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Evaluation of Two Isolation and Two Nonisolation Methods for Detecting Naturally Occurring Salmonellae from Broiler Flock Environmental Drag-Swab Samples

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ABSTRACT

One hundred and fifty-five environmental drag-swab samples, collected from 15 broiler flocks, were examined for the presence of salmonellae using a conventional and modified isolation method and two nonisolation assays. The conventional isolation method consisted of primary overnight enrichment in Hajna's tetrathionate broth at 41°C followed by inoculation to xylose-lysine desoxycholate and brilliant green agar plating medium. The modified isolation method employed the same overnight enrichment broths inoculated to xylose-lysine-tergitol 4 agar and brilliant green agar with novobiocin plating media. In the modified isolation method, any samples found *Salmonella* negative after primary enrichment were further evaluated using delayed secondary enrichment. The two commercial nonisolation assays examined were an enzyme-linked immunosorbent assay (ELISA) and a colorimetric DNA hybridization assay (DNAHA). Salmonellae were detected in 60 samples using the conventional isolation method and in 73 samples using the modified isolation method. Salmonellae were presumptively detected in 72 samples using the ELISA and in 67 samples using the DNAHA. The improved selectivity observed with the modified isolation method was primarily due to the incorporation of the xylose-lysine-tergitol 4 plating medium. The results from the modified isolation method revealed that both the ELISA and DNAHA were more specific, but less sensitive, than was indicated by the results from the conventional isolation method.

Recently, several nonisolation assays have been evaluated for their ability to presumptively detect *Salmonella* in a variety of foods and food environments. These include enzyme-linked immunosorbent assays (ELISA) (*1,2,4,7,9,11,15*) and DNA hybridization assays (DNAHA) (*1,3-8,17*). The use of a sensitive and reliable culture isolation method is critically important in assuring valid cross-comparisons with these newer nonisolation assays (*11,18*).

Many of these studies have evaluated ELISA and DNAHA kits for *Salmonella* detection using culture isolation methods to verify the results of the nonisolation assays. Unfortunately, the evaluations employed a variety of plating media that appeared less sensitive than the nonisolation assays being assessed. Consequently, the validity of these comparisons was compromised in situations where the nonisolation assay gave a positive result and the isolation method gave a negative result.

In a nonspiked naturally contaminated specimen, a false-positive nonisolation result cannot be differentiated from a false-negative isolation result. A number of studies have raised concerns about which detection method was correct (*1,2,4,7,11*). In general, these researchers concluded that many of the isolation-negative samples actually contained salmonellae, but the plating media were inadequate for isolation due to overgrowth of competitive bacteria. However, the reverse concern must also be recognized in that nonisolation assays have been shown to cross-react with nonsalmonellae *Enterobacteriaciae* (*2,4,7*) or react with cellular debris from nonviable salmonellae or other antigens within the biological specimen (*7,15*) causing false-positive results.

Several studies (*14,16,18,19*) have shown that delayed secondary enrichment (DSE) improved the *Salmonella* recovery rate from poultry and other samples. Recently, the use of brilliant green agar containing 20 mcg novobiocin per ml (BGAN) (*18*) and xylose-lysine-tergitol 4 (XLT4) (*12,13*) plating media have both been shown to significantly increase the isolation rate of non-*typhi Salmonella* from poultry specimens. These modified plates, and the use of DSE, improved the detection sensitivity of the cultural schemes in which they were employed.

The present study was undertaken to evaluate a conventional and modified *Salmonella* isolation method and to compare each to two commercially available nonisolation assays: an ELISA kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and a colorimetric DNAHA kit (GENE-TRAK Systems, Framingham, MA). These comparisons were done to determine the sensitivity and specificity of each nonisolation assay based on the individual results of the conventional and modified isolation methods.

MATERIALS AND METHODS

Primary enrichment of naturally contaminated drag-swab samples

One hundred and fifty-five environmental drag-swab (*10-12,18*) samples were collected from 15 broiler flocks. Each drag-

swab was premoistened with 7-8 ml of double-strength skim milk (Difco Laboratories, Detroit, MI). After collection, all drag-swab samples were held in sterile whirlpak bags for 1 to 5 d at 4-5°C before analyses. Each drag-swab was then placed in 60 ml of Hajna's tetrathionate broth (TT broth, Difco) and incubated for 22-24 h in a 41°C air incubator.

Isolation methods

Conventional. Each primary overnight TT broth sample was streaked onto xylose-lysine-desoxycholate agar (Difco) and brilliant green agar (BBL Inc., Cockeysville, MD) plating medium.

Modified. The same primary overnight TT broths were streaked onto XLT4 and BGAN plating media. In addition, any samples found *Salmonella* negative after primary enrichment underwent the following DSE procedure. The primary TT broths were held at room temperature an additional 5-7 d after which 0.5 ml of each TT broth sample was transferred to 10 ml of fresh TT broth. The secondary TT broths were incubated at 41°C overnight and restreaked to the XLT4 and BGAN plating media.

Examination of plates. All plates were examined after 24 and 48 h at 35°C. Representative colonies resembling *Salmonella* were selected and transferred to slants of triple sugar iron agar (Difco) and lysine iron agar (Difco). Slide-agglutination serologies (A-I polyvalent O antisera, Difco) were performed on those culture slants that biochemically indicated salmonellae. The API 20E identification system (Analytab Products, Inc., Plainview, NY) was used to identify suspect colonies when the serologies showed negative reactions. A representative number of *Salmonella*-positive cultures were submitted to the Veterinary Services Laboratory (Ames, IA) for serotyping.

Nonisolation methods

The same 155 primary overnight TT broth samples were used to evaluate both the ELISA and DNAHA kits. The manufacturer's instructions were followed for both nonisolation assays with the following exception to the DNAHA procedure. Selenite-cystine broth was not used in conjunction with TT broth as a primary enrichment broth. Only overnight TT broth cultures were used to inoculate the DNAHA postenrichment gram negative (Difco) broths, as described in the DNAHA method below. All reagents and equipment were supplied by the manufacturers.

ELISA. The ELISA colorimetric reactions were read using a Titertek Multiscan Multichannel Spectrophotometer (Flow Laboratories, McClean, VA) at a wavelength of 405 nm to determine the optical density (OD) values. The average positive and negative control values were determined. The corrected positive control value was determined by subtracting the average negative control OD from the average positive control OD. Sample-to-positive (S/P) control ratios were calculated in accordance with the following formula recommended by the ELISA kit manufacturer:

$$S/P = \frac{\text{Avg. sample OD - Avg. negative control OD}}{\text{Corrected positive control}}$$

S/P ratio values of <1.0 were considered negative and values of 1.0 and greater were considered presumptive positive based on numerous previous comparisons with similar drag-swab samples.

DNAHA. For the postenrichment step, one ml from each of the 155 primary TT broths was transferred to 10 ml of gram-negative broth and incubated 16-18 h at 35°C prior to DNAHA analysis.

The DNAHA colorimetric optical density measurements were read using a GENE-TRAK® spectrophotometer at a wavelength of 450 nm. Based on the manufacturer's recommendations, OD readings of less than 0.1 (after subtracting the negative control OD reading) were considered negative and OD readings of 0.1 and greater were considered presumptive positive.

For the ELISA and DNAHA results, false positives (FP) are defined as those samples displaying OD readings in the positive range, from which *Salmonella* is not found by a given isolation method. False negatives (FN) are defined as those samples displaying OD readings in the negative range, from which *Salmonella* is found by a given isolation method. True positives (TP) are defined as those samples displaying OD readings in the positive range from which *Salmonella* is found by a given isolation method. True negatives (TN) are defined as those samples displaying OD readings in the negative range from which *Salmonella* is not found by a given isolation method.

$$\text{Definition of ELISA/DNAHA sensitivity: } \frac{TP}{TP + FN} \times 100\%$$

$$\text{Definition of ELISA/DNAHA specificity: } \frac{TN}{TN + FP} \times 100\%$$

RESULTS AND DISCUSSION

Salmonellae were detected in 60 samples using the conventional isolation method and in 73 samples using the modified isolation method. The 73 samples found positive by the modified isolation method included all 60 samples shown positive by the conventional isolation method. For the 73 isolation-confirmed positive samples, the conventional isolation method gave 13 false-negative results and the modified isolation method gave 0 false-negative results. Using the modified isolation method, 72 of the 73 positive samples were detected on the XLT4 plating medium and 68 were detected on the BGAN plating medium. Only one negative primary TT broth sample was found to be *Salmonella* positive after delayed secondary enrichment. For that one sample, none of the four detection methods detected *Salmonella* from the primary enrichment broth.

The ELISA presumptively detected *Salmonella* in 72 samples and the DNAHA presumptively detected *Salmonella* in 68 samples. Table 1 describes the number of ELISA and DNAHA results considered false positive or negative based on the results from the conventional isolation method. Table 2 compares the same ELISA and DNAHA results based on the results from the modified isolation method. Tables 1 and 2 also show the sensitivity and specificity of each nonisolation assay based on the results from the conventional and modified isolation method, respectively.

As described in these two tables, the number of false-positive ELISA and DNAHA results dramatically decreased when they were compared to the results from the modified isolation method. Therefore, the modified isolation method results revealed that the ELISA and DNAHA were both more *Salmonella*-specific than the conventional isolation method results indicated. Conversely, the number of false-negative ELISA and DNAHA results increased when they were compared to the modified isolation method results. Therefore, the modified isolation method results revealed that the ELISA and DNAHA were both less sensitive in detecting *Salmonella* than the conventional isolation method results indicated.

This study demonstrates the need for using a more sensitive isolation method when evaluating the detection capabilities of nonisolation assays such as ELISA and

TABLE 1. *Evaluation of the ELISA and DNAHA results based on results from the conventional isolation method showing 60 positive and 95 negative drag-swab samples.*

	No. +/No. -	No. FP (%)[1]	No. FN (%)[2]	Specificity (%)[3]	Sensitivity (%)[4]
ELISA	72/83	12 (16.7)	0 (0)	87.4	100
DNAHA	67/88	9 (13.4)	2 (2.3)	90.5	96.8

[1] FP = false positive.
[2] FN = false negative.
[1] Specificity = $\frac{\#\text{ True negative (TN)}}{\#\text{ TN} + \#\text{ FP}}$ X 100%.
[2] Sensitivity = $\frac{\#\text{ True positive (TP)}}{\#\text{ TP} + \#\text{ FN}}$ X 100%.

TABLE 2. *Evaluation of the ELISA and DNAHA results based on results from the modified isolation method showing 73 positive and 82 negative drag-swab samples.*

	No. +/No. -	No. FP (%)[1]	No. FN (%)[2]	Specificity (%)[3]	Sensitivity (%)[4]
ELISA	72/83	3 (4.2)	4 (4.8)	96.3	94.5
DNAHA	67/88	0 (0)	6 (6.8)	100	91.8

[1] As described in note 1, Table 1.
[2] As described in note 2, Table 1.
[3] As described in note 3, Table 1.
[4] As described in note 4, Table 1.

DNAHA. As shown in this study, the modified isolation method was more sensitive than the conventional isolation method in detecting *Salmonella*.

These findings also indicate that the sensitivity of the modified isolation method was equal to or slightly exceeded those of the ELISA and DNAHA. This increased parity was underscored by the better overall data agreement between the modified isolation method and the two nonisolation assays. The increased sensitivity of the modified isolation method was mainly due to the incorporation of XLT4 plating medium. Unlike other studies (*14,16,18,19*), the use of DSE did not significantly improve the *Salmonella* recovery rate, as only one additional negative primary enrichment broth became positive after DSE culture. Most likely, this was a reflection of the increased isolation efficiency of the XLT4 agar (*12,13*), which provided better recovery from the primary overnight TT broths.

Because the modified isolation method displayed greater sensitivity than did the conventional isolation method, comparisons between the ELISA and DNAHA were more interpretable. Beckers et al. (*2*) stated that, when using naturally contaminated samples, "a positive ELISA and a negative isolation test do not necessarily indicate a false positive ELISA, even when the presence of *Salmonella* could not be confirmed. Instead, such a result may indicate a false-negative isolation test". As indicated in this study, this assumption was shown to be valid when the comparisons were made based on the results of the less sensitive conventional isolation method. However, when the more sensitive modified isolation method results were used as a basis for comparisons, a dramatic decrease in ELISA/DNAHA false positives and an increase in ELISA/DNAHA false negatives was observed. In these latter instances, since the presence of *Salmonella* was confirmed by colony isolation, there was no disputing that the nonisolation assays failed to detect *Salmonella*. Hence, the modified isolation method provided more definitive data on the detection capabilities of the nonisolation assays because the number of conflicting nonisolation-positive, isolation-negative results was greatly reduced.

It is recommended that the described modified isolation method be used for isolating *Salmonella* from poultry and other nonspiked, naturally contaminated specimens, especially those that are likely to contain high numbers of nonsalmonellae. In addition, the authors suggest using the modified isolation method when performing cross-comparative evaluations of other nonisolation assays, such as ELISA and DNAHA.

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