Comparison of Hemagglutinin and Agglutinin Schemes for the Serological Classification of *Haemophilus paragallinarum* and Proposal of a New Hemagglutinin Serovar

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Ninety-five isolates of *Haemophilus paragallinarum* were classified serologically by a hemagglutinin serotyping scheme, and the results were compared with results from agglutinin serotyping of the same isolates. Of the isolates, 65 were from Australia, 5 were from Japan, 6 were from the Federal Republic of Germany, 7 were from the United States, and 12 were from South Africa. Seven hemagglutinin serovars, HA-1 to HA-7, corresponding to agglutinin serovars A (HA-1, HA-2, and HA-3), C (HA-4, HA-5, and HA-6), and B (HA-7) have been described. Only one of the seven hemagglutinin serovars was found among the Australian isolates. This was serovar HA-5, comprising 49 isolates. Fifteen Australian isolates, all from southeast Queensland and northern New South Wales, were found to belong to a new hemagglutinin serovar. This was designated HA-8 and represented a fourth subgroup of agglutinin serovar A. Of the 95 isolates examined, only 1 did not produce a hemagglutinating antigen and was nonserotypeable by the hemagglutinin system. This compared favorably with the agglutinin scheme, which serotyped only 60 of the 95 isolates, with 29 nonserotypeable and 6 autoagglutinating.

*Haemophilus paragallinarum* is the causative agent of infectious coryza, generally an acute, but sometimes a chronic, disease of chickens. Nasal discharge and swelling of the face, eyes, nasal sinuses, and wattles are features of the clinical disease (17). Major economic effects are increased culling and a marked reduction in egg production of laying and breeding hens, particularly on multiage farms (17).

Page (8), in the United States, first serotyped isolates of *H. paragallinarum* and reported three agglutinin serovars: A, B, and C. Rimler et al. (11) confirmed the serovar A of Page and described the Modesto strain as a new agglutinin serovar C-type isolate, the original type C isolate of Page having been lost.

Sawata et al. (13) used an agglutination test to serotype isolates of *H. paragallinarum* in Japan and found two serovars, designated 1 and 2. In a further study (14), they found that serovars 1 and 2 were equivalent to serovars A and C, respectively, of Page (8) and Rimler et al. (11).

The agglutinin schemes of Page (8) and Rimler et al. (11) were used at our laboratory in two studies on Australian and South African isolates of *H. paragallinarum*. Both studies indicated the presence of serovars A and C, with serovar C predominating. No serovar B isolates were found (3, 16). A significant number of strains were either nonserotypeable or autoagglutinating, resulting in only 85 and 62%, respectively, of strains being serotyped in each study. Hyaluronidase treatment, used to remove any masking hyaluronic acid capsule (11, 12), failed to significantly improve the results (3, 16).

Kume et al. (6) found that untreated antigens prepared from serovar 1 isolates agglutinated fresh chicken erythrocytes (RBC) and induced hemagglutination inhibition (HI) antibodies, while a similar antigen from serovar 2 isolates lacked these properties. However, Sawata et al. (15) were able to produce an antigen from serovar 2 isolates that agglutinated RBC which had been fixed previously with glutaraldehyde (GA) by means of treatment with potassium thiocyanate (KSCN) and sonication. Following this, Kume et al. (5) proposed a new serotyping system for *H. paragallinarum* based on hemagglutinins prepared by treatment with KSCN and sonication and using GA-fixed RBC. In examining 17 isolates with this scheme, Kume et al. (5) recognized three serogroups, which corresponded to the three agglutinin serovars of Page (8) and Rimler et al. (11). These three hemagglutinin serogroups were subdivided into a total of seven serovars by cross-absorbing sera (5).

The present study was undertaken to determine the occurrence of hemagglutinin serovars of *H. paragallinarum* in Australia and to compare the results with those obtained by agglutinin serotyping in this and in previous studies. Of the Australian isolates of *H. paragallinarum*, 65 were classified serologically by the hemagglutinin scheme of Kume et al. (5) and 18 were classified by the agglutinin schemes of Page (8) and Rimler et al. (11). Thirty isolates of *H. paragallinarum* from four other countries were available for testing and were included to provide some information on the geographic occurrence of the hemagglutinin serovars.

**MATERIALS AND METHODS**

Reference bacteria. Table 1 shows the sources and hemagglutinin serovars assigned by Kume et al. (5) to the seven reference hemagglutinin serovar isolates used in this study. Their agglutinin serovars, as determined at both the originating laboratory and this institute, are also shown.

**Field isolates.** Isolates of *H. paragallinarum* from chickens in eastern Australia were supplied by laboratories in Queensland, New South Wales, and Victoria. Of the isolates, 5 from Japan, 6 from the Federal Republic of Germany, 7 from the United States, and 12 from South Africa were supplied by K. Kume (Kitasato Institute, Tokyo, Japan), K. H. Hinz (Institute for Poultry Diseases, Hannover, Federal Republic of Germany), R. B. Rimler (National Animal Disease Center, 1510

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TABLE 1. Hemagglutinin serovars for seven reference isolates of \textit{H. paragallinarum}

<table>
<thead>
<tr>
<th>Isolate (agglutinin serovar)*</th>
<th>Source*</th>
<th>Hemagglutinin serovar</th>
<th>Agglutinin serovar*</th>
</tr>
</thead>
<tbody>
<tr>
<td>221 (HA-1)</td>
<td>Rimler</td>
<td>HA-1</td>
<td>NT*</td>
</tr>
<tr>
<td>2403 (HA-2)</td>
<td>Hinz</td>
<td>HA-2</td>
<td>A</td>
</tr>
<tr>
<td>E-3C (HA-3)</td>
<td>Kume</td>
<td>HA-3</td>
<td>A</td>
</tr>
<tr>
<td>H-18 (HA-4)</td>
<td>Kume</td>
<td>HA-4</td>
<td>C</td>
</tr>
<tr>
<td>Modesto (HA-5)</td>
<td>Rimler</td>
<td>HA-5</td>
<td>C</td>
</tr>
<tr>
<td>SA-3 (HA-6)</td>
<td>Kume</td>
<td>HA-6</td>
<td>NT</td>
</tr>
<tr>
<td>2671 (HA-7)</td>
<td>Kume</td>
<td>HA-7</td>
<td>B</td>
</tr>
</tbody>
</table>

* Agglutinin serovar according to Sawata and Page or Rimler (A, B, and C) schemes as reported by originating laboratory.
* Hinz, K. H. Hinz, Institute for Poultry Diseases, Hannover, Federal Republic of Germany; Kume, K. Kume, Kitasato Institute, Tokyo, Japan; Rimler, K. B. Rimler, National Animal Disease Center, Ames, Iowa.
* As determined at this institute.

**TABLE 2. Protocol used for the absorption of antisera to \textit{H. paragallinarum}**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Hemagglutinin serovar</th>
<th>Hemagglutinin used for antisera absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA1</td>
<td>HA-1</td>
<td>Expt 1</td>
</tr>
<tr>
<td>2403</td>
<td>HA-2</td>
<td>Expt 2</td>
</tr>
<tr>
<td>E-3C</td>
<td>HA-3</td>
<td>Expt 3</td>
</tr>
</tbody>
</table>

**TABLE 3. Hemagglutination test results**

Antigenic reactions were determined with 50-μl volumes of reagent in a microtitration method. Dubbling dilutions of antigen were made with PBS (pH 7.2) diluted 1:10 with normal saline (PBSS) containing 0.1% (wt/vol) bovine serum albumin and 0.0015% (wt/vol) gelatin. GA-fixed RBC (1% in PBSS-bovine serum albumin-gelatin) were added to each well, and the plates were incubated for 1 h at room temperature. The hemagglutination titer was the highest dilution of antigen causing agglutination of the GA-fixed RBC.

**HI tests.** Fifty-microliter volumes of doubling dilutions of antisera at 1:40 to 1:1,280 were prepared in PBSS-bovine serum albumin-gelatin. An equal volume of antigen containing 4 hemagglutination units, followed by 50 μl of 1% GA-fixed RBC, was added to each well. Plates were read after 1 h at room temperature. The HI titer was the highest dilution of antisera inhibiting hemagglutination.

**Antiserum absorption.** The rabbit antisera were absorbed with hemagglutinins of other reference serovar isolates to produce monospecific reagents. Five volumes of antigen containing 64 hemagglutination units was centrifuged and suspended in 1 volume of a 1:40 dilution of antisera in PBSS-bovine serum albumin-gelatin. The suspension was stirred at room temperature for 2 h and then at 4°C overnight. After centrifugation, the absorbed antisera was removed, and its HI titers to homologous and heterologous antigens were determined. Antisera were absorbed up to three times, until all reactions with heterologous antigens were removed. The absorption procedures used for the various antisera are detailed in Table 2. Antisera to isolates SA-3 and 2671 (hemagglutinin serovars HA-6 and HA-7, respectively) did not require any absorption. Finally, all of the antisera were absorbed with 5 volumes of GA-fixed RBC by the procedure described above. They were stored at -20°C as a 1:40 dilution of absorbed antisera.

**RESULTS**

With the agglutinin serotyping scheme, 24 isolates were serotyped: 10 were nonserotypeable and 2 were autoagglutinating. Fourteen isolates were assigned to serovar A, two were assigned to serovar B, and eight were assigned to serovar C.
TABLE 3. Results of hemagglutinin serotyping of 95 field isolates of *H. paragallinarum*

<table>
<thead>
<tr>
<th>Hemagglutinin serovar</th>
<th>Australia</th>
<th>Japan</th>
<th>USA</th>
<th>FRG</th>
<th>RSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-1</td>
<td></td>
<td>5</td>
<td>6</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>HA-5</td>
<td>49</td>
<td></td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HA-6</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HA-7</td>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HA-8</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*No isolates belonging to serovars HA-2, HA-3, or HA-4 were found. FRG, Federal Republic of Germany; RSA, Republic of South Africa; USA, United States of America.*

Of the 65 Australian isolates, 49 were serotyped as serovar HA-5 by the hemagglutinin serotyping system. One isolate could not be serotyped, as the antigen failed to agglutinate GA-fixed RBC. The remaining 15 Australian isolates could not be serotyped as any of the existing 7 hemagglutinin serovars. Although they reacted with unabsorbed antisera to isolates 221, 2403, and E-3C (serovars HA-1, HA-2, and HA-3, respectively) at 1:160 or greater, hemagglutinins prepared from these isolates were not inhibited by any of the absorbed antisera. Two of these isolates, HP14 and HP62, were selected, and an antiserum to each was prepared in rabbits. HP14 and HP62 antisera had reciprocal HI titers of 10,240 and 2,560, respectively, when tested against either HP14 or HP62 hemagglutinins. When tested against serovars HA-1, HA-2, and HA-3 hemagglutinins, HP14 had HI titers ranging from 320 to 1,280 and HP62 titers ranging from 320 to 640. No HI activity against serovars HA-4 to HA-7 hemagglutinin was detected. Following absorption with E-3C (serovar HA-3) hemagglutinin (Table 2), the HI activity against serovars HA-1 to HA-3 hemagglutinins was removed. HP14 then had a reciprocal HI titer of 320, and HP62 had a titer of 80 when tested against either HP14 or HP62 hemagglutinins. These absorbed antisera inhibited agglutination of GA-fixed RBC by hemagglutinins prepared from all of the 15 Australian isolates that could not be serotyped with the seven reference-absorbed antisera. A new hemagglutinin serovar, designated HA-8, was identified, with HP14 as the serovar reference isolate.

The results of hemagglutinin serotyping of the 95 field isolates of *H. paragallinarum* are presented in Table 3, and they are compared with agglutinin serotyping results from both this and the two previous studies in Table 4.

**DISCUSSION**

Of the 95 isolates tested in this study, 47 from Australia and 13 from South Africa were included in two previous agglutinin-serotyping studies in which 85 and 62%, respectively, of isolates were serotyped (3, 16). Similar results were obtained in this study, since 24 of the 36 isolates (67%) could be serotyped by the agglutinin system following hyaluronidase treatment of nonserotypeable antigens. Thus, by the agglutinin system, only 60 of the 95 isolates (63%) were serotypeable, with 29 being nonserotypeable and 6 being autoagglutinating. This result may be due to the presence of some substance other than a hyaluronic acid capsule, which masks the serospecific antigens detected in the agglutinin test (3).

Only one of the seven hemagglutinin serovars designated by Kume et al. (5) was found among the Australian isolates. This was serovar HA-5, equivalent to a subgroup of agglutinin serovar C. It was the most common Australian serovar, accounting for 75% of field strains. Fifteen other Australian isolates belonged to a new serovar, designated HA-8. The discovery of this new hemagglutinin serovar confirms the prediction by Kume et al. (5) that the number of hemagglutinin serovars would increase as more isolates from different parts of the world were typed.

Kume et al. (5) thought that there could be a correlation between hemagglutinin serovars and geographic origins of isolates. This was supported by the occurrence of only two serovars, HA-5 and HA-8, in Australian isolates, with the new serovar HA-8 found in a relatively small geographic area of Australia comprising southeast Queensland and northern New South Wales. However, in contrast, other serovars, such as HA-1, HA-5, and HA-7, were found in isolates from at least three countries (Table 3).

The use of an extracted antigen in a HI test avoids the problems of autoagglutination and nonreaction due to masked surface antigens, which occur in agglutination tests. Sawata et al. (15) suggested that the treatment of serovar 2 (serovar C of Page) isolates with KSCN and sonication might act to remove a surface substance surrounding the organism which inhibits hemagglutinating activity, but the mechanism is still unclear. The absence of hemagglutinating activity in the antigen prepared from one Australian isolate would suggest either a failure to remove such a surface inhibitor or the absence of a hemagglutinin in this isolate. The serovar-specific hemagglutinins HA-L1 and HA-L2 of Sawata (serovars 1 and 2, respectively) have been shown to correlate with protection against challenge by the homologous isolate (15). Further examination of the nonhemagglutinating isolate is warranted to determine both whether it is pathogenic and whether it could be used to prepare an effective vaccine.

Experimental, inactivated, aluminum hydroxide gel, infectious coryza vaccines have been produced in this laboratory and shown to be protective (2, 9) only against the agglutinin serovars present in the vaccine (4). Similar findings have been reported by others (6, 7, 11). Because of the predominance of agglutinin serovar C, a monovalent serovar C vaccine is now commercially available in Australia. However, hemagglutinin serovar HA-8 isolates have been repeatedly isolated from a multiage layer flock in Queensland over the last 12 months (unpublished data). As this hemagglutinin...
serovar was identified as an agglutinin serovar A subgroup, the occurrence of agglutinin serovar A isolates is more widespread in Australia than was recognized previously when only the agglutinin scheme was used. Thus, there is a need in Australia for a vaccine which will protect against challenge from agglutinin serovar A isolates of \textit{H. paragallinarum}.

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**LITERATURE CITED**