Development of a New Serological Test for Serotyping \textit{Haemophilus parasuis} Isolates and Determination of Their Prevalence in North America

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\textit{Haemophilus parasuis} causes polyserositis in swine. Fifteen serovars have been characterized by immunodiffusion test, but many field strains are not typeable. Isolates ($n = 300$) of \textit{H. parasuis} from animals in North America were serotyped by a new indirect hemagglutination test. The test was rapid and effective for serotyping of \textit{H. parasuis}, and serovars 4, 5, 13, and 7 were the most prevalent serotypes.

Porcine polyserositis (Glasser’s disease) caused by \textit{Haemophilus parasuis} is a disease of increasing economic importance, causing high morbidity and mortality in specific-pathogen-free or high-health-status pigs (12). Heterogeneity among \textit{H. parasuis} isolates was demonstrated by serotyping (4, 9), morphology (10), and protein profiles of whole-bacterial-cell suspensions (4, 10) and outer membranes (12a, 15). An association between serovar, protein pattern, presence of capsule, and pathogenicity of an isolate was demonstrated. An immunodiffusion test with heat-stable antigens (9) is used for typing of \textit{H. parasuis}, and 15 serotypes have been described (5, 13). However, approximately 30% of field isolates of \textit{H. parasuis} are untypeable by immunodiffusion, and cross-serotype reactivity is a problem with this test. Antigenic characterization of prevalent strains of \textit{H. parasuis} is essential for developing effective vaccines and serodiagnostic tests. The aim of the present study was to develop and evaluate an improved test for serotyping of \textit{H. parasuis} and to determine the prevalence of the various serotypes in a collection of North American isolates.

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Reference strains of \textit{H. parasuis} serovars 1 to 15 were provided by R. F. Ross (College of Veterinary Medicine, Ames, Iowa), and A. Raßbach (Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Jena, Germany). Field isolates of \textit{H. parasuis} from Canada ($n = 250$) and the United States ($n = 50$) from 1991 to 2002 were evaluated. Isolates were biochemically characterized as \textit{H. parasuis} as previously described (7, 8) and cultured on pleuropneumonia-like organism agar medium with overnight incubation at 37°C (8, 13).

Antiserum against the 15 reference strains were prepared as described by Morozumi and Nicolet (9) with some modifications. Overnight growth of reference strains on pleuropneumonia-like organism agar was harvested with phosphate-buffered saline solution, pH 6.8, containing 0.5% formalin, and was kept at room temperature for 2 days. Formaldehyde-fixed-whole-cell (FWC) suspensions were adjusted to an optical density of 1 at 540 nm, and 2 ml of the suspensions and an equal volume of Freund’s incomplete adjuvant were injected subcutaneously at four sites. Three weeks later, rabbits were given an intravenous inoculation of 0.5 ml of FWC suspension, followed by seven doses given intravenously in increasing doses twice a week. Rabbits were bled 7 days after the last injection. Antisera were separated and stored at $-20^\circ$C. Sera showing weak reactions in an immunodiffusion test were concentrated with an SV3/H speedvac concentrator (Savant). However, considerable difficulty was encountered in producing antisera for some serovars, mainly against reference strain N4 (serotype 1) and strain 174 (serotype 7). Thus, strain SW35 of serotype 1 (9) and field strain 85-665 of serotype 7 (13) were used to produce hyperimmune sera in rabbits.

The FWC suspension of each reference strain was boiled for 30 min followed by centrifugation at 1,500 \times g for 10 min. The resulting supernatant was referred to as boiled whole-cell supernatant and was used directly as an antigen in the immunodiffusion test and also to coat sheep red blood cells for the indirect hemagglutination test as described previously (6). The immunodiffusion test (9) and indirect hemagglutination tests (6) were conducted as described previously.

To evaluate serovar specificity of the antisera prepared against FWC antigens in rabbits, immunodiffusion and indirect hemagglutination tests were performed with each antisera using each of the boiled whole-cell supernatant antigens prepared from each of the reference strains of \textit{H. parasuis}. Results of this analysis with antigens from reference strains showed that with both serotyping tests the reference antisera was serovar specific and only minor cross-reactivity was observed.

When the immunodiffusion test was used to serotype field isolates, extensive cross-reactives were observed. With some isolates, the cross-reactions were too strong to distinguish between the serovar-specific and species-specific reactions, and some isolates completely failed to react. More than 30% of the field isolates were nontypeable by immunodiffusion. Attempts to adsorb the cross-reacting antibody with antigens of the cross-reacting serovars eliminated the serovar-specific reactiv-
ity as well. In contrast, more than 90% of the field strains of *H. parasuis*, including those with cross-reactivity by immunodiffusion, were typeable by the indirect hemagglutination test. Some isolates were not typeable by either method, including isolates from Canada and the United States.

Analysis of 250 field isolates from Canada indicated a high prevalence of serovar 4 (27% of isolates), followed by serotypes 5 (15%), 13 (14%), 7 (12%), 2 (8%), and 12 (5%). Of 50 isolates from the United States, serotype 4 was the most prevalent (25%), followed by serotypes 12 (23%) and 5 (15%).

Serotyping of *H. parasuis* is important in both epidemiological and immunological studies of *H. parasuis* infection. Different antigen preparations of *H. parasuis* are used in different serological tests for serotyping and serodiagnosis. The cellular localization of serotype-specific antigens of *H. parasuis* has not been well defined, although studies have indicated that these antigens may be polysaccharides associated with capsule or outer membrane components (4, 9).

In this study, immunodiffusion tests using a boiled-whole-cell extract as antigen and antisera prepared against *H. parasuis* reference strains were serotype specific except for a one-way cross-reaction of serotype 5 with serotype 1. Cross-reactivity was not a problem with the indirect hemagglutination test. However, when the tests were applied to field isolates, cross-reactivity was a significant problem with the immunodiffusion test but not with the indirect hemagglutination test. These results demonstrate the usefulness of the indirect hemagglutination test for typing field isolates and suggest that heat-stable, serotype-specific antigens present in boiled cell extracts are selectively adsorbed onto the surface of erythrocytes.

Bacterial lipopolysaccharides are adsorbed directly onto the surface of sheep red blood cells, but protein antigens require pretreatment of red blood cells with tannic acid, bisdiabenzi-
dine, chromium chloride, etc., for adsorption (2, 3, 16). Based on this information, we speculate that the serotype-specific *H. parasuis* antigens selectively adsorbed onto the surfaces of sheep red blood cells may be lipopolysaccharide in nature. This may explain why the indirect hemagglutination test was found to be more specific than the immunodiffusion test. The antigens reactive in the immunodiffusion test are soluble and are of a precipitating nature, whereas in the indirect hemagglutination test, the antigens are of a particulate nature. The sensitivity of the indirect hemagglutination test for detection of antibodies in sera is much higher than that for the immunodiffusion test (17). It is likely that the increased test sensitivity is the reason that some sera reacted in the indirect hemagglutination assay but not in the immunodiffusion test.

In an attempt to decrease cross-reactivity of rabbit antisera in the immunodiffusion test, antisera were absorbed with heterologous antigens. However, this procedure removed reactivity against both serotype-specific and species-specific antigens, as previously reported (13).

Initially, serotype 5 was reported to be the most prevalent serotype in North America, Europe, and Australia, followed by serotype 4 (1, 5, 13, 14). Results of our study confirm those of Oliveira et al. (11), who described serotype 4 as being the most prevalent serotype in North America. Serotyping information is still the key to understanding the epidemiology and control of *H. parasuis* infection.

**ADDENDUM**

This paper gave the first description of the use of this indirect hemagglutination test for serotyping of *H. parasuis*. Subsequent to the submission of this paper, another paper reporting on the same procedure was published (M. L. Del Rio, C. B. Gutierrez, and F. E. F. Rodriguez, J. Clin. Microbiol. 41: 880–882, 2003).

**REFERENCES**