An Adhesion-Hemadsorption Inhibition Test for the Detection of Serum Antibody to *Mycoplasma gallisepticum*

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**ABSTRACT.** A simple adhesion-hemadsorption inhibition (AHAI) test was developed for the detection of antibodies to *Mycoplasma gallisepticum* in the chicken sera. The AHAI antibody was detected simultaneously with HI antibody from sera of chickens intratracheally inoculated with viable cells of *M. gallisepticum*. A good correlation between HI and AHAI antibody titers was obtained with 382 (84.7%) of 451 sera from chickens reared on farms spontaneously contaminated with *M. gallisepticum*, whereas the remainder, 69 sera, was positive for HI but negative for AHAI test. It was not apparent whether the latters exhibited a non-specific reaction or the discrepancy was due to the lower sensitivity of AHAI reaction. The AHAI test does not require a great amount of antigen, special reagents or instruments, or pre-absorption treatment of test sera, and, therefore, it may serve as a simple serological test for detecting antibodies to *M. gallisepticum.*—**KEY WORDS:** adhesion-hemadsorption, AHAI test, *Mycoplasma gallisepticum.*


In a previous paper [2] we reported that strains of *Mycoplasma gallisepticum* adhering on the plastic surfaces adsorbed chicken and sheep red blood cells (RBC). This reaction was referred to as an adhesion-hemadsorption (AHA). The AHA of *M. gallisepticum* MR3 was specifically inhibited by rabbit antiserum when it was incorporated before the addition of RBC.

In this paper, we describe the inhibition of both adhesion and AHA of *M. gallisepticum* by antiserum and results of application of these inhibition tests for detecting antibodies to this microorganism in the sera of infected chickens.

**MATERIALS AND METHODS**

*Mycoplasma strain:* *M. gallisepticum* MR3, isolated from airsacculitis of a chicken and maintained under lyophilized condition in this laboratory, was used throughout this study. *M. synoviae* K7, an isolate from airsacculitis of another bird, was used as an inoculum for chickens.

*Medium:* A modified Hayflick medium was prepared by the same manner as described previously [2].

*Rabbit antisera:* Polyvalent antisera against 13 avian Mycoplasma species used in a previous study [2] were employed for the present investigation.

*Chicken sera:* Fifteen conventionally reared 3-week-old male broiler chickens from a mycoplasma free flock were divided into 3 groups. Sera were obtained from these birds before inoculation and were confirmed to be free from antibodies to *M. gallisepticum* and *M. synoviae* by the routine rapid slide agglutination test and hemagglutination inhibition (HI) test. A group of 5 chickens were inoculated intratracheally with $6.2 \times 10^7$ CFU of MR3 in 1 ml of phosphate buffered saline (PBS, pH 7.2). The second group of 5 birds were inoculated through the same route with $3.4 \times 10^6$ CFU of *M. synoviae* K7, whereas the third group was inoculated with $5.0 \times 10^7$ heat-
inactivated cells of *Staphylococcus aureus* MORI, an isolate from dermatitis of a chicken. Sera were obtained from all chickens at a week intervals for 5 weeks and then at 7 and 9 weeks postinoculation. All sera were used for HI test before frozen, while other three serological tests were performed with sera kept frozen at −30°C. In addition, a total of 451 sera were obtained from commercial layer and broiler chickens kept in 7 farms which were proved to be spontaneously infected with *M. gallisepticum* by a routine rapid slide agglutination test. These sera were frozen and sent to this laboratory. All chicken sera tested were used without heat-inactivation.

**Adhesion-hemadsorption inhibition (AHAI) test:** An overnight liquid culture of MR3 was diluted 100-fold in fresh medium, and 25 µl each of the dilution was distributed to the wells of U-shaped microtiter plates (Corning). After incubating plates at 37°C for 18 hr, wells were rinsed with 50 µl of PBS twice and finally supplemented with 25 µl each of PBS. The wells were then added with test sera diluted in two fold series with PBS on transfer plates (Dynatech). Wells served as negative controls were treated with PBS in lieu of serum dilution. After incubating plates at 37°C for 30 min, fluid was removed from wells which were rinsed twice as before, and were added with 25 µl each of 0.2% chicken RBC suspension in PBS. Plates were again incubated at 37°C for 30 min, and wells were observed for the formation of hemagglutination-like patterns which were regarded as negative, while sedimentation of RBC to the bottom of wells to form a pack of cells was judged as positive for AHAI. Reciprocal of the highest dilution of a test serum positive for AHAI was designated as an AHAI titer.

**Adhesion inhibition (ADI) test:** Two fold dilutions of test sera were made in 25 µl quantities of liquid medium on U-shape microtiter plates (Corning). Each well was added with 25 µl of a 100-fold dilution in medium of an 18-hr culture of MR3. Plates were inoculated at 37°C for 18 hr, and each well was rinsed three times with 50 µl of PBS. Fluid was removed from wells and 25 µl of 0.2% chicken RBC was added to each well. After incubating plates at 37°C for 30 min, each well was observed for the occurrence or inhibition of AHA as mentioned above.

**Colonial hemadsorption inhibition (CHAI) test:** Inhibition of adsorption of chicken RBC on the surfaces of colonies of MR3 was tested by the method of Goren [1].

**HI test:** Inhibition of hemagglutination with *M. gallisepticum* MR3 antigen having an HA titer of 1:80 was tested by the method of Windsor *et al* [3] by using test sera previously adsorbed with chicken RBC.

**RESULTS**

Among rabbit hyperimmune sera against 13 avian Mycoplasma species, only homologous serum inhibited hemagglutination, AHA, adhesion, and colonial hemadsorption of *M. gallisepticum* MR3, and exhibited inhibition titers as shown in Table 1. Both AHAI and ADI titers were higher than HI titer with anti-MR3 rabbit serum. In contrast, 5 sera of layer chickens which were proved to be infected with *M. gallisepticum* exhibited slightly or much lower AHAI and ADI titers than HI titer (Table 1), and all of them were negative for CHAI.

To confirm these discrepancies, sera from experimentally inoculated chickens were subjected to four inhibition tests with MR3. Rise of inhibition titers in these tests among 5 chickens challenged with viable cells of MR3 is as shown in Table 2. The HI antibody appeared earliest in all birds, followed by AHAI antibody. The ADI titers elapsed low during 9 weeks postinoculation. CHAI was never exhibited by any
of test sera during the experimental period. Sera of chickens inoculated with either viable cells of \textit{M. gallisepticum} or heat-killed cells of \textit{S. aureus} did not inhibit any reaction with MR3 throughout the experimental period and, therefore, results with these sera are excluded from the table.

A total of 451 chicken sera obtained from 7 farms and proved to be strongly or weakly positive for anti-\textit{M. gallisepticum} agglutinin were used for both HI and AHAI tests. Of these, 116 (25.7\%) were negative for both HI (<10) and AHAI (<4) tests, while 69 (15.3\%) were positive for HI but negative for AHAI test. The remainder, 266 sera, exhibited a good correlation between their HI and AHAI titers, as shown in Fig. 1.

**DISCUSSION**

Results of the present study with rabbit hyperimmune sera indicated that factors exhibiting both AHAI and ADI were, like HI and CHAI, species specific antibodies. Chickens inoculated with \textit{M. gallisepticum} MR3 developed AHAI and ADI antibodies as well as HI antibodies, although CHAI by these sera were never proved. In contrast, no inhibitory action on 4 tests was shown with sera from chickens inoculated with either \textit{M. synoviae} or \textit{S. aureus}. These findings suggested that both AHAI and ADI with chicken sera were also specific for Mycoplasma species. Although the reason of absence of CHAI antibodies in the infected chicken sera is unknown at present, it is clear that fairly high antibody titer is required for CHAI [1], and chickens experimentally or spontaneously infected with \textit{M. gallisepticum} do not develop such a high level of antibodies as in the case of repeatedly immunized rabbits.

It was probable that the occurrence of ADI by rabbit or chicken serum antibodies was due to the inhibition of growth of mycoplasmal cells. Results of ADI test with chicken sera restricted itself as a diagnostic tool for \textit{M. gallisepticum} infection due to the

### Table 1. Antibody titers exhibited in 4 serological tests by anti-MR3 rabbit hyperimmune serum and sera of 5 chickens naturally infected with \textit{M. gallisepticum}

<table>
<thead>
<tr>
<th>Serum</th>
<th>HI</th>
<th>AHAI</th>
<th>ADI</th>
<th>CHAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>640</td>
<td>2560</td>
<td>1280</td>
<td>4</td>
</tr>
<tr>
<td>Chicken 1</td>
<td>320</td>
<td>64</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Chicken 2</td>
<td>160</td>
<td>64</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Chicken 3</td>
<td>160</td>
<td>64</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Chicken 4</td>
<td>80</td>
<td>32</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Chicken 5</td>
<td>80</td>
<td>16</td>
<td>&lt;2</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Abbreviations. HI, hemagglutination inhibition. AHAI, adhesion-hemadsorption inhibition. ADI, adhesion inhibition. CHAI, colonial hemadsorption inhibition. These apply to the following tables.

### Table 2. Move of HI, AHAI and ADI titers in the sera of 5 chickens intratracheally inoculated with 6.2×10^7 CFU of \textit{M. gallisepticum} MR3

<table>
<thead>
<tr>
<th>Chicken No.</th>
<th>Week postinoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>-/-/-/-</td>
</tr>
<tr>
<td>2</td>
<td>-/-/-/</td>
</tr>
<tr>
<td>3</td>
<td>-/-/-/</td>
</tr>
<tr>
<td>4</td>
<td>-/-/-/</td>
</tr>
<tr>
<td>5</td>
<td>-/-/-/</td>
</tr>
</tbody>
</table>

a) HI/AHAI/ADI titers in this order. A - indicates <10, <4 and <2 for HI, AHAI and ADI titer, respectively. All sera were negative for CHAI and omitted from the table.
slow appearance and low titers of ADI antibody.

In contrast, mechanism of AHAÏ seemed to resemble the CHAI of *M. gallisepticum* although chicken serum antibodies negative for the latter test were easily detected by the former test. In fact, the AHAÏ antibodies were developed in chickens within 2 weeks after infection with *M. gallisepticum* and rose to considerably high titers. Of 451 individual chicken sera from spontaneously infected farms, 382 (84.7%) exhibited a good correlation in AHAÏ and HI tests. However, the remainder, 69 sera, was positive for HI but negative for AHAÏ test (Fig. 1). It is not apparent whether such a discrepancy is due to a lower sensitivity of AHAÏ reaction or it is due to a nonspecific reaction of HI. At any rate, the AHAÏ test is easy to perform and is much more sensitive than CHAI. Unlike HI test, the AHAÏ technique does not require pre-absorption treatment of test sera with chicken RBC or considerable amount of antigen; indeed, a 5 ml of fresh liquid culture is sufficient to perform AHAÏ test with more than 100 test sera. In addition, the AHAÏ test can be performed with microtiter plates carrying adhered antigen and stored at 4°C for at least two weeks without losing hemadsorption property (unpublished data). It does not require any special reagents or instruments which are necessary for ELISA etc.

It is suggested from these properties and results of the present study that the AHAÏ test may serve as a new tool for detecting antibodies against *M. gallisepticum* in the chicken sera. It should be studied further if the same technique is applicable for diagnosing *M. pneumoniae* infections of men.

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REFERENCES

要約

付着・血球吸着阻止反応による抗 Mycoplasma gallisepticum 抗体の検出：清水高正・永友寛司（宮崎大学農学部家畜衛生学教室）——M. gallisepticum MR3 株のマイクロタイターウェルへの付着と、付着菌体による鶏赤血球の吸着は、共に抗血清により特異的に阻止された。MR3 株接種鶏の血清中には、付着・血球吸着阻止（AHAI）抗体の方が付着阻止（ADI）抗体に比べて早期かつ高価に出現した。野外で本菌に感染した7鶏群由来451例の鶏血清を対象に、HI 抗体価と AHAI 抗体価を測定したところ、382例（84.7%）については互いに高い相関関係が得られた。他の69検体は HI 反応陽性、AHAI 反応陰性であった。AHAI 試験はごく少量の抗原で実施でき、被検血清の吸収処理や特殊な試薬、器具類を必要とせず、抗 M. gallisepticum 抗体の検出法として有用と考えられる。