

# Naturally Occurring Pasteurellosis in Laboratory Rabbits: Chemical and Serological Studies of Whole Cells and Lipopolysaccharides of *Pasteurella multocida*

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Whole cells and lipopolysaccharides (LPS) of 10 isolates of *Pasteurella multocida* from laboratory rabbits were subjected to chemical and serological analysis. LPS of most of these isolates possessed pyrogenic potency comparable to LPS from *Salmonella minnesota* 9700, although their average ketodeoxyoctonate content was only 18% of that of salmonella. A gel diffusion precipitin test for somatic antigens extracted in a formal-saline solution demonstrated several isolates with three to four somatic antigens, with some variation in the major somatic type from one test to another. Conversely, the use of LPS as antigen in the gel diffusion precipitin test (i) eliminated cross-reactivity with reference antisera and (ii) often resulted in the organism being typed as serotype 12 even when the type 12 antigen was a minor antigen in the formal-saline extracts. Antisera from specific pathogen-free rabbits immunized with either whole cells or LPS of two isolates were tested against whole cells or LPS of the 10 isolates by enzyme immunoassay and indirect hemagglutination. Both whole cells and LPS of one of the isolates (isolate 2) were serologically specific, whereas those of the other isolate (isolate 1) were moderately to strongly cross-reactive with other isolates. The data indicate that although LPS is the major antigen responsible for typing based on the gel diffusion precipitin test, substances other than LPS (probably capsular polysaccharide) are responsible for the type specificity that forms the basis for the A, B, D, or E classification of this organism.

Infection by *Pasteurella multocida* is enzootic in virtually all conventionally housed colonies of rabbits, including colonies maintained by animal suppliers and research laboratories (15). The acute or pneumonic form of the disease occurs sporadically and is usually precipitated by stressful stimuli including shipping and experimental manipulation. The chronic form of pasteurellosis is usually more troublesome to researchers because affected rabbits are often being used in long-term studies which are at high risk of being prematurely terminated as a result of the infection. Recurrent purulent rhinitis (snuffles), the principle clinical sign of chronic pasteurellosis, often results in sequelae such as conjunctivitis, otitis media (often accompanied by torticollis), furunculosis, and pulmonary abscesses. Treatment of pasteurellosis with antibiotics, usually penicillin, may temporarily retard progress of the disease but is not curative. Recent studies on the pathogenesis of rabbit pasteurellosis have emphasized the morphological changes in infected tissues (15), granulocyte function (18), and in vivo colonization by mutant strains (12, 22). Although a few studies have addressed the serological features of rabbit isolates of *P. multocida* (4, 13, 23, 24), most investigations of the serology of *P. multocida* have involved the study of isolates from cattle and fowl in which the organism causes hemorrhagic septicemia and fowl cholera, respectively. Serological studies of these isolates have resulted in a more precise characterization of outbreaks of spontaneous disease and have contributed to improved strategies of prevention and control. In anticipation of a more thorough understanding of pasteurellosis in laboratory rabbits, we have undertaken to study selected chemical and serological characteristics of cell wall materials of rabbit isolates of *P. multocida*.

## MATERIALS AND METHODS

**Typing of isolates.** Isolates of *P. multocida* were obtained from rabbits with various clinical manifestations of pasteurellosis (Table 1). The isolates were streaked onto nutrient agar containing 5% sheep blood and incubated overnight at 37°C. Fresh colonies were identified by standard procedures (8) and typed by acriflavine flocculation (11), hyaluronidase inhibition (10), and by a gel diffusion precipitin test (GDPT) (16). In the latter procedure, a formal-saline extract of the organisms is heated at 100°C for 1 h; the extract is reacted in a GDPT against each of 16 reference antisera obtained by immunizing chickens with a bacterin comprised of 1 of 16 serotypes of *P. multocida*. The results of the GDPT were recorded at 24, 48, and 72 h of incubation at room temperature. Each GDPT was performed at least twice. Our isolates were designated as either capsular type A or D followed by Arabic numerals indicating the antiserum type(s) against which the formal-saline extracts or lipopolysaccharides (LPS) reacted, e.g., A:3, D:12. Since extracts of most isolates reacted with two or more antisera, the antiserum against which the extract reacted strongest was listed first, and minor (faintly visible and occurring usually after 24 h) reactions were indicated by enclosure within parentheses.

**Bacterial cultures, extraction, and chromatographic procedures.** Strains of *P. multocida* were maintained at -70°C in the lyophilized state. Before preparation of batches of organisms, the lyophilate was reconstituted in 10 to 20 volumes of Trypticase soy broth, and after 12 to 14 h of incubation at 37°C, 0.1 ml of the broth culture was injected intraperitoneally into Swiss-Webster mice. The mice were killed 4 h later in a CO<sub>2</sub> chamber, and the organisms were washed from the

peritoneal cavity with Trypticase soy broth which was then incubated overnight at 37°C. Batches of the organism were obtained by heavily streaking the isolate in the broth culture onto 32 to 50 large (150-mm diameter) Mueller-Hinton agar plates with 5% sheep blood (Diagnostic Inc., St. Paul, Minn.). The plates were incubated at 37°C overnight, and growth from each plate was gently removed with a glass spatula. The colonies were pooled in distilled water and mixed thoroughly, and the bacteria were packed by centrifugation. The bacterial pellet was then extracted in hot phenol and water essentially as described by Carter and Rappay (9) except the aqueous phases were washed four times in 3 volumes of ether to remove traces of phenol before ethanol precipitation, and ethanol precipitation was repeated three times rather than twice. The precipitates were air dried and dissolved as a 0.2% solution in 0.025 M Tris-acetate buffer (pH 7.5) after which 100 Kunitz units of RNase A (Sigma Chemical Co., St. Louis, Mo.) per mg was added, and the mixture was incubated at 37°C for 18 h. The sample was then dialyzed in cold, flowing tap water for 24 h, and degraded nucleic acid was removed by centrifugation. The supernate was centrifuged at  $105,000 \times g$  at 4°C for 18 h. The gel-like sediment was removed, dissolved in 12 volumes of distilled water, and again subjected to ultracentrifugation for 12 h.

Gel filtration chromatography was performed at room temperature in glass columns (110 by 0.8 cm) containing agarose (Ultragel A4; LKB, Bromma, Sweden), which were washed and equilibrated in distilled water. Lyophilate (2 to 7 mg) in 5 to 12 ml of distilled water was applied to columns with a gel bed height of 95 cm. The column flow rate was maintained at 5 ml/h per  $\text{cm}^2$  with a peristaltic pump (LKB model 2132), and the absorbance of the eluant was monitored at 206 nm (LKB model 2138 UV monitor) and recorded. Fractions of 5 to 15 ml were collected; fractions comprising a single peak were combined, concentrated by membrane filtration (membrane A30; Amicon Corp., Lexington, Mass.), and lyophilized. This material was designated LPS.

**ELISA.** The antigen used for enzyme-linked immunosorbent assay (ELISA) was either boiled cells or lyophilized LPS. Peroxidase-conjugated goat antirabbit immunoglobulin G (IgG) Fc fragment (gamma chain specific; Cappel Laboratories, West Chester, Pa.) was diluted 1:250 in a buffer of 0.3% gelatin-0.2 M phosphates-0.15 M NaCl-1% bovine serum albumin. *o*-Phenylenediamine (Sigma) was prepared as a 0.05% solution in a buffer comprised of 0.05 M citric acid, 0.05 M sodium citrate, and 0.01% hydrogen peroxide.

A 0.1-ml volume of either boiled cells corresponding to 30% transmission at 610 nm or lyophilized LPS dissolved in distilled water at 0.4 mg/ml was placed into flat-bottomed wells of an uncovered polystyrene plate (Costar, Cambridge, Mass.) and incubated at 37°C overnight. The wells were washed three times with 0.4 ml of phosphate-buffered saline after which 0.2 ml of a fetal calf serum buffer (0.02 M phosphate buffer with 10% fetal calf serum, 1% bovine serum albumin, and 0.3% gelatin) was added and allowed to stand at room temperature for 15 min. The wells were then washed three times with 0.4 ml of phosphate-buffered saline. Then, 0.1 ml of the serum dilutions (primary antisera) in fetal calf serum buffer was added to the wells. The plates were covered and incubated at 37°C for 60 min, washed three times in 0.4 ml of phosphate-buffered saline, and 0.1 ml of diluted conjugated secondary antibody was added and incubated at 37°C for 1 h. The conjugate was aspirated and the wells were washed seven times with phosphate-buffered saline before addition of 0.1 ml of *o*-phenylenediamine substrate. The plates were covered and incubated at 37°C for

1 h, and the titers were expressed as the highest serum dilution resulting in an optical density greater than 2 standard deviations from the average optical density of the contents of control wells. Control wells contained all reagents except primary antisera. Optical densities were determined at 410 nm in a Microelisa reader (Dynatech Laboratories, Inc., Alexandria, Va.).

***Limulus* ameocyte lysate assay.** The gelation potency of LPS of isolates of *P. multocida* were compared with similar extracts of *Salmonella minnesota* (Sigma) and *Escherichia coli* O55:B5 by the *Limulus* ameocyte lysate assay (Pyrogen; Mallinckrodt Inc., St. Louis, Mo.). The assay was performed according to instructions supplied by the manufacturer. Pyrogen-free reagents were used, and all glassware was chemically cleaned, repeatedly rinsed in distilled water, and heated at 500°F for 1 h before use. The assay tubes were examined after 1 h of incubation at 37°C, and the results were recorded as complete (+) or incomplete (-) gelation of the lysate as determined by inversion of the tubes.

**Animals and immunizations.** *Pasteurella*-free New Zealand White female rabbits (Hazelton/Dutchland Inc., Denner, Pa.) were used for immunization. The rabbits were housed individually in stainless steel cages in a laminar flow enclosure (Lab Products Inc., Rochelle Park, N.J.) in rooms kept at  $21 \pm 1^\circ\text{C}$  with 15 to 17 air changes hourly and an automatically controlled light cycle with 12 h of fluorescent light. Fresh food and water were available at all times. Cultures of the nasal passages of the rabbits were taken on their arrival and at 6- to 10-week intervals thereafter. Nasal isolates were identified by standard techniques (8). Whole cell antigens were prepared from organisms grown on nutrient agar with 5% sheep blood for 14 to 18 h. Colonies were scraped from the surface of the agar with a glass spatula, mixed thoroughly in 0.85% saline solution, sedimented by centrifugation at  $1,500 \times g$  for 20 min, and resuspended in saline solution. The suspension was placed in a boiling water bath for 2 h and then adjusted by addition of saline solution to a concentration corresponding to 22% transmission at 610 nm. Rabbits were initially injected intramuscularly in each thigh with 0.5 ml of a 1:1 mixture of the bacterial suspension (whole cell antigen) in complete Freund adjuvant. The next four injections were done similarly every 10 to 14 days with whole cell suspensions in incomplete Freund adjuvant. Animals immunized with lyophilized LPS were handled identically except that the complete Freund adjuvant and incomplete Freund adjuvant were mixed with equal volumes of saline solution containing 2 mg of lyophilate per ml. Each animal was injected with 0.5 ml in each thigh muscle. One to 2 weeks after final immunization, blood was collected from the ear artery of the rabbits. The blood was allowed to clot at room temperature for 2 h and then centrifuged at  $1,500 \times g$  for 5 min. Serum was aspirated and thimensonol was added to a final concentration of 1:10,000; samples of serum were stored at -4°C.

**Chemical analysis and spectral properties of LPS bacterial extracts.** All chemical analyses were performed on lyophilized LPS reconstituted in distilled water. Spectrophotometric readings were made in a Beckman DBG spectrophotometer against appropriate reagent blanks. All chemicals used were analytical grade or better. Values shown in the tables are the result of duplicate determinations.

The presence of capsular polysaccharides as a contaminant in the LPS extracts was determined by dissolving 10 to 50  $\mu\text{g}$  of LPS in 1 ml of a 2.5% solution of carbocyanine dye (Eastman Chemicals, Rochester, N.Y.) prepared as described by Janda and Work (19). The absorbance spectrum

TABLE 1. Capsular and somatic types of *P. multocida* isolates from laboratory rabbits

Isolate no.	Source	Capsular type	Somatic type <sup>a</sup>		
			NADC	Lab	LPS
1	Skin abscess	A	3(4)	4(7,12,3)	12(4,3)
2	Scrotal abscess	D	3(4)	3(12,4)	3
3 <sup>b</sup>	Lung	A	12	12(4,5,7)	12
5	Middle ear	A,D	4	4(5,7,12)	12
6	Mesenteric abscess	D	4	12(4,5,7)	12
7	Nasal passage	A	ND <sup>c</sup>	Untypable	12
8	Nasal passage	Non-A,D	1(4,5)	1	— <sup>d</sup>
9	Nasal passage	A	ND <sup>c</sup>	Untypable	(12)
10 <sup>e</sup>	Unknown	D	1(3,4,5)	1	—
11 <sup>e</sup>	Unknown	A	15	Untypable	(15)

<sup>a</sup> Minor (faint) or delayed (faint and occurring after 24 h) precipitin lines are included within parentheses. Somatic typing was done in a GDPT in which type-specific chicken antiserum (kindly provided by the National Animal Disease Center, Ames, Iowa) was reacted against a formal-saline heat-stable extract of the isolate either at the Ames laboratory (column labeled NADC) or in my laboratory (column labeled Lab) with chicken antisera kindly provided by Billy Blackburn of the National Animal Disease Laboratory. In the last column, LPS was used as the source of antigen.

<sup>b</sup> Kindly provided by Y. S. Lu, University of Texas Health Science Center, Dallas, Tex.

<sup>c</sup> ND, Not done.

<sup>d</sup> Repeated extraction of isolates 8 and 10 yielded no material with absorbance at 460 to 470 nm (a characteristic of LPS-carbocyanine solution).

<sup>e</sup> Kindly provided by Microbiology Laboratory, National Animal Disease Center.

of dye-extract solution was recorded from 440 to 675 nm in a scanning spectrophotometer. Only extracts that possessed a single absorbance peak at 460 to 472 nm characteristic of LPS and were free of absorbance at 600 to 640 nm characteristic of acidic polysaccharides (14) were used in subsequent chemical determinations.

Protein content was determined by a microassay (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, Calif.) procedure in which protein forms a stable complex with Coomassie brilliant blue G-250 (3). Optical density readings were made at 595 nm, and protein concentration was calculated based upon standard curves obtained with bovine serum albumin (Armour Pharmaceutical, Phoenix, Ariz.). Ketodeoxyoctonate (KDO; Sigma) was measured by the method of Karkhanis et al. (20). Standard curves were established with purified KDO. This method permitted accurate detection of as little as 0.7 µg of KDO.

**Other bacterial extracts.** For comparative purposes, LPS of *S. minnesota* 9700 and *E. coli* O55:B5 (Difco Laboratories, Detroit, Mich.) were subjected to the same analytical procedures as were performed on the *Pasteurella* extracts.

**IHA.** The indirect hemagglutination assay (IHA) was done with a microtiter system slightly modified from the procedures described by Sawada et al. (31). Before use in the IHA, all sera were inactivated at 56°C from 30 min and were absorbed with unsensitized glutaraldehyde-treated sheep erythrocytes to remove heterophile antibodies by mixing 0.1 ml of lightly packed erythrocytes with 1.0 ml of rabbit antisera and allowing the mixture to stand at room temperature for 2 h. Serial twofold dilutions of 0.1 ml of antisera in bovine serum albumin-phosphate-buffered saline were added to 0.1 ml of a 1% suspension of sensitized glutaraldehyde-treated sheep erythrocytes in V-bottomed plates. The plates were shaken once to thoroughly mix the antisera and erythrocytes and placed on a flat surface. After 3 h at room temperature the plates were examined, and the IHA titer was expressed as the reciprocal of the highest dilution of serum showing a diffuse mat of erythrocytes as compared to

negative controls showing a clearly delineated spot of erythrocytes within the vertex of the well.

## RESULTS

**Capsular and somatic types.** The source of the isolates and the results of capsular and somatic typing procedures are given in Table 1. Five of 10 isolates were capsular type A, i.e., mucoid colonies whose growth was inhibited by hyaluronidase, and three isolates were type D in that they formed a coarse aggregate in a 0.1% acriflavine solution. Isolate 8 was unresponsive to either the acriflavine or hyaluronidase procedures, and isolate 5 reacted positively to both procedures.

The results of somatic antigen typing by the GDPT indicate that formal-saline extracts of whole cells of several isolates reacted with up to four different typing antisera. Moreover, the GDPT as performed in two different laboratories gave only generally similar results. Five of the seven isolates tested by both laboratories were found to possess the same major somatic antigen, but the results of the GDPT in my laboratory often revealed the presence of several somatic serotypes in one organism, e.g., isolates 1, 2, 3, 5, and 6. Isolates 1 and 6 were determined to possess a different major somatic antigen by the two laboratories. When LPS from each isolate was used as antigen in the GDPT, the predominant and often only precipitin reaction was against type 12 antisera. In isolates 1 and 5, somatic antigen 12 was one of three minor cross-reacting antigens in the formal-saline extract, whereas the LPS of these isolates reacted predominantly with antisera to type 12. Similarly the formal-saline extracts of isolates 7 and 9 were unreactive with any of the 16 antisera, but the LPS of these isolates reacted with the type 12 antisera.

**Qualitative features of LPS.** The results of protein and KDO measurements as well as Limulus amoebocyte lysate assays on LPS are summarized in Table 2. The mean protein content of several of the extracts was similar to that of reference strain *S. minnesota* 9700. The KDO content of the

TABLE 2. KDO, protein content, and *Limulus* amebocyte lysate gelation potency of LPS of rabbit isolates of *P. multocida*

Isolate	% KDO	% Protein	Gelation potency (pg/ml) <sup>a</sup>
1	1.50	4.1	50
2	0.80	2.1	12
3	0.1	2.9	12
5	0.36	1.6	25
6	0.93	1.7	>100
7	0.36	1.7	25
8	— <sup>b</sup>	—	ND <sup>c</sup>
9	0.22	0.4	ND
10	—	—	ND
11	0.40	1.8	ND
<i>S. minnesota</i> 9700	0.57 ± 0.17 <sup>d</sup>	2.04 ± 0.38 <sup>d</sup>	12
<i>E. coli</i> O55:B5	ND	ND	12

<sup>a</sup> Picograms of LPS per milliliter causing complete gelation within 1 h.

<sup>b</sup> Repeated extraction of isolates 8 and 10 yielded no material with absorbance at 460 to 470 nm (a characteristic of LPS-carbocyanine solution).

<sup>c</sup> ND, Not done.

<sup>d</sup> Mean ± standard error of the mean.

isolates averaged 0.57% and was substantially less than that of the *Salmonella* reference strain, 3.2%. Although isolates of capsular type A had higher mean values for both KDO and protein than isolates of capsular type D, the differences were not statistically significant ( $P > 0.05$ ).

The amebocyte lysate assay provides a comparative *in vitro* estimate of the pyrogenicity of extracts containing endotoxin. LPS of *P. multocida* isolates 1, 2, 3, 5, 6, and 7 were compared with similar extracts from reference strains *S. minnesota* 9700 and *E. coli* O55:B5. No clear differences among most of these extracts emerged in this assay in that the threshold pyrogenic concentration of LPS from isolates 2 and 3 was identical to those of the reference strains at 12 pg/ml, and two others were one dilution (25 pg/ml) greater. LPS of isolate 6 did not induce gelation even at a concentration of 100 pg/ml.

**ELISA results.** Pasteurella-free rabbits were immunized with either killed whole cells or LPS of isolate 1 or 2. These antisera were tested by ELISA against either whole cell antigens or LPS of several isolates. The results are summarized in Tables 3 and 4. Titers of preimmune sera were less than 1:100. Antiserum to whole cells of isolate 1 reacted in moderate to high titer (Table 3) to whole cell antigens of all the isolates, whereas antiserum to whole cells of isolate 2 reacted strongly with isolate 2 whole cells and only weakly with all the other whole cell isolates except isolate 6, which possess both the same capsular type and similar somatic antigens. Antiserum to LPS of isolate 1 reacted in highest titer to the homologous whole cell antigen but also reacted in moderately high titer to whole cell antigen of all the other isolates (Table 3), whereas antiserum to LPS of isolate 2 reacted only with the homologous cell bodies. When LPS served as antigen rather than whole cells (Table 4), roughly parallel results were observed, although the antibody titers were considerably lower. Rabbit antibody to whole cells of isolate 1 reacted in rather high titer to LPS from all isolates, whereas antisera to LPS of isolate 1 reacted (though in modest titer) most strongly to both homologous LPS and LPS of isolate 3 (which possesses similar somatic antigens) and weakly with LPS of other isolates. Conversely, antisera

to both isolate 2 whole cells and LPS reacted strongly with homologous LPS and very weakly to LPS of other isolates.

**IHA.** Pasteurella-free rabbits were immunized with whole cells, LPS from isolates 1 and 2, or *S. minnesota* LPS. The antibody response was determined by IHA (Table 5). Neither of the two LPS preparations from isolates 1 or 2 induced detectable antibody titer, whereas *S. minnesota* LPS was quite antigenic. Whole cell preparations of both isolates 1 and 2 induced antibodies which were reactive with determinants on LPS of several isolates. Although antiserum from the rabbit injected with whole cells of isolate 1 was cross-reactive in moderate to high titer with LPS of several isolates, antiserum against isolate 2 whole cells was strongly reactive with homologous LPS and only weakly cross-reactive with LPS of other isolates.

DISCUSSION

The serology of *P. multocida* has been the subject of several studies beginning 4 decades ago. Many of the early methods of serologic typing including slide agglutination (21), passive protection tests (21, 30), and IHA (7) have received little use in recent years. Contemporary methods of typing *P. multocida* isolates in the United States are the GDPT (16) and nonserological tests for detection of "capsular" types A and D by hyaluronidase inhibition and acriflavine flocculation (10, 11). Brogden and Packer (5) were unable to reliably correlate the results of the several methods used to serotype *P. multocida*. They proposed that this lack of correlation derived from the antigenic complexity of *P. multocida* and the nature of the antigens involved in each test. This antigenic complexity undoubtedly applies to rabbit isolates of *P. multocida* as well.

The distribution of capsular types among our 10 isolates as 60% type A, 30% type B, and 10% untypable (Table 1) is in general agreement with data presented in several larger surveys which indicate a predominance of type A organisms (13, 23, 24). Although only 1 of our 10 isolates was not typable as either A or D, nearly 30% of 42 rabbit isolates in another study (24) were untypable by these methods. The basis of capsular typing of *P. multocida* is an IHA which identifies four mutually exclusive capsular types designated A, B, D, and E, (or untypable) (7). Although we have retained the designation of "capsular" types in this report, the appropriateness of this designation is uncertain (25).

TABLE 3. ELISA titers of rabbit antisera to whole cells of *P. multocida*

Isolate no. (whole cell)	Capsular type:somatic type <sup>a</sup>	Rabbit antiserum titer <sup>b</sup> to isolate no.:			
		1		2	
		Whole cell	LPS	Whole cell	LPS
1	A:4(7,12,3)	52	6.5	0.4	0
2	D:3(12,4)	26	1.6	6.5	1.6
3	A:12(4,5,7)	26	3.2	0.8	0
5	A,D:(4,5,7,12)	26	1.6	0.4	0
6	D:(12,4,5,7)	26	1.6	1.6	0
7	A:untypable	26	1.6	0.4	0
8	Untypable:1	6.5	1.6	0.4	0
9	A:untypable	26	3.2	0.4	0
10	D:1	13	1.6	0.4	0
11	A:untypable	6.5	0.8	0.4	0

<sup>a</sup> Determined in my laboratory (see Table 1).

<sup>b</sup> Reciprocal of serum antibody titer (×10<sup>3</sup>).

Some investigators have demonstrated that the major antigen involved in the IHA of capsular typing is LPS (2, 9) and thus is presumably of somatic rather than capsular origin. Other workers have shown that capsular polysaccharides rather than endotoxins, presumably LPS, confer type specificity (27). Although it would appear that type specificity of *P. multocida* can derive from either capsular or somatic antigens, much of previously contradictory results stem from the use of crude extracts including formal-saline extracts and phenol-water extracts containing both polysaccharides and LPS. Although we made no attempt in this study to isolate and characterize capsular substances which may confer type specificity in our *P. multocida* isolates, we were able to obtain LPS demonstrably free of polysaccharide as determined by the sensitive carbocyanine test (14, 19) and thus eliminate or minimize the effects of capsular polysaccharides. If LPS were responsible for type specificity among isolates typed as A or D, a clear difference in both the ELISA and IHA would be expected. Thus, isolates 1, 3, 7, 9, and 11 (type A) would be expected to demonstrate antigenic homology as would type D isolates 2, 6, and 10. However, our ELISA and IHA data indicate that both whole cells and LPS of isolate 1 (type A) cross-reacted considerably with type D organisms, especially to antigens of isolates 2 and 6. This cross-reactivity was due to common somatic antigens as determined by the GDPT; these antigens are seemingly unrelated to those substances responsible for the A, B, D, and E classification system.

The 16 type-specific somatic antigens of *P. multocida* (16) have been demonstrated to be LPS (6). In our hands the GDPT often yields variable results with the same isolate as shown in Table 1. The Ames laboratory records the results of the GDPT after 24 h, whereas we record the results at 24, 48, and 72 h. Several precipitin lines not seen within 24 h occur within the next 48 h. This phenomenon is likely an expression of the heterogeneity of the antigens and antibodies involved. Although the major somatic type is usually the same from test to test, occasionally a precipitin reaction recorded in one test as minor (and designated within parentheses) is observed to be the major type in another test, e.g., isolates 1 and 6 (Table 1). Some results of the GDPT in which LPS rather than formal-saline extract was used as antigen were quite unexpected. GDPT of formal-saline extracts of

TABLE 4. ELISA titers of rabbit antisera to *P. multocida* LPS

Isolate no. (LPS)	Capsular type:somatic type <sup>a</sup>	Rabbit antiserum titer <sup>b</sup> to isolate no.:			
		1		2	
		Whole cell	LPS	Whole cell	LPS
1	A:4(7,12,3)	13	0.8	0.1	0.1
2	D:3(12,4)	26	0.1	0.8	0.8
3	A:12(4,5,7)	13	0.8	0.1	0.1
5	A,D:(4,5,7,12)	26	0.1	0.1	0.1
6	D:12(4,5,7)	13	0.2	0.1	0.1
7	A:untypable	13	0.1	0.2	0.1
8	— <sup>c</sup>				
9	A:untypable	13	0.1	0.4	0.1
10	— <sup>c</sup>				
11	A:untypable	13	0.1	0.1	0.1

<sup>a</sup> Determined in my laboratory (see Table 1).

<sup>b</sup> Reciprocal of serum antibody titer ( $\times 10^3$ ).

<sup>c</sup> Phenol-water extraction of isolates 8 and 10 yielded no material with absorbance at 460 to 470 nm (a characteristic of LPS-carbocyanine solution).

TABLE 5. Serum antibody titers against LPS from several isolates of *P. multocida* in rabbits injected with either killed whole cells or LPS of isolates 1 or 2 as determined by IHA

Sheep erythrocytes sensitized to LPS of isolate <sup>a</sup> :	Antibody titer <sup>a</sup> in rabbits immunized with:				
	Isolate 1 (whole cells)	Isolate 2 (whole cells)	Isolate 1 (LPS)	Isolate 2 (LPS)	<i>S. minnesota</i> (LPS)
1	640	80	0	0	20
2	640	640	0	0	20
3	640	160	0	0	80
5	160	80	0	0	80
6	40	0	0	0	20
7	320	80	0	0	80
9	40	0	0	0	20
11	160	80	0	0	20
<i>Salmonella</i> LPS	40	20	0	0	640

<sup>a</sup> See Tables 1 and 2 for characteristics of isolates.

<sup>b</sup> Expressed as the reciprocal of the highest serum dilution resulting in complete agglutination.

isolates 1 and 5 consistently resulted in a major precipitin reaction with reference antiserum number 4. Substitution of LPS for formal-saline extract resulted in strong precipitin reactions with antiserum number 12. Moreover, we were unable to type isolates 7 and 9 when formal-saline extracts were used; both isolates were type 12 when LPS was used. Notably, the use of LPS as antigen in the GDPT eliminated virtually all cross-reactivity with other typing antisera except for isolate 1. Although additional studies will be required to determine the reason(s) for these observations, we suspect that antigenic determinants on the LPS may be altered by the method of extraction, ionic strength of the solution (8.5% NaCl solution versus distilled water), the presence of non-LPS in the extract, and the degree of aggregation of constituents of the extracts. Other surveys of somatic antigens from rabbit isolates of *P. multocida* consistently reveal that type 12 is the most prevalent serotype (4, 13, 22). That rabbits harbor unique serotypes of *P. multocida* is suggested by the studies demonstrating that several isolates of *P. multocida* are not typable with the 16 reference sera used in the GDPT (4, 13, 26).

The concentrations of KDO among our rabbit isolates of *P. multocida* were considerably lower than that of reference strain *S. minnesota*, although the pyrogenic potency of LPS from most of the isolates as assessed by the lysate gelation assay was comparable to that of *S. minnesota*. We were unable to find reference to KDO concentrations in previously published reports on isolates of *P. multocida* from several animal species, except for a report in which the KDO content of the one isolate was estimated at 2% (29). Also, we did not detect the presence of dideoxyhexose (1) in any of our LPS preparations from *P. multocida* or *S. minnesota* 9700, whereas this compound as well as KDO were present in reference strain *E. coli* O55:B5 (results not shown).

On the basis of the data presented in Table 1, it could be predicted that only isolate 2 (and perhaps isolate 11) possesses a serologically specific LPS, whereas the other isolates share common somatic antigens and thus antisera to the latter would be very cross-reactive. The ELISA bears out this prediction in that antiserum to isolate 1 did indeed cross-react with all the isolates in moderate to high titer, and antiserum to isolate 2 reacted only with homologous whole

cell or LPS antigen. As expected, whole cells were considerably more antigenic than LPS. Also, antibody induced by injection of LPS is reactive in much higher titer to native LPS, i.e., LPS on whole cells, than to purified LPS, which may reflect altered antigenic determinants by phenol extraction (32). The spectrum of antigens of *P. multocida* as determined by GDPT is doubtlessly influenced by the complex nature of the compounds in the formal-saline extract. Our results with LPS in the GDPT confirm and extend the observations of others (17, 28) that LPS is a principle reactant in the precipitin reaction of the GDPT.

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