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# Use Of Secondary Enrichment To Improve The Risk Assessment Of Salmonella In Broilers

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# USE OF SECONDARY ENRICHMENT TO IMPROVE THE RISK ASSESSMENT OF SALMONELLA IN BROILERS

By

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ASSESSMENT OF SALMONELLA IN BROILERS

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Sampling in on-farm production environments presents challenges that must be considered when doing hazard analysis. The sensitivity and specificity of the test used and the sample types chosen will have an impact on the food safety outcome and food safety decisions made during the interpretation of results. In this work, broiler houses were sampled for the presence of *Salmonella spp.* using two different sampling strategies and four different microbiological isolation procedures. The study was undertaken after complications arose during a field study evaluating the role darkling beetles play in the transmission of foodborne pathogens. It was determined that, based on this work, incorporating a secondary enrichment procedure into the isolation protocol significantly increased the isolation rate from the various sample types, including

drag swabs and litter samples. It was also determined that when attempting to characterize the *Salmonella*-status of a particular broiler house, no one sampling strategy is superior. The results of this study demonstrate that both drag swabs and litter samples need to be utilized to accurately determine if the pathogen is present in a flock.

Not only did the secondary enrichment procedure have a higher isolation frequency than the other three methods compared, it also highlighted the discrepancies of the other methods. Two commonly used isolation procedures, tetrathionate and Rappaport-Vassiliadis, were found to disagree on a significant number of samples analyzed. While the isolation frequencies for these procedures were not found to be statistically different, the analysis for agreement, kappa, did indicate that the procedures did not identify the same samples as positive. Overall, the secondary enrichment procedure identified all the samples positive that were also found to be positive by either of the other methods used.

Since the secondary enrichment method is a modified version of the traditional delayed secondary enrichment procedure, which requires five additional day of incubation, this study also compared these two procedures. It was determined that the secondary enrichment protocol was as effective for isolating *Salmonella* from broiler house samples as

the delayed secondary enrichment procedure. The secondary enrichment procedure, did however, provide for a quicker turn around for results.

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# CHAPTER I

# INTRODUCTION

The future of the U. S. food animal industry was dramatically altered in the 1990s by the consuming public's reactions to highly publicized incidences of death and disease from dangerous foodborne bacteria. It is estimated that foodborne illnesses within the U.S. cost an average of $6.9 billion per annum (Anonymous, 2000), resulting from five different etiological agents. *Salmonella* alone accounts for 1.3 million cases (Mead, et al., 1999) resulting in a $2.4 billion cost per year (Anonymous, 2003). These statistics along with changes in consumers' attitudes toward the safety of America's food supply resulted in new federal legislation which significantly impacted the future manner by which foods of animal origin will be processed, inspected, and ultimately produced. The food industry is now legally responsible for determining foodborne hazards, including microbes, "before, during and after entrance into the establishment" (USDA/FSIS, 1996).

To address these key issues in animal production food safety we must begin by expanding our knowledge base concerning the particular

organisms involved and how they function in both the production and processing environments. In recent years, Supreme Beef, Inc. in Texas (Spiritas, 2000) and Hudson Foods in Nebraska (Fallik, 1997) failed to control regulated bacteria, *Salmonella* and *E. coli* O157:H7 respectively, in their finished raw ground hamburger. This failure was beyond their control in that it resulted directly from the presence of microbes on the meat products entering the plants. This highlights the need to fill the current lack of understanding that is evident in the area of pre- and post- harvest food safety concerning the ecology of these pathogens. These gaps in knowledge make the food animal industry susceptible to failure to comply with new federal regulations and vulnerable to punitive regulatory action.

Now more than ever, the need for science based risk assessment from which to do science based risk management decisions is evident. Once this is done, science-based determinations can be made of the true efficacy of critical control points and intervention methods along the food animal production and processing continuum as they relate to the reduction of pathogens on the product.

The research presented in this document is of the utmost importance to not only the $28 billion U. S. poultry industry, but to the Mississippi poultry industry as well (Anonymous, 2005). Poultry are

Mississippi's largest commodity, and had an estimated 2005 gate value of $1.98 billion (Breazeale, 2005). Mississippi ranked fourth nationally in number of broilers produced. Obviously the success of the poultry industry is of paramount importance both nationally and to the state.

We recently conducted a field trial to establish the prevalence of *Salmonella* in broiler grow-out houses as well as the relationship between the darkling beetle (*Alphatobius diapernius*) and *Salmonella*. During this trial, it became evident that the ability of currently accepted isolation and sampling methods (used in our laboratory) to consistently identify the organism within a culture positive house, over multiple sampling times, warranted closer examination. Given the inherent difficulties encountered with interfering organisms when culturing field-derived samples, we scrutinized five different *Salmonella* isolation techniques on actual field samples from multiple broiler grow-out houses through out the entire grow-out period.

Conventional microbiological isolation methods used to identify *Salmonella spp.* from within poultry samples differ greatly. Many of the protocols employ a nonselective pre-enrichment followed by a selective enrichment, incorporating either *Salmonella* selective broths and/or agar plates. Many studies have been conducted to evaluate the sensitivity

and specificity of each of the protocols; however, the results of these studies are contradictory. Therefore, this present work was designed to determine which selective enrichment broth, tetrathionate (TT) or Rappaport-Vassiliadis (RV), in combination with two different plating media, brilliant green agar (BG) and xylose-lysine tergitol-4 (XLT4) would provide the highest recovery rate when compared in pure culture, inoculated samples, and in actual field samples. This study also compared standard incubation time (24 to 48 hs) using the TT and RV to that of a secondary enrichment method.

Several published reports have been generated indicating that a delayed secondary enrichment (DSE) protocol provides for increased sensitivity when attempting to isolate *Salmonella spp.* from samples. It has been shown that the isolation rate for *Salmonella* is dramatically increased when using a secondary enrichment during sample processing (Edel and Kampelmacher, 1973;Pourciau and Springer, 1978;Waltman, et al., 1991). Therefore, in order to accurately report on the sensitivity and specificity of TT and RV in combination with the two different plating media, a modified DSE was used to provide a definitive positive or negative result for each sample tested.

Not only did this work comparing the use of currently accepted *Salmonella* isolation methods, it also incorporated the use of a novel,

rapid method. Here, the efficacy of a commercially available immunomagnetic separation assay (IMS), specific for *Salmonella*, was compared to the other three methods, TT, RV and the modified DSE. It has been demonstrated that IMS is a rapid and reliable method for detecting *Salmonella* in a variety of samples (Cudjoe, et al., 1994a;Cudjoe, et al., 1994b;Mansfield and Forsythe, 1996;Cudjoe and Krona, 1997;Shaw, et al., 1998).

In order for the poultry industry to accurately assess the pathogen status of a particular broiler house, and ultimately the *Salmonella* load entering the processing plant, the methods used must be reliable and accurate. A proper risk assessment can only be accomplished if the tests used have near perfect sensitivity and specificity. Determining which *Salmonella* isolation protocol provides the most sensitivity and specificity will allow the poultry industry to better assess their risk for *Salmonella spp.*, as well as provide more accurate data for risk management decision making.

In this research, the sensitivity and specificity of four microbiological isolation protocols specific for *Salmonella spp.* were evaluated. This research was divided into four generalized objectives, which were to:

1. Determine which isolation protocol provided the highest sensitivity when using pure culture of *Salmonella* and determine which isolation protocol provided the highest sensitivity and specificity when using poultry samples inoculated with a known concentration of a *Salmonella* culture
2. Determine which isolation protocol provided the highest sensitivity and specificity when attempting to isolate *Salmonella* from actual poultry samples obtained from multiple broiler grow-out houses sampled during the entire grow-out period
3. Compare two sampling strategies commonly used to determine the *Salmonella* status of broiler growout houses
4. Compare the use of a modified secondary enrichment protocol for the isolation of *Salmonella* to that of a traditional delayed secondary enrichment procedure

# CHAPTER II

# LITERATURE REVIEW

## POULTRY AND *SALMONELLA*

*Salmonella* has become a major concern for the food industry over the past few years. This Gram-negative organism is of more concern to the food animal industry, especially beef and poultry. Since the increased concern, control and possible eradication programs have been studied (Edel, 1994). However, the sources of this organism must first be elucidated prior to developing a control mechanism.

To determine the possible sources of *Salmonella* within an integrated poultry company, evaluation of the *Salmonella* status of various stages of the production system have been evaluated. In 1991, it was found that 13% of the samples collected at the breeder facilities were positive (Jones, et al., 1991).

It has also been suggested that the hatcheries can serve as a possible source of *Salmonella* to the broiler industry. A study reporting on possible sources of *Salmonella* found that 75.4% of all samples from hatcheries (eggshells, belting, and tray pads) were positive (Cox, et al.,

1990). These studies were repeated in 1991 and 1997 and indicated positive samples were again isolated from the hatcheries, however, at a lower percentage (Cox, et al., 1991;Cox, et al., 1997). Others have substantiated these reports (Dougherty, 1976;Byrd, et al., 1999).

Some investigators have collected tray pads, cardboard sheets lining the chick transport crates from the hatchery to the grow-out houses, and evaluated them for *Salmonella*. One study found that 37.5% of all broiler chick transport crates were *Salmonella*-positive via tray pad sampling (Dougherty, 1976). Another study has demonstrated that 12.1% of sampled tray pads were positive (Byrd, et al., 1999). These reports were contradicted by Limawongpranee *et al.* (1999b) who found no positive tray pads from birds that were delivered from a positive hatchery.

Although some researchers have suggested either the breeder facilities or hatcheries are sources of *Salmonella*, other scientists suggest that broiler houses and their environment serve as a source. It has been suggested that the "resident" *Salmonella* strains in the houses will be the primary strains isolated from the birds (Lahellec, et al., 1986). Limawongpranee *et al.* (1999a) studied whether the environment could serve as a source and reported that 38.5% of environmental samples collected were positive for *Salmonella*.

Others have suggested feed as a possible source of *Salmonella*. Jones *et al.* (1991) found a 20.8% positive rate in samples collected at the feed mill. However, others found feed was not a source (Morgan-Jones, 1982;Bhargava, et al., 1983). The water supply was found to be the source of *Salmonella* in a study conducted by Morgan-Jones (1982).

Although variation in the sources of *Salmonella* has been reported, studies evaluating the survivability of this Gram-negative organism in the broiler production continuum have been reported. It has been demonstrated that *Salmonella* can survive for long periods of time in inoculated poultry feed (up to 26 months) (Davies and Wray, 1996). A high $a_w$, water activity, is associated with *Salmonella*'s presence in broiler houses (Carr, et al., 1995).

The dissemination of *Salmonella* within the broiler industry has also been evaluated. It has been suggested that as the poultry are subjected to stressful situations (i.e. feed withdrawal and harvest) *Salmonella* are shed in the feces more rapidly (Higgins, et al., 1981;Line, et al., 1997). Further, no seasonal variation effects for *Salmonella* were observed by Jacobs-Reitsma, et. al (1994).

Since the exact sources of *Salmonella* within the broiler industry may vary, control and/or elimination of this pathogen from the continuum seems highly unlikely. It has also been suggested that,

within the turkey industry, several critical control points (CCPs) for *Salmonella* exist, such as feeders, drinkers, litter and air (Hoover, et al., 1997). Therefore, each unit of the broiler industry can serve as a possible source of contamination and effective control mechanisms should be incorporated for each component.

## TRADITIONAL *SALMONELLA* METHODOLOGY

Tetrathionate (TT) is an enrichment broth that is selective for *Salmonella spp.* (Anonymous, 1998). The broth gets its name from one of its components, tetrathionate. For organisms to proliferate in this broth, they must produce the enzyme tetrathionate reductase, which will reduce the tetrathionate to thiosulfate. Tetrathionate is produced in the broth with the addition of a potassium iodine solution prior to dispensing into sample tubes. This *Salmonella* broth also contains bile salts, which are inhibitory to many Gram-positive organisms. Sodium thiosulfate is also incorporated into the formulation of this broth, allowing for further suppression of commensal organisms. A toxic by-product neutralizer, calcium carbonate, is also contained in this broth to prevent accumulation of toxic compounds (Anonymous, 1998).

This broth has been used for isolating *Salmonella* from various sample types including those from poultry. It is also used as a

secondary enrichment in the Food and Drug Administration's Bacteriological Analytical Manual (BAM) (Andrews, et al., 1995). The use of TT in the reference manual has resulted from reports published on the efficacy of the medium.

Smyser and Snoeyenbos (1976) determined that direct inoculation of poultry litter samples into TT resulted in 98% of all positive samples being detected. Subsequently, it was shown that a pre-enrichment prior to inoculation of TT resulted in better recovery of *Salmonella*; therefore it has been suggested that pre-enrichment should be used (Thomason and Dodd, 1978).

Rappaport-Vassiliadis (RV) broth is used for isolation of *Salmonella spp.* from meat and dairy products as well as from feces and sewage samples (Anonymous, 1998). One of the main selective compounds in the formulation of RV is malachite green (MG). This dye is inhibitory to most enteric bacteria except *Salmonella*. Another inhibitory component is the inclusion of magnesium chloride ($MgCl_2$), which provides high osmotic pressure. Both the MG and $MgCl_2$ inclusions allow for specific selection of *Salmonella spp.* when isolating this Gram-negative from samples. RV was originally formulated by Rappaport but has been modified by Vassiliadis to produce a medium more conducive for *Salmonella* isolation (Anonymous, 1998).

The growth of *Salmonella* and *Shigella* species have been evaluated for their ability to grow in RV. It was found that no *Shigella* was able to proliferate and *Salmonella* growth was allowed (Vassiliadis, 1968). Because the RV was slightly inhibitory to some *Salmonella* strains, Vassiliadis and coworkers (1979) compared RV/R10 to RV/R25, which contained decreased MG concentrations, 10-mL per 1110-mL and 25-mL per 1125-mL, respectively. It was found that the R10 version was slightly more sensitive than the R25 version as well as more specific due to the increased incubation temperature (43°C) of the R10 version. These findings were confirmed by Xirouchaki *et al.* (1982).

Due to the above results, increased use of RV in sample processing has been seen. For selective enrichment using raw flesh foods, highly contaminated foods and animal feeds, it is recommended that RV be used in place of Selenite Cystine (SC) broth (Andrews, et al., 1995).

## DELAYED SECONDARY ENRICHMENT

The use of a second enrichment broth after an extended incubation period in the primary enrichment medium has been referred to as delayed secondary enrichment (DSE) (Pourciau and Springer, 1978). Most DSE protocols require extended incubation of primary enrichment samples from 5 to 10 days at ambient temperatures. After primary

enrichment for 24 hs, samples are left at room temperature for an extended time and subsequently an aliquot is transferred to fresh selective enrichment broth and further incubated at elevated temperatures overnight. Samples are then plated onto selective agar plates. This method has been shown to be highly effective.

Delayed secondary enrichment was evaluated by Pourciau and Springer (1978) and compared to the standard method using tetrathionate (TT) as the primary enrichment broth. Incorporating DSE into the protocol increased the isolation rate from 45% with a single enrichment broth to 67% using DSE. Similar results were reported by Rigby and Pettit (1980), who evaluated direct enrichment of samples in TT, pre-enrichment prior to primary selective enrichment with TT, and use of a secondary enrichment with extended incubation time. Other reports confirming both of these findings have also been published (Tate, et al., 1990;Waltman, et al., 1991;Waltman, et al., 1993;Nietfeld, et al., 1998;O'Carroll, et al., 1999;Davies, et al., 2000)

Waltman and coworkers (1991) demonstrated that using DSE allowed for a higher isolation rate in both drag swabs and litter when using TT as both the primary and secondary enrichment broths. Others have also found agreement with these reports (Tate, et al., 1990;Nietfeld, et al., 1998). Although the cost is increased when using DSE, it is out

weighted because the number of samples could be decreased, due to the increased sensitivity, yet the confidence will remain high (Nietfeld, et al., 1998).

## RAPID METHODS

### Immunomagnetic Separation

Immunomagnetic separation (IMS) technology has been used for many bacterial organisms, including *Listeria sp.* and *E. coli* (Lund, et al., 1988;Skjerve, et al., 1990). Immunomagnetic isolation and detection of *Salmonella* is accomplished using magnetized particles coated with anti-*Salmonella* antibodies, which are attached covalently. When the beads come into contact with a *Salmonella* cell, the antibodies will bind to the antigen on the cell and will form a bead-bacteria complex. The cells bound to the beads can then be isolated from the sample matrix using the magnetic properties of the beads.

Bacterial cells can be isolated and identified by applying a magnetic field to the samples. By placing the sample tubes containing the sample and magnetic beads into a magnetic field, the beads will concentrate onto the side of the tube taking the bound *Salmonella* cells with them. The bead-bacteria complex can then be plated directly onto a plating medium such as brilliant green agar or xylose-lysine-tergitol 4

(XLT4) agar plates. Because this novel methodology reduces the time required for results to be generated, this methodology has been evaluated under a variety of conditions in an attempt to validate it.

Many reports that characterize the IMS methodology have been published with varying results. While some researchers have found that IMS is lacking in either sensitivity or specificity (Vermunt, et al., 1992;Fierens and Huyghebaert, 1996), others state that this technique is either superior to or equal to the current conventional methods of *Salmonella* isolation and identification (Cudjoe, et al., 1994a;Cudjoe, et al., 1994b;Mansfield and Forsythe, 1996;Cudjoe and Krona, 1997;Shaw, et al., 1998).

Cudjoe and coworkers (1994b) reported that the Dynal anti-*Salmonella* Dynabeads® IMS method was superior to the International Standards Organizations (ISO) method, using various poultry samples including cloacal/fecal swabs, meat, eggshell, and liver in the assay. They also found that the plates from the IMS method were overgrown with non-*Salmonella* and that when RV was used the plates were near pure culture; however, there were significantly fewer *Salmonella* positive samples found when using RV. They suggested that since RV inhibits some *Salmonella*, the use of IMS would overcome the selective pressure of RV (Cudjoe, et al., 1994b). Cudjoe and Krona (1997) also reported

that they found 100% agreement between directly plating bead-bacteria complex and selectively enriching bead-bacteria in RV.

Mansfield and Forsythe (1996) reported that when evaluating IMS using herbs and spices, the new method agreed at almost 100% with conventional methods (TT, RV, and SC). Both the Dynabeads® and conventional method detected 41.7% of all samples as positive. They noted a decreased detection time when using the IMS methodology, which was later concurred by Shaw *et al.* (1998).

Shaw *et al.* (1998) found a 100% agreement was obtained between the conventional methods and IMS. Here, they analyzed environmental samples taken from a processing establishment, as well as cheeses, eggs, and animal feeds. They suggested that there were no false-positives or false-negatives with the IMS method (Shaw, et al., 1998). However, this report and the above mentioned reports are contradicted with other published reports.

As reported by Vermunt *et al.* (1992), IMS may have been an appropriate method for *Salmonella* isolation from a complex matrix; however, the methodology needed to be improved. They evaluated the method using inoculated minced meat and suggested that the recovery rate was dependent upon the concentration of not only the target organism, but also on the concentration of the beads present. It has

been suggested that IMS may not be suitable for all sample types in that some components of the sample matrix may be inhibitory (Skjerve and Olsvik, 1991). In another report Fierens and Huyghebaert (1996), suggested that a commercially available IMS method, Dynabeads ®, was inferior to four other methods evaluated.

In the above mentioned work, five commercially available rapid *Salmonella* isolation and identification kits were compared to conventional methods when sampling animal feeds. The rapid methods included Modified Semisolid Rappaport-Vassiliadis (MSRV) method, Salmosyst-Rambach, *SALMONELLA*-TEK, Dynabeads, and EIAFOSS. These methods were compared to the conventional method using buffered peptone water pre-enrichment followed by selective enrichment in RV. Samples were thereafter selectively plated onto BG and xylose-lysine deoxycholate plates. They reported that the Dynabeads® was inferior to all rapid methods as well as to the conventional methods detecting only 33.3% of the positive samples (Fierens and Huyghebaert, 1996).

Nucleic acid based methods

Polymerase chain reaction (PCR) has been used in various studies comparing its ability to detect *Salmonella* in different sample matrices. Pillai *et al.* (1994) evaluated PCR's ability to detect *Salmonella* in chicken

cecal contents. They reported that the PCR method seemed reliable. This report was concurred by others (Cohen, et al., 1994;Soumet, et al., 1997;Soumet, et al., 1999). In these studies, Cohen *et al.* (1994) evaluated PCR methodology in drag swab samples. They found PCR to be a more sensitive method than the conventional culture methods, reporting that PCR detected 47 of 50 samples to be positive and conventional methods detected 29 of 50. Soumet *et al.* (1999) compared different sample preparation methods before using PCR in environmental swabs from poultry houses. They reported PCR to be efficient, although the sample preparation methods lacked sensitivity. They later reported that PCR in combination with probe hybridization yielded 100% specificity as well as 93.2% sensitivity (Soumet, et al., 1997). These reports indicated PCR may offer an advantage over traditional culture methods not only because this method has been found to be reliable, but also because it is a rapid method. However, further work using different sample types needs to be conducted before using PCR methodology (Soumet, et al., 1997;Soumet, et al., 1999).

As noted by Pillai *et al.* (1994), the PCR method is a reliable alternative to culture methodology, but some sample types may not be as appropriate as others when choosing PCR. Many inhibitory components are present in fecal material and if adequate steps are not taken to

remove these compounds, false-negative results may be generated. Also, when determining which method to employ, future work to be done on the isolated culture needs to be decided. PCR methodology identifies DNA from the specific organism, therefore, the cells must be lysed prior to PCR rendering the cells non-viable. Cohen and coworkers (1994) also mentioned that PCR will detect any *Salmonella* DNA present whether from a viable organism or non-viable, therefore culture methods may also need to be used.

## COMPARISON OF *SALMONELLA* ISOLATION METHODOLOGY

In a recent nationwide survey, it was determined that variation between methodologies used in various laboratories for isolation of *Salmonella* exists. It was found that 17 different selective broths and 14 different plating media are used and no one lab uses the same method as another (Waltman and Mallinson, 1995). A current review of the literature suggests that no particular method has superiority over another, in all cases, and that the sensitivity and specificity of the methods depends on the sample type as well as the isolation conditions.

Most of the studies reviewed have concentrated on comparing various selective enrichment broths, specifically tetrathionate (TT), Rappaport-Vassiliadis (RV), and selenite-cystine (SC) (Vassiliadis, et al., 1974;Vassiliadis, et al., 1976;Vassiliadis, et al., 1978a;Cox, et al.,

1982;Davies and Wray, 1994;Read, et al., 1994b;Hammack, et al., 1998;Huang, et al., 1999). However, a few have evaluated the validity of pre-enrichment prior to the use of a selective enrichment broth.

Vassiliadis, *et al.* (1974) evaluated the growth of *Salmonella* within RV incubated at 37°C and TT incubated at 37°C and 43°C. Here it was reported that TT is inhibitory to the *Salmonella* strains used and that the addition of 5% fecal material is needed to overcome this effect, were as RV is less inhibitory without the addition of any fecal material.

Vassiliadis, *et al.* (1976) compared the uses of both pre-enrichment and selective enrichment as well as selective enrichment without pre-enrichment in minced meat samples. Here, TT and RV were used as selective enrichment broths and buffered peptone water (BPW) was used as a pre-enrichment medium. Also within this study, the authors reported on the use of a secondary enrichment procedure, which included incubation of TT broth for 24 hs followed by sample transfer into RV broth and incubation for an additional 24 hs prior to plating. Of the different protocols employed in this study, it was found that pre-enrichment increased the isolation rate of *Salmonella*. It was also found that using a secondary enrichment broth increased the isolation rate (Vassiliadis, et al., 1976). However, it is curious as to what the isolation rate would have been if the sample had been pre-enriched and TT had

been incubated for 48 hs prior to incorporation of a secondary broth, as it has been shown that incubation of TT for 48 hs dramatically increases the *Salmonella* isolation rate (Edel and Kampelmacher, 1973).

Vassiliadis, *et al.* (1978a) also reported on the use of a new formulation of RV broth, R10. This formulation has a reduced concentration of malachite green, from 25-mL per 1125-mL of broth to 10-mL per 1110-mL. It was reported that the new formulation allows for more *Salmonella* isolation along with increased incubation temperature to decrease the growth of other non-*Salmonella*.

Studies evaluating the usefulness of SC broth compared to TT have also been performed. Cox *et al.* (1982) performed an inoculation study using feed samples and SC and TT as selective enrichment broths. Here, samples were pre-enriched prior to selective enrichment in both broths. It was reported that TT had a higher isolation rate than did SC. Different incubation temperatures were also evaluated and it was found that no significant difference was seen.

More recently, two studies reported on the use of RV, TT and SC. It was shown that, after incubation of inoculated samples in TT at either 35°C or 43°C, SC incubated at 35°C, and RV incubated at 42°C, RV had a lower level of detection among all foods, although non-significant (Hammack, et al., 1998). A non-significant difference between RV and TT

was also reported when examining fecal samples from layers (Braun, et al., 1998).

Huang *et al.* (1999) reported on the use of TT supplemented with brilliant green and RV in combination with an ELISA method. It was reported that the ELISA was effective; however, variation in the growth of *Salmonella* in the different broths existed and needs to be evaluated.

Other studies have evaluated several rapid methods of *Salmonella* isolation. The different methods used include a membrane filter immunoimmobilization procedure, modified semisolid RV, an enzyme immuno-assay, Reveal, BIND, and a filter monitor method (Greenwood and Swaminathan, 1981;Davies and Wray, 1994;Read, et al., 1994b;Dusch and Altwegg, 1995;Wegener and Baggesen, 1997;Peplow, et al., 1999).

The evaluation of the modified semisolid Rapport-Vassiliadis (MSRV) method has shown that this procedure for *Salmonella* isolation and identification is a possible alternative to the selective enrichment procedures. Davies and Wray (1994) found that the MSRV method was more sensitive than RV enrichment. This report has been corroborated by Read *et al.*(1994b) and Dusch *et al.* (1995) both of whom suggest that the MSRV method was highly sensitive.

Peplow *et al.* (1999) reported on the use of three rapid methods, originally developed for food samples, for isolation *Salmonella* in drag swab samples. The authors compared a gold-labeled antibody ELISA, a bacterial ice nucleation detection assay, and a filter monitor method to conventional methods using drag swabs from poultry house. It was reported that none of these methods should be used for broiler house environmental samples at the current development stage of the individual tests.

## SAMPLING BROILER HOUSES

In order to assess the *Salmonella* status of broiler chicken houses, various sample types have been collected. Traditionally and prior to 1981, researchers and industry have relied on litter samples, fecal dropping, and cloacal swabs as indicator of the *Salmonella* status of a particular flock. Of these methods, it has been found that litter sampling was the most appropriate.

### Litter samples

Long and coworkers (1980) sampled 60 broiler houses by collecting litter and suggested litter sampling was the most appropriate method. This report was in agreement with previous work by Olesiuk *et al.* (1969), who compared litter sampling to cloacal samples and

environmental samples. A more recent report showed that of 85 litter samples collected from 13 broiler houses, 57% (48/85) were positive for *Salmonella* (Sasipreeyajan, et al., 1996).

Drag swabs

Although litter was proven to be an effective means of sampling broiler houses, Kingston (1981) reported on another sampling method, which was determined to be simple and less laborious. Here he showed that drag swabbing poultry houses with sterile cotton gauze tied to a kite string was equally effective as sampling via litter. He demonstrated that when sampling thirteen broiler grow-out houses, his drag swabbing method detected seven to be positive where as the litter sampling method only identified five as positive. It was also demonstrated that cecal swabs taken and cultured agreed with the drag swabbing method. He also reported on the ease and simplicity of this method, suggesting that drag swabs samples can offer an alternative sampling regime when used in broiler houses.

When drag swabbing became the gold standard for sampling, other researchers evaluated the method and have found it to be effective. Mallinson *et al.* (1989) combined drag swabbing with a *Salmonella* antigen capturing method. He reported that drag swabbing can be effective when four swabs are used per house. This was confirmed by

Caldwell *et al.* (1994) who suggested more drag swabs could provide a better assessment of the *Salmonella* status of a particular house. Opengart *et al.* (1991) used drag swabbing in turkey houses and suggested that this method was simpler than litter sampling since turkeys shed *Salmonella* periodically.

Sampling broiler houses has been primarily done using the previously discussed drag swabbing method. Therefore, many studies were conducted to evaluate the most appropriate pre-moistening medium for the drag swabs. Kingston (1981) performed this step using buffered peptone water; however, other reports offer other media are more effective (Opara, et al., 1992;Opara, et al., 1994;Byrd, et al., 1997;Rolfe, et al., 2000).

In order to allow for effective sampling, pre-moistening the cotton gauze with a sterile medium seemed necessary. Double strength skim milk (2xSM) has been included in evaluation of different media (Opara, et al., 1992;Opara, et al., 1994;Byrd, et al., 1997;Rolfe, et al., 2000), although disagreement exists. Opara (1992) evaluated 2xSM with that of buffered peptone water, modified Cary Blair transport broth, and lactose broth. In this study, 2xSM provided the highest *Salmonella* recovery rate. This has been concurred by Byrd *et al.* (1997) and again by Opara

*et al.* (1994). However, Rolfe *et al.* (2000) determined that 2xSM was an effective medium, but chicken broth was more sensitive.

# CHAPTER III

# COMPARISON OF FOUR *SALMONELLA* ISOLATION TECHNIQUES IN FOUR DIFFERENT INOCULATED MATRICES[1]


## ABSTRACT

The poultry industry is now operating under increased regulatory pressure following the introduction of the Pathogen Reduction and HACCP rule in 1996. This new operation scheme has greatly increased the need for on-farm food safety risk management of foodborne bacteria, such as *Salmonella*. Information needed to make informed food safety risk management decisions must be obtained from accurate risk assessments, which rely on the sensitivity of the isolation techniques used to identify *Salmonella* in the production environment. Therefore, better characterization of the *Salmonella* isolation and identification


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[1] Reprint with permission (Appendix C) from Rybolt, M. L., R. W. Wills, J. A. Byrd, T. P. Doler and R. H. Bailey. 2004. Comparison of four *Salmonella* isolation techniques in four different inoculated matrices. Poultry Sci. 83:1112-1116.

techniques is warranted. One new technique, immunomagnetic separation (IMS), may offer a benefit to the poultry industry, as it has been shown to be efficacious in the isolation of *Salmonella* from various sample matrices, including some poultry products. In this work, we compared the isolation ability of four *Salmonella*-specific protocols: IMS, tetrathionate (TT) broth, Rappaport-Vassiliadis R10 (RV) broth, and a secondary enrichment (TR) procedure. All four methods were compared in four different spiked sample matrices: Butterfield's solution, poultry litter, broiler crops, and carcass rinses. IMS was able to detect *Salmonella* at a level of 3.66, 2.09, 3.06, and 3.97 $\log_{10}$ CFU/ml in Butterfield's solution, poultry litter, carcass rinse, and broiler crop matrices, respectively. In the broiler litter and Butterfield's solution, there were no ($p>0.05$) differences among the four isolation protocols. However, in the carcass rinse and crop samples, there were no differences between the isolation of *Salmonella* using RV, TR, or TT, but all three were more successful ($p\leq0.05$) at recovering *Salmonella* than the IMS method.

## INTRODUCTION

Over the years, numerous reports have been published comparing various methods for isolating and identifying *Salmonella* from various

sample types. Many of these publications have focused on isolation of *Salmonella* from within poultry samples, either meat products or pre-harvest environmental samples. A current review of the literature suggests that no one method has superiority over another and that the sensitivity and specificity of the methods depends on the sample type as well as the isolation conditions. Most of the studies reviewed have concentrated on comparing various selective enrichment broths, specifically tetrathionate, Rappaport-Vassiliadis, and selenite-cystine (Vassiliadis, et al., 1974;Vassiliadis, et al., 1976;Vassiliadis, et al., 1978b;Cox, et al., 1982;Davies and Wray, 1994;Read, et al., 1994b;Hammack, et al., 1998;Huang, et al., 1999).

Also, when analyzing samples for the presence or absence of *Salmonella* the samples matrix composition should be considered when attempting to interpret the results of the analysis (Davies, et al., 2000). It has been demonstrated that sample makeup can reduce the sensitivity and specificity of an isolation protocol (Skjerve and Olsvik, 1991). An understanding of the characteristics of an isolation method employed is essential when making production/processing risk management decisions, such as strategic scheduling (Long, et al., 1980;Hargis, et al., 2000). These decisions are based on risk assessments which require accurate results obtained from sample analysis.

To obtain accurate results from various sample matrices, identifying the most appropriate methodology for microbial evaluation of samples containing low levels of *Salmonella* is crucial. Additionally, methodologies that provide for rapid screening are essential. New technologies, such as immunomagnetic separation (IMS), may offer an opportunity for detecting *Salmonella* at lower levels in various pre-harvest sample matrices in less time when compared to traditionally used isolation methods. The use of IMS has been reported in examining raw eggs, where it was shown to be efficient when the egg samples were supplemented with ferrous sulphate to aid the *Salmonella* growth prior to IMS (Cudjoe, et al., 1994a). It was also shown that the ability of IMS, in combination with flow cytometry (Wang and Slavik, 1999) and with immuno-optical absorption (Liu, et al., 2001), to isolate *Salmonella* from poultry carcass rinses could detect the pathogen at low levels. However, there are no reports in the literature that indicate the efficacy of IMS in the analysis of pre-harvest poultry samples, such as litter or crops.

The purpose of this work is to characterize four *Salmonella* isolation methods, two traditional, one IMS, and one secondary enrichment method, in matrices where the pathogen is commonly found in the poultry production continuum.

# MATERIALS AND METHODS

The two traditional *Salmonella* selective isolation broths used were tetrathionate (TT)[2] and Rappaport-Vassiliadis R10 (RV)[3]. The IMS method used was Dynal Biotech anti-*Salmonella* Dynabeads ® (DB)[4]. For the secondary enrichment method (TR), tetrathionate was used as the primary enrichment and Rappaport-Vassiliadis was used as the secondary enrichment broth. The four matrices used were Butterfield's solution (PC), broiler litter, carcass rinses, and crops from market age chickens.

## Bacterial Culture

A pure culture of *Salmonella enterica* serotype Typhimurium NN (obtained from the National Veterinary Services Laboratory, Ames, Iowa), which is resistant to nalidixic acid (NA) and novobiocin (NO), was used as the test organism. The culture was maintained on brilliant green agar[4] (BG) plates supplemented with 25-µL novobiocin and 20-µL nalidixic acid at refrigerated temperatures until needed.

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[2] Remel Inc., Lenexa, KS 66215

[3] Difco Laboratories, Detroit, MI 48232

[4] Neogen Corporation, Lansing, MI 48912

To prepare the inoculum for each sample matrix, an overnight culture of S. Typhimurium NN picked from a single isolated colony was grown in sterile brain heart infusion broth[4] containing both novobiocin and nalidixic acid (BHI-NN). The broth was incubated at 37°C in an environmental shaker[5]. A 1-mL aliquot of the overnight culture was transferred to 75-mL of BHI-NN broth and the optical density measured at 600 nm ($OD_{600}$)[6]. The freshly inoculated culture was incubated at 37°C in the environmental shaker until an $OD_{600}$ of 0.7 was reached, at which point a ten-fold serial dilution ($10^{0}$-$10^{-10}$ CFU/mL) was made. Enumeration plate counts were performed on the serial dilutions to determine the culture concentration at the time of inoculation.

Sample Inoculation

To compare the four isolation protocols (RV, TT, TR, and DB) without interference from confounding factors likely to be present in litter, carcass rinse, and crop sample, 1-mL aliquots of each inoculum dilution ($10^{0}$-$10^{-10}$) were used to inoculate sterile Butterfield's solution

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[5] Series 25, New Brunswick Scientific, Edison, NJ

[6] Virian DMS 200 UV Visible Spectrophotometer

(first sample matrix). This procedure was repeated so that a total of ten replicates were performed using the same bacterial culture.

A second sample matrix was crop samples obtained from a local poultry processing facility. One hundred ten (110) crops from market age broilers were collected aseptically, placed into sterile WhirlPak® bags[7], transported on wet ice to the laboratory, and stored at 4°C until used.

In the laboratory, 1-mL aliquots from each of the *Salmonella* culture dilutions were used to inoculate eleven crop samples (weighing an average of 8.2 g). Each inoculated sample was mixed vigorously by hand for 30 s. This process was repeated nine more times to provide a total of ten replicate sets of crops inoculated with $10^{0}$-$10^{-10}$ dilutions of the *Salmonella* culture.

Litter served as the third sample matrix for the inoculation study. A pooled sample was collected from a broiler grow-out house located at Mississippi State University South Farm following harvest of the birds and transported to the laboratory for further processing. The litter was divided into 110 25-g aliquots and placed into sterile filtered WhirlPak®

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[7] Nasco FT Atkinson

bags. One-mL aliquots of each of the *Salmonella* culture dilutions were used to inoculate eleven bags containing litter and mixed vigorously by hand for 30 s. This process was repeated nine times to provide a total of ten replicates of litter matrix samples inoculated with $10^{0}$-$10^{-10}$ dilutions of the *Salmonella* culture.

The fourth sample matrix, carcass rinse, was obtained from a local poultry processing plant as part of the plant's routine sampling program. The Butterfield's rinse sample was divided into 110 9-mL aliquots and placed into sterile 50-mL conical bottom centrifuge tubes. One-mL aliquots of each of the *Salmonella* culture dilutions were used to inoculate eleven rinse tubes. This process was repeated nine times to provide a total of ten replicates of carcass rinse matrix samples inoculated with $10^{0}$-$10^{-10}$ dilutions of the *Salmonella* culture.

Pre-enrichment, Selective Enrichment and Isolation

Non-selective pre-enrichment broth, Butterfield's solution (0.00031 M $KH_2PO_4$, pH 7.2) was added to the litter and crop samples at a 1:10 wt/vol ratio to allow microorganisms to recover from injury resulting from sample preparation and/or deleterious effects of the environment. Although this step was not necessary for this study, it was included to fully simulate normal practice when dealing with field samples. No additional Butterfield's solution was added to either the PC or carcass

rinse samples since both sample types already contained the pre-enrichment broth. All samples were incubated overnight at 42°C before being subjected to each of the selective enrichment/isolation protocols.

For both RV and TT protocols, the broths were prepared according to the manufacturer's directions. Nine-mL aliquots of both broths were aseptically transferred to eleven 50-mL conical bottom centrifuge tubes and inoculated with 1-mL of each sample for each replicate. Tubes were vortexed and incubated at 42°C overnight.

Dynal anti-*Salmonella* Dynabeads ® (DB) were obtained from Neogen Corporation and stored at 4°C until used. Following the manufacturer's suggested protocol, 20 µL aliquots of magnetic bead complex were aseptically added to 1.5-mL microfuge tubes. One-mL of each sample was added to corresponding tubes. Tubes were vortexed and incubated at room temperature for 30 mins with intermittent shaking. Tubes were placed into a magnetic particle concentrator[8] and left undisturbed for 10 mins to allow magnetic beads to concentrate onto the side of the tubes. The supernatant was aspirated using sterile Pasteur pipettes, leaving the beads concentrated onto the side of the

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[8] Product No. Z5342, Promega Inc., Madison, WI 53711

tubes. A 1-mL volume of sterile phosphate buffered saline-Tween 20 wash solution (PBS-Tween 20) (0.15M NaCl, 0.01M $Na_2HPO_4$, pH 7.4, 0.05% Tween 20) was added to each tube. Tubes were shaken to evenly distribute the beads in the wash solution and allowed to sit undisturbed for 10 mins. Samples were washed two more times following the same procedure. After the third wash, beads were resuspended in 100 µL of PBS-Tween 20.

For the secondary enrichment method (TR), the original TT tubes were re-incubated an additional 24 hs at 42°C. After re-incubation, 0.1-mL aliquots of each tube was transferred to 9.9 mL fresh RV and incubated at 42°C for 24 hs. This method is a slight modification of a previously published method (Barber, et al., 2002).

Following incubation, a loop-full of the RV, TT, and TR samples and 50-µL of the DB samples were streaked onto xylose-lysine tergitol 4 (XLT4)[4] plates supplemented with 25 µL novobiocin and 20 µL nalidixic acid, followed by overnight incubation at 37°C. XLT4 plates containing suspect *Salmonella* colonies, which were red with black centers, were further characterized by observing the typical biochemical reactions on triple sugar iron agar and lysine iron agar slants. Isolates producing

positive results on the slants were also tested using serological testing (*Salmonella* O Antiserum Poly A-I & Vi[9]).

Statistical Analysis

For each of the four sample matrices (PC, litter, carcass rinse and crop), differences in $\log_{10}$ CFU/mL of *Salmonella* detected among the four isolation protocols were evaluated by analysis of variance (PROC GLM, SAS version 8.0[10]). Least square means using Tukey's adjustment for multiple comparisons was used to determine the significance of differences among treatment means.

RESULTS

The initial concentration of the inoculum cultures for each of the matrices was $10^8$ CFU/mL as determined by enumeration plate counts.

For the PC matrix, the TR protocol demonstrated the lowest level of *Salmonella* detection with a mean of 2.56 log10 CFU/mL (Table 3.1); however, there were no statistically significant differences ($p>0.05$) among the four isolation protocols evaluated.

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[9] Difco Laboratories, Detroit, MI 48232

[10] SAS Institute, Inc., Cary, NC

When evaluating the four isolation protocols in the litter matrix, TR demonstrated the lowest level of detection (1.79 $\log_{10}$ CFU/mL); however, there were no statistically significant differences ($p>0.05$) among the four isolation protocols.

In the crop samples, TR again provided the lowest level of detection (2.07 log10 CFU/mL) compared to the other three protocols. There were no statistically significant differences ($p>0.05$) among the TR, TT, and RV protocols; however, there was a significant difference ($p<0.001$) in the isolation ability of the DB protocol when compared to the other three methods.

Results in the forth sample matrix, carcass rinses, were similar to results as those found in the crop samples. The TR method again provided the lowest level of detection with a mean of 0.76 $\log_{10}$ CFU/mL. There were no statistically significant differences detected among the TR, TT, and RV protocols; however, there was a significant difference ($p<0.0001$) between DB and the other three methods.

Table 3.1. Minimum concentration of *Salmonella* detected in four different spiked matrices using four different isolation protocols.

| Protocol | Sample Matrix | | | |
|---|---|---|---|---|
| | Pure Culture | Litter | Crop | Carcass Rinse |
| | ($Log_{10}$ CFU/ml)[1] | | | |
| Dynabeads® | 3.66±1.51[a] | 2.09±0.79[a] | 3.97±1.43[a] | 3.06±0.63[a] |
| Rappaport-Vassiliadis | 2.96±0.48[a] | 1.89±0.52[a] | 2.17±0.42[b] | 0.96±0.48[b] |
| Tetrathionate | 3.46±0.92[a] | 1.99±0.48[a] | 2.47±0.32[b] | 0.86±0.52[b] |
| Secondary Enrichment | 2.56±1.25[a] | 1.79±0.53[a] | 2.07±0.48[b] | 0.76±0.53[b] |

[1]Mean values (n=10) within a column with the same superscript are not significantly different ($p > 0.05$) using Tukey's adjustment of least square means.

## DISCUSSION

When performing pre-harvest risk assessment, identification of the most appropriate sites that give the highest probability of isolating *Salmonella* is essential. Previously published work has demonstrated that two primary sites positive for *Salmonella* within the production continuum are poultry litter and broiler crops (Fanelli, et al., 1971;Snoeyenbos, et al., 1982;Corrier, et al., 1991;Hargis, et al., 1995;Ramirez, et al., 1997;Corrier, et al., 1999b, 1999a;Byrd, et al., 2001;Byrd, et al., 2002). For that reason, these two matrices were

chosen for study in this work. The carcass rinse samples were used for the inoculation study to simulate an in-plant *Salmonella* monitoring program. The litter was studied because it has been demonstrated, when compared to cloacal sampling and other environmental samples, that litter samples provide a better assessment of the *Salmonella* status of a house pre-harvest (Olesiuk, et al., 1969;Sasipreeyajan, et al., 1996).

Analyzing the ability of the four protocols within this study, DB did not provide the lowest level of *Salmonella* detection in the four matrices studied. Nonetheless, this protocol allowed for more rapid results. Therefore, since there were no statistically significant differences among the isolation abilities of these four protocols in inoculated Butterfield's solution and poultry litter, it is anticipated that DB may be useful when certain matrices are tested in a pre-harvest *Salmonella* monitoring regimen.

The reduced efficacy of *Salmonella* isolation by DB from spiked crop and carcass rinse samples may well be directly related to the composition of these matrices. Previously, in using IMS in various food products, it was determined that a sample matrix could affect the isolation ability of a method (Skjerve and Olsvik, 1991).

The crops consist of muscle and fat, the components of which are proteins and lipids. Any of the components of the crops, alone or in

combination with other constituents, could provide a physical and/or a chemical barrier that could interfere with the binding sites on the anti-*Salmonella* antibodies attached to the magnetic beads. Another factor to consider is that the direct physical attachment of the bacteria to the components of the matrix could be stronger than the attraction to the antibodies on the magnetic beads in this context. Similarly, for the carcass rinse samples, fatty components that were washed from the carcass during the rinse process may contribute to the decreased level of detection for the DB protocol. The use of cheese cloth has been used to remove such inhibitory components when evaluating IMS in carcass rinse samples (Wang and Slavik, 1999). In the current study, no sample filtration techniques were used because it can potentially remove *Salmonella* that may be present within the sample. Wang and Slavik demonstrated an average isolation rate of 4.36 $\log_{10}$ CFU/mL with filtration, and in the current study the DB average isolation rate was 3.06 $\log_{10}$ CFU/mL without the use of cheese cloth.

The use of delayed secondary enrichment (DSE) has been shown to dramatically increase the *Salmonella* isolation from various sample matrices (Pourciau and Springer, 1978;Waltman, et al., 1991;Nietfeld, et al., 1998). Most DSE protocols require extended incubation of primary enrichment samples from 5 to 10 days at ambient temperatures if no

suspect colonies are present after plating the initial enrichment. After primary enrichment for 24 hs, samples are left at room temperature for an extended time, and an aliquot is subsequently transferred to fresh selective enrichment broth and further incubated at elevated temperatures overnight. Samples are then re-plated onto selective agar plates. A shortened modification of the DSE method was used as the fourth *Salmonella* isolation method for this study.

This methodology, secondary enrichment, used tetrathionate as the primary enrichment broth and Rappaport-Vassiliadis as the secondary enrichment. Also, the original tetrathionate was only incubated for 48 hs at elevated temperatures as opposed to 5-10 days at ambient temperatures as common DSE methodology does. The tetrathionate, which contains calcium carbonate, provides an optimal environment for the *Salmonella* to proliferate, but at the same time other microorganisms that may be present may also grow. Therefore, the use of Rappaport-Vassiliadis, which contains malachite green, a substance which is toxic to many bacterial species, eliminates the competing organisms when this secondary enrichment is utilized.

In this work, there were no statistically significant differences in the isolation abilities of the four protocols in two of the four matrices (PC and litter) studied. However, the shorter time required to obtain results

when using the DB method could provide an advantage in certain matrices from a commercial operation. On the other hand, the DB method may not be as effective as the other methods in samples from chicken tissues. Therefore, further work is warranted to determine whether the DB isolation protocol would provide the same advantage seen in this study when examining actual field samples contaminated with naturally occurring levels of *Salmonella.*

It should be noted here that the cost of the various *Salmonella* isolation methodologies used is varied. The DB is considerably more expensive than the other three methods used. However, if the DB method can reduce the analysis time by one to three days and proves to be at least as sensitive as the other methods, it could be worth the additional cost in a production/process risk assessment scenario.

This work demonstrates that when doing microbial risk assessment, attention should be given to the type of matrix that the *Salmonella* is to be isolated from as well as the microbiological isolation methods employed. It is essential that risk management decisions be based on well-defined and characterized risk assessment methods.

# CHAPTER IV

# USE OF SECONDARY ENRICHMENT FOR ISOLATION OF *SALMONELLA* IN NATURALLY CONTAMINATED ENVIRONMENTAL SAMPLES[11]


## ABSTRACT

Since the implementation of HACCP, the need for on-farm food safety risk assessment/management has greatly increased. In order to provide accurate risk assessments, attention should be focused on better characterization of the *Salmonella* isolation and identification techniques. In this work, we compared the isolation ability of four *Salmonella*-specific protocols: immunomagnetic separation (DB), tetrathionate (TT) broth, Rappaport-Vassiliadis R10 (RV) broth, and a secondary enrichment (TR) procedure, as well as two selective solid media (brilliant green agar, BG; xylose-lysine tergitol 4, XLT4). All four methods were compared in both litter and drag swab samples collected weekly during the broiler grow out


[11] Reprinted with permission (Appendix C) from Rybolt, M. L., R. W. Wills and R. H. Bailey. 2005. Use of secondary enrichment for isolation of *Salmonella* from naturally contaminated environmental samples. Poultry Sci. 84:992-997.

period in seven houses. There were 65/126 (51.6%) pooled litter samples positive and 115/304 (37.8%) drag swab samples positive for *Salmonella* by at least one method. Of the 65 positive litter samples, DB, RV, and TT isolated 1 (2.7%), 31 (47.7%), and 23 (35.4%) of the samples as positive when using brilliant green agar, respectively. The TR protocol identified 83.1% (54/65) of the positive samples as positive when using brilliant green agar. In the drag swab samples, DB did not identify any samples as positive, whereas TT and RV found 28 (25.7%) and 26 (23.9%) of the 109 samples to be positive when using brilliant green agar, respectively. Again, the TR protocol identified the highest percentage of positive samples (94.5%). An analysis of agreement, kappa, revealed that TT and RV do not always agree on which samples were positive, although the number of samples identified as positive by both were not different. A comparison between the two agar plates used, brilliant green and xylose-lysine tergitol 4, showed that they had high agreement when the secondary enrichment protocol was used, but agreement was only moderate to low when the other three methods were used.

## INTRODUCTION

Over the years, numerous reports have been published comparing various methods for isolating and identifying *Salmonella* from different

sample types. Many of these publications have focused on isolation of *Salmonella* from various poultry samples, either meat products or pre-harvest environmental samples. A current review of the literature suggests that no one method is superior and that the sensitivity and specificity of the methods depends on the sample type as well as the isolation conditions. Most of the studies reviewed have concentrated on comparing various selective enrichment broths, specifically tetrathionate, Rappaport-Vassiliadis, and selenite-cysteine (Vassiliadis, et al., 1974;Vassiliadis, et al., 1976;Vassiliadis, et al., 1978b;Cox, et al., 1982;Davies and Wray, 1994;Read, et al., 1994a;Hammack, et al., 1998;Huang, et al., 1999).

It is essential to understand the characteristics of the isolation method employed when making production/processing risk management decisions, such as strategic scheduling of poultry flocks for processing (Long, et al., 1980;Hargis, et al., 2000). However, when analyzing samples for the presence or absence of *Salmonella,* the sample's matrix composition should also be considered when attempting to interpret the results of the analysis (Davies, et al., 2000), as it has been demonstrated that sample makeup can affect the sensitivity and specificity of an isolation protocol (Skjerve and Olsvik, 1991). Therefore, for the risk

management decisions to be effective, they must be based on accurate risk assessments, which ultimately requires correct sample analysis.

To obtain accurate results from various sample matrices, identifying the most appropriate methodology for microbial evaluation of samples containing low levels of *Salmonella* is crucial. Previously, this laboratory has characterized four *Salmonella* isolation methods in artificially contaminated matrices simulating conditions where the pathogen is commonly found in the poultry production continuum (Rybolt, et al., 2004). The next logical step would be to employ these methods with samples acquired under actual production conditions.

The purpose of this work is to examine these four previously used isolation methods in samples obtained from naturally contaminated environments. Samples were acquired from broiler houses by two different sampling methods, drag swabs and floor litter. The *Salmonella* selective isolation broths used were Muller Kauffman tetrathionate (TT), Rappaport-Vassiliadis R10 (RV), and a secondary enrichment protocol (TR). An immunomagnetic bead method, Dynal Biotech anti-*Salmonella* Dynabeads® (DB) was also used.

# MATERIALS AND METHODS

## Experimental Design

Thirteen individual broiler grow-out houses managed by a single production company were selected for this study. The houses were divided between two farms (six on farm A and seven on farm B). At the initial time of sampling, all houses were empty and being prepared for new flocks. The houses had conventional tunnel ventilation with dirt base floors. There were no other farm animals present on either farm A or B; however, the presence of feral animals was evident (tracks and fecal droppings) around the houses on both farms. Pine shavings, which had not been changed from the previous flock, were used for floor litter in the houses. Four drag swabs and two pooled litter samples were collected in each house. Farm A houses were sampled only once by drag swabs, whereas, Farm B houses were sampled for 10 consecutive weeks by drag swabs and 9 consecutive weeks via litter. This gave a total of 304 drag swab samples and 126 litter samples to use for method comparison.

## Sampling

Sampling procedure for drag swabs was followed as previously described (Caldwell, et al., 1994). Drag-swab assemblies were prepared prior to use in the poultry houses. Each swab was constructed with 4 x 4 –

inch (10.2 cm x 10.2 cm) cotton gauze[12] tied to 6-ft (182.9 cm) cotton-polyester twine[13]. The assemblies were sterilized with steam and aseptically transferred to sterile WhirlPak ® bags containing 20-mL sterile double strength skim milk for a pre-soaking medium. The skim milk was prepared according to the manufacturer's directions[14], except the concentration of powder to water was doubled (from 45.36 g per 500-mL to 90.72 g per 500-mL). In the houses, each swab was removed from the bag and dragged through the house and returned after sampling. All swabs were stored on wet ice until processing in the laboratory.

Two litter samples were collected in each grow-out house starting at week 0 (placement). The houses were divided lengthwise, and floor shavings were collected from three different locations per sample equidistant from each other at each end and in the middle. Collection was done using examination gloves and samples were placed into sterile WhirlPak ® bags and sealed. Samples were placed on wet ice and transported to the laboratory and were processed in less than 2 hs.

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[12] Abco Dealers, Inc., Nashville, TN

[13] The Lehigh Group, Macungie, PA

[14] Wal-Mart Stores, Inc., Bentonville, AR

Enrichment and Isolation

All samples were mixed with Butterfield's solution (0.00031 M $KH_2PO_4$, pH 7.2) at a 1:10 wt/vol ratio. After addition of Butterfield's solution, samples were incubated overnight at 42°C before being subjected to each of the selective enrichment/isolation protocols, as described below.

Both Rappaport-Vassiliadis R10 (RV) broth[15] and tetrathionate (TT) broth[16] were prepared according to the manufacturer's directions. Nine-mL aliquots were aseptically transferred to eleven 50-mL conical bottom centrifuge tubes and inoculated with 1-mL of each sample. Tubes were vortexed and incubated at 42°C overnight.

Dynal anti-*Salmonella* Dynabeads ® (DB) were obtained from Neogen Corp.[17] and stored at refrigeration temperatures until used. Following the manufacturer's suggested protocol, 1.5-mL microfuge tubes were numbered and 20 µL aliquots of magnetic bead complex were aseptically added. One-mL of each sample was added to corresponding tubes. Tubes were vortexed and incubated at room temperature for 30 mins with intermittent shaking.

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[15] Difco Laboratories, Detroit, MI 48232

[16] Remel Inc., Lenexa, KS 66215

[17] Neogen Corp., Lansing, MI 48912

Tubes were placed into a magnetic particle concentrator[18] and left undisturbed for 10 mins to allow magnetic beads to concentrate onto the side of the tubes. The supernatant was aspirated using sterile Pasteur pipettes, leaving the beads concentrated onto the side of the tubes. A 1-mL volume of sterile phosphate buffered saline-Tween 20 wash solution (PBS-Tween 20) (0.15M NaCl, 0.01M $Na_2HPO_4$, pH 7.4, 0.05% Tween 20) was added to each tube. Tubes were shaken to evenly distribute the beads in the wash solution and allowed to sit undisturbed for 10 mins. Samples were washed two more times following the same directions. After the third wash, beads were resuspended in 100 μL of PBS-Tween 20.

For the secondary enrichment method (TR), the original TT tubes were incubated an additional 24 hs at 42°C. After incubation, 0.1-mL aliquots of each tube were transferred to 9.9 mL fresh RV and incubated at 42°C for 24 hs.

Following incubation, a loop-full of each of the RV, TT, and TR samples and 50-μL of the DB samples were streaked onto individual brilliant green (BG) and xylose-lysine tergitol 4 (XLT4) plates, followed by overnight incubation at 37°C. BG and XLT4 plates containing suspect

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[18] Product No. Z5342, Promega Inc., Madison, WI 53711

*Salmonella* colonies were further characterized using triple sugar iron agar and lysine iron agar slants. Isolates producing positive results on the slants were also tested using serological testing (anti-*Salmonella* poly A-I and Vi antibodies).

Statistical Analysis

The PROC FREQ procedure of SAS[19] was used to generate a chi-square test statistic to determine if there was an association between the four *Salmonella* isolation protocols used. Separate analyses were conducted on litter and drag swab samples for both BG and XLT4 results. To minimize the opportunity of finding an association due to chance, Bonferroni's correction for multiple comparisons was used with an initial alpha level of 0.05. Also, the kappa coefficient of agreement was generated to determine the degree of agreement among the results of the four protocols, and interpretations followed that of Landis and Koch (Landis and Koch, 1977). Briefly, a kappa value of 0.0 or less is considered to be poor agreement, and kappa values above 0.81 indicate almost perfect agreement. For the values between 0.0 and 0.81,

[19] SAS Institute, Inc., Cary, NC

interpretations are slight, fair, moderate and substantial for 0.00-0.20, 0.21-0.40, 0.41-0.60, and 0.61-0.80, respectively.

## RESULTS

### Frequencies

There were 65 of 126 (51.6%) litter samples positive for *Salmonella sp.* as determined by at least one method and streaked onto either selective agar plates. For the drag swab samples, when using BG plates, there were 109 of 304 (35.9%) samples determined to be *Salmonella* positive. When using XLT4 plates with the four protocols in evaluating the drag swab samples, there was a 37.8% (115/304) *Salmonella* isolation rate (Table 4.1). After three weeks of evaluation, the DB protocol was discontinued because of insufficient performance; therefore, only 56 litter samples and 164 drag swab samples were evaluated with this method.

Of the 56 litter samples that were tested by DB, 37 and 36 samples were positive for *Salmonella* by one or more methods when plated on BG or XLT4, respectively. DB determined 1 (2.7%) of the 37 samples to be positive for *Salmonella* when plated onto BG and XLT4 (11.1%) of 36 samples to be positive for *Salmonella* when plated onto XLT4 plates (Table 4.1). When plated on BG plates, DB was statistically different from the next best method, TT ($p = 0.0002$). When plated on XLT4, DB had a significantly

lower isolation of *Salmonella* than TT and TR ($p < 0.0125$), but no difference was detected between DB and RV ($p = 0.0376$; using Bonferroni's correction for multiple comparisons, the alpha level for this set of comparisons was $0.05/4 = 0.0125$).

Table 4.1. Total positive drag swab and litter samples by at least one method.

| | BG | XLT4 |
|---|---|---|
| Litter | 65/126 (51.6%) | 65/126 (51.6%) |
| Drag Swabs | 109/304 (35.9%) | 115/304 (37.8%) |

Of the 65 positive litter samples, RV had a *Salmonella* isolation rate of 47.7% (31/65) and TT had an isolation rate of 35.4% (23/65) when plated onto BG plates (Table 4.2). There was no statistically significant difference ($p = 0.1545$) found between these methods. When plated onto XLT4 selective agar, RV had an isolation rate of 29.2% (19/65), and TT had a *Salmonella* isolation rate of 35.4% (23/65). Again, no significant difference ($p = 0.4531$) was detected for this comparison.

The fourth protocol used to evaluate the litter samples, TR, provided the highest isolation rates compared to the other three methods evaluated. TR had a statistically significant ($p < 0.0001$) higher *Salmonella* isolation rate, 83.1% (54/65), when compared to the next highest protocol (RV) using BG plates. The isolation rate for TR when

plated onto XLT4 plates was 80.0% (52/65), which was significantly higher ($p < 0.0001$) than the next highest protocol, TT.

Table 4.2. Comparison between four *Salmonella* isolation protocols in two different sampling mediums using two different selective agars.

| | Litter | | Drag Swab | |
|---|---|---|---|---|
| | BG* | XLT4 | BG | XLT4 |
| DB | 1/37 (2.7)[a] | 4/36 (11.1)[a] | 0/85 (0)[a] | 3/90 (3.3)[a] |
| RV | 31/65 (47.7)[b] | 19/65 (29.2)[a,b] | 26/109 (23.9)[b] | 26/115 (22.6)[b] |
| TT | 23/65 (35.4)[b] | 23/65 (35.4)[b] | 28/109 (25.7)[b] | 34/115 (29.6)[b] |
| TR | 54/65 (83.1)[c] | 52/65 (80.0)[c] | 103/109 (94.5)[c] | 107/115 (93.0)[c] |

*Values within a column with the same superscript are not significantly different ($p > 0.0125$). No. Positive/No. positive by at least one method (%).

For the drag swab samples when plated onto BG plates, DB did not isolate any *Salmonella* from the 164 samples evaluated. This protocol's isolation rate was lower at a statistically significant level ($p = 0.0001$) than RV, the protocol with the next lowest isolation rate. Only three samples

were positive by DB plated onto XLT4. The protocol's isolation rate was significantly lower ($p = 0.0040$) than RV, the protocol with the next lowest isolation rate.

Using BG plates, RV had a 23.9% (26/109) isolation rate and TT had a 25.7% (28/109) *Salmonella* isolation rate. On XLT4 plates, RV found 26/115 (22.6%) samples to be positive and TT found 34/115 (29.6%) samples to be positive. There were no significant differences (BG: $p = 0.7537$; XLT4: $p = 0.2296$) found between these two methods on either type of plate.

The secondary enrichment protocol (TR) provided the highest isolation rates compared to the other three methods. When using BG as the selective enrichment agar, TR isolated *Salmonella* from 103/109 (94.5%) samples and was significantly different ($p < 0.0001$) from TT, the protocol with the next highest isolation rate. TR plated onto XLT4 agar had a 93.0% (107/115) *Salmonella* isolation rate and was significantly different ($p < 0.0001$) from TT, the protocol with the next highest isolation rate.

Kappa Analysis

The kappa analysis assesses the agreement between two protocols at a time i.e. how many samples were classified as positive by both protocols, negative by both protocols or positive by one protocol and negative by the other. For instance, the comparison between TT and RV,

when drag swabs were plated onto XLT4 plates, demonstrated that both broths agreed that 8 samples were *Salmonella* positive and 252 samples were *Salmonella* negative. However, there were 44 discordant pairs (those samples the protocols disagreed on) of the 304 samples; therefore yielding a low kappa value of 0.19 ($p = 0.0009$) (Table 4.3). A second comparison between TT and TR, when drag swabs were plated onto XLT4, demonstrated that both of these enrichment protocols agreed on the status of 225 samples (31 *Salmonella* positive and 194 *Salmonella* negative). There were 79 discordant pairs leading to a fair kappa of 0.32 ($p < 0.0001$) (Table 4.4). A comparison was also made between RV and TR when drag swabs were plated onto XLT4 plates. These protocols agreed on 215 samples (22 positive and 193 negative) and there were 89 discordant pairs. The kappa value for this comparison was 0.22 ($p < 0.0001$) (Table 4.5), which is considered fair agreement.

For the drag swab samples, no comparison was made between DB and the other three protocols when BG was used because DB did not isolate *Salmonella* from any of the samples (Table 4.6). The highest agreement was found comparing TT to TR ($\kappa = 0.29$; $p < 0.0001$) and the lowest was between RV and TT ($\kappa = 0.19$; $p = 0.0011$). When XLT4 plates were used the lowest agreement ($\kappa = 0.01$; $p = 0.6484$) was found in the

comparison between DB and TR and the highest ($\kappa = 0.32$; $p < 0.0001$) was between TT and TR.

Making comparisons in litter samples using BG agar, DB compared to RV had the lowest agreement with a kappa value of -0.03 ($p = 0.5602$) and RV compared to TR had the highest agreement with a kappa value of 0.40 ($p < 0.0001$) (Table 4.6). For comparisons using XLT4 plates, the lowest agreement with a kappa value was found in the comparison between DB and TR ($\kappa = 0.09$; $p = 0.0905$). The highest agreement with a kappa value of 0.27 ($p = 0.0109$) was found comparing DB to TT.

An agreement comparison was also made between the two selective agars, BG and XLT4 (Table 4.7). For the litter samples, BG and XLT4 agreement was highest when using TR ($\kappa = 0.94$, $p < 0.0001$). For the other three protocols, DB, RV, and TT, the kappa agreement values were -0.03, 0.46, and 0.42, respectively. In the drag swab samples, the kappa agreement comparison yielded a near perfect value of 0.91 ($p < 0.0001$) when using TR as the protocol. The other three protocols' values were 0.00, 0.87, and 0.53 for DB, RV, and TT, respectively.

Table 4.3. Comparison between tetrathionate (TT) and Rappaport-Vassiliadis (RV) when plated on xylose-lysine tergitol 4 (XLT4) plates for the isolation of *Salmonella* from drag swab samples (DS).

| | | Tetrathionate | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| Rappaport-Vassiliadis | Positive | 8 | 18 | 26 |
| | Negative | 26 | 252 | 278 |
| | Total | 34 | 270 | 304 |

Table 4.4. Comparison between tetrathionate (TT) and secondary enrichment (TR) when plated on xylose-lysine tergitol 4 (XLT4) plates for the isolation of *Salmonella* from drag swab samples (DS).

| | | Tetrathionate | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| Secondary Enrichment | Positive | 31 | 76 | 107 |
| | Negative | 3 | 194 | 197 |
| | Total | 34 | 270 | 304 |

Table 4.5. Comparison between Rappaport-Vassiliadis (RV) and secondary enrichment (TR) when plated on xylose-lysine tergitol 4 (XLT4) plates for the isolation of *Salmonella* from drag swab samples (DS).

| | | Rappaport-Vassiliadis | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| Secondary Enrichment | Positive | 22 | 85 | 107 |
| | Negative | 4 | 193 | 197 |
| | Total | 26 | 278 | 304 |

Table 4.6. Kappa agreement results between all sample isolation comparisons.

| | Comparison | BG Plates | | XLT4 Plates | |
|---|---|---|---|---|---|
| | | Kappa | Pr > \|Z\| | Kappa | Pr > \|Z\| |
| Litter Samples | DB : RV | -0.03 | 0.5602 | 0.16 | 0.1484 |
| | DB : TT | 0.11 | 0.0665 | 0.27 | 0.0109 |
| | DB : TR | 0.02 | 0.4344 | 0.09 | 0.0905 |
| | RV : TT | 0.25 | 0.0042 | 0.20 | 0.0228 |
| | RV : TR | 0.40 | <0.0001 | 0.15 | 0.0355 |
| | TT : TR | 0.25 | 0.0009 | 0.20 | 0.0099 |
| Drag Swab Samples | DB : RV | nc* | nc | 0.07 | 0.2112 |
| | DB : TT | Nc | nc | 0.13 | 0.0080 |
| | DB : TR | Nc | nc | 0.01 | 0.6484 |
| | RV : TT | 0.19 | 0.0011 | 0.19 | 0.0009 |
| | RV : TR | 0.24 | <0.0001 | 0.22 | <0.0001 |
| | TT : TR | 0.29 | <0.0001 | 0.32 | <0.0001 |

*nc= no comparison made.

Table 4.7. Kappa agreement values between brilliant green and Xylose Lysine tergitol 4 plates.

| | Litter | | Drag Swabs | |
|---|---|---|---|---|
| | Kappa | P > \|Z\| | Kappa | P > \|Z\| |
| Dynabeads® | -0.03 | 0.7796 | * | * |
| Rappaport-Vassiliadis | 0.46 | < 0.0001 | 0.87 | <0.0001 |
| Tetrathionate | 0.42 | < 0.0001 | 0.53 | <0.0001 |
| Secondary Enrichment | 0.94 | < 0.0001 | 0.91 | <0.0001 |

* No positive samples detected by this method using BG, therefore no value calculated.

# DISCUSSION

In this study, four *Salmonella* specific isolation protocols were evaluated for their ability to detect *Salmonella* in naturally contaminated broiler grow-out house samples. The secondary enrichment (TR) protocol provided the highest *Salmonella* isolation rate when using either BG or XLT4 selective agar plates. The lowest isolation rate on either agar plate was found when using DB. The TT and RV methods on either BG or XLT4 plates had similar isolation rates but did not have a high degree of agreement (did not find the same samples positive or negative). Therefore, of the four protocols evaluated in this study, the TR protocol appears to be the method of choice when conducting a *Salmonella* risk assessment in broiler grow-out houses analyzing litter or drag swab samples.

*Salmonella* isolation methodology has been evaluated in many studies (Knox, et al., 1942;Vassiliadis, et al., 1974;Vassiliadis, et al., 1976;Vassiliadis, et al., 1978a;Cox, et al., 1982;Davies and Wray, 1994;Peplow, et al., 1999). Some research has focused on development of rapid methodologies, such as polymerase chain reaction (Huang, et al., 1999;Peplow, et al., 1999), whereas others have concentrated on improvements to conventional methods (Davies and Wray, 1994;Read, et al., 1994a;Hammack, et al., 1998). Regardless of the methodology of

choice, the physical and/or chemical composition of the sample matrix has been shown to affect the isolation ability of a protocol; therefore, the methodology must be evaluated prior to selection and used with a particular sample matrix (Skjerve and Olsvik, 1991;Davies, et al., 2000).

In this study, the immunomagnetic separation technique employed did not provide an advantage in the isolation of *Salmonella*, contrary to previously published work (Cudjoe, et al., 1994b;Cudjoe and Krona, 1997;Hsih and Tsen, 2001). Reasons for the DB protocol's failure may be attributed to the composition of the sample matrix (inclusion of inhibitory substances and/or physical composition) and the presence of low concentrations of *Salmonella* cells (Skjerve and Olsvik, 1991;Davies, et al., 2000). Previously, cheese cloth has been used to remove such inhibitory components when evaluating IMS in carcass rinse samples (Wang and Slavik, 1999). In the current study, no sample filtration techniques were used because they can potentially remove *Salmonella* that may be present within the sample and were not recommended in the manufacturer's guidelines provided with product.

For the TT and RV protocols, no significant differences were found between them in either of the matrices, litter and drag swabs, evaluated. However, one very interesting and significant finding was the kappa

analysis indicated that these methods, although having no difference in isolation frequencies, do not agree on the status of the same samples. In other words, these two methods did not always identify the same sample as positive or negative. This variation may be ascribed to the possible variations of *Salmonella* serotypes present in the various samples (Vassiliadis, et al., 1974). Nonetheless, these findings do highlight the importance of assessing the agreement between tests rather than simply comparing isolation rates when evaluating test methods.

The findings above could have important implications in conducting an on-farm risk assessment of *Salmonella* in broiler grow-out houses. If using one of these commonly used microbiological isolation methods under the described conditions of this study, it is possible that the risk assessment will not be valid and potential risk management decisions based on the assessment could be erroneous.

The most likely scenarios would involve false-negative results. The decreased sensitivity, not detecting *Salmonella* when present, of either TT or RV compared to that of TR results in an increased false-negative rate, which, under an on-farm HACCP type program, would lead to no action when a corrective action should be employed. The lower sensitivity of these protocols can also result in missing an important association between *Salmonella* and any specific risk factors. Attempts to evaluate a

production system for potential risk factors affecting the presence or absence of *Salmonella* may be further complicated when using either of these lower sensitivity protocols.

The lack of agreement between TT and RV indicates that these protocols are potentially identifying different *Salmonella* subpopulations. Therefore, different risk factors might be identified depending on the protocol used. However, using TR appears to help identify the most complete set of risk factors associated with *Salmonella*. The use of TR under the described conditions of this study provided the highest isolation rate and therefore a low false-negative rate. Similar results were previously demonstrated in meat products (Vassiliadis, et al., 1976). The TR protocol also provided for a higher isolation rate for both the selective agar plates used.

The comparison between the two selective agar plates employed revealed that the plates have a high sensitivity and agreement when TR protocol is used; however, this was not true when using any of the other three protocols. For both TT and RV, the kappa values were 0.42 and 0.46, respectively, which indicates that when using either of these broths, the plates only had moderate agreement. Similar results were seen when the comparisons were made in drag swab samples.

It is important to note the differences in kappa values between the protocols, not only within a sample type but also across sample types. When looking at the kappa values in both litter and drag swab samples for the same broth, the kappa values vary. For the TT and RV broths, the kappa values are higher in drag swab samples than in the litter samples, indicating that the plates have higher agreement when using either of these broths in drag swab samples as opposed to litter samples. This reiterates the point made earlier that the sample matrix can affect the isolation ability of a protocol. Although for the secondary enrichment protocol (TR), the kappa values, assessing agreement between BG and XLT4 plates, are consistently higher than the other methods for both litter and drag swab samples.

There is an increase in discussions that to best control foodborne pathogens, the next step is to implement a control system on the farm (Sanchez, et al., 2002;Luedtke, et al., 2003). While it is beyond the scope of this paper to debate the merit of such an action, our findings could have significant bearing on the process, and especially when conducting an on-farm risk assessment of foodborne pathogens. This study has demonstrated that when attempting to conduct an on-farm risk assessment, the sample analysis should include an evaluation of not only the microbiological method to be used but also the sample

composition itself when using a specific method. It has also been shown that tetrathionate when used with Rappaport-Vassiliadis broth as a secondary enrichment detected more *Salmonella* when testing samples obtained from broiler grow-out houses. Although this protocol required more time than either of the broths used alone, the increase (or the decreased false-negative rate) in the number of *Salmonella* positive samples provides for a more accurate risk assessment for this foodborne pathogen in broiler chicken grow-out houses.

# CHAPTER V

# EVALUATION OF SAMPLING METHODS OVER SEVEN GROWOUT HOUSES


## ABSTRACT

Determining the *Salmonella* status of broiler houses not only requires the use of validated microbiological methods, it also requires the use of sampling methods that provide the highest sensitivity. In this study, two methods for sampling broiler houses were compared for their ability to accurately assess the *Salmonella* status of each house. Seven broiler grow-out houses were sampled for nine consecutive weeks via drag swabbing and litter samples. Both sample types were compared using chi-square and kappa analysis. The chi-square test determined no significant difference ($p=0.2597$) in the isolation frequencies for either method for all samples and weeks. The kappa analysis, however, revealed that the two sampling strategies had only slight agreement in determining which houses were positive. There were 27 house/week combinations that the two strategies did not agree on the *Salmonella* status. Of these 27 house/week combinations, 15 were found to be

positive via drag swabbing and negative by litter sampling. There were also 12 combinations determined to be positive via litter sampling that were found to be negative by drag swabbing. The results here demonstrate that not only do the microbiological methods need to be evaluated, but also the sampling methods should be evaluated prior to conducting a risk assessment in animal production environments.

## INTRODUCTION

The promulgation of new food safety regulations in the late nineties significantly impacted the manner by which foods of animal origin are produced, processed, and inspected in the U.S. These new regulations dictate that the industry is now, by definition, legally responsible for determining foodborne hazards, including microbes, "before, during and after entrance into the establishment" (USDA/FSIS, 1996). The effort to address the central issues in animal production food safety has prompted the expansion of the knowledge base concerning the particular organisms involved and how they function in both the production and processing environments. Specifically, the commercial poultry industry was challenged with addressing the presence of *Salmonella* throughout their operations.

The introduction of *Salmonella* performance standards in the red meat and poultry industry by FSIS prompted the development of new

rapid methods of pathogen isolation and identification (USDA/FSIS, 1996). The incorporation of a rapid diagnostic test to determine the *Salmonella* status of a particular broiler flock may potentially aid the poultry industry by allowing for scheduling of known positive flocks at the end of a processing day to help reduce the chance of cross contamination to *Salmonella* negative flocks (Long, et al., 1980). However, knowledge of the *Salmonella* status of a flock requires sampling the flock prior to harvest.

In order to assess the *Salmonella* status of broiler chicken houses, various sample types have been collected. There are a number of different methods that are utilized when trying to determine if *Salmonella* is present in a broiler flock that range from environmental to direct bird sampling. Several sampling methods that have been shown to be effective include cloacal swabs or fecal samples (Higgins, et al., 1981), litter sampling (Davies and Wray, 1996;Sasipreeyajan, et al., 1996;Limawongpranee, et al., 1999a),and boot covers (Caldwell, et al., 1998). Another means of sampling broiler houses, as described initially by Kingston (1981), is the drag swab method. This method has since become widely used in sampling houses (Mallinson, et al., 1989;Opengart, et al., 1991;Caldwell, et al., 1994). Prior to 1981, it was

thought that litter sampling offered the most appropriate method (Olesiuk, et al., 1969;Long, et al., 1980;Sasipreeyajan, et al., 1996).

Although litter was proven to be an effective means of sampling broiler houses, Kingston's (1981) drag swab method was determined to be simple and less laborious. The author reported that drag swabbing poultry houses with sterile cotton gauze tied to a kite string was equally as effective as sampling via litter. He demonstrated that when sampling thirteen broiler grow-out houses, his drag swabbing method detected seven to be positive where as the litter sampling method only identified five as positive. It was also demonstrated that cecal swabs taken and cultured agreed with the drag swabbing method. Kingston (1981) reported on the ease and simplicity of this method, suggesting that drag swabs samples could offer an alternative sampling regime when used in broiler houses.

When drag swabbing became the gold standard for sampling, other researchers evaluated the method and found this method to be effective. Mallinson *et al.* (1989) combined drag swabbing with a *Salmonella* antigen capturing method. He reported that drag swabbing can be effective when four swabs are used per house. This was confirmed by Caldwell *et al.* (1994) who suggested more drag swabs could provide a better assessment of the *Salmonella* status of a particular house.

Opengart *et al.* (1991) used drag swabbing in turkey houses and suggested that this method was simpler than litter sampling since turkeys shed *Salmonella* periodically.

For a number of years, sampling broiler houses has been primarily done using the previously discussed drag swabbing method. Therefore, many studies were conducted to evaluate the most appropriate pre-moistening medium for the drag swabs. Kingston (1981) performed this step using buffered peptone water; however, other reports offered that other media are more effective (Opara, et al., 1992;Opara, et al., 1994;Byrd, et al., 1997;Rolfe, et al., 2000).

In order to allow for effective sampling, pre-moistening the cotton gauze with a sterile medium seemed necessary. Double strength skim milk (2xSM) has been included in evaluation of different media (Opara, et al., 1992;Opara, et al., 1994;Byrd, et al., 1997;Rolfe, et al., 2000), although disagreement exists. Opara (1992) evaluated 2xSM with that of buffered peptone water, modified Cary Blair transport broth, and lactose broth. In this study, 2xSM provided the highest *Salmonella* recovery rate. This has been concurred by Byrd *et al.* (1997) and again by Opara *et al.* (1994) . However, Rolfe *et al.* (2000) determined that 2xSM was an effective medium, but chicken broth was better.

The study presented here was designed to evaluate the effectiveness of two *Salmonella* sampling methods for use in broiler growout houses. The two methods, litter sampling and drag swabs, were chosen based on relative ease of sample collection and processing. Samples were analyzed for *Salmonella* based on the method previously described (Rybolt, et al., 2004;Rybolt, et al., 2005).

## MATERIALS AND METHODS

### Experimental Design

Seven individual broiler grow-out houses managed by a single production company were selected for this study. At the initial time of sampling, all houses were empty and being prepared for new flocks. The houses were curtain-sided and had conventional tunnel ventilation with dirt base floors. There were no other farm animals present; however, the presence of feral animals was evident (tracks and fecal droppings) around the houses. Pine shavings, which had not been changed from the previous flock, were used for floor litter in the houses. Four drag swabs and two pooled litter samples were collected in each house for 9 consecutive weeks. This gave a total of 252 drag swab samples and 126 litter samples for use in this method comparison.

Sampling

*Drag Swabs*. Drag-swab assemblies were prepared prior to use in the poultry houses. Each swab was constructed with 4 x 4 – inch (10.2 cm x 10.2 cm) cotton gauze[20] tied to 6-ft (182.9 cm) cotton-polyester twine[21]. The assemblies were sterilized with steam and aseptically transferred to sterile WhirlPak ® bags containing 20-mL sterile double strength skim milk for a pre-soaking medium. The skim milk was prepared according to the manufacturer's directions[22], except the concentration of powder to water was doubled (from 45.36 g per 500-mL to 90.72 g per 500-mL). In the houses, each swab was removed from the bag and returned after sampling. Four swabs were used to drag the entire length of each house, one up and down the north side, two up and down the middle avoiding crossovers, and one up and down the south side. The swabs were returned to the WhirlPak® bags and transported back to the laboratory on wet ice for further processing. In the laboratory, 100-mL sterile Butterfield's solution was aseptically added to each swab. Samples were incubated overnight at 42°C.

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[20] Abco Dealers, Inc., Nashville, TN

[21] The Lehigh Group, Macungie, PA

[22] Wal-Mart Stores, Inc., Bentonville, AR

*Litter.* From each house, two litter samples were collected weekly. The samples were pooled from six different sites (three sites per pooled sample). Litter was placed in WhirlPak ® bags, stored on wet ice, and shipped back to the laboratory for further processing. In the laboratory, 25-g were weighed and added to 225-mL Butterfield's solution and incubated overnight at 42°C.

Isolation and Enrichment

Following the method described by Rybolt *et al.* (2004), samples were incubated overnight in Butterfield's solution at 42°C. A one-mL aliquot was transferred to tetrathionate broth (TT) and incubated for 48 hrs at 42°C before transferring a 0.1-mL aliquot to 9.9 mL Rappaport-Vassiliadis R10 broth (RV) for secondary enrichment. RV tubes were incubated overnight at 42°C before plating.

After incubation, a loop-full of the RV samples was streaked onto xylose-lysine tergitol 4 (XLT4) plates, which were incubated overnight at 37°C. Samples were considered positive if any suspect *Salmonella* colonies, determined by lactose-negative, hydrogen sulfide production, and morphological appearance, were present. Suspect colonies were transferred to both triple sugar iron agar (TSI) and lysine iron agar (LIA) for biochemical confirmation. For further confirmation, samples

providing positive reactions via both TSI and LIA were confirmed serologically using anti-*Salmonella* Poly A-I and Vi serum.

Statistical Analysis

The PROC FREQ procedure of SAS[23] was used to generate a chi-square test statistic to determine if there was an association between the two sampling strategies used. Also, the kappa coefficient of agreement was generated to determine the degree of agreement between the two methods, and interpretations followed that of Landis and Koch (1977). Briefly, a kappa value of 0.0 or less is considered to be poor agreement, and kappa values above 0.81 indicate almost perfect agreement. For the values between 0.0 and 0.81, interpretations are slight, fair, moderate and substantial for 0.00-0.20, 0.21-0.40, 0.41-0.60, and 0.61-0.80, respectively.

## RESULTS

Seven broiler grow-out houses were monitored via drag swab and litter samples for nine consecutive weeks (from day of chick placement until after birds were harvested). Four drag swab samples and two

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[23] SAS Institute, Inc., Cary, NC

pooled litter samples (from three sites each) were collected in each house. A house was determined to be positive if *Salmonella sp.* was isolated from any one of the samples. For both sampling strategies, if any one of the drag swab samples or one of the litter samples was positive for *Salmonella sp.*, the house was considered to be positive by the respective sampling strategy. The houses' status as determined by each sampling strategy was compared to determine which strategy was most effective for determining house status. Tables 5.1 and 5.2 demonstrate the results of both sampling strategies by house and week.

Descriptive

*Drag swabs.* During the week of bird placement, all seven houses under evaluation tested positive by at least one drag swab sample. On the following week, again, all houses were positive. Subsequent weeks showed a slight drop in the number of houses positive via drag swab samples. On subsequent sampling weeks, the *Salmonella*-status of each house varied. On week 7, all houses were negative for *Salmonella* via drag swab samples; however on the last week of sampling (week 8), two of the seven houses were again positive.

*Litter samples.* Unlike the drag swab samples the litter sampling results were more varied. Only two houses were positive via the litter

samples on the first week. The following week, all but one house was positive. One week 3, all houses tested positive for *Salmonella* via litter sampling. During week 8, all houses tested negative for *Salmonella* via litter samples.

Comparisons

Using chi-square analysis, there was no overall significant difference between the two sampling strategies. Of the 63 possible events (seven houses sampled for nine weeks), the drag swabs identified 55.6% of the houses as positive, and the litter samples identified 50.8% as positive. The comparison yielded a chi-square value of 0.2869 ($p=0.5922$). However, when making the comparison within each week, there was a significant difference between the two methods the first week of sampling (Figure 5.1). On week 0, only 2 (28.6%) of the houses were positive via litter samples and all seven (100%) of the houses were positive by drag swabbing, therefore, yielding a significant difference ($p=0.0053$). There was also a statistically significant ($p=0.0507$) difference on week 3 between the two sampling methods.

Table 5.1. Drag Swab results for each house and each week.

| Week | 9 | 10 | 11 | 12 | 13 | 14 | 15 | Total |
|---|---|---|---|---|---|---|---|---|
| 0 | 2/4 (50) | 1/4 (25) | 4/4 (100) | 3/4 (75) | 1/4 (25) | 3/4 (75) | 2/4 (50) | 16/28 (57) |
| 1 | 1/4 (25) | 2/4 (50) | ¾ (75) | 1/4 (25) | 2/4 (50) | 2/4 (50) | 2/4 (50) | 13/28 (46) |
| 2 | 1/4 (25) | 4/4 (100) | 0/4 (0) | 0/4 (0) | 1/4 (25) | 2/4 (50) | 1/4 (25) | 9/28 (32) |
| 3 | 2/4 (50) | 3/4 (75) | ¼ (25) | 0/4 (0) | 0/4 (0) | 4/4 (100) | 0/4 (0) | 10/28 (36) |
| 4 | 1/4 (25) | 2/4 (50) | 2/4 (50) | 0/4 (0) | 0/4 (0) | 0/4 (0) | 0/4 (0) | 5/28 (18) |
| 5 | 1/4 (25) | 2/4 (50) | 2/4 (50) | 0/4 (0) | 0/4 (0) | 3/4 (75) | 0/4 (0) | 8/28 (29) |
| 6 | 1/4 (25) | 0/4 (0) | 0/4 (0) | 0/4 (0) | 0/4 (0) | 1/4 (25) | 1/4 (25) | 3/28 (11) |
| 7 | 0/4 (0) | 0/4 (0) | 0/4 (0) | 0/4 (0) | 0/4 (0) | 0/4 (0) | 0/4 (0) | 0/28 (0) |
| 8 | 0/4 (0) | 0/4 (0) | ¼ (25) | 0/4 (0) | 0/4 (0) | 0/4 (0) | 2/4 (50) | 3/28 (11) |
| Total | 9/36 (25) | 14/36 (39) | 13/36 (36) | 4/36 (11) | 4/36 (11) | 15/36 (42) | 8/36 (22) | 67/252 (27) |

House*

* Number of samples positive/number of samples (%)

Table 5.2. Litter sampling results for each house and each week.

| Week | 9 | 10 | 11 | 12 | 13 | 14 | 15 | Total |
|---|---|---|---|---|---|---|---|---|
| 0 | 0/2 (0) | 0/2 (0) | ½ (50) | 2/2 (100) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 3/14 (21) |
| 1 | 1/2 (50) | 2/2 (100) | 2/2 (100) | 1/2 (50) | 0/2 (0) | 2/2 (100) | 2/2 (100) | 10/14 (71) |
| 2 | 0/2 (0) | 2/2 (100) | 2/2 (100) | 2/2 (100) | 0/2 (0) | 2/2 (100) | 2/2 (100) | 10/14 (71) |
| 3 | 1/2 (50) | 2/2 (100) | 2/2 (100) | 1/2 (50) | 1/2 (50) | 2/2 (100) | 2/2 (100) | 11/14 (79) |
| 4 | 0/2 (0) | 2/2 (100) | 2/2 (100) | 2/2 (100) | 0/2 (0) | 1/2 (50) | 0/2 (0) | 7/14 (50) |
| 5 | 0/2 (0) | 1/2 (50) | 2/2 (100) | 1/2 (50) | 0/2 (0) | 2/2 (100) | 2/2 (100) | 8/14 (57) |
| 6 | 0/2 (0) | 1/2 (50) | 0/2 (0) | 1/2 (50) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 2/14 (14) |
| 7 | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 1/2 (50) | 1/14 (7) |
| 8 | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/14 (0) |
| Total House* | 2/18 (11) | 10/18 (56) | 11/18 (61) | 10/18 (56) | 1/18 (3) | 9/18 (50) | 9/18 (50) | 52/126 (41) |

* Number of samples positive/number of samples (%)

When treating each sampling week and house combination as a separate event, the sampling methods have very low agreement when determining the house *Salmonella* status. There were a total of 63 house/week combinations evaluated by both sampling methods. Of the 63 possible events, the two sampling methods only had 36 concordant pairs (those events that the strategies agreed on the status) and therefore 27 discordant pairs (those events that the strategies disagreed on the status) (Table 5.3). Using kappa analysis to determine the degree of agreement, the two sampling strategies evaluated had a kappa of 0.1413, which is described as slight agreement.

## DISCUSSION

Evaluation of the sampling methods used to determine the *Salmonella* status of poultry houses is necessary in order to fully characterize the risk this organism poses throughout the production continuum. In this study, the drag swab method had relatively consistent results, when evaluating a house based on all four swabs. The litter samples had just the opposite results. This method's results varied over the entire study period. There were 52 of 126 (41%) individual litter samples found to be *Salmonella*-positive, and 67 of 252 drag swab samples positive.

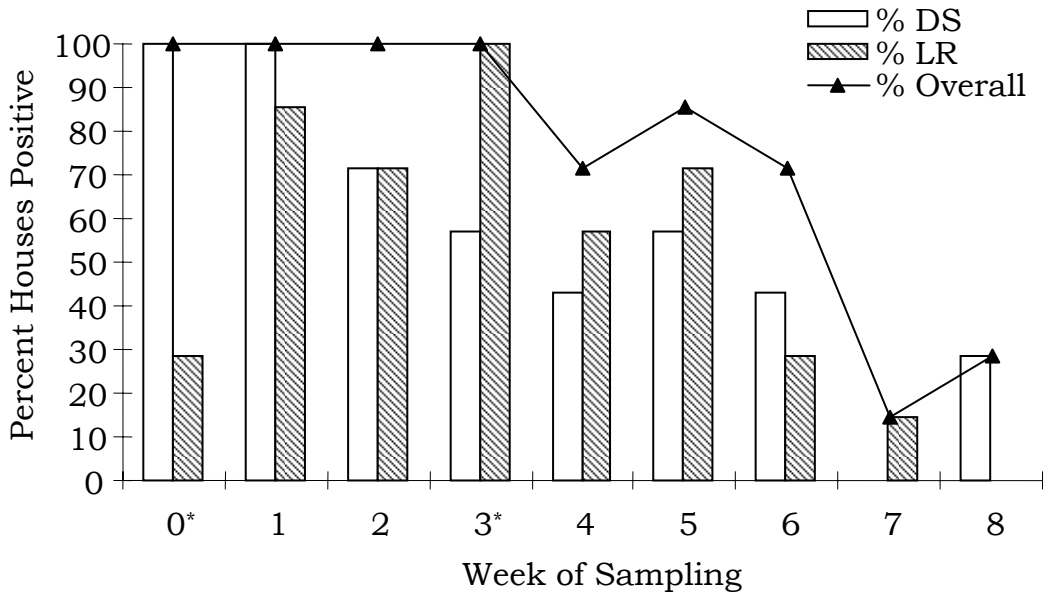


Figure 5.1. Percent houses determined to be positive by either DS or LR samples[1].

[1]Overall % includes houses determined positive by either protocol.
*Statistically significant difference was found between the two sampling strategies in week 0 (p=0.0053) and week 3 (p=0.0507).

Table 5.3. Comparison between drag swab sampling and litter sampling for determining the *Salmonella* status of a broiler house.

| | | Drag Swabs | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| Litter Samples | Positive | 20 | 12 | 32 |
| | Negative | 15 | 16 | 31 |
| | Total | 35 | 28 | 63 |

Figure 5.1 displays the overall results when determining a houses status based on the outcome of either sampling method. When

evaluating the *Salmonella*-status of these broiler houses, a house was more likely to test positive if both sampling methods were used. On week 0, if sampling had only included litter, there would have been 5 houses that were classified as negative, although they were positive when the drag swabs were used. Conversely on week 3, if only drag swabs were taken, three houses would have been misclassified as negative. On the other weeks of sampling, only 1 or 2 houses would have been missed. Of most importance is the variation between the two sampling methods used for determining the *Salmonella*-status of each house.

Both the variation between each of the sampling strategies' results and the lack of consistency of each method calls to question any assessment based only on one of these methods. When evaluating the status of a particular house or conducting a risk assessment for *Salmonella* within the broiler production continuum, it is imperative that not only are the laboratory methods compared (Rybolt, et al., 2004;Rybolt, et al., 2005), but also that the sampling strategy is properly analyzed and characterized. Proper risk analysis is only as good as the methods utilized to conduct the assessment. An establishment attempting to use strategic process scheduling (Long, et al., 1980) as a means to prevent cross contamination of *Salmonella* from one flock to

another in the processing plant may loose control of their system if the status of the house is not properly determined.

# CHAPTER VI

# THE EFFICACY OF SECONDARY ENRICHMENT FOR ISOLATING AND DETECTING *SALMONELLA* IN BROILER FLOCKS


## ABSTRACT

Methods of isolating and identifying *Salmonella* from poultry production samples have been studied for many years. Most studies have focused on the use of specific selective enrichment broths where others have concentrated on the specific parameters for using such broths. To ensure isolation, some authors have described a protocol using delayed incubation time along with the use of a secondary enrichment, which has been shown to dramatically increase isolation rates. In this study, a method that uses secondary enrichment with a slightly extended incubation has proven to be as effective as the traditional delayed secondary enrichments (DSE). When comparing the modified secondary enrichment method (TR) to that of the DSE in tray pads, gastrointestinal tracts, drag swab, litter, ceca, crops, whole carcass rinses, pre-chill rinses and post-chill rinses, it was found that the TR had

an overall isolation rate of 40% (282/700) and the DSE isolation rate was 42% (296/700). In the three different sampling segments (hatchery, grow-out and processing plant), the isolation rates for TR were 45.6%, 32.6%, and 44.8%, for, respectively. For the same three segments, the isolation frequency for the DSE was 52.9%, 31.1%, and 47.3%, respectively. There was no statistically significant difference between the two methods in either segment or sample type. A kappa analysis revealed substantial agreement between the two protocols overall and in all segments. The kappa coefficient generated for the sample types was interpreted as substantial agreement for all types except for litter samples during the hatchery segment ($\kappa$=0.00, p=0.0000). Overall, the agreement between the two protocols was substantial. Combined with the similar isolation frequencies, the kappa values indicate that the TR protocol, as described in this study, is a shorter, viable alternative to the delayed secondary enrichment protocols. The TR method provided at least a 5-d quicker turn around time in sample results, compared to the 10-d turn around time for the DSE protocol used here.

## INTRODUCTION

*Salmonella* remains a significant concern for poultry processing establishments. This organism has been attributed to nearly 1.3 M

human illnesses per year with poultry products serving as a possible vehicle for human salmonellosis (Mead, et al., 1999). The poultry industry, which is regulated by the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS), has recently been put under increased regulatory pressure with regards to the *Salmonella* incidence in poultry products. FSIS recently held meetings in Athens and Atlanta, GA to discuss the methods of pre-harvest and post-harvest *Salmonella* control, respectively, currently being used by the industry (FSIS Pre-Harvest Interventions Conference, Athens, GA, August 2005 and FSIS Post-Harvest Interventions Conference, Atlanta, GA, February 2006). However, before the industry can truly assess their food safety control systems and effectively understand the prevalence of the organism throughout the poultry processing continuum, an efficacious isolation method must be well characterized and validated.

Various authors have studied numerous *Salmonella* isolation methods over the years. Most of the studies reviewed have concentrated on comparing various selective enrichment broths, specifically tetrathionate, Rappaport-Vassiliadis, and selenite-cystine (Vassiliadis, et al., 1974;Vassiliadis, et al., 1976;Vassiliadis, et al., 1978a;Cox, et al., 1982;Davies and Wray, 1994;Read, et al., 1994b;Hammack, et al., 1998;Huang, et al., 1999).

Vassiliadis (1976) reported on the use of a secondary enrichment procedure, which used tetrathionate as the primary enrichment for 24 hs followed by sample transfer to RV broth for 24 hs. The author reported that the use of secondary enrichment increased the isolation rate of *Salmonella*. It was also reported that incubating the samples in TT an additional 24 hs (for a total of 48 hs) increased the *Salmonella* isolation rate. The author did not report on the inclusion of a secondary enrichment along with the use of TT for 48 hs.

The use of a second enrichment broth after an extended incubation period in the primary enrichment medium has been referred to as delayed secondary enrichment (DSE) (Pourciau and Springer, 1978). Most DSE protocols require extended incubation of primary enrichment samples from 5 to 10 days at ambient temperatures. After primary enrichment for 24 hs, samples are left at room temperature for an extended time and subsequently an aliquot is transferred to fresh selective enrichment broth and further incubated at elevated temperatures overnight. Samples are then plated onto selective agar plates. This method has been shown to be highly effective (Pourciau and Springer, 1978;Rigby and Pettit, 1980;Tate, et al., 1990;Waltman, et al., 1991;Nietfeld, et al., 1998)

The objective of this study was to compare a modified secondary enrichment protocol (TR) to that of a traditional delayed secondary enrichment protocol (DSE). The TR method, as described previously by Rybolt *et al.* (2004;2005), utilizes both TT and RV; however, as the primary selective enrichment broth, samples are incubated in TT for 48 hs before being transferred to RV, which is incubated for 24 hs.

## MATERIALS AND METHODS

### Experimental Design

To compare the two isolation methods in samples collected during normal production, two commercial broiler houses managed by a single integrator were selected for intensive sampling. The two houses were located on a single farm adjacent to each other and were tunnel ventilated with pine shaving for litter, which was not changed prior to the broiler chick placement. Samples were collected from the first day of bird placement until the birds were harvested for processing.

Immediately prior to placement, the houses were sampled via 4 drag swabs and 4 litter samples per house. As the birds were placed, 30 randomly selected chicks were humanely euthanized and placed into individual plastic bags. The tray pad from the delivery crate for the

selected bird was also collected and placed into individual plastic bags. Sampling via 4 drag swabs and 4 litter samples continued weekly until the birds were harvested for processing. On week 7, thirty broilers were randomly collected and humanely euthanized, rinsed and the ceca and crops were collected.

The flocks were again sampled at the processing plant. Thirty mature broilers were randomly collected for each house and humanely euthanized. The birds were rinsed and the crop and ceca were removed. During the processing, the birds from the individual houses were identified and thirty birds were removed from the lines and rinsed prior the antimicrobial treatment and after exiting the chiller.

Sampling

*Drag Swabs.* Drag-swabs assemblies were prepared prior to use in the poultry houses. Each swab was constructed with 4 x 4 – inch (10.2 cm x 10.2 cm) cotton gauze[24] tied to 6-ft (182.9 cm) cotton-polyester twine[25]. The assemblies were sterilized with steam and aseptically transferred to sterile WhirlPak ® bags containing 20-mL sterile double

[24] Abco Dealers, Inc., Nashville, TN

[25] The Lehigh Group, Macungie, PA

strength skim milk for a pre-soaking storage medium. The skim milk was prepared according to the manufacturer's directions[26], except the concentration of milk powder to water was doubled (from 45.36 g per 500-mL to 90.72 g per 500-mL). In the houses, each swab was removed from the bag and returned after sampling. Two swabs, one in each hand of the sample collector, were dragged down the north side of house and then returned. This was also done on the south side of the house. All swabs were stored on wet ice until further processing in the laboratory. In the laboratory, 100-mL sterile buffered peptone water (BPW) was aseptically added to each swab. Samples were incubated overnight at 42°C.

*Litter.* From each house, two litter samples were collected weekly. The samples were pooled from six different sites (three sites per pooled sample). Litter was mixed in WhirlPak ® bags, stored on wet ice until processed in the laboratory.

*Chick GI.* Day-old chicks were collected on the farm at the time of arrival. Upon euthanasia, individual birds were placed into sterile WhirlPak ® bags and stored on wet ice until processed in the laboratory.

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[26] Wal-Mart Stores, Inc., Bentonville, AR

In the laboratory, the entire gastrointestinal track of the chicks was removed aseptically and stomached with 22-mLs of buffered peptone water. The samples were incubated overnight at 42°C before undergoing the selective enrichment protocol listed below.

*Whole carcass.* Broilers were euthanized and individual birds were placed into a sterile plastic bag and mixed vigorously for 60 s with 300-mL of Butterfield's solution. The rinsate was collected, placed on ice, returned to the laboratory and mixed with 10X BPW to provide a final concentration of 1X BPW. One-mL was then transferred to 9-mL TT tubes following the protocol described below.

*Crop.* The crops were removed aseptically from euthanized mature broilers and placed into individual sterile WhirlPak® bags. Samples were stored on ice until processed in the laboratory. In the laboratory, samples were constituted with 22-mLs buffered peptone water and stomached for 1 min before incubating at 42°C for 24 hs. Each sample was then subjected to the isolation method described below.

*Ceca.* Each cecum was aseptically removed from the euthanized mature broilers and placed into individual sterile WhirlPak ® bags. Each sample was weighed and stomached with TT broth, mixed at a 1:9 wt/vol ratio. Since these samples were already placed into TT broth, they were

incubated at 42°C for 48 hs before being aliquoted into RV for secondary enrichment.

*Pre-chill.* Samples were aseptically collected from the processing plant immediately after evisceration and before any antimicrobial rinse. Broiler carcasses were removed from the line aseptically, placed into sterile bags and shaken by hand with 100-mL Butterfield's solution. The rinsate was collected, placed on ice, returned to the laboratory, mixed with 10X BPW to provide a final concentration of 1X BPW and incubated overnight at 42°C. Samples were subsequently subjected to the selective enrichment as described below.

*Post-chill.* Broiler carcasses were aseptically collected at the processing plant immediately after exiting the chiller. Carcasses were placed into sterile bags and shaken by hand with 100-mL Butterfield's solution. The rinsate was collected, placed on ice, returned to the laboratory, mixed with 10X BPW to provide a final concentration of 1X BPW and incubated overnight at 42°C. Samples were subsequently subjected to selective enrichment as described below.

Isolation and Enrichment

*Secondary Enrichment.* Following the method described by Rybolt *et al.* (2004;2005), samples were incubated overnight at 42°C. A one-mL aliquot was transferred to tetrathionate broth (TT) and incubated for 48

hs at 42°C before transferring a 0.1-mL aliquot to 9.9 mL RV for secondary enrichment. RV tubes were incubated overnight at 42°C before plating (Figure 6.3).

After incubation, a loop-full of the RV samples were streaked onto xylose-lysine tergitol 4 (XLT4) plates, which were incubated overnight at 37°C. Samples were considered positive if any suspect *Salmonella* colonies, determined by lactose-negative and morphological appearance, were present. Suspect colonies were transferred to both triple sugar iron agar (TSI) and lysine iron agar (LIA) for biochemical confirmation. For further confirmation, samples providing positive reactions via both TSI and LIA were confirmed serologically using anti-*Salmonella* Poly A-I and Vi[27] serum.

*Delayed Secondary Enrichment.* After transferring aliquots to the RV broth for the TR protocol, the original TT tubes were set aside at room temperature for an additional 5 days. After room-temperature incubation, 1-mL aliquots were transferred to fresh TT and incubated for an additional 24 hrs before plating onto XLT4 plates (Figure 6.3). All plates were incubated at 37°C and any suspect *Salmonella* colonies were

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[27] Difco Laboratories, Detroit, MI 48232

confirmed using both TSI and LIA. Those isolates that were biochemically confirmed were also subjected to the anti-*Salmonella* Poly A-I and Vi serum for further confirmation.

Statistical Analysis

The PROC FREQ procedure of SAS[28] was used to generate a chi-square test statistic to determine if there was an association between the two *Salmonella* isolation protocols used. Analysis was conducted for all samples by segment, sample type, and sample type within each segment. Also, the kappa coefficient of agreement was generated to determine the degree of agreement among the results of the two protocols, and interpretations followed that of Landis and Koch (1977). Briefly, a kappa value of 0.0 or less is considered to be poor agreement, and kappa values above 0.81 indicate almost perfect agreement. For the values between 0.0 and 0.81, interpretations are slight, fair, moderate and substantial for 0.00-0.20, 0.21-0.40, 0.41-0.60, and 0.61-0.80, respectively.

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[28] SAS Institute, Inc., Cary, NC

# RESULTS

## Frequency

Overall, there was a 45% (315/700) isolation rate when combining the results of both methods. Individually, the TR protocol had an isolation rate of 40% (282/700) regardless of sample type and the DSE method identified 296 (42%) of the 700 samples to be positive (Figure 6.1). When comparing the isolation frequencies of the two methods, regardless of sample type, there was no statistically significant ($p=0.4473$) difference found.

When comparing the sampling protocols by sampling segment, hatchery, grow-out or processing plant, there, again, was no statistically significant difference found. Figure 6.1 displays the comparison between the two isolation protocols by sampling segment. During the hatchery sampling segment, the TR and DSE protocols had a 45.6% (62/136) and 52.9% (72/136) isolation rate, respectively. In the grow-out segment, the TR had a 32.6% (86/264) isolation rate and the DSE protocol had a 31.1% (82/264) isolation rate. At the processing segment, the isolation rates for both protocols were 44.8% (134/300) for TR and 47.3% (142/300) for DSE.

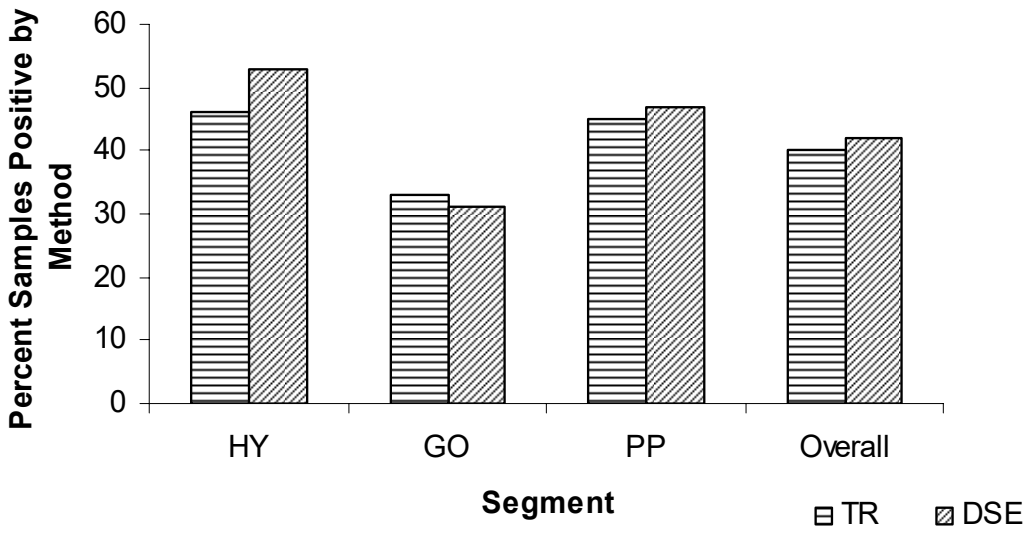


Figure 6.1. Percent samples positive by segment and overall*.

* No statistically significant differences were found between the two sampling protocols in either of the segments.

To see if the sample type affected the isolation frequency of the two protocols, a comparison was made controlling for sample type (Figure 6.2). There were nine total sample types collected, five of which (drag swabs, litter, whole carcass rinses, ceca, and crops) were collected at more than one sampling segment. No significant differences between the TR and DSE were found ($p \leq 0.05$). For the chick tray pads, the isolation rate for the TR was 78.3% (47/60) and the DSE protocol had an 83.3% (50/60) isolation rate. For the 60 gastrointestinal tracts collected, TR

found 6 (10%) to be positive and DSE found 8 (13.3%) to be positive. For the drag swab samples, there was only one sample difference between the two protocols, TR isolated 37 of the 64 samples (57.8%) as positive and DSE isolated 36 of the 64 samples (56.3%) as positive. Similar to the drag swab samples, there was only one litter sample difference between the two protocols; TR had a 52.8% (19/36) isolation rate while the DSE protocol's rate was 50.0% (18/36).

The comparison between the protocols in the ceca samples yielded an isolation rate of 10.8% (13/120) and 13.3% (16/120) for TR and DSE, respectively. For the crop samples, TR found 28 of the 120 samples (23.3%) to be positive while the DSE found 29 (24.2%) of the crop samples to be positive. For the whole carcass rinse samples, there was a 48.3% (58/120) and 54.2% (65/120) isolation rate for TR and DSE, respectively. The pre-chill rinse samples were found to be positive at a rate of 86.7% (52/60) by TR and 85.0% (51/60) by DSE. For the final sample type, 60 post-chill rinses, 22 (36.7%) and 23 (38.3%) were found to be positive by TR and DSE, respectively.

When comparing the protocols in the different sample types divided by the three different segments, the only notable comparison was in the litter samples during the hatchery segment. Here, there were a total of eight samples and the DSE protocol identified all eight (100%) to

be positive, while the TR method only found 4 (50%) to be positive for *Salmonella*. The chi-square analysis determined there was a statistically significant difference (p=0.0209) between the two protocols. For the other comparisons, there was no statistically significant difference found.

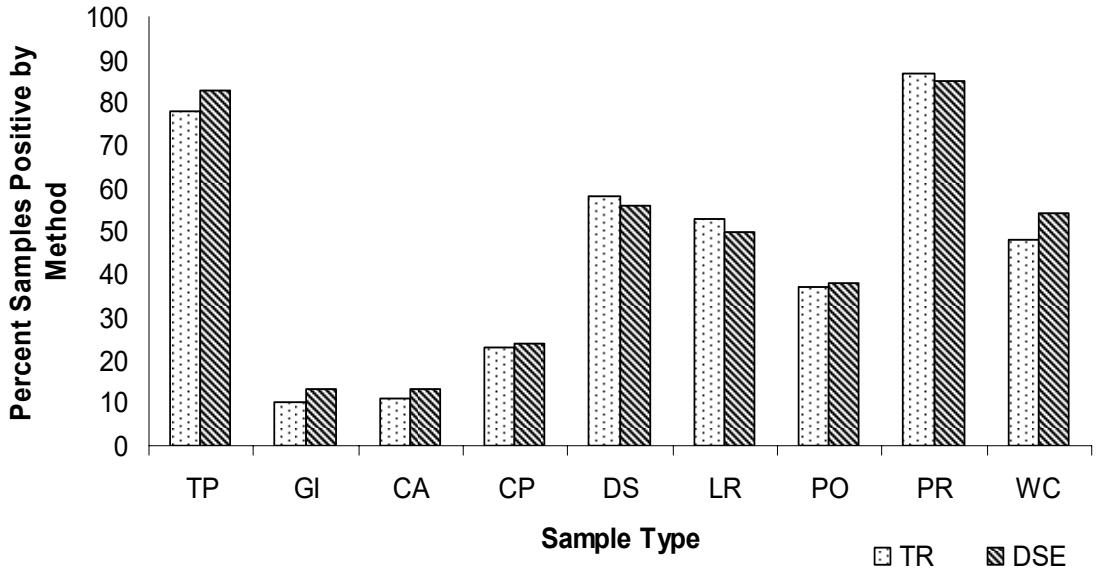


Figure 6.2. Percent samples positive by sample type*.

* No statistically significant differences were found between the two protocols in any of the sample types

Kappa

Table 6.1 presents the kappa analysis between the two protocols evaluated in this study. The kappa analysis assesses the agreement between two protocols at a time, i.e. how many samples were classified

as positive by both protocols, negative by both protocols, or positive by one protocol and negative by the other. For instance, the comparison between the two protocols using all samples demonstrates that both methods agreed that 263 (37.6%) of the 700 samples were positive for *Salmonella* and 385 (55.0%) were negative (Table 6.2). However, there were 52 (7.4%) discordant pairs (those samples that the protocols disagreed on). The analysis yielded a 0.85 ($p<0.0001$) kappa coefficient.

Table 6.1. Kappa analysis comparing TR to DSE by segment and overall.

| Segment | kappa | Pr > \|Z\| |
|---|---|---|
| PP | 0.8257 | <0.0001 |
| GO | 0.8953 | <0.0001 |
| HY | 0.7952 | <0.0001 |
| Overall | 0.8468 | <0.0001 |

Table 6.2. Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from all sample types.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 263 | 33 | 296 |
| | Negative | 19 | 385 | 404 |
| | Total | 282 | 418 | 700 |

The agreement comparison between the two protocols in the three different sampling segments has yielded significant kappa coefficients.

During the hatchery segment, there were 122 concordant pairs (those that protocols agreed on the status) and 14 discordant pairs (those that the protocols disagree on the status) (Table 6.3). This resulted in a kappa value of 0.80 ($p<0.0001$). During the grow-out segment, the kappa value was 0.90 ($p<0.0001$) with 252 concordant pairs (78 *Salmonella* positive and 174 negative) and 12 discordant pairs (Table 6.4). In the processing sampling segment, the kappa coefficient was 0.82 ($p<0.0001$). In this comparison, there were 125 samples that the protocols agreed were *Salmonella* positive and 149 agreed to be negative. There were also 26 discordant pairs (Table 6.5).

Table 6.3. Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from hatchery samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 60 | 12 | 72 |
| | Negative | 2 | 62 | 64 |
| | Total | 62 | 74 | 136 |

Table 6.4. Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from grow-out samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 78 | 4 | 82 |
| | Negative | 8 | 174 | 182 |
| | Total | 86 | 178 | 264 |

Table 6.5. Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from processing plant samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 125 | 17 | 142 |
| | Negative | 9 | 149 | 158 |
| | Total | 134 | 166 | 300 |

When evaluating the two isolation protocols in the nine different sample types, the highest agreement was found in the crop samples and the lowest agreement was found when sampling in the litter samples (Table 6.6). The kappa analysis yielded a coefficient of 0.8850 ($p<0.0001$) in the crop samples and 0.5000 ($p=0.0027$) in the litter samples. The litter was the only sample type that yielded a kappa below the substantial agreement category, based on the interpretation by Landis and Koch (1977).

For the other sample types, the kappa coefficients were interpreted as substantial agreement. The second highest agreement was found in the ceca samples, with a kappa coefficient of 0.8825 ($p<0.0001$). The drag swab samples yielded a kappa coefficient of 0.8406 ($p<0.0001$) and the whole carcass rinses' kappa coefficient was 0.8172 ($p<0.0001$). The agreement between the two protocols, when isolating *Salmonella* from the gastrointestinal tracts, was substantial with a kappa coefficient of 0.8387 ($p<0.0001$). While the agreement was not as strong with the pre-chill rinses, post-chill rinses and the chick tray pads, the kappa coefficients (0.7945, 0.7512 and 0.6250, respectively) were substantial and statistically significant ($p<0.0001$).

When evaluating the sample types in the different segments, the agreements for nearly all the sample types was again interpreted as substantial. However, for the litter samples in the hatchery segment, the kappa agreement coefficient was 0.0 since of the eight samples, the TR method only identified 4 to be positive and the DSE determined all eight samples were positive for *Salmonella*.

## DISCUSSION

Over the years, many reports have been published comparing various methods for isolating and identifying *Salmonella* from various sample types. Most of the studies reviewed have concentrated on

comparing various selective enrichment broths, specifically tetrathionate (TT), Rappaport-Vassiliadis (RV), and selenite-cystine (SC). These broths have been the focus of many studies (Vassiliadis, et al., 1974;Vassiliadis, et al., 1976;Vassiliadis, et al., 1978a;Cox, et al., 1982;Davies and Wray, 1994;Read, et al., 1994b;Hammack, et al., 1998;Huang, et al., 1999). A current review of the literature suggests that no one method has superiority over another, in all cases, and that the sensitivity and specificity of the methods depends on the sample type as well as the isolation conditions (Rybolt, et al., 2004;Rybolt, et al., 2005).

Different methods of sample processing are available for use when processing samples from broiler houses and attempting to isolate *Salmonella*. Typical practice prescribes incubation of samples overnight in a nonselective broth after which a selective broth is inoculated with the pre-enriched sample and then incubated for 24-48 hs before plating. After plating, if no suspect colonies are present, the samples are classified as negative. However, it has been shown that using a second enrichment broth after incubation in the primary broth allows for higher *Salmonella* recovery rates.

Table 6.6. Kappa coefficient for comparison of TR and DSE in each sample types within each sampling segment.

| | | | Grow-out | | Processing Plant | | Overall | |
|---|---|---|---|---|---|---|---|---|
| Type | kappa | Pr > \|Z\| | kappa | Pr > \|Z\| | kappa | Pr > \|Z\| | kappa | Pr > \|Z\| |
| TP | 0.6250 | <0.0001 | | | | | 0.6250 | <0.0001 |
| Hatchery | | | | | | | | |
| GI | 0.8387 | <0.0001 | | | | | 0.8387 | <0.0001 |
| DS | 0.7143 | <0.0001 | 0.8557 | <0.0001 | | | 0.8406 | <0.0001 |
| LR | 0.0000 | 0.0000 | 0.6500 | 0.0002 | | | 0.5000 | 0.0027 |
| CA | | | 0.9138 | <0.0001 | 0.8561 | <0.0001 | 0.8825 | <0.0001 |
| CP | | | 1.0000 | <0.0001 | 0.8276 | <0.0001 | 0.8850 | <0.0001 |
| WC | | | 0.9334 | <0.0001 | 0.7013 | <0.0001 | 0.8172 | <0.0001 |
| PR | | | | | 0.7945 | <0.0001 | 0.7945 | <0.0001 |
| PO | | | | | 0.7512 | <0.0001 | 0.7512 | <0.0001 |

The use of a second enrichment broth has been referred to as delayed secondary enrichment (DSE). Most DSE protocols require extended incubation of primary enrichment samples from 5 to 10 days at ambient temperatures. After primary enrichment for 24 hs, samples are left at room temperature for an extended time and subsequently an aliquot is transferred to fresh selective enrichment broth and further incubated at elevated temperatures overnight. Samples are then plated onto selective agar plates. This method has been shown to be highly effective.

DSE was evaluated by Pourciau and Springer (1978) who compared it to a standard method using tetrathionate (TT) as the primary enrichment broth. Incorporating DSE into the protocol increased the isolation rate from 45% with a single enrichment broth to 67% using DSE. Waltman *et al.* (1991) demonstrated that using DSE allowed for a higher isolation rate in both drag swabs and litter when using TT as both the primary and secondary enrichment broths. Similar results have been demonstrated by others (Rigby and Pettit, 1980;Tate, et al., 1990;Nietfeld, et al., 1998). Nietfeld *et al.* (1998) reported on the use of RV as the primary selective enrichment broth as opposed to TT and found similar results.

Vassiliadis (1976) compared the uses of pre-enrichment, selective enrichment, secondary selective enrichment, and various incubation times in minced meat samples. Here, TT and RV were used as selective enrichment broths and buffered peptone water (BPW) was used a pre-enrichment medium. The secondary enrichment procedure included incubation of TT broth for 24 hs followed by sample transfer into RV broth and incubation for an additional 24 hs prior to plating. It was found that pre-enrichment increased the isolation rate of *Salmonella* as did the use of 48-hour incubation for primary enrichment with TT. It was also found that using a secondary enrichment broth increased the isolation rate. However, what the author did not evaluate was the effect of using a pre-enrichment followed by a 48-hour incubation of the primary enrichment TT and the use of a secondary enrichment.

In this present study, two *Salmonella* specific isolation protocols were evaluated for their ability to detect *Salmonella* in nine different, naturally contaminated sample types collected during three segments of the broiler production process. Overall, there was no statistically significant difference in isolation frequency between the TR method and the DSE method. Likewise, the level of agreement as measured by the kappa analysis was substantial and statistically significant. The results here demonstrate that the TR method is a reliable isolation method that

can be used in place of a traditional delayed secondary enrichment protocol, thereby, saving considerable time (Figure 6.3) between sample collection and obtaining results.

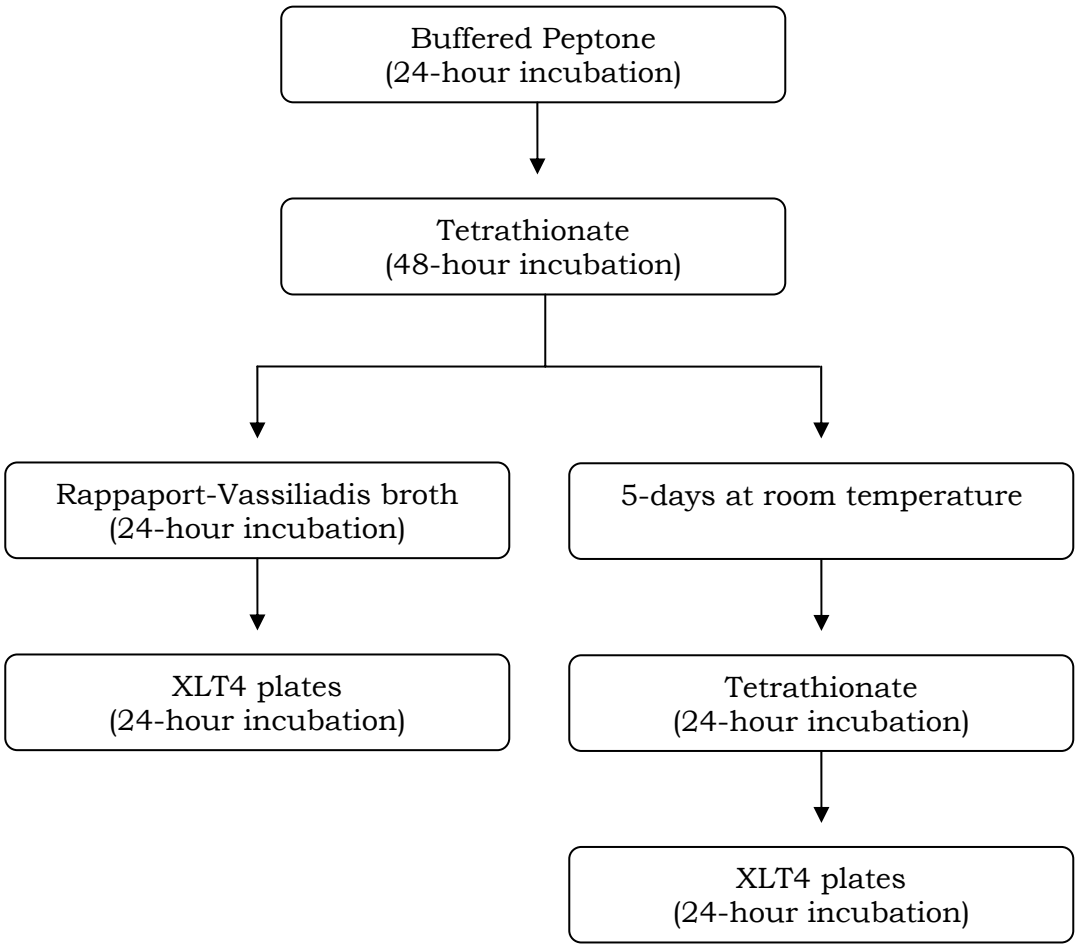


Figure 6.3. Sample processing flow chart for TR and DSE protocols.

# CHAPTER VII

# CONCLUSIONS

## SUMMARY

The purpose of this work was to more fully characterize both the sampling methods and the microbiological methods, currently used in the commercial poultry industry, to assess the *Salmonella*-status of broiler houses. This work was undertaken after complications arose during a field study designed to estimate the prevalence of *Salmonella* in the broiler houses and to evaluate the role the darkling beetle (*Alphatobius diapernius*) plays in the transmission of *Salmonella* and other foodborne pathogens between broiler flocks (Appendix A). The results presented in this work demonstrate that the use of a secondary enrichment further enhances the isolation of *Salmonella* from broiler grow-out house samples. It was also found that the various sampling strategies may need to be used simultaneously to ensure that a broiler house is accurately characterized as positive or negative for *Salmonella sp.*

The initial project compared the secondary enrichment (TR) method with that of two commonly used methods, tetrathionate (TT) and Rappaport-Vassiliadis (RV), and a rapid method, immunomagnetic separation (IMS). This work was conducted using inoculated samples to determine the lowest level of detection for each method. It was determined that in all sample matrices evaluated, the TR method was able to detect *Salmonella* at the lowest level. The levels of detection were calculated as 2.56, 1.79, 2.07, and 0.76 $\log_{10}$ cfu/mL for pure culture, litter, crop and carcass rinse samples, respectively. The results of this portion of the work demonstrated that the characterization of isolation methods should be conducted in various sample matrices before being adopted and that the TR method was able to detect *Salmonella* at very low levels in all inoculated samples tested.

In the second phase of the project, the isolation methods were compared in naturally contaminated broiler grow-out house samples. Thirteen broiler houses were sampled using both drag swabs and litter samples. The samples were evaluated for the presence of *Salmonella* using TT, RV, IMS, and TR. The IMS method was discontinued during this phase due to lack of performance and cost. The evaluation also included the use of two different *Salmonella*-specific selective plating media, brilliant green (BG) and xylose-lysine tergitol-4 (XLT4).

Overall, it was determined that the TR method had the highest isolation frequency of 94.5% on BG plates and 93.0% on XLT4 plates in drag swab samples. Similarly, TR was determined to have the highest isolation rate in the litter samples as well. It was also found that the current methods commonly used for isolating *Salmonella,* TT and RV, could provide contradictory results. While the isolation frequencies for both TT and RV were not statistically different, it was found that the two broths sometimes were identifying different samples as positive and negative. This lead to the occurrence of false-negative results, which were overcome using the TR method.

The second phase of the study not only compared the use of the four different isolation broths, but also evaluated the two selective plating media, BG and XLT4. It was determined that either plating media is effective for isolating *Salmonella*; however, using the TR method increased the isolation rate of both plates. A kappa analysis revealed a high agreement between the plating media as well.

After determining the validity of the *Salmonella* isolation protocols, the next step was to determine which broiler house sampling strategy was most appropriate to evaluate the status of individual houses. Here, litter sampling and drag swab sampling was used for comparison. It was found that overall, either sampling method is effective in determining the

status of a broiler house. However, when evaluating the sample type results within each week of sampling, variation occurred. This variation resulted from some houses being classified as negative one week by one sampling method and positive by the other sampling method the same week. It is concluded that in order to accurately classify a house's *Salmonella*-status, both methods should be used simultaneously.

In assessing the degree of agreement, the kappa coefficient resulted in a low level of agreement. There were 43% discordant pairs (those events that the strategies disagreed on the status) between these two sampling strategies. It was determined that using either drag swabbing or litter sampling alone, may also lead to falsely classifying a house as negative. Based on these finding, use of both the litter sampling and drag swabbing method will provide a better assessment of a houses status.

The final objective of this study was to evaluate the use of the TR method described herein to that of a traditional delayed secondary enrichment (DSE) method in naturally contaminated samples. The DSE method used required 10 days from the time the samples were processed until the results were obtained, whereas the TR method used only required 5 days. Not only does the TR method provide considerably less

turn-around time, it also proved to be as effective as the DSE in isolating *Salmonella* from the various samples tested.

Overall, the TR method had an isolation rate of 40% for all samples, compared to 42% of the DSE protocol. There was no statistically significant difference found between these methods. In this study, the methods were also compared in samples collected from different segments of the broiler production continuum. In each segment, again no difference was found between the isolation frequencies of the two methods. The isolation frequencies for the two methods were also similar when compared in the nine different sample types. The only notable comparison is in the litter samples collected during the hatchery segment, in which there was a significant difference in the isolation frequencies.

The analysis of agreement for these two methods was interpreted to be substantial, overall, and in each sampling segment. Overall, there were 52 (7.4%) discordant pairs. This provided a kappa value of 0.85. In each sampling segment, the kappa analysis provided a value of 0.82, 0.90, and 0.80, for processing plant, grow out and hatchery, respectively. In comparing the isolation methods in the nine different sample types, all kappa values generated were interpreted as substantial except for litter samples. The level of agreement between TR and the DSE methods when

sampling litter was 0.5, still considered moderate agreement. The lower level of agreement results from the higher number of discordant pairs in the litter samples collected during the hatchery segment.

The results of the fourth phase of this work further add to the credence of the TR method described here. Not only has the TR protocol provided for a lower level of detection (cfu/mL) when compared to the other methods in inoculated samples, it has also been proven to be superior to the other methods in field-derived samples. The TR method has also been proven to be an efficacious replacement for the traditional DSE used in this study; therefore, allows for obtaining quicker results.

## SIGNIFICANCE OF WORK

Since the promulgation of the Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems; Final Rule regulations in 1996, the U.S. red meat and poultry industries have been forced to operate under tighter standards. The new regulations prompted the industries to begin assessing their entire operations for the presence of foodborne pathogens and to implement a science-based food safety and process control program, HACCP. The rules of operation were essentially changed from a command-and-control system to an industry driven and agency verification system. Additionally, the HACCP regulations also subject the broiler chicken industry to “random”

sampling of both whole broiler carcasses and raw ground chicken for the presence of *Salmonella sp.* to allow the USDA Food safety and Inspection Service (agency) to verify that an establishment's HACCP and food safety systems are effective.

The HACCP rule established a performance standard for *Salmonella* by which the agency evaluates an establishment's food safety control program. The measure of performance for broiler operation was set at a 20% incidence on whole carcasses exiting the chiller, based on the national baseline data captured by the agency (USDA/FSIS, 1995). In the PR/HACCP rule, the agency indicated it had selected *Salmonella* as the measure of performance because:

> *…(1) it is the most common bacterial cause of foodborne illness; (2) FSIS baseline data show Salmonella colonizes a variety of mammals and birds, and occurs at frequencies which permit changes to be detected and monitored; (3) current methodologies can recover Salmonella from a variety of meat and poultry products; and (4) intervention strategies aimed at reducing fecal contamination and other sources of Salmonella on raw product should be effective against other pathogens.*

The HACCP rule not only required establishments to develop a control process within their slaughter establishments, but it also

obligated the industry to address the condition of incoming animals. In fact, the regulations established by the HACCP rule, at 9 C.F.R. § 417.2, describe a hazard analysis to include hazards before entry into the establishment. This in essence required establishments to address the presence of potential hazards, such as *Salmonella sp.*, on incoming broiler carcasses, therefore, necessitating the use of pre-harvest food safety monitoring and control practices.

Nearly a decade since the promulgation of the HACCP rule, the reduction and control of *Salmonella sp.* on broiler carcasses remains a significant concern for the agency and industry, alike. While the incidence of *Salmonella* on broiler carcasses has remained below the baseline level, the agency has recognized an increase over the years. Figure 7.1 demonstrates the *Salmonella* incidence from the regulatory samples collected by the agency and the percent of sets passed. The incidence of *Salmonella* has increased from 10.8% in 1998 to 16.3% in 2005, with an exception in 2000 when the incidence was 9.1%. The percent sets passed has fluctuated from 90.9% in 1998 to the lowest passing rate of 81.3% in 2005. Since 2000, the passing rate declined fairly rapidly with an exception in 2004, when the passing rate was 90.3%.

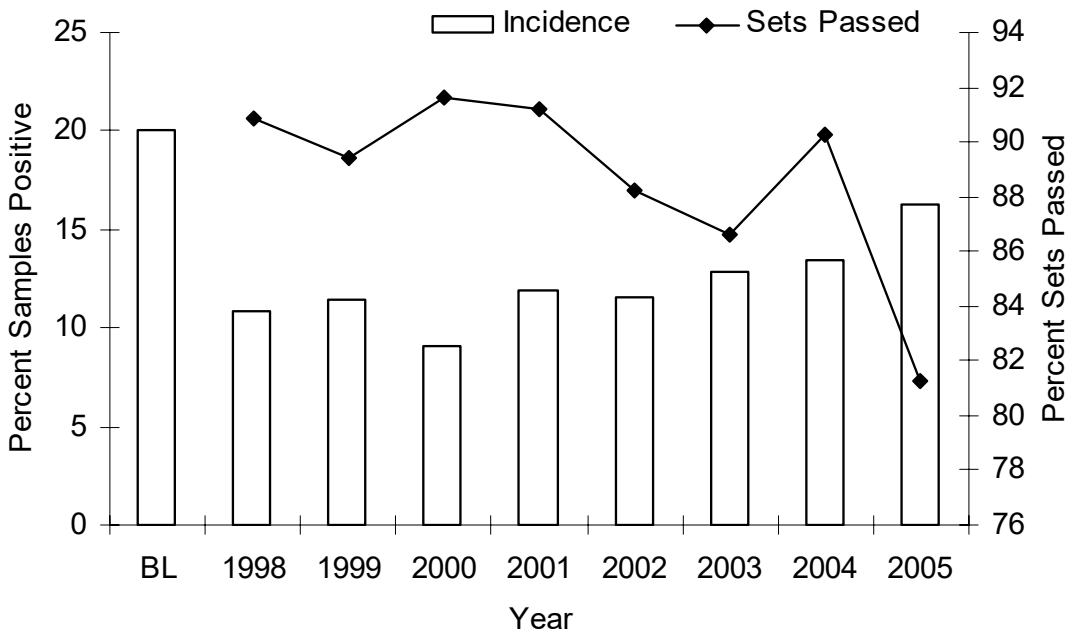


Figure 7.1 Percent positive *Salmonella* test from USDA/FSIS HACCP verification sampling (set A) and percent sets passed (1998-2005)[1].

[1]BL=baseline incidence; Sets Passed is plotted on the secondary y-axis

The increase in the incidence of *Salmonella* in the agency's regulatory samples is not the only reason for the heightened concern with *Salmonella* in the broiler industry. The agency has concerns because the overall incidence in the human population has not declined, specifically in relation to the Healthy People 2010 Objective. According to the agency's recent *Federal Register* notice (USDA/FSIS, 2006), the incidence of reported salmonellosis in the U.S. is 14.7 cases per 100,000.

The Healthy People 2010 Objective is 6.8 cases per 100,000. While there is no true attribution data to implicate poultry products as a significant source of salmonellosis, the agency has expressed its concern with particular serotypes common to the poultry industry. Therefore, it is acting to reduce the incidence on poultry products.

More specifically, the agency indicated in its notice that it will be focusing greater attention to establishments that do not maintain a consistent incidence of *Salmonella* in its regulatory samples. The agency suggested that it will begin categorizing establishments into three classifications based on the results of its two most recent sets. If an establishment is below half the performance standard, 20% for broilers, in two of its most recent sets, it will be classified as category 1. If an establishment is between half of the standard and the standard, it will be category 2. Establishment above the standard will be a category 3.

Based on its categorization, an establishment's regulatory sampling will vary. For example, the agency suggested that an establishment in category 1 should expect to have its food safety system evaluated at least once in a two year period. However, if an establishment is a category three plant, the agency indicated it will focus its resources more heavily toward those plants. As part of the agency's focus, it will perform comprehensive food safety assessments, including

primary concern with how the establishment addresses the potential occurrence of *Salmonella sp.*

During a comprehensive food safety assessment, the agency inspection force evaluated and verifies that an establishment's system design and implementation appropriately addressed potential hazards. Recent concern with *Salmonella sp.* has prompted the agency to specifically question, during the assessments, how an establishment addresses the potential for *Salmonella*. Questions from the agency included if an establishment considers the presence or absence of *Salmonella* on incoming birds and what live production programs the establishment had to address *Salmonella* pre-harvest.

Now, more than ever, broiler processing establishments need pre-harvest risk assessments for *Salmonella*. However, in order for control practices to be developed and evaluated for efficacy, more fully characterized sampling and isolation methods need be utilized. A recent report by the American Academy of Microbiology (Isaacson, et al., 2004) highlighted the need for increased understanding of microbial ecology on farm and how it relates to contamination of raw food products and ultimately public health. The report also stressed that, for this to be accomplished, risk factors associated with particular pathogens should be evaluated using sampling and detection methods that are efficacious.

While the microbiological method described in the study presented here is not rapid, it does, however, provide for increased sensitivity and specificity over the commonly used methods. Use of this method in future studies, such as pre-harvest risk assessments, will ultimately add confidence to the results of such studies.

# REFERENCES


Andrews, W. H., G. A. June, P. S. Sherrod, T. S. Hammack and R. M. Amaguana. 1995. *Salmonella*. 8th Edition FDA Bacteiological Analytical Manual.

Anonymous. *Poultry: Production and Value of Production by Year, Us*. 2005 [cited 2006 February 14]; Available from: http://www.nass.usda.gov/Charts_and_Maps/Poultry/valprdbetc.asp.

Anonymous, *Difco Manual*, in *Difco Manual*. 1998, Decton Dickinson and Company: Sparks, MD. p. 862.

Anonymous. *Economics of Foodborne Disease: Feature*. [internet] 2000 [cited 2005 February 14]; Available from: http://www.ers.usda.gov/briefing/FoodborneDisease/features.htm.

Anonymous. *Foodborne Illness Cost Calculator: Salmonella* 2003 [cited 2005 February 14]; Available from: http://www.ers.usda.gov/Data/FoodBorneIllness/salm_Intro.asp?pathogen=*Salmonella*.

Barber, D. A., P. B. Bahnson, R. Isaacson, C. J. Jones and R. M. Weigel. 2002. Distribution of *Salmonella* in Swine Production Ecosystems. J Food Prot. 65:1861-1868.

Bhargava, K. K., J. B. O'Neil, M. G. Prior and K. E. Dunkelgod. 1983. Incidence of *Salmonella* Contamination in Broiler Chickens in Saskatchewan. Can. J. Comp. Med. 47:27-32.

Braun, C., V. Kostka, E. Balks, T. Redmann and R. Helmuth. 1998. Comparative Studies of Diagnostic Bacteriological Methods for the Recovery of *Salmonella* from Faecal Samples from Flocks of Layers. Zentralbl Veterinarmed [B]. 45:245-250.

Breazeale, L. *State's Agriculture Exceeds $6 Billion*. Mississippi Agricultural News 2005 [cited 2006 February 14]; Available from: msucares.com/news/print/agnews/an05/051215all.html.

Byrd, J. A., D. E. Corrier, J. R. DeLoach and D. J. Nisbet. 1997. Comparison of Drag-Swab Environmental Protocols for the Isolation of *Salmonella* in Poultry Houses. Avian Dis. 41:709-713.

Byrd, J. A., J. R. DeLoach, D. E. Corrier, D. J. Nisbet and L. H. Stanker. 1999. Evaluation of *Salmonella* Serotype Distributions from Commercial Broiler Hatcheries and Grower Houses. Avian Dis. 43:39-47.

Byrd, J. A., B. M. Hargis, D. J. Caldwell, R. H. Bailey, K. L. Herron, J. L. McReynolds, R. L. Brewer, R. C. Anderson, K. M. Bischoff, T. R. Callaway and L. F. Kubena. 2001. Effect of Lactic Acid Administration in the Drinking Water During Preslaughter Feed Withdrawal on *Salmonella* and Campylobacter Contamination of Broilers. Poultry Sci. 80:278-283.

Byrd, J. A., B. M. Hargis, D. E. Corrier, R. L. Brewer, D. J. Caldwell, R. H. Bailey, J. L. McReynolds, K. L. Herron and L. H. Stanker. 2002. Fluorescent Marker for the Detection of Crop and Upper Gastrointestinal Leakage in Poultry Processing Plants. Poultry Sci. 81:70-74.

Caldwell, D. J., B. M. Hargis, D. E. Corrier, J. D. Williams, L. Vidal and J. R. DeLoach. 1994. Predictive Value of Multiple Drag-Swab Sampling for the Detection of *Salmonella* from Occupied or Vacant Poultry Houses. Avian Dis. 38:461-466.

Caldwell, D. J., B. M. Hargis, D. E. Corrier and J. R. DeLoach. 1998. Frequency of Isolation of *Salmonella* from Protective Foot Covers Worn in Broiler Houses as Compared to Drag-Swab Sampling. Avian Dis. 42:381-384.

Carr, L. E., E. T. Mallinson, C. R. Tate, R. G. Miller, E. Russek-Cohen, L. E. Stewart, O. O. Opara and S. W. Joseph. 1995. Prevalence of *Salmonella* in Broiler Flocks: Effect of Litter Water Activity, House Construction, and Watering Devices. Avian Dis. 39:39-44.

Cohen, N. D., D. E. Wallis, H. L. Neibergs, A. P. McElroy, E. D. McGruder, J. R. DeLoach, D. E. Corrier and B. M. Hargis. 1994.

Comparison of the Polymerase Chain Reaction Using Genus-Specific Oligonucleotide Primers and Microbiologic Culture for the Detection of *Salmonella* in Drag-Swabs from Poultry Houses. Poultry Sci. 73:1276-1281.

Corrier, D. E., B. M. Hargis, A. Hinton, D. Lindsey, D. J. Caldwell, J. Manning and J. R. Deloach. 1991. Effect of Cecal Colonization Resistance of Layer Chicks to Invasive *Salmonella Enteritidis*. Avian Dis. 35:337-334.

Corrier, D. E., J. A. Byrd, B. M. Hargis, M. E. Hume, R. H. Bailey and L. H. Stanker. 1999a. Presence of *Salmonella* in the Crop and Ceca of Broiler Chickens before and after Preslaughter Feed Withdrawal. Poultry Sci. 78:45-49.

Corrier, D. E., J. A. Byrd, B. M. Hargis, M. E. Hume, R. H. Bailey and L. H. Stanker. 1999b. Survival of *Salmonella* in the Crop Contents of Market-Age Broilers During Feed Withdrawal. Avian Dis. 43:453-460.

Cox, N. A., J. S. Bailey and J. E. Thomson. 1982. Effect of Various Media and Incubation Conditions on Recovery of Inoculated *Salmonella* from Poultry Feed. Poultry Sci. 61:1314-1321.

Cox, N. A., J. S. Bailey, J. M. Mauldin and L. C. Blakenship. 1990. Presence and Impact of *Salmonella* Contamination in Commercial Broiler Hatcheries. Poultry Sci. 1606-1609.

Cox, N. A., J. S. Bailey, J. M. Mauldin, L. C. Blakenship and J. L. Wilson. 1991. Extent of *Salmonellae* Contamination in Breeder Hatcheries. Poultry Sci. 416-418.

Cox, N. A., J. S. Bailey and M. E. Berrang. 1997. Diminishing Incidence and Level of *Salmonellae* in Commercial Broiler Hatcheries. J. Appl. Poultry Res. 6:90-93.

Cudjoe, K. S., R. Krona, B. Gron and E. Olsen. 1994a. Use of Ferrous Sulphate and Immunomagnetic Separation to Recover *Salmonella Enteritidis* from Raw Eggs. Inter. J. Food Microbiol. 23:149-158.

Cudjoe, K. S., R. Krona and E. Olsen. 1994b. Ims: A New Selective Enrichment Technique for Detection of *Salmonella* in Foods. Inter. J. Food Microbiol. 23:159-165.

Cudjoe, K. S. and R. Krona. 1997. Detection of *Salmonella* from Raw Food Samples Using Dynabeads Anti-*Salmonella* and a Conventional Reference Method. Inter. J. Food Microbiol. 37:55-62.

Davies, P. R., P. K. Turkson, J. A. Funk, M. A. Nichols, S. R. Ladely and P. J. Fedorka-Cray. 2000. Comparison of Methods for Isolating *Salmonella* Bacteria from Faeces of Naturally Infected Pigs. J. Appl. Microbiol. 89:169-177.

Davies, R. H. and C. Wray. 1994. Evaluation of a Rapid Cultural Methods for Identification of *Salmonella*s in Naturally Contaminated Veterinary Samples. J. Appl. Bacteriol. 77:237-241.

Davies, R. H. and C. Wray. 1996. Persistence of *Salmonella* Enteritidis in Poultry Units and Poultry Food. Br. Poultry Sci. 37:589-596.

Dougherty, T. J. 1976. A Study of *Salmonella* Contamination in Broiler Flocks. Poultry Sci. 55:1811-1815.

Dusch, H. and M. Altwegg. 1995. Evaluation of Five New Plating Media for Isolation of *Salmonella* Species. J. Clin. Microbiol. 33:802-804.

Edel, W. and E. H. Kampelmacher. 1973. Comparative Studies on the Isolation of Sublethally Injured *Salmonellae* in Nine European Laboratories. Bull. World Health Organiz. 48:167-174.

Edel, W. 1994. *Salmonella* Enteritidis Eradication Programme in Poultry Breeder Flocks in the Netherlands. Inter. J. Food Microbiol. 21:171-178.

Fallik, D., *Ibp to Buy Hudson Plant Closed after Beef Recall.*, in *Washington Post.* 1997: Washington, D.C. p. E02.

Fanelli, M. J., W. W. Sadler, C. E. Franti and J. R. Brownell. 1971. Localization of *Salmonellae* within the Intestinal Tract of Chickens. Avian Dis. 15:366-375.

Fierens, H. and A. Huyghebaert. 1996. Screening of *Salmonella* in Naturally Contaminated Feeds with Rapid Methods. Inter. J. Food Microbiol. 31:301-309.

Greenwood, D. E. and B. Swaminathan. 1981. Rapid Detection of *Salmonellae* in Mechanically Deboned Poultry Meat. Poultry Sci. 60:2253-2257.

Hammack, T. S., R. M. Amaguana, G. A. June and P. S. Sherrod. 1998. Relative Effectiveness of Selenite Cystine Broth, Tetrathionate Broth, and Rappaport-Vassiliadis Medium for the Recovery of *Salmonella* Spp. From Foods with a Low Microbial Load. J. Food Prot. 62:16-21.

Hargis, B. M., D. J. Caldwell, R. L. Brewer, D. E. Corrier and J. R. Deloach. 1995. Evaluation of the Chicken Crop as a Source of *Salmonella* Contamination for Broiler Carcasses. Poultry Sci. 1548-1552.

Hargis, B. M., D. J. Caldwell and J. A. Byrd, *Microbiological Pathogens I: Live Poultry Considerations.*, in *Poultry Meat Processing*, A. R. Sams, Editor. 2000, CRC Press: Boca Raton, FL. p. 344.

Higgins, R., R. Malo, E. Rene-Roberge and R. Gauthier. 1981. Studies on the Dissemination of *Salmonella* in Nine Broiler-Chicken Flocks. Avian Dis. 26:26-33.

Hoover, N. J., P. B. Kenney, J. D. Amick and W. A. Hypes. 1997. Preharvest Sources of *Salmonella* Colonization in Turkey Production. Poultry Sci. 1232-1238.

Hsih, H. Y. and H. Y. Tsen. 2001. Combination of Immunomagnetic Separation and Polymerase Chain Reaction for the Simultaneous Detection of *Listeria Monocytogenes* and *Salmonella Spp*. In Food Samples. J. Food Prot. 64:1744-1750.

Huang, H., M. M. Garcia, B. W. Brooks, K. Nielsen and S.-P. Ng. 1999. Evaluation of Culture Enrichment Procedures for Use with *Salmonella* Detection Immunoassay. Inter. J. Food Microbiol. 51:85-94.

Isaacson, R. E., M. Torrence and M. R. Buckley. 2004. Preharvest Food Safety and Security. American Academy of Microbiology. Washington, DC

Jacobs-Reitsma, W. F., N. M. Bolder and R. W. A. W. Mulder. 1994. Cecal Carriage of Campylobacter and *Salmonella* in Dutch Broiler Flocks at Slaughter: A One-Year Study. Poultry Sci. 73:1260-1266.

Jones, F. T., R. C. Axtell, D. V. Rives, S. S. E., F. R. Tarver, R. L. Walker and M. J. Wineland. 1991. A Survey of *Salmonella* Contamination in Modern Broiler Production. J. Food Prot. 54:502-507.

Kingston, D. J. 1981. A Comparison of Culturing Drag Swabs and Litter for Identification of Infections with *Salmonella* Spp. In Commercial Chicken Flocks. Avian Dis. 25:513-516.

Knox, R., P. H. Gell and M. R. Pollock. 1942. Selective Media for Organisms of the *Salmonella* Group. J. Pathol. Bacteriol. 54:469-483.

Lahellec, C., P. Colin and G. Bennejean. 1986. Influence of Resident *Salmonella* on Contamination of Broiler Flocks. Poultry Sci. 2034-2039.

Landis, J. R. and G. G. Koch. 1977. The Measurement of Observer Agreement for Categorical Data. Biometrics. 33:159-174.

Limawongpranee, S., H. Hayashidani, A. T. Okatani, C. Hirota, K.-i. Kaneko and O. Masuo. 1999a. Contamination of *Salmonella* Blockley in the Environment of a Poultry Farm. Avian Dis. 43:302-309.

Limawongpranee, S., H. Hayashidani, A. T. Okatani, K. Ono, C. Hirota, K. Kaneko and M. Ogawa. 1999b. Prevalence and Persistence of *Salmonella* in Broiler Chicken Flocks. J. Vet. Med. Sci. 61:255-259.

Line, J. E., J. S. Bailey, N. A. Cox and N. J. Stern. 1997. Yeast Treatment to Reduce *Salmonella* and Campylobacter Populations Associated with Broiler Chickens Subjected to Transport Stress. Poultry Sci. 76:1227-1231.

Liu, Y., Y. Che and Y. Li. 2001. Rapid Detection of *Salmonella* Typhimurium Using Immunomagnetic Separation and Immuno-Optical Sensing Method. J. Sens. Act. 72:214-218.

Long, J. R., W. F. DeWitt and J. L. Ruet. 1980. Studies on *Salmonella* from Floor Litter of 60 Broiler Chicken Houses in Nova Scotia. Can. Vet. J. 21:91-94.

Luedtke, A. N., B. Chapman and D. A. Powell. 2003. Implementation and Analysis of an on-Farm Food Safety Program for the Production of Greenhouse Vegetables. J Food Prot. 66:485-489.

Lund, A., A. L. Hellemann and F. Vartdal. 1988. Rapid Isolation of K88+ Escherichia Coli by Using Immunomagnetic Particles. J. Clin. Microbiol. 26:2572-2575.

Mallinson, E. T., C. R. Tate, R. G. Miller, B. Bennett and E. Russek-Cohen. 1989. Monitoring Poultry Farms for *Salmonella* by Drag-Swab Sampling and Antigen-Capture Immunoassay. Avian Dis. 684-690.

Mansfield, L. and S. Forsythe. 1996. Collaborative Ring-Trial of Dynabeads Anti-*Salmonella* for Imunomagnetic Separation of Stressed *Salmonella* Cells from Herbs and Spices. Inter. J. Food Microbiol. 29:41-47.

McAllister, J. C., C. D. Steelman, L. A. Newberry and J. K. Skeeles. 1995. Isolation of Infectious Bursal Disease Virus from the Lesser Mealworm, Alphitobius Diaperinus (Panzer). Poultry Sci. 74:45-49.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin and R. V. Tauxe. 1999. Food-Related Illness and Death in the United States. Emerg. Infect. Dis. 5:607-625.

Morgan-Jones, S. C. 1982. The Presence of *Salmonellae* During the Rearing of Turkeys. Br. Poultry Sci. 23:481-486.

Nietfeld, J. C., B. Kelly, S. S. Dritz, I. Feder and J. C. Galland. 1998. Comparison of Conventional and Delayed Secondary Enrichment for Isolation of *Salmonella* Spp. From Swine Samples. J. Vet. Diag. Invest. 10:285-287.

O'Carroll, J. M., P. R. Davies, M. T. Correa and B. D. Slenning. 1999. Effects of Sample Storage and Delayed Secondary Enrichment on Detection of *Salmonella* Spp in Swine Feces. Am J Vet Res. 60:359-362.

Olesiuk, O. M., V. L. Carlson, G. H. Snoeyenbos and C. F. Smyser. 1969. Experimental *Salmonella* Typhimurium Infection in Two Chicken Flocks. Avian Dis. 13:500-508.

Opara, O. O., E. T. Mallinson, C. R. Tate, L. E. Carr, R. G. Miller, L. Stewart, C. Kelleher, R. W. Johnston and S. W. Joseph. 1992. The Effect of Exposure, Storage Times, and Types of Holding Media on the Drag-Swab Monitoring Technique for *Salmonella*. Avian Dis. 36:63-68.

Opara, O. O., L. E. Carr, C. R. Tate, R. G. Miller, E. T. Mallinson, L. E. Stewart and S. W. Joseph. 1994. Evaluation of Possible Alternatives to Double-Strength Skim Milk Used to Saturate Drag Swabs for *Salmonella* Detection. Avian Dis. 38:293-296.

Opengart, K. N., C. R. Tate, R. G. Miller and E. T. Mallinson. 1991. The Use of the Drag-Swab Technique and Improved Selective Media in the Recovery of *Salmonella* Arizona (7:1,7,8) Fromturkey Breeder Hens. Avian Dis. 228-230.

Peplow, M. O., M. Correa-Prisant, M. E. Stebbins, F. Jones and P. Davies. 1999. Sensitivity, Specificity, and Predictive Values of Three *Salmonella* Rapid Detection Kits Using Fresh and Frozen Poultry Environmental Samples Versus Those of Standard Plating. Appl. Environ. Microbiol. 65:1055-1060.

Pillai, S. D., S. C. Ricke, D. J. Nisbet, D. E. Corrier and J. R. DeLoach. 1994. A Rapid Method for Screening for *Salmonella* Typhimurium in a Chicken Cecal Microbial Consortium Using Gene Amplification. Avian Dis. 38:598-604.

Pourciau, S. S. and W. T. Springer. 1978. Evaluation of Secondary Enrichment for Detecting *Salmonellae* in Bobwhite Quail. Avian Dis. 22:42-45.

Ramirez, G. A., L. L. Sarlin, D. J. Caldwell, C. R. Yezak, Jr., M. E. Hume, D. E. Corrier, J. R. DeLoach and B. M. Hargis. 1997. Effect of Feed Withdrawal on the Incidence of *Salmonella* in the Crops and Ceca of Market Age Broiler Chickens. Poultry Sci. 654-656.

Read, S. C., R. J. Irwin, C. Poppe and J. Harris. 1994a. A Comparison of Two Methods for Isolation of *Salmonella* from Poultry Litter Samples. Poultry Sci. 73:1617-1621.

Read, S. C., R. J. Irwin, C. Poppe and J. Harris. 1994b. A Comparison of Two Methods for Isolation of *Salmonella* from Poultry Litter Samples. Poultry Sci. 1617-1621.

Rigby, C. E. and J. R. Pettit. 1980. Delayed Secondary Enrichment for the Isolation of *Salmonellae* from Broiler Chickens and Their Environment. Appl. Environ. Microbiol. 40:783-786.

Rolfe, D. L., H. P. Riemann, T. B. Farver and S. Himathongkham. 2000. Drag Swab Efficiency Factors When Sampling Chicken Manure. Avian Dis. 44:668-675.

Rybolt, M. L., R. W. Wills, J. A. Byrd, T. P. Doler and R. H. Bailey. 2004. Comparison of Four *Salmonella* Isolation Techniques in Four Different Inoculated Matrices. Poultry Sci. 83:1112-1116.

Rybolt, M. L., R. W. Wills and R. H. Bailey. 2005. Use of Secondary Enrichment for Isolation of *Salmonella* from Naturally Contaminated Environmental Samples. Poultry Sci. 84:992-997.

Safrit, R. D. and R. C. Axtell. 1984. Evaluations of Sampling Methods for Darkling Beetles (Alphitobius Diaperinus) in the Litter of Turkey and Broiler Houses. Poultry Sci. 63:2368-2375.

Sanchez, M. X., W. M. Fluckey, M. M. Brashears and S. R. McKee. 2002. Microbial Profile and Antibiotic Susceptibility of Campylobacter Spp. And *Salmonella* Spp. In Broilers Processed in Air-Chilled and Immersion-Chilled Environments. J. Food Prot. 65:948-956.

Sasipreeyajan, J., J. Jerngklinchan, C. Koowatananukul and K. Saitanu. 1996. Prevalence of *Salmonellae* in Broiler, Layer and Breeder Flocks in Thailand. Trop. Anim. Hlth. Prod. 28:174-180.

Shaw, S. J., B. W. Blais and D. C. Nundy. 1998. Performance of the Dynabeads Anti-*Salmonella* System in the Detection of *Salmonella* Species in Foods, Animal Feeds, and Environmental Samples. J. Food Prot. 61:1507-1510.

Skjerve, E., L. M. Rorvik and O. Olsvik. 1990. Detection of Listeria Monocytogenes in Foods by Immunomagnetic Separation. Appl. Environ. Microbiol. 56:3478-3481.

Skjerve, E. and O. Olsvik. 1991. Immunomagnetic Separation of *Salmonella* from Foods. Int. J. Food Microbiol. 14:11-17.

Smyser, C. F. and G. H. Snoeyenbos. 1976. Examination of Poultry Litter for *Salmonellae* by Direct Culture and Flourescent Antibody Technique. Avian Dis. 20:545-551.

Snoeyenbos, G. H., A. S. Soerjadi and O. M. Weinack. 1982. Gastrointestinal Colonization by *Salmonella* and Pathogenic Escherichia Coli in Monozenic and Holoxenic Chicks and Poults. Avian Dis. 26:566-575.

Soumet, C., G. Ermel, G. Salvat and P. Colin. 1997. Detection of *Salmonella* Spp. In Food Products by Polymerase Chain Reaction and Hybridization Assay in Microplate Format. J. Appl. Microbiol. 24:133-116.

Soumet, C., G. Ermel, N. Rose, D. P., G. Salvat and P. Colin. 1999. Evaluation of a Multiplex Pcr Assay for Simultaneous Identification of *Salmonella* Sp., *Salmonella* Enteritidis and *Salmonella* Typhimurium from Environmental Swabs of Poultry Houses. Lett. Appl. Microbiol. 113-117.

Spiritas, S. F. *Supreme Beef President Announces Decision to Cease Operations*. [listserv] 2000 [cited 2000 September 26]; Available from: http://archives.foodsafetynetwork.ca:16080/fsnet/2000/9-2000/fs-09-26-00-02.txt.

Tate, C. R., R. G. Miller, E. T. Mallinson, L. W. Douglas and R. W. Johnston. 1990. The Isolation of *Salmonellae* from Poultry Environmental Samples by Several Enrichment Procedures Using Plating Media with and without Novobiocin. Poultry Sci. 69:721-726.

Thomason, B. M. and D. J. Dodd. 1978. Enrichment Procedures for Isolating *Salmonellae* from Raw Meat and Poultry. Appl. Environ. Microbiol. 36:627-628.

USDA/FSIS. 1995. Nationwide Broiler Chicken Microbiological Baseline Data Collection Program U.S. Department of Agriculture. Washington, DC

USDA/FSIS. 1996. Pathogen Reduction: Hazard Analysis and Critical Control Point (Haccp) Systems; Final Rule. Fed. Reg. 61:38805-38889.

USDA/FSIS. 2006. *Salmonella* Verification Sample Result Reporting: Agency Policy and Use in Public Health Protection. Fed Reg. 71:9772-9777.

Vassiliadis, P. 1968. Shigella Spp., *Salmonella* Choleraesuis and Arizona in Rappaport's Medium. J. Appl. Bacteriol. 31:367-372.

Vassiliadis, P., E. Pateraki, J. Papadakis and D. Trichopoulos. 1974. Evaluation of the Growth of *Salmonellae* in Rappaport's Broth and in Muller-Kauffmann's Tetrathionate Broth. J. Appl. Bacteriol. 37:411-418.

Vassiliadis, P., J. A. Papadakis, D. Karalis and D. Trichopoulos. 1976. Enrichment in Muller-Kauffmann's Broth and Rappaport's Broth from Buffered Peptone Water in the Isolation of *Salmonellae* from Minced Meat. J. Appl. Bacteriol. 40:349-354.

Vassiliadis, P., D. Trichopoulos, A. Kalandidi and E. Xirouchaki. 1978a. Isolation of *Salmonellae* from Sewage with a New Procedure of Enrichment. J. Appl. Bacteriol. 44:233-239.

Vassiliadis, P., D. Trichopoulos, E. Pateraki and N. Papaiconomou. 1978b. Isolation of *Salmonella* from Minced Meat by the Use of a New Procedure of Enrichment. Zbl. Bakt. Hyg., I. Abt. Orig. B. 166:81.86.

Vassiliadis, P., D. Trichopoulos, G. Papoutsakis and Pallandiou. 1979. A Note on the Comparison of Two Modifications on Rappaport's Medium with Selenite Broth in the Isolation of *Salmonella*s. J. Appl. Bacteriol. 46:567-569.

Vermunt, A. E. M., A. A. J. M. Franken and R. R. Beumer. 1992. Isolation of *Salmonella*s by Immunomagnetic Separation. J. Appl. Bacteriol. 72:112-118.

Waltman, W. D., A. M. Horne, C. Pirkle and T. G. Dickson. 1991. Use of Delayed Secondary Enrichment for the Isolation of *Salmonella* in Poultry and Poultry Environments. Avian Dis. 35:88-92.

Waltman, W. D., A. M. Horne and C. Pirkle. 1993. Influence of Enrichment Incubation Time on the Isolation of *Salmonella*. Avian Dis. 37:884-887.

Waltman, W. D. and E. T. Mallinson. 1995. Isolation of *Salmonella* from Poultry Tissue and Environmental Samples: A Nationwide Survey. Avian Dis. 45-54.

Wang, X. and M. F. Slavik. 1999. Rapid Detection of *Salmonella* in Chicken Washes by Immunomagnetic Separation and Flow Cytometry. J. Food Prot. 62:717-723.

Wegener, H. C. and D. L. Baggesen. 1997. Comparison of Conventional Culture Methods and Two Commercial Enzyme Immunoassays for Detection of *Salmonella* in Porcine Fecal Samples and Cecal Contents. J. Vet. Diag. Invest. 352-356.

Xirouchaki, E., P. Vassiliadis, D. Trichopoulos and C. H. Mavrommati. 1982. A Note on the Performance of Rappaport's Medium, Compared with Rappaport-Vassiliadis Broth, in the Isolation of *Salmonella*s from Meat Products, after Pre-Enrichment. J. Appl. Bacteriol. 52:125-127.

# APPENDIX A

# PRELIMINARY STUDY: DETERMINATION OF THE ROLE THE DARKLING BEETLE (*ALPHITOBIUS DIAPERINUS*) PLAYS IN THE TRANSMISSION OF *SALMONELLA* IN BROILER HOUSES.

ABSTRACT

Proper sampling for analysis of food safety hazards within farm production environments is challenging. The sensitivity, specificity, and repeatability of sampling techniques for foodborne pathogens are not well documented. In this work, broiler grow-out houses were sampled by various techniques to test for the presence of *Salmonella* and *Campylobacter*. Thirteen broiler houses were sampled by the drag swab method (four swabs/house) to identify *Salmonella* positive houses for further study. Three houses were found to be positive for *Salmonella*; none tested positive for *Campylobacter*. Subsequently, the *Salmonella* positive houses were intensely sampled by drag swabs, litter samples, and darkling beetle traps 11 days after the initial drag swabs were collected. On day eleven, the three previously *Salmonella* positive houses were negative by drag swabs (0/12). One of the houses had positive litter samples (3/48) but no beetle traps were positive (0/48). Beetle traps were all negative for the other houses as well. For *Campylobacter*, all drag swabs (0/12) and litter samples (0/144) were negative. *Campylobacter* was recovered from darkling beetle trap contents in one house (7/48). The results of this and previous experiments demonstrate the difficulty encountered when trying to accurately assess broiler houses for the presence of *Salmonella* and *Campylobacter*. More fully

characterized sampling techniques need to be developed to investigate the ecology and prevalence of the pathogens on the farm so that effective food safety risk management strategies can be developed and assessed.

## INTRODUCTION

The USDA FSIS has issued various directives requiring stronger regulation of the food industry. Under the Pathogen Reduction/Hazard Analysis Critical Control Point: Final Rule, industry is to "identify, prevent and take corrective action on food safety hazards that are reasonably likely to occur in the production process, before, during, and after entry into the establishment." This directive essentially requires industry to identify critical control points (CCP) or best practices on the farm. However, in order to accurately identify the CCPs, the methods used to identify foodborne pathogens, such as *Salmonella*, must be accurate and reliable.

In order for accurate and reliable risk management decisions to be made concerning the pathogen status of a poultry house, assessment methods need to be validated and confirmed. In the present study, we evaluated three possible methods for determining the *Salmonella* status of a house. Here, we examined the possibilities of the drag swab, litter culturing, and darkling beetle traps methods.

Prior to 1980, litter samples were used as the gold standard sample in assessing the pathogen status of a poultry house, particularly *Salmonella sp.* and *Campylobacter sp.* However, Kingston (1981) reported that the drag swab method served as a fast and reliable protocol for identifying *Salmonella sp.* within a poultry house and subsequently, many people began using this method which soon replaced litter culturing as the gold standard.

Darkling beetles (*Alphitobius diaperinus*) are a ubiquitous insect found in the poultry establishment. This insect is nocturnal and feeds off dead, dying, and decaying chicken carcasses. They have been shown to harbor *Salmonella* as well as many other pathogens. McAllister *et al.* (1995) demonstrated the ability of darkling beetle adults to shed *S.* Typhimurium for up to 28 days allowing for the possible reintroduction of the pathogen into the poultry house during the next flock. This possibility as well as other documented studies, has drawn much attention to the beetles as possible sources of pathogens.

This study was designed to not only accurately assess the *Salmonella* and *Campylobacter* status of each house, but also to determine the role the darkling beetle plays in the transmission of these particular pathogens from flock to flock.

# MATERIALS AND METHODS

## Drag Swabs

Drag-swabs assemblies were prepared prior to use in the poultry houses. Each swab was constructed with 4 x 4 – inch (10.2 cm x 10.2 cm) cotton gauze[29] tied to 6-ft (182.9 cm) cotton-polyester twine[30]. The assemblies were sterilized with steam and aseptically transferred to sterile WhirlPak ® bags containing 20-mL sterile double strength skim milk for a pre-soaking storage medium. The skim milk was prepared according to the manufacturer's directions[31], except the concentration of milk powder to water was doubled (from 45.36 g per 500-mL to 90.72 g per 500-mL). In the houses, each swab was removed from the bag, swabbed following Figure A.1 and returned to its bag after sampling. All swabs were stored on wet ice until further processing in the laboratory. In the laboratory, 100-mL sterile buffered peptone water (BPW) was aseptically added to each swab. Samples were incubated overnight at 42°C.

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[29] Abco Dealers, Inc., Nashville, TN

[30] The Lehigh Group, Macungie, PA

[31] Wal-Mart Stores, Inc., Bentonville, AR

Beetle Samples

Darkling beetles (*Alphitobius diaperinus*) were collected using the Arend's tube trap (Safrit and Axtell, 1984). The collection tubes consisted of a 30.5 cm by 5.1 cm diameter polyvinyl chloride (PVC) pipe with a 43.2 cm by 22.9 cm piece of corrugated cardboard roll inserted. Forty-eight traps were placed equidistant from each other on the floor of the poultry houses, with 16 down each of the north and south sides and 16 down the center (Figure A.1). The traps were left for seven days, after which, the contents and the cardboard roll were collected in sterile plastic bags. The bags were sealed and transported back to the laboratory for further processing.

Each beetle trap bag was individually opened and all contents extracted and collected. The cardboard rolls were separated to remove all darkling beetles and larvae. The collected contents were pooled into a single WhirlPak® bag, weighed, and sterile PBS added to give a 1:10 dilution. They were then incubated at 37°C for 18 hs.

Litter Samples

Forty-eight litter samples were collected, one at each beetle trap site, in each house. At each of the beetle tube trap locations, a litter sample was collected. Approximately 50-g of litter was collected for each site and placed into sterile WhirlPak® bags and sealed. The bags were

then placed on wet ice and transported back to the laboratory for further processing.

For each litter sample, 25-g was measured and placed into sterile bags. To each sample, 225-mL of PBS (1:10 wt/vol ratio) was added and the sample stomached for 2 mins at normal speed in the Seward Stomacher 400 and then incubated for 18 hs at 37°C.

*Salmonella* screening

For each of the above-mentioned samples, a 10-mL aliquot was transferred into a sterile fifty-mL centrifuge tube containing 10 mL of double strength tetrathionate broth (TT) and incubated at 37°C for 18 hs. After incubation, the samples were streaked onto brilliant green agar[32] and the plates incubated at 37°C for 18 hs. The colonies that appeared typical of *Salmonella sp.* were carefully picked and streaked onto Triple Sugar Iron (TSI) agar[33] and Lysine Iron agar (LIA)[34] slants and incubated for 18 hs at 37°C. Colonies appearing as *Salmonella sp.* were confirmed using the anti-*Salmonella* Poly A-I and Vi serum for further confirmation.

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[32] Difco Laboratories, #0285-17-7

[33] Difco, #0265-17-1

[34] Difco, #0849-17-6

Confirmed *Salmonella* positives were submitted to the National Veterinary Services Laboratory in Ames, Iowa for serotyping.

Campylobacter screening

For each of the above-mentioned samples, a 10-mL aliquot was transferred into a sterile 50-mL centrifuge tube containing 10-mL of double strength Bolton's broth[35] prepared according to the manufacturer's guidelines and the tubes were then incubated at 42°C for 18 hs. After incubations, the samples were streaked onto *Campylobacter* Blood-Free Selective agar (MCCDA)[36] prepared according to the manufacturer's guidelines and then incubated at 42°C for 48 hs in a microaerophilic atmosphere (5% oxygen, 10% carbon dioxide, and 85% nitrogen). The colonies appearing as typical *Campylobacter sp.* were picked and confirmed using the Oxoid *Campylobacter* Test Kit[37].

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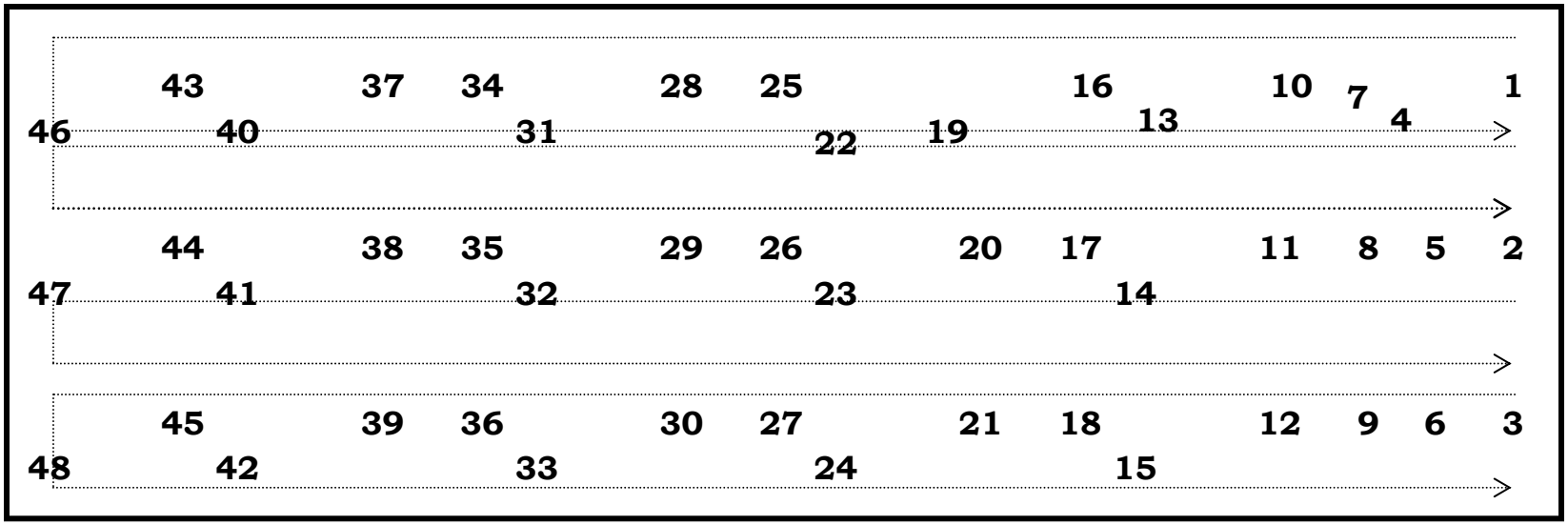
[35] Oxoid, #CM983

[36] Oxoid, #CM739

[37] Oxoid, #DR150M

Figure A.1. Schematic diagram of poultry house sampling pattern.




The numbers indicate location of the Arend beetle traps. The dotted lines indicate the drag swabbing patterns.

# RESULTS

Eleven days prior to sampling, thirteen commercial broiler houses were drag swabbed to determine the presence of *Salmonella sp.* and *Campylobacter sp.* From these houses, three were found to be *Salmonella* positive, where as none were found be *Campylobacter* positive. The three *Salmonella* positive houses were subsequently, intensely sampled, collecting drag swabs, darkling beetle trap content, and litter samples, eleven days after initial drag swab testing. Each of these samples was tested for *Salmonella sp.* and *Campylobacter sp.*

## Salmonella

Of the three *Salmonella* positive houses, none were determined to be positive by the drag swab method eleven days (seven days post introduction of tube traps) after the initial testing. All beetle traps were also negative. One house had three litter samples positive. The three positive litter samples were collected from sites 28, 42, and 45 in House 1 (Figure A.1). Serotyping results from two of the litter-isolated *Salmonella* samples obtained from House 1 were *S.* Montevideo and the third was unknown. The cultures isolated from the initial drag swabs were all *S.* Montevideo except one, which was *S.* Infantis.

Campylobacter

The samples collected from the three *Salmonella* positive houses were also screened for the presence of *Campylobacter sp.* No *Campylobacter* was recovered from any of the drag swabs or the litter. However, seven of the beetle trap contents were positive from House 3. The Oxoid *Campylobacter* Test Kit gives positive results for *C. jejuni*, *C. coli*, and *C. upsaliensis*. Variable results are obtained for *C. fetus* subsp. *fetus*. Beetle traps found to be positive were located at positions 21, 30, 39, 40, 42, 43, and 45. From Figure A.1, it can be seen that theses sites are within close proximity to each other on the southwest end of house 3. It was observed that the extreme west end of the house was very wet on both the north and south sides.

## DISCUSSION

In attempts to make accurate risk management decisions concerning the pathogen status of a particular poultry house, one must rely upon the diagnostic tests as an indicator. However, when these tests are not accurate, this could lead to poor management and possible severe outcomes, financially or otherwise. For this reason, the accuracy of the tests must be validated and confirmed before the risk management decisions are made. We have shown here that the methods used as "gold

standards" in the poultry industry to help determine the *Salmonella* and *Campylobacter* presence in a particular house are not completely accurate and that further work is needed to develop a more sensitive and reliable test.

Previous studies indicating the difficulty in recovering pathogens have been well documented. *Campylobacter spp.* are very fastidious organisms and do not adapt well to desiccation, therefore requiring higher litter moisture content for survival. Our results showed *Campylobacter* was only being isolated from the wettest areas of a particular house. Over growth of many organisms may also compete for nutrients therefore excluding *Campylobacter* growth. The fact that the only isolated *Campylobacter* was found in the beetle traps is not understood. However, this could be a result of the cycling of pathogen shedding and the beetle's ability to harbor the organism for an extended period of time.

All the houses that tested positive for *Salmonella sp.* on day 0 via the drag swab method tested negative by this method on day 11, or seven days post introduction of the beetle traps. This phenomenon perhaps can be explained also by the cyclic shedding of pathogens. In trying to determine the best course of action, we determined the methods may lack the sensitivity desired. Likewise, since the beetles were also found

to be negative on day eleven, we conclude that the assessment methods are not as reliable as presumed. *Salmonella spp.* are very tolerant organisms and can be isolated from very harsh environments. Therefore, it is our assessment that the methods employed to isolate the organism are not very reliable and need to be more fully characterized.

Table A.1. Sampling Timeline.

| | Sampling Timeline | | |
|---|---|---|---|
| Day | Drag Swabs | Litter | Beetle Traps |
| Day 0 | + | - | - |
| Day 4 | - | - | Placed |
| Day 11 | + | + | + |

(+) indicates samples were collected and – indicates no samples were collected. On Day 4, the beetle traps were placed and collected on Day 11.

Table A.2. Results of the *Salmonella* testing from each house.

| | Sample | | | |
|---|---|---|---|---|
| | Drag Swab | | Litter | Beetle Trap |
| Day | 0 | 11 | 11 | 11 |
| House 1 | 1/4 (25%) | 0/4 (0%) | 3/48 (6.3%) | 0/48 (0%) |
| House 2 | 2/4 (50%) | 0/4 (0%) | 0/48 (0%) | 0/48 (0%) |
| House 3 | 2/4 (50%) | 0/4 (0%) | 0/48 (0%) | 0/48 (0%) |

Table A.3. Serotype results for *Salmonella* isolates.

| | | *Salmonella* Serotypes Isolated | |
|---|---|---|---|
| Day | House | Sample | Serotype |
| 0 | 1 | Drag swab | *S.* Montevideo |
| 0 | 2 | Drag swab | unknown |
| 0 | 2 | Drag swab | *S.* Infantis |
| 0 | 3 | Drag swab | *S.* Montevideo |
| 0 | 3 | Drag swab | *S.* Montevideo |
| 11 | 1 | Litter | unknown |
| 11 | 1 | Litter | *S.* Montevideo |
| 11 | 1 | Litter | *S.* Montevideo |

Table A.4. Results of the *Campylobacter* testing from each house.

| | Sample | | | |
|---|---|---|---|---|
| | Drag Swab | | Litter | Beetle Trap |
| Day | 0 | 11 | 11 | 11 |
| House 1 | 0/48 (0%) | 0/4 (0%) | 0/48 (0%) | 0/48 (0%) |
| House 2 | 0/48 (0%) | 0/4 (0%) | 0/48 (0%) | 0/48 (0%) |
| House 3 | 0/48 (0%) | 0/4 (0%) | 0/48 (0%) | 7/48 (14.6%) |

# APPENDIX B

# SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE FOR CHAPTER 4

Table B.1 Comparison between immunomagnetic separation (DB) and Rappaport-Vassiliadis (RV) for the isolation of *Salmonella* from litter samples[1].

| DB | | RV | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| on BG plates | Positive | 0 | 1 | 1 |
| | Negative | 14 | 41 | 55 |
| | Total | 14 | 42 | 56 |
| on XLT4 plates | Positive | 2 | 2 | 4 |
| | Negative | 10 | 42 | 52 |
| | Total | 12 | 33 | 56 |

[1]BG, brilliant green; XLT4, xylose-lysine tergitol 4

Table B.2 Comparison between immunomagnetic separation (DB) and tetrathionate (TT) for the isolation of *Salmonella* from litter samples[1].

| DB | | TT | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| on BG plates | Positive | 1 | 0 | 1 |
| | Negative | 12 | 43 | 55 |
| | Total | 13 | 43 | 56 |
| on XLT4 plates | Positive | 3 | 1 | 4 |
| | Negative | 10 | 42 | 52 |
| | Total | 13 | 43 | 56 |

[1]BG, brilliant green; XLT4, xylose-lysine tergitol 4

Table B.3 Comparison between immunomagnetic separation (DB) and secondary enrichment (TR) for the isolation of *Salmonella* from litter samples[1].

| DB | | TR Positive | TR Negative | Total |
|---|---|---|---|---|
| on BG plates | Positive | 1 | 0 | 1 |
| | Negative | 34 | 21 | 55 |
| | Total | 35 | 21 | 56 |
| on XLT4 plates | Positive | 4 | 0 | 4 |
| | Negative | 30 | 22 | 52 |
| | Total | 34 | 22 | 56 |

[1]BG, brilliant green; XLT4, xylose-lysine tergitol 4

Table B.4 Comparison between Rappaport-Vassiliadis (RV) and tetrathionate (TT) for the isolation of *Salmonella* from litter samples[1].

| RV | | TT Positive | TT Negative | Total |
|---|---|---|---|---|
| on BG plates | Positive | 11 | 20 | 31 |
| | Negative | 12 | 83 | 95 |
| | Total | 23 | 103 | 126 |
| on XLT4 plates | Positive | 7 | 12 | 19 |
| | Negative | 16 | 91 | 107 |
| | Total | 23 | 103 | 126 |

[1]BG, brilliant green; XLT4, xylose-lysine tergitol 4

Table B.5 Comparison between Rappaport-Vassiliadis (RV) and secondary enrichment (TR) for the isolation of *Salmonella* from litter samples[1].

| RV | | TR Positive | TR Negative | Total |
|---|---|---|---|---|
| on BG plates | Positive | 12 | 7 | 19 |
| | Negative | 40 | 67 | 107 |
| | Total | 52 | 67 | 126 |
| on XLT4 plates | Positive | 25 | 6 | 31 |
| | Negative | 29 | 66 | 65 |
| | Total | 54 | 72 | 126 |

[1]BG, brilliant green; XLT4, xylose-lysine tergitol 4

Table B.6 Comparison between tetrathionate (TT) and secondary enrichment (TR) for the isolation of *Salmonella* from litter samples[1].

| TT | | TR Positive | TR Negative | Total |
|---|---|---|---|---|
| on BG plates | Positive | 17 | 6 | 23 |
| | Negative | 37 | 66 | 103 |
| | Total | 54 | 72 | 126 |
| on XLT4 plates | Positive | 15 | 8 | 23 |
| | Negative | 37 | 66 | 103 |
| | Total | 52 | 74 | 126 |

[1]BG, brilliant green; XLT4, xylose-lysine tergitol 4

Table B.7 Comparison between immunomagnetic separation (DB) and Rappaport-Vassiliadis (RV) on xylose-lysine tergitol 4 (XLT4) plates for the isolation of *Salmonella* from drag swab samples.

| | | RV | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DB | Positive | 1 | 2 | 3 |
| | Negative | 17 | 144 | 161 |
| | Total | 18 | 146 | 164 |

Table B.8 Comparison between immunomagnetic separation (DB) and tetrathionate (TT) on xylose-lysine tergitol 4 (XLT4) plates for the isolation of *Salmonella* from drag swab samples.

| | | TT | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DB | Positive | 2 | 1 | 3 |
| | Negative | 21 | 140 | 161 |
| | Total | 23 | 141 | 164 |

Table B.9 Comparison between immunomagnetic separation (DB) and secondary enrichment (TR) on XLT4 plates for the isolation of *Salmonella* from drag swab samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DB | Positive | 2 | 1 | 3 |
| | Negative | 86 | 75 | 161 |
| | Total | 88 | 76 | 164 |

Table B.10 Comparison between Rappaport-Vassiliadis (RV) and tetrathionate (TT) on brilliant green plates for the isolation of *Salmonella* from drag swab samples.

| | | TT | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| RV | Positive | 7 | 19 | 26 |
| | Negative | 21 | 257 | 278 |
| | Total | 28 | 276 | 304 |

Table B.11 Comparison between Rappaport-Vassiliadis (RV) and secondary enrichment (TR) on brilliant green plates for the isolation of *Salmonella* from drag swab samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| RV | Positive | 22 | 4 | 26 |
| | Negative | 81 | 197 | 278 |
| | Total | 103 | 201 | 304 |

Table B.12 Comparison between tetrathionate (TT) and secondary enrichment (TR) on brilliant green plates for the isolation of *Salmonella* from drag swab samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| TT | Positive | 26 | 2 | 28 |
| | Negative | 77 | 199 | 376 |
| | Total | 103 | 201 | 304 |

Table B.13 Comparison between brilliant green (BG) and xylose-lysine tergitol 4 (XLT4) plates using immunomagnetic separation for the isolation of *Salmonella*.

| | | XLT4 | | |
|---|---|---|---|---|
| BG | | Positive | Negative | Total |
| Litter | Positive | 0 | 1 | 1 |
| | Negative | 4 | 51 | 55 |
| | Total | 4 | 52 | 56 |
| Drag Swabs | Positive | 0 | 0 | 0 |
| | Negative | 3 | 161 | 164 |
| | Total | 3 | 161 | 134 |

Table B.14 Comparison between brilliant green (BG) and xylose-lysine tergitol 4 (XLT4) plates using Rappaport-Vassiliadis (RV) for the isolation of *Salmonella*.

| | | XLT4 | | |
|---|---|---|---|---|
| BG | | Positive | Negative | Total |
| Litter | Positive | 14 | 17 | 31 |
| | Negative | 5 | 90 | 95 |
| | Total | 19 | 107 | 126 |
| Drag Swabs | Positive | 23 | 3 | 26 |
| | Negative | 3 | 275 | 278 |
| | Total | 26 | 278 | 304 |

Table B.15 Comparison between brilliant green (BG) and xylose-lysine tergitol 4 (XLT4) plates using tetrathionate for the isolation of *Salmonella*.

| | | XLT4 | | |
|---|---|---|---|---|
| BG | | Positive | Negative | Total |
| Litter | Positive | 12 | 11 | 23 |
| | Negative | 11 | 92 | 103 |
| | Total | 23 | 103 | 126 |
| Drag Swabs | Positive | 18 | 10 | 28 |
| | Negative | 16 | 260 | 276 |
| | Total | 34 | 270 | 304 |

Table B.16 Comparison between brilliant green (BG) and xylose-lysine tergitol 4 (XL4) plates using secondary enrichment for the isolation of *Salmonella*.

| | | XLT4 | | |
|---|---|---|---|---|
| BG | | Positive | Negative | Total |
| Litter | Positive | 51 | 3 | 54 |
| | Negative | 1 | 71 | 72 |
| | Total | 52 | 74 | 126 |
| Drag Swabs | Positive | 99 | 4 | 103 |
| | Negative | 8 | 193 | 201 |
| | Total | 107 | 193 | 304 |

SUPPLEMENTAL FIGURE FOR CHAPTER 6

Table B.17 Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from ceca samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 13 | 3 | 16 |
| | Negative | 0 | 104 | 104 |
| | Total | 13 | 107 | 120 |

Table B.18 Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from crop samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 26 | 3 | 29 |
| | Negative | 2 | 89 | 91 |
| | Total | 28 | 92 | 120 |

Table B.19 Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from drag swab samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 34 | 2 | 36 |
| | Negative | 3 | 25 | 28 |
| | Total | 37 | 27 | 64 |

Table B.20 Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from GI samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 6 | 2 | 8 |
| | Negative | 0 | 52 | 52 |
| | Total | 6 | 54 | 60 |

Table B.21 Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from litter samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 14 | 4 | 18 |
| | Negative | 5 | 13 | 18 |
| | Total | 19 | 17 | 36 |

Table B.22 Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from postchill rinse samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 19 | 4 | 23 |
| | Negative | 3 | 34 | 37 |
| | Total | 22 | 38 | 60 |

Table B.23 Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from pre-chill rinse samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 50 | 1 | 51 |
| | Negative | 2 | 7 | 9 |
| | Total | 52 | 8 | 60 |

Table B.24 Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from tray pad samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 45 | 5 | 50 |
| | Negative | 2 | 8 | 10 |
| | Total | 47 | 13 | 60 |

Table B.25 Comparison between secondary enrichment (TR) and delayed secondary enrichment (DSE) for the isolation of *Salmonella* from whole carcass rinse samples.

| | | TR | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| DSE | Positive | 56 | 9 | 65 |
| | Negative | 2 | 53 | 55 |
| | Total | 58 | 62 | 120 |

# APPENDIX C

# PERMISSION TO PUBLISH

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March 7, 2006



To Whom It May Concern:

Permission is granted by the Poultry Science Association to M. L. Rybolt to include the following two papers in his dissertation, provided appropriate credit is given to the original publication in *Poultry Science*:

Rybolt, M. L., R. W. Wills, J. A. Byrd, T. P. Doler, and R. H. Bailey. 2004. Comparison of four *Salmonella* isolation techniques in four different inoculated matrices. Poultry Sci. 83:112-1116.

Rybolt, M. L., R. W. Wills, and R. H. Bailey. 2005. Use of Secondary enrichment for isolation of *Salmonella* from naturally contaminated environmental samples. Poultry Sci. 84:992-997.

Sincerely,

Susan Pollock
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