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Determination of Antigenic Specificity and Relationship Among *Haemophilus pleuropneumoniae* Serotypes by an Indirect Hemagglutination Test

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Serological properties of antigens extracted from strains of *Haemophilus pleuropneumoniae* belonging to seven different serotypes were investigated. Antisera were prepared in rabbits against Formalin-treated whole cell suspensions as well as autoclaved cell suspensions. Saline and heat extracts and their alcohol precipitate antigens of *H. pleuropneumoniae* were used in the indirect hemagglutination test. All the antigens used were easily adsorbed directly onto sheep erythrocytes. Saline extract antigen showed maximum type specificity. Heating of the whole cell suspension revealed the cross-reactive minor antigenic determinants. Thus, the heat extract preparations had both type-specific and species-specific antigens. It is suggested that the indirect hemagglutination test may be useful for both serotyping and serodiagnosis of *H. pleuropneumoniae* infections in pigs.

Porcine infections with *Haemophilus pleuropneumoniae* have been mainly associated with pleuropneumonia. When the disease occurs in the acute form in nonimmune herds, it causes high mortality. The chronic form of the infection also causes significant economic losses (4, 10, 12). The disease has been reported from many countries (4, 6, 8, 10, 11, 14, 15).

Serological classification schemes of *H. pleuropneumoniae* involving tube agglutination (6), slide agglutination (8), immunodiffusion (10), immunofluorescence (10, 14), and ring precipitation (8) have been described. At present, five serotypes of *H. pleuropneumoniae* have been well recognized (6). Two more new serotypes (namely, serotypes 6 and 7) have recently been proposed (13).

Nicolet (10) reported two types of type-specific antigens, thermolabile and thermostable. Gunnarsson and co-workers (5, 7) characterized both common and type-specific antigens for the five serotypes of *H. pleuropneumoniae* by means of agglutination, immunodiffusion, and absorption tests. They also identified heat-stable and heat-labile type-specific antigens.

The indirect hemagglutination (IHA) test in various modifications has been extensively applied for the detection of antibodies to various bacterial species in human and animal sera (9) and also for the antigenic analysis of certain bacteria (16). The object of the present study was to characterize the type-specific antigens and the antigenic relationship among various serotypes of *H. pleuropneumoniae*. A comparative study of seven serologically distinct strains of *H. pleuropneumoniae* was undertaken to measure differences and similarities in antigenic properties. Thus, this paper reports on the application of the IHA test to the antigenic analysis of *H. pleuropneumoniae*.

MATERIALS AND METHODS

Bacteria. Strains representing serotypes 1 through 5 of *H. pleuropneumoniae* were obtained from A. Gunnarsson of the National Veterinary Institute, Uppsala, Sweden. Strains representing serotypes 6 and 7 were provided by S. Rosendal, University of Guelph, Guelph, Ontario, Canada. Only one reference strain representing each serotype was used for the preparation of antibodies. Cultures were suspended in tryptic soy broth and were grown on chocolate blood agar supplemented with IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). The plates were incubated for 24 h in 10% CO_2 at 37°C. Cultural and biochemical characterization of the bacteria was carried out by the method of Biberstein et al. (2).

Antigens for immunizing rabbits. Antigens for immunizing rabbits were prepared from a mucoid growth 18 h old on chocolate blood agar plates. The growth from each plate was harvested gently in 5 ml of physiological saline containing 0.3% Formalin and was washed twice. A cell suspension of approximately 10% was used for immunizing rabbits. This antigen is referred to as formalinized whole cell antigen. A portion of the whole cell antigen was autoclaved for 2 h and was used as antigen for immunizing another batch of rabbits.

This antigen is referred to as autoclaved antigen.

Preparation of antisera in rabbits. Antisera were prepared in rabbits by intravenous inoculations of formalinized whole cell antigens or autoclaved antigens of different serotypes of *H. pleuropneumoniae* as described by Mittal et al. (8). Two young adult rabbits were injected twice a week with each antigen preparation of each serotype. The rabbits were bled 7 days after the last injection. The sera were separated and stored at −20°C until used.

Antigens for IHA test. The overnight growth from chocolate blood agar supplemented with IsoVitaleX was washed off in 0.85% saline, pH 11.0. The suspension was adjusted to 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 at 15 lb [6.8 kg] of pressure) for 1 h. All three preparations were centrifuged at 8,000 × *g* for 30 min. The clear supernatants were pipetted off and are referred to as saline extract, boiled extract, and autoclaved extract, respectively. These extracts were used directly to coat the sheep red blood cells (SRBC) in the IHA test.

To obtain alcohol precipitates, one part each of saline extract and boiled extract was treated with five parts of chilled absolute alcohol and was kept at 4°C overnight. The mixtures were centrifuged at 8,000 × *g* for 30 min. The white precipitate was dissolved in saline to the original volume and was used as a sensitizing antigen.

Sensitization of SRBC with various extracts and alcohol precipitates of *H. pleuropneumoniae*. Checkerboard titrations were carried out to find the optimum dilutions of various antigens. Fresh SRBC were washed three times in physiological saline and were sensitized with antigens as follows. A volume of 0.2 ml of well-washed packed SRBC was added to a tube containing 2 ml of an optimally standardized dilution of the antigen and was incubated at 37°C for 1 h. The sensitized SRBC were again washed three times in saline to remove unbound antigen and were suspended in saline, pH 7.0, to a concentration of 0.5%. The sensitized cells were used on the same day.

Performance of the test. The IHA test was performed with a microtiter system (Dynatech Laboratories, Inc.). The test sera were heat inactivated at 56°C for 30 min. The heterophile antibodies were removed by absorption of sera with unsensitized SRBC at room temperature for 1 h. Serial twofold dilutions of the sera from 1/10 to 1/10,240 were made in 0.05-ml volumes of saline in U-bottom microplates. The same volume of sensitized SRBC suspension was added to each well. The contents of the wells were thoroughly mixed by gently tapping the plates and were incubated at 37°C for 2 h before reading. The IHA titer was expressed as the reciprocal of the highest dilution of serum showing a definite positive pattern (flat sediment) as compared with the pattern of negative control (smooth dot in the center of the well). Controls consisted of unsensitized SRBC plus test serum, sensitized SRBC plus positive serum, and sensitized SRBC plus diluent.

RESULTS

Initial experiments were carried out to find the optimum dilution of antigen for the sensitization of SRBC by box titrations against homologous rabbit antiserum. The undiluted supernatants obtained from 10% cell concentration showed the highest titer in the homologous antiserum. Almost identical titers were obtained with the same antisera when several different lots of sensitized SRBC were tested.

IHA cross-tests were performed to determine the specificity of the reaction with various antigen preparations. The results of IHA tests with saline extracts and alcohol precipitates of boiled extract and rabbit antisera are shown in Table 1. Capsular group-specific reactions were demonstrated for each serotype. The titers varied from a low of 20 to a high of 10,240. The autoclaved extract showed the maximum antigenic activity. The saline extract seemed to possess more antigenic activity than the boiled extract for all the serogroups except for serotype 3, for which the saline extract could not detect any antibody activity.

The antigenic activity of alcohol precipitate of boiled extracts was found to be both more specific and higher than that of alcohol precipitate of saline extract in the case of serotypes 1, 3 and 5. Serotype 2 showed the maximum cross-reactivity, especially with autoclaved extract antigen. Saline extracts of all the serotypes showed maximum specificity.

IHA tests involving various soluble antigens and rabbit hyperimmune sera produced against autoclaved antigens of reference serotypes were also carried out. Low levels of antibody titers varying from 20 to 160 were observed with autoclaved antigens in almost all the test sera. Boiled extract antigen also detected lower antibody titers in some sera, but saline extract antigen failed to detect any antibody levels.

DISCUSSION

IHA tests have been employed successfully for the identification of serological types of *Pasteurella multocida* (3) and *Pasteurella haemolytica* (1). Nielsen (11) used the IHA test in the diagnosis of *H. pleuropneumoniae* of swine. He used goat erythrocytes sensitized with the supernatant of a 6-h-old disintegrated culture of *H. pleuropneumoniae* of serotype 2. He reported that the cross-reactions occurred between *Haemophilus parasuis* and *H. pleuropneumoniae*, thus suggesting the nonspecificity of the IHA test in field diagnostic work.

The data shown in Table 1 clearly indicate that saline extract of the known seven serotypes never showed any reactivity with heterologous sera. The test was negative when saline extract of whole cell antigen or its alcohol precipitate of serotype 3 was used with homologous antisera. However, the same antigen after boiling or auto-

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

Serotype[a]	Test antigen[b]	Antibody titer against antigen of reference serotype[c]:						
		1	2	3	4	5	6	7
1 (4074)	SE	>10,240	0	0	0	0	0	0
	AP-BE	10,240	0	0	0	0	0	0
2 (4226)	SE	0	2,560	0	0	0	0	0
	AP-BE	0	2,560	20	40	20	0	0
3 (1421)	SE	0	0	0	0	0	0	0
	AP-BE	0	0	2,560	0	0	0	0
4 (M62)	SE	0	0	0	1,280	0	0	0
	AP-BE	0	0	0	320	0	0	0
5 (K17)	SE	0	0	0	0	640	0	0
	AP-BE	0	0	0	0	1,280	0	0
6 (Femϕ)	SE	0	0	0	0	0	2,560	0
	AP-BE	20	0	0	0	20	2,560	0
7 (WF83)	SE	0	0	0	0	0	0	1,280
	AP-BE	0	0	0	0	20	0	2,560

[a] Reference strain given in parentheses.
[b] SE, Saline extract; AP-BE, alcohol precipitate of boiled extract.
[c] Titers, determined in rabbit antisera against whole cell antigens of reference serotypes, are expressed as the reciprocals of the final dilutions of serum giving positive reactions.

claving was able to detect a significantly high level of antibody. These observations suggest that surface antigens of the reference serotype 3 either lacked the antigenicity necessary to provoke antibodies that are reactive with saline extract antigen or failed to be adsorbed on the SRBC. Heating the cell suspension or saline extract did reveal certain deeper antigens which were highly antigenic. In conclusion, it appears that this phenomenon of IHA tests in the present system is brought about by the surface antigens as well as antigens in the deeper layers of the cells. Our results clearly show that in general, saline extracts, boiled extracts, and autoclaved extracts are adsorbed promptly on the SRBC and are able to detect antibodies produced in rabbits against whole cell antigens. However, the autoclaved cells proved weakly antigenic in rabbits as the antibody level in rabbits was very low when tested against the homologous autoclaved antigen. These findings lead to the following speculations: the saline extract of the whole cell suspension contains two types of antigens, namely, heat-labile antigens associated with type specificity and heat-stable antigens, which may be both type-specific and species-specific common antigens. The autoclaved antigens are not capable of producing type-specific antibodies when injected into rabbits. On the other hand, they produce weak antibody responses which react only with heat-stable common antigens present in the autoclaved extract. However, the same antigen is capable of detecting high levels of antibodies in sera produced against whole cell antigens. Thus, these findings indicate that the autoclaved antigens could be hapten in nature and could require the presence of heat-labile antigens as carriers to be able to provoke antibodies against both heat-labile and heat-stable type-specific antigens.

Minor cross-reactions occurred among various serogroups with boiled and autoclaved antigens. All such cross-reactions were not very marked. Homologous titers were always higher than heterologous ones. Hence, none of the cross-reactions led to confusion, nor did they detract from the usefulness of the procedure.

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