

Value of Indirect Hemagglutination and Coagglutination Tests for Serotyping *Haemophilus parasuis*

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An indirect hemagglutination test (IHA) and a coagglutination test (CA) were evaluated using saline, boiled, and autoclaved extracts for serotyping *Haemophilus parasuis*. CA showed several cross-reactions, whereas IHA gave rise to specific reactions, with minor exceptions. IHA was further compared with the immunodiffusion test (the “gold standard”) for the serotyping of 67 field isolates. As a conclusion, IHA is recommended as a useful method for sensitive and specific serotyping of *H. parasuis*.

Haemophilus parasuis, a small pleomorphic gram-negative rod of the family *Pasteurellaceae*, is the causative agent of Glässer's disease, which has historically been considered a sporadic, stress-associated disease of young pigs (19). However, since the establishment of specific-pathogen-free herds, increased spread of the disease and increased mortality rates have been described for the swine industry worldwide (20).

Although some other methods have been used for the serotyping of *H. parasuis* (5, 12), the Kielstein-Rapp-Gabrielson scheme (based on heat-stable cell antigens tested by agar gel precipitation) is now the internationally recognized test (1, 6, 14). At present, 15 serovars have been proposed, with most recent serotyping studies reporting the existence of nontypeable isolates, which seems to indicate the possibility of a larger number of serovars (6, 14). It is essential to know which are the most prevalent serovars in a given area to effectively control Glässer's disease, because the vaccine immunity confers a reduced cross-protection among serovars (7). In addition, several molecular methods (restriction endonuclease fingerprinting [19], multilocus enzyme electrophoresis [2], enterobacterial repetitive intergenic consensus-PCR [13], and outer-membrane-protein profiling [17]) have also been developed for typing *H. parasuis*.

Coagglutination (CA) and indirect hemagglutination (IHA) are accessible tests at any diagnostic laboratory, and they have been proven to be reliable and suitable for serotyping clinical isolates of a variety of gram-positive and gram-negative organisms. The aim of this study was to evaluate the use of these two tests in comparisons of different antigens as screening procedures for serotyping *H. parasuis*.

Reference strains representing serotypes 1 through 15 of *H. parasuis* (H409, H410, H411, H412, H413, H780, H643, H494, H553, H555, H465, H425, H793, H792, and H790) were used. The production of antisera in rabbits has already been described elsewhere (6). Antisera were absorbed with an equal volume of a mixture of 10% of whole cells of the 14 heterologous serovars (bacterial suspensions from an 18-h culture on chocolate agar), as described previously (8).

For the preparation of antigens for CA and IHA, the overnight growth from chocolate agar was washed off in 0.15 M NaCl. The suspension was adjusted to a 10% cell concentration and was divided into three parts. The first part was left overnight at room temperature, the second part was boiled for 1 h, and the third portion was autoclaved (121°C for 2 h). All three preparations were then centrifuged at 8,000 × *g* for 10 min. The clear supernatants which were pipetted off the first, second, and third preparations are referred to in this work as saline extract, boiled extract, and autoclaved extract, respectively.

Details of the preparation of CA reagent (antibodies bound to *Staphylococcus aureus* strain Cowan 1 [NCTC 8530]) and details of the test procedure have been described previously (10). For the IHA test, fresh sheep red blood cells (SRBC) were sensitized as described previously (4, 9). The IHA test was performed with a microtiter system as reported by Mittal et al. (9).

Concerning CA test results, saline extracts gave strong positive reactions with all the homologous reagents. However, several cross-reactions appeared, especially for serovars 10, 13, 14, and 15, some of which were of the same intensity as that seen with the homologous reagent. Cross-reactions appeared for all tests except when serovars 1 and 7 were tested. The use of boiled extracts decreased the amount of cross-reactions, but even so, they were observed for serovars 2 to 4, 6, and 10 to 15. These findings indicate that the heat-labile nature of some of the cross-reacting antigens but not of others was detected by CA testing. Finally, autoclaved extracts did not give rise to positive reactions with any of the homologous or heterologous reagents. CA is a simple, specific, and sensitive test (10); however, given the large amount of cross-reactions detected in this study, use of this test cannot be recommended for serotyping *H. parasuis*. These results clearly contrast with those reported for other members of the *Pasteurellaceae* (e.g., *Actinobacillus pleuropneumoniae*, *Mannheimia haemolytica*, and *Pasteurella multocida*), for which CA testing proved to be useful for detecting type-specific antigens (3, 10, 11, 16, 18).

When saline extracts were used as antigens in IHA, specific reactions were demonstrated for all serovars, with the exception of those resulting in a small number of cross-reactions (Table 1). As the homologous antibody titers observed in these

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TABLE 1. IHA test results with various soluble antigens and rabbit hyperimmune sera produced against whole-cell antigens

Serovar (type of antigen ^a)	Antibody titer against antigen of reference serovar ^b														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
SE	20														
BE	20														
AE	10														
2															
SE		40													
BE	20	40													
AE															
3															
SE			160												
BE			160												
AE															
4															
SE				20											
BE				20											
AE				10											
5															
SE					80										
BE					80										
AE	10				20										
6															
SE						160									
BE						80									
AE					10	20									
7															
SE							160								
BE							160								
AE															
8															
SE								320							
BE								80							
AE								40							
9															
SE									320						
BE									80						
AE									40						
10															
SE	10									40					
BE	20									40					
AE															
11															
SE											40				
BE									10		40				
AE															
12															
SE												320			
BE												80			
AE															
13															
SE						20								40	
BE						20								40	
AE															
14															
SE														320	
BE														160	
AE															
15															
SE															160
BE															160
AE					20										40

^a SE, saline extract; BE, boiled extract; AE, autoclaved extract.

^b Titers are expressed as the reciprocals of the final dilutions of sera giving positive reactions.

cross-reactions were always higher than the heterologous ones and the cross-reactions were one way only, neither type of result led to confusion. Using boiled extracts, serovars 1, 3 to 9, 12, 14, and 15 did not show any cross-reactions, whereas the remaining serovars exhibited one-way cross-reactions (Table 1). Anyway, we were able to differentiate between homologous and heterologous reactions, again with the help of the antibody titers. The fact that the cross-reactions detected when using saline extracts were also present after boiling indicates the heat-stable nature of the cross-reacting antigens. The autoclaved extracts showed the lowest antigenic activity, and they did not reveal any antibody activity for serovars 2, 3, 7, and 10 to 14. Some new one-way cross-reactions were observed with these extracts (Table 1). The dilution of antisera allowed us to differentiate again between homologous and heterologous reactions.

Investigators using other serological tests for *H. parasuis* have reported the presence of specific, thermostable, soluble antigens of a polysaccharide nature associated with the cell capsule or outer membrane (5, 6, 14). Surface polysaccharides from gram-negative bacteria adsorb naturally to SRBC (22); for this reason, these antigens are very suitable for IHA. In light of the results obtained, use of the IHA test with any of the saline or boiled extracts appears to be a good choice for serotyping *H. parasuis*. Our study also confirmed that the heat-stable antigens adsorbed on the SRBC are serovar specific. However, strong heat treatments are not necessary, because the antigen preparations consisting of supernatants obtained from unheated cells are equally serovar specific. This observation seems to suggest that not only deeper but also surface antigens are involved in the specificity of *H. parasuis* serovars, a finding already reported for *A. pleuropneumoniae* (9). Even stronger heat treatments, such as autoclaving, are not useful (despite the fact that autoclaved extracts are the antigens of choice in the immunodiffusion test [ID] [12, 14]), probably because heating at 121°C for 2 h is capable of modifying the thermostable antigens in a such manner as to eliminate their adsorption to SRBC in some serovars and decrease it in others.

To validate the usefulness of the IHA test, a total of 67 *H. parasuis* field isolates recovered in Spain between 1998 and 2002 were further tested using saline extracts (the extract showing maximum specificity), and results were compared to those of the ID using autoclaved extracts (6, 14). All of the isolates that were serotyped by ID were also correctly typed by the proposed IHA test, with the exception of one isolate of serovar 2 (Table 2). Of the 25 isolates that were considered nontypeable by ID, 80% were typed with IHA while 5 isolates remained nontypeable. Slight cross-reactions, none of which led to confusion when selecting the correct serovar, were shown by only three isolates (Table 2).

A total of 91% of the isolates were serotyped with IHA versus the almost 63% that were serotyped with ID, thus suggesting that the former test is a more sensitive procedure for serotyping *H. parasuis*. The presence of five isolates nontypeable by both IHA and ID, a finding reported at higher levels in other investigations in which the isolates were serotyped only by ID (14, 15, 21), confirms the antigenic heterogeneity of *H. parasuis* and suggests the existence of additional serovars. Other possible topics for speculation include that of the low

TABLE 2. Results of serotyping of 67 *H. parasuis* field isolates by ID and the proposed IHA test

Serovar	No. (%) of positive strains by:		Cross-reacting serovar (no. of strains)
	ID	IHA test	
1	2	6 ^b	— ^a
2	3	4 ^c	—
3	1	2 ^d	—
4	11	13 ^e	15 (1)
5	12	15 ^f	—
6	1	1	—
7	4	7 ^g	9 (1)
9	2	2	—
12	2	6 ^h	—
13	2	2	—
14	1	1	—
15	1	2 ⁱ	—
Nontypeable	25	5	2 (1)
Total	42 (62.7)	61 (91.0)	

^a —, no cross-reaction.

^b Four of the strains identified by the IHA test were untypeable by ID.

^c Two of the strains identified by the IHA test were untypeable by ID.

^d One of the strains identified by the IHA test was untypeable by ID.

^e Two of the strains identified by the IHA test were untypeable by ID.

^f Three of the strains identified by the IHA test were untypeable by ID.

^g Three of the strains identified by the IHA test were untypeable by ID.

^h Four of the strains identified by the IHA test were untypeable by ID.

ⁱ One of the strains identified by the IHA test was untypeable by ID.

polysaccharide content in these isolates, the main type-specific antigen involved in IHA.

Although the reliability of the classical typing methods is strongly dependent on the characteristics of the serovar-specific antisera used, the sensitivity and specificity revealed by the proposed IHA test recommend its use as an additional method for reliable serotyping of *H. parasuis*.

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