Detecting Infections of Chickens with Recent Salmonella pullorum Isolates Using Standard Serological Methods

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ABSTRACT Despite extensive testing for Salmonella pullorum infections in commercial poultry, occasional outbreaks of pullorum disease still occur. In some recent instances, questions have been raised about whether standard serological assays, which employ antigen strains selected several decades ago, are still able to detect currently prevalent S. pullorum strains. The present study evaluated the ability of standard rapid whole-blood plate agglutination test and serum tube agglutination test antigens to detect infection in Single Comb White Leghorn hens inoculated with large oral doses of six recent S. pullorum isolates. Two commercially available plate test antigens were obtained and three tube test antigens were prepared. All five antigens identified most inoculated hens as seropositive, although some differences in sensitivity were evident between the two assay types and between the two plate test antigens. The antigenic composition of the tube test antigens did not affect their ability to detect infections with the various inoculum strains. Regardless of the antigen used, hens infected with antigenically intermediate or variant S. pullorum strains were detected as seropositive less often than were hens infected with antigenically standard strains. Positive culturing results for S. pullorum in the livers and ovaries of infected hens were nearly always predicted by positive serological test results.

(Key words: Salmonella pullorum, pullorum disease, chicken, serology)

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INTRODUCTION

Infections with Salmonella pullorum can result in acute systemic disease and a high incidence of mortality in young poultry. Vertical transmission of S. pullorum from infected parent flocks, magnified by horizontal transmission in the hatcher, can cause economically devastating losses among chicks or poults (Snoeyenbos, 1991). In many developing nations, S. pullorum infections of poultry are common and pullorum disease remains among the principal disease threats to poultry producers (Bhattacharyya et al., 1984; Bouzoubaa and Nagaraja, 1984; Majid et al., 1991; Nabbut, 1993). In most developed nations, however, the incidence of pullorum disease in commercial poultry has remained low for many years. In the U.S., the National Poultry Improvement Plan (NPIP) was inaugurated in 1935. This plan, which is a voluntary effort involving the cooperation of the poultry industry, state agencies, and the USDA, relies on the testing of breeding flocks to prevent disease transmission to progeny (USDA, 1994). The implementation of the NPIP testing program resulted in a dramatic decline in the incidence of pullorum disease in U.S. commercial flocks (Snoeyenbos, 1991). Most U.S. pullorum disease outbreaks in recent years have been reported in small or backyard poultry flocks, which may serve as potential reservoirs for disease transmission to commercial flocks (Erbeck et al., 1993).

Although the principal current economic significance of S. pullorum in developed nations is the cost of testing programs, reminders of the potential for catastrophic losses have been provided by the occasional appearance of pullorum disease in commercial flocks. In 1990, a major pullorum disease outbreak was reported in an integrated broiler operation in the U.S. (Salem et al., 1992). The outbreak involved 22 parent flocks and more than 150 roaster flocks in five states, and was ultimately traced back to an infected grandparent male-line breeding flock (Johnson et al., 1992). One particularly disturbing outcome of the investigation of this outbreak was the isolation of S. pullorum from cultured tissues taken from parent flocks that had earlier tested negative in NPIP-mandated serological screening (Johnson et al., 1992). This finding suggests that either these flocks became infected after testing (which would be inconsistent with the usual vertical transmission paradigm for pullorum disease) or the serological testing failed to detect existing infections within the flocks.

The NPIP program for controlling S. pullorum is based principally on the detection of infected breeding flocks before they begin producing eggs (USDA, 1994). Breed-
**TABLE 1. Salmonella pullorum isolates used to inoculate chickens**

<table>
<thead>
<tr>
<th>Isolate designation</th>
<th>Identification number</th>
<th>Antigenic category</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8862</td>
<td>Variant</td>
<td>Delaware (1)</td>
</tr>
<tr>
<td>B</td>
<td>3142</td>
<td>Standard</td>
<td>Missouri (1)</td>
</tr>
<tr>
<td>C</td>
<td>51-0071</td>
<td>Intermediate</td>
<td>Maryland (2)</td>
</tr>
<tr>
<td>D</td>
<td>50-1048</td>
<td>Standard</td>
<td>Maryland (2)</td>
</tr>
<tr>
<td>E</td>
<td>50-1313</td>
<td>Intermediate</td>
<td>Maryland (2)</td>
</tr>
<tr>
<td>F</td>
<td>32460</td>
<td>Standard</td>
<td>Texas (1)</td>
</tr>
</tbody>
</table>

1Standard *S. pullorum* strains express somatic antigens 9, 121, and 123; intermediate strains express antigens 9, 121, 122, and 123; and variant strains express antigens 9, 121, and 122.

2*Salmonella pullorum* isolates were provided by L. Thomas, USDA-APHIS-NVSL (1) and G. Stein, Maryland Department of Agriculture (2). All isolates were obtained from tissues of infected chickens in commercial flocks.

ING flocks are screened for specific serum antibodies, using a rapid whole-blood plate agglutination test, at about 16 wk of age. This test employs commercially available stained *S. pullorum* antigen preparations and can be performed relatively quickly in poultry housing facilities. Serum samples from birds that yielded positive results in the screening test are frequently submitted to diagnostic laboratories for further serological evaluation, most often by a tube agglutination test with an antigen prepared locally according to NPIP specifications. Various internal tissues from seropositive birds are then excised and cultured for *S. pullorum* to confirm the diagnosis.

The possibility that serological testing for *S. pullorum* failed to detect infected birds during the 1990 pullorum disease outbreak raises questions about the adequacy of the traditional serological methods and antigens for detecting infections with current field strains of *S. pullorum*. Moreover, *S. pullorum* exists in three somewhat unstable antigenic forms (designated standard, variant, and intermediate), which express different proportions of somatic antigens 121 and 123 (Edwards and Bruner, 1946; Edwards *et al.*, 1948; Snoeyenbos *et al.*, 1950). The commercial rapid whole-blood plate agglutination test antigens incorporate both standard and variant strains, but tube agglutination test antigens are generally prepared using three antigenically standard reference strains. The effect of antigenic form variation on the detection of *S. pullorum* infections by serological testing remains unclear. The objective of the present study was to determine whether the rapid whole-blood plate agglutination test and the tube agglutination test, conducted according to NPIP specifications, could effectively identify chickens experimentally infected with strains of *S. pullorum* isolated from commercial poultry during recent disease outbreaks.

**MATERIALS AND METHODS**

**Hens**

In each of two trials, 78 Single Comb White Leghorn hens from the specific-pathogen-free flock at our laboratory were housed in single-bird cages in a disease-containment isolation building. All hens were derived from the same hatch and were 28 and 38 wk old at the beginning of the first and second trials, respectively. Each bird was provided *ad libitum* access to water and an antibiotic-free layer-breeder ration (16.7% CP, 2,968 kcal ME/kg, 2.9% Ca, 0.39% P).

**Salmonella pullorum Cultures**

All nine *S. pullorum* isolates used (Table 1) were originally stored at −20 C on Protect cryopreservative storage beads. These isolates were cultured from tissues of infected commercial chickens in four states during recent pullorum disease outbreaks, and included at least one representative of each of the three antigenic forms. Inoculum cultures were prepared by transferring one frozen storage bead into 9 mL of tryptone soya broth1 and incubating the culture for 24 h at 37 C. Each culture was then diluted in 0.85% saline to contain approximately 1.5 x 10⁸ cfu/mL (confirmed by subsequent plate counts).

**Experimental Design**

In each trial, the hens were allocated to three treatment groups of 24 hens each plus an uninfected negative control group of 6 hens. Each group of hens was housed in a separate room. Three-fourths of the hens in each treatment group were orally inoculated with 1 mL of diluted *S. pullorum* culture. Every fourth hen in each treatment group was left uninoculated to evaluate the possibility of horizontal contact transmission of infection. Isolates A, B, and C were each administered to treatment groups in the first trial and isolates D, E, and F were similarly administered in the second trial.

Blood was collected from the brachial vein of every bird before inoculation and at 6 weekly postinoculation intervals. Each blood sample was immediately assayed for specific antibodies in rapid whole-blood plate agglutination tests conducted according to NPIP specifications.

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1Unipath Co., Oxoid Division, Ogdensburg, NY 13669.
(USDA, 1994), using commercially-prepared Solvay\(^2\) and Vineland\(^3\) S. pullorum antigens. Each blood sample was tested with each of the two plate test antigens. Serum extracted from each blood sample was also assayed for specific antibodies in tube agglutination tests conducted according to NPIP specifications (USDA, 1994), using three S. pullorum antigens prepared in our laboratory. Antigen 1 incorporated the three antigenically standard S. pullorum reference strains (numbers 17, 19, and 20) most often used to prepare tube test antigens for diagnostic purposes. Antigen 2 was prepared using S. pullorum inoculum Strains A, B, and C (and thus included one antigenically standard, one intermediate, and one variant strain). Antigen 3 was composed of S. pullorum inoculum Strains D, E, and F (and thus included two antigenically standard strains and one intermediate strain). Each serum sample was tested with each of the three tube test antigens.

At 2 wk and again at 6 wk postinoculation, eight hens from each treatment group (including six hens orally inoculated with S. pullorum and two contact-exposed hens) plus two negative control hens were euthanatized by cervical dislocation. Samples (approximately 5 to 10 g each) of the liver and ovary of each hen were removed, mixed with 50 mL tetrathionate broth,\(^1\) stomached for 30 s in a Stomacher Model 80 Lab Blender,\(^4\) and incubated 24 h at 37 C. Loopfuls of broth were then streaked onto brilliant green agar\(^5\) supplemented with 0.02 mg/mL novobiocin\(^6\) and incubated 24 h at 37 C. The tetrathionate broth cultures were also retained for delayed secondary enrichment (Waltman and Horne, 1993) by incubating them for an additional 5 d at 25 C, after which 1 mL was transferred to a fresh 9-mL tube of tetrathionate broth, incubated for 24 h at 37 C, and streaked onto brilliant green agar as described above. The identity of suspected S. pullorum colonies on brilliant green agar plates was confirmed biochemically and serologically (Mallinson and Snoeyenbos, 1989).

**Statistical Analysis**

Significant differences (P < 0.05) between the mean frequencies of detection of S. pullorum infection by the various serological tests and antigens were determined by applying Fisher’s exact test to data organized into 2 × 2 contingency tables (Snedecor and Cochran, 1980).

**RESULTS**

**Detection of Serum Antibodies to S. pullorum by Agglutination Tests**

Antibodies to S. pullorum were more often detected by rapid whole-blood plate tests using the Solvay antigen than by similar tests using the Vineland antigen during the first 2 wk after inoculation (Table 2). The frequency of detection of antibodies in plate tests was significantly greater using the Solvay antigen than using the Vineland antigen among hens infected with Strain A and for all six inoculum groups combined (58.0% for the Vineland antigen and 60.8% for the Solvay antigen; Table 3). No significant differences in detection frequency were observed between the two plate test antigens (58.0% for the Vineland antigen and 60.8% for the Solvay antigen; Table 3).

## Table 2. Detection of antibodies by standard serological tests 1 to 2 wk after experimental infection of hens with recent Salmonella pullorum isolates

<table>
<thead>
<tr>
<th>S. pullorum inoculum strain(^4)</th>
<th>Rapid whole-blood plate test(^2)</th>
<th>Tube agglutination test(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vineland antigen</td>
<td>Solvay antigen</td>
</tr>
<tr>
<td>A</td>
<td>18.8(^a)</td>
<td>41.7(^b)</td>
</tr>
<tr>
<td>B</td>
<td>50.0(^b)</td>
<td>56.3(^b)</td>
</tr>
<tr>
<td>C</td>
<td>35.4(^b)</td>
<td>45.8(^b)</td>
</tr>
<tr>
<td>D</td>
<td>54.2(^a)</td>
<td>64.6(^a)</td>
</tr>
<tr>
<td>E</td>
<td>50.0(^b)</td>
<td>54.3(^b)</td>
</tr>
<tr>
<td>F</td>
<td>56.3(^b)</td>
<td>70.8(^ab)</td>
</tr>
<tr>
<td>All strains</td>
<td>44.0(^b)</td>
<td>55.6(^b)</td>
</tr>
</tbody>
</table>

\(^a-c\)Values within rows with no common superscript differ significantly (P < 0.05).

\(^1\)Twenty-four hens per group, each tested at 7 and 14 d postinoculation, for a total of 48 samples per group.

\(^2\)Salmonella pullorum plate test antigens were obtained from Vineland Laboratories, Vineland, NJ 08360 and Solvay Animal Health, Mendota Heights, MN 55120.

\(^3\)Salmonella pullorum tube test antigens were prepared in our laboratory: Antigen 1 from standard reference strains 17, 19, and 20; Antigen 2 from inoculum strains A, B, and C; and Antigen 3 from inoculum strains D, E, and F.

\(^4\)Seventy-five percent of the hens in each group were orally inoculated with 10\(^8\) cfu of the indicated strain.
TABLE 3. Detection of antibodies by standard serological tests 5 to 6 wk after experimental infection of hens with recent
Salmonella pullorum isolates (standard, intermediate, and variant antigenic forms). Blood samples were collected at 5 and 6 wk after oral
inoculation of 75% of groups of 24 hens with three standard strains, two intermediate strains, and one variant strain of S. pullorum. Rapid whole
blood plate tests were performed using two commercially available antigens made from standard and variant S. pullorum strains (V =
Vineland Laboratories, S = Solvay Animal Health). Tube agglutination tests were performed using S. pullorum antigens prepared in our
laboratory: Antigen 1 was made from three standard reference strains; Antigen 2 from one standard, one intermediate, and one variant
inoculum strain; and Antigen 3 from the other three inoculum strains (two standard and one intermediate).

Antigen 1 (P = 0.04). No other significant differences in
detection frequency were noted between the three tube
test antigens at either 1 to 2 wk (Table 2) or 5 to 6 wk (Table
3) postinoculation. The frequency of antibody detection by
tube tests (for all six inoculum strains combined) ranged
from 67.9 to 73.1% at 1 to 2 wk postinoculation and from
76.2 to 81.0% at 5 to 6 wk postinoculation.

At both 1 to 2 wk and 5 to 6 wk postinoculation, the
frequency of detection of serum antibodies to S. pullorum
was often higher using tube tests than using plate tests. At
1 to 2 wk postinoculation (Table 2), tube tests conducted
using any of the three antigens under consideration
detected seropositive hens significantly more frequently
(for all six inoculum strains combined) than did plate tests
using either the Vineland (P < 0.001) or Solvay (P < 0.006)
antigens. During this interval, various significant differences in antibody detection frequency were observed
between tube and plate tests for five of the six inoculum
strains. At 5 to 6 wk postinoculation (Table 3), similarly,
tube tests using any of the three antigens provided a significantly higher frequency of antibody detection (for
all six inoculum strains combined) than did plate tests
with either the Vineland (P < 0.001) or Solvay (P < 0.002)
antigens. During this latter interval, various significant
differences in antibody detection frequency were evident
between tube and plate tests for four of the six inoculum
strains. No specific antibodies were detected in sera from
uninfected negative control hens using any of the
agglutination tests or antigens.

For both plate and tube tests, all antigens under
consideration detected a higher proportion of hens as
seropositive among groups infected with antigenically
standard S. pullorum strains than among groups infected
with antigenically intermediate or variant strains. At 5 to 6
wk postinoculation (Figure 1), the overall frequency of
detection of specific antibodies among the three groups
inoculated with standard strains was significantly higher
than among the groups inoculated with variant or
intermediate strains for all five combinations of tests and
antigens (P < 0.005).

Correlation of Antibody Test Results
with the Isolation of S. pullorum
from Internal Organs

Five of the six S. pullorum inoculum strains demonstrated
a high degree of invasiveness as measured by the
frequency of subsequent isolation from internal organs
(Table 4). In all groups except those given Strain A, more
than half of the sampled hens were found to harbor S.
SEROLOGICAL DETECTION OF *Salmonella pullorum*

### TABLE 4. Using standard serological tests to predict internal organ culture results in hens experimentally infected with recent isolates of *Salmonella pullorum*

<table>
<thead>
<tr>
<th><em>S. pullorum</em>-positive organ cultures/total</th>
<th>Culture-positive hens with positive antibody test results/total culture-positive hens&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pullorum inoculum strain&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Livers</td>
</tr>
<tr>
<td>A</td>
<td>1/16</td>
</tr>
<tr>
<td>C</td>
<td>9/16</td>
</tr>
<tr>
<td>D</td>
<td>6/16</td>
</tr>
<tr>
<td>E</td>
<td>4/16</td>
</tr>
<tr>
<td>F</td>
<td>7/16</td>
</tr>
<tr>
<td>All strains</td>
<td>36/96</td>
</tr>
</tbody>
</table>

<sup>1</sup>Antibody test results are based on samples taken within 7 d before each culture date.

<sup>2</sup>*Salmonella pullorum* plate test antigens were obtained from Vineland Laboratories, Vineland, NJ 08360 and Solvay Animal Health, Mendota Heights, MN 55120.

<sup>3</sup>*Salmonella pullorum* tube test antigens were prepared in our laboratory: Antigen 1 from standard reference strains 17, 19, and 20; Antigen 2 from inoculum strains A, B, and C; and Antigen 3 from inoculum strains D, E, and F.

<sup>4</sup>Eight hens from each group were cultured at 14 d postinoculation and eight more at 42 d.

<sup>5</sup>Seventy-five percent of the hens in each group were orally inoculated with 10<sup>8</sup> cfu of the indicated strain.

*S. pullorum* in either the liver or the ovary. Overall, for both sampling dates and all six strains combined, *S. pullorum* was isolated from 37.5% of livers and 54.2% of ovaries. All culture-positive internal organ samples came from orally inoculated hens (*S. pullorum* was not isolated from any tissues from either contact-exposed hens or uninfected negative control hens). Despite the large inoculum doses administered, no characteristic signs of clinical pullorum disease were observed in any of the infected hens.

Of the 54 hens from which *S. pullorum* was isolated from either liver or ovaries, nearly all (ranging from 92.6 to 98.1% for the various combinations of tests and antigens) were also determined to be seropositive (Table 4). No significant differences between tests or antigens were observed in the percentage of culture-positive hens that was detected as antibody-positive. On the other hand, less uniformity between serological tests was observed in the proportion of all antibody-positive hens that was also found to be culture-positive (data not shown). Whereas *S. pullorum* was recovered from internal organs of 80.3 and 83.3% of hens determined to be seropositive in plate tests using the Solvay and Vineland antigens, respectively, significantly (*P < 0.025*) fewer hens identified as seropositive in tube tests were also found to be culture-positive (64.2% for Antigen 1, 63.8% for Antigen 2, and 63.1% for Antigen 3).

**DISCUSSION**

Recent *S. pullorum* outbreaks, in which serological testing of pullets was sometimes apparently unable to detect antibodies in flocks that would subsequently be identified as infected (Johnson *et al*., 1992), have stimulated renewed interest in assessing the current effectiveness of the traditional serological methods. The presence of *S. pullorum* infection in mature flocks could result from horizontal transmission of the pathogen after serological screening or from inadequate performance of the screening test. Because many prior studies have indicated that vertical transmission to chicks is the principal route of introduction of *S. pullorum* into flocks (Snoeyenbos, 1991), the possibility of horizontal transmission at later ages has not often been extensively investigated. The current NPIP testing program is based on the assumption that testing breeder pullet flocks before they begin egg production provides an effective control point for preventing later transmission of *S. pullorum* to progeny. In the present study, *S. pullorum* was seldom isolated from samples of voided feces from inoculated hens and was never found in feces of contact-exposed hens (data not shown), suggesting that horizontal transmission by the conventional fecal-oral route may not occur very often.

Infections of chickens with all six recent *S. pullorum* isolates were detected by both plate and tube tests in the present study, although the frequency of positive results was consistently greater with tube tests than with plate tests. Some prior investigations likewise showed serum tube tests to detect *S. pullorum*-infected chickens more frequently than did whole-blood plate tests (Williams and Whittemore, 1979). However, comparative conclusions about tube and plate test results should also take into account the possibility that the production of tube test antigens in individual diagnostic laboratories is a potential source of variability in performance, whereas plate test antigens are produced commercially by a much more limited number of suppliers. Various microagglutination and microantiglobulin tests for *S. pullorum* antibodies have been reported to be more sensitive (Williams and Whittemore, 1979; Thain and...
infected with the three standard reference strains detected chickens using three antigenically standard reference strains. However, as tube test antigens are routinely prepared, each contain both standard and variant strains. 1950). The commercial plate test antigen preparations of birds as seropositive that were apparently not actively infected when the blood samples were drawn. This result could be explained either as the persistent detection of antibodies in hens that had already cleared an earlier transient S. pullorum infection or as the inaccurate identification of uninfected hens as seropositive. Although tube tests frequently identified contact-exposed hens as seropositive (Tables 2 and 3 show numerous instances where the frequency of antibody detection exceeded the initial 75% frequency of oral inoculation), S. pullorum was never isolated from the livers or ovaries of contact-exposed hens. However, no positive tube test reactions were obtained with sera from negative control hens. In a survey of commercial flocks over a 3-yr period, Waltman and Horne (1993) isolated S. pullorum from the tissues of less than 6% of serological reactors (as determined in a tube agglutination test), whereas other salmonellae were found in about 27% of such birds.

In the present study, all of the various combinations of tests and antigens under investigation detected antibodies in chickens infected with any of a variety of S. pullorum isolates. However, these experiments employed relatively large inoculum doses that were administered to all hens on the same day. In naturally infected flocks, far fewer birds exhibit high, easily detectable antibody titers at any one time. The performance of serological tests under commercial poultry production conditions, therefore, cannot be expected to equal that obtained in experimental settings. Nevertheless, the results of the present study suggest that the traditional NPIP serological strategy (relying principally on the whole-blood plate test as a screening tool) remains an effective and economical method of detecting infections of chickens with current field strains of S. pullorum.

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REFERENCES
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