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Evaluation of Slide Agglutination and Ring Precipitation Tests for Capsular Serotyping of *Haemophilus pleuropneumoniae*

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Rapid slide agglutination (RSA), quantitative plate agglutination, slow tube agglutination (STA), and ring precipitation (RP) tests were performed on 200 isolates of *Haemophilus pleuropneumoniae* by using the type sera produced in rabbits against five known serotype strains and one strain 202. RSA and RP tests both yielded the same results as those by STA. None of the agglutination procedures could be used for serotyping isolates that autoagglutinated in saline. The RP test was successfully used for serotyping such strains. The specificity of the RSA and RP tests was confirmed by cross-absorption studies. All of the isolates except two had strong serotype-specific activities. The most common serotype isolated in Quebec was serotype 1, followed by serotypes 5 and 2. None of the isolates belonged to serotypes 3 and 4. Only two isolates were found to be untypable; they could possibly belong to serotype(s) not yet defined. The RSA and RP tests may be at least as reliable as the STA test, but easier to perform, less expensive, and much more rapid than any of the other methods reported. Of all the procedures studied by us, the RP test proved to be the method of choice for serotyping *H. pleuropneumoniae*; hence, it should replace the STA test for serotyping *H. pleuropneumoniae*.

Pleuropneumonia in swine is caused by *Haemophilus pleuropneumoniae*, formerly *H. parahaemolyticus*, and has been reported in many countries with intensive swine-producing industries (2, 3, 7, 8, 11, 13). In herds which have never been in contact with the disease, peracute infections and death are commonly observed. In more chronically infected herds, subclinical infections tend to dominate, seriously affecting the growth rate (2, 11).

Olander (H. J. Olander, Ph.D. thesis, University of California at Davis, Davis, Calif., 1963) performed cross-agglutination tests between different California isolates of *H. pleuropneumoniae* and one of Shope's Argentinian isolates. All of the California isolates except one agglutinated in the same group. The Argentinian strain did not cross-agglutinate with any of the California strains. Nicolet (9) serogrouped strains of *H. pleuropneumoniae* into three types by agglutination and precipitation tests, Newfeld capsular swelling reaction, and immunofluorescence. Recently, Gunnarsson (5) reported that capsular antigens of *H. pleuropneumoniae* could be divided into five serotypes (serotypes 1, 2, 3, 4, and 5) by means of a tube agglutination test.

We describe a simple method for typing capsular antigens of *H. pleuropneumoniae* by an RP test.

MATERIALS AND METHODS

Reference stains of *H. pleuropneumoniae*. Lyophilized cultures of the five known serotypes of Gunnarsson et al. (5) and strain 202 were kindly supplied by A. Gunnarsson of the National Veterinary Institute, Uppsala, Sweden. The cultures were suspended in tryptic soy broth and grown on chocolate blood agar supplemented with IsoVitaleX (BBL Microbiology Systems). The plates were incubated for 24 h in 10% CO_2 at 37°C.

Field isolates of *H. pleuropneumoniae*. A total of 200 strains of *H. pleuropneumoniae* were isolated from pulmonary tissues of feeder pigs which had died of acute pleuropneumonia. Each of the isolates originated from a different herd. All of the strains were identified by the method of Biberstein et al. (1).

Antigens for immunization of rabbits. Antigens for immunization of rabbits were prepared from 18-h-old mucoid growth on chocolate blood agar plates. The growth from each plate was harvested gently in 3 ml of physiological saline containing 0.3% Formalin and washed two times. An approximate 10% cell suspension was used for inoculation of rabbits.

Antigens for agglutination tests. Antigens for agglutination tests were also prepared in the same manner as described for the immunization of rabbits. A dense suspension of the bacterial growth in Formol-saline was standardized in the presence of a known homologous antiserum and used for rapid slide agglutination (RSA) and quantitative plate agglutination (QPA) tests. The same antigen was standardized to an optical density of 0.40 at 550 nm (Spectronic 20, Bauch &

Lomb, Inc.) for the STA test. The formalinized bacterial suspension obtained from 18-h-old mucoid growth on chocolate agar was referred to as whole-cell antigen. The whole-cell antigen was boiled for 1 h and used without washing; it was referred to as heat-treated formalinized antigen.

Antigens for the RP test. The growth from an 18-h-old culture on chocolate agar plate was suspended in 5 ml of Formol-saline. The suspension was centrifuged in a clinical centrifuge at 3,000 rpm for 10 min. A clear supernatant was obtained and used as antigen for the test. This antigen was referred to as saline extract. The heat extracts were prepared either by boiling the whole-cell suspension for 1 h or by autoclaving it for 2 h. The clear supernatants were obtained after centrifugation as described above and used as antigens for the test. These antigens were referred to as boiled or autoclaved extracts.

Preparation of antisera in rabbits. Antisera to each of the six reference strains of Gunnarsson and five local isolates of *H. pleuropneumoniae* were prepared in rabbits. Two young adult rabbits were injected twice a week, beginning with a single 0.5-ml dose of antigen injected subcutaneously. The subsequent seven injections were given intravenously. The doses were 1, 2, and 3 ml. The last four doses consisted of 3 ml each. The rabbits were bled 7 days after the last injection. The sera were separated and stored at −20°C until used. Preimmunization sera of rabbits were tested for their antibody contents to different type strains of *H. pleuropneumoniae*. Only rabbits that were negative for antibodies were used for preparation of type sera.

Serological tests. (i) STA test. The STA procedure was used as described by Gunnarsson et al. (5). Serial twofold dilutions of rabbit antiserum, beginning at 1 in 10, were prepared in 0.2-ml volumes of saline. Equal volumes of test antigen were added to each tube. The tubes were incubated in a water bath at 52°C for 24 h. The titer was determined as the highest serum dilution giving visible agglutination.

(ii) RSA test. For the RSA test a drop of each type of undiluted serum (approximately 0.01 ml) was placed on a clean glass slide with a Pasteur pipette. A small amount of an *H. pleuropneumoniae* colony from the chocolate agar plate was collected on an inoculating needle and mixed with serum. Alternatively, one drop of a standardized bacterial suspension in Formol-saline was added to each drop of antiserum and also to a drop of normal rabbit control serum and mixed with a single wooden applicator stick to form a uniform suspension. A strong positive reaction in the form of clumping and clearing occurred within a few seconds as the mixture was stirred. A negative reaction remained turbid.

(iii) QPA test. For the QPA test serial twofold dilutions of rabbit antisera were prepared in normal saline solution beginning at 1 in 2 in a 0.1-ml volume, and one drop of each dilution was tested on the plate as for the whole serum. One drop of plate antigen was added to each dilution and mixed. The positive reaction occurred within a few seconds to 1 min. The titers were expressed as the reciprocal of the last serum dilution at which positive agglutination occurred.

(iv) RP test. For the RP test each of the reference type sera was sucked into Pasteur pipettes by capillary action. The pipettes were inverted, and the serum was allowed to stay in the neck of the pipette after its tip was sealed with plasticine. The Pasteur pipettes were kept in a vertical position in a rack at room temperature. The test antigen was layered slowly over the surface of the serum with a Pasteur pipette. A positive reaction, in the form of a clear and sharp ring of precipitation, appeared at the interface within a few seconds to 1 min and was easily seen by inspection against dark background. Known positive and negative control sera were always kept simultaneously.

Cross-absorptions. The 18-h-old growth of each reference strain was harvested from the chocolate blood agar plates very gently in saline and centrifuged at 3,000 rpm in a clinical centrifuge for 10 min. The supernatant was removed with a Pasteur pipette. One milliliter each of the homologous as well as the heterologous undiluted type sera was added to the sediment to make a final concentration of approximately 10% cells in the mixture. The serum was mixed well with the cells, using a Pasteur pipette. The mixture was incubated at 37°C for 2 h and then at 4°C overnight. After centrifugation, the same absorption procedure was repeated for the second time. The clear supernatant was taken and used as absorbed serum.

Serotyping of the field isolates. All of the 200 isolates of *H. pleuropneumoniae* were serotyped by the standard STA procedure of Gunnarsson et al. (3), using rabbit antisera to all five serotypes and strain 202. RSA, QPA, and RP tests were also performed on all of the isolates.

RESULTS

Agglutination tests with whole-cell antigens. The six reference strains were tested with whole-cell antigens and rabbit anti-whole cell sera by the RSA, QPA, and STA procedures. No cross-reactions were observed by the QPA test, and only limited cross-reactions were seen by the RSA, RP, and STA tests. Undiluted serum against strain 202 gave a positive reaction with serotype 1 by RSA and RP; this reaction disappeared when diluted serum was used. It is noteworthy that serum against serotype 1 did not cross-react with strain 202 in any of the tests. By using STA, a slight cross-reaction was observed between serotypes 3 and 4.

Agglutination tests with heat-treated formalinized antigens. The heat-treated antigens of the six reference strains were tested with the same antisera as above. The heat-treated antigens of serotype 1, 2, and 5 strains did not show any cross-reactions with any of the heterologous type sera, whereas serotype 3 and 4 strains and strain 202 cross-reacted. Maximum cross-reactions were observed by RSA; however, with further dilution of type sera by QPA, we were better able to differentiate between homologous and heterologous reactions.

RP test. Various saline extracts of the reference strains were used for the evaluation of the RP test as a system for typing *H. pleuropneumoniae* strains. When unheated saline extract antigens were used, a sharp ring of precipitation

TABLE 1. Results of agglutination and precipitation tests between reference strain whole-cell antigens and rabbit anti-whole-cell sera

Antigens of reference serotype (strain)	Test used[a]	Rabbit anti-sera against whole-cell antigens of reference serotypes:					
		1	2	3	4	5	Strain 202
1 (4074)	RSA	+	−	−	−	−	+
	QPA[b]	16	−	−	−	−	−
	STA[b]	1,280	−	−	−	−	−
	RP	+	−	−	−	−	+
2 (4226)	RSA	−	+	−	−	−	−
	QPA	−	8	−	−	−	−
	STA	−	640	−	−	−	−
	RP	−	+	−	−	−	−
3 (1421)	RSA	−	−	+	−	−	−
	QPA	−	−	16	−	−	−
	STA	−	−	2,560	80	−	−
	RP	−	−	+	−	−	−
4 (M62)	RSA	−	−	−	+	−	−
	QPA	−	−	−	16	−	−
	STA	−	−	80	640	−	−
	RP	−	−	−	+	−	−
5 (K17)	RSA	−	−	−	−	+	−
	QPA	−	−	−	−	16	−
	STA	−	−	−	−	640	−
	RP	−	−	−	−	+	−
Strain 202	RSA	−	−	−	−	−	+
	QPA	−	−	−	−	−	64
	STA	−	−	−	−	−	2,560
	RP	−	−	−	−	−	+

[a] RSA, Rapid slide agglutination test; QPA, quantitative plate agglutination test; STA, slow tube agglutination test; RP, ring precipitation test.

[b] The results are expressed in titer, which is the reciprocal of the last serum dilution at which positive agglutination occurred.

was observed only with the homologous systems, except for a cross-reaction between serotype 1 and serum against strain 202 (Table 1). However, when heated extracts were used, this cross-reactivity was abolished.

Cross-absorptions. The type sera were tested for agglutinating and precipitating activities by RSA and RP tests before and after absorption with both homologous and heterologous whole cells. Absorption of test sera with any of the heterologous cells did not cause any reduction in the intensity or degree of the reaction in both RSA and RP tests except in anti-202 serum absorption, of which, with either serotype 1 or strain 202, whole cells completely removed the cross-reactivity with serotype 1. Cross-absorptions of the test sera with 10 field isolates collected at random yielded similar results. Absorption of the type sera with homologous cells completely removed both the agglutinating and precipitating activities in RSA and RP tests, respectively.

Agglutination and precipitation tests with rabbit antisera produced against the field isolates. Five local isolates belonging either to serotype 1, 2, or 5 and one untypable, presumably new, serotype were selected; antisera were produced in rabbits against whole cells described. The sera were tested by RSA, STA, and RP, using the antigens of the six reference strains and the five local isolates. The sera produced against the local field strains gave similar results to those of the sera produced against the reference strains.

Serotyping of the field isolates. A total of 200 isolates were serotyped by using type sera against the known five serotypes and one strain 202. The results are given in Table 2. All of the isolates except two were typable. There were no cross-reactions among any of the isolates except the one-way cross-reaction between serotype 1 and anti-202 serum by the RSA and RP tests. There was no apparent difference in the reaction between the long-term stored cultures or the freshly isolated strains by any of the serological methods used. A total of 66 isolates were nonmucoid on chocolate blood agar and thus were unstable in saline, with a tendency to autoagglutinate. It was, therefore, not possible to serotype them by any of the agglutination procedures. The RP test was the only test found to be efficient in determining their serotype. None of the isolates belonged to serotypes 3 or 4 or to the strain 202 group. Eighty-three percent of the isolates belonged to serotype 1, followed by

TABLE 2. Results of serotyping of 200 strains of field isolates of *H. pleuropneumoniae* in Quebec

Type of strain	Test used	No. of strains belonging to serotype:					
		1	2	3	4	5	UT[a]
Mucoid strains	RSA QPA STA RP	104	2	0	0	26	2
Autoagglutinating strains	RP	62	0	0	0	4	0

[a] Untypable serotype.

serotype 5 (15%) and serotype 2 (1%). We came across only two strains (1%) which did not react with any of the type sera.

Reproducibility of the serological procedures. Serological typing of 50 isolates selected at random was repeated after cultures suspended in Formol-saline had been stored at 4°C for 9 to 12 months. There was no difference in the results obtained before as well as after storage in both agglutination and precipitation tests. Lyophilized cultures stored for more than 1 year also showed similar results.

DISCUSSION

Gunnarsson et al. (5) classified various isolates into six distinct serotypes by the tube agglutination test. Serotype 6 was represented by a single isolate (strain 202), which was subsequently found to have additional characteristics; it is now uncertain whether it belongs to the same species. Thus, up to now, we have only five recognized serological groups, which are designated as serotypes 1 to 5.

The results of agglutination tests (Table 1) showed that it was possible to group the isolates into five distinct serotypes by a simple plate agglutination test, using unheated formalinized whole-cell antigen and rabbit anti-whole-cell serum. There was a one-way cross-reaction between serotype 1 and undiluted rabbit anti-202 serum by RSA, which was completely abolished when the antigen used was a heat-treated preparation. When the type serum was diluted, as in the QPA and STA tests, no cross-reaction was observed.

The use of heat-treated formalinized whole-cell antigens in the RSA, QPA, and STA tests resulted in cross-reactions, especially among serotypes 3, 4, and strain 202. This observation suggests that some of the type-specific antigens involved in agglutination tests may be heat labile in nature and seem to be present on the surface of *H. pleuropneumoniae*.

Gunnarson et al. (4–6) reported that the type specificity of *H. pleuropneumoniae* is usually located in their capsular substance. Thus, serotyping was based on whole-cell agglutination and required cells in the encapsulated mucoid phase. Dissociation of mucoid cells to smooth or rough forms reduced typability.

With unheated saline extract antigen, similar results as those observed by RSA were observed by RP. Boiling or autoclaving the extract abolishes the one-way cross-reaction between serotype 1 antigen and anti-202 serum, but not the type-specific reaction. The latter observation indicates that heat-stable antigen is involved in the serotyping of *H. pleuropneumoniae* in the RP test.

Rabbit antisera produced against the whole-cell preparations of five field isolates representing serotypes 1 (two strains), 2, 5, and one untypable serotype were used in all of the serological tests with the homologous as well as the reference strains. The results suggest that field isolates in Quebec are antigenically similar to the reference strains isolated elsewhere.

With saline extracts from all of the 200 field isolates, including 66 autoagglutinating ones, all but 2 of the isolates could be typed by RP. The results suggest that the RP test can be used not only for typing mucoid isolates but also for the autoagglutinating ones (Table 2). Evidently all of the autoagglutinating strains remained untypable by agglutination tests. The RP test is rapid as well as easy to perform and interpret and always gave consistent, reliable, and reproducible results. The test was specific as demonstrated by absorption experiments and extremely sensitive as shown by its detection of type-specific antigen, even in the autoclaved extract of rough strains. The potential benefit of this method for epidemiological studies and for reference laboratories processing large numbers of isolates is obvious. Hence we believe that the RP test is the method of choice for routine serotyping of *H. pleuropneumoniae* and should replace the STA test.

Serological studies of *H. pleuropneumoniae* have been reported from different parts of the world. Swedish strains studied have been assigned to serotypes 2, 3, and 4 (3, 10), serotypes 4 and 5 have been reported in the United States (5), and serotypes 1, 2, 3, and 5 have been reported in Canada (3, 12). The present studies report serotypes 1, 2, and 5 isolated from pigs which died of acute pleuropneumonia in the region of Quebec. Serotype 4 seems to be confined to the United States and has not been reported yet in Canada.

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