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Identification of Salmonella enteritidis from Experimentally Infected Hens Using a Colorimetric DNA Hybridization Method

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SUMMARY. Identification of Salmonella enteritidis from cloacally challenged commercial laying hens was studied by comparing bacterial isolations using conventional methods with detection by the use of the GENE-TRAK® colorimetric DNA probe assay. More positive test results were obtained using the latter on days 14, 28, and 42 postchallenge, but the difference between the two methods was not statistically significant. Over the duration of the experiment, positive cloacal samples were statistically more frequent from a commercial strain of white leghorn hens when compared with a commercial brown egg-producing strain (28/60 vs. 9/57; chi-square 1 df = 12.9, \( P < 0.001 \)). Eggs having various shell defects were produced by the infected hens only after Salmonella challenge. These defects included, in order of frequency, elongated shape, thin shells, off-white color (tints), small size, wrinkles, and pimples. No Salmonella could be recovered from 193 defective eggs, nor were positive isolates made from additional tests performed on 50 normal eggs. Proteus sp. was isolated from 10 eggs, however. Our observations demonstrate that the GENE-TRAK colorimetric method is comparable with conventional bacteriology for the identification of Salmonella in cloacal samples taken from laying hens. Moreover, the two methods demonstrate the existence of breed differences in susceptibility to S. enteritidis challenge.

RESUMEN. Identificación de Salmonella enteritidis en gallinas infectadas experimentalmente mediante la utilización de un método colorimétrico de hibridación del ácido desoxirribonucleico.

Se estudió la identificación de Salmonella enteritidis a partir de gallinas ponedoras de salmónidas por la vía cloacal. Se compararon los métodos convencionales de aislamiento bacteriano con el uso de la sonda colorimétrica del ácido desoxirribonucleico, Gene-Track®. Con este último método se obtuvieron mayores resultados positivos a los 14, 28 y 42 días después del desafío; pero no hubo una diferencia estadísticamente significante entre los dos métodos. Durante el experimento, las muestras cloacales positivas fueron estadísticamente más frecuentes en las gallinas de una estirpe comercial leghorn blanca que en una estirpe comercial de huevo marrón (28/60 vs. 9/57; Chi-cuadrado 1 gr = 12.9 \( P < 0.001 \)).

En las gallinas infectadas, se obtuvieron huevos con varios defectos en la cáscara solamente después del desafío. Estos defectos incluyeron huevos alargados, con cáscara delgada, pérdida de color, tamaño reducido y huevos con cáscaras arrugadas y granulosas. No se pudo aislar la Salmonella de ninguno de los 193 huevos defectuosos ni de un grupo de 50 huevos normales. Sólomente se aisló Proteus sp. de 10 huevos.

Los resultados demostraron que el método colorimétrico Gene-Trak® es comparable con el método bacteriológico convencional para la identificación de Salmonella en muestras tomadas de la cloaca de ponedoras comerciales. Además, los dos métodos demostraron diferencias en la susceptibilidad entre las estirpes frente al desafío con S. enteritidis.

Key words: Salmonella enteritidis, DNA hybridization, cloacal samples
Abbreviations: CB = conventional bacteriology; GT-DNAH = GENE-TRAK® DNA hybridization; TSB = trypticase soy broth

Conventional methods for identification of *Salmonella* that employ selective and differential culture media for their isolation, and carbohydrate fermentation patterns for the specification of suspect bacteria (13) have been supplemented by newer more rapid techniques. One such method is GENE-TRAK® DNA hybridization (GT-DNAH), in which the presence of *Salmonella* in a sample is determined by the capture and detection of a species-specific rRNA target unique to *Salmonella* (4,5). The early version of this method that employed a short-lived radioactive reporter probe has been supplanted by a second generation procedure that is colorimetric, which obviates some of the difficulties associated with radioactivity (2).

The recovery of *Salmonella* from experimentally infected hens and their eggs was studied by comparing the relative efficiency of the early, radioactive GT-DNAH method to one using conventional bacteriology (CB). In those studies, more *Salmonella* were recovered using the GT-DNAH method than by CB, but the overall rate of infection was low (9,10). Additionally, some of the hens exposed to *S. enteritidis* via the cloacal route produced eggs with defective shells, 20% (10/49) of which also contained *Salmonella* (10).

The objectives of the present study were to compare CB with the newer colorimetric GT-DNAH by measuring the frequency of identification of *S. enteritidis* by both methods from cloacal swabs and eggs produced by hens after a cloacal challenge. Both a commercial white leghorn strain and a commercial brown egg-producing strain were included because susceptibility differences between breeds have been reported with other salmonellae (12). The duration of such infections was also studied, as was the postchallenge production of eggs having various shell defects.

**MATERIALS AND METHODS**

**Hens.** Adult laying hens were housed in an isolation facility and were placed individually in wall-mounted, wire egg-collection cages having sloped bottoms. Twenty brown and 20 white leghorn commercial strain egg-production types were included in the study. They were fed a commercial formula layer ration containing 20% crude protein and 2,860 kcal ME/kg. Feed and water were available *ad libitum*. All experimental hens were prechallenge negative for *S. pullorum* antibody, and negative for the presence of *Salmonella* bacteria as determined by sampling of their cloacas using both CB and GT-DNAH assay methods. Because of the frequency of isolating *Proteus* sp. from the eggs of hens challenged by *S. enteritidis* in our earlier studies (9,10), half of the hens (10) of each strain were challenged with *S. enteritidis* alone or combined with a *Proteus* sp. It was our hope that such a combined challenge would result in a higher incidence of eggs positive for *Salmonella*.

Eggs from each hen were saved for approximately 14 days prior to the challenge. They were examined for the presence of the types of shell defects associated with *S. enteritidis* as described in our earlier experiments (9,10). None were found.

**Bacteria and challenge.** A clinical isolate of *S. enteritidis* (CDC strain no. 1406-82) from human stool and a cloacal isolate of *Proteus* sp. obtained from a healthy hen were used as the challenge microorganisms. Log-phase trypticase soy broth (TSB) (BBL, Bethesda, Md.) cultures of each bacteria were diluted with additional TSB to the turbidity of a MacFarland No. 1 standard (Difco Laboratories, Detroit, Mich.), giving an estimated $3 \times 10^8$ bacteria per milliliter. One milliliter of *S. enteritidis* used alone, or combined with an additional 1 ml of the *Proteus* sp. culture were dosed directly into the cloaca of each manually immobilized hen. The inocula were delivered using a syringe fitted with a blunt catheter at a rate that allowed for the retention of nearly all of the liquid.

**Sample collection.** Cloacal samples were collected on postchallenge days 14, 28, and 42, using sterile cotton swabs that were placed immediately into tubes containing 10 ml of 1% peptone broth (Difco). Tissue sample sections were obtained aseptically at the termination of the experiment (day 50) from the ovary, the ceca, and the large bowel of each hen.

Eggs were collected daily, marked with the hen number, dated, and stored at room temperature until testing at the end of each of three collection intervals over the course of the experiment; on postchallenge days 14, 28, and 42. This practice was deemed necessary for pragmatic reasons so that sufficient numbers would be available to justify the expenditure of resources needed to conduct both CB and GT-DNAH. Groups of three unwashed eggs from individual hens were placed into a small plastic bag, broken by crushing, and their entire contents (shell included) were homogenized manually for 30 sec. One-milliliter aliquots of such homogenates were used for further bacteriological testing.
Table 1. Identification of *Salmonella* from postchallenge cloacal swabs using CB and GT-DNAH.

<table>
<thead>
<tr>
<th>Hen type</th>
<th>Testing method</th>
<th>Days postchallenge</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>White</td>
<td>CB</td>
<td>11/20*</td>
<td>7/20</td>
</tr>
<tr>
<td>Brown</td>
<td>CB</td>
<td>4/19</td>
<td>0/19</td>
</tr>
<tr>
<td>White</td>
<td>GT-DNAH</td>
<td>14/20</td>
<td>9/20</td>
</tr>
<tr>
<td>Brown</td>
<td>GT-DNAH</td>
<td>5/19</td>
<td>1/19</td>
</tr>
</tbody>
</table>

*No. positive cloacal isolates/no. hens in group.

*Chi-square, total cloacal positives white *vs* brown hens using CB = 9.6, *P* < 0.01.

*Chi-square, total cloacal positives white *vs* brown hens using GT-DNAH = 6.8, *P* < 0.01.

**Conventional bacteriology.** Ten milliliters of 1% peptone broth cultures were incubated for 18 hr at 39°C, the pre-enrichment phase. One milliliter aliquots including the swab (where applicable) were transferred to 10 ml tetrathionate broth (Difco) containing 0.01% brilliant green and sulfathiazole (Sigma Chemicals, St. Louis, Mo.) (1.25 μg/ml) and incubated for an additional 24 hr the selective enrichment phase. Brilliant green agar (Difco) and Hektoen enteric agar (Difco) plates were streaked from the tetrathionate broths and these were incubated at 37°C for 24 hr. Suspect *Salmonella* colonies were transferred into triple sugar iron agar (Difco) and lysine iron agar (Difco) slants and incubated overnight at 37°C. Slants showing reactions consistent with known positive controls were tested for carbohydrate fermentation reactions typical of *Salmonella* (3) using the MiniTek® disk system (BBL). Positive isolates were serotyped using group-specific antisera (Difco) for somatic factors 4, 5, and 9.

**Colorimetric DNA hybridization assay.** GN broths (Difco) were inoculated from the same tetrathionate broths as those used for CB, and assayed according to the instructions provided by the manufacturer (GENE-TRAK Systems, Framingham, Mass.). Briefly, the GN broth cultures were incubated at 35°C for 6 hr. then they were treated with alkaline lysis solution, which releases the target nucleic acids. After neutralization, DNA probes were added that are capable of hybridizing specifically with *Salmonella* rRNA. One of these is tailed with poly deoxyadenylic acid, whereas the other is labeled with a fluorescein, acting as a hapten in a later detection step. A plastic dipstick coated with long polymers of deoxythymidinonic acid is then added. The probe–target complex binds to the dipstick, which is then reacted with enzyme-labeled anti-fluorescein. Color was generated by exposing the dipstick to a substrate–chromogen mixture, and its intensity was compared to that of both positive and negative controls included with the kit.

**Statistical analyses.** Data were analyzed by the contingency chi-square or analysis of variance methods using the Minitab® statistical software package (Minitab Inc. State College, Pa.).

**RESULTS**

The duration of infection was measured by determining if *Salmonella* could be identified from cloacal swabs taken on three postchallenge occasions (Table 1). Both CB and GT-DNAH methods were applied to each specimen by using the tetrathionate broths as the source of material for further assay. Thus the data presented in Table 1 represent the comparative capability of the two methods (CB *vs* GT-DNAH) to detect any *Salmonella* present at the point of selective-enrichment. Because there were no statistically significant differences in recoveries (chi-square 1 df = 0.65, 0.5 > *P* > 0.75) between hens challenged with both *Salmonella* and the *Proteus* sp. versus those challenged with *Salmonella* alone, these results were combined.

Although there was no statistically significant difference between the two assay methods in detecting *Salmonella* based on the total number of cloacal recoveries (26 by CB and 37 by GT-DNAH), the latter method was numerically superior at each sample occasion (chi-square, 1 df = 2.63, 0.75 > *P* > 0.90). Cloacal recovery of *S. enteritidis* was consistently higher from the white leghorns compared with the brown egg producers (chi-square, 1 df = 12.9, *P* < 0.001). The total egg production, the number of defective eggs, and the number of eggs from which bacteria other than *Salmonella* could be isolated are given in Table 2. There were no statistically significant differences between brown versus white egg types for total egg production, or the number of defective eggs (ANOVA, *F* = 3.97, *P* = 0.08; and *F* = 2.51, *P* = 0.15, respectively). Egg defects included, in order of frequency, elongated shape, thin shells, off-white
Table 2. Total and defective egg production and bacteriological test results from experimental hens cloacally challenged with S. enteritidis.

<table>
<thead>
<tr>
<th>Hen type</th>
<th>No. eggs</th>
<th>No. defective</th>
<th>%</th>
<th>No. assayed</th>
<th>%</th>
<th>No. Salmonella positive</th>
<th>No. bacteria positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td>490*</td>
<td>116*</td>
<td>24</td>
<td>102</td>
<td>21</td>
<td>0</td>
<td>5c</td>
</tr>
<tr>
<td>Brown</td>
<td>570</td>
<td>77</td>
<td>13</td>
<td>139</td>
<td>24</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

*ANOVA total egg production, $F = 3.97$, $P = 0.08$.
*ANOVA defective egg production, $F = 2.51$, $P = 0.15$.

The few with gross fecal contamination were excluded. Additional assays where performed on some normal-looking eggs collected from hens having a history of a positive cloacal swab sample.

No Salmonella were identified from any of the eggs by either assay method; however, other bacteria were recovered from 10 of 241 (4.6%) eggs assayed. Ten of these isolates were identified as Proteus sp. using CB. Nine of the 10 Proteus isolates came from hens challenged with Salmonella enteritidis plus Proteus sp., but it was not determined if these isolates were identical to the inoculated species. The remaining isolate was not identified.

Gross necropsy results performed on all experimental hens on day 50 are given in Table 3. Tissue samples from the ceca, ceca, and colon were removed aseptically and assayed for the presence of Salmonella using CB only. All results were negative. However, the tetrathionate of two of the colon specimens taken from white leghorns became suspiciously cloudy after prolonged (>7 day) incubation at room temperature. Salmonella were subsequently identified from both using GT-DNAH. One of these specimens was obtained from a hen with a history of two previous positive cloacal swab samples, and the other from a hen with no prior positive cloacal samples.

DISCUSSION

The results of the present study agree with our earlier observations that showed that GT-DNAH is comparable with CB in detecting Salmonella from cloacal samples (9,10). Although more samples were found to be positive using the GT-DNAH method, the difference was not significant. Moreover, there was no instance in which a specimen found positive by CB was not
also found positive by the GT-DNAH method. Alternately, there were nine cloacal samples identified as positive using GT-DNAH from which *S. enteritidis* was not identified by CB. Although speed was not considered in the experimental design, our observations imply that the potentially more rapid GT-DNAH method offers some advantage in obtaining results, while not sacrificing the sensitivity of CB.

The apparent infection rate declined from a high of 19 of 39 total hens (49%) on postchallenge day 14 to a low of 8 of 39 (20%) by day 42 as shown in Table 1. A similar decline in the apparent infection rate was observed by us earlier (9,10) and was found by Shivaprasad et al. (11) who observed fecal shedding for 17–21 days, depending on the trial, after intracloacal inoculations of commercial white leghorns. Moreover, Gast and Beard (6) reported a significant decline in recoveries after 14 days in orally challenged hens.

Positive tests were more frequently obtained from the white leghorn strain throughout the experimental period. Moreover, they produced fewer eggs during the post-challenge period (Fig. 1), a numerically higher proportion of which were defective. Two of the leghorns were found by necropsy to be out of production, and three were found to contain an internal egg (Table 3). Taken together these observations suggest they were more susceptible to the challenge as compared to the brown egg strain.

There were several noteworthy observations regarding the cloacal swab recoveries. First, there were two hens from which a negative sample was bracketed by two positive samples (+, −, +). There was a single example of a positive sample occurring only at the last sample (−, −, +), and an additional case of three negative cloacal samples but with a positive necropsy sample from colon tissue. Such observations are difficult to interpret but might be due to the transient presence (intermittent shedding) of the bacteria in the colon, or they may reflect deficiencies in either the sampling techniques (the use of a swab) or the detection methods.

The occurrence of the delayed positive samples from two tetrathionate broths, as mentioned above, suggests that on occasion the number of viable bacteria in a sample is initially very small. Reaching the minimal level required for detection may not be possible in the time usually allotted to either the pre-enrichment or selective enrichment phases of the assay methods.

The production of eggs having various shell defects after cloacal exposure to *S. enteritidis* was reported in our earlier study (9,10) and was repeated in the present study. *Salmonella enteritidis* could not be recovered from any of the 195 defective or 50 normal eggs tested by either assay method, however. The presence of four internal eggs detected at necropsy (Table 3) may explain in part the production of shell abnormalities. Their presence in the abdomen may cause an unusual pressure to be exerted on other eggs during their time in the shell gland. Internal eggs were detected at necropsy in our earlier study, some of which were positive for *S. enteritidis*, others were not. Moreover, in one instance, 13 normal-appearing salmonella-negative eggs were recovered from a single hen in which a salmonella-positive internal egg was detected at necropsy (9,10). Misshapen ovules, and egg peritonitis were reported by Hopper and Mawer (7) from hens identified epidemiologically as the source of raw eggs that caused an outbreak of human food poisoning. No mention of shell defects was made in that report, nor were such defects reported by Lister (8) who found similar lesions in a broiler flock positive for *S. enteritidis*.

Ten eggs were positive for the presence of *Proteus* sp. and one additional unidentified isolate was made from another egg using CB. These could have been fecal contaminants, but the low (11/1060, 1%) level of bacteria in the eggs suggests that our method of including the shell along with the internal contents does not result in gross bacterial contamination of the sample.

<table>
<thead>
<tr>
<th>Hen type</th>
<th>No. of observations*</th>
<th>No. of <em>Salmonella</em> isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NVL FL OP IE</td>
<td>Ceca Colon Ovary</td>
</tr>
<tr>
<td>White</td>
<td>20 11 1 2 3</td>
<td>0 0/2* 0</td>
</tr>
<tr>
<td>Brown</td>
<td>19 13 5 0 1</td>
<td>NEC NEC</td>
</tr>
</tbody>
</table>

*NVL = no visceral lesions; FL = fatty liver; OP = out of production; IE = internal egg.

*Positive by GT-DNAH only after prolonged incubation at room temperature.

NE = not examined.

Table 3. Gross necropsy observations and conventional bacteriology results from hens cloacally challenged by *S. enteritidis* obtained at postchallenge day 50.
Our earlier experience (10) in isolating Proteus sp. from eggs prompted the decision to include it in half of the challenge inoculations. However, there was no statistically detectable effect on any of the experimental parameters due to the use of the mixed inoculum.

In conclusion, our present results support our earlier demonstration of the usefulness of the GT-DNAH method in detecting Salmonella in cloacal samples taken from poultry. This method seems to be at least as sensitive as the CB method, and offers a potential advantage in the time needed to complete the assay. Moreover, the colorimetric procedure is free of any disadvantage associated with the requirement for a radioactive probe. Our observations with experimental Salmonella enteritidis infection in laying hens may be of interest to those investigating this emerging human health problem (1).

REFERENCES