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Neuraminidase Activity of *Pasteurella haemolytica* Isolates

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Neuraminidase activities of *Pasteurella haemolytica* cell suspensions were measured. Type isolates of the 12 established serotypes and bovine and ovine field isolates were included in the study. Neuraminidase activities ranged from 0 to 0.87 U per mg (dry weight) of *P. haemolytica*. Activity levels among the isolates studied were serotype associated.

Pasteurella haemolytica is the bacterium most commonly isolated from pneumonic lungs of cattle and sheep with acute respiratory disease. In spite of the pathogenicity of *P. haemolytica* in nature, most attempts to produce a *P. haemolytica* pneumonia experimentally in the absence of viral agents or stress have not been successful (6). *P. haemolytica* has low pathogenicity for laboratory animals (15, 18), and thus far no virulence markers are known. However, different serotypes (2–4, 8) and biotypes (11) have been described and associated with various disease conditions.

We recently found and measured neuraminidase activity in the culture fluid of a bovine lung isolate of *P. haemolytica* serotype 1. Neuraminidase has been implicated as a virulence factor in other species of bacteria (10–12, 15) and may serve as a marker for determining virulent *P. haemolytica* isolates.

Our objective was to quantitate the neuraminidase activity of different *P. haemolytica* isolates. Type isolates of the 12 established serotypes and bovine and ovine field isolates representing the various serotypes were included.

MATERIALS AND METHODS

***P. haemolytica* isolates.** Lyophilized type strains of the 12 established *P. haemolytica* serotypes were obtained from E. L. Biberstein, University of California, Davis (8). Five isolates of each of the serotypes 2, 5, 6, 7, 9, 11, and 12 and four isolates of serotypes 1 and 8 were collected from nasopharyngeal swabs from healthy lambs originating from 63 flocks from several midwestern states. One isolate each of serotypes 1 and 8 were collected from the lungs of lambs that died from acute respiratory disease.

Bovine isolates included 9 of serotype 1, 10 of serotype 2 (5 hemolytic and 5 nonhemolytic), and 10 untypable isolates (7) from nasopharyngeal swabs collected from calves immediately after arrival at a feedyard. Calves from which the serotype 1 isolates were collected originated from four different farms; those from which serotype 2 isolates were collected originated from seven different farms. One additional bovine serotype 1 isolate was from the lung of a calf that died from acute respiratory disease the previous year. The untypable isolates were collected from calves originating from nine different farms on 2 different years. All field isolates had been passaged twice on blood agar for isolation and then stored frozen on blood agar base slants.

Determination of neuraminidase activity. *P. haemolytica* cultures were grown overnight at 36°C on blood agar base (Difco Laboratories, Detroit, Mich.) supplemented with 5% bovine blood. A suspension of bacteria approximating an optical density of 0.36 at 575 nm was made in 2 ml of 0.1 M sodium acetate, pH 5.0, in a screw-cap tube (13 by 100 mm) by transferring the fresh growth to the buffer with a loop. Culture suspensions were assayed for neuraminidase activity as follows, according to a modification of described methods (1, 17). Fifty microliters of bacterial suspension was transferred to a screw-cap tube (13 by 100 mm), 50 μl of fetuin (40 mg/ml) was added, and the mixture was incubated with frequent shaking for 30 min in a 37°C water bath. The reaction was stopped by adding 100 μl of phosphotungstic acid (5% in 2.5 N HCl). Oxidation of released *N*-acetylneuraminic acid was initiated by adding 50 μl of 0.2 M periodic acid in 9 M phosphoric acid, and the mixture was incubated for 20 min at room temperature.

Oxidation was stopped by adding 0.5 ml of sodium arsenite (100 mg/ml in 0.5 M sodium sulfate and 0.1 N H_2SO_2). The mixture was shaken to help liberate iodine, as evidence by disappearance of the resulting yellow color. To develop the chromogen, 1.5 ml of thiobarbituric acid (6 mg/ml in 0.5 M sodium sulfate) was added; the reaction mixture was boiled in a water bath for 15 min and then cooled in ice water. The resulting pink chromogen was extracted by adding 4.0 ml of *n*-butanol containing 5% 12 N HCl followed by vigorous shaking on a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.). The absorbance of the upper (*n*-butanol-chromogen) phase was read at 549 nm against the same phase from a control mixture which contained 50 μl of 0.1 M sodium acetate buffer instead of the *P. haemolytica* suspension. All precipitates formed during the extraction procedure remained in the lower phase; therefore, no centrifugation was necessary. Final optical density readings of bacterial suspensions for dry cell weight determination were made in matched cuvettes, and dry cell weights were determined from absorption values.

Determination of bacterial concentration for assay. Extreme variation in the amount of chromogen

released by *P. haemolytica* isolates made it necessary to determine the linear range of the relationship between bacterial concentration and chromogen released to establish a standard concentration of bacteria to use in the assay. Acetate buffer suspensions of two *P. haemolytica* isolates, one (73-80) with a very high level of neuraminidase activity and the other (48–80) with a medium level of activity, were diluted twofold in acetate buffer and incubated with fetuin as described. The dry cell weights of both original suspensions were determined, and the dry cell weight was plotted against absorbance of the released *N*-acetylneuraminic acid.

Calculation of neuraminidase activity. The amount of *N*-acetylneuraminic acid released was obtained from a standard curve of absorbance versus nanomoles of authentic samples of *N*-acetylneuraminic acid (Sigma Chemical Co., St. Louis, Mo.). Units of neuraminidase activity per milligram of dry *P. haemolytica* cells were determined from the definition of unit (the amount of neuraminidase that liberates 1 μmol of *N*-acetylneuraminic acid from a substrate in 1 min).

RESULTS

Bacterial concentrations used in the assay. Absorption values of the standard *N*-acetylneuraminic acid curve were linear to 60 μg, representing an absorbance of 2.24 at 549 nm. Absorption values of bacterial concentration plotted against released chromogen were linear to an absorbance of 2.0. For assay, we used a suspension of bacteria that approximated the optical density of isolate 73-80, which resulted in a released chromogen absorption of 1.4 at 549 nm. This bacterial suspension had an absorption of 0.36 at 575 nm, which was calculated to be 7.06×10^{-3} mg of dry cells used in the neuraminidase assay. Actual absorption values of bacterial suspensions tested ranged from 0.25 to 0.49 at 575 nm.

Neuraminidase activity of *P. haemolytica* isolates. Neuraminidase activity of the various *P. haemolytica* isolates studied ranged from undetectable levels to 0.87 U per mg of dry cells (Table 1). All of the released chromogen absorption values fell within the linear portion of the standard *N*-acetylneuraminic acid curve. Of the type strains, serotypes 2, 3, 4, 10, and 11 had no measurable neuraminidase activity. This included all biotype T isolates of the type strains (serotypes 3, 4, and 10); however, no field biotype T isolates were available for the study. None of the ovine field isolates of serotype 11 had neuraminidase activity, but ovine field isolates of serotype 2 had low levels (Table 2).

Neuraminidase activity levels of the ovine field isolates varied among the isolates within each serotype, as did those of the bovine field isolates (Table 2). To determine whether neur-

TABLE 1. *Neuraminidase activity of the type strains of P. haemolytica*

Serotype	Culture no.	Neuraminidase activity (U/mg of dry cells)[a]
1	I 29	0.09
2	J 28	—[b]
3	863	—
4	S	—
5	G 13	0.21
6	A 30	0.34
7	H 1	0.14
8	H 21	0.11
9	B 1	0.87
10	JF 2	—
11	KC 282	—
12	S 209	0.28

[a] One unit of neuraminidase activity equals the release of 1 μmol of *N*-acetylneuraminic acid per min.
[b] —, No detectable neuraminidase activity.

TABLE 2. *Neuraminidase activity of ovine and bovine field isolates[a] of P. haemolytica*

Serotype	Neuraminidase activity (U/ mg of dry cells)[b]				
	Mean[c]	Standard deviation	Range		95% Confidence interval
			Minimum	Maximum	
Ovine					
1	0.20	0.10	0.07	0.32	(0.08, 0.32)
2	0.06	0.02	0.03	0.09	(0.04, 0.08)
5	0.35	0.06	0.26	0.42	(0.28, 0.42)
6	0.18	0.02	0.15	0.20	(0.16, 0.20)
7	0.17	0.11	0.10	0.36	(0.03, 0.25)
8	0.34	0.10	0.21	0.46	(0.22, 0.46)
9	0.42	0.05	0.38	0.48	(0.36, 0.48)
11	—[d]	—	—	—	(—, —)
12	0.29	0.05	0.23	0.34	(0.23, 0.35)
Bovine					
1	0.17[e]	0.04	0.10	0.24	(0.14, 0.20)
2	0.04	0.04	0.01	0.09	(—, 0.09)
2N[f]	0.02	0.02	—	0.04	(—, 0.04)
UT[g]	—	—	—	—	(—, —)

[a] Isolates were from nasopharyngeal swabs, except for one serotype 1 ovine lung isolate (activity = 0.28), one serotype 8 ovine lung isolate (activity = 0.21), and one serotype 1 bovine lung isolate (activity = 0.13).
[b] One unit of neuraminidase activity equals the release of 1 μmol of *N*-acetylneuraminic acid per min.
[c] Mean of five isolates per serotype, except where indicated.
[d] —, No detectable neuraminidase activity.
[e] Mean of 10 isolates.
[f] Nonhemolytic on both bovine and ovine blood agar.
[g] Isolates untypable by the indirect hemagglutination procedure.

aminidase activity levels were serotype related, comparison intervals (9) of the neuraminidase activity levels of all 11 groups of isolates (Tables 1 and 2), representing 8 serotypes, were simultaneously determined. Due to the conservative nature of the comparison intervals (9), they were determined at the 80% confidence level (Fig. 1).

Results showed four levels of serotype-associated neuraminidase activity. Serotype 11 isolates had no activity, serotype 2 isolates had low levels of activity, serotypes 1, 6, and 7 had intermediate levels, and serotypes 5, 8, and 9 had the highest levels. Serotype 12 isolates had intermediate to high levels of activity (Fig. 1).

Bovine and ovine field isolates of serotype 1 had the same neuraminidase activity levels. Some bovine serotype 2 field isolates had no activity, but others had levels similar to those of the ovine isolates (Table 2). Hemolytic and nonhemolytic bovine serotype 2 isolates had the same general activity ranges. The 10 untypable bovine isolates, all biotype A and representing 3 serotypes (7), had no neuraminidase activity (Table 2).

Populations of individual colonies from a single isolate were closer in neuraminidase activity levels than were various isolates within a serotype (Table 3), as shown with populations from five selected colonies from a single isolate each of serotypes 2, 5, and 9.

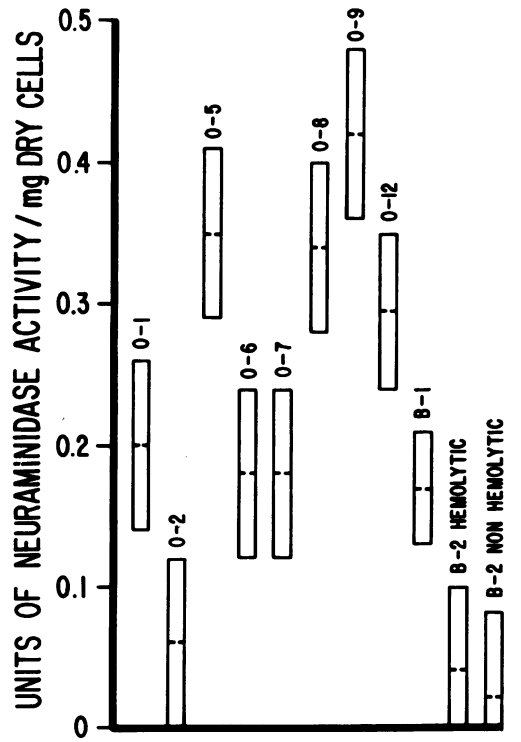

FIG. 1. *Comparison intervals at the 80% confidence level of the neuraminidase activity levels of 11 groups of ovine (O) and bovine (B) P. haemolytica isolates, representing 8 serotypes. Serotype is designated by number. The dashed line represents the mean, and nonoverlapping intervals between two serotypes indicate a significant difference between them.*

TABLE 3. *Neuraminidase activity of five populations derived from each of three isolates[a] representing three serotypes of P. haemolytica*

Serotype	Neuraminidase activity (U/mg of dry cells)[b]				
	Mean	Standard deviation	Range		95% Confidence interval
			Minimum	Maximum	
1	0.15	0.02	0.12	0.18	(0.13, 0.17)
2	0.06	0.02	0.04	0.08	(0.04, 0.08)
9	0.67	0.06	0.58	0.73	(0.60, 0.73)

[a] Isolates were the type strains (Table 1).

[b] One unit of neuraminidase activity equals the release of 1 μmol of *N*-acetylneuraminic acid per min.

DISCUSSION

Neuraminidase activity levels of *P. haemolytica* isolates varied, and the activity levels among the isolates studied were serotype associated. The biotype T isolates (serotypes 3, 4, and 10) and some of the biotype A isolates (serotype 11, untypable isolates and some bovine serotype 2 isolates) had no measurable activity.

Scharmann et al. (13) found neuraminidase activity in 102 of 104 *P. multocida* isolates and in 3 of 5 untyped *P. haemolytica* isolates. The neuraminidase was cell associated, with usually less than 5% of the activity found in the culture supernatant. Our assay procedure, with suspensions of unwashed *P. haemolytica* colonies, measured mostly cell-bound enzyme, because much of the released enzyme would have diffused into the agar. We found the specific activity of neuraminidase in soluble extracellular antigens produced by a *P. haemolytica* serotype 1 isolate to be 0.53 U per mg of protein (L. B. Tabatabai and G. H. Frank, Curr. Microbiol., in press). The specific activity of the whole-cell suspension of the same isolate was 0.13 U per mg of dry cells, a relatively low level of activity when compared with that of other *P. haemolytica* isolates. The neuraminidase content of *P. haemolytica* is difficult to compare with that of other species of bacteria, because different preparations, methods of standardization, and substrates have been used.

Neuraminidase is produced by several species of bacteria and has been associated with virulence (10–12, 15). Although some *P. haemolytica* serotypes have been more frequently associated with pneumonia in sheep and cattle than others, the relationship between neuraminidase activity and ability to produce pneumonia is not known. Smith (14) divided the ovine isolates into two biotypes, A and T. He associated biotype A with septicemia of newborn lambs and enzootic pneumonia, and he associated biotype T with septicemia of older lambs. Later, biotype T was found to consist of serotypes 3, 4, and 10, and biotype A was found to consist of the other serotypes (3). Serotypes most commonly associated with ovine pneumonia are 2, 1, and 6 (5, 16), even though all serotypes are found in sheep. Serotypes 1 and 2 are the predominant serotypes isolated from the nasal passages of cattle (6). Serotype 1 is most frequently isolated from calves with respiratory disease and is also most frequently found in pneumonic lungs (6).

In sheep, a direct relationship may not exist between neuraminidase activity and pneumonia, because serotype 2 *P. haemolytica* may have less activity than other serotypes. However, a possible relationship may exist in cattle because serotype 1 *P. haemolytica* may have higher neuraminidase activity than serotype 2. Neuraminidase activities of numerous *P. haemolytica* isolates from field cases of bovine and ovine pneumonia must be determined before neuraminidase can be associated with virulence.

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