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Identification of Type D *Pasteurella multocida* by Counterimmunoelectrophoresis

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A counterimmunoelectrophoresis (CIE) test was applied to serotype 35 isolates of type D *Pasteurella multocida* recovered from 32 cases of atrophic rhinitis (in swine) and 3 cases of snuffles (in rabbits). The CIE test was compared with the indirect hemagglutination (IHA) and acriflavine (AF) tests. Results of the CIE test correlated 100% with those of the IHA test whereas results of the AF test correlated 91.43% with those of the IHA test. The CIE test was rapid and simpler to perform compared with the IHA test and more sensitive than the AF test. Cross-reactions were not encountered with capsular antigens of *P. multocida* types A, B, and E in the CIE test. The CIE test was not found to be suitable for typing type A *P. multocida* strains.

The four capsular types of *Pasteurella multocida* designated A, B, D, and E were first recognized by an indirect hemagglutination (IHA) procedure (3, 4). Because the IHA procedure is somewhat involved, less involved serologic (6, 11) and nonserologic (7, 8) tests for typing *P. multocida* types B and E, and A and D were developed, respectively. In addition, Sawada et al. (12) have described a modified IHA procedure that uses glutaraldehyde-fixed sheep erythrocytes sensitized with extracts of *P. multocida* types B and E. Most cultures of *P. multocida* recovered from diseased farm and other animals in North and South America are type A (5). Capsular type D cultures are recovered frequently from swine but less frequently from other animals (5).

A nonserologic procedure, the acriflavine (AF) test (8), is a commonly used procedure for typing type D *P. multocida*. However, the test seemed to give false-negative results when blue colony variants of *P. multocida* were used. The blue colony variants were the smallest, and constituent organisms had little if any capsule and a tendency to autoagglutinate (5).

Counterimmunoelectrophoresis (CIE) has been used to recognize capsular antigens of *Haemophilus influenzae* (10), *P. multocida* (6), *Pasteurella haemolytica* (9), pneumococci and meningococci (13), and some other bacteria (1). In this report we describe the successful application of the CIE test to the identification of type D *P. multocida*. Because the IHA test is somewhat involved and the AF test is not sensitive enough to detect the capsular antigens of blue variants, it was thought that the simple, yet sensitive CIE test might be advantageous for typing type D *P. multocida*.

MATERIALS AND METHODS

Strains of *P. multocida*. Cultures included 35 type D isolates obtained in the field (swine, 32; rabbit, 3); 12 type A strains (turkey, 5; chicken, 2; human, 2; cattle, 1; pine siskin, 1; herring gull, 1 [2]); 4 type B isolates (cattle, 3; bison, 1); and 4 type E isolates from cattle. Sources of cultures were the National Animal Disease Center, Ames, Iowa; G. R. Carter, Virginia Polytechnic Institute and State University, Blacksburg, Va.; and J. F. Prescott, University of Guelph, Guelph, Ontario, Canada. Types B and E were hemorrhagic septicemia strains.

Typing antisera. Smooth, iridescent colonies of types A, B, D, and E were streaked onto glucose starch agar (GSA; GIBCO Diagnostics, Madison, Wis.) plates. Following 18 h of incubation at 37°C, the growth was harvested with 0.02 M phosphate-buffered saline (PBS; pH 7.2), and the turbidity of the cell suspension was adjusted to a no. 10 McFarland standard nephelometer tube. The cells were killed with 0.3% Formalin and stored in a refrigerator until use. Three adult New Zealand white rabbits were used for each antigenic preparation. The immunizing schedule was as follows: 0.1 ml subcutaneously and then 0.2, 0.4, 0.6, 0.8, and 1.0 ml intravenously at 4-day intervals. At 6 days after the final antigen dose, the rabbits were exsanguinated and antisera were collected and stored at −60°C.

IHA test. The IHA test was performed with a microtiter system (Dynatech Laboratories, Inc., Alexandria, Va.). Each of the type D cultures was grown on 2 GSA plates at 37°C for 18 h. The growth from each plate was harvested with 2 ml of PBS containing 100 U of hyaluronidase (Sigma Chemical Co., St. Louis, Mo.). The mixture was incubated at 37°C for 1 h and then centrifuged at 20,000 × *g* for 20 min. The supernatant (4 ml) was diluted with an equal volume of PBS and mixed with 1 ml of prewashed (three times in PBS) packed equine erythrocytes. The mixture was incubated for 2 h at 37°C in a water bath. Following incubation the mixture was centrifuged at 3,000 × *g* for 10 min; the sensitized erythrocytes were washed three times in PBS containing 0.5% bovine serum albumin (BSA; Sigma). The sedimented cells were suspended to a 0.25% (vol/vol) concentration in PBS containing 0.5% BSA. Serial twofold dilutions of antiserum were made in PBS-BSA solution, and 0.05 ml of the sensitized erythrocytes was added to 0.05 ml of the antiserum dilution in U-bottom plates. The plates were shaken and incubated for 2 h at 25°C before results were read. The IHA titer was expressed as the reciprocal of the highest dilution of serum showing uniform layers of erythrocytes evenly lining the bottom of the wells. Dense red buttons at the lowest points of the wells indicated the absence of agglutination. Controls consisted of unsensitized erythro-

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cytes plus test serum and sensitized erythrocytes plus diluent.

AF test. The AF test was performed by the method of Carter and Subronto (8). Strains were grown for 18 h in 3 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and concentrated to 0.5 ml by centrifugation at 10,000 × *g* for 20 min. A total of 0.5 ml of 1:1,000 freshly prepared aqueous solutions of neutral AF (Sigma) was added to the concentrated 0.5 ml of broth culture. After mixing to suspend bacteria, the tube was left stationary at room temperature. Type D strains gave a heavy flocculent precipitate and began to settle within 5 min. After 30 min the precipitate settled, leaving a distinct clear supernatant, whereas other serotypes remained unaffected.

CIE test. The CIE test was performed by the method of Carter and Chengappa (6) with slight modifications. Antigen preparation for the CIE procedure consisted of the same capsular extract as that used for the IHA test described above, with the exception that the cells were treated with hyaluronidase for 30 min. Antigen preparations were adjusted to 8 mg (dry weight) per ml with PBS. The electrophoresis plates were prepared by coating glass plates (10 by 8 cm) with 15-ml volumes consisting of 0.5% agarose (Seakem; Marine Colloids, Rockland, Maine)–0.5% Bacto-Agar (Difco)–0.015% sodium azide in 0.025 M high-resolution barbital buffer (pH 8.8; Gelman Instrument Co., Ann Arbor, Mich.). Wells (3 mm in diameter and 7 mm from center to center) were prepared with a template (Grafar Corp., Detroit, Mich.). A 20-μl quantity of antigen was placed in the cathodal well, and an equal quantity of 1:4-diluted antiserum was placed in the anodal well. The electrophoresis tank (Gelman) contained 0.05 M barbital buffer (pH 8.8). The reagents were electrophoresed at room temperature for 25 min at 50 V. The plates were examined for distinct precipitation lines, washed in 2% NaCl, and stained with 0.1% amido black (Sigma).

RESULTS

Serum titration. The IHA titers of *P. multocida* types A, B, D, and E were 1:128, 1:2,048, 1:1,024, and 1:2,048, respectively. In addition, type D antiserum was titrated by CIE. A clear, sharp line of precipitation was observed with 1:4-diluted type D antisera.

IHA, AF, and CIE tests. The distribution of the 55 isolates of *P. multocida* among the three tests with the type D antiserum is presented in Table 1. Cross-reactions were not observed in the CIE test between the capsule antigens of types A, B, and E and 1:4-diluted type D antiserum (Fig. 1). However, cross-reactions in the form of very thin precipitin lines were noticed in the CIE test between the undiluted type D antiserum and 4 of 12 type A antigens. Cross-reactions were also not noticed in the CIE test with the undiluted antisera of types A, B, and E against the antigens of 35 type D strains. Results of the CIE test correlated 100% with those of the IHA test, whereas results of the AF test correlated 91.43% with those of the IHA test (Table 1).

TABLE 1. Comparisons of IHA, AF, and CIE tests of 55 isolates of *P. multocida* tested against the type D antiserum

No. of strains	Capsular type	No. of isolates testing positive by the following tests:		
		IHA[a]	AF	CIE
35	D	35	32[b]	35
12	A	0	0	0
4	B	0	0	0
4	E	0	0	0

[a] Titers ranged from 1:8 to 1:1,024.
[b] Strains negative by the AF test had a very low IHA titer (1:8).

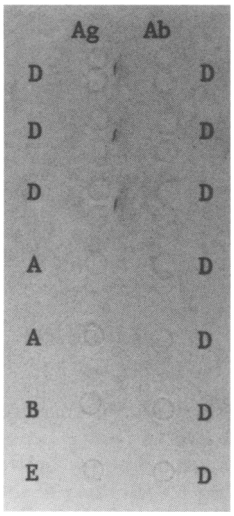

FIG. 1. CIE patterns of homologous and heterologous systems of four capsular types (A, B, D, and E) of *P. multocida*. Abbreviations: Ag, antigen in cathodal well; Ab, antibody in anodal well.

The three strains that tested negative by the AF test had predominantly (>90%) blue colony types on GSA plates. Further analysis of these three strains revealed that the AF test is not suitable for testing blue colony types of *P. multocida* (Table 2).

DISCUSSION

The data presented in Table 1 suggest that the CIE test is as sensitive as the IHA test. This is in agreement with results of our previous studies with *P. multocida* types B and E (6) and *P. haemolytica* (9). Although cross-reactions were reported between type D antiserum and type A antigen in our previous study (6), they were not encountered in this study. This is probably due to the adaptation of a modified CIE procedure, especially the antigen and antibody preparation techniques and the voltage at which the CIE test was run.

Results of this study also indicate that the AF test is less sensitive than the other two tests. The strains that tested negative by the AF test consisted predominantly of blue colony types. This suggests that the AF test is not suitable for those organisms with little or insufficient capsular material. The IHA titers for the blue colony types were lower

TABLE 2. IHA, AF, and CIE test results with iridescent and blue colony types of three type D *P. multocida* strains testing negative by the AF test

Strain no.	Colony type[a]	Test results		
		IHA	AF	CIE
304	Iridescent	1:64	+	+
	Blue	1:2	−	+
966	Iridescent	1:128	+	+
	Blue	1:2	−	+
2182	Iridescent	1:128	+	+
	Blue	1:4	−	+

[a] Colony types were selected with the aid of an oblique light and propagated on GSA plates for serotyping.

than those for the iridescent colony types of type D *P. multocida* (Table 2). This was attributed to the lack of sufficient capsular material on organisms that give rise to blue colony types. In the case of the CIE test, inadequacy of capsular antigen did not seem to interfere with the results (Table 2). This seemed to be a major advantage over the IHA or the AF tests. In addition, the CIE test was found to be simple and rapid in comparison with the IHA test and yielded essentially the same result as the IHA test. Cultures consisting of mostly blue colony variants can be restored to a state of predominantly smooth and iridescent colony variants by passage in mice (5). This time-consuming step can be avoided with the CIE procedure, as the test seems to be very sensitive in detecting type-specific capsular antigens.

Based on the results of this study, it was concluded that the CIE test is a rapid, simple, and yet specific method for identifying type D *P. multocida* of both colony variants. In addition, cross-reactions were not observed in the CIE test between the antisera of types A, B, and E and the antigens of all 35 type D. *P. multocida* isolates. The cross-reactions, if any, in the CIE test can probably be eliminated by diluting the type D antiserum. The CIE test was not suitable for typing type A *P. multocida*. The use of the CIE test in its original or modified form for titrating antibodies seems worthy of investigation. Also, further work is needed to determine whether a modification of the CIE test will identify type A *P. multocida*.

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