SIMPLIFIED IDENTIFICATION OF
SOMATIC VARIETIES OF PASTEURELLA
MULTOCIDA CAUSING FOWL CHOLERA

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SUMMARY
Representative type strains as well as field strains of Pasteurella multocida from fowl cholera were examined by the indirect hemagglutination procedure and by a rapid plate agglutination test. The former method identified the specific capsular or K substance, and the latter identified the somatic antigens. Inagglutinable organisms were made agglutinable for the rapid procedure by treatment with hyaluronidase. The results confirmed the identity of representative type strains. Of the 25 field strains examined, one was identified as a type D of low pathogenicity, and the other 25 were type A: 19 serotype 8:A (Heddleston type 3), 3 serotype 5:A (Heddleston type 1), 2 Heddleston’s type 4, and 1 serotype 1:A.

INTRODUCTION
Fowl cholera continues to be a major cause of losses among chickens, ducks, turkeys, and other fowl. Serologic studies of the strains of Pasteurella multocida causing fowl cholera have been prompted mainly by a desire to produce more effective active immunizing agents. Knowledge of the occurrence of serotypes is also essential for a full understanding of the epizootiology of fowl cholera. It is not yet known with certainty whether fowl cholera can result from strains carried by sparrows, pigeons, starlings, rats, raccoons, and other animals. Heddleston et al. (8) found that some serologic varieties that cause fowl cholera in domestic fowl also occurred in wildfowl. Evidence is still sparse that certain strains from cattle, swine, and other domestic animals can cause epizootic fowl cholera. A serotype that causes fowl cholera (5:A) was recovered by Namioka and Bruner (10) from swine with pneumonia.

The first clear evidence that different immunotypes of P.
multocida could cause fowl cholera was obtained by Roberts (11) in mouse-protection tests. He found that at least three immunotypes occurred. Later, Heddleston (5) showed by vaccination and challenge experiments in chickens and turkeys that two different immunologic types occurred. The strains identified as X73 and P1059 were respectively designated types 1 and 3 (see Table 1).

Namioka and Bruner (10) employed the indirect hemagglutination (IHA) technique and a tube agglutination procedure to identify capsular types and O groups of fowl cholera strains. They designated a serotype by listing the O group followed by the type, e.g., 5:A. They identified only one capsular type (A) and five different O groups, of which 5 and 9 were the most prevalent.

It was subsequently found that Heddleston's X73 strain was serotype 5:A. Except for serotypes 5:A and 8:A the relationship between serologic identity and immunologic behavior of the serotypes of Namioka and Bruner (10) is not known. Heddleston (6) found a close immunologic and serologic relationship between strains representing serotypes 8:A and 9:A.

Heddleston (7) recently identified two new types from fowl cholera (types 4 and 5) by vaccination and challenge experiments and an agar precipitin test. Strains respectively representing these immunotypes have been numbered P1662 and P1702. Three additional serotypes, designated types 6, 7, and 8, were later identified from wildfowl by the agar precipitin test (8).

Langpap and Matisheck (9) examined fowl cholera strains by plate and tube agglutination tests, but did not present the dilutions and some other details of the procedures. Of 60 strains examined, 21 were Heddleston's type 4, 34 belonged to his type 3, and 5 were type 1. Four isolates were studied by "cross-challenge" in chickens, and the results confirmed the identity of serotype and immunotype. Their study and studies of Heddleston have not included identification of capsular antigens.

*P. multocida* has a great capacity for colony variation (2). The principal colony variants are designated mucoid, smooth or iridescent, intermediate, and blue. Organisms from mucoid and iridescent variants are capsulated and inagglutinable. Agglutinable organisms can sometimes be obtained from predominantly capsulated cultures by colony selection and repeated plating. Mucoidness, which is especially marked with type A strains, is due to large amounts of capsular hyaluronic acid. Organisms from mucoid and smooth colonies of type A cultures are agglutinable after treatment with hyaluronidase (3).
Table 1. Serotypes and/or immunotypes of avian Pasteurella multocida identified by other investigators.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Origin</th>
<th>Serotype</th>
<th>Capsule</th>
<th>Heddeleston type</th>
</tr>
</thead>
<tbody>
<tr>
<td>X73</td>
<td>Chicken</td>
<td>5</td>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td>P1059</td>
<td>Turkey</td>
<td>8</td>
<td>A</td>
<td>3</td>
</tr>
<tr>
<td>P1662</td>
<td>Turkey</td>
<td>5</td>
<td>A</td>
<td>4</td>
</tr>
<tr>
<td>P1702</td>
<td>Turkey</td>
<td>8</td>
<td>A</td>
<td>5</td>
</tr>
<tr>
<td>VA3</td>
<td>Chicken</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS8</td>
<td>Chicken</td>
<td>5</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Turkey</td>
<td>9</td>
<td>A</td>
<td>3</td>
</tr>
</tbody>
</table>

The present study was done mainly to apply the previously described rapid plate agglutination technique (3) to identification of the somatic or O antigens of serotypes and immunotypes from fowl cholera described by other workers. To complete the serotype identification of strains the capsular or K antigen was identified by the IHA procedure.

MATERIALS AND METHODS

Strains examined. The strains examined included serotypes and immunotypes kindly supplied by Mr. Heddleston and Dr. Namioka (see Table 1) and field strains from outbreaks of fowl cholera in various parts of the United States.

Pasteurella antisera. Pasteurella antisera were produced in rabbits as described previously (1). Some antisera were prepared by the inoculation of formalin-killed tryptose phosphate broth (Difco) cultures. The schedule of doses was as follows: 2 ml subcutaneously followed in a week by intravenous doses given at 4-day intervals. The latter began at 0.5 ml and was increased by 0.5-ml increments until a 3.0-ml dose was administered. The 3-ml dose was repeated three times at 4-day intervals.

Absorption of agglutinating sera. It was generally not necessary to absorb sera used in the plate agglutination test. Cross reactions were sometimes observed if the antiserum lacked specificity or the antigen suspension lacked stability. Sera that gave such reactions were absorbed with the heterologous strains with which the reaction was observed. If the bacteria for absorption was inagglutinable they were treated with hyaluronidase as described previously (3). The growth from 3–10 plates was used to absorb 2.5 ml of a 1:10 dilution of antiserum. The absorption was carried out at 37 C for 3–4 hours and continued overnight at 4 C.

Indirect hemagglutination procedure. This test was carried out
Table 2. Agglutination titers (reciprocals) obtained with unabsorbed antipasteurella sera and representative avian types of *Pasteurella multocida*.

<table>
<thead>
<tr>
<th>Sera against</th>
<th>Titers with representative type strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X73</td>
</tr>
<tr>
<td>X73</td>
<td>320</td>
</tr>
<tr>
<td>P1059</td>
<td>neg</td>
</tr>
<tr>
<td>P1662</td>
<td>neg</td>
</tr>
<tr>
<td>P1702</td>
<td>neg</td>
</tr>
</tbody>
</table>

as described earlier (1) with the modifications, including hyaluronidase treatment, described recently (4).

**Rapid plate agglutination.** This procedure was carried out as described earlier (3). Blood agar (5–6% defibrinated blood) plates were found to be the most satisfactory for the preparation of antigen suspensions. Because moisture considerably influences the growth and capsulation of *P. multocida* the plates were stored in plastic bags and incubated in a humid incubator to keep them from drying out. Hyaluronidase treatment was employed for strains that were inagglutinable. Strains with a tendency to autoagglutination could sometimes be rendered more stable by passage in embryonated eggs or mice and washing with phosphate-buffered saline (pH 7.2). Generally the most specific antigens were obtained from freshly isolated smooth or mucoid strains.

Twofold dilutions of the antisera (1:10 to 1:160) were prepared in formol (0.3%/5) phosphate-buffered saline (pH 7.2). The tests were carried out on a glass plate of a Standard Testing Box, although microscope slides and an ordinary glass plate were equally satisfactory. The lighting of the Standard Testing Box facilitates reading of the reactions.

A drop of each serum dilution was delivered to the squares of the plate by a Pasteur pipette. To each of these drops was added a drop of the bacterial suspension; thus the final dilutions ranged from 1:20 to 1:320. The combined drops were thoroughly mixed with a wooden applicator, after which the plate was rotated periodically to promote clumping. The tests were read as soon as agglutination was appreciable, usually within 5 to 10 minutes. A positive reaction consisted of marked agglutination of the type seen in plate agglutination tests with *Brucella*, *Salmonella* and *E. coli*.

**RESULTS**

The results of the IHA tests confirmed the identity of strains X73, P1059, VA3, TS8, and “Liver” as type A. Strain P1702 was not typable, and P1662 was identified as type A.

Table 2 gives the results of the agglutination tests with un-
Identifying strains of Pasteurella multocida

Identifying strains of Pasteurella multocida absorbed antisera prepared from representative type strains. The occurrence of different somatic antigens as reported by Namioka and Bruner (10) was confirmed. Also demonstrated was the serologic difference between Heddleston's new types (types 4 and 5). Cross-reactions were minimal when tests were read within 5 to 10 minutes. On later readings some cross-reactions were noted, e.g., between serum P1702 and antigen X73. The strain "Liver" (9:A) was found to be serologically identical to strain P1059 (8:A).

The specificity of the reactions depended to a considerable extent on the freshness of suspensions and their growth on dehydrated blood agar plates. Suspensions stored for several weeks in the refrigerator lost considerable specificity.

Table 3 presents the results of the examination of 26 fowl cholera strains. Recovered besides the three types identified by Heddleston (5,7) and the two varieties of Namioka and Bruner (10) was a type D strain of unidentified somatic antigen. This strain was found to be only weakly pathogenic for chickens on experimental inoculation. One chicken yielded a strain of serotype 1:A that occurs most commonly in swine, according to Namioka and Bruner (10).

**DISCUSSION**

Serologic identification of type strains of Heddleston and Namioka and Bruner was confirmed with the IHA procedure and the rapid agglutination test. The latter procedure should contribute to the ease of recognition of O group or somatic antigens of strains of *P. multocida* from various hosts. In view of previous studies it seems likely that the specificity of the O antigens resides in the carbohydrate determinants of the lipopolysaccharides.

There would appear to be a real advantage in identifying both the capsular and somatic antigens of *P. multocida*, as Namioka and Bruner (10) have done and as is done with other gram-negative bacteria. That identification of the somatic antigen alone is not sufficient is shown by the fact that strains with the same predominant

<table>
<thead>
<tr>
<th>No. of strains</th>
<th>Origin</th>
<th>Serotype</th>
<th>Heddleston type</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Chicken</td>
<td>5:A</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>Chicken</td>
<td>8:A</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Turkey</td>
<td>8:A</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Turkey</td>
<td>8:A</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>Chicken</td>
<td>1:A</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Chicken</td>
<td>-D</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Serologic identification of 26 strains of *Pasteurella multocida* from outbreaks of fowl cholera.
somatic antigenic determinant may have different capsular substances, e.g., serotypes 6:B and 6:E, and 3:A and 3:D.

A number of systems have been used over several decades for the designation of serologic varieties. The failure of investigators to settle on one system has considerably hampered the conduct and application of research. Because the scheme evolved by Namioka and associates is the most comprehensive to date, its adoption as a standard for the species is strongly recommended.

It has been clear for some time that a number of serotypes of P. multocida may cause fowl cholera. In fact the distribution of serologic varieties in fowl cholera bears some similarity to the occurrence of serotypes in salmonellosis in poultry. Both diseases have several more commonly occurring serotypes as well as a number of less frequently encountered varieties. The antigenic heterogeneity of P. multocida strains no doubt accounts in part for the irregular results obtained from the use of current bacterins.

REFERENCES

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