bacteria, as well as have an indicator of some type to show if a colony has the metabolic property characteristic of *Salmonella*. Brilliant Green differentiates lactose-fermenting bacteria. Bacteria that do not ferment lactose, like *Salmonella*, form a pale transparent colony with a red halo. When choosing suspect colonies from XLT4, H₂S producing, black colonies are indicative of *Salmonella*. SS agar also identifies H₂S producing bacteria by their black colonial phenotype. BS agar not only monitors H₂S producing bacteria, but also has an indicator within the media that produces a golden/metallic appearance with *Salmonella* colonies. Advantages of any of these differential isolation methods include the ability to identify suspect *Salmonella* colonies. Disadvantages, however, lie within the phenotypic indicators in the media and the presence of other microorganisms that produce similar colonial phenotypes similar to *Salmonella*. For example, *Proteus* is a common contaminant that can survive through
pre-enrichment processes, and produce similar colony phenotypes, as *Salmonella* on all indicator plates, except BS agar.

Once suspect colonies are identified on the differential media, confirmation tests are performed to identify *Salmonella*. Common tests include a triple sugar iron (TSI) slant and lysine iron slants (28). Biochemical confirmation can then be performed using a battery of tests for sugar utilization, identification of fermentation, and end-products (65). Slide agglutination test with *Salmonella*-specific, poly O antiserum is often used to verify colony type as *Salmonella* (65).

After colonies are confirmed as *Salmonella*, isolates are generally typed through serotyping. *Salmonella* are grouped based on antigenic differences in their lipopolysaccharide (LPS). The O antigen of the LPS is a common starting point for serotyping bacterial species belonging to *Enterobacteriaceae* (32). There are 64 *Salmonella* O serogroups (28). Most *Salmonella* serotypes are biphasic producing two antigenically distinct flagellins. However, there are monophasic *Salmonella* serotypes like *S. enterica* Enteritidis that express only the phase 1 antigen (28). Therefore, due to antigenic differences in phase 1 (H1) and phase 2 (H2) flagellins, *S. enterica* can be differentiated into over 2,000 serotypes based on the O, H1, and H2 antigenic formula that define each serotype. Spicer *et al* developed four polyvalent *Salmonella* antisera to rapidly identify more than 17 of the more commonly occurring H antigens belonging to *S. enterica* serotypes that are often isolated in most diagnostic microbiology laboratories (93). Edwards *et al* modified Spicer’s method so that no single H antigen reacted with all four *Salmonella* H antisera developed, meaning potential serotypes can be narrowed
down to a few or eliminated altogether based on the reactivity with the four typing antisera (32).

The idea of using *Salmonella* surface antigens for genus-specific ELISA and agglutination tests has been conducted with success except for serological cross-reactivity due to low specificity experienced with these tests (19, 34, 56, 61, 101). In 1993, Doran et al pioneered serological tests using AfgA as the fimrial antigen, in a genus-specific diagnostic test that would offer a quicker and more specific test than those using lipopolysaccharide or other outer membrane antigens (29). Though the study was able to detect the genus *Salmonella*, it also detected other bacterial types that shared the AfgA antigen including some *E. coli* strains, *Citrobacters, Shigellas* and *Entertobacters*. Practical applications like serotype distinction has made a more specific serological diagnostic test high in demand for confirmatory purposes. Kilger et al concluded that restriction of amplified *Salmonella* H1 genes could distinguish between serotypes like Gallinarum-Pullorum (57). Flagellar antigens are indeed unique and if applied correctly, can accurately detect and identify *Salmonella* in poultry without cross reacting with other genera.

Current serological tests for identifying *Salmonella* have advantages over conventional culture methods: time, sensitivity, and specificity. While many serological tests can be done in one day’s time, conventional serological tests, like the Pullorum test, may require additional confirmatory tests that can take more than one day because results may not be true due to known sensitivity issues (14). A serological test that can accurately detect a target *Salmonella* pathogen will not only help the poultry industry increase the quality of their products, but also decrease the chances of cross
contamination of incoming flocks and production facilities, thus raising consumer confidence in food safety. A closer look at the history of the pullorum test demonstrates the utility for such a serological test.

Pullorum disease (PD) is caused by *Salmonella enterica* Pullorum, a serogroup D *Salmonella* according to the Kaufman-White scheme (18, 78). While not normally known to cause illness in humans, Pullorum can be a serious problem on a poultry farm. *S. enterica* Pullorum is highly adapted for chickens and mortalities are noted in the first 3 weeks of age (91). PD is transmitted vertically to progeny by a carrier hen and can also infect birds by entering through a wound or contaminated feed (18). In younger birds, PD is an acute systemic infection and in adult birds it is usually characterized as a localized and chronic infection. Infected chicks are poorly developed, weak, and have poor appetites. Symptoms include painful excretions (49), blindness (62), and lameness (86). Reduction in egg production is also observed (5). Once a bird is infected and survives, it can continue shedding the *Salmonella enterica* Pullorum for the duration of its life (18).

In 1913, a tube agglutination test was developed by Jones *et al* to detect birds infected with *S. enterica* Pullorum (54). The test is conducted by diluting the serum (1:25) with the test antigen and observed for agglutination after 24 h of incubation, indicative of positive status of the bird or flock for Pullorum. To eliminate the need to separate the serum before use, Schaffer *et al* modified Jones’ serological test to a whole-blood test in 1931 (89). This test involves mixing the screening antigen with a drop of blood from the bird and the two components are observed for agglutination (40, 89). This test has been noted for false positive because antigens being used are commonly
found on other *Salmonella* serotypes (14). Runnels *et al* described a rapid serum test that is conducted by mixing the screening antigen (<1:50) with serum from a bird to be tested and looking for agglutination after two min for a positive result (85). There is also a microagglutination assay that is performed by diluting serum (1:20) from a test bird in a microtiter plate using antigen prepared by the NPIP laboratories (100).

Problems were seen with detection of *S. enterica* Pullorum when the traditional antigen test failed to identify Pullorum positive birds (33). Tests of the Pullorum-positive birds resulted in false negatives because the antigen used in the agglutination tests was different from that of *S. enterica* Pullorum isolated from this flock, meaning *S. enterica* Pullorum had undergone an antigenic change. The dependability of this test, either as a screen or confirmatory tests was drastically compromised. False negatives would be decreased if a serological test was developed that could detect all possible variants of *S. enterica* Pullorum.

A more modern serological test used for detecting viral or bacterial pathogens is an enzyme linked immunosorbent assay (ELISA). An ELISA is an immunological assay that involves the detection of specific antigens or antibodies using a detecting antibody bound with an enzyme like alkaline phosphatase or horseradish peroxidase that produces a color change in the presence of the appropriate enzymatic substrate (45). An ELISA can detect antibodies to a particular antigen using antigen-coated plates and passing sera over the antigen and looking for bound antibody (45). In a sandwich-ELISA, antigen can be detected in a sample using capture antibody attached to ELISA plates to bind the antigen from the sample and then detecting bound antigen using a second antibody (45). Bell *et al* developed a sandwich ELISA for detection of *E. coli* fimbriae and compared its
specificity and sensitivity to a PCR-based detection system (13). Out of 36 positive identifications from PCR, 14 samples were also positive by ELISA (13). The assay was specific because none of the positives of the ELISA assays were negative on PCR, but lacked the sensitivity of the PCR (13). Yap et al developed an ELISA that used purified Enteritidis g,m antigen for detection (102). While S. enterica Enteritidis is monophasic, does not undergo phase variation, other Salmonella serotypes that also share the g,m antigen are biphasic. How does Yap’s test determine whether the detected serotype is Enteritidis or not since many serotypes share the same antigen? This is the question that newly designed serological tests need to answer. If the test was able to determine that there was not a second phase of antigen, then it is more likely to be Enteritidis. If the test detected a second phase of antigen then the detected Salmonella is not Enteritidis.

Serological tests are important tools when attempting to detect harmful Salmonella. Having a good screen and confirmatory test for harmful Salmonella serotypes will increase the producer’s value and profits because successful tests can stop harmful Salmonella from spreading to other flocks or facilities. Production obviously benefits when pathogenic Salmonella can be controlled, but the ultimate advantage is a high standard in food safety. By using the best detection schemes in farms, the bar is raised and a better quality and standard for poultry production and food safety is achieved. From the producers to the consumers, better Salmonella detection methods benefit everyone by identifying the source of contamination within the integrated, poultry production system.
References


CHAPTER 2

Development of Recombinant Flagellar Antigens for Serological detection of

Salmonella enterica Serovars Enteritidis, Hadar, Heidelberg, and

Typhimurium in Poultry

\(^1\)

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Abstract

Accurate and fast serological detection of harmful *Salmonella* in poultry is a major concern of food safety. Common *Salmonella* serotypes responsible for human associated foodborne outbreaks are Enteritidis, Hadar, Heidelberg, and Typhimurium. Recombinant flagellin for phase 1 (H1) and phase 2 (H2) were cloned into Pinpoint-T® protein expression vector and purified to be used as antigens in an ELISA. An ELISA was developed for the serological detection of *S. enterica* Enteritidis in this study. Once optimized through checkerboard titration of antigen and positive-control sera, 500 ng of purified recombinant g,m antigen and a 1:64 dilution was determined to be optimal for testing field serum samples. A negative baseline cutoff was calculated to be an optical density (OD) of 0.35. All sera from birds with history of *S. enterica* Enteritidis exposure tested positive and all sera from chickens with no exposure tested negative to this *Salmonella* serotype. Current ELISA for serological detection of *Salmonella* suffers from cross reactivity inherent in lipopolysaccharide (LPS) or whole cell antigen based serological tests. This new ELISA eliminates common cross reactivity by focusing specifically on the H1 and H2 phase combination of flagellin found on *Salmonella* serotypes present in poultry and associated with foodborne outbreaks in man.
Introduction

*Salmonella* is one of the leading causes of foodborne outbreaks in the United States, responsible for approximately 40,000 cases each year (30). Poultry and poultry products have been implicated as a major source of *Salmonella* infection in humans (37). Due to public health concerns, the Food Safety and Inspection Service (FSIS) mandated that meat-processing plants implement Hazard Analysis Critical Control Point (HACCP) program and meet USDA approved limits for *Salmonella* contamination on poultry and other meat products (2). Since the implementation of HACCP program, U.S. poultry plants have reduced *Salmonella* contamination of carcasses to 11.5% in 2002 (http://www.fsis.usda.gov). Some consumer groups call for additional measures to further reduce the level of *Salmonella* entering processing plants through live animals (48). Further reduction of *Salmonella* contamination on the final product will require pre-harvest, on-farm intervention strategies. When *Salmonella* is present on the farm, extensive testing and culling of positive flocks, as well as implementation of biosecurity control measures, is necessary in its eradication (13, 46). Continued on-farm surveillance for *Salmonella* becomes an important component to National Poultry Improvement Plan for maintaining a “*Salmonella*-free” status (1).

Over 2,000 serovars have been identified for *Salmonella enterica*, based on antigenic differences in O, H1 and H2 antigens (39). Despite the diversity of serotypes, only a few of these *Salmonella* serotypes are associated with disease in man and domestic animals. In the United States, the *S. enterica* serotypes commonly associated with human foodborne outbreaks are Typhimurium, Enteritidis, Heidelberg, and Hadar (36), the same *Salmonella* serotypes that colonize poultry (10, 44, 47). During the 1980’s, *S. enterica*
serotype Enteritidis displaced *S.* ser. Typhimurium in its frequency of isolation from outbreaks in the eastern United States (11, 27), primarily through its contamination of USDA grade A table eggs (17). In response to this emerging problem, the poultry industry and USDA implemented a series of steps to monitor layer flocks for *S.* ser. Enteritidis, implement a *Salmonella* eradication program, and divert table eggs contaminated with this *Salmonella* serotype for pasteurization (1). Though pasteurization is an acceptable resolution to Enteritidis for food safety, the producers lose valuable income when this method is necessary due to costs. Therefore, identification of *S. enterica* Enteritidis positive flocks can have an important economic impact for the table egg producer.

Different methods have been developed to detect *Salmonella* in an effort to identify positive flocks. Current techniques include culture and isolation (9, 15, 21, 31), PCR (24, 28), agglutination tests (50, 54), and enzyme-linked immunosorbent assays (ELISA) (16, 20). Current culture-based methods for detecting *Salmonella* vary in their ability to isolate all *Salmonella* serotypes and identify salmonellae with atypical colony type on differential/selective agar (7, 21, 22, 38). It takes at least 5-to-7 days to identify *Salmonella* from culture (35) and an additional 4-to-5 days if a secondary enrichment is included (40, 43, 55). Certain sampling methods like drag swabs (8) are also likely to miss salmonellae, especially on farms with intermediate to low-level contamination (33). Simpler and cost effective methods for monitoring birds involve serological screens that measure antibody responses to specific microbes.

Serological screens have proven both practical and useful in the eradication of important veterinary pathogens from US poultry farms (1) and at least in one study, a
serological screen was useful in the eventual elimination of *S. enteritidis* from poultry breeders in the Netherlands (18). The National Poultry Improvement Plan has instituted several serological agglutination tests to insure that birds are free of economically devastating microbes like *Mycoplasma gallisepticum* and *S. enterica* Pullorum (1). Several of these tests are simple and routinely done by the veterinarian in the field. The slide-agglutination test has been adapted to the serological detection of different *Salmonella* serotypes (19, 54), including Enteritidis (50). These serological assays, however, have not been able to compete with the sensitivity of ELISA tests (6, 34). Previous ELISA immunoassays have focused on *Salmonella* lipopolysaccharide (LPS) as the detecting antigen (19). LPS is a powerful B-cell mitogen that induces a strong humoral response, directed mostly to O-antigen component of this polysaccharide (41). Serology using *Salmonella* LPS as the detecting antigen, only determines whether or not the animal has been exposed to one of one hundred *S. enterica* serotypes with distinctive O-antigen type present in the LPS.

Current ELISA tests for *Salmonella* have moved towards the use of purified flagellin as the antigen for the serological detection of specific *S. enterica* serotypes (53). The *Salmonella* flagellins are a more appropriate antigen for serology due to reduced cross-reactivity observed in ELISA (4). Since *Salmonella* flagellin is not difficult to clone or over-express (25, 32), we were able to express and purify recombinant *Salmonella* flagellar H1 antigens g,m; i; r; z10 and H2 antigens 1,2 and e,n,x using current protein fusion technology, and develop an ELISA assay for monitoring poultry for exposure to *S. enterica* serotypes Enteritidis, Hadar, Heidelberg, and Typhimurium. A serological test for specific, human *Salmonella* pathogens will be an important tool for
the industry to assess the effectiveness of their *Salmonella* eradication programs as well as identify farms where *Salmonella* contamination is a significant problem.

**Materials and Methods**

**PCR amplification and cloning of *S. enterica* flagellin genes, *fliC* and *fljB* from serotypes Enteritidis, Hadar, Heidelberg, and Typhimurium.** Whole cell template for PCR was made for *S. enterica* serovars Enteritidis, Hadar, Heidelberg, and Typhimurium as described by Hilton et al (23). Template was diluted 1:10 and 1µl of each dilution was used in PCR reaction. PCR primers were designed, using Gene Runner 3.04 (Hastings Software, Hastings, NY), to amplify the complete H1 and H2 flagellin genes *fliC* and *fljB*, respectively, and allows for its insertion in-frame with the biotin purification tag on Promega’s Pinpoint Xa-T protein purification system (Promega, Madison, WI). A *Bam* HI restriction enzyme recognition site was engineered into the reverse *fliC* and *fljB* PCR primers to determine the DNA inserts orientation with respect to the vector’s biotin gene tag. Oligonucleotides, used for PCR and DNA sequencing, are listed in Table 1. PCR was performed using the Idaho Technologies Rapidcycler, hot-air thermocycler (Idaho Falls, ID) (57). The PCR reaction consisted of 3 mM MgCl₂, 50 mM Tris (pH 7.4), bovine serum albumin at 0.25 mg/ml, 0.1 mM primer, 0.2 mM deoxynucleoside triphosphates (Boehringer Mannheim, Indianapolis, IN), 0.5 U of *Taq* polymerase (Boehringer Mannheim), and 1 µl of whole cell template. Program parameters for the *fliC* PCR consisted of a hold at 94°C for 15 s; 30 cycles of 94°C for 15 s (denaturing), 60°C for 15 s (annealing), and 72°C for 35 s (extension) with a slope of 2.0; and a final extension at 72°C for 4 min, while the program parameters for the *fljB* PCR was a hold at 94°C for 15 s; 30 cycles of 94°C for 15 s (denaturing), 55°C for 15 s
(annealing), and 72°C for 35 s (extension) with a slope of 2.0; and a final extension at 72°C for 4 min. PCR amplicands were visualized by gel electrophoresis using a 0.8% agarose gel containing 5 µg/ml ethidium bromide and run at 100V for 45 min in TAE buffer (45). The PCR products were purified using a Qiagen PCR purification kit (Qiagen Inc. Valencia, CA).

Amplified PCR products were cloned into Promega’s Pinpoint Xa1-T protein expression vector. PCR amplicands were ligated to vector at a 4.5:1 insert to vector DNA molar ratio with T4 DNA ligase according to the manufacturer’s instructions (Promega). Ligated plasmid DNA was transformed into Escherichia coli BL21 Gold (DE3) chemically competent cells according to the manufacturer’s instructions (Stratagene, La Jolla, CA), and transformants were selected on LB agar with 100 µg/ml ampicillin (Bristol-Myers Squibb Company, Princeton, NJ). E. coli transformants were screened for fliC or fljB DNA inserts by DNA: DNA hybridizations of colony blots. DNA: DNA hybridization was performed according to procedures described by Ausubel (3). DNA was covalently bound to the membranes using an optimal setting of 1,200 X 200 µJ/cm² for 15-17 seconds on a Fisher Biotech UV crosslinker (Fisher Scientific, Pittsburgh, PA). Probes specific for either fliC or fljB Salmonella serovar, were produced using the same primers for cloning except the amplicon was labeled with digoxygenin-tagged nucleotides present in the PCR reaction (Roche Diagnostics, Mannheim, Germany). Nylon membranes hybridized with probe at 68°C overnight in prehybridization buffer containing 0.1% sodium chloride/sodium citrate buffer and 0.1% sodium dodecyl sulfate. Probe bound to membranes were detected with an anti-DIG antibody-alkaline phosphatase conjugate with color substrate solution of 4-nitro blue
tetrazolium chloride (NBT) and X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche Diagnostics, Mannheim, Germany). Positive colonies for *fliC* or *fljB* were traced back to the master plates for further screening by restriction analysis following plasmid DNA extraction.

Plasmid DNA was extracted using an alkaline lysis method (45) and digested with restriction enzyme *Bam* HI (New England Biolabs, Beverly, MA) at 1 U per µg plasmid DNA. DNA was quantified by spectrophotometric measurement at OD λ 260 nm. Plasmid DNA was visualized by gel electrophoresis on a 0.8% agarose gel containing 5 µg/ml ethidium bromide. Extracted plasmid DNA representative of phase 1 (H1) and phase 2 (H2) of flagellar antigens of *S. enterica* Enteritidis, Heidelberg, Hadar, and Typhimurium are described in Table 2. At position 404 of the Pinpoint Xa1-T vector is a *Bam* HI restriction site. The engineered *Bam* HI site on the PCR amplicon is 1.5 kb opposite the *Bam* HI restriction site in the cloning vector only when the inserted PCR product is in this correct orientation. Only when the PCR product is in the correct orientation, will the *Bam* HI liberate the DNA insert from the Pinpoint Xa1-T vector. DNA sequencing primers were designed, using Gene Runner 3.04, to confirm in-frame fusion of *Salmonella* flagellin gene with the vector’s biotin protein tag (Table 1).

**Expression *S. enterica* flagellin, protein fusions in *E. coli* cloning host BL21.**

*E. coli* with prospective protein fusion constructs were grown in BHI media (Difco, Detroit, MI) with ampicillin 100 µg/ml, with aeration, to an optical density (OD) (λ 600 nm) of 0.5 before inducing expression of the recombinant protein fusion with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) (Fisher Scientific) for 10 h at 37°C. Cells were
then centrifuged 5,000 x g for 10 min at room temperature, supernatant was decanted and cell pellets stored at -80°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Lammeli et al (26). Polyacrylamide gels (3% stacking & 8% resolving) were cast and run using Bio-Rad Mini-PROTEAN II electrophoresis system with Kaleidoscope Prestained Standards (Bio-Rad Laboratories, Hercules, CA) serving as molecular weight standards. Prior to induction with IPTG, one milliliter of cells was removed and served as a pre-induction control. Following 10 h of induction with IPTG, another one milliliter aliquot was taken as a post-induction sample. Total protein concentrations were determined using a BCA protein quantification kit (Pierce Chemical Company, Rockford IL). Equal amounts of protein (approximately 400 µg) was added to SDS-PAGE solubilization buffer (25% Tris base, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 20% glycerol, and 0.0025% bromophenyl blue) and boiled (100°C) for fifteen min. Samples were loaded (15 µl per sample) into the SDS-PAGE lanes and run at 26 mA, constant amperage for 2 h using Bio-Rad Mini-PROTEAN II electrophoresis system (Bio-Rad). Gels were run in duplicate for Coomassie brilliant blue staining and electrophoretic transfer to nitrocellulose membrane (Osmonics Inc., Westborough, MA) for Western analysis (Bio-Rad). SDS-PAGE gels were transferred to nitrocellulose using constant voltage of 75 volts for 45 min and prepared according to the manufacturer’s instructions (Bio-Rad). Western analysis was performed as described by Towbin et al (51). Westerns blots were performed for each recombinant protein fusion construct to verify expression of fusion protein identified on SDS-PAGE gel stained with Coomassie blue, using either anti-biotin antibody-alkaline phosphatase conjugate (Roche
Diagnostics) or Spicer-Edwards, *Salmonella* typing, antisera (Difco) (1:5,000 dilution). Anti-rabbit antibody-alkaline phosphatase conjugate (Sigma, St. Louis, Mo) was used as the secondary antibody for detecting bound, *Salmonella* typing-antibody (1:5,000 dilution). Western blots were examined for detected, protein bands similar in size to the theoretical size of the recombinant protein fusion, 70.8 kDa.

**Purification of recombinant *Salmonella* flagellin proteins with biotin tag.**

IPTG induced cells, containing the recombinant protein fusion constructs, were resuspended in lysis solution (15 ml per gram cell paste) consisting of 6 M guanidine hydrochloride (Fisher Scientific) and 4 mg/ml lysozyme (Roche Diagnostics) and incubated at 37°C overnight with shaking (200 rpm). Cellular debris was removed by centrifugation (5000 x g for 10 min at 4°C) and supernatant was transferred to 22 mm dialysis tubing with a 3.5 kDa molecular weight exclusion limit (Snakeskin®, Pierce Chemical Company, Rockford IL). Guanidine hydrochloride was removed from the supernatant via dialysis against two liters of dialysis buffer containing 50mM Tris-HCl pH 7.5, 50mM NaCl, and 0.1% Triton X-100. Dialysis tubing (approximately 4 inches) was cut for dialysis of volumes no more than 15 ml. No more than two dialysis bags were present per two liters of dialysis buffer. Dialysis was performed at 4°C with constant stirring with a magnetic stir bar. Dialysis buffer was changed once at 4 h and bags were removed the following day. The dialysate was frozen at -20°C if purification was not performed immediately following dialysis. Softlink Soft Release Avidin Resin (Promega) was prepared and batch capture purification was performed as per the manufacturer’s instructions. Purified recombinant proteins were dispensed as 1 ml aliquots into 1.5 ml capacity microfuge tubes and frozen at -20°C.
Enzyme-Linked Immunosorbent Assay. Sera were collected from an adult broiler breeder hen farm of chickens to establish a negative baseline cutoff for the ELISA. Eight milliliters of blood, at most, was collected per bird before releasing back into the house. Birds were wing-bled using 1 inch 20 gauge needles (PrecisionGlide®, Becton Dikinson and Company, Franklin Lakes, NJ). Five environmental drag swabs were collected for poultry houses (8). Drag swabs were incubated overnight at 42°C in Tetrathionate broth (Difco, Detroit, MI), then plated on XLT-4 plates (Difco, Detroit, MI) and incubated overnight at 37°C. Suspect colonies indicative of *Salmonella* were tested with O serogroup typing antisera (Difco, Detroit, MI) to identify O serogroup. PCR analysis was used for serotype identification (24).

A checkerboard titration was performed to determine the optimal antisera dilution and antigen concentration to use in our ELISA. Twenty, 10 week old specific pathogen free (SPF) white leghorn chickens were immunized using 1 inch 20 gauge needles (PrecisionGlide®, Becton Dikinson and Company, Franklin Lakes, NJ) with 0.5 ml of *S. enterica* Enteritidis bacterin (Maine Biological Laboratories, Waterville MN) subcutaneously in the cervical region. Two weeks later, birds were subcutaneously boosted using 1 inch 20 gauge needles (PrecisionGlide®, Becton Dikinson and Company, Franklin Lakes, NJ) with 0.5 ml of pre-purified *S.* ser. Enteritidis recombinant g.m flagellin preparation (1.68 mg/ml) in 5 mM biotin elution buffer (Promega, Madison, WI) in the cervical region. Birds were then bled the following week using 3 inch 20 gauge needles (PrecisionGlide®, Becton Dikinson and Company, Franklin Lakes, NJ) via cardiac puncture through the thoracic inlet. Volumes of at least 5 ml per bird were obtained. Birds were euthanized by cervical dislocation following the procedure. Fifty
microliters of purified recombinant g,m flagellin protein at 250ng, 500ng, 750ng, and 1µg in carbonate/bicarbonate coating buffer (pH 9.6) (25 mM sodium carbonate, 35 mM sodium bicarbonate) was placed in Costar flat bottom E.I.A./R.I.A 8 well strips (Costar Corporation, Cambridge, MA). Plates were wrapped in Saran-Wrap® and incubated over-night at 4°C. Plates were then washed once with PBS with 0.05% Tween 20. Fifty microliters of conjugate buffer (PBS, 0.05% Tween 20, 5% powered skim milk) was added to each well and serum was added serially to create a 10-fold range from 1:10 to 1:100,000 in triplicate. Plates were incubated at 37°C for 45 min then washed with PBS with 0.05% Tween 20. Fifty microliters of anti-chicken horseradish peroxidase conjugate (Sigma) was then added to each well diluted to 1:10,000 and incubated at room temperature for 45 min. Plates were washed four times with PBS with 0.05% Tween 20. HRP color substrate, O-Phenil-Dyamine (5 mg per ml) (Sigma), solution was added to wells and developed for 10 min at room temperature. The reaction was terminated with 12.5 µl, 40% HCl and the OD for ELISA plates were read at λ492 nm.

Results

Expression and purification of recombinant Salmonella phase 1 and phase 2 antigens. Flagellin genes fliC and fljB from Salmonella enterica serotypes Enteritidis, Typhimurium, Heidelberg, and Hadar were amplified by PCR, cloned into Pinpoint Xa-1 T vector, and introduced into E. coli BL 21. The Pinpoint Xa-1 T vector is designed for PCR cloning of foreign genes in-frame with a biotin purification tag (BPT), which is used for the purpose of purifying recombinant proteins by affinity column chromatography with avidin-bound resin. The expression of biotin-tagged flagellin was observed upon induction with 0.1 mM IPTG for all cloned Salmonella flagellin genes, producing the
expected, 70.8 kD proteins, as determined by SDS-PAGE and confirmed by western analysis (Fig. 1).

Purification of our recombinant antigens from *E. coli* BL21 was achieved through standard sonication and Triton-X 100 solubilization procedures commonly used in purification of recombinant, protein fusions (2). Our protein tended to be insoluble and modifying the Triton X-100 concentration did not improve the solubility of the recombinant *Salmonella* protein fusion (data not shown). Analysis of the recombinant fusion protein, amino acid sequence revealed extensive hydrophobic regions throughout the protein as determined using Kyte-Doolittle hydropathy plot (Fig 2), that might explain the insoluble nature of our protein in Triton X-100. In order to solubilize the protein, the chaotropic agent, guanidine HCl (6 M) was used in place of Triton X-100. While the guanidine HCl did release the protein from the insoluble fraction, the avidin-resin was not compatible with this chaotropic agent. It was therefore necessary to dialyze against 50 mM Tris, pH 7.5 with 0.1% Triton X-100 to maintain solubility and compatibility with the purification column. SDS-PAGE and western analysis demonstrated the protein was removed from insoluble fractions following dialysis with Triton X-100 (Fig. 3).

**Development and evaluation of *S. enterica* Enteritidis, specific ELISA using recombinant FiC<sub>g,m</sub> protein fusion as the detecting antigen.** A rapid format of direct ELISA based on the Enteritidis g,m antigen was developed for detection of Enteritidis-infected birds. A checkerboard titration revealed that 500 ng of purified, recombinant phase 1 g,m antigen per well was optimal antigen concentration for our ELISA (Fig. 4). We also chose 1:100, dilution of chicken sera for determining quality control ranges for
our negative and positive control sera (Fig. 5). We used sera from *S. ser.* Enteritidis, bacterin-vaccinated birds and un-vaccinated, *Salmonella* culture-negative, birds as our positive and negative controls, respectively. We set the criteria for acceptable ELISA runs from seven trials at optical densities (OD) (490nm) between 0.78 to 1.07 (mean = 0.93, 2 standard deviation = 0.15) and 0.03 to 0.11 (mean = 0.07, 2 standard deviation = 0.04) for the positive and negative control sera, respectively.

Sera were collected from a broiler breeder farm that was environment, culture-positive for *Salmonella enterica* Typhimurium but negative for *S. enterica* Enteritidis. These sera were used to determine the negative baseline background from a *S. ser.* Enteritidis culture-negative flock (Fig. 6). Criteria for determining the appropriate baseline titer was defined as the highest titer of serum that could discriminate between known positive and negative samples. Since background was most apparent at the lower dilutions, we chose the dilution of 1:64 as the minimal dilution for the detection of g,m antibody titers. Determination of the Enteritidis-negative baseline yielded optical densities (490nm) that ranged from 0.19 to 0.21 (mean = 0.19, standard deviation = 0.082, 2 standard deviations = 0.16) at a 1:64 dilution. A net absorbance value of 0.35 at the 1:64 dilution was calculated to be the threshold value for a positive serum titer according to the method of Balfour and Harford (5). This value was determined by adding the mean absorbance value of the sera from known negative serum samples at a 1:64 dilution, plus two times the standard deviation. Any values at or near the baseline criteria will be considered “borderline” and additional tests may need to be administered to either confirm or deny whether the sample is indeed positive or negative.
Titers were determined for known positive serum samples. Of five serum samples from birds colonized with *S. enterica* Enteritidis, orally challenged with $10^6$ CFU, four had titers of 1:128, and the “pooled” sera a titer of 1:256 for our recombinant phase 1 g,m antigen (Table 3).

Western blots were performed to ensure that test sera were reacting with the recombinant protein fusions. The positive control sera and sera from *S. enterica* Enteritidis colonized birds reacted with a protein band at approximately 70.8 kD, the expected size for our recombinant protein antigen. There was no reactivity with the protein antigen with sera from *S. ser.* Enteritidis, culture negative birds (Figure 7).

**Discussion**

In this study we produced biotin-flagellin protein fusions in order to develop an ELISA for detecting the presence of specific *Salmonella* serotypes in poultry. Previous studies have targeted flagellin for detection methods of *Salmonella* (29, 59), however were unable to detect specific serotypes because the first phase of flagellin (*fliC*) was only considered in the development of their *S. enterica* Enteritidis ELISAs. While successful in detecting the presence of g,m-reactive antibodies, the tests were unable to distinguish between monophasic g,m Entertidis and other bi-phasic serotypes that share g,m as a phase 1 antigen. Positive identification of targeted serotypes can be detected by addressing the combination of phase 1 and 2 antigens. Our study differs in that both phase 1 FliC and phase 2 FljB flagellin proteins are considered in the development of our ELISA. The ELISA was effective because it can distinguish between other Salmonella serovars that can be found in the poultry environment. The fact that the ELISA negative
baseline was determined from Enteritidis-free chickens, but were positive for Typhimurium, proved that the test could be used as a discriminatory serological screen for different serovars of *Salmonella*. This means a new serological test with serotype-specific capabilities was developed by creating an ELISA that focuses on the bi-phasic nature of the flagellin.

Serological monitoring of poultry has many advantages. Poultry producers can easily screen and confirm the presence of specific *Salmonella* serotypes in poultry due to the speed and accuracy of these tests. A serotype-specific serological test can be applied when screening breeder flocks, as an initial screen of the flock for exposure to important serotypes Typhimurium and Enteritidis. A serological test is especially useful for identifying *Salmonella* positive flocks where *Salmonella* is present intermittantly, low prevalence, or at low levels in the poultry flocks. if environmental culture screens indicate *Salmonella*-like colonies are present. A fast and accurate test to screen these flocks will be useful to producers since the National Poultry Improvement Plan has strict guidelines for the monitoring of specific serotypes of *Salmonella*, like Entertidis (52). Targeting parent lines of poultry is beneficial to producers because there are far fewer numbers of chickens to screen, and vertical transmission of *Salmonella* can be intercepted. If caught early, producers will be able to prevent the transmission to progeny flocks, thus ultimately improve the overall safety of their consumable product as to *Salmonella* status of layer flocks for Enteritidis. Applications of serological tests may also have use as confirmatory measure because of serotype-specific nature of he tests. Using serological screens can assist the producer in decisions concerning fate of flocks or their poultry product. If a layer flock is serologically positive for *S. enterica* Enteritidis,
the eggs can be pasteurized and used in pre-manufactured products like cake mixes. Manufacturer processing facilities can use a serotype-specific test to screen farms for *S. enterica* Enteritidis to prevent potential cross-contamination of Enteritidis-free flocks via contaminated equipment; if a flock is confirmed to be positive for *Salmonella*, processing of the flock’s progeny can be scheduled last in the production schedule to prevent cross contamination. By using these screens, producers can prevent infected poultry and poultry products from entering the food chain or accidentally contaminating the processing facilities.

One of the biggest disadvantages of current detection methods is the time needed to screen and identify an infecting *Salmonella* serotype. Culture and enrichment processes can take over a week for identification, especially if delayed enrichment is used (42). Initial confirmation can be completed in 3 days. If pooled sera samples of a flock are available, a serological test can be used as a screen. Though more specific as a confirmatory test because of calculated baseline cutoffs, more standard deviations of the baseline criteria can be used in the detection range to make the test more sensitive and thus more effective as a screen. Recalibrating a serological test for screening purposes can save time for producers because the test can be repeated the same day.

The ability to detect *Salmonellas* responsible for human foodborne outbreaks is essential because of the criteria set by today’s standards in food safety (52). Guidelines set by the NPIP are based on the producers’ ability to be able to determine not only whether *Salmonella* is present in their breeder flocks, but also have the ability to label the farm as “U.S. *S. enterica* Enteritidis Monitored” so their products can be shipped interstate (52). Standards to determine quality of poultry and poultry products are based
on rules of the NPIP. Since producers aim for the highest grade of poultry and poultry products, they need to have an effective detection system that can be accurately used in a manner set by the NPIP’s guidelines. An effective screen followed by an accurate and fast serological confirmation test can keep poultry farmers producing high quality and best-value products. This means highest profit while keeping harmful *Salmonella* from the consuming public.

**Conclusions**

Our study provides a quick and easy serological method for screening breeder flocks of poultry for common serotypes of *Salmonella* responsible for human foodborne outbreaks in the United States. Current serological methods lack the specificity to determine the serotypes of *Salmonellas* that have been exposed to poultry due to common cross reactivity of antigens prepared for the tests. These cross reactivity issues can be reduced by focusing on the combination of phase 1 (H1) and phase 2 (H2) of the flagellar antigens of common serotypes responsible for human foodborne outbreaks. The fact that the common serotypes found in the United States that cause food poisoning do not share the same flagellin phase combinations make this study useful to breeders. Following a sensitive environmental culture, breeders can determine whether the cultured *Salmonellas* are serovars Enteritidis, Typhimurium, Heidelberg, or Hadar. Breeder farms will be able to use this test to help keep their products under the strict NPIP regulations in food safety.

Problems with the incidence of *Salmonella* have already been decreased due to the regulations of the NPIP. Developing a serotype-sensitive serological screen may be able to decrease the incidence even more by focusing on the disadvantages and
challenges of current methods. With continuing efforts in research and efficiency, it may be possible to expand the limits of detection to include incidence of *Salmonella* on broiler farms in the near future. Food safety is always going to be important to producers and consumers alike. Accurate and quick tests are essential to maintain the high quality of food safety standards set by government regulations. Our serotype-specific serological ELISA will help improve current *Salmonella* screens by implementing a quick and accurate procedure while possibly expanding the area of usage to the progeny and products of the breeder flocks.
Table 1. PCR and DNA Sequencing Primers

<table>
<thead>
<tr>
<th>Target/Name</th>
<th>Sequence¹</th>
<th>Expected Size (bp)</th>
<th>Distance from Fusion²</th>
</tr>
</thead>
<tbody>
<tr>
<td>fliC</td>
<td>F: ACA AGT CAT TAA TAC AAA CAG CC</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>R: GGA TCC AGT AAA GAG AGG ACG TTT TG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fljB</td>
<td>F: GGC ACA AGT AAT CAA CAC</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>R: GGA TCC TTA ACT TAA CAG AGA CAG CA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3′BPT</td>
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<td>5′fliC</td>
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<tr>
<td>5′fljB</td>
<td>GGT CAG CAG CGA CAG ACT GT</td>
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¹ Underline denotes engineered Bam HI site.

² Primer distance from the junction of the biotin purification tag and fliC gene.
## Table 2. Bacterial Strains and Plasmids.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>$F^-$, <em>dcm, ompT</em>, <em>hsdS(rBmB)</em>, <em>gal λ(DE3)</em></td>
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<tr>
<td>JM 109</td>
<td><em>endA1, recA1, gyrA96, thi, hsdR17 (rk−, mk+)</em>, <em>relA1</em>, <em>supE44, Δ(lac-proAB)</em>, [F’, traD36, proAB, laqIQZM15]</td>
<td>(58)</td>
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<tr>
<td><em>Salmonella enterica</em> Typhimurium</td>
<td>Poultry Isolate</td>
<td>(49)</td>
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<tr>
<td><em>Salmonella enterica</em> Enteritidis</td>
<td>Poultry Isolate</td>
<td>(49)</td>
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<tr>
<td><em>Salmonella enterica</em> Heidelberg</td>
<td>Poultry Isolate</td>
<td>(49)</td>
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<tr>
<td><em>Salmonella enterica</em> Hadar</td>
<td>Poultry Isolate</td>
<td>(49)</td>
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</table>

### Plasmid

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>PinPoint™ Xa-1 T</td>
<td><em>bla</em>&lt;sub&gt;TEM&lt;/sub&gt;, pUC based vector with a biotin purification tag for protein fusions (Promega, Madison, WI)</td>
<td>(12, 14)</td>
</tr>
<tr>
<td>pJMZ 63</td>
<td>PinPoint™ Xa-1 T with in-frame biotin fusion with <em>S. ser</em> Typhimurium <em>fliC</em> (i)</td>
<td>This Study</td>
</tr>
<tr>
<td>pJMZ 42</td>
<td>PinPoint™ Xa-1 T with in-frame biotin fusion with <em>S. ser</em> Enteritidis <em>fliC</em> (g,m)</td>
<td>This Study</td>
</tr>
<tr>
<td>pJMZ 2</td>
<td>PinPoint™ Xa-1 T with in-frame biotin fusion with <em>S. ser</em> Heidelberg <em>fliC</em> (r)</td>
<td>This Study</td>
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<tr>
<td>pJMZ 47</td>
<td>PinPoint™ Xa-1 T with in-frame biotin fusion with <em>S. ser</em> Hadar <em>fliC</em> (z10)</td>
<td>This Study</td>
</tr>
<tr>
<td>pJMZ 5</td>
<td>PinPoint™ Xa-1 T with in-frame biotin fusion with <em>S. ser</em> Typhimurium <em>fljB</em> (1,2)</td>
<td>This Study</td>
</tr>
<tr>
<td>pJMZ 18</td>
<td>PinPoint™ Xa-1 T with in-frame biotin fusion with <em>S. ser</em> Hadar <em>fljB</em> (c,n,x)</td>
<td>This Study</td>
</tr>
</tbody>
</table>
Table 3. Antibody titers of chickens experimentally colonized with *S. enterica* Enteritidis using ELISA and recombinant phase 1 g,m as the detecting antigen.

<table>
<thead>
<tr>
<th>Serum Sample #</th>
<th>Salmonella Status</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 9-5&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td><em>S. enterica</em> Enteritidis Challenged</td>
<td>1:128</td>
</tr>
<tr>
<td>39 4-5&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td><em>S. enterica</em> Enteritidis Challenged</td>
<td>1:128</td>
</tr>
<tr>
<td>40 9-5&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td><em>S. enterica</em> Enteritidis Challenged</td>
<td>1:128</td>
</tr>
<tr>
<td>40 8-29&lt;sup&gt;1,3&lt;/sup&gt;</td>
<td><em>S. enterica</em> Enteritidis Challenged</td>
<td>1:128</td>
</tr>
<tr>
<td>Pool&lt;sup&gt;4&lt;/sup&gt;</td>
<td><em>S. enterica</em> Enteritidis Challenged</td>
<td>1:256</td>
</tr>
</tbody>
</table>

<sup>1</sup> Week-old chickens were orally challenged with 10<sup>6</sup> *Salmonella enterica* Enteritidis.

<sup>2</sup> Serum collected 24 days post challenge.

<sup>3</sup> Serum collected 17 days post challenge.

<sup>4</sup> Pooled sera from varying ranges of *Salmonella enterica* Enteritidis challenged chickens.
Fig. 1. SDS-PAGE and western analysis of IPTG induced and non-induced recombinant *Salmonella* fliC clones for phase 1: i, g,m, r, and z_{10} antigens. *E. coli* B21 with the recombinant fliC alleles i (lanes 2, 3), g,m (lanes 4, 5), r (lanes 6, 7) and z_{10} (lanes 8, 9) were allowed to grow to an optical density (λ 600 nm) of 0.5 before inducing expression of the protein fusion with 0.1mM IPTG for 10 h (lanes 3, 5, 7 & 9). Lanes 2, 4, 6, & 8: non-induced cells. Lane 1: molecular weight standards. (A) SDS-PAGE of whole cell lysates of induced and non-induced *E. coli* B21 with recombinant fliC alleles. Recombinant *Salmonella* flagellin proteins were identified by western analysis (B-D) using Spicer-Edwards typing sera for i, gm (B), r (C) and z10 (D) phase 1 antigens.
Fig. 2  Kyte-Doolittle hydropathy plot of *Salmonella enterica* Enteritidis g,m protein fusion. Negative hydropathy score values indicate hydrophobic regions of the fusion protein.
Fig. 3. Purification of recombinant, biotin-tagged, *S. enterica* Enteritidis g,m antigen. Molecular weight markers (lane 1), *S. enterica* Enteritidis wild type, whole cells (lane 2), *E. coli* BL21 whole cells (lane 3), *E. coli* BL21 with pJMZ42 induced with 0.1mM IPTG for 10 h: whole cells (lane 4); insoluble debris, pellet, following removal of guanidine HCl via dialysis (lane 5); soluble fraction following removal of guanidine HCl via dialysis (lane 6); protein remaining in soluble fraction following binding of proteins to affinity chromatography resin (lane 7); and purified g,m recombinant antigen following elution from avidin beads with 5mM biotin (lane 8). The arrow indicates the correct size for the g,m protein fusion. (A) SDS-PAGE. (B) Western Blot using Spicer-Edwards anti-i, g,m sera to detect recombinant phase 1 g,m antigen.
Fig. 4. Checkerboard titration of recombinant phase 1 g,m and positive control sera for *Salmonella enterica* Enteritidis
Fig. 5. Establishing quality control for positive and negative control sera in *S. enterica* Enteritidis ELISA using the recombinant phase 1 g,m antigen. Sera was obtained from known Enteritidis-exposed birds for the positive, and specific pathogen free (SPF) birds for the negative.
Fig. 6. Establishing negative baseline for *S. enterica* Enteritidis ELISA using the recombinant phase 1 g,m antigen. A-J sera are individual sera collected from a farm that cultured positive for *S. enterica* Typhimurium and negative for *S. enterica* Enteritidis.
Fig. 7. Western analysis demonstrates specificity of recombinant g,m antigen.

Positive control sera (lane 1), negative control sera (lane 2), *S. enterica* Enteritidis challenged specific pathogen free leghorn birds (37 9-5, 39 4-5, 40 9-5, 40 8-29, and pooled sera) (lanes 3-7). Sera samples from birds prior to *S. enterica* Enteritidis challenge (1638, 1636, 1637, 37 8-8, 39 8-8, 1659, 1660, and 1670) (Lanes 8-15), Sera samples collected from *S. enterica* Enteritidis-culture negative chickens (A, B, C, D, K, L, G, and J) (Lanes 16-23).
References


distribution of *Salmonella* isolates from food animals after slaughter differs from that of isolates found in humans. J. Infect. Dis. **183**:1295-1299.


