

## Serotyping and Enzyme Characterization of *Pasteurella haemolytica* and *Pasteurella multocida* Isolates Recovered from Pneumonic Lungs of Stressed Feeder Calves

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**Abstract.** Ninety-one isolates of *Pasteurella multocida* (Pm) and 124 of *Pasteurella haemolytica* (Ph) were recovered from the lungs of calves that died of bovine respiratory tract disease (BRTD). Nine Pm enzyme profiles (A through I) and 9 Ph enzyme profiles (J through R) were determined for the *Pasteurella* isolates. The Pm isolates were relatively evenly divided among the enzyme profiles, with one exception, profile I. The Ph isolates were not evenly distributed among the profiles. Fifty of the 91 Pm isolates were serotyped. Forty-two Pm isolates were positive for capsule type A, and 8 were untypable. Five somatic type antigen profiles (3; 3,4; 3,7; 3,4,7; and 4) were identified among the 50 serotyped Pm isolates; one isolate was untypable. The Ph isolates were further divided through serotyping and grouped as follows: 74 (60%) *Pasteurella haemolytica* A1 (PhA1), 12 (10%) PhA2, 4 (3%) PhA5, and 34 (27%) PhA6. Eighty-one percent of the Ph serotypes were clustered in the M and N enzyme profile. The P enzyme profile was almost unique to PhA2 (8 of 12, 67% of PhA2 isolates). Results of this study indicate a need to collect more data on Ph serotypes at the state veterinary diagnostic laboratories.

Bovine respiratory tract disease (BRTD) continues to be a major economic problem in the cattle feeding industry. *Pasteurella haemolytica* (Ph) is the most important causative agent in acute and chronic BRTD [23]. Carter [4] demonstrated that Ph serotype 1 was more commonly isolated than other Ph serotypes from pneumonia in calves. Identification of Ph biovars and serovars by animal species [1] was an important advance. It was determined that Ph A1 (PhA1) was frequently isolated from market-stressed feeder calves [10]. However, Yates [27] reported that *Pasteurella multocida* (Pm) biotype A was frequently isolated from pneumonic calves. State veterinary diagnostic laboratories frequently isolated both Ph and Pm from BRTD calves. However, these laboratories do not routinely serotype Ph or Pm isolates, owing to the time and expense involved. This is unfortunate

because serotypes other than PhA1 may be contributing to the feeder calf disease problem.

Many researchers are trying to determine which PhA1 whole bacterins [6, 17] and subunit antigens [5, 7, 19] are immunogenic. However, the involvement of other Ph serovars in the bovine species has not been addressed to the same degree. Cross protection among Ph serovars has not been studied with a challenge model. There are a number of Ph bacterins on the market; however, most are exclusively limited to PhA1. The purpose of this study was to characterize Ph and Pm lung isolates from calves with BRTD, obtained from 10 state diagnostic laboratories, by determining enzyme profiles and serotypes.

### Materials and Methods

***Pasteurella* isolates.** Ph (n = 51) and Pm (n = 61) isolates were obtained from the Upjohn Co (Kalamazoo, Mich.) Upjohn had originally procured the bacteria from 10 different state veterinary diagnostic laboratories (CA, CO, IA, IL, NE, OK, PE, TX, WA, and WY) in 1991

Table 1. Definition of profiles by the unique sequence of enzyme activities in 91 *Pasteurella multocida* and 107 *Pasteurella haemolytica* (Ph) isolates recovered from the lungs of BRD calves

<i>Pasteurella</i> sp.	Profile (no.)	Enzymes																			
		1 <sup>a</sup>	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Pm	A (14)	0 <sup>b</sup>	+ <sup>c</sup>	+	+	0	+	+	0	0	0	+	+	0	0	0	0	0	0	0	0
Pm	B (11)	0	+	+	+	0	+	+	0	0	0	+	+	0	0	0	+	0	0	0	0
Pm	C (10)	0	+	+	+	0	+	0	0	0	0	+	+	0	0	0	+	0	0	0	0
Pm	D (6)	0	+	+	+	0	+	0	0	0	0	+	+	0	0	0	0	0	0	0	0
Pm	E (6)	0	+	0	+	0	+	+	0	0	0	+	+	0	0	0	0	0	0	0	0
Pm	F (7)	0	+	0	+	0	+	+	0	0	0	+	+	0	0	0	+	0	0	0	0
Pm	G (24)	0	+	0	+	0	+	0	0	0	0	+	+	0	0	0	+	0	0	0	0
Pm	H (12)	0	+	0	+	0	+	0	0	0	0	+	+	0	0	0	0	0	0	0	0
Pm	I (1)	0	+	0	0	0	+	0	0	0	0	+	+	0	+	0	+	0	0	0	0
Total	9 (91)	0	91	41	90	0	91	38	0	0	0	91	91	0	1	0	53	0	0	0	0
Ph	J (1)	0	+	0	+	0	+	+	0	0	0	+	+	0	+	0	0	0	0	0	+
Ph	K (1)	0	+	+	0	0	+	0	0	0	0	+	+	0	+	0	0	0	0	0	+
Ph	L (5)	0	+	+	0	0	+	+	0	0	0	+	+	0	0	0	0	0	0	0	+
Ph	M (49)	0	+	+	0	0	+	+	0	0	0	+	+	0	+	0	0	0	0	0	+
Ph	N (35)	0	+	+	+	0	+	+	0	0	0	+	+	0	+	0	0	0	0	0	+
Ph	O (4)	0	+	+	+	0	+	+	0	0	0	+	+	0	+	0	0	0	0	0	0
Ph	P (9)	0	+	+	+	0	+	+	0	0	0	+	+	0	0	0	0	0	0	0	+
Ph	Q (2)	0	+	0	0	0	+	+	0	0	0	+	+	0	0	0	0	0	0	0	+
Ph	R (1)	0	+	+	0	0	+	+	0	0	0	+	+	0	+	0	0	0	0	0	0
Total	9 (107)	0	107	104	49	0	107	106	0	0	0	107	107	0	91	0	0	0	0	0	102

<sup>a</sup> The numbers represent the enzymes tested for: 1, control; 2, alkaline phosphatase; 3, esterase (C4); 4, esterase lipase (C8); 5, lipase (C14); 6, leucineaminopeptidase; 7, valine aminopeptidase; 8, cystine aminopeptidase; 9, trypsin; 10, chymotrypsin; 11, acid phosphatase; 12, phosphohydrolyase; 13,  $\alpha$ -galactosidase; 14,  $\beta$ -galactosidase; 15,  $\beta$ -glucuronidase; 16, 2-naphthyl-2-D-glucopyranosidase; 17,  $\beta$ -glucosidase; 18, N-acetyl- $\beta$ -D-glucopyranosidase; 19,  $\alpha$ -mannosidase; 20,  $\alpha$ -naphthyl- $\alpha$ -L-fucopyranosidase.

<sup>b</sup> 0 means no enzyme activity.

<sup>c</sup> + means enzyme activity.

in order to study antibiotic susceptibility patterns. The Colorado State University Veterinary Diagnostic Laboratory (CSUVDL) (Fort Collins, CO) supplied 35 Ph isolates which were originally isolated from calves in two local feedyards from 1985 to 1988. The Texas A&M University Veterinary Diagnostic Laboratory (TAMVDL) (Amarillo, TX) supplied 38 Ph and 30 Pm isolates obtained in 1993 from feeder or stocker beef calves. All Ph and Pm isolates from each source were from different calves. All *Pasteurella* isolates were supplied to us on blood agar base plates with 5% bovine erythrocytes and identified as Ph or Pm.

**Bacteriologic culture and identification procedures.** *Pasteurella* cultures were subcultured (first subculture) on a second blood agar plate, incubated in air with 5% CO<sub>2</sub> at 37°C for 24 h, then identified as Ph or Pm by use of standard bacteriologic identification procedures [25]. If the first subculture was pure, it was overlaid with 1 ml of skim milk prepared twice the strength suggested by the manufacturer, mixed with an inoculating loop, absorbed on filter paper, then stored at -85°C [18]. The Ph were identified as PhA1, PhA2, PhA5, or PhA6 with specific serotyping antiserum [11]. Serotyping of the Pm isolates was done by the methods of Rimler and Brogden [21], and somatic typing was completed by using the methods of Heddleston et al. [13].

**Enzyme assays.** Each Ph or Pm isolate was tested for its enzyme activity against 19 substrates with a commercial semiquantitative enzyme system (API ZYM System, API Analytab Products, Division of Sherwood Medical, Plainview, NY) as described by the manufacturer. A piece of thawed filter paper with the bacteria was streaked on the surface of a blood agar plate. From this subculture, enzyme characteristics of

the isolate were determined. Colonies from the blood agar plate, after incubation at 37°C for 20 h, were suspended in 3 ml of sterile distilled water to a McFarland No. 6 standard. Inoculation of the enzyme strip, incubation, and light exposure have been described [18]. Color intensity of the reaction was compared with the manufacturer's interpretation scheme, and reactions were graded as follows: 0, no color change; trace,  $\leq 5$  nmol of hydrolyzed substrate; 1, 5 nmol of hydrolyzed substrate; 2, 10 nmol of hydrolyzed substrate; 3, 20 nmol of hydrolyzed substrate; 4, 30 nmol of hydrolyzed substrate; and 5,  $\geq 40$  nmol of hydrolyzed substrate. Grades 1 to 5 were scored as positive reactions. All Ph and Pm isolated were profiled, and a letter was assigned to each profile (Table 1).

**Reproducibility of enzyme assay system.** The commercial enzyme assay system was tested for its reproducibility, by use of an identified PhA1 and Pm isolate. Each isolate was replicated five times, as previously described [18].

**Statistical analysis.** The frequency of enzyme profiles was analyzed by  $\chi^2$  analysis with one-way and two-way contingency tables. Enzyme profiles or Ph serovars with less than five occurrences were deleted from the analysis. The frequencies of enzyme profiles within *Pasteurella* species and serovars were tested with one-way tables. Two-way tables were used to test the frequency of enzyme profiles among isolate age, source, and serovars. The null hypothesis for the one-way and two-way tests considered that distribution among groups was homogeneous or uniform. The null hypothesis was accepted if they were of equal

Table 2. Sources and serotypes of *Pasteurella haemolytica* isolates

Ph serotype <sup>a</sup>	CSUVDL		Upjohn		TAMUVDL		Total	Percent
	Number	Percent	Number	Percent	Number	Percent		
PhA1	14	(19)	43	(58)	17	(23)	74	(60)
PhA2	6	(50)	2	(17)	4	(33)	12	(10)
PhA5	3	(75)	0	(0)	1	(25)	4	(3)
PhA6	12	(35)	6	(18)	16	(47)	34	(27)
Total	35	(28)	51	(41)	38	(31)	124	(100)

<sup>a</sup> Determined by the method of Frank and Wessman [11].

Table 3. Average enzyme activity of *Pasteurella* isolates by species and sources

Source of isolates	No.	Species	Enzymes									
			2 <sup>a</sup>	3	4	6	7	11	12	14	16	20
TAMUVDL	30	Pm	5 <sup>b</sup>	0.03	1.2	2.5	0.03	4.97	1.43	0.03	0.77	0
Upjohn	61	Pm	5	0.69	1.7	4.3	0.61	5	2.34	0	0.98	0
CSUVDL	14	PhA1	5	1.4	0.64	4.9	1	5	4.9	1.2	0	5
TAMUVDL	17	PhA1	5	1.8	0.47	5	1	5	5	1.4	0	5
Upjohn	43	PhA1	5	1.5	0.51	4.9	1	5	4.9	1	0	4.9
TAMUVDL	2	PhA2	5	1.5	0	5	1	5	4	0.5	0	5
Upjohn	4	PhA2	5	1	0.75	4.8	1	5	5	0.25	0	5
CSUVDL	6	PhA2	5	1.7	0.83	5	1.5	5	5	0	0	5
CSUVDL	3	PhA5	5	1	0.7	5	1	5	5	1	0	5
Upjohn	1	PhA5	5	1	0	5	1	5	5	1	0	5
TAMUVDL	6	PhA6	5	1.5	0.33	4.8	1	5	5	1.2	0	5
Upjohn	16	PhA6	5	1.3	0.44	5	1	5	4.8	0.8	0	5
CSUVDL	12	PhA6	5	2.3	0.67	5	1.2	5	4.7	1.2	0	3.3

<sup>a</sup> The numbers represent the enzymes tested for; see Table 1 for the enzyme code.

<sup>b</sup> The numbers under the enzyme code represent the amount (nanomoles) of substrate hydrolyzed by the indicated enzyme. 1, 5 nanomoles; 2, 10 nanomoles; 3, 20 nanomoles; 4, 30 nanomoles; 5,  $\geq 40$  nanomoles.

frequency. Chi-square values were considered significant at  $P \leq 0.05$  [22].

## Results

### Enzyme and serotyping data from sample specimens.

*Pasteurella multocida* isolates ( $N = 91$ ) fit into nine (A through I) enzyme profiles (Table 1). *Pasteurella haemolytica* isolates ( $N = 107$ ) fit into nine (J through R) enzyme profiles (Table 1). Even though 124 Ph isolates were examined, 17 of them had enzyme profiles with less than five occurrences, so they were not included in Table 1, for a total of 107 Ph isolates described. The Ph isolates were identified by serotype (74 PhA1, 12 PhA2, 4 PhA5, and 34 PhA6) and by source (Table 2). Forty-two Pm isolates were identified as capsular type A, and 8 were untypable. Five somatic antigen types (3; 3,4; 3,7; 3,4,7; and 4) were identified from 49 isolates, and one was untypable.

The average color intensity developed for each of the substrates tested is presented by isolate source and

species in Table 3. Within each *Pasteurella* species, the average color intensities developed for each enzyme tended to be similar across isolate source. There also appeared to be little difference among Ph serovars in average color development for each enzyme. The most active Ph enzymes hydrolyzed 30 to  $\geq 40$  nanomoles of these substrates: 2-naphthyl-phosphate (enzyme 2), L-leucyl-2-naphthylamide (enzyme 6), 2-naphthyl-phosphate (enzyme 11), naphthol-AS-BI-phosphate (enzyme 12), and 2-naphthyl- $\alpha$ -L-fucopyranoside (enzyme 20) [except 4 CSUVDL PhA6 isolates and 1 PhA1 from Upjohn that were inactive against the latter substrate]. Least active Ph enzymes hydrolyzed 5–10 nanomoles of these substrates: 2-naphthyl-butyrate (enzyme 3), 2-naphthyl-caprylate (enzyme 4), L-valyl-2-naphthylamide (enzyme 8), and 2-naphthyl- $\beta$ -D-galactopyranoside (enzyme 14). None of the following Ph enzyme activity was detected: lipase, cystine aminopeptidase, trypsin, chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase, 2 naphthyl-2-D-glucopyranosidase,  $\beta$ -glucosidase, N-acetyl- $\beta$ -D-gluco-

Table 4. 124 *Pasteurella haemolytica* isolates identified by source, serotype, and enzyme profiles

Enzyme profile <sup>a</sup>	CSUVDL				Upjohn				TAMUVDL		
	PhA1	PhA2	PhA5	PhA6	PhA1	PhA2	PhA5	PhA6	PhA1	PhA2	PhA6
J	— <sup>b</sup>	—	—	—	1	—	—	—	—	—	—
K	—	—	—	—	1	—	—	—	—	—	—
L	—	1	—	—	1	—	—	2	—	1	—
M	5	—	1	2	19	1	1	8	9	1	4
N	9	—	2	5	20	—	—	4	8	—	2
O	—	—	—	3	1	—	—	—	—	—	—
P	—	5	—	—	—	3	—	1	—	—	—
Q	—	—	—	1	—	—	—	1	—	—	—
R	—	—	—	1	—	—	—	—	—	—	—
Total (124)	14	6	3	12	43	4	1	16	17	2	6

<sup>a</sup> Defined in Table 1.

<sup>b</sup> — denotes no strains with that enzyme profile.

pyranosidase, and  $\alpha$ -mannosidase (Table 1). The Pm isolates were similar in enzyme activity, except against the substrate 2-naphthyl- $\alpha$ -D-glucopyranoside (enzyme 16) (no hydrolysis of this substrate was detected in the Ph isolates). Thirty-eight of 91 Pm isolates had no detectable  $\beta$ -glucosidase activity against the naphthol-AS-BI- $\beta$ -D-glucuronide (Table 1). Only one Pm isolate had  $\beta$ -galactosidase which hydrolyzed 5 nanomoles of 2-naphthyl- $\beta$ -D-galactopyranoside. Alpha-naphthyl- $\alpha$ -L-fucopyranosidase, which hydrolyzes 2-naphthyl- $\alpha$ -L-fucopyranoside, was never active in the Pm isolates. This enzyme readily differentiated Pm from Ph isolates, except for the four aberrant PhA6 hemolytic isolates from CSUVDL and one PhA1 from Upjohn which were also inactive for this enzyme.

The  $\chi^2$  test for the one-way table analysis was significantly different ( $P \leq 0.005$ ) for Pm enzyme profiles A through H; thus, the null hypothesis of equal frequency was rejected. The frequency of enzyme profiles M and N were similar for PhA1 ( $P \leq 0.65$ ) and PhA6 ( $P \leq 0.5$ ).

The  $\chi^2$  two-way tables of analysis indicated there were significant differences ( $P \leq 0.0001$ ) in the frequency of Pm enzyme profiles among isolate sources and serotypes. In the most recently acquired isolates (new-1993-TAMUVDL), profiles A through F predominated, while in earlier acquired Pm isolates (old-1991-Upjohn), profiles G and H predominated. The frequencies of Ph enzyme profiles M or N were not significantly different among sources for PhA1 ( $P \leq 0.61$ ) and PhA6 ( $P \leq 0.23$ ). The frequency of enzyme profiles L, M, N, and P of PhA2 isolates were significantly different ( $P \leq 0.0001$ ) across sources. In the PhA2 isolates, enzyme profiles P and L predominated; while in the PhA1 and PhA6 isolates, enzyme profiles M and N predominated. The number of

Ph isolates in each serotype, enzyme profile, and source are presented in Table 4.

**Enzyme replication study.** Reproducibility of the enzyme assay system was shown to be reliable. A Pm and a PhA1 isolate were replicated five times each. The enzyme profile for each replication per isolate remained the same (data not shown).

## Discussion

Efficacy of vaccines for PhA1 may be very dependent on the amount of BRTD induced by this serotype in feeder/stocker calves. In this study, 40% (Table 2) of 124 Ph isolates from calf pneumonic lungs from 10 different states were not serotype PhA1, but were PhA2 ( $n = 12$ , 10%), PhA5 ( $n = 4$ , 3%), and PhA6 ( $n = 34$ , 27%). If no cross-protection is afforded by vaccination, a 100% effective PhA1 vaccine would be only 60% effective in this group of 124 calves that died of BRTD. *Pasteurella haemolytica* A2 is usually not found during severe stress in calves [10, 17]. The PhA2 serotype is infrequently found in pneumonic calves [26], but it is frequently isolated from goat [9] and sheep [8, 24] respiratory infections. It is also the most common serotype that we isolate from the upper respiratory tract of goats [19]. Little is known about the incidence of PhA5 and PhA6 in calves, although both have been recovered from pneumonic calves [11, 20]. The high incidence of PhA6 in feeder calves dying of BRTD in 1985 ( $n = 12$ ), 1991 ( $n = 6$ ), and 1993 ( $n = 16$ ) should be of concern to researchers developing Ph vaccines, large animal veterinary practitioners concerned with vaccine management, ranchers, and to the feeder/stocker calf industry. Attention was called to the different Ph serovars capable of

inducing respiratory infections in sheep when developing vaccines [3]. A Ph vaccine failure in sheep in Kenya may have been in part due to lack of multivalent serotypes in the product [15]. It appears that a more comprehensive account of Ph serotypes found in pneumonic calves would be desirable. This information should be considered before developing Ph vaccines for cattle.

The high incidence of Pm isolates should be of concern to those dedicated to decreasing the impact of BRTD on the feeder/stocker calf industry. The importance of Pm in BRTD is unclear; however, experimental bovine respiratory disease has been induced by aerosol exposure to Pm alone and/or in combination with bovine herpesvirus 1 [12, 14]. It has been suggested that Pm should receive more recognition as a primary pathogen [12].

The number of different enzyme profiles were fewer than previously reported [16]. This may be because nasal isolates from healthy calves are more heterotypic than those isolated from pneumonic calves. It may also imply that certain Ph enzyme profiles like M and N are more pathogenic and, therefore, have a higher incidence of recovery from pneumonic calves. No Pm isolate had the same enzyme profile as any of the Ph isolates. The enzyme profiles for the 91 Pm isolates were very similar in their average enzyme activity for each of the 19 substrates regardless of the source, time collected, serotype, or enzyme profile identified.

The Ph profiles were scattered among J through R (Table 4). However, the enzyme profiles M (n = 51) and N (n = 50) accounted for 81% of the total Ph isolates. Enzyme profile P was almost unique to PhA2 (8 of 12 isolates) recovered prior to 1993. The enzyme profiles for the 124 Ph isolates were very similar in average enzyme activity (Table 3) for each of the 19 different substrates regardless of the source, time collected, serotype, or enzyme profile identified. All Ph isolates had  $\alpha$ -fucosidase enzyme activity with the exception of four PhA6 isolates from CSUVDL and one PhA1 isolate from Upjohn. This was very unusual, since most Ph isolates in this study use this enzyme to hydrolyze  $\geq 40$  nanomoles of 2-naphthyl- $\alpha$ -L-fucopyranoside substrate. In a previous report [18], only 63 of 143 PhA1 isolates were positive for the  $\alpha$ -naphthyl- $\alpha$ -L-fucopyranosidase enzyme. The amount of hydrolyzed substrate indicated by color intensity was not reported; however, after reviewing the data of the previous paper, it was determined that of those 63 positive isolates, few had hydrolyzed more than 5 nanomoles of 2-naphthyl- $\alpha$ -L-fucopyranoside substrate.

Enzyme profiling is a relatively new technique to characterize Pm and Ph. It was used in swine to determine that Pm strains isolated from the kidneys had spread from

primary lung infections [2]. We have used enzyme profiles as an epidemiological tool to trace PhA1 isolates in market-stressed feeder calves back to their farm of origin. Certain PhA1 enzyme profiles were more associated with the calves' farm of origin than with feedyard commingling [18]. In the present study we identified 9 Pm and 9 Ph enzyme profiles from a group of 215 isolates recovered from the lungs of pneumonic calves. We assume that all of these *Pasteurella* isolates were pathogenic and were responsible for their deaths. We also reported some peculiarities in four PhA6 isolates and that enzyme profile P was almost unique to PhA2 isolates recovered prior to 1993. It is hoped that as these data bases become available, we will have a better understanding of the *Pasteurella* group of bacteria from calves in health and in sickness. The question of why the Pm isolates were more evenly divided among profiles while the Ph isolates were clustered in the M and N enzyme profiles remains unanswered.

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