Coagglutination Test for Serotyping *Pasteurella haemolytica*

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A coagglutination test was described for simple, fast, and reliable detection of *Pasteurella haemolytica* type-specific antigens in lung lesions even in the absence of viable *P. haemolytica*. The coagglutinating reagents were prepared by coating protein A-producing *Staphylococcus aureus* cells with hyperimmune sera raised against *P. haemolytica* type strains. Bacterial suspensions, saline extracts, and boiled saline extracts of the bacteria were used as antigens. Homologous reactions with all types of antigens were precise. Some cross-reactions were similar to those obtained by the indirect hemagglutination test, and some additional one-way cross-reactions were identified. The coagglutination test was used for serotyping 65 *P. haemolytica* field strains and for the detection of *P. haemolytica* type-specific antigens in the lung specimens of 62 calves and 78 sheep. Ninety-four percent of the field strains could be serotyped by the coagglutination test. *P. haemolytica* type-specific antigens were detected in the lung specimens of 3 calves and 5 sheep that had succumbed to naturally occurring *P. haemolytica* pneumonia and in the lungs of 20 calves experimentally infected with *P. haemolytica* A1. The coagglutination test detected type-specific antigens in 36% of the lung specimens of slaughtered field sheep but not in the lungs of slaughtered field cattle with small chronic lung lesions. No reaction occurred in the case of nonpneumonic calves and sheep or when pneumonic lesions were caused by other bacteria. No *P. haemolytica* strains could be isolated from lung samples that were coagglutination test negative. This test is recommended as an additional method for fast and reliable serotyping of *P. haemolytica*.

Pasteurella haemolytica is a well-known pathogen of ruminants worldwide. *P. haemolytica* has two biotypes (31): biotype A, which causes pneumonia in cattle and sheep (13, 17, 34), mastitis in ewes (17, 33), and occasionally arthritis and meningitis in sheep (17); and biotype T, which is responsible for ovine systemic pasteurellosis of feeding lambs (9). Some authors have suggested the creation of a new species, *Pasteurella trehalosi*, for strains of the latter biotype (32).

Several methods have been used for the serologic typing of *P. haemolytica*. The tube agglutination test, used for the examination of somatic antigens, did not prove to be practical because of numerous cross-reactions (3, 4). Biberstein et al. (3, 4) introduced the indirect hemagglutination (IHA) test for serotyping *P. haemolytica* and were able to identify 12 serotypes. Modifications to the IHA test made the test easier and faster (15). IHA became the most widespread method for the examination of *P. haemolytica* serotypes. The double diffusion test and countercurrent immunoelectrophoresis can also be used for serotyping *P. haemolytica* (10). For the rapid serotyping of *P. haemolytica* strains, Frank and Wessman (14) introduced a slide agglutination test, which gave results similar to those obtained with the IHA test. However, some *P. haemolytica* strains could not be serotyped with the IHA test. The double gel diffusion test and countercurrent immunoelectrophoresis proved to be suitable for grouping of these “untypeable” strains, resulting in the identification of nine serogroups (8). The IHA test has been used to identify 17 serotypes of *P. haemolytica* (3, 11, 16, 28, 35). With the exception of the slide agglutination test, the methods presented above are time-consuming and laborious and generally need viable bacterium

cultures. Since immunity against *P. haemolytica* is serotype specific, the correct serotyping of the causative *P. haemolytica* strain on a farm is a requirement for effective vaccination (18).

The aim of the present work was to produce a fast and reliable method for serotyping *P. haemolytica* that was capable of detecting *P. haemolytica* type-specific antigens in extracts from lung lesions, even in the absence of viable *P. haemolytica* strains, or in suspensions of *P. haemolytica*.

Coagglutination tests with hyperimmune rabbit sera bound to protein A-producing *Staphylococcus aureus* proved to be reliable and suitable for serotyping *Pasteurella multocida* groups (29), streptococci (6, 20), *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* (22–25), *Mycoplasma synoviae* (26), brucellae (2, 7), klebsiellae (27), and *Taylorella equigenitalis* (5).

Protein A of *S. aureus* is a unique cell wall-associated protein which has high affinity to the Fc part of immunoglobulin G (12). Antibodies of the immunoglobulin G class bind to protein A-producing *S. aureus* cells via their Fc fragment, leaving the Fab structures capable of binding to the homologous antigen. Staphylococci with type-specific antibodies on their surface will only be agglutinated by the corresponding antigen (21).

MATERIALS AND METHODS

Type strains. Type strains of *P. haemolytica* were obtained from the Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia (*P. haemolytica* A1–A12), and the Moredun Research Institute, Edinburgh, Scotland (*P. haemolytica* A13–T15). The suggested type strain of serotype A16 was an isolate of our institute. The proposed type strain of serotype A17 was kindly provided by M. Younan (University of Hohenheim, Stuttgart, Germany). *S. aureus* Cowan 1 (NCTC 8530) was obtained from the Hungarian National Collection of Medical Bacteria, Budapest, Hungary (no. 110006).

Field isolates. Out of 65 isolates, 49 *P. haemolytica* strains were isolated from the lungs of cattle, sheep, and goats which had died from pneumonia in the field and from nasal discharges of cattle and sheep in Hungary. Sixteen strains isolated postmortem from ruminants, representing each serotype, were kindly received from W. Donachie, Moredun Research Institute.

Lung samples. Lung specimens were collected postmortem from 62 calves and 78 sheep as follows. Of the calves, four died from acute pneumonia (group A). Three succumbed, but not from pneumonia, with chronic lobular pneumonic

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TABLE 1. Results of the coagglutination test with suspensions of *P. haemolytica* type strains as antigens

Type strain	Result with immune serum																
	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	A16	A17
A1	++++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A2	—	++++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
T3	—	—	++++	—	—	—	—	—	—	—	—	—	—	—	++	—	—
T4	—	—	—	++++	—	—	—	—	—	—	—	—	—	—	—	—	—
A5	—	—	—	—	++++	—	—	—	—	—	—	—	—	—	—	—	—
A6	—	—	—	—	—	++++	—	—	—	—	—	(+)	—	—	—	—	—
A7	—	—	—	—	—	—	++++	—	—	—	—	—	—	—	—	—	—
A8	—	—	—	—	—	—	—	++++	—	—	—	—	—	—	—	—	—
A9	—	—	—	—	—	—	—	—	++++	—	—	—	—	—	—	—	—
T10	—	—	—	—	—	—	—	—	—	+++	—	—	—	—	—	—	—
A11	—	—	—	—	—	—	—	—	—	—	++++	—	—	—	—	—	—
A12	—	+++	—	—	—	—	—	—	—	—	—	++++	—	—	—	—	—
A13	—	—	—	—	++	—	—	—	—	—	—	—	+++	—	—	—	—
A14	—	—	—	—	—	—	—	—	—	—	—	—	—	++++	—	—	—
T15	—	—	+++	—	—	—	—	—	—	—	—	—	—	—	++++	—	—
A16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	++++	—
A17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	++++

lesions, generally in the apical lobe (group B). Three died without pneumonic lesions (group C). Twenty calves were sacrificed, after having been experimentally infected intranasally with *P. haemolytica* A1 and then developing acute pneumonia (30) (group D). Ten calves were slaughtered normally. They were clinically healthy but were found to have small chronic lobular pneumonic lesions, mainly in the apical lobe (group E). Twenty-two calves were slaughtered normally and were clinically healthy and without pneumonic lesions (group F).

Of the sheep, six succumbed from acute pneumonia (group G). Five died with small chronic lobular pneumonic lesions (group H). Three succumbed without pneumonic lesions (group I). Sixty-four sheep were slaughtered normally. They were clinically healthy but were found to have small chronic lobular pneumonic lesions in the apical lobe (group J).

Samples from all lung tissues were inoculated on blood agar and chocolate agar containing 0.5% yeast extract and 5% sheep blood. The blood agar plates were incubated at 37°C for 2 days; the chocolate agar plates were incubated at 37°C for 3 days in 15% CO₂. The plates were examined daily. The *P. haemolytica* strains isolated were identified and serotyped by the IHA (3).

Preparation of antisera. Antisera against the type strains of *P. haemolytica* were raised in rabbits (3).

Preparation of antigens. Bacterial suspensions were prepared from both the type strains of *P. haemolytica* and the field isolates. Blood agar cultures were resuspended in 3 ml of physiologic saline to a MacFarland tube 4 density.

Bacterial suspensions were left at room temperature for 5 min and centrifuged at 8,000 × g for 15 min; the clear supernatant is referred to as the saline extract. A sample of this extract was boiled for 10 min and is referred to as the boiled extract (22). Lung extracts were prepared as described by Mittal et al. (23). Briefly, a small piece of lung (about 2 g) was homogenized in 3 ml of saline in a mortar, boiled for 10 min, and centrifuged at 8,000 × g for 30 min. The undiluted supernatants were used as antigens.

Preparation of the coagglutinating reagents. The coagglutinating reagents were prepared according to the methods of Kronvall (20) and Mittal et al. (22).

Coagglutination test. Equal volumes (50 µl) of antigen and coagglutinating reagents were mixed on a glass slide and rotated by hand for 2 min. In the case of a positive reaction, a clear agglutination was observed and quantitated as +, ++, +++, or +++++. The reaction was regarded as negative (—) (20) if no agglutination occurred within 2 min.

RESULTS

The results of the coagglutination tests with bacterial suspensions of *P. haemolytica* type strains as antigens and coagglutinating reagents made of hyperimmune sera produced against the type strains of *P. haemolytica* are presented in Table 1. Suspensions of the 17 type strains of *P. haemolytica* gave strong positive reactions with the homologous coagglutinating reagents; however, some cross-reactions appeared, although they could be abolished by cross-absorbed sera. When saline extracts were used as antigens, the coagglutination between type strain *P. haemolytica* A12 and immune serum anti-A2 became weaker, while no cross-reaction appeared between serotype A13 and immune serum anti-A5. The use of

boiled extracts as antigens abolished all cross-reactions, except those between serotype T3 and immune serum anti-T15 and those between serotype T15 and immune serum anti-T3.

The results of a comparative evaluation of IHA and coagglutination tests examining 65 field isolates of *P. haemolytica*, representing each serotype, are summarized in Table 2. Sixty-one strains (93.8%) could be correctly typed by both tests. The coagglutination reagents failed to react in four cases (6.2%), of which three strains belonged to *P. haemolytica* A2 and one strain belonged to *P. haemolytica* A5. The latter strain also showed autoagglutination.

P. haemolytica type-specific antigens were detected in the lung specimens of three of four calves and from five of six sheep which had died in the field from acute pneumonia (groups A and G). The coagglutination test was negative from lung samples of animals found to have small lobular pneumonic lesions (groups B and H). In the case of nonpneumonic

TABLE 2. Serotyping *P. haemolytica* strains with coagglutination test

Serotype (no. of strains)	No. of positive strains by:	
	IHA	Coagglutination test
A1 (15)	15	15
A2 (16)	16	13
T3 (1)	1	1
T4 (1)	1	1
A5 (2)	2	1
A6 (5)	5	5
A7 (1)	1	1
A8 (5)	5	5
A9 (5)	5	5
T10 (2)	2	2
A11 (3)	3	3
A12 (1)	1	1
A13 (2)	2	2
A14 (1)	1	1
T15 (2)	2	2
A16 (1)	1	1
A17 (1)	1	1
Not typeable (1)	1	1
Total (65)	65	61

TABLE 3. Detection of *P. haemolytica* antigens in lungs of calves and sheep by coagglutination test

Sample group (characteristics)	No. of animals	No. of <i>P. haemolytica</i> coagglutination test-positive samples										No. of negative samples
		A1	A2	T4	A6	A8	A9	T10	A11	A12		
Calves												
A (Died from acute pneumonia)	4	3										1
B (Died with chronic lobular lung lesions)	3											3
C (Died without pneumonic lesions)	3											3
D (Sacrificed, experimentally infected)	20	20	2									
E (Slaughtered, chronic lobular lung lesions)	10											10
F (Slaughtered without pneumonic lesions)	22											22
Sheep												
G (Died from acute pneumonia)	6		5									1
H (Died with chronic lobular lung lesions)	5											5
I (Died without pneumonic lesions)	3											1
J (Slaughtered, chronic lobular lung lesions)	64 ^a	1	3	1	2	5	1	1	2	9		41

^a Note that in two lung specimens each, two serotypes (A8, T10, and A8, A12) were detected.

calves and sheep that had died or were slaughtered (groups C, F, and I), the coagglutination test was negative. Type-specific antigens of the *P. haemolytica* serotype A1 strains used for experimental infection of 20 calves were detected in the lung samples of the sacrificed animals. In addition to serotype A1 antigens, serotype A2 antigens were also detected in the lungs of two animals (group D).

The coagglutination test detected *P. haemolytica* type-specific antigens in 23 (35.9%) lung specimens of clinically healthy, slaughtered sheep with small chronic lobular lung lesions (group J). No *P. haemolytica* type-specific antigens were detected in the lung samples from slaughtered calves with similar lesions (group E) (Table 3).

P. haemolytica strains of the same serotype could be isolated from lung samples of calves and sheep that died from acute pneumonia that were coagglutination test positive and from the calves challenged with *P. haemolytica* that died from pneu-

monia. No *P. haemolytica* strains could be cultured from slaughtered animals. Several bacterium species were isolated from coagglutination test-negative lung samples of animals that died or were slaughtered and were found to have chronic lobular lung lesions. Most lung specimens without lesions were free from or contained lung pathogenic bacteria in very low numbers (Table 4).

DISCUSSION

The coagglutination test described above proved to be useful for detecting *P. haemolytica* type-specific antigens, as previously shown for other bacterium species and genera (2, 5, 20, 22, 26, 27, 29), even in the absence of viable *P. haemolytica*.

Results of the coagglutination test with suspensions of *P. haemolytica* type strains as antigens were in close correlation with those obtained by IHA (3). Cross-reactions between se-

TABLE 4. Comparison of direct bacterium isolation and the coagglutination test

Serotype	No. coagglutination positive	Sample group ^a	Organism(s) isolated (no. of strains)
A1	3	A	<i>Pasteurella haemolytica</i> A1 (3)
	20	D	<i>Pasteurella haemolytica</i> A1 (20)
	1	J	<i>Pasteurella multocida</i> (1)
A2	5	G	<i>Pasteurella haemolytica</i> A2 (5)
	2	D	Negative (2)
	3	J	<i>Corynebacterium</i> sp. (1), negative (2)
T4	1	J	<i>Pasteurella multocida</i> (1)
A6	2	J	<i>Pasteurella multocida</i> (1), negative (1)
A8	5	J	<i>Escherichia coli</i> (1), <i>Bacillus</i> sp. (1), negative (3)
A9	1	J	<i>Staphylococcus epidermidis</i> (1)
T10	1	J	<i>Escherichia coli</i> (1)
A11	2	J	<i>Staphylococcus epidermidis</i> (2)
A12	9	J	<i>Pasteurella multocida</i> (4), negative (5)
Negative	1	A	<i>Pasteurella multocida</i> (1)
	3	B	<i>Corynebacterium</i> sp. (2), <i>Bacillus cereus</i> (1)
	1	G	<i>Pasteurella multocida</i> (1)
	5	H	<i>Actinomyces pyogenes</i> (2), <i>Corynebacterium</i> sp. (3)
	3	C	Negative (3)
	3	I	<i>Staphylococcus epidermidis</i> (1), negative (2)
	10	E	<i>Pasteurella multocida</i> (5), <i>Corynebacterium</i> sp. (2), negative (3)
	22	F	<i>Staphylococcus epidermidis</i> (2), <i>Corynebacterium</i> sp. (2), <i>Pasteurella multocida</i> (2), negative (16)
	41	J	<i>Bacillus</i> sp. (9), <i>Pasteurella multocida</i> (8), <i>Staphylococcus epidermidis</i> (6), <i>Corynebacterium</i> sp. (1), <i>Neisseria</i> sp. (1), <i>Enterobacterium</i> sp. (1), negative (15)

^a Groups are described in Materials and Methods and in Table 3.

rototypes T3 and T15 are well known in the case of IHA as well (16). Only two one-way cross-reactions between *P. haemolytica* serotype A12 and anti-A2 serum and *P. haemolytica* serotype A13 and anti-A5 serum appeared, which could be abolished after cross-absorption of the sera, thus causing no difficulties in serotyping. When saline extracts were used as antigens, only one of the cross-reactions mentioned above was observed; however, after boiling of the extracts, neither of these two cross-reactions occurred, indicating the heat-labile nature of the cross-reacting antigens.

Over 93% of the *P. haemolytica* field strains were serotyped with the coagglutination test. Apart from one autoagglutination, three serotype A2 strains failed to be typed. In some cases, this can be explained by the low capsule content of serotype A2 strains (19). The capsule has been demonstrated to contain the type-specific antigens (1, 3).

The coagglutination test gave very fast and reliable results when the antigens were prepared from lungs of ruminants that had died from acute pneumonia caused by *P. haemolytica* both in the field and in experimentally infected calves. The results of the coagglutination test and of the culture methods were parallel.

The coagglutination test proved to be sensitive. *P. haemolytica* type-specific antigens were detected in 36% of the clinically healthy sheep but not in the lungs of calves with small chronic lobular pneumonic lesions. No viable *P. haemolytica* bacteria could be cultured from the lung samples of slaughtered animals. A similar phenomenon was observed when swine lungs were infected with *A. pleuropneumoniae* biotype 1 in Hungary (25). Mittal et al. (23) found that no *A. pleuropneumoniae* strains could be isolated from 28 coagglutination-positive samples out of 240 acute pneumonic samples.

The coagglutination test was specific. All lung samples that gave viable *P. haemolytica* cultures were positive in the coagglutination test. No false-positive reactions were seen in lung samples from animals without pneumonic lesions. Lung samples collected from calves and sheep which had died from acute pneumonia caused by bacteria other than *P. haemolytica* (e.g., *P. multocida*) were negative in the coagglutination test. The presence of different pathogenic, facultatively pathogenic, or saprophytic bacteria in the lung tissue of slaughtered animals did not result in a false-positive coagglutination test. Similar bacterium species were isolated from coagglutination-positive and -negative lung samples collected at the slaughterhouse, which showed that the positive coagglutination reactions were not caused by these bacteria, indicating that the positive test results must have resulted from the type-specific antigens of *P. haemolytica*.

On the basis of the results presented, the coagglutination test proved to be capable of serotyping *P. haemolytica* strains or extracts from *P. haemolytica*-containing samples. The treated staphylococci were stable for at least 1 year when stored at 4°C (22). The test is simple, specific, and sensitive, allowing the serotypes of *P. haemolytica* to be determined faster than with the IHA or the rapid IHA. The serotype of a *P. haemolytica* strain can be determined within about 4 min when bacterial suspensions are used as antigens or 45 min in lung specimens. The coagglutination test gave reliable results even in the absence of viable *P. haemolytica*. Therefore, the coagglutination test is recommended as an additional method for the fast and reliable serotyping of *P. haemolytica*.

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