Use of Secondary Enrichment for Isolation of *Salmonella* from Naturally Contaminated Environmental Samples

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ABSTRACT Since the implementation of Hazard Analysis Critical Control Point (HACCP), the need for on-farm food safety risk assessment and 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 4 *Salmonella*-specific protocols: immunomagnetic separation (DB), tetrathionate (TT) broth, Rappaport-Vassiliadis R10 (RV) broth, and a secondary enrichment (TR) procedure as well as 2 selective solid media (brilliant green agar, BG; and xylose-lysine tergitol 4, XLT4). All 4 methods were compared in litter and drag swab samples that were collected weekly during the broiler grow out period in 7 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 BG agar, respectively. The TR protocol identified 83.1% (54/65) of the positive samples as positive when using BG 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 BG agar, respectively. Again, the TR protocol identified the highest percentage of positive samples (94.5%). An analysis of agreement, κ, revealed that TT and RV did 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 2 agar plates used, BG and XLT4, showed that they had high agreement when the secondary enrichment protocol was used, but agreement was only moderate to low when the other 3 methods were used.

(*Key words*: *Salmonella*, methodology, broiler, environmental sample)

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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 as meat products or preharvest environmental samples. A current review of the literature suggests that no 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 (TT), Rappaport-Vassiliadis (RV), and selenite-cysteine (Vassiliadis, et al., 1978, 1978b, 1976; Cox et al., 1982; Davies and Wray, 1994; Read et al., 1994; Hammack, et al., 1998; Huang, et al., 1999).

It is essential to understand the characteristics of the isolation method employed when making production and 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 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, identification of the most appropriate methodology for microbial evaluation of samples containing low levels of *Salmonella* is crucial. Previously, this laboratory has

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Abbreviation Key: BG = brilliant green; DB = Dynadeads; RV = Rappaport-Vassiliadis; TR = secondary enrichment; TT = tetrathionate; XLT4 = xylose-lysine tergitol 4.

characterized 4 *Salmonella* isolation methods in artificially contaminated matrices simulating conditions in which 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 4 previously used isolation methods in samples obtained from naturally contaminated environments. Samples were acquired from broiler houses by 2 different sampling methods, drag swabs and floor litter. The *Salmonella* selective isolation broths used were Muller Kauffman TT, 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 2 farms (6 on farm A and 7 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 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 2 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 wk consecutively by drag swabs and 9 wk consecutively via litter. This gave a total of 304 drag swab samples and 126 litter samples to use for method comparison.

Sampling

The 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 10.2 × 10.2 cm (4 × 4 in) cotton gauze[2] tied to 182.9 cm (6-ft) cotton-polyester twine.[3] The assemblies were sterilized with steam and aseptically transferred to sterile WhirlPak bags containing 20 mL of sterile double strength skim milk as a presoaking medium. The skim milk was prepared according to the manufacturer's directions,[4] except the concentration of powder to water was doubled (from 45.36 g/500 mL to 90.72 g/500 mL). In the houses, each swab was removed from the bag, 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 wk 0 (placement). The houses were divided lengthwise, and floor shavings were collected from 3 different locations per sample, equidistant from each other at each end and in the middle. Samples were collected using examination gloves and then 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 h.

Enrichment and Isolation

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

The RV broth[5] and TT[6] broth were prepared according to the manufacturer's directions. Nine-milliliter aliquots were aseptically transferred into eleven 50-mL conical bottom centrifuge tubes and inoculated with 1 mL of each sample. The tubes were vortexed and incubated at 42°C overnight.

The DB were obtained from Neogen Corp.[7] 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 added aseptically. One milliliter of each sample was added to the corresponding tube. Tubes were vortexed and incubated at room temperature for 30 min with intermittent shaking and then placed into a magnetic particle concentrator[8], and left undisturbed for 10 min to allow the magnetic beads to concentrate onto the side of the tubes. The supernatant was aspirated using sterile Pasteur pipettes, leaving the beads concentrated on the side of the tubes. A 1-mL volume of sterile PBS-Tween 20 wash solution (0.15 *M* NaCl, 0.01 *M* 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 set undisturbed for 10 min. Samples were washed 2 more times following the same directions. After the third wash, beads were resuspended in 100 μL of PBS-Tween 20.

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

After 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 *Salmonella* colonies were further characterized using triple sugar iron

[2]Abco Dealers Inc., Nashville, TN.
[3]Lehigh Group, Macungie, PA.
[4]Wal-Mart Stores, Inc., Bentonville, AR.
[5]Difco Laboratories, Detroit, MI.
[6]Remel Inc., Lenexa, KS.
[7]Neogen Corp., Lansing, MI.
[8]Product Z5342, Promega Inc., Madison, WI.

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[9] was used to generate a chi-squared test statistic to determine if there was an association between the 4 *Salmonella* isolation protocols used. Separate analyses were conducted on litter and drag swab samples for the 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 α level of 0.05. Also, the κ coefficient of agreement was generated to determine the degree of agreement among the results of the 4 protocols, and interpretations followed that of Landis and Koch (Landis and Koch, 1977). Briefly, a $\kappa \leq 0.0$ is considered to be poor agreement, and $\kappa > 0.81$ indicate almost perfect agreement. For the values between 0.0 and 0.81, interpretations are slight, fair, moderate, and substantial for 0.00 to 0.20, 0.21 to 0.40, 0.41 to 0.60, and 0.61 to 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 positive for *Salmonella*. When using XLT4 plates with the 4 protocols in evaluating the drag swab samples, there was a 37.8% (115/304) *Salmonella* isolation rate. After 3 wk 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 4 (11.1%) of 36 samples to be positive for *Salmonella* when plated onto XLT4 plates (Table 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 α level for this set of comparisons was $0.05/4 = 0.0125$).

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 1). 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 with the other 3 methods evaluated. TR had a statistically significant ($P < 0.0001$) higher *Salmonella* isolation rate, 83.1% (54/65), when compared with 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.

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 3 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.

With 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 2 methods on either type of plate.

The TR provided the highest isolation rates compared with the other 3 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.

κ Analysis

Table 2 presents the κ analysis among the 4 protocols evaluated in this study. The κ analysis assesses the agreement between 2 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 κ value of 0.19 ($P = 0.0009$; Table 2). 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 lead-

[9]SAS User's Guide, Version 9.1, SAS Institute Inc., Cary, NC.

TABLE 1. Comparison among 4 *Salmonella* isolation protocols in 2 different sampling mediums with 2 different selective agars[1]

	Litter		Drag swab	
Medium	Brilliant green	Xylose-lysine tergitol 4	Brilliant green	Xylose-lysine tergitol 4
Dynabeads	1/37 (2.7)[a]	4/36 (11.1)[a]	0/85 (0)[a]	3/90 (3.3)[a]
Rappaport-Vassiliadis	31/65 (47.7)[b]	19/65 (29.2)[a,b]	26/109 (23.9)[b]	26/115 (22.6)[b]
Tetrathionate	23/65 (35.4)[b]	23/65 (35.4)[b]	28/109 (25.7)[b]	34/115 (29.6)[b]
Secondary enrichment	54/65 (83.1)[c]	52/65 (80.0)[c]	103/109 (94.5)[c]	107/115 (93.0)[c]

[a–c]Values within a column with the same superscript are not significantly different ($P > 0.0125$).

[1]Values are number positive/number positive by at least one method (%).

ing to a fair κ of 0.32 ($P < 0.0001$; Table 2). 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 κ value for this comparison was 0.22 ($P < 0.0001$; Table 2), which is considered fair agreement.

For the drag swab samples, no comparison was made between DB and the other 3 protocols when BG was used because DB did not isolate *Salmonella* from any of the samples. The greatest agreement was found comparing TT to TR ($\kappa = 0.29$; $P < 0.0001$), and the least was between RV and TT ($\kappa = 0.19$; $P = 0.0011$). When XLT4 plates were used the least agreement ($\kappa = 0.01$; $P = 0.6484$) was found in the comparison between DB and TR, and the greatest ($\kappa = 0.32$; $P < 0.0001$) was between TT and TR.

When making comparisons of litter samples with BG agar, DB compared with RV had the least agreement with a κ value of –0.03 ($P = 5602$), and RV compared with TR had the greatest agreement with a κ value of 0.40 ($P < 0.0001$). For comparisons using XLT4 plates, the least agreement with a κ value was found in the comparison between DB and TR ($\kappa = 0.09$; $P = 0.0905$). The highest agreement with a κ value of 0.27 ($P = 0.0109$) was found comparing DB to TT.

An agreement comparison was also made between the 2 selective agars, BG and XLT4 (Table 3). For the litter samples, BG and XLT4 agreement was greatest when using TR ($\kappa = 0.94$, $P < 0.0001$). For the other 3 protocols, DB, RV, and TT, the κ agreement values were –0.03, 0.46, and 0.42, respectively. In the drag swab samples, the κ agreement comparison yielded a near perfect value of 0.91 ($P < 0.0001$) when using TR as the protocol. The values for the other 3 protocols were 0.00, 0.87, and 0.53 for DB, RV, and TT, respectively.

DISCUSSION

In this study, 4 *Salmonella* specific isolation protocols were evaluated for their abilities to detect *Salmonella* in naturally contaminated broiler grow-out house samples. The TR protocol provided the highest *Salmonella* isolation rate when using 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 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 4 protocols evaluated in this study, the TR protocol appears to be the method of choice when conducting a *Salmonella* risk assessment of broiler grow-out houses and analyzing litter or drag swab samples.

Salmonella isolation methodology has been evaluated in many studies (Knox et al., 1942; Vassiliadis et al., 1974, 1976, 1978a; Cox et al., 1982; Davies and Wray, 1994; Peplow et al., 1999). Some research has focused on devel-

TABLE 2. Kappa agreement values among all comparisons[1]

		BG plates		XLT4 plates	
Sample origination	Comparison	κ	$P > \|Z\|$	κ	$P > \|Z\|$
Litter	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	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

[1]DB = Dynabeads; RV = Rappaport-Vassiliadis; TT = tetrathionate; TR = secondary enrichment; NC = no comparison made; BG = brilliant green; XLT4 = xylose-lysine tergitol 4.

TABLE 3. Kappa agreement values between brilliant green and xylose lysine tergitol 4 plates

	Litter		Drag swabs	
Medium	κ	$P > \|Z\|$	κ	$P > \|Z\|$
Dynabeads	–0.03	0.7796	—	—
Rappaport-Vassilaides	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

[1]No positive samples were detected by this method using brilliant green; therefore, no value was calculated.

opment 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., 1994; Hammack et al., 1998). Regardless of the method of choice, the physical and chemical compositions of the sample matrix has been shown to affect the isolation ability of a protocol; therefore, the method 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., 1994; Cudjoe and Krona, 1997; Hsih and Tsen, 2001). The reasons for the failure of the DB protocol may be attributed to the composition of the sample matrix (inclusion of inhibitory substances, physical composition, or both) and the presence of low concentrations of *Salmonella* cells (Skjerve and Olsvik, 1991; Davies et al., 2000). Previously, cheesecloth has been used to remove such inhibitory components when evaluating immunomagnetic separation 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, which is not recommended in the manufacturer's guidelines provided with the 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 that the κ analysis indicated that these methods, although having no difference in isolation frequencies, did not agree on the status of the same samples. In other words, these 2 methods did not always identify the same sample as positive or negative. This variation could 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 common 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 with that of TR results in an increased false-negative rate, which under an on-farm Hazzard Analysis Critical Control Point (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 selective agar plates used.

The comparison between the 2 selective agar plates employed revealed that the plates had high sensitivity and agreement when the TR protocol was used; however, this was not true when with any of the other 3 protocols. For TT and RV, the κ values were 0.42 and 0.46, respectively, which indicated that when using either of these broths, the plates only had moderate agreement. Similar results were observed when the comparisons were made with drag swab samples.

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

There are increased discussions that to best control foodborne pathogens, the next step is to implement a control system on the farm (Luedtke et al., 2003; McKee, 2004). Although 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, 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 when using a specific method. It has also been shown that TT when used with RV 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 provided for a more accurate risk assessment for this foodborne pathogen in broiler chicken grow-out houses.

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