Identification and Serotyping of *Haemophilus pleuropneumoniae* by Coagglutination Test

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A coagglutination test for the identification and serotyping of *Haemophilus pleuropneumoniae* is described. A total of 360 *H. pleuropneumoniae* strains were isolated from pulmonary tissues of feeder pigs which died of acute pleuropneumonia. All of the isolates were serotyped by coagglutination, and the results were confirmed by the ring precipitation test. The most common serotype isolated in Quebec was serotype 1, followed by serotypes 5, 2, and 7. None of the isolates belonged to serotypes 3, 4, or 6. Mixed infections due to *H. pleuropneumoniae* of more than one serotype in the same animal were encountered. Serotype 1 was the only common isolate among the mixed-serotype infections. The coagglutination test was more sensitive than the ring precipitation test. Serotyping by the coagglutination test is inexpensive, rapid, reliable, and easy to perform.

In pigs pleuropneumonia which is caused by *Haemophilus pleuropneumoniae* is worldwide in distribution and results in severe economic losses to the swine industry. At present, five serotypes are well recognized (5). Two more new serotypes, namely serotypes 6 and 7, have recently been proposed (14). Serotypes 1, 3, 4, and 5 seem to be predominant in the United States (3, 5; R. A. Shultz and R. F. Ross, Proc. Int. Pig Vet. Soc. Congr., p. 80, 1982), whereas serotype 2 is most significant in Europe (10, 11). Serotypes 1, 2, 3, 4, and 7 have been reported in Canada (4, 7–9, 13, 14).

Serotyping is based on tube agglutination (5), slide agglutination (7), immunodiffusion (10), immunofluorescence (10, 13), ring precipitation (RP) (7), and indirect hemagglutination (8) tests. Although all of these methods are reliable, they present some disadvantages either in the time needed to perform the test or in the cost of sera and reagents. Moreover, readings are not always easy to interpret. Autoagglutinating strains cannot be serotyped by the agglutination test. We describe a simple method for serotyping *H. pleuropneumoniae* by the antibody-coated staphylococcal coagglutination test.

**MATERIALS AND METHODS**

**Type strains.** Five strains of *H. pleuropneumoniae*, representing serotypes 1 to 5, were received from A. Gunnarsson of the National Veterinary Institute, Uppsala, Sweden. Strains representing serotypes 6 and 7 were received from S. Rosendal of the University of Guelph, Ontario, Canada.

**Field isolates.** A total of 360 *H. pleuropneumoniae* strains were isolated from lung tissues of feeder pigs which died of acute pleuropneumonia. Lung tissues with pleuropneumonia were cultured on chocolate blood agar supplemented with IsoVitaleX (BBL Microbiology Systems). The plates were incubated for 24 h in 10% CO₂ at 37°C. All of the strains were identified by the method of Biberstein et al. (1).

**Preparation of antisera.** Antisera against type strains were prepared in rabbits intravenously inoculated with formalinized whole-cell antigens of each serotype, as described earlier (7).

**Preparation of antigen for serotyping.** The growth from an 18-h-old culture on chocolate blood agar plates (diameter, 9 cm) was suspended in 3 ml of Formol saline containing ca. 10% bacterial cells. The saline suspension was left at room temperature for about 5 min and centrifuged at 8,000 × g for 15 min; the clear supernatant referred to as saline extract was used for the RP test. The whole-cell suspension or its saline extract was used for serotyping smooth, nonautoagglutinating strains by the coagglutination test. Only the saline extract was used for serotyping when the bacterial cell suspension showed autoagglutination tendencies.

**RP test.** The RP test was performed in Pasteur pipettes by using the saline extract of the culture as the antigen; the reference type serum was that prepared in rabbits. Details of this technique have been described earlier (7).

**Coagglutination test.** (i) **Preparation of reagents.** *Staphylococcus aureus* strain Cowan 1 (NCTC 8530), capable of producing large amounts of protein A, was treated by the method of Kronvall (6). The strain was grown on tryptic soy agar overnight at 37°C. The growth was harvested in phosphate-buffered saline (PBS), pH 7.4, containing 0.15 M NaCl, 0.01 M Na₂HPO₄, and 0.01 M KH₂PO₄ and was washed twice.
with PBS. The bacteria were suspended in 0.5% Formalin in PBS, kept at room temperature for 3 h, washed once in PBS, and adjusted to a concentration of 10% (vol/vol). The suspension was further treated at 80°C in a water bath for 5 min.

To 1 ml of this staphylococcal suspension, 0.025 ml of serotype-specific antiserum was added. After being mixed, the suspension was allowed to stand at room temperature for 30 min and then was washed twice with PBS. After the final washing, the bacteria were resuspended to a concentration of 10% (vol/vol) in PBS containing 0.05% sodium azide and 0.1% bovine serum albumin (Sigma Chemical Co.). Staphylococci treated this way were stable for at least 1 year when stored at 4°C. Pool agglutination reagents were prepared as follows. Equal volumes of antisera were mixed in three different combinations as shown in Table 1. The staphylococcal cell suspensions were then treated with pool antiserum in the same proportions as for individual rabbit antiserum.

(ii) Test procedure. One drop (0.05 ml) of the staphylococcal coagglutination reagent was mixed on a glass slide with an equal volume of bacterial suspension or its saline extract. The mixture was stirred thoroughly with a wooden applicator stick. The slide was rotated by hand, examined against a dark background, and considered negative if macroscopic agglutination did not occur within 2 min. A positive reaction was characterized by a distinct agglutination usually occurring within a few seconds. Reaction strength was quantitated as 0, +, 2+, 3+, or 4+, based on the degree of agglutination and the size of the aggregates formed. Any antigen giving a 2+ reaction was considered positive. Controls consisting of unlabeled staphylococcal cell suspensions as well as staphylococcal cell suspensions coated with normal rabbit serum were used when bacterial cell suspensions were used for serotyping, they were always checked for possible autoagglutination.

Evaluation of coagglutination and RP tests for sensitivity. The growth from an 18-h-old culture of reference strains as well as that of field strains of *H. pleuropneumoniae* on chocolate blood agar plates (diameter, 9 cm) was suspended in 3 ml of Formol-saline. The suspension was centrifuged, and a clear supernatant (saline extract) was obtained. Tenfold or twofold dilutions of this extract were prepared in saline in 0.5-ml volumes. Different antigen dilutions were tested against homologous antiserum or coagglutination reagents in the RP and coagglutination tests, respectively. The reciprocal of the highest dilution of antigen giving a positive reaction was considered the titer.

### RESULTS

Serotyping of the known serotypes by coagglutination test. We first checked the proposed method with a set of reference strains belonging to known serotypes. The results obtained by the coagglutination test were in perfect agreement with those of the capsular serotypes of the known strains (Table 1). A set of 100 other stock strains belonging to known serotypes was also tested by the coagglutination test, and the results agreed quite well with those of the RP test. Both whole-cell suspensions or their saline extracts gave similar results in coagglutination tests.

There were no cross-reactions among various serotypes when whole-cell suspensions or their saline extracts were used in coagglutination tests. However, when the bacterial suspensions in saline were kept at 4°C for a long time or boiled for 15 min, all of the antigens showed serotype-specific reactions in the coagglutination test, except that serotype 6 shared a minor cross-reaction with serotypes 3 and 5 but not vice versa.

The pooled staphylococcus reagents in three different combinations also gave satisfactory results with the reference strains representing each of the seven serotypes. Pools of whole-cell antigens belonging to different serotypes were mixed in equal proportions and tested by the coagglutination reagents. The results are shown

### TABLE 1. Reactivity of *H. pleuropneumoniae* (serotypes 1 to 7) with pooled and individual coagglutination reagents

<table>
<thead>
<tr>
<th>Coagglutination reagents (sero-type)</th>
<th>Reactivity of <em>H. pleuropneumoniae</em> serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Pool A (1, 2, and 5)</td>
<td>4+</td>
</tr>
<tr>
<td>Pool B (3, 4, 6, and 7)</td>
<td></td>
</tr>
<tr>
<td>Pool C (1 to 7)</td>
<td>4+</td>
</tr>
<tr>
<td>1</td>
<td>4+</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>
<tr>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

*For control tests (C1, unlabeled coagglutination reagent; C2, coagglutination reagent labeled with normal rabbit serum), no reactivity was assessed.*
TABLE 2. Reactivity of coagglutination reagents with different combinations of *H. pleuro pneumoniae* antigens

<table>
<thead>
<tr>
<th>Antigens (serotype mixture)</th>
<th>Coagglutination reagents</th>
<th>Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1, 2, 3, 4, 5, 6, and 7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1, 2, 3, 4, and 5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1, 2, 3, and 4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1, 2, and 3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1, 2, and 2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* For control tests (C1, unlabeled coagglutination reagent; C2, coagglutination reagent labeled with normal rabbit serum), no reactivity was assessed.

in Table 2. None of the combinations showed any evidence of cross-reactions among various serotypes by coagglutination test. Comparative endpoint titrations of the type strains and many field isolates with mixed serotypes showed that the coagglutination test was consistently more sensitive than the RP test in detecting serotype-specific antigen (Table 3).

Initially, the whole growth from the chocolate blood agar plate was suspended in saline. About 12% of such suspensions were found to contain more than one serotype. However, when single colonies were picked up and subcultured, all 150 single colonies tested proved positive only for a single serotype.

A total of 360 *H. pleuro pneumoniae* strains isolated from porcine pneumonia were serotyped by using the coagglutination test (Table 4).

The results of serotyping were always confirmed by the RP test. The serotypes encountered most frequently were 1 and 5, representing 71 and 15% of the total isolates, respectively. Serotypes 2 and 7 represented about 1% each of the isolates. Of the isolates, 12% had a mixture of two or more serotypes. The most common combinations were 1 and 5 (7%), followed by 1 and 2 (3%). Serotype 1 was found to be the only common serotype among the mixed isolates. None of the isolates were untypable.

**DISCUSSION**

Mittal et al. (7) reported that about 30% of the isolates in Quebec are autoagglutinating and that the RP test could replace the agglutination test for serotyping both mucoid and nonmucoid autoagglutinating strains. The coagglutination test with antibody-coated staphylococci has been used for serotyping different bacteria (2, 6, 12). In the present investigation, the coagglutination test was successfully used to serotype *H. pleuro pneumoniae*.

Results of the comparative sensitivity of coagglutination and RP tests showed that the coagglutination test was 2- to 32-fold more sensitive than the RP test in detecting type-specific antigens in bacterial suspensions. Besides, it is much easier to read and interpret the coagglutination reaction than the RP test.

The agglutination test cannot be used for serotyping autoagglutinating nonsmooth strains. The RP test requires a clear cell-free saline extract as the antigen. The immunofluorescence technique is a costly and time-consuming procedure.

Our experience with serotyping *H. pleuro pneumoniae* showed that both nonmucoid as well as mucoid strains and particulate as well as soluble antigens could easily be serotyped by the coagglutination test. Unlike the agglutination test, the coagglutination test can be used for serotyping autoagglutinating strains by using saline cell extracts as the antigens instead of bacterial cells. A high correlation between coagglutination and RP tests is strong evidence that both tests are detecting the same antigens. We found the coagglutination procedure to be a rapid, simple, specific, and sensitive method for serotyping *H. pleuro pneumoniae*.

The predominant serotype isolated in Quebec is serotype 1, as reported earlier (7), along with
serotype 5. An interesting observation is the ca. 12% prevalence of mixed infections, with isolates of more than one serotype from the same animal. The most frequent combination was serotype 1 with 5, followed by serotype 1 and 2. There seems to be no existing report on the involvement of multiple serotypes in the etiology of *Haemophilus pleuropneumoniae* infection in pigs. Characterization of the most common *H. pleuropneumoniae* serotypes in pneumonic swine lungs is needed for implementation of future vaccination policies.

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**LITERATURE CITED**