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Serological Groups Among Untypable Bovine Isolates of *Pasteurella haemolytica*

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Ten *Pasteurella haemolytica* isolates from the nasal passages of cattle, untypable by the indirect hemagglutination procedure, were grouped into three serotypes by the rapid plate agglutination procedure. Serological specificity and serum absorption results showed that the 10 indirect hemagglutination-negative isolates belonged to three distinct serotypes and were not members of established serotypes that lack the specific soluble antigen that forms the basis of serotyping by indirect hemagglutination. All isolates were biotype A.

Pasteurella haemolytica isolates from sheep and cattle can be divided into 12 established serotypes by an indirect hemagglutination (IHA) procedure (3-5), and 2 new serotypes have been reported (8). However, many isolates from sheep and cattle do not group with the 12 serotypes (1-4, 6, 7, 10) and do not give a positive IHA reaction against homologous antiserum (1, 2, 6); these isolates are considered IHA negative or untypable. A rapid plate agglutination (RPA) procedure that allows *P. haemolytica* colonies to be serotyped directly from an agar plate yields essentially the same results with the 12 established serotypes as does the IHA procedure (6).

An untypable bovine isolate that did not react by IHA with homologous antiserum agglutinated with the antiserum by RPA (G. H. Frank and L. B. Tabatabai, Abstr. Annu. Meet. Am Soc. Microbiol. 1979, C205, p. 344). The reaction was specific, indicating that the IHA-negative isolate might have a serotype-specific antigen and that other IHA-negative isolates might react by RPA.

Ten bovine IHA-negative isolates were serotyped by RPA. Their serological specificities, their possible antigenic relationships to the 12 established serotypes and to each other, and their biotypes were determined.

MATERIALS AND METHODS

***P. haemolytica* isolates.** Ten IHA-negative isolates were collected from the nasal passages of 10 calves during field studies on bovine respiratory disease. At isolation, some calves were experiencing a mild respiratory disease, whereas others were healthy. The isolates were identified as *P. haemolytica* because of their colonial morphology and biochemical characteristics as described by Biberstein (2). None of the 10 isolates could be placed into an established serotype by either IHA or RPA.

Antiserum. Rabbit antiserum was prepared against Formalin-killed whole cells as described previously (6). When an antiserum did not react by RPA with an IHA-negative isolate, an antiserum to the isolate was prepared by the same procedure (see Table 1).

Preparation of heat-stable antigens. Heat-stable antigens were prepared by two methods. In one method, *P. haemolytica* cells were centrifuged from a 20-h tryptose broth (Difco Laboratories, Detroit, Mich.) culture, washed, and suspended in 0.15 M NaCl. The cells were heated to 100°C for 30 min, washed twice at 5,000 × *g* in 0.15 M NaCl, and then suspended in a volume of 0.15 M NaCl with 1:10,000 thimerosal equal to that of the pellet. The other method was similar to that of Biberstein et al. (4) in which a 20-h tryptose broth culture was autoclaved for 15 min and the cells were washed twice in 0.3% formalinized buffered saline (pH 7.0) and suspended as were the boiled cells.

Serum absorption. Cells were harvested from 20-h tryptose broth cultures, washed in 0.15 M NaCl, and suspended in 0.15 M NaCl with 1:10,000 thimerosal. Three milliliters of cell suspension (equivalent to 10 times an optical density of 0.5 at 575 nm) was centrifuged at 4,340 × *g* for 30 min. The cell pellet was suspended in 0.5 ml of a 1:16 dilution of the antiserum to be absorbed. After 2 h of incubation at 37°C with frequent shaking, the mixture was refrigerated overnight, and the cells were centrifuged from the serum at 4,340 × *g* for 30 min.

Serological tests. Whole, live cells picked from a colony on blood agar were mixed with antiserum and observed for agglutination by direct RPA as previously described (6). Heat-stable antigens were tested for antigenic specificity by direct RPA in which 5 μl of concentrated heat-stable antigen was mixed with 10 μl of antiserum. The mixture was observed for agglutination within 1 min during mixing.

Both boiled and autoclaved antigen preparations were tested for RPA with unabsorbed antisera to all 12 established serotypes of *P. haemolytica*. In addition, boiled antigens prepared from all 12 established serotypes were tested for RPA with unabsorbed antisera to the IHA-negative isolates.

Whole cells and heat-stable antigen preparations of each IHA-negative isolate were tested for RPA with homologous antisera absorbed with each of the 12 established serotypes and the other IHA-negative isolates.

Biotyping. As originally described by Smith (9), biotypes of the 10 IHA-negative isolates were determined by their ability to ferment arabinose, trehalose, xylose, lactose, and salicin (2). Fermentation medium was phenol red broth base (Difco) containing 1% carbohydrate. Both bromocresol purple broth base (Difco) and phenol red broth base were used for salicin fermentation. Carbohydrate broths were inoculated with colonies from blood agar, incubated at 36°C, and observed for growth and pH change for 14 days. For comparison, cultures of the 12 established serotypes were tested by the same procedure. Catalase production was determined by mixing part of a colony from a blood agar plate with a drop of 3% H_2O_2 on a glass slide and observing for immediate effervescence of the mixture.

RESULTS

The IHA-negative isolates were grouped into three serotypes by RPA with whole live cells and unabsorbed antisera (Table 1). Boiled antigen preparations of the IHA-negative isolates reacted only with the antisera with which their whole cells reacted (Table 1). Autoclaved antigen preparations reacted not only with the antisera with which their whole cells reacted, but also eight of them reacted with serotype 2 antiserum, six of the eight reacted with serotype 9 antiserum, and one reacted with serotype 9 antiserum and not with serotype 2 antiserum. To confirm the observation, two other serotype 2 antisera (one prepared against the same isolate and the other prepared against another isolate) and one other serotype 9 antiserum (prepared against the same isolate) were tested (Table 1). None of the whole cells or boiled antigen preparations of the 12 established serotypes reacted with antisera to the IHA-negative isolates.

TABLE 1. *RPA of whole cells and boiled or autoclaved antigen preparations of IHA-negative bovine P. haemolytica isolates with homologous and heterologous antisera*

Antigens of culture no.	Agglutination with antisera[a]				
	282-76	194-76	148-78	Serotype 2[b]	Serotype 9[b]
282-76	+	0	0	+[c]	+
88-78	+	0	0	+[c]	+
141-78	+	0	0	+[d]	+
194-76	0	+	0	+[d]	0
171-76	0	+	0	0	0
253-76	0	+	0	+[e]	0
275-76	0	+	0	+[c]	+
288-76	0	+	0	+[e]	+
290-76	0	+	0	0	+
148-78	0	0	+	+[c]	+

[a] +, Agglutination; 0, no agglutination.

[b] Positive RPA with autoclaved antigen preparations only. The antigens were tested with three different serotype 2 antisera and two different serotype 9 antisera.

[c] Positive RPA with three of three different serotype 2 antisera.

[d] Positive RPA with two of three different serotype 2 antisera.

[e] Positive RPA with one of three different serotype 2 antisera.

Whole cell absorption of antisera to IHA-negative isolates with heterologous serotypes did not remove the agglutinating antibody for either whole cells or heat-stable antigens, whereas absorption with homologous whole cells did. On the basis of the cross-absorption experiments, the IHA-negative serotypes were distinct and not related to each other or to the standard serotypes.

All of the IHA-negative isolates were biotype A (Table 2). *P. haemolytica* had grown in all carbohydrate media by day 2. Established serotypes 1 to 12 reacted characteristically for their biotypes. Serotypes 3, 4, and 10 were biotype T, and the others were biotype A. All of the IHA-negative isolates and the established serotypes of the biotype A were catalase positive. Established serotypes 3, 4, and 10 of biotype T were catalase negative, using both 3 and 30% H_2O_2.

DISCUSSION

Ten IHA-negative *P. haemolytica* isolates from cattle were grouped into three serotypes by RPA. Results of whole-cell and boiled-antigen RPA with absorbed sera support the contention that the three new serotypes are formerly unrecognized and distinct serotypes among the IHA-negative isolates.

Investigators have speculated that IHA-negative isolates are members of the established IHA-positive serotypes and that their non-IHA reactivity results from a lack of soluble serotype-specific antigen on the outer cell surface (1, 2, 4). Supportive evidence should have been provided by agglutination studies with heat-stable antigens of the IHA-negative isolates, but such studies have been inconclusive. Biberstein et al. (4) reported that autoclaved cells of IHA-negative isolates sometimes agglutinated readily in one or more of the grouping sera, whereas other cultures remained unclassifiable. Åarsleff et al. (1) tested autoclaved antigens from 80 IHA-negative ovine isolates for agglutination against somatic antisera to 10 of the established serotypes. Overall results were inconclusive, because some of the preparations autoagglutinated, some reacted with all antisera, and some reacted with one or none.

TABLE 2. *Fermentation by P. haemolytica isolates in phenol red broth base with 1% carbohydrate*

Culture	Fermentation reaction[a]				
	Arabinose	Trehalose	Xylose	Lactose	Salicin
ST-1	Neg	Neg	1	6W	Neg
ST-2	Neg	Neg	1	Neg	Neg
ST-3	Neg	1	Neg	Neg	Neg[b], 7[c]
ST-4	Neg	1	Neg	Neg	1
ST-5	3W	Neg	1	6	Neg
ST-6	3W	Neg	1	6W	Neg
ST-7	7W	Neg	1	6W	Neg
ST-8	Neg	Neg	1	6	Neg
ST-9	3W	Neg	1	6	Neg
ST-10	Neg	1	Neg	Neg	1
ST-11	Neg	Neg	1	3	1
ST-12	3W	Neg	1	6W	Neg
282-76	3	Neg	1	Neg	Neg
88-78	7W	Neg	1	Neg	Neg
141-78	3	Neg	1	Neg	Neg
194-76	3	Neg	1	Neg	Neg
171-76	3	Neg	1	Neg	Neg
253-76	3W	Neg	1	Neg	Neg
275-76	3	Neg	1	Neg	Neg
288-76	3	Neg	1	Neg	Neg
290-76	3	Neg	1	Neg	Neg
148-78	3	Neg	1	Neg	Neg

[a] Numbers indicate the day the reaction was read as positive. Neg, Negative reaction; W, weak reaction.
[b] Phenol red broth base.
[c] Bromocresol purple broth base.

At least some IHA-negative isolates are distinct serotypes, but some may be established IHA-positive serotypes that lack the soluble serotype-specific antigen because heat-stable antigens of some IHA-negative isolates do react with antisera to established serotypes (1, 4). We can reasonably assume that both situations exist in nature. However, RPA should prove to be a very useful tool in determining the category of each IHA-negative isolate.

Fermentation patterns identified all 10 bovine IHA-negative isolates as biotype A; this finding agrees with those in other studies on such isolates (1–3, 5, 10, 11). Fermentation results (especially with arabinose and salicin) have varied among investigators, depending upon the fermentation medium used (1, 3, 11, 12). The subject is well covered in a recent review by Biberstein (2). Unexpected findings included the lack of salicin fermentation by serotype 3 in phenol red broth base and by all of the IHA-negative isolates in both bases. Using a bromothymol blue broth base, Åarsleff et al. (1) found a large number of salicin fermenters among IHA-negative isolates from sheep. From the results of Wessman and Hilker (11), one might expect some of the A biotypes to have fermented salicin in phenol red broth base. Note that in the present study the 10 IHA-negative isolates fermented arabinose. This finding agrees with that of Åarsleff et al. (1).

In the present study, all 10 of the bovine IHA-negative isolates were catalase positive; this finding agrees with those of others (1, 3, 4). Unexpectedly, the S isolate of serotype 4 was catalase negative, whereas the same isolate was found earlier to be catalase positive, and serotypes 3 and 10 were found to be catalase negative (4). Catalase testing of a large number of biotype T isolates may reveal catalase production to be a distinguishing feature between biotypes A and T.

The relationship between serotype-specific antigens of the IHA-positive and IHA-negative *P. haemolytica* isolates is unknown at present. The same or completely different antigens could be involved, the antiserum reacting with the outermost antigen on the cell. I suggest that RPA is a method for classifying IHA-negative or untypable strains, but that it is premature to suggest a separate system of classification at present. However, in establishing serological groups among the IHA-negative or untypable *P. haemolytica* isolates from widely separated geo-

graphical areas, the problem of distinguishing the serotypes in a useful manner will arise.

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