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Studies on *Pasteurella multocida*: Comparison of typing systems and description of certain ultrastructural features

by

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INTRODUCTION

Pasteurella multocida is the causative agent of disease in a variety of animal hosts. This broad spectrum of hosts makes it an important organism to consider. P. multocida is the etiological agent of fowl cholera in poultry, waterfowl and feral birds; hemorrhagic septicemia, shipping fever and mastitis in cattle and buffalo; snuffles in rabbits; and cat and dog-bite infections in humans. It also may be associated with diseases in other animals including sheep, swine, and wildlife. Pasteurellosis is of considerable economic importance due to its worldwide occurrence and severe losses resulting from high morbidity and mortality rates (Bhandari, 1972; USDA, 1965). For example, an estimated thirteen million turkeys were affected by fowl cholera in the United States in 1969 resulting in an estimated loss of near fourteen million dollars (Bhandari, 1972).

Since the first isolation of P. multocida, strains from various hosts and disease conditions have been compared in order to determine the extent of similarities and diversities. As a result, a great deal of variation has been found. The correlation of these characteristics with respect to hosts, specific disease conditions and epizootiology of Pasteurellosis has always been under study. Attempts have been made to group isolates on the basis of immunological and serological properties. A satisfactory serological classification, with a relationship to the immunological properties, also has been the object of considerable study.

A variety of methods are now available for serotyping P. multocida. These serotyping methods involve different antigens and preparations; whole cell antigens, soluble antigens, and extracted antigens. Because of

the variety of antigens employed in these classification systems, a comparative serological examination of the type strains is needed to determine the degrees of similarity or difference in these systems.

A fimbriated strain of P. multocida has recently been reported in the literature. This strain possessed characteristics and appendages not previously described. The presence of external appendages on P. multocida may be of serological and immunological importance as well as play a role in pathogenicity. Since the detailed physical structure of P. multocida is only vaguely described in the literature, an investigation is needed to examine the ultrastructure of P. multocida.

This thesis consists of 2 separate yet related parts: a serological comparison of Pasteurella multocida reference strains including an investigation into the cross reactivity of two type cultures in immunodiffusion tests and examination of Pasteurella multocida in the electron microscope. Specific literature reviews pertaining to each part will be presented in the respective introductions.

HISTORY

Diseases resembling Pasteurellosis were reported long before the isolation of the etiological agent. The early histories of fowl cholera and hemorrhagic septicemia were reviewed by Gray (1913) and Rosenbusch (1937) respectively. During the seventeenth century, large epornitics in poultry were reported throughout Europe. The etiology of these early epornitics such as fowl cholera is questionable since many investigators, some inadequately trained, could only rely upon pathologic and clinical diagnosis (Gray, 1913). This disease was studied in France by Chabert in 1782 and by Maillet in 1835 who termed it fowl cholera. The transmissibility of fowl cholera was later examined by Renault, Reynal, and Delafond in 1851 at the Alfort Veterinary School. Although Reynal was unable to transmit fowl cholera by feeding infected tissue to poultry and rabbits (Gray, 1913), Delafond demonstrated transmission by injection of infected blood, secretions, and tissue (Salmon, 1899). Later, in 1851, Benjamin considered fowl cholera to be a contagious disease (Salmon, 1899), and warned people about eating infected carcasses. Benjamin accurately described fowl cholera and demonstrated experimental infection by cohabitation (Gray, 1913).

Clinical symptoms identical to fowl cholera were reported in Washington, IA as early as 1867.

> Often the first evidence we have of the existence of the disease is in finding chickens lying dead in the morning where they had fallen from their roost. My turkeys and chickens have just got through with it. I watched the symptoms closely. The eyes look dull and heavy, and the small feathers on the top of the head ruffle up and appear to stand on end. The sick huddle closely together. When the fever comes on they pant considerably and

> want water. Their dung is quite soft, and about two-thirds of it as white as chalk, the remainder being a bright green. They usually die in from twenty-four to forty-eight hours; sometimes they lose the power of moving when they live longer (USDA, 1867).

Fifty to 80% mortality was sometimes recorded in the epornitics (USDA, 1869, 1870).

In 1869, Moritz investigated fowl cholera and reported on hematological differences in the blood of diseased birds. Later, in 1878, Perroncito of Italy and Semmer of Russia observed a rounded, bipolar staining microbe in the tissues of infected birds (Salmon, 1899; Gray, 1913). Toussaint (1879) confirmed these observations and was the first to isolate and describe P. multocida from the blood. Toussaint was able to cultivate this organism in neutralized urine and prove that it was the causative agent of the disease (Gray, 1913).

Pasteur (1880a,b) extensively studied the causative agent of fowl cholera. He isolated and cultivated this organism in pure culture in chicken broth. Pasteur examined the virulence and immunological properties of this isolate. He discovered that: 1) recovery from infection would protect against subsequent challenge, 2) attenuation of the organism would affect the virulence, and 3) immunity could be overwhelmed by a large challenge dose.

In the United States, Salmon (1899) investigated fowl cholera giving special attention to vaccination and the effect of disinfectants. A series of experiments determined the communicability of fowl cholera (Salmon, 1881). These included direct inoculations, contact exposures, effect of various disinfectants, immunological studies, and pathological

investigations. Other studies (Salmon, 1881, 1882) included work on virulence, cultivation, disinfectants, and the effect of toxin.

In 1885, Kitt examined the organisms producing fowl cholera, rabbit septicemia, swine septicemia, and cattle septicemia referring to them as *Bacterium bipolare multocidum* (Rosenbusch, 1937). Later in 1886, Huppe included the organism of fowl cholera in the hemorrhagic septicemia group (Gray, 1913). In honor of Pasteur, Trevisan in 1887 recommended the genus name *Pasteurella* for this class of organisms.

In 1900, Lingieres included the pasteurellas in his zoological classification. Under this system, each animal disease was regarded as a separate species. The species name was originated from the host it infected. Names such as *P. avicida* (fowl), *P. boviseptica* (cattle), *P. suiseptica* (swine), and *P. oviseptica* (ovine) have been used.

Wilson and Miles in 1929 (Buchanan et al., 1966) suggested that these pasteurellas be referred to as *P. septica*. Rosenbusch and Merchant (1939) proposed the name *P. multocida* for all typical strains. This is the present classification.

PART 1. A SEROLOGICAL COMPARISON OF *PASTEURELLA MULTOCIDA* TYPING CULTURES

INTRODUCTION

Investigators have tried to group P. multocida in a variety of ways. Due to the ability to infect a broad spectrum of hosts, grouping or classifying this organism is important epidemiologically. Grouping of similar types of P. multocida and correlating them with specific diseases has been a continuing area of study. The organism has been typed on the basis of biochemical reaction and fermentation patterns, serotypes, immunological types, phage susceptibility, and even on the basis of host specificity.

This research is a study of reference strains constituting the four serological classification systems of P. multocida. An extensive review of literature pertaining to the serological classification is included as are the sources and histories of some of the typing cultures involved.

Early work on the serological classification of P. multocida was reviewed by Cornelius (1929). Agglutination and complement-fixation tests were two of the first techniques employed. The varying agglutinability of some strains was encountered by Chamberland and Jouan in 1906. In 1910, Matsuda verified the zoological classification of P. multocida with the complement fixation test. He used 16 strains from various animal species. Using the agglutination test, Miessner and Schern could not find any relationship between ovine isolates and strains originating from other species. Jones (1921) examined P. multocida types encountered in a dairy herd. He was able to divide 16 isolates into three groups with a tube agglutination test. Roderick in 1922 examined cattle, swine, bird, sheep, guinea pig, and rabbit cultures by means of the complement-fixation test. He was able to divide these strains into two groups based on antigenic

differences. The first group consisted of cattle and swine isolates and the second group were those isolated from sheep, birds, rabbits, and guinea pigs. Fitch and Nelson (1923) were able to divide 28 strains of P. multocida into 4 groups depending on their agglutinability in rabbit antiserum. They could not find any relationship between these serological groupings and their biochemical reactions. Numi, in 1924, found cross-agglutination among the zoological types very complex. From a comparison of 26 Pasteurella cultures from various species, Tanaka (1926) concluded that the strains were identical in their cultural, biochemical, and serological properties. In 1927, Lal compared his strains by complement-fixation methods and found 3 groups: bovine and ovine strains, avian and porcine strains, and rabbit strains. Cornelius (1929) divided 17 of 26 strains into 4 groups by the means of an agglutinin-absorption test. Nine of these strains were not typable. No relationship between the serological groups and the animal of origin could be found. In 1934, Ochi examined 72 hemorrhagic septicemia strains isolated from various species of animals. He was able to divide these pasteurellas into 4 groups on the basis of serological, immunological, and pathogenic properties. The 4 groups were designated A, B, C, and D. Avian isolates were in group A; cattle, buffalo, and swine isolates were in group B; sheep, mice, and swine isolates were in group C; and some sheep isolates were in group D. Like Cornelius, Ochi concluded that there was no relationship between his types and the host of origin. A precipitin-absorption technique was used by Yusef (1935) to classify 21 strains of P. multocida. Fourteen of the 21 strains were found to fall into 3 serological groups. The results of this test correlated with those of Cornelius. Rosenbusch (1937) divided

the nonhemolytic pasteurellas into 4 groups based on agglutination tests. These groups were observed to correlate with the fermentation of xylose, arabinose, and dulcitol. Little and Lyon (1943) subdivided P. multocida into 3 types using slide agglutination and passive protection tests. Serological studies of 30 strains of nonhemolytic pasteurellas demonstrated that these organisms could be divided into 3 distinct types. The type specificity, virulence, and host of origin of these strains were found to be unrelated.

Up to this point, the lack of consistency and standardization on these various typing methods is evident. Most researchers have concluded, however, that there is more than one serological type based on results with agglutination and complement-fixation tests. It was also noted that these types generally did not have any relationship to virulence, fermentation patterns, and host of origin.

Carter has studied P. multocida by a variety of methods including precipitation, capsular swelling, and indirect hemagglutination. He has suggested that nonserological methods can replace serological procedures (1976) suggests that biotypes be established on the basis of host of origin, capsular type, colonial morphology, and fermentation patterns.
capsular type, colonial morphology, and fermentation patterns.

Carter (1952) reported three distinct serological types of P. multocida based on precipitation tests involving a soluble capsular polysaccharide. These were designated as A, B, and C from cattle, buffalo, and canine origin respectively. The type specificity of these groups was confirmed by a capsular swelling technique. Sixty-eight strains of P. multocida were examined by precipitation and capsular swelling tests

(Carter and Byrne, 1953). Fourteen cultures were type A, 23 were type B, and 4 were type C. No type was reported for 24 cultures. An additional serotype, D, was proposed. Three cultures were of this type.

Serotypes A, B, C, and D were confirmed with the indirect hemagglutination test (IHA) by Carter (1955). This technique involved the absorption of heat extracted antigen to human type O erythrocytes. Sensitized erythrocytes were then agglutinated in the presence of type specific antiserum. Carter thought that this antigen was associated with the capsule.

With the IHA test, 122 strains of P. multocida from various geographic locations and animal species were examined (Carter, 1955). Twenty strains were found to be type A, 3 strains were found to be type B, 6 strains were type C, and 20 strains were type D. Seventy-two strains were not typable. In another study (Carter, 1957b), 197 bovine and porcine strains from various parts of the world were examined. It was discovered that there was a predominance of types A and B from cattle and types A and D from swine. Fourteen strains could not be typed. Yet another study involving the serological types from species other than cattle and swine revealed 62 type A strains, 14 type B strains, and 23 type D strains (Carter, 1959). The type C category was subsequently dropped because of difficulties in recognition (Carter, 1959, 1963). An additional serotype, E, was reported later (Carter, 1961). This strain was recovered from an outbreak of hemorrhagic septicemia in African cattle.

Nonserological methods were developed to identify the type of mucoid and noncapsulated colonial variants that could not be typed by the indirect hemagglutination procedure. The capsule of type A and mucoid

strains were found to contain hyaluronic acid as well as the type specific capsular antigen (Carter and Annan, 1953). The capsular hyaluronic acid on these strains could be depolymerized by testicular and staphylococcal hyaluronidase (Carter 1958b; Carter and Annan, 1953). The presence of hyaluronic acid in the capsule was thought to interfere with the release of specific antigen located within or below it (Carter, 1972c). Since hyaluronic acid was present in encapsulated type A or mucoid strains, a nonserological procedure was developed to detect it (Carter and Rundell, 1975). This simple procedure, involving the incubation of mucoid strains with a hyaluronidase-producing strain of *Staphylococcus aureus*, could be used to replace the IHA test in identifying type A strains.

Carter (1957a) reported that the colonial and antigenic characteristics of type D strains enabled them to produce a distinctive reaction when suspended in acriflavine. A tube test was later developed and used to examine more than 100 cultures (Carter and Subronto, 1973). It was concluded that this simple nonserological procedure could also be substituted for the involved IHA test for the identification of type D strains.

Recently Carter (1976) has proposed a system of classifying *P. multocida* into biotypic groups based on colonial appearance, host of origin, hyaluronidase decapsulation, acriflavine flocculation, fermentation of several carbohydrates, mouse pathogenicity, and serum protection tests. Five different biotypes were recognized: the mucoid, the hemorrhagic septicemia, the porcine, the canine, and the feline biotypes. The mucoid biotype was frequently associated with poultry, cattle, sheep, and swine infections. The hemorrhagic septicemia biotype was associated with

epizootic hemorrhagic septicemia in cattle and water buffalo. The porcine biotype was reported to occur in swine and less frequently in cattle and other species. The canine and feline biotypes could be found in the mouth and throat of dogs and cats, and associated with cat and dog bite infections in humans. It was suggested that these biotypes could be recognized as subspecies within the species P. multocida.

Namioka and Murata (1961a) reported a slide agglutination test as a simplified method for capsular typing. Eleven cultures were examined with both the indirect hemagglutination and slide agglutination tests. It was concluded that the slide agglutination tests could be used to replace the IHA test. Further examination of 91 cultures typed by this method revealed 27 strains to be type A, 4 to be type B, and 20 strains to be type D. Forty strains could not be typed.

To explain differences in virulence and host specificity that occurred within the capsular types described by Namioka (1970), the somatic antigen (O antigen) of P. multocida was investigated (Namioka and Murata, 1961b). A number of antigenic preparations were evaluated. Organisms treated with 1 N HCl were found to possess both common and specific somatic antigens. Twenty-four strains were divided into six groups based on absorption tests (Namioka and Murata, 1961c) from the capsular and somatic antigen results.

In another study, the capsular and somatic antigens from 156 cultures were examined with absorbed O group factor sera (Namioka and Bruner, 1963). Of these cultures, 118 strains could be typed and 29 could not. Nine strains were not classified due to the presence of undescribed

antigens. The cultures fell into ten somatic groups. Matched with the capsular typing results 12 serotypes could be demonstrated. Five somatic antigen groups were found in type A (1:A, 3:A, 5:A, 7:A, and 9:A), 5 somatic antigen groups in type D (1:D, 2:D, 3:D, 4:D, and 10:D), and one group in type B (6:B). The capsular type of somatic group 8 was not reported. A correlation between epizootiology and these serotypes was made (Namioka and Murata, 1964). Three somatic groups in type A were shown to be the causative agent of fowl cholera (5:A, 8a:A, and 9:A). Of the two somatic groups 6 and 11 in capsule type B, only 6:B could produce hemorrhagic septicemia in cattle. Cultures belonging to 1:A, 3:A, 1:D, 2:D, and 4:D were associated with pneumonia in swine and sheep (Namioka and Bruner, 1963). Serotype 7:A was isolated from a number of cattle in the Philippines, Burma, and Viet Nam.

Studies on pathogenicity demonstrated that Carter's classification, based on capsular antigens, was inadequate in etiological studies of P. multocida (Murata et al., 1964).

With the combination of capsular and somatic antigen groups, 15 serotypes have been reported (Namioka, 1973). There are 6 O-groups in Carter's type A, 2 O-groups in type B, 6 O-groups in type D, and 1 O-group in type E.

Thirty-seven strains of P. multocida were studied by Roberts (1947) to determine the existence of immunological types. In cross-protection experiments, these strains were placed into at least 4 groups according to immunological differences in mice. Bovine and porcine isolates were in type I; avian, bovine, and porcine isolates were in type II; a bovine and

a deer isolate were in type III, and a bovine and a turkey isolate were in type IV. Three isolates were shown to be different from each other as well as from the previous 4 types. Although the majority of strains in this study were in types I and II, Roberts thought that seven or eight types were probable in this study.

MacLennan and Rondle (1957) extracted type specific lipopolysaccharide from Roberts' immunotypes I, II, III, IV, and V. This antigen and another type specific thermostable antigen was examined. On the basis of the heat stable antigens, types I and III were shown to be different from each other as well as from types II, IV, and V. Differences in II, IV, and V were not investigated.

Perreau and Petit (1963) examined the Westphal extracted lipopolysaccharide of Carter's type E. It was found to be identical to the type B lipopolysaccharide in composition, toxicity, and serological characteristics. Baxi et al. (1970) reported differences in the extracted lipopolysaccharide and heat-stable antigen in immunoelectrophoresis and the gel diffusion precipitin test. They reported the lipopolysaccharide from Carter's type B to be identical to that of type E.

Prince and Smith (1966a) investigated the antigenic structure of P. multocida with immunodiffusion techniques. Using immunoelectrophoresis, 18 soluble antigens were identified and labelled. Two antigens, designated alpha and beta, were shown to be composed of protein and lipopolysaccharide respectively. The beta antigen was found to be stable after heating at 100°C for 15 minutes. In another study by Prince and Smith (1966b), 17 avian and bovine strains were examined. Using immunoelectro-

phoresis, 16 of the 18 soluble antigens were common to all of the strains. The alpha and beta antigens, present in phenol extracts, were found to be type-specific. However, the alpha antigens would sometimes cross-react. The beta antigen was shown to be heat-stable as well as type specific. Unknown strains could be typed in the gel diffusion precipitin test by this antigen alone.

Heddleston et al. (1972a) reported on a gel diffusion precipitin test for serotyping avian strains of P. multocida. Two hundred and fifty-eight field isolates were divided into 5 serotypes based on differences in the heat-stable antigens. Thirty-three avian isolates were found to be type 1, 157 were type 3, 28 were type 4, 4 were type 5, and 4 were type 6. Twenty-seven of the remaining isolates were type 3 cross reacting with type 4. Five avian strains could not be typed. Further studies revealed additional types that could be of epidemiological importance in examining epornitics of fowl cholera among avian species (Heddleston et al., 1972b). In this study, 73 isolates of P. multocida were examined from various avian species. The results had shown that 3 serotypes (1, 3, and 7) were associated with epornitics in waterfowl, 4 serotypes (1, 3, 6, and 8) with feral birds, 3 serotypes (1, 3, and 4) with chickens, one serotype (3) with turkeys, and one serotype (7) with domestic ducks. The diversity of P. multocida was shown in a study of 762 isolates from 20 different species of animals from 23 states (Blackburn et al., 1975). Serotype 3 was shown to be the most common. Since the establishment of this typing system, numerous investigations dealing with the distribution of serotypes

by states and animal species have been reported (Baroutchieva and Feinhaken, 1974; Derieux, 1974; Langpap and Matischeck, 1970; and Donahue and Olson, 1971).

Pasteurella multocida possesses a variety of antigens that have been used in serotyping the organism. The purpose of this investigation is to examine the reference strains constituting the four serological classification systems of *P. multocida* and to determine the correlation between them.

MATERIALS AND METHODS

Cultures

The 39 cultures of P. multocida used in this study are listed in Table 1. These are the typing cultures maintained in the stock culture collection at the National Animal Disease Center (NADC). A representative strain of each type is included. Records maintained with this collection indicate that each strain represented here was among the first subcultures of the original isolate. These strains have been stored at 4°C in a lyophilized state since their arrival at NADC.

Little and Lyon (1943) reported American Type Culture Collection (ATCC) strains 7707, 6530, and 6535 to be representatives of their types 1, 2, and 3 respectively. P. avicida strain USDA 2050 (ATCC 7707) was isolated in February of 1940 by Dr. Walter J. Hall, Beltsville, MD. This isolate is from an acute outbreak of fowl cholera in turkeys.[1] Actions on diagnostic media, complement-fixation tests and agglutination tests were reported to be typical for Pasteurella. Transplants of this isolate were then sent to the American Type Culture Collection by Dr. Hall. P. bubaliseptica strain USDA H-1054 (ATCC 6530) is a subculture of the buffalo B strain. This strain was originally isolated by Dr. L. T. Giltner, Pathological Division, Bureau of Animal Industry, U.S. Department of Agriculture (Stein et al., 1949). It was isolated from an acute case of

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[1]Personal communication from Dr. Stein, Bacterial and Mycotic Diseases Section, ADP-RB, Washington, DC to Dr. Walter Hall, Beltsville, MD, August 5, 1955.

Table 1. Pasteurella multocida typing cultures

Type	NADC culture number of type strains	Host	Colonial appearance[a]	Original and reference designations
Heddleston:				
1	X-73	Chicken	Iridescent	ATCC 11039
2	M-1404	Bison	Iridescent	Buffalo "B"
3	P-1059	Turkey	Iridescent	ATCC 15742
4	P-1662	Turkey	Iridescent	1224-6
5	P-1702	Turkey	Iridescent	1284
6	P-2192	Chicken	Iridescent	5-7-72
7	P-1997	Herring Gull	Bluish-Iridescent	S-2866
8	P-1581	Pine Siskin	Bluish-Iridescent	146
9	P-2095	Turkey	Iridescent	BSP 1001
10	P-2100	Turkey	Iridescent	S0743
11	P-903	Swine	Iridescent	4950
12	P-1573	Human	Watery-Mucoid	831
13	P-1591	Human	Blue	848
14	P-2225	Cattle	Iridescent	1440
15	P-2237	Turkey	Blue	230L
16	P-2723	Turkey	Iridescent	74-144-9
Carter:				
A	P-1201	Fowl	Watery-Mucoid	ATCC 12945
B	P-932	Buffalo	Bluish-Iridescent	ATCC 12946
D	P-934	Swine	Bluish-Iridescent	ATCC 12948
E	P-1234	Bovine	Iridescent	
Namioka:				
1A	P-1249	Swine	Iridescent-Mucoid	KOBE 5
2D	P-1175	Chicken	Iridescent	27
3A	P-1392	Swine	Iridescent-Mucoid	P-8
4D	P-1254	Sheep	Iridescent	M-17
5A	P-1182	Swine	Iridescent-Mucoid	TS-8
6B	P-1248	Bovine	Iridescent	R488
7A	P-1255	Bovine	Iridescent	PBII
8A	P-1197		Iridescent	23639
9A	P-1380	Turkey	Iridescent	Liver

[a]Obliquely transmitted light.

Table 1. (Continued)

Type	NADC culture number of type strains	Host	Colonial appearance	Original and reference designations
Roberts:				
V	P-2251		Iridescent	C.N. 4020
I	P-2252		Iridescent	C.N. 5124
IV	P-2253		Iridescent	C.N. 5127
V	P-2254	Porcine	Iridescent-Mucoid	C.N. 4840
II	P-2255		Iridescent-Mucoid	C.N. 5125
V	P-2256		Iridescent-Mucoid	C.N. 4988
III	P-2263		Iridescent-Mucoid	C.N. 5126
Little and Lyon:				
1	P-3262	Turkey	Blue	ATCC 7707
2	P-3263	Buffalo	Blue	ATCC 6530
3	P-3264	Mouse	Blue	ATCC 6535

hemorrhagic septicemia occurring in March of 1922 in an epizootic in buffalo in Yellowstone National Park. Gochenour designated it as the hemorrhagic septicemia buffalo B strain (Stein et al., 1949). From 1922 to 1932 the strain was maintained in the USDA culture collection by serial passage in nutrient broth every two weeks and its virulence was maintained by frequent passage through large animals, principally horses and mules (Heddleston et al., 1967; Stein et al., 1949). _P. bubaliseptica_ strain USDA H-1054 was isolated on February 18, 1938 from the blood of horse 1054 inoculated intramuscularly with 15 ml of an 18 hour broth culture of the buffalo B strain.[1] Transplants of this culture were then sent to the

[1]Personal communication from Dr. Stein, Bacterial and Mycotic Diseases Section, ADP-RB, Washington, DC to Dr. Walter Hall, Beltsville, MD, August 5, 1955.

American Type Culture Collection. P. muriseptica strain USDA 60-L (ATCC 6535) was isolated by Dr. Charles Grey on March 19, 1938 from a natural infection in a mouse. Transplants were also sent to the American Type Culture Collection.[1]

Cultures representing the capsular types of P. multocida, as reported by G. R. Carter, are in the American Type Culture Collection. Strain P-931 (ATCC 12945) is capsular type A of avian origin, strain P-932 (ATCC 12946) is capsular type B from buffalo, and P-934 (ATCC 12948) is type D of swine origin. A fifth type, E, was reported later (Carter, 1961). This culture, designated P-1234, was received at NADC from Dr. Perreau on February 16, 1963. It was isolated in central Africa (Cameroons) during the rainy season (August-September) of 1962, from Zebu cattle.[2] Culture P-1234 was lyophilized after only two subcultures on tryptose-yeast extract medium with 5 percent horse serum.

The reference strains of P. multocida used to determine the somatic antigen groups are listed in Table 1. Lyophilized subcultures from these strains have been stored at 4°C since their arrival in 1962 and 1963. Nine of the 12 O-groups reported by Namioka (1973) are represented. Cultures representing somatic groups 10, 11, and 12 were not available. When

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[1]Personal communication from Dr. Stein, Bacterial and Mycotic Diseases Section, ADP-RB, Washington, DC to Dr. Walter Hall, Beltsville, MD, August 5, 1955.

[2]Personal communication from Dr. Perreau, Institut D'elevage et de Medecine, Veterinaire des Pays Tropicaux, 10, Rue P. Curio, Alfont, Seine, France to K. L. Heddleston, National Animal Disease Center, Ames, IA, February 6, 1963.

matched with the capsular types, 12 of Namioka's 15 serotypes can be represented.

Seven cultures of Roberts' (1947) five immunotypes were included in the serological study. Records maintained at the NADC indicate that these strains were received in June of 1972 from the Wellcome Research Laboratories, Beckenham, Kent, England.[1] Subcultures were lyophilized and stored at 4°C. Strains CN 5124 to 5127 (P-2252, P-2255, P-2263, and P-2253) are Roberts' types I, II, III, and IV. These strains were sent to the Wellcome Research Laboratories by R. S. Roberts, F.R.C.V.S., Evans Biological Institute, Runcorn, Cheshire.[1,2] No information on these types was available from the Wellcome Research Laboratories.[2] The accession date for these cultures was June 3, 1962. The history on the remaining 3 type V strains was available.[2] Strain CN 4020 (P-2251) was isolated from the lung of a pig at the Ministry of Agriculture and Fisheries Veterinary Laboratory at Weybridge, Surrey. The accession date was May 16, 1956. Strain CN 4840 (P-2254) was isolated by a veterinarian from a pig on a farm near Canterbury, England. This culture was sent directly to the Wellcome Research Laboratories. The accession date on this culture was August 8, 1960. Strain CN 4988 (P-2256) was from M.A.A.F. Laboratory in Weybridge. The accession date was December 12, 1960.

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[1]Personal communication from Dr. C. W. Penn, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England to K. L. Heddleston, National Animal Disease Center, Ames, IA, May 18, 1972.

[2]Personal communication with Dr. J. F. Spilsbury, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England, April 28, 1977.

Based on the results of gel diffusion precipitin tests, 16 serotypes of P. multocida have been demonstrated (Heddleston et al., 1972a). Representatives of these sixteen types are listed in Table 1. Strain X-73 (type 1) was isolated from a chicken with peracute fowl cholera in a larger breeder flock (Heddleston and Hall, 1958). Strain M-1404 (type 2) is a subculture of the strain isolated in 1922 from an acute case of hemorrhagic septicemia in a buffalo in Yellowstone National Park (Stein et al., 1949). This isolate was maintained by serial passage in nutrient broth every two weeks (Heddleston et al., 1967). The virulence of this culture was maintained by frequent passage through horses and mules. M is the army designation for mule. Strain M-1404 represents the last passage of the buffalo B strain through an army mule serial number 1404.[1] Since 1939, the culture has been stored in a lyophilized state at 5°C. Strain P-1059 (type 3) was isolated in West Virginia by K. L. Heddleston in October of 1959 from a 16-week-old turkey that had died of acute fowl cholera (Heddleston, 1966). This turkey was from a flock of 8,000 birds vaccinated with strain X-73. Strain P-1662 (type 4) and P-1702 (type 5) were received from Dr. B. W. Bierer, Clemson University in 1967 (Heddleston et al., 1970) and from C. B. Reubush, Jr., Virginia Department of Agriculture in 1969 (Heddleston et al., 1972a) respectively. Serotype 6 (P-2192) was isolated from an outbreak of fowl cholera in 23-week-old turkeys. This culture was received May of 1972 from Dr. T. O. McMillan, Plantation Foods, Inc., Waco, TX.

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[1] Personal communication from Dr. Stein, Bacterial and Mycotic Diseases Section, ADP-RB, Washington, DC to Dr. Walter Hall, Beltsville, MD, August 29, 1955.

Serotype 7 (P-1997) was from the spleen of a one-year-old herring gull (*Larus argentatus*).[1] This bird, heavily parasitized by large unidentified nematodes in the thoracic cavity, was found dead on a duck farm which was having a fowl cholera problem. *P. multocida* was recovered from the heart, liver, and spleen of this bird. Strain P-1581 (type 8) was isolated from an adult, female pine siskin.[2] This infection was septicemic and diagnosed as the primary cause of death. Strains P-2095 (type 9) and P-2100 (type 10) are turkey isolates received at the NADC in September of 1971 from Dr. J. Scherbe, St. Paul, MN and Dr. L. R. Barnes, Indianapolis, IN respectively. Human isolates P-1573 (type 12) and P-1591 (type 13) were received April 6, 1967 from Dr. D. P. Nicholson, University of Iowa.[3] Strain P-1573 was isolated from the throat of one patient and P-1591 from the peritoneum of another. Serotype 14 (P-2225), a bovine isolate, was submitted in July of 1972 by Dr. J. Vogel, National Animal Disease Center, Ames, IA. P-2237 (type 15) and P-2723 (type 16) were from turkeys. P-2237 was originally isolated by Dr. Filion at the School of Veterinary Medicine, St. Hyacinthe, Quebec. It was sent to Salsbury Laboratories, Inc. in Canada, then forwarded to the laboratory in Charles City, IA. Dr.

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[1]Personal communication from Dr. L. Leibovitz, Duck Research Laboratory, Cornell University, Ithica, NY to K. L. Heddleston, National Animal Disease Center, Ames, IA, May 10, 1971.

[2]Personal communication from Dr. G. P. Faddoul, University of Massachusetts, Waltham Field Station, Waltham, MA to K. L. Heddleston, National Animal Disease Center, Ames, IA, April 17, 1967.

[3]Personal communication from Dr. D. P. Nicholson, University of Iowa, College of Medicine, Iowa City, IA to K. L. Heddleston, National Animal Disease Center, Ames, IA, March 31, 1967.

J. W. Zerfas submitted this culture to the NADC in November of 1972 where it was examined and designated serotype 15. P-2723 was isolated from 12 to 13-week-old turkeys. This isolate produced a 2 to 4% morbidity rate and a 0.5 to 0.75% mortality rate per day. It was sent for serological examination in June of 1974 by Dr. D. M. Wenger, Central Soya Company, Inc., Decatur, IN.

Antisera

Avian antisera were prepared in 12 to 16-week-old New Hampshire roosters. Each strain of P. multocida was grown 18 to 24 hours at 37°C on Dextrose Starch Agar[1] (DSA), harvested 0.85% saline containing 0.3% formalin, held overnight, and adjusted to 10 times the McFarland number 1 density. A McFarland number 1 density is equivalent to 80% transmittance at 600 nm in the spectrophotometer.[2] This suspension was emulsified with equal amounts of Bayol F[3] containing 3.0% Arlacel A.[4] One milliliter of this emulsified bacterin was injected subcutaneously in the neck region. Three weeks later, 1 ml of bacterin was injected intramuscularly in the breast; 0.5 ml on each side of the sternum. The birds were exsanguinated one week later. Antisera were preserved with 0.01% thimersol and 0.06% phenol.

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[1]Difco Laboratories, Inc., Detroit, MI.

[2]Coleman Jr. spectrophotometer (Model 6-A), Coleman Instruments, Inc., Maywood, IL.

[3]A light mineral oil, Esso Standard Oil Co., Linden, NJ.

[4]Mannide monooleate, Atlas Powder Co., Wilmington, DE.

Rabbit antisera were prepared in 5-month-old female New Zealand rabbits. The bacterin for the strains used was prepared as described above. Four milliliters of emulsified bacterin was injected intradermally in 4 sites (1 ml per site) on the back. Inoculations were given at monthly intervals for 3 months. One week following the last injection, 1.0 ml of aqueous suspension (10 times the McFarland number 1 density) was given intravenously. The rabbits were bled 1 week later by cardiac puncture. The antisera were preserved as before.

Gel Diffusion Precipitin Test (GDPT)

The gel diffusion precipitin test was conducted as described by Heddleston et al.(1972a). Eighteen to 24 hour growth from a heavily inoculated DSA plate was suspended in 1.0 ml of 0.02 M phosphate, 8.5% NaCl, and 0.3% formalin, pH 7.0. The cells were heated in a boiling water bath for one hour and centrifuged for 30 minutes at 4,000 rpm in a table top centrifuge.[1] The supernatant was used as the GDPT antigen.

The agar gel consisted of 0.9% Noble agar,[2] and 8.5% NaCl in distilled water. Five milliliters of molten agar were pipetted onto standard 25 x 75 mm microscope slides. Three patterns consisting of 7 wells (6 peripherally located around a center well) were cut on a single slide. The wells, 3.5 mm in diameter, are separated approximately 5.5 mm from center to center. Antisera, prepared to Heddleston's typing strains, were

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[1]Precision Vari-Hi-Speed Centricone, Precision Scientific Co., Chicago, IL.

[2]Difco Laboratories, Inc., Detroit, MI.

placed in the outer wells and antigens in the center wells. The slides were placed in covered petri dishes, incubated overnight at 37°C, and examined under a magnifying glass with background illumination (Glazier, 1966).

Rapid Agglutination Test

A slide agglutination test was performed as reported by Little and Lyon (1943). Whole cell antigens were prepared by suspending the overnight growth from bovine blood agar or DSA in 1.0 ml of sterile physiological saline.

Reactions were carried out on agglutination slides[1] 52 x 76 mm in size with twelve 16 mm diameter concavities. Inoculating loops 5 mm and 1 mm in diameter were used to measure and transfer the antigen and antiserum respectively. Agglutination tests were also conducted by using Pasteur pipettes to deliver one drop of antigen and one drop of antiserum on a glass plate divided into 40 x 50 mm squares in a Brucella plate agglutination testing box. Antigens and antisera were mixed well and examined. Reactions occurred within 1 to 2 minutes.

Due to the nature of the capsule, some cultures were not typable by this procedure. The antisera of those cultures were then examined with antigens prepared from Little and Lyon's type cultures.

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[1]Clay Adams, Parsippany, NJ.

Determination of Somatic-Capsular Serotypes

Examination of the somatic and capsular types was performed as described by Namioka and Murata (1961a). Capsular type was determined by agglutination using whole cell saline suspensions and antisera prepared to Carter's types A, B, D, and E. The agglutinating antigen consisted of a sufficient quantity of organisms grown 18 to 24 hours on Namioka's yeast proteose cystine agar (YPC; Namioka and Murata, 1961a) harvested in 0.85% sterile saline to make a heavy suspension. Agglutination tests were performed by placing one drop of antigen and one drop of antiserum, diluted 1:2, on a glass plate on a Brucella plate agglutination testing box. Reactions were recorded after 3 minutes. Due to the nature of the capsule, some cultures did not agglutinate. Those cultures, treated with hyaluronidase, were then typed.

Bacterial suspensions used for the determination of somatic antigens were prepared by suspending the growth from a heavily seeded YPC plate in 10 ml of 1 N HCl. After overnight incubation at 37°C, the suspensions were centrifuged and the cells washed twice in 10 ml of 0.3% formalinized buffered saline. These were resuspended in 20 ml of phosphate buffered saline and adjusted to pH 7.0 with 10% sodium bicarbonate. The density of the suspensions were adjusted to match a McFarland number 1 density in the spectrophotometer. Auto-agglutinating cultures were discarded as being unsuitable for use in agglutination tests.

Antisera prepared to Namioka's 10 somatic types were absorbed as reported (Namioka and Murata, 1961b). Three milliliters of a 1:10 dilution of antiserum were absorbed with HCl treated, packed cells from 2 YPC agar plates. After 2 hours incubation at 37°C, the mixture was centrifuged and

the supernatant antiserum used in the tube agglutination tests. In some instances the exact culture was not available for absorption of the antiserum. A similar culture of the appropriate somatic-capsular serotype was used.

The tube agglutination test was performed in 11 x 100 mm test tubes. Nine-tenths milliliter of PBS, pH 7.0 was placed in the first tube and 0.5 ml in the rest. One-tenth milliliter of antiserum was added and diluted 2-fold to 1:640. Five-tenths milliliter of bacterial suspension was added to each tube which was then shaken and incubated at 37°C overnight. Normal serum and saline controls were included with each group of tests.

Determination of Capsular Serotypes

The indirect hemagglutination test (IHA), acriflavine flocculation test, and hyaluronidase decapsulation test were performed as described by Carter (1955, 1957a), Carter and Subronto (1973), and Carter and Rundell (1975) to determine the capsular serotype.

Human type O erythrocytes[1] were used in the hemagglutination tests. Cells were washed 3 times with 6 volumes of PBS, pH 7.2, before use.

Chicken antisera prepared against Carter's standard typing strains were inactivated in a 56°C waterbath for 30 minutes and absorbed with human erythrocytes before use. One and a half milliliters of antisera were absorbed with 0.2 ml of washed, packed erythrocytes for 2 hours at room temperature, centrifuged, and adjusted to the appropriate concentration.

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[1]Courtesy of the Blood Bank, Mary Greeley Hospital, Ames, IA.

Two procedures were reported for the IHA test: the original (Carter, 1955) and a modified procedure for the detection of type A strains (Carter 1972c).

Original indirect hemagglutination test

Growth from 1 to 2 bovine blood agar plate cultures was scraped from the surface and placed in 3 ml of 0.85% saline. This suspension was heated 30 minutes in a 56°C waterbath to assist in the removal of the antigen to be used for typing. The suspension was then centrifuged at 7,000 rpm for 20 minutes in a Servall centrifuge (SS-34 rotor). The supernatant was used as the capsular extract. To each extract, 0.2 ml of washed, packed erythrocytes was added, mixed, and incubated 2 hours in a 37°C waterbath. The erythrocytes thus coated with capsular extract were then washed 3 times in 10 ml of PBS, pH 7.2. The cells were adjusted to a 1.0% suspension.

This test was performed in 12 x 75 mm test tubes. Sixty-four hundredths milliliter of saline was placed in the first tube and 0.4 ml in the rest. Sixteen hundredths milliliter of antiserum was added to the first tube and diluted 2-fold to 1:80. Four tenths milliliter of the 1.0% suspension of coated erythrocytes was added, shaken, and the tubes incubated 2 hours at room temperature. The test was read after the 2 hours incubation and again after 18 to 24 hours at 4°C. The formation of a shield of erythrocytes on the tube bottom and/or agglutination of the erythrocytes when the tubes were shaken constituted a positive reaction.

Modified indirect hemagglutination test for the detection of type A strains

A later modified procedure (Carter, 1972c) involved the incubation of the culture to be typed with bovine testicular hyaluronidase. Growth from 1 to 2 bovine blood agar plates was scraped off and suspended in 3 ml of 0.1 M phosphate buffered saline (PBS), pH 6.0. One milliliter of 0.1 M PBS, pH 6.0, containing 50 viscosity-reducing units of testicular hyaluronidase,[1] was added and heated 3 hours in a 37°C waterbath to enhance the activity of the enzyme. It was then heated for 30 minutes in a 56°C waterbath to assist in the removal of the capsular antigen. Each culture was then centrifuged at 7,000 rpm for 20 minutes in a Servall centrifuge (SS-34 rotor). The supernatant was used as the capsular extract. To each bacterial extract, 0.1 ml of washed, packed erythrocytes was added, mixed, and incubated 2 hours in a 37°C waterbath. The erythrocytes were washed as before and adjusted to a 0.5% suspension. This test was also performed in 12 x 75 mm test tubes. Four-tenths milliliter of 0.85% saline with 0.3% formalin was placed in the first tube and 0.25 ml in the rest. One-tenth milliliter of antiserum was added to the first tube and diluted 2-fold to 1:80. Twenty-five hundredths milliliter of the 0.5% coated erythrocytes were added to each tube and the test performed and read as previously described.

A nonserological test was substituted for the involved IHA test in the identification of type D strains of P. multocida (Carter and Subronto,

[1]Wydase, Wyeth Laboratories, Inc., Philadelphia, PA.

Table 2. Brief descriptions of the four typing procedures compared

Investigator	Preparation of antigen	Test	Nature of the test
Little and Lyon	Organisms were suspended in physiological saline.	Plate Agglutination	Identification of surface antigens
Carter	Organisms were suspended in saline and heated at 56 C for 30 minutes. The supernatant was attached to erythrocytes.	Indirect Hemagglutination Test	Identification of capsular antigens
	Broth cultures centrifuged and resuspended in Acriflavine Neutral Dye.	Acriflavine Flocculation	Identification of rough colonial variants
	Mucoid strains streaked perpendicular to a hyaluronidase producing strain of _S. aureus_.	Hyaluronidase Decapsulation	Detection of hyaluronic acid
Namioka	Organisms suspended in saline	Plate Agglutination	Identification of surface antigens to determine capsular types
	Organisms were incubated overnight at 37 C in 1 N HCl and washed with buffered formalinized saline.	Tube Agglutination	Determination of somatic antigens
Heddleston	Growth was suspended in high salt buffered saline and heated for 1 hour at 100 C. The supernatant is used.	Gel Diffusion Precipitin Test	Identification of the heat stable antigen

1973). This test involved the flocculation or precipitation of the type D organisms when suspended in acriflavine. A single colony of the Pasteurella was transferred from bovine blood agar to 3 ml of Brain Heart Infusion broth.[1] After overnight incubation at 37°C, each culture was centrifuged and 2.5 ml of the supernatant removed. Five-tenths milliliter of a 1:1000 aqueous solution of acriflavine neutral dye[2] was added to the remaining 0.5 ml, mixed, and observed. Type D strains produced a flocculent precipitate in 3 to 5 minutes that was distinctly different from the other capsular types. At 30 minutes a clear supernatant was evident.

Another nonserological test replaced the original IHA and the modified IHA procedures for the identification of type A strains (Carter and Rundell, 1975). This test involved the depolymerization of the capsular hyaluronic acid. Decapsulation of type A strains with staphylococcal-produced hyaluronidase was performed on DSA. P. multocida cultures were streaked once across the agar surface. A hyaluronidase-producing strain of Staphylococcus aureus[3] was streaked at a right angle to the P. multocida culture. After 24 hours at 37°C, the type A mucoid strains showed a diminution in colonial size and a change in appearance in obliquely transmitted light in the region adjacent to the staphylococcal streak. Colonies of other capsular types were unaffected.

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[1]Difco Laboratories, Inc., Detroit, MI.

[2]Matheson, Coleman, and Bell, Norwood, OH.

[3]Strains A-801 and A-795, courtesy of Dr. John McDonald, National Animal Disease Center, Ames, IA.

Immunoelectrophoresis (IEP)

Immunoelectrophoresis was used to evaluate the cross reactivity of Heddleston's serotypes 2 and 5 to determine if they represent the same serotype or are just closely related serotypes. Representatives of the serotypes examined in this study are listed in Table 10, Appendix A.

Two different antigenic preparations of these types were examined with both avian and rabbit antisera. To obtain more antigen, 3 Roux bottles of M-1404 and 3 Roux bottles of P-1702 were used. After heating, the cells were centrifuged for 20 minutes at 7,000 rpm (Servall centrifuge, SS-34 rotor). The heat-stable antigen was pelleted from the supernatant at 40,000 rpm for 2 hours (Spinco centrifuge, 40.2 rotor). The small gelatinous pellet was resuspended in the barbital buffer used in IEP and stored at 4°C.

The Westphal extracted lipopolysaccharides were prepared as described by Rebers and Heddleston (1974). The 18 to 24 hour growth from 3 Roux bottles of strains M-1404 and P-1702 was removed with 60 ml of 0.85% saline containing 0.3% formalin. After stirring continuously for 2 days, the cell suspensions were centrifuged for 1 hour at 10,000 rpm. The cells, about 2 grams wet weight, were washed 3 times in 50 ml of 0.02 M phosphate buffered saline and recentrifuged for 1 hour at 10,000 rpm. The packed cells were resuspended in 10 ml of distilled water and heated to 65-68°C in a waterbath. Ten milliliters of 65°C glass-distilled phenol were added and incubated for 10-15 minutes in the waterbath. The solution was then centrifuged at 2,000 rpm for 30 minutes. The aqueous phase was

removed and dialyzed against distilled water for 3 days to remove the phenol.

Both rabbit and avian antisera were used. An effective method for use of avian antisera in immunoelectrophoresis was examined. Immunoelectrophoresis must be conducted in a medium containing low or physiological salt concentrations. However, maximal precipitation of avian antiserum occurs in the presence of 8 to 12% NaCl (Goodman et al., 1951).

Immunoelectrophoresis was conducted in an agar medium containing 1% Noble agar (Difco) and 0.1 M barbital buffer, pH 8.2. Five milliliters of molten agar was pipetted onto microscope slides previously coated with 0.1% Noble agar. Fifty milliamperes of current at 14-16 volts was applied for 2 hours.

The following technique was used to produce a high salt gel following the electrophoretic treatment of antigens in the low salt gel. Following electrophoresis, gel slides containing the antigens to be examined against avian antiserums were immersed in various saline concentrations. Slides were immersed in 8.5%, 12.75%, and 17.0% NaCl solutions for 10 and 15 minute periods immediately after electrophoresis and before the addition of antiserum. Slides were also immersed under these conditions 18 to 24 hours after the addition of antiserum to the slide. In each case, a control slide was immersed in low salt barbital buffer.

Slides with avian antisera were placed in covered petri dishes, incubated at 37°C 18 to 24 hours, and held for 2 to 3 days at room temperature before discarding. Low salt slides with rabbit antisera were

incubated 3 to 4 days at room temperature in covered petri dishes. All slides were examined under a magnifying glass with background illumination (Glazier, 1966).

RESULTS

The comparison of the serological types of *P. multocida* is listed in Table 3. These serotypes were derived from the results of the individual typing systems listed in Tables 11-17, Appendix B.

Numerous cross reactions were evident in typing systems involving agglutination tests. Interpretation of the results from cultures typed with the plate agglutination test and tube agglutination test was often difficult. In Little and Lyon's plate agglutination test, encapsulated strains failed to agglutinate in either chicken or rabbit antiserum. In this instance, antiserum prepared to the encapsulated strains were examined with whole cell antigens prepared from Little and Lyon's noncapsulated strains. Strain P-3264 (Little and Lyon's type 3), grown on bovine blood agar, would spontaneously autoagglutinate when suspended in 0.85% saline. Agglutination reactions are reported with this strain grown on Dextrose Starch Agar.

Numerous cross reactions were observed in the plate agglutination test reported by Namioka to replace the indirect hemagglutination test for determining capsular type. The agglutination test, performed as reported, was not successful due to the insufficient growth after 6 hours of incubation on the YPC agar plates. Due to the presence of a capsule, some cultures were not typable in this system. These cultures were first treated with hyaluronidase and then typed. The antisera of all the cultures were also examined with antigens prepared from Carter's type cultures.

Table 3. A summary of the comparison of *Pasteurella multocida* typing cultures

NADC culture number of type strains	Type	Little & Lyon: agglutination test	Carter: capsular-typing methods	Namioka: capsular-somatic test	Heddleston: gel diffusion precipitin test
Heddleston:					
X-73	1	1	A	3:A	1
M-1404	2	2	B,A	7:A	2,5
P-1059	3	1	A	8:A	3
P-1662	4	1	A	3:A,8:A	4
P-1702	5	2	A	4:D	5,2
P-2192	6	1	A	9:A	6
P-1997	7	1	A,B,E	7,9:-	7
P-1581	8	1	A	3:A	8
P-2095	9	1,3	A	5:A,7:A	9
P-2100	10	1	NR	1:A,7:A	10
P-903	11	1,3	D	2:D,4:D	11
P-1573	12	NR	A	1:A	12
P-1591	13	1	A,B,E	NR	13
P-2225	14	1,2	A	5:A	14
P-2237	15	1	D	1:D,2:D	15
P-2723	16	1	A	9:A	16
Carter:					
P-1201	A	1	A	1:A,9:A	3
P-932	B	2	B,slight A	6:B	2,5
P-934	D	NR	D	1:D,3:D	11
P-1234	E	NR	E	6:E	2,5

Table 3. (Continued)

NADC culture number of type strains	Type	Little & Lyon: agglutination test	Carter: capsular-typing methods	Namioka: capsular-somatic test	Heddleston: gel diffusion precipitin test
Namioka:					
P-1249	1:A	1	A	1,7,9:A	4
P-1175	2:D	1	D	2,4:D	3
P-1392	3:A	3	A	3:A,D,7:A	10,11
P-1254	4:D	1,2	D	4:D,8:A	3
P-1182	5:A	1	A	5:A	1
P-1248	6:B	1,2	B,A	6:B	2,5
P-1255	7:A	1,3	A	3,7:A,3:D	10
P-1197	8:A	1	A	8:A,4:D	3
P-1174	9:A	1	A	9:A	3
P-3394	11:B				
Little and Lyon:					
P-3262	1	1	D	2,4:D	3
P-3263	2	2	NR	7:A,4:D	2,5,slight 12
P-3264	3	3	NR	7:A	10,11
Roberts:					
P-2252	I	1,2	B,A,E	6:B,E	2,5
P-2255	II	2,3	A,D,E	5:A	14
P-2263	III	1	A	2,4:D	3
P-2253	IV	2	NR	1,2:-	1
P-2251	V	1	D	-:D	3
P-2254	V	1	A	-:A	3,slight 12
P-2256	V	1	D	-:D	3,slight 12

The tube agglutination test was used in determining the somatic serotype. Both unabsorbed antisera and absorbed antisera were used. A comparison of the absorbed and unabsorbed antisera in homologous and heterologous reactions revealed a reduction in cross reactions with the absorbed antisera. However, in a few instances the homologous reaction was lost completely. This can be seen with culture P-1175 in Appendix B, Tables 13 and 14. The titer with unabsorbed antisera was 160. No reaction was recorded with the absorbed antisera.

Namioka used pooled antisera prepared from more than one strain of a particular somatic type. Absorption of the antisera with the original strain was not possible in some instances. In such instances a similar culture of the appropriate somatic-capsular type was used.

The somatic type was determined by agglutination of the HCl-treated organisms with antisera prepared to Namioka's types. Some strains, in the presence of the saline buffer and antisera, settled out of suspension in the 24 hour incubation period. This was observed in the typing antisera, normal control serum, and the saline control tubes. The cross reactions listed under the somatic type in Appendix B, Tables 13 and 14 may be a result of a specific serological reaction or nonspecific settling of the antigen.

Namioka's serotypes were determined by combining the results of the capsular type and the results of the somatic type. Cross reactions occurring in either type or both were considered (Table 16, Appendix B). Those combinations not conforming to Namioka's serotypes were not considered. The somatic and capsular types of strain P-932 were type 6

crossing with type 7 and type B crossing with type D and E respectively. Of these types, the only conforming combination is serotype 6:B. However, with some strains more than one combination was possible. All of the types were considered in evaluating the strain in this comparison.

Carter's capsular types were determined, and checked by 3 different procedures: indirect hemagglutination, acriflavine flocculation, and hyaluronidase decapsulation. Numerous cross reactions were observed with the indirect hemagglutination test. The capsular type, determined by acriflavine flocculation and hyaluronidase decapsulation was used rather than the type from the IHA test. The results obtained from Namioka's plate agglutination test usually coincided with the results from Carter's techniques.

Grouping cultures on the basis of common serotypes revealed common types within a system containing specific, separate types of another system. In some cases, numerous cultures could be grouped together by similarities in the serotypes derived from all four typing systems. An example of this is shown in Table 4. The cultures listed here are Little and Lyon's and Carter's types 1 and A respectively. They can be further subdivided on the basis of Namioka's somatic antigens and Heddleston's serotypes. The distribution of all serotype designations of all the cultures of each typing system employed is listed in Appendix C. Some strains, due to the multiplicity of cross reactions in some of the tests, are placed in more than one category. Strain P-1662, for example, reacted with both Namioka's 3A and 8A. This strain was then listed in Appendix C under both serotypes.

Table 4. An example of the distribution of serotypes within serotypes

Little & Lyon	Carter	Namioka	Heddleston	Cultures
1	A	7A	4	P-1249
			7	P-1997
			10	P-2100, P-1255
		8A	3	P-1059, P-1197
			4	P-1254
				P-1662

Some patterns occurred more frequently than others. In the example from Table 4, cultures P-1059, P-1197, and P-1254 were all Little and Lyon's type 1, Carter's type A, Namioka's type 8A, and Heddleston's type 3. Other frequently occurring patterns in this comparison are listed in Table 5.

Table 5. Frequently occurring patterns of serotypes of P. multocida

Little & Lyon	Carter	Namioka	Heddleston	Cultures
1	A	8A	3	P-1059, P-1197 P-1254
	D	2,4D	3	P-1175, P-1197 P-3262, P-1254 P-2263
2	A	5A	14	P-2225, P-2255
	B	6B	2,5	P-932, P-2252 P-1248
3	A	7A	10,11	P-1255, P-1392 P-3264

Two serotypes of P. multocida, Heddleston's types 2 and 5, commonly cross react in the gel diffusion precipitin test. Different antigenic preparations of these types were examined with both avian and rabbit antiserums in the GDPT to determine if they represent the same serotype or just closely related serotypes.

The heat stable antigens and the Westphal extracted lipopolysaccharides of strains M-1404 and P-1702 were examined in the GDPT against antiserum prepared to all 16 serotypes. The heat stable antigens of strain M-1404 and P-1702 cross reacted with type 5 and type 2 antiserums respectively. These lines were of equal intensity. The lipopolysaccharide extracted from these strains were then examined against antisera prepared to all 16 serotypes. With this antigen, the reaction of these two strains was identical. The gel diffusion precipitin pattern of strains M-1404 and P-1702 are shown in Figures 1A and 1B.

Field isolates representing serotypes 2 and 5 were typed with the GDPT. The characteristics and serotypes of these strains are listed in Table 10, Appendix A. The heat stable antigens from isolates P-2547 and P-2651 reacted strongly with type 5 antiserum and weakly with type 2 antiserum. The gel diffusion precipitin pattern of isolate P-2651 is shown in Figure 3A. Isolate P-2910 was serotype 2 with a slight cross reaction with types 4, 13, and 14 (Figure 1D). No reaction was observed with type 5 antiserum. The reaction of strain P-2101 was similar to that observed with M-1404 and P-1702. This is shown in Figure 1C. Although strain P-1458 was a serotype 3, there was a strong cross reaction with type 2 antiserum.

Immunoelectrophoresis was used in an attempt to separate possible cross-reacting components of the heat stable antigens. An effective method using avian antiserum was devised before examining the cross reactions between strain M-1404 (Heddleston's type 2) and strain P-1702 (Heddleston's type 5). Since maximum precipitation of antigen and avian antibody occurs at high salt concentrations (Goodman et al., 1951), agar gel slides were immersed in various saline concentrations for different time intervals. Following electrophoresis of the antigens, the gel slides were immersed in the salt concentrations before and 24 hours after the addition of avian antiserum.

Slides were immersed in 8.5%, 12.75%, and 17.0% NaCl concentrations for 10 and 15 minutes immediately after electrophoresis and before the addition of antiserum. After incubating the slides at 37°C overnight in a moist chamber, faint, diffuse precipitin lines were evident. Of these slides, the best lines although still weak occurred after immersion in 17-0% NaCl for 15 minutes. Slides immersed under the same conditions 18 to 24 hours after the addition of antiserum to the slides produced cloudy, hazy precipitin lines.

Antigen and antiserum were concentrated 5 times and 4 times respectively to increase the intensity of the lines. Slides involving various combinations of concentrated and normal antigens and antiserums were immersed in 17.0% NaCl for 15 minutes 24 hours after incubation with the antiserum. Excellent precipitin lines were observed when the antigen, concentrated 5 times, was examined against unconcentrated antiserum. When strains M-1404 and P-1702 were examined with both avian and rabbit

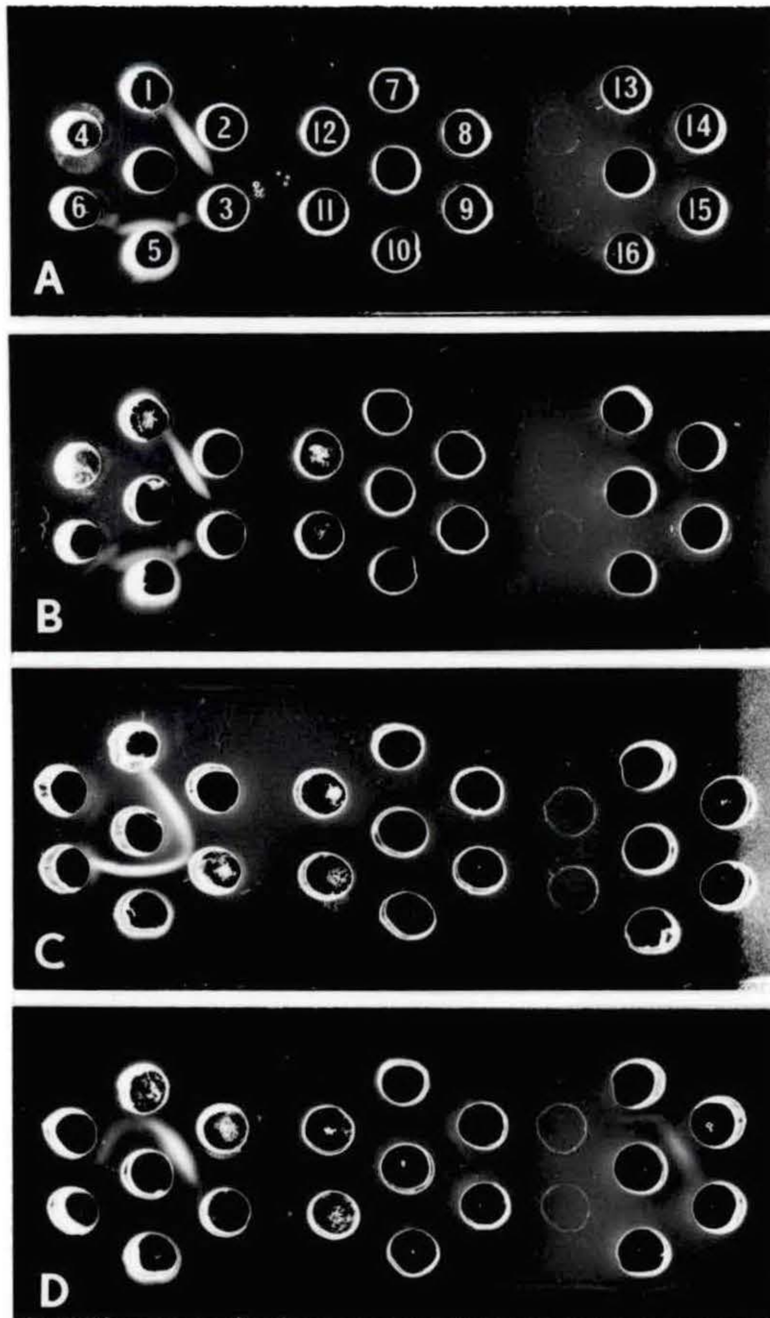
Figure 1. Gel diffusion precipitin pattern of *Pasteurella multocida* antigens (center wells) and avian antiserum prepared against each of the 16 serotypes (peripherally located).

A. Gel precipitin pattern of the Westphal lipopolysaccharide extracted from strain M-1404.

B. Gel precipitin pattern of the Westphal lipopolysaccharide extracted from strain P-1702. Note the similarity of this reaction and the reaction in A.

C. Gel diffusion precipitin pattern of the heat stable antigen of strain P-2101. This reaction was recorded as type 2 crossed with 5.

D. Gel diffusion precipitin pattern of the heat stable antigen of strain P-2910. Note the specificity of this reaction. This strain was recorded as type 2 slight crossing with types 4, 13, and 14.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
A
B
C
D

antisera in immunoelectrophoresis, the homologous and cross reacting heterologous precipitin lines were nearly identical. When the heat stable antigens of M-1404 and P-1702 were electrophoresed and examined against type 2 antiserum, two precipitin lines were observed in the homologous reaction and only one long line in the heterologous reaction. The two homologous precipitin lines consisted of a short, wide diffuse band closest to the antigen well preceded by a thin sharp line. The heterologous reaction consisted of a long, diffuse line arching toward the antiserum trough. When the antigens were examined against type 5 antiserum, only one line was observed with both the homologous and heterologous reactions. These patterns are shown in Figures 2A and 2B.

One of the field isolates reacting with type 2 antiserum in the gel diffusion precipitin test was examined with immunoelectrophoresis. The electrophoretic pattern of the heat stable antigen of isolate P-2910 is illustrated in Figure 3B. In this slide, the only reaction observed is with type 2 antiserum. The halo surrounding the lower trough containing the type 5 antiserum is a result of the diffusion of normal serum constituents and not an antibody-antigen reaction.

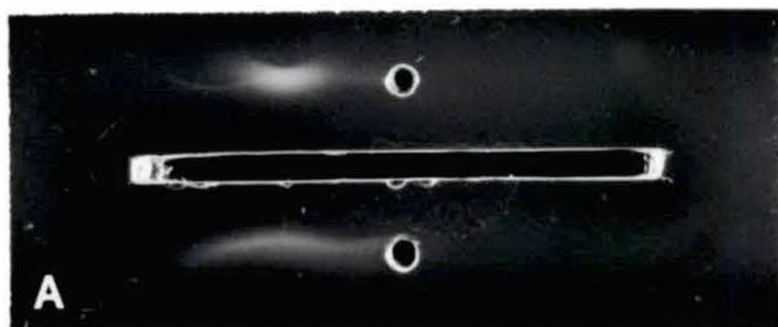
Figure 2. Immunoelectrophoresis patterns (IEP) of the heat stable antigens of strains M-1404 and P-1702 located in the upper and lower wells respectively.

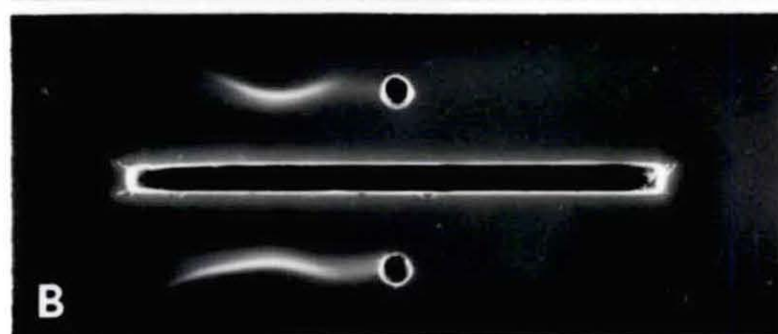
A. IEP pattern of types 2 and 5 incubated with type 2 avian antiserum. Notice the presence of an additional precipitin line.

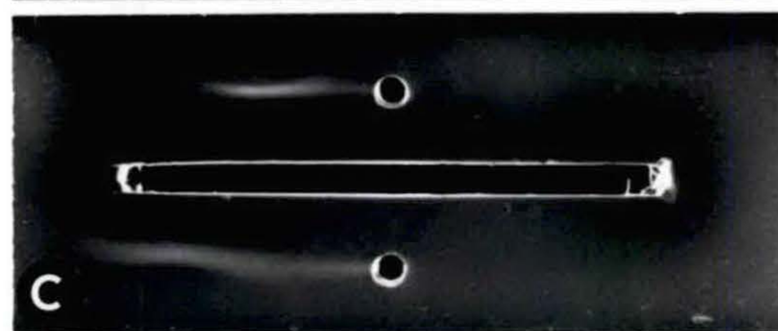
B. IEP pattern of types 2 and 5 incubated with type 5 avian antiserum.

C. IEP pattern of types 2 and 5 incubated with type 2 rabbit antiserum.

D. IEP pattern of types 2 and 5 incubated with type 5 rabbit antiserum.


A

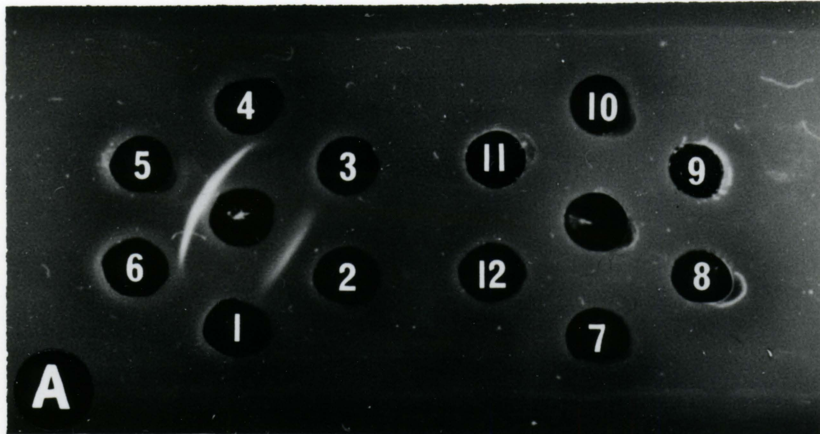

B

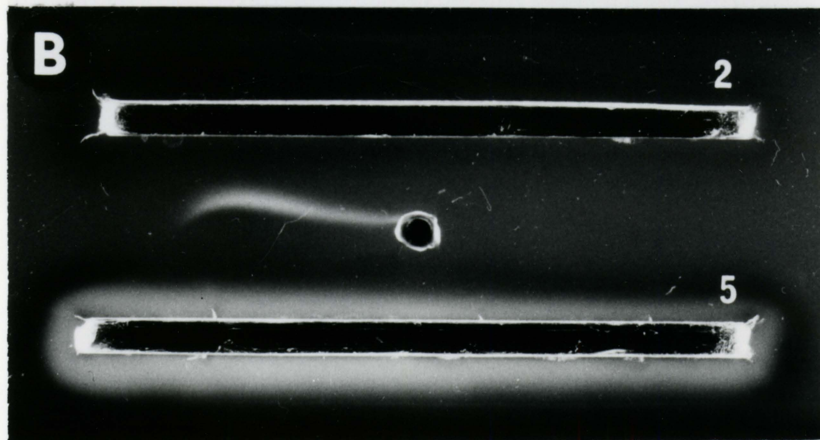

C

D

Figure 3A. Gel diffusion precipitin pattern of *Pasteurella multocida* antigen P-2651 (center wells) and avian antisera prepared against the first 12 serotypes (peripherally located).

Figure 3B. Immunoelectrophoresis pattern of strain P-2910. Types 2 and 5 avian antisera are located in the upper and lower troughs respectively. Note the specific reaction with type 2 antiserum. The halo surrounding the lower trough containing the type 5 antiserum is a result of the diffusion of normal serum constituents and not an antigen-antibody reaction.


4
5
3
10
11
9
6
2
12
8
1
7
A


B
2
5

DISCUSSION

The serological varieties of *P. multocida* were examined in four typing systems: (1) Little and Lyon's plate agglutination test, (2) Carter's indirect hemagglutination test, hyaluronidase decapsulation test, and acriflavine reaction, (3) Namioka's plate and tube agglutination tests, and (4) Heddleston's gel diffusion precipitin test. The advantages and disadvantages of each typing system, shown in the results, are discussed below. These observations were generally in accord to those reported in the literature.

Numerous cross reactions were evident in the agglutination tests constituting Little and Lyon's and Namioka's typing systems. Although the plate agglutination tests were simple, rapid, and easy to interpret, inadequacies were evident. Mucoid and iridescent strains were inagglutinable in specific antiserum while blue, noncapsulated strains readily agglutinated and occasionally autoagglutinated. Tube agglutination tests, to determine the somatic type, were also subject to cross reactions and nonspecific agglutination in the presence of saline. Other investigators reported similar results with these tests (Carter, 1958a, 1972a; Heddleston et al., 1972a).

The capsular type was determined by four methods. Carter employed the indirect hemagglutination test, reaction with acriflavine and effect of staphylococcal hyaluronidase to determine it. Namioka used a plate agglutination test. Grouping these strains by the IHA test was often difficult due to the multiplicity of cross reactions, weak or negative reactions with mucoid strains, and the lack of hemagglutinating antigens

on uncapsulated cells. Similar problems were sometimes reported by others (Heddleston et al., 1972a; Bain and Knox, 1961).

Decapsulation with hyaluronidase and flocculation with acriflavine are two nonserological tests developed to recognize characteristics associated with capsular types A and D respectively. Although decapsulation with staphylococcal hyaluronidase is used to identify type A strains, this test only detects the presence of hyaluronic acid associated with this type. It cannot be used to identify type A strains that have dissociated to noncapsulated mutants. The flocculation reaction observed with acriflavine neutral dye can only identify the colonial variation of the culture. Therefore, a positive reaction indicated a smooth colonial variant (S^R) with rough antigenic properties and rough variants (R) associated with the type D strains.

Discrepancies between capsular types determined by the indirect hemagglutination test and Namioka's simplified plate agglutination test have been reported in the literature (Carter, 1967). Differences of this type were also apparent in this study. Strains M-1404, P-2100, P-1591, P-3263, P-2251, and P-2256 differed significantly in capsular types determined by these procedures (Appendix B). Besides this inconsistency, numerous cross reactions were evident.

The procedures for determing Namioka's somatic types have not been used widely. This may be due to the difficulty in preparing the "specific factor sera" (Carter, 1972a). Absorption of these antisera with the reported strain was not possible in some instances. Although a similar culture of the appropriate somatic-capsular type was used, numerous cross reactions plagued the test.

P. multocida strains that could not be differentiated by agglutination, indirect hemagglutination, and a combination of the two could be subdivided on the basis of the heat stable antigen in the GDPT. This arrangement can be seen in Table 18 in Appendix C.

The serotype in Heddleston's typing scheme is related to the immunological antigens in the serotypes examined thus far (Heddleston et al., 1972a). The total number of immunotypes within the 16 serotypes has not yet been determined. Whether the occasional cross reactions represent similarities in the LPS of the heat-stable antigens of common immunotypes of similarities in different immunotypes is not known.

Each serotype in this study represents a separate type based on the antigen used in that typing system. Some of these cultures are infrequently isolated. Therefore, the characteristics listed for future isolates of this serotype may not be the same as those listed in this study. For example, since strain P-2723 was first isolated, 652 other cultures of P. multocida have been examined with the gel diffusion precipitin test at the National Animal Disease Center. None of the heat stable antigens prepared from the 652 cultures reacted to serum prepared to P-2723. It is evident that this serotype is not widely distributed. The characteristics listed for P-2723 may not be the same for other type 16 isolates. P-2723 is the only isolate of serotype 16. The strains used in this study do not represent the frequency of serotypes on isolation but they represent the total antigenic diversity of the species to date.

Although no comparative serological studies have been carried out to examine the strains within these groups, limited comparisons have been reported in the literature. These comparisons have been limited to Little

and Lyon's, Carter's, and Roberts' typing designations. Although there are a few discrepancies in the present comparison, the results generally confirm those reported since discrepancies also appear among the comparisons within the literature. A comparison of the serotypes and the discrepancies are illustrated in Table 6.

Table 6. Serotype designations and the discrepancies that appear in the literature[a]

Roberts	Carter	Little & Lyon
I	B	2
II	A	1
	D	
III	A	
IV	A	1
	D	1
V	D	
No equivalent	E	

[a]Carter, 1963; Carter et al., 1953; Ochl, 1956; Prodjoharjono et al., 1974.

The results in the present study generally support those listed in Table 6. Roberts' type strains, Carter's type strains, and Little and Lyon's type strains are shown in Tables 7, 8, and 9 respectively. In Table 7, Roberts' types I and II cross reacted in both Carter's and Little and Lyon's typing systems. In each case, except for Little and Lyon's designation for strain P-2255, a combination can be arranged from these cross

Table 7. Carter's and Little and Lyon's serotype designations for Roberts' typing strains

Roberts	Carter	Little & Lyon
I (P-2252)	B, A, E	1, 2
II (P-2255)	A, D, E	2, 3
III (P2263)	A	1
IV (P-2253)	-	2
V (P-2251)	D	1
V (P-2254)	A	1
V (P-2256)	D	1

Table 8. Little and Lyon's serotype designations for Carter's typing strains

Carter	Little & Lyon
A (P-1201)	1
B (P-932)	2
D (P-934)	-
E (P-1234)	-

reactions that corresponds to that reported in the literature. Roberts' type II (P-2252) reacted with both Carter's types A and D. According to the literature, either of these types is possible (Prodjoharjono et al., 1974; Carter, 1963; Harshfield, 1965; Ochi, 1956; Carter and Byrne, 1953). Little and Lyon's type 2 crossing with 3 did not agree however. Roberts' types II, III, and IV have been reported to be decapsulated with

Table 9. Carter's serotype designations for Little and Lyon's typing strains

Little & Lyon	Carter
1 (P-3262)	D
2 (P-3263)	-
3 (P-3264)	-

hyaluronidase (Carter and Bain, 1960). These strains should then be classified as type A by Carter's proposal. Strains P-2255 (II) and P-2253 (IV) did not react with hyaluronidase in this study. Strain P-2254, reported to be Carter's type D (Prodjoharjono et al., 1974; Carter, 1963), was one of the three type V strains that was decapsulated with hyaluronidase. This suggests that there are other capsular types within Roberts' type V. By these examples, it is apparent that there is more than one capsular type within some of Roberts' immunological designations. The immunological antigen may be related to something other than the capsule.

In Table 8, Little and Lyon's serotype designations are listed for Carter's type strains. Only two of Carter's four strains were typable. Although no reaction was determined for Carter's type D, it has been reported to be Little and Lyon's type 1 (Ochi, 1956). In the overall comparison, 19 out of the 25 cultures that reacted with Little and Lyon's type 1 reacted with Carter's type A. The other 6 type 1 cultures were Carter's type D. Of the 10 cultures involving the reaction of Little and Lyon's type 2, 5 reacted with Carter's type A, 3 with Carter's type B, 3

with Carter's type D, and one with Carter's type E. Since some of these types were involved in cross reactions, the culture was placed in each group in which the reaction occurred. Of the 5 cultures involving Little and Lyon's type 3, 4 cultures were Carter's type A and 2 were Carter's type D.

Little and Lyon's serotype 3 has been equated with Carter's type C (Ochi, 1956; Carter and Byrne, 1953; Harshfield, 1965). The absence of type C in most typing systems accounts for the limited occurrence of Little and Lyon's type 3. The removal of type C from Carter's capsular typing system may be the reason some cultures in the other typing systems do not have a capsular type.

The results from Little and Lyon's three serotypes examined by Carter's typing methods are in Table 9. Since these are noncapsulated, blue strains, they are not typable in the IHA and hyaluronidase decapsulation tests. Strain P-3262 was identified as Carter's type D on the basis of the acriflavine reaction. This is in agreement with the report of Ochi (1956).

Examination of Little and Lyon's serotypes in the gel diffusion precipitin test revealed Little and Lyon's type 1 to be Heddleston's type 3, type 2 to be type 2 and 5 (slight crossing with 12) and Little and Lyon's type 3 to be type 10 and 11 (slight crossing with 2 and 5) Heddleston's serotypes were given arabic numbers as a continuation of Little and Lyon's system based on serum plate agglutination and passive immunity tests[1]

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[1] Personal communication with K. L. Heddleston, Lakeview, AR, August 18, 1977.

(Heddleston et al., (1972a). Heddleston's types 1 (X-73), 2 (M-1404), and 3 (P-1059), antigenically and immunologically distinct (Carter, 1967; Rebers et al., 1967; Heddleston et al., 1972), originally correlated with Little and Lyon's types 1, 2, and 3 respectively. Although strains X-73 and P-1059 can be separated by agglutination, they both reacted with antisera to P-3262 (Table 18, Appendix C). Even though the cultures in the American Type Culture Collection are Little and Lyon's serotypes, they are replacements from Lederle Labs (ATCC, 1974). It is doubtful that they represent the strains examined by Heddleston when originating the GDPT. This is possible since numerous serotypes are listed as being Little and Lyon's type 1. Of the 39 cultures, those of Little and Lyon's type 1 could be subdivided into at least 14 serotypes by Heddleston's system, type 2 could be subdivided into at least 4 serotypes, and type 3 into at least 3 serotypes (Table 18, Appendix B). More cultures would have to be examined to determine if these are the only serotypes within Little and Lyon's groups.

Limited comparisons of Heddleston's and Namioka's serotyping designations have appeared in the literature. Heddleston's type 1 (X-73) was reported to be type 5A and type 3 (P-1059) was type 8A (Carter, 1967; Heddleston, 1966). P-1662 was type -A (Carter, 1972b). In the present study, however, X-73 was serotyped as 3A.

The discovery of a number of O-groups demonstrated the complexity of the somatic components (Carter, 1967; Prodjoharjono et al., 1974). Namioka's serotype was redesignated as 8abA to recognize 2 subgroups. Heddleston's type 3 (P-1059) was redesignated as type 8aA (Carter, 1972b).

Examination of the heat stable antigens from cultures with Namioka's 8A serotype revealed 2 serotypes present: types 3 and 4. Namioka's other serotypes can also be subdivided by Heddleston's typing system. Although Namioka's serotypes could be subdivided by Heddleston's serotypes, no obvious correlation existed between specific somatic types and specific types in Heddleston's system.

On the basis of heat stable antigens, Roberts' immunotypes were reported to be different from each other (MacLennan and Rondle, 1957). Types I and III were shown to be different from each other as well as from types II, IV, and V. Differences in II, IV, and V were not examined. In the present comparison, types I and III were different serotypes in the GDPT serotype 2 crossing with 5 and serotype 3 respectively. These types were different from types II, IV, and V with one exception. Roberts' type III and type V were both Heddleston's serotype 3. Whether other type III and V strains possessed this same type or other types has not been determined.

A variety of methods for serotyping P. multocida were examined and the type strains of each system compared. Due to the variety of classifications and different antigenic preparations, serotypes of one typing scheme cannot be equated with those of another. Examples in this study have shown that cultures with one or two serotyping antigens in common may differ in the other types.

Pasteurella multocida possesses a variety of antigens that can be used in serotyping the organism. A comparison of the type strains in each typing scheme indicated that there are specific group antigens, represented by Little and Lyon's and Carter's systems, and specific, separate

antigens, represented by Heddleston's and Namioka's systems, within these cultures. From this study, it is apparent that these antigens appear in various arrangements. In some instances (Table 5) definite patterns were observed. In other cases, patterns were not as obvious.

Each serotype in this study was characterized by the serotype designations of the other tests. Whether all of the field isolates of a certain serotype conform to the same serological breakdown as the type strain has not been determined on all of the serotypes. Heddleston's P-2723 (type 16) was Little and Lyon's type 1, Carter's type A, and Namioka's type 9A. These characteristics may not be the same for other type 16 isolates. However, in some serotypes, a great diversity was evident. Little and Lyon's type 1 was found to contain 3 capsular types, 7 somatic types, and 10 of Heddleston's types (Appendix C, Table 18). Various attempts have been made to equate serotypes within each of the typing systems. Due to the antigenic complexity of the organism and the nature of the antigens involved in each test, a reliable correlation or equality between serotypes of different typing systems cannot be made.

Two of Heddleston's serotypes were examined in the gel diffusion precipitin test and immunoelectrophoresis to determine if they represent the same serotype or just closely related serotypes. Two antigen preparations each for M-1404 and P-1702 were examined with both avian and rabbit antiserum. Strains M-1404 and P-1702 were found to react with equal intensity to homologous and heterologous antisera with both the heat stable antigen and the Westphal lipopolysaccharide. When other type 2 and type 5 field isolates were examined, some results were identical and some were

different when treated with homologous and heterologous antisera. The reaction of isolate P-2101 was identical in specificity and intensity to the reaction observed with strains M-1404 and P-1702. Another isolate, P-2910, reacted only with type 2 antiserum. Isolate P-1458 was a serotype 3 crossing with 2. These examples demonstrate a difference in the specificity of serotype 2.

A quantitative difference in the cross reactions was shown with isolates P-2547 and P-2651. The heat stable antigen prepared to these strains reacted strongly with type 5 antiserum and moderately with type 2 antiserum. The higher affinity of the type 5 antiserum to the heat stable antigens of these cultures also suggests a difference in the antigenic structure between type 2 and 5.

Although no differences were observed in the GDPT with strains M-1404 and P-1702, differences were seen with immunoelectrophoresis. An additional precipitin line was observed in the homologous reaction of M-1404. A line of this nature was not observed in the other reactions. When isolate P-2910 was examined, only one precipitin line was observed in the reaction with the type 2 antiserum. The occurrence of an additional precipitin line in IEP and a field isolate that reacts with type 2 antiserum only suggests that an additional antigenic determinate may be present on M-1404 that does not occur on P-1702. This determinate site is separate from the site that is evidently common between type 2 and type 5. Serotypes 2 and 5 were shown to be closely related on the basis of IEP lines and the specificity and intensity of the precipitin lines from selected field isolates.

SUMMARY

Thirty-nine reference strains representing the four typing systems for the classification of Pasteurella multocida were compared by procedures as originally described for: (1) Little and Lyon's plate agglutination test, (2) Carter's indirect hemagglutination test, hyaluronidase decapsulation test, and acriflavine reaction, (3) Namioka's plate and tube agglutination tests, and (4) Heddleston's gel diffusion precipitin test. When the reference strains were examined by the typing system from which they were described, the results correlated with those published with occasional exceptions. Carter's and Namioka's typing results correlated quite closely as would be expected since both involve the same capsular antigen. Again, some discrepancies were noted between Carter's indirect hemagglutination test and Namioka's plate agglutination test. Carter's type A and D were typed as Little and Lyon's type 1 and type B was type 2. Little and Lyon's strains did not react with Carter's type E.

Heddleston's type 2 crossing with 5 reacted with Little and Lyon's type 2. This serotype is usually associated with outbreaks of hemorrhagic septicemia. Heddleston's other two types failed to correlate with Little and Lyon's types 1 and 3 as originally designated.

Although Roberts' typing system is based on immunological (protective) antigens, cultures representing the five types were included in this serological comparison. More than one capsular type was found to occur within some of the immunological types. Therefore, immunological types should not be equated with capsular types in most instances.

When the distributions of all four serotype designations of all the cultures from each typing system were compared, most cultures could be grouped on the basis of common serotypes. Serotypes indistinguishable by one serotyping system often could be differentiated by the results obtained by using another system. In some cases, cultures could not be differentiated. Such cultures could, however, be grouped according to similarities in the results derived from all four systems.

Various attempts have appeared in the literature to equate serotypes between the four systems. Examples from this study have shown that cultures with one or two serotyping antigens in common may differ in the other antigens. Due to the antigenic complexity of P. multocida and the nature of the antigens involved in each test, it was thought that a reliable correlation or equality between serotypes of different systems could not be made.

Two of Heddleston's serotypes, 2 and 5, were examined in the gel diffusion precipitin test and immunoelectrophoresis to determine if they represent the same serotype or just closely related serotypes. Although no differences were observed in the gel diffusion precipitin test with two antigenic preparations of strains M-1404 and P-1702, differences were observed in the heat stable antigen when subjected to immunoelectrophoresis. Serotypes 2 and 5 were shown to be closely related on the basis of the immunoelectrophoresis of strains M-1404 and P-1702 and the specificity and intensity of the precipitin lines from selected field isolates in the gel diffusion precipitin test.

PART 2. EXAMINATION OF *PASTEURELLA MULTOCIDA* IN THE ELECTRON MICROSCOPE AND THE DESCRIPTION OF SOME ULTRASTRUCTURAL FEATURES

INTRODUCTION

Pasteurella multocida is a Gram negative, nonmotile, nonsporulating, coccobacillary-shaped rod (0.2-0.4 x 0.6-2.5 micrometers) occurring singly, in pairs, and occasionally in short chains (Heddleston, 1972). Repeated subculture in broth often results in the dissociation of some encapsulated organisms from iridescent colonies to noncapsulated organisms from blue and gray colonies (Heddleston et al., 1964). The cells from gray colonies appear as noncapsulated, long filamentous chains.

Capsules present on P. multocida can only be seen by special staining procedures. These procedures may involve direct staining of the capsule (Carter, 1952) or by demonstration of its presence indirectly with India ink (Heddleston, 1972).

India ink preparations were examined in the electron microscope to demonstrate the presence of a capsule around organisms from iridescent colonies (Heddleston, 1972). Since the India ink particles could not penetrate the capsular material, the capsule corresponded to a clear electron transparent space between the electron opaque Indian ink particles and cell. In the noncapsulated organisms India ink particles were lying next to the cell surface.

An electron microscopic examination of an encapsulated strain of P. multocida before and after exposure to 1 N HCl was briefly described by Namioka (1970). The cell, extracted with 1 N HCl, was calculated to be one-fifth the size of the untreated cells. The treated cell was thought to correspond with the somatic or cell wall antigen. Its composition was hypothesized as a lipopolysaccharide combined with protein. The HCl

extract was found to contain the surface or capsular substance and various intracellular components.

An examination of the particulate antigens from two immunogenic types of P. multocida was described by Heddleston et al. (1966). Negatively stained and shadowed preparations of purified, high molecular weight, polysaccharide complexes revealed small spherical, sac-like particles ranging in size from less than 15 nm to greater than 100 nm. Most of these membranous sacs had diameters of 20 to 50 nm.

Henriksen and Frøholm (1975) reported a strain of P. multocida, isolated from a human ear infection, to possess fimbriae. In this report, Henriksen described a fimbriated strain of P. multocida that produced spreading and corroding colonies, formed surface growth on static media, and spread by twitching motility.

Electron microscopic examination of the spreading growth was evaluated. Here it was discovered that approximately 10% of the cells possessed fimbriae, or similar structures. Examination of the nonspreading, noncorroding colonies did not reveal these structures. Henriksen concluded that the spreading and corroding properties of this strain may be associated with fimbriation as is reported for members of the Moraxella species.

The physical structure of P. multocida is only vaguely described in the literature. The purpose of this investigation is to further characterize the structure of P. multocida and to investigate the presence of external appendages.

MATERIALS AND METHODS

Cultures

Three cultures of P. multocida were examined in the electron microscope. Some characteristics of these cultures are listed below. Negatively stained preparations of P-895 and P-1234 were examined for surface structure. Thin section preparations of P-1059 were examined for internal detail.

Culture	Host of origin	Colonial appearance[1]	Serotype
P-895	Turkey	Blue	3
P-1234	Bovine	Iridescent	2 cross with 5
P-1059	Turkey	Iridescent	3

Serial passage of cultures in static liquid medium is selective for bacteria possessing pili or fimbriae (Duguid, 1968; Duguid and Gilles, 1956). Broth cultures of fimbriated organisms may form a pellicle or aggregation on the broth surface (Ottow, 1975). Strain P-895 was discovered to produce a pellicle in modified broth used to detect H_2S production while strain P-1234 did not. Strain P-895 and P-1234 were passed six times every 48 hours in this broth in an attempt to enhance pellicle production. Subcultures after each transfer were checked for purity.

[1]Obliquely transmitted light.

Media

Lyophilized cultures of P. multocida were reconstituted in tryptose broth[1] and plated on dextrose starch agar[1] from which a single colony was transferred to Heddleston's modified hydrogen sulfide broth consisting of the following (Heddleston et al., 1967):

Beef Extract	3.0 gm
Proteose Peptone No. 3	10.0 gm
Ferric Ammonium Citrate	0.2 gm
Sodium Citrate	2.0 gm
Sodium Chloride	5.0 gm
L-cysteine 2% dissolved in 2% NaOH solution	10.0 ml
Distilled Water	990.0 ml
(final pH 7.2)	

Strain P-895 readily produced pellicles within 24 hours when grown in this medium.

Antiserum

Antiserum was prepared in 12 to 16 week old New Hampshire roosters as described in Part 1. Serological Comparison of Pasteurella multocida Typing Cultures.

A procedure for the isolation of gamma globulin from serum involving repeated precipitation with ammonium sulfate has been described (Campbell et al., 1970). This procedure was modified in order to utilize available materials.

Five milliliters of saturated ammonium sulfate was added dropwise to 10 ml of P-1234 antiserum. This mixture was stirred 20-30 minutes and

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[1]Difco Laboratories, Inc., Detroit, Michigan.

centrifuged 30 minutes at 1400 x g. The precipitate was dissolved in 10 ml of saline. A second identical precipitation was then performed on this solution.

The precipitate was redissolved in saline to a final volume of 5 ml. The ammonium sulfate was removed by dialyzing against saline at 4°C. The gamma globulin was tested in the gel diffusion precipitin test against a saline suspension of strain P-1234 cells.

Negative Staining

For negative contrast staining, the following procedure was utilized.[1] P. multocida strains P-895 and P-1234 were grown 48 hours and 72 hours in Heddleston's modified H_2S broth. The broth cultures were lightly centrifuged and the cells resuspended in distilled water. A portion of the cells of P-1234 were incubated 18-24 hours in the presence of purified gamma globulin after which they were centrifuged and resuspended in distilled water. Strain P-895 also was grown 18-24 hours on DSA.

A drop of the bacterial suspension or a cotton-tipped swab containing organisms from a single colony on agar was mixed in the well of a porcelain plate containing 3-4 drops of 3% neutralized phosphotungstic acid, 1-2 drops of a 1% bovine serum albumin solution, and approximately 20 drops of distilled water. This suspension is mixed gently with a Pasteur pipette and applied as an aerosol to a carbon-coated collodion-filmed grid

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[1] A. E. Ritchie, National Animal Disease Center, Ames, Iowa.

with an all-glass nebulizer.[1] The grids were immediately examined[2] in a Phillips EM-200 electron microscope using double condensor illumination.

Thin Sectioning

A technique which prepared bacterial cells for thin sectioning has been described (Swanson et al., 1969). This procedure was modified to utilize available materials and solutions. P. multocida strain P-1059, passed nine times on DSA and seven times in tryptose broth, was centrifuged and fixed in 2.5% glutaraldehyde. Bacterial cells, fixed 2 hours at 4°C, were centrifuged and washed twice in sodium cacodylate buffer, pH 7.4. The glutaraldehyde-fixed cells were stained for an hour with 1% osmium tetroxide. These were washed and mixed with 4% aqueous molten Noble agar and then pipetted into the wells of a microtiter plate. The small agar blocks, containing the cells, were removed from the wells, washed in buffer, and dehydrated in a series of increasing concentrations of ethanol. Dehydrated cells were infiltrated with propylene oxide for 20 minutes and propylene oxide-epon (1:1) for 2 hours. Samples were embedded in epon and hardened at 60°C for 24-48 hours.

Polymerized blocks were rough trimmed to a size suitable for 1 µm sections. In the ultramicrotome, glass knives were used to cut 1-2 µm sections for light microscopy. These were mounted on a microscope slide, dried, and stained with toluidine blue.

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[1]Vaponefrin Company, New York, New York.

[2]A. E. Ritchie, National Animal Disease Center, Ames, Iowa.

Epon blocks were trimmed to an area of interest selected after viewing the 1 μm sections. This size was less than a quarter of that used in thick sectioning. Diamond knives were used to cut thin sections.[1]

Sections of the appropriate thickness were mounted on copper grids and stained in lead citrate, dehydrated in 50%, 70%, 95%, and absolute methanol. These were placed in uranyl acetate and washed in absolute methanol. Samples were examined with a Phillips EM-200 electron microscope.

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[1]Doris Buck, National Animal Disease Center, Ames, Iowa.

RESULTS

Organisms

Strain P-895 was found to produce good pellicles when incubated in stationary broth at 37°C after three passes in modified hydrogen sulfide broth. Strains P-1234 and P-1059 did not produce pellicles. It was discovered, however, after 7 passes that P-1234 could produce a faint pellicle. Upon plating, it was noted that the culture had dissociated since blue and gray colonies were observed in addition to the iridescent type. After each transfer in broth, a subculture was plated and examined for purity.

Negative Staining

The external surface of _P. multocida_ is illustrated in Figures 4 and 5A. The contoured, rugated surface of the cell envelope can be seen.

Negative stained preparations of broth and agar cultivated cultures revealed the presence of microfilaments on the surface of _P. multocida_.[1] These filamentous appendages were observed on two strains of _P. multocida_, P-895 and P-1234, which were quite different in colonial morphology, pellicle formation on broth, host of origin, serotype, or conditions of cultivation.

Measurements of the filamentous structures were determined from prints enlarged from the negatives. Estimates show their size to be 40-50 nm in length and 15-30 A in diameter. These filaments were located

[1] A. E. Ritchie, National Animal Disease Center, Ames, Iowa.

Figure 4. Negative stained preparation of *P. multocida* strain P-895. Seventy-two hour modified hydrogen sulfice broth culture with a pellicle. Filamentous appendages completely cover the cell. (Prepared by A. E. Ritchie, A.R.S., National Animal Disease Center, Ames, Iowa.)

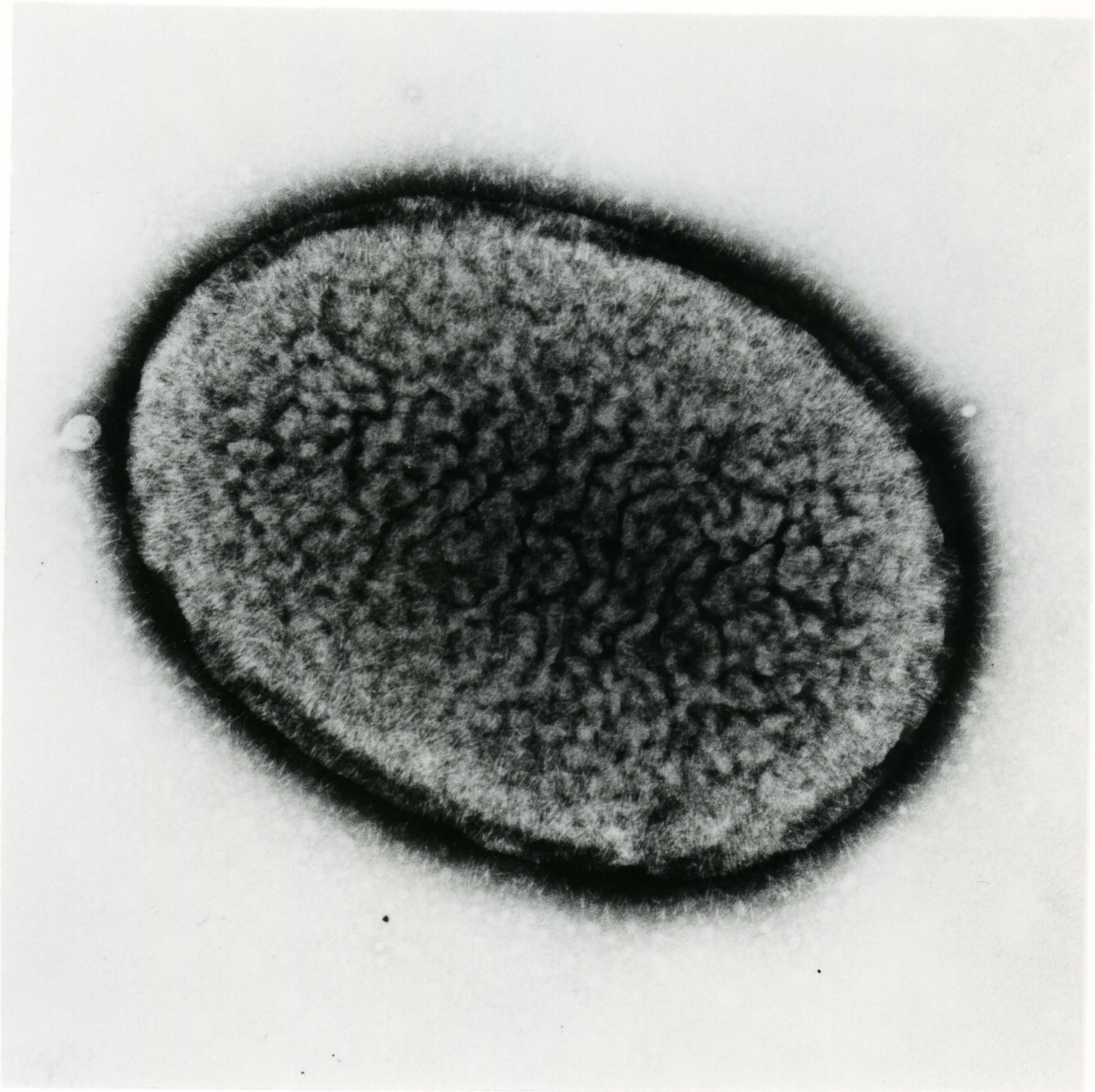
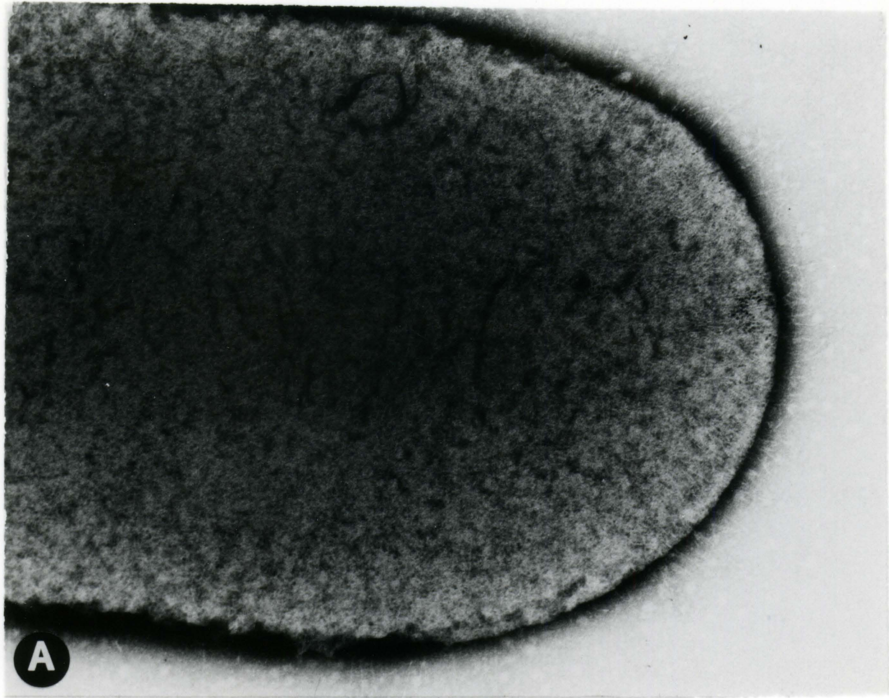
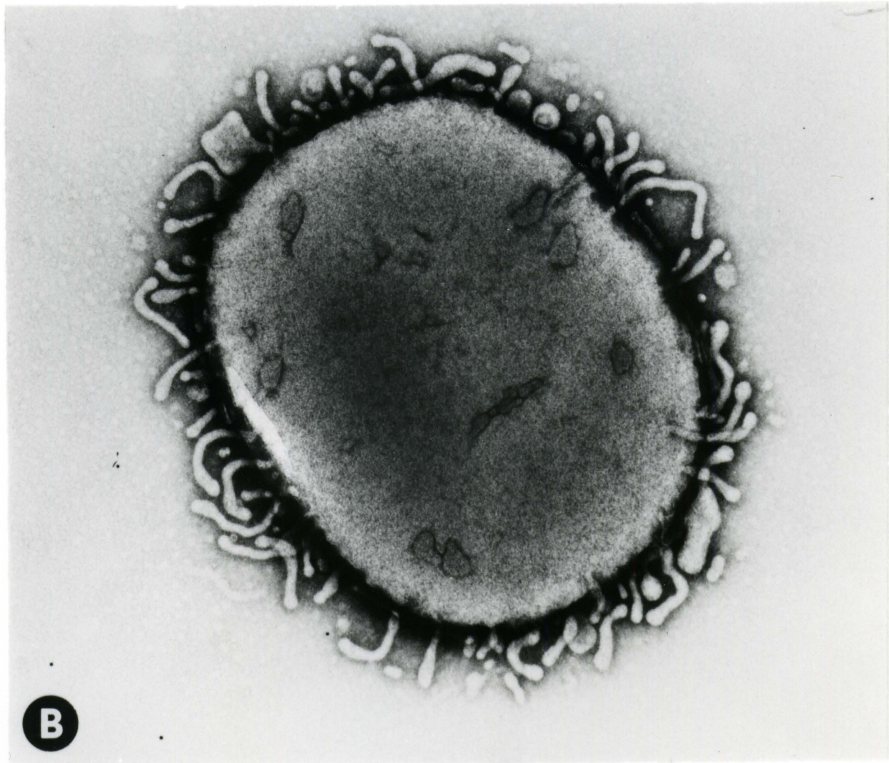


Figure 5A. Negative stained preparation of *P. multocida* strain P-1234. Forty-eight hour modified hydrogen sulfide broth culture without a pellicle. Note the convoluted surface and numerous microfilaments protruding peripherally. X108,000. (Prepared by A. E. Ritchie, A.R.S., National Animal Disease Center, Ames, Iowa.)

Figure 5B. Negative stained preparation of *P. multocida* strain P-895. Twenty-four hour DSA culture. Blebs protrude or stream from the surface of the cell. X97,000. (Prepared by A. E. Ritchie, A.R.S., National Animal Disease Center, Ames, Iowa.)


A


B

peripherally on the cell. They seemed to originate from the outer membrane but the lack of detail in this area could not confirm this.

Broth cultivated strain P-1234 was incubated in the presence of whole antiserum and ammonium sulfate-precipitated gamma globulin. Negative stained preparations revealed antibody attached to the microfilaments.[1] In Figure 7B, numberous antibody-antigen complexes are present. Within these aggregates structures resembling the microfilaments could not be seen.

Numerous organisms possessed small, spherical blebs on the cell surface and extracellularly in the surrounding area. Figure 5B illustrates a cell with large, irregular surface projections radiating from the cell. The projections ranged in size from approximately 7-75 nm for the spherical blebs and approximately 12-24 nm by 85-180 nm for the larger surface projections. These structures were observed[1] on both agar and broth cultivated organisms. The close proximity of the blebs did not allow easy definition of the microfilaments on some cells.

Thin Sectioning

The contoured, rugated surface of P. multocida can be seen in thin section of cells as a loose fitting membranous envelope consisting of four layers (Figure 6). The envelope consists of a triple layered outer membrane (Om), a closely associated dense intermediate layer, an electron transparent interspace (Is), and the cytoplasmic membrane (Cm). The outer

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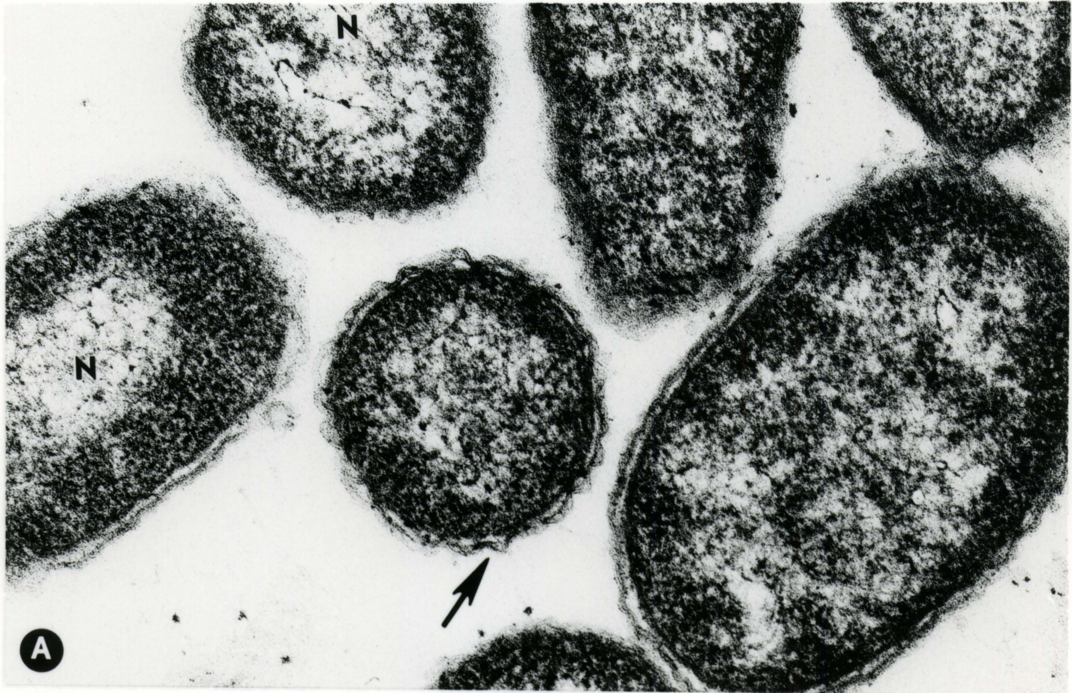
[1]A. E. Ritchie, National Animal Disease Center, Ames, Iowa.

Figure 6A. Thin section of glutaraldehyde-fixed cells of P. multocida strain P-1059. Twenty-four hour DSA culture stained with uranyl acetate and lead citrate. The cell envelope is contoured and rugae are evident (arrow). The nuclear material (N) can be seen in some cells. X109,000.

Figure 6B. Thin section of glutaraldehyde-fixed cells of P. multocida strain P-1059. Twenty-four hour tryptose broth culture stained with uranyl acetate and lead citrate. The cell envelope consists of a rugated, multilayered outer membrane (Om), an electron transparent interspace (Is), and the cytoplasmic membrane (Cm). X109,000.


N
N
A

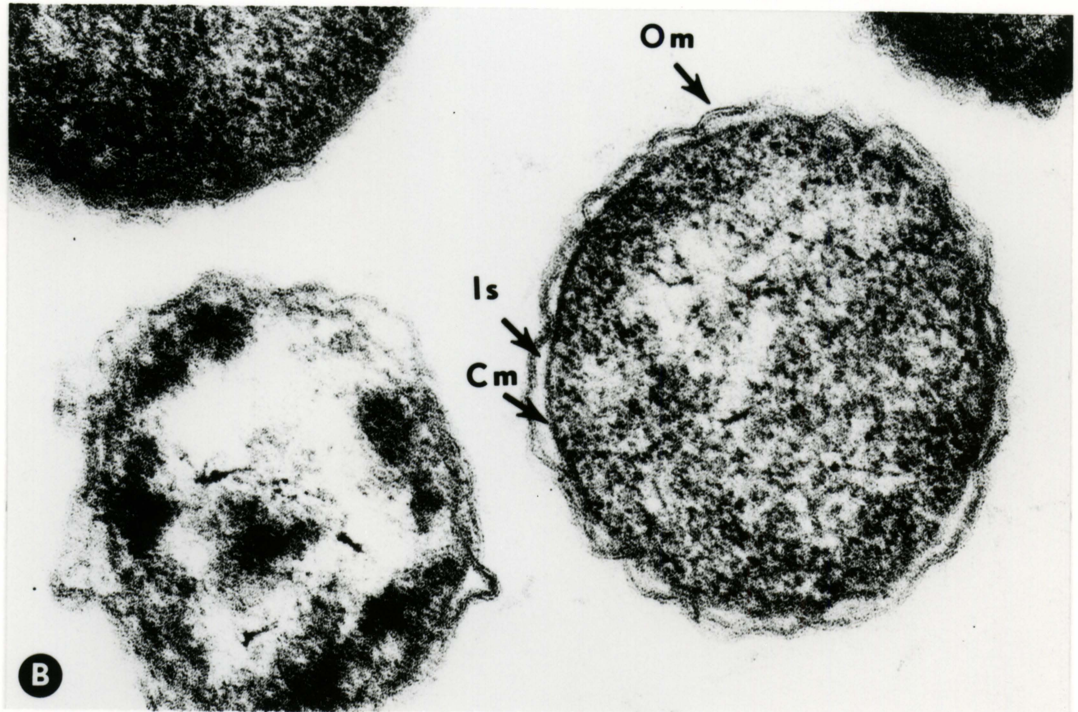
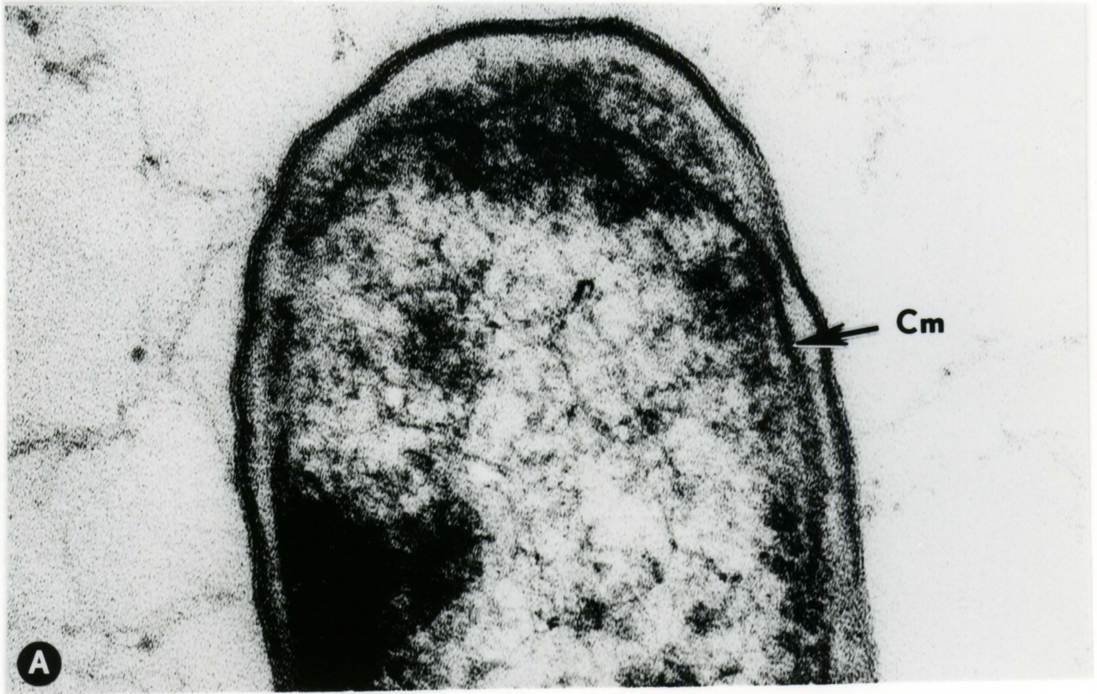
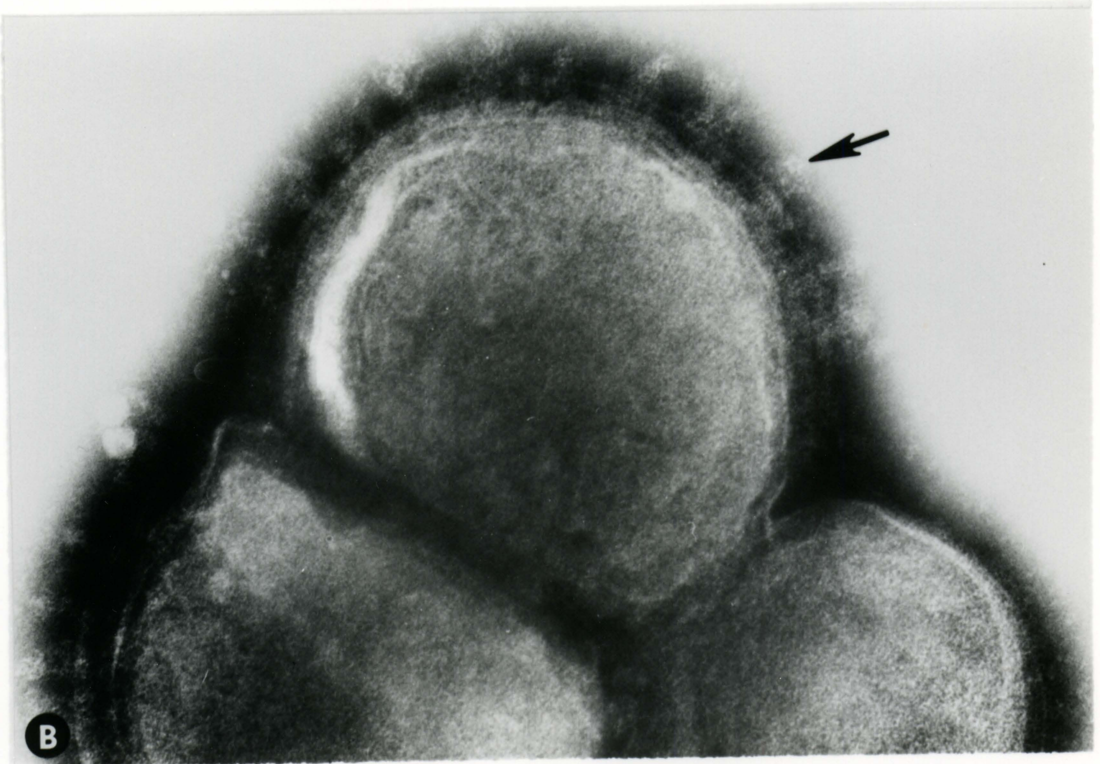

Om
Is
Cm
B

Figure 7A. Thin section of glutaraldehyde-fixed cells of strain P-1059. Twenty-four hour tryptose broth culture stained with uranyl acetate and lead citrate. The electron-dense inner cytoplasmic membrane (Cm) is evident in this cell. X147,000.

Figure 7B. Negative stained preparation of *P. multocida* strain P-1234 incubated in the presence of ammonium sulfate-precipitated gamma globulin. Note the presence of antibody (arrow) completely covering the filamentous structures. (Prepared by A. E. Ritchie, A.R.S., National Animal Disease Center, Ames, Iowa.)


Cm
A


B

membrane consists of two electron-dense layers separated by another electron-transparent layer.

An examination of some of the spherical and elongated blebs present in the thin sections revealed that they too consist of the trilaminar arrangement found in the outer membrane. It is possible that they may be evaginations of this membrane that have or are in the process of budding-off.

The appearance of the cytoplasm may vary with the fixative and plane of sectioning. The cytoplasm of these glutaraldehyde-fixed cells (Figures 6A, 6B, and 7A) is poorly defined. Within the cytoplasm the darker inter-connected areas correspond with the ribosomes and the lighter fibrous areas correspond to the nuclear material.

Thin section preparations of P. multocida were examined for the microfilaments that appeared in negatively stained preparations. Numerous cells were examined from both agar grown and broth cultivated cultures. Structures resembling microfilaments could not be seen.

DISCUSSION

The physical structure of P. multocida (except for the microfilaments) closely resembles the structure of other Gram negative bacteria. The contoured, rugated outer membrane, which resembles a wavy outline in thin section is not uncommon among Gram negative bacteria (Galvert and Thornley, 1969; Richter and Kress, 1967; Salton, 1961).

In thin sections, the cell wall of P. multocida has a multilaminar structure. A trilaminar outer membrane, a dense, closely associated intermediate layer, an electron transparent interspace and the cytoplasmic membrane were distinct. This arrangement is in accord with that described for most Gram negative bacteria (Mergenhagen et al., 1966; Morse and Morse, 1960). Glauert and Thornley (1969) discuss the composition of the outer membrane and dense intermediate layer in Gram negative bacteria. The outer membrane is composed of lipopolysaccharides and lipoprotein (DePetris, 1967; Mergenhagen et al., 1966) and the dense intermediate layer contains mucopeptide constituents.

The occurrence of spherical and elongated blebs radiating from the surface of the cell has been described in a number of Gram negative bacteria. Devoe and Gilchrist (1973) examined the release of cell wall blebs in Neisseria meningitidis. These blebs originated as evaginations of the outer membrane. They were present in substantial quantities in culture supernatant fluids and could be isolated by ultracentrifugation. Morse and Morse (1969) described membranous particles, derived from long projections on the organism, to be composed of the same trilaminar structure as the outer membrane. The origin of these blebs and elongated

projections at the outer membrane is important. This membrane is thought to be the site of endotoxin in the cell (DePetris, 1967; Glauert and Thornley, 1969; Mergenhagen et al., 1966).

The spherical blebs observed on the surface of P. multocida are thought to be evaginations of the outer membrane. In thin sections the blebs possess a trilaminar membrane similar to that of the outer membrane. If this membrane is the site of endotoxin in the cell as with other Gram negative bacteria, these blebs may represent naturally occurring particulate antigen (Heddleston et al., 1966) or endotoxin.

Uniformly distributed filamentous appendages (15-30 Å in diameter x 40-50 nm in length) were observed[1] on the periphery of negative stained cells of P. multocida. Filaments were present on two strains of P. multocida but were not correlated with other characteristics of these strains. This suggests that these structures may appear on any strain.

The measurements of the microfilaments are far below the range reported for normal pili (Ottow, 1975). Morse and Morse (1970) reported the presence of small surface projections on Bordetella pertussis, B. parapertussis and B. bronchiseptica. These filamentous appendages had a diameter of approximately 20 Å and ranged in length from 40-70 nm. These filaments were readily observed on the bacterial cells in 48 hour broth cultures. Examination of the culture after 96 hours revealed a diminished number of filaments on the cells and an increased number in supernatant fluids. Agar-grown organisms examined at various time periods revealed similar changes to those seen in broth cultures.

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Morse and Morse (1970) concluded that the microfilaments, arising from the cell wall of B. pertussis, were not readily comparable with any previously described bacterial structure.

Farrington (1974) examined the microfilaments on B. bronchiseptica and estimated the diameter to be less than 3 nm.

Broth cultivated strain P-1234, incubated in the presence of ammonium sulfate-precipitated gamma globulin, was negative stained and examined. The microfilaments were shown to be antigenic by the attachment of specific antibody. This can be seen in Figure 7B.

The outer membrane is thought to be the origin of the microfilaments. Although negative stained cells did not provide direct evidence, a few spherical blebs were found to possess the filamentous appendages.[1] If these blebs originate from evaginations of the outer membrane, then it can be assumed, indirectly, that this is the site of attachment.

Microfilaments were not observed in thin section preparations of P. multocida propagated on agar or in broth. The size of the filamentous appendages would greatly reduce the chances of being detected. Further studies involving labelled, specific immunoglobulins might detect the presence of these structures in thin section preparations.

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SUMMARY

Three strains of Pasteurella multocida were investigated by means of electron microscopy. Two strains, P-895 and P-1234, were examined after negative staining with phosphotungstate and one strain after glutaraldehyde fixation followed by embedding, sectioning, and staining.

A rugated, convoluted outer surface peripherally covered with small (15-30 Å x 40-50 nm), filamentous structures was evident on negatively stained cells from broth and agar. Small (7-75 nm), spherical blebs were also observed on the surface of most cells and extracellularly in the surrounding area. The filamentous appendages were observed on two strains of P. multocida which were quite different in colonial morphology, pellicle formation in broth, host of origin, serotype, and condition of cultivation. The microfilaments were observed to be antigenic and reacted with specific gamma globulin precipitated from antiserum prepared to the whole organism.

The contoured, rugated surface of P. multocida can be seen in thin section preparations as a loose fitting membranous envelope consisting of at least four layers: a trilaminar outer membrane, a closely associated dense intermediate layer, an electron transparent interspace and the cytoplasmic membrane. Examination of the spherical and elongated blebs revealed a trilaminar arrangement similar to the outer membrane. Thin section preparations of organisms propagated on agar or in broth did not reveal structures resembling the microfilaments that have been seen in negatively stained preparations.

Bacterial cell membranes and cytoplasm were similar to the structure of other Gram-negative bacteria. The structure and arrangement of the filamentous appendages closely resembled the structures present on members of the genus *Bordetella*.

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APPENDIX A: SEROTYPE 2 AND SEROTYPE 5 CULTURES OF *PASTEURELLA MULTOCIDA*

Table 10. *P. multocida* cultures representatives of Heddleston's serotypes 2 and 5

Cultures	Origin	Serotype	Colonial morphology
M-1404	Buffalo	2 X $\bar{c}$ 5	Iridescent
P-1702	Turkey	5 X $\bar{c}$ 2	Iridescent
P-2547	Turkey	5 X $\bar{c}$ 2	Iridescent
P-2651	Mocking Bird	5 X $\bar{c}$ 2	Blue
P-2910	Bovine	2, sl X 4, 14	Blue
P-2101	Turkey	2 X $\bar{c}$ 5	Iridescent
P-1458	Buffalo	3 X $\bar{c}$ 2	Iridescent

APPENDIX B: RESULTS OF THE COMPARISON OF *PASTEURELLA MULTOCIDA* TYPING CULTURES IN EACH OF THE SEROTYPING SYSTEMS

Table 11. The results of the type cultures in Heddleston's gel diffusion precipitin test

NADC culture number of type strains		Heddleston's gel diffusion precipitin test
Heddleston:		
1	X-73	1
2	M-1404	2 crossing with 5
3	P-1059	3
4	P-1662	4
5	P-1702	5 crossing with 2
6	P-2192	6
7	P-1997	7
8	P-1581	8
9	P-2095	9
10	P-2100	10
11	P-903	11
12	P-1573	12
13	P-1591	13
14	P-2225	14
15	P-2237	15
16	P-2723	16
Carter:		
A	P-1201	3
B	P-932	2 crossing with 5
D	P-934	11
E	P-1234	2 crossing with 5

Table 11. (Continued)

NADC culture number of type strains		Heddleston's gel diffusion precipitin test
Namioka:		
1:A	P-1249	4
2:D	P-1175	3
3:A	P-1392	10 crossing with 11
4:D	P-1254	3
5:A	P-1182	1
6:B	P-1248	2 crossing with 5
7:A	P-1255	10
8:A	P-1197	3
9:A	P-1380	3
Little and Lyon:		
1	P-3262	3
2	P-3263	2 crossing with 5, slight 12
3	P-3264	10 crossing with 11
Roberts:		
I	P-2252	2 crossing with 5
II	P-2255	14
III	P-2263	3
IV	P-2253	1
V	P-2251	3
V	P-2254	3 slight crossing with 12
V	P-2256	3 slight crossing with 12

Table 12. Results of the type cultures in Little and Lyon's agglutination test

NADC culture number of type strains		Little and Lyon's agglutination test Serotype[a]	Serotype[b]
Heddleston:			
1	X-73	1	1
2	M-1404	-	2
3	P-1059	1	1
4	P-1662	-	1
5	P-1702	-	2
6	P-2192	1	1
7	P-1997	1	1
8	P-1581	1	1
9	P-2095	-	1,3
10	P-2100	-	1
11	P-903	1,3	1
12	P-1573	-	-
13	P-1591	1	1
14	P-2225	1	1,2
15	P-2237	1	1
16	P-2723	-	1
Carter:			
A	P-1201	-	1
B	P-932	2	-
D	P-934	-	-
E	P-1234	-	-

[a]Culture antigens typed with antiserum (chicken and rabbit) prepared to Little and Lyon's strains.

[b]Type culture antiserum examined with antigens made from Little and Lyon's strains.

Table 12. (Continued)

NADC culture number of type strains		Little and Lyon's agglutination test Serotype	Serotype
Namioka:			
1:A	P-1249	-	1
2:D	P-1175	-	1
3:A	P-1392	-	3
4:D	P-1254	-	1,2
5:A	P-1182	-	1
6:B	P-1248	1,2	1
7:A	P-1255	1,3	-
8:A	P-1197	-	1
9:A	P-1380	1	1
Little and Lyon:			
1	P-3262	1	1
2	P-3263	2	2
3	P-3264	3	3
Roberts:			
I	P-2252	1,2	1,2
II	P-2255	-	2,3
III	P-2263	1	1
IV	P-2253	-	2
V	P-2251	-	1
V	P-2254	-	1
V	P-2256	-	1

Table 13. Titers obtained in the tube agglutination test by reacting each culture with unabsorbed antisera prepared against Namioka's typing strains

NADC culture number of type strains		Antisera to Namioka's strains								
		1A	2D	3A	4D	5A	6B	7A	8A	9A
Heddleston:										
1	X-73	20	20	40	80	20	20	20	20	20
2	M-1404	-[a]	-	-	-	-	-	20	-	-
3	P-1059	-	80	-	40	-	-	-	160	80
4	P-1662	-	-	80	40	-	-	20	20	20
5	P-1702	20	-	20	20	-	-	-	-	-
6	P-2192	-	-	20	40	20	-	20	20	40
7	P-1997	-	-	40	20	20	-	40	-	-
8	P-1581	-	-	40	20	-	-	-	-	-
9	P-2095	-	-	20	40	20	-	20	20	-
10	P-2100	80	-	-	-	-	-	80	-	-
11	P-903	-	40	-	80	-	-	80	-	-
12	P-1573	40	-	-	20	-	-	-	-	-
13	P-1591	20	-	20	40	-	-	-	-	-
14	P-2225	-	40	-	-	40	-	-	-	-
15	P-2237	40	40	-	-	-	-	-	-	-
16	P-2723	-	-	-	-	-	-	-	-	40
Carter:										
A	P-1201	80	-	-	20	-	-	-	-	40
B	P-932	-	-	-	20	-	80	80	-	-
D	P-934	-	-	-	-	-	-	-	-	-
E	P-1234	-	-	-	-	40	-	-	20	-

[a]No titer recorded.

Table 13. (Continued)

NADC culture number of type strains		Antisera to Namioka's strains								
		1A	2D	3A	4D	5A	6B	7A	8A	9A
Namioka:										
1:A	P-1249	160	20	20	20	10	-	40	20	20
2:D	P-1175	20	160	10	40	-	-	10	10	-
3:A	P-1392	20	-	160	20	10	-	40	20	10
4:D	P-1254	-	-	-	80	-	-	10	20	10
5:A	P-1182	-	20	10	20	80	-	20	-	-
6:B	P-1248	40	20	40	40	40	160	20	40	40
7:A	P-1255	-	-	-	-	-	-	80	-	-
8:A	P-1197	-	-	-	20	20	10	10	80	-
9:A	P-1380	-	20	20	40	10	10	20	20	160
Little and Lyon:										
1	P-3262	-	20	-	80	-	-	-	-	-
2	P-3263	-	-	-	20	-	-	40	-	-
3	P-3264	-	-	-	-	-	-	80	-	-
Roberts:										
I	P-2252	40	80	40	20	40	320	20	-	320
II	P-2255	-	20	-	-	80	-	-	-	-
III	P-2263	20	40	-	40	-	-	-	-	40
IV	P-2253	-	180	-	-	40	-	-	-	-
V	P-2251	-	40	-	-	-	-	-	-	-
V	P-2254	20	160	40	40	-	80	-	-	-
V	P-2256	-	-	-	-	-	-	-	-	-

Table 14. Titers obtained in the tube agglutination test by reacting each culture with unabsorbed antisera prepared against Namioka's typing strains

NADC culture number of type strains		Antisera to Namioka's strains								
		1A	2D	3A	4D	5A	6B	7A	8A	9A
Heddleston:										
1	X-73	-[a]	-	40	-	-	-	-	-	-
2	M-1404	-	-	-	-	-	-	20	-	-
3	P-1059	-	80	-	80	-	-	-	80	80
4	P-1662	-	-	40	20	-	-	-	20	-
5	P-1702	-	-	-	-	-	-	-	-	-
6	P-2192	-	-	-	-	-	-	-	-	80
7	P-1997	-	-	-	-	-	-	20	-	160
8	P-1581	-	-	40	20	-	-	-	-	-
9	P-2095	-	-	-	20	40	-	80	-	-
10	P-2100	40	-	-	-	-	-	80	-	-
11	P-903	-	40	-	80	-	-	160	-	-
12	P-1573	40	-	-	-	-	-	-	-	-
13	P-1591	-	-	-	-	-	-	-	-	-
14	P-2225	-	40	-	-	-	-	-	-	-
15	P-2237	20	20	-	-	-	-	-	-	-
16	P-2723	-	-	-	-	-	-	-	-	40
Carter:										
A	P-1201	40	20	-	40	-	-	-	-	-
B	P-932	-	-	-	-	-	160	80	-	-
D	P-934	160	-	80	-	-	-	-	-	-
E	P-1234	-	-	-	-	-	40	-	-	-

[a]No titer recorded.

Table 14. (Continued)

NADC culture number of type strains		Antisera to Namioka's strains								
		1A	2D	3A	4D	5A	6B	7A	8A	9A
Namioka:										
1:A	P-1249	160	-	-	-	-	-	40	-	20
2:D	P-1175	-	-	-	80	-	-	-	20	-
3:A	P-1392	-	-	160	-	-	-	20	-	-
4:D	P-1254	-	-	-	40	-	-	-	40	-
5:A	P-1182	-	-	-	-	20	-	-	-	-
6:B	P-1248	20	-	-	-	-	320	20	-	20
7:A	P-1255	-	-	40	-	-	-	80	-	-
8:A	P-1197	-	-	-	20	-	-	-	80	-
9:A	P-1380	-	80	-	80	-	-	-	20	80
Little and Lyon:										
1	P-3262	-	-	-	40	-	-	-	-	-
2	P-3263	-	-	-	-	-	-	40	-	-
3	P-3264	+[b]	+	+	+	+	+	+	+	+ rough
Roberts:										
I	P-2252	-	-	-	-	-	160	20	-	40
II	P-2255	+	+	+	+	+	+	+	+	+ rough
III	P-2263	-	40	-	40	-	-	-	20	-
IV	P-2253	20	20	-	-	-	-	-	-	-
V	P-2251	20	40	-	40	-	40	20	20	80
V	P-2254	-	20	-	40	-	-	-	40	40
V	P-2256	20	40	-	40	-	20	20	20	20

[b]Settling of the antigen in all tubes.

Table 15. Results of the type cultures in Namioka's agglutination test to determine the capsular type

NADC culture number of type strains		Namioka's agglutination test Serotype[a]	Serotype[b]
Heddleston:			
1	X-73	A	A
2	M-1404	D	D, slight A,B
3	P-1059	A	A
4	P-1662	-	A
5	P-1702	D	D, slight A,B
6	P-2192	A, slight E	-
7	P-1997	E	-
8	P-1581	A, slight E	-
9	P-2095	-	A
10	P-2100	A, slight E	-
11	P-903	-	D
12	P-1573	A, slight E	A
13	P-1591	-	-
14	P-2225	-	Slight A,B
15	P-2237	D, slight A	D
16	P-2723	-	A
Carter:			
A	P-1201	A, slight D	A
B	P-932	B	B, slight D,E
D	P-934	D	D, slight B
E	P-1234	E	E, slight B,D

[a]Culture antigens typed with antiserum prepared to Carter's strains.

[b]Type culture antisera examined with antigens made from Carter's strains.

Table 15. (Continued)

NADC culture number of type strains		Namioka's agglutination test Serotype	Serotype
Namioka:			
1:A	P-1249	A	A
2:D	P-1175	D	D
3:A	P-1392	A	A
4:D	P-1254	D	D, slight A
5:A	P-1182	A	A
6:B	P-1248	B, slight A,E	B
7:A	P-1255	A	A,D slight B
8:A	P-1197	A, slight E	A, slight D
9:A	P-1380	A	A
Little and Lyon:			
1	P-3262	A, slight E	A,D
2	P-3263	E, slight A,B	B,D
3	P-3264	A,B,E	D
Roberts:			
I	P-2252	E	B
II	P-2255	-	A,D
III	P-2263	A, slight E	D
IV	P-2253	-	-
V	P-2251	-	A
V	P-2254	A	A
V	P-2256	-	A

Table 16. Determination of Namioka's serotype from the results of the tube agglutination and plate agglutination tests of the type cultures

NADC culture number of type strains		Tube agglutination results	Plate Agglutination results	Serotype
Heddleston:				
1	X-73	3	A	3:A
2	M-1404	7	D, slight A,B	7:A
3	P-1059	8	A	8:A
4	P-1662	3,8	A	3:A, 8:A
5	P-1702	4	D, slight A,B	4:D
6	P-2192	9	A, slight E	9:A
7	P-1997	7,9	E	7,9:
8	P-1581	3	A, slight E	3:A
9	P-2095	7,4 slight 5	A	7:A, 5:A
10	P-2100	1,7	A, slight E	1:A, 7:A
11	P-903	2,4,7	D	2:D, 4:D
12	P-1573	1,4	A, slight E	1:A
13	P-1591	untypable	untypable	untypable
14	P-2225	2,5	A,B	5:A
15	P-2237	1,2	D, slight A	1:D, 2:D, 1:A
16	P-2723	9	A	9:A
Carter:				
A	P-1201	1,9	A	1:A, 9:A
B	P-932	6,7	B	6:B
D	P-934	1,3	D	1:D, 3:D
E	P-1234	6	E	6:E

Table 16. (Continued)

NADC culture number of type strains		Tube agglutination results	Plate agglutination results	Serotype
Namioka:				
1:A	P-1249	1, slight 7,9	A	1:A, 7:A, 9:A
2:D	P-1175	2,4	D	2:D, 4:D
3:A	P-1392	3, slight 7	A, slight D	3:A, 3:D, 7:A
4:D	P-1254	4,8	D, slight A	4:D, 8:A
5:A	P-1182	5	A	5:A
6:B	P-1248	6	B	6:B
7:A	P-1255	7,3	A,D slight B	7:A, 3:A, 3:D
8:A	P-1197	8,4	A, slight D	8:A, 4:D
9:A	P-1380	9,2,4	A	9:A
Little and Lyon:				
1	P-3262	4, slight 2	A,D, slight E	2:D, 4:D
2	P-3263	7, slight 4	A,B,D,E	2:D, 4:D
3	P-3264	7	A,B,D,E	7:A
Roberts:				
I	P-2252	6,9	E,B	6:B, 6:E
II	P-2255	5	A,D, slight E	5:A
III	P-2263	2,4	A,D, slight E	2:D, 4:D
IV	P-2253	1,2	untypable	1:- or 2:-
V	P-2251	untypable	A	-:A
V	P-2254	untypable	A	-:A
V	P-2256	untypable	A	-:A

Table 17. Results of the type cultures with Carter's capsular typing methods

NADC culture number of type strains		Indirect hemagglutination	Acriflavine flocculation	Hyaluronidase decapsulation
Heddleston:				
1	X-73	A	-	A
2	M-1404	B,A	-	-
3	P-1059	A	-	A
4	P-1662	Slight A	-	A
5	P-1702	A,B, slight E	-	A
6	P-2192	A	-	-
7	P-1997	A,B,E	-	-
8	P-1581	A	-	-
9	P-2095	A	-	A
10	P-2100	-	-	-
11	P-903	A,B,E	D	-
12	P-1573	A	-	A
13	P-1591	A,B,E	-	-
14	P-2225	A	-	-
15	P-2237	A,B,E	D	-
16	P-2723	A	-	A
Carter:				
A	P-1201	A, slight D	-	A
B	P-932	B, slight A	-	-
D	P-934	D,A	D	-
E	P-1234	E	-	-

Table 17. (Continued)

NADC culture number of type strains		Indirect hemag-glutination	Acriflavine flocculation	Hyaluronidase decapsulation
Namioka:				
1:A	P-1249	A	-	A
2:D	P-1175	D	D	-
3:A	P-1392	A	-	A
4:D	P-1254	D,A	D	-
5:A	P-1182	A	-	A
6:B	P-1248	B,A	-	-
7:A	P-1255	A, slight D	-	A
8:A	P-1197	A	-	A
9:A	P-1380	A	-	A
Little and Lyon:				
1	P-3262	-	D	-
2	P-3263	-	-	-
3	P-3264	-	-	-
Roberts:				
I	P-2252	B, slight A,E	-	-
II	P-2255	A, slight D,E	-	-
III	P-2263	A	-	-
IV	P-2253	-	-	-
V	P-2251	-	D	-
V	P-2254	-	-	A
V	P-2256	-	D	-

APPENDIX C: THE DISTRIBUTION OF ALL THE SEROTYPES OF THE TYPE STRAINS INTO EACH OF THE FOUR TYPING SYSTEMS

Table 18. The distribution of type cultures grouped on the basis of Little and Lyon's serotypes

Little and Lyon's serotype	Carter's serotype	Namioka's serotype	Heddleston's serotype	NADC culture number
1	A	1A	3	P-1201
			4	P-1249
			10	P-2100
		3A	1	X-73
			4	P-1662
			8	P-1581
			10	P-1255
		5A	1	P-1182
			9	P-2095
			14	P-2225
		7A	4	P-1249
			7	P-1997
			10	P-2100, P-1255
		8A	3	P-1059, P-1197, P-1254
			4	P-1662
		9A	3	P-1201, P-1380
			6	P-2192
			7	P-1997
			16	P-2723
		-A	3	P-2254
	B	6B	2,5	P-1248, P-2252
	D	-D	3	P-2251, P-2256
		2D	3	P-1175, P-3262, P-2263
			11	P-903
			15	P-2237

Table 18. (Continued)

Little and Lyon's serotype	Carter's serotype	Namioka's serotype	Heddleston's serotype	NADC culture number
		4D	3	P-1175, P-3262, P-2263, P-1197, P-1254
			11	P-903
		1D	15	P-2237
2	-	1-	1	P-2253
		2-	1	P-2253
	A	5A	14	P-2225, P-2255
		7A	2,5	M-1404
			2,5 sl 12	P-3362
		8A	3	P-1254
	B	6B	2,5	P-932, P-2252, P-1248
	D	4D	3	P-1254
			2,5	P-1702
			2,5 sl 12	P-3263
3	A	3A	10	P-1255
			10,11	P-1392
		5A	9	P-2095
		7A	9	P-2095
			10	P-1255
			10,11	P-1392
			10,11	P-3264
	D	2D	11	P-903
		3D	10	P-1255
			10,11	P-1392
		4D	11	P-903

Table 19. The distribution of type cultures grouped on the basis of Carter's serotypes

Carter's serotypes	Little and Lyon's serotypes	Namioka's serotypes	Heddleston's serotypes	NADC culture number
A	1	1A	3	P-1201
			4	P-1249
			12	P-1573
		3A	1	X-73
			4	P-1662
			8	P-1581
			10	P-1255
		5A	1	P-1182
			9	P-2095
			14	P-2225
		7A	2,5	P-3263
			4	P-1249
			7	P-1997
			9	P-2095
		8A	3	P-1059, P-1197
			4	P-1662
		9A	3	P-1201, P-1380
			4	P-1249
			6	P-2192
			7	P-1997
			16	P-2723
		-A	3,12	P-2254
	2	5A	14	P-2225, P-2255
		7A	2,5	P-3263
		4D	2,5	P-1702, P-3263
	3	3A	10,11	P-1255, P-1392
		5A	9	P-2095
			14	P-2255
		7A	10,11	P-3264, P-1255, P-1392
			9	P-2095

Table 19. (Continued)

Carter's serotypes	Little and Lyon's serotypes	Namioka's serotypes	Heddleston's serotypes	NADC culture number
		3D	10,11	P-1255, P-1392
B	1	-	13	P-1591
		6B	2,5	P-1248, P-2252
	2	6B	2,5	P-932, P-1248, P-2252
D	1	1D	15	P-1591
		2D	3 11 15	P-1175, P-3262 P-903 P-1591
		4D	3 11	P-1254, P-3262, P-1175 P-903
		-D	3,12	P-2251, P-2256
	2	4D	3	P-1254,
	3	2D, 4D	11	P-903
E	1	6E	2,5	P-2252, P-1234
		-	13	P-1591

Table 20. The distribution of type cultures grouped on the basis of Namioka's serotypes

Namioka's serotypes	Carter's serotypes	Little and Lyon's serotypes	Heddleston's serotypes	NADC culture number
1A	A	1	3	P-1201
			4	P-1249
			10	P-2100
		NR	12	P-1573
1D	D	NR	11	P-934
		1	15	P-2237
2D	D	1	3	P-1175, P-3262, P-2263
			15	P-2237
		1,3	11	P-903
3A	A	1	1	X-73
			4	P-1662
			8	P-1581
		3	10	P-1255
			10,11	P-1392
3D	D	NR	11	P-934
		1,3	10	P-1255
4D	D	1	3	P-1175, P-1197, P-3262, P-2263, P-1254
		2	2,5	P-1702, P-3263
		3	11	P-903
5A	A	1	1	P-1182
			9	P-2095
		2	14	P-2225, P-2255
		3	9	P-2095
6B	B	2	2,5	P-932, P-1248, P-2252

Table 20. (Continued)

Namioka's serotypes	Carter's serotypes	Little and Lyon's serotypes	Heddleston's serotypes	NADC culture number
6E	E	1,2	2,5	P-2252
		NR		P-1234
7A	A	1	4	P-1249
			7	P-1997
			9	P-2095
			10	P-2100, P-1255
		2	2,5	M-1404, P-3263
		3	9	P-2095
			10	P-1255
			10,11	P-1392, P-3264
8A	A	1	3	P-1059, P-1254, P-1197
			4	P-1662
9A	A	1	3	P-1201, P-1380
			4	P-1249
			6	P-2192
			7	P-1997
			16	P-2723

Table 21. The distribution of type cultures grouped on the basis of Heddleston's serotypes

Heddleston's serotypes	Little and Lyon's serotypes	Carter's serotypes	Namioka's serotypes	NADC culture number
1	1	A	3A	X-73
			5A	P-1182
2,5	2	A	7A	M-1404, P-3263
		B	6B	P-932, P-1248, P-2252
		E	6E	P-2252, P-1234
3	1	A	1A 8A 9A -A	P-1201 P-1254, P-1059 P-1201, P-1380 P-2254
		D	2D 4D	P-3263, P-1175, P-2263 P-3262, P-1175, P-2263, P-1254
4	1	A	1A 3A 7A 8A 9A	P-1249 P-1662 P-1249 P-1662 P-1249
6	1	A	9A	P-2192
7	1	A	7A, 9A	P-1997
8	1	A	3A	P-1581
9	1,3	A	5A, 7A	P-2095
10	1	A	7A	P-1255, P-2100, P-1255, P-3264
10,11	3	A	7A	P-3264, P-1392
11	1,3	D	2D, 4D	P-903
	-	D	1D, 3D	P-934

Table 21. (Continued)

Heddleston's serotypes	Little and Lyon's serotypes	Carter's serotypes	Namioka's serotypes	NADC culture number
12	-	A	1A	P-1573
13	1	A,B,E	-	P-1591
14	2	A	5A	P-2225, P-2255
15	1	D	1, 2D	P-2237
16	1	A	9A	P-2723