A New Organism Resembling P. tularensis Isolated From Water

By CARL L. LARSON, M.D., WILLIAM WICHT, and WILLIAM L. JELLISON, Ph.D.

THE DEMONSTRATION of the presence of *Pasteurella tularensis* in ponds, rivers, and streams in northwestern United States (*1*) prompted continued study on the distribution of *P. tularensis* in such waters, especially where there have been reports of epizootics in aquatic mammals.

During the course of the studies, an organism, apparently not described in the literature, was detected in a water sample obtained from Utah. The purpose of this paper is to describe this organism, for which we propose the name *Pasteurella novicida* sp. nov. In correspondence and in the exchange of cultures, this organism has been referred to as Utah 112.

Isolation of the Organism

A turbid water sample collected from Ogden Bay Bird Refuge, near Ogden, Utah, was received September 12, 1950, from Dr. Jessup B. Low of the Utah Cooperative Wildlife Research Unit. Muskrats had been found dead in the immediate area where the sample was collected. Two guinea pigs were each injected intraperitoneally with 10 ml. of this sample, 4 white mice with 2 ml. each intraperitoneally, and 4 white mice with 2 ml. each subcutaneously.

One guinea pig was found dead 6 days after inoculation. Indistinct foci on the liver and spleen were suggestive of tularemia. The other guinea pig, moribund later in the day, was sacrificed and its heart blood placed on glucose-cystine-blood agar. On necropsy, numerous foci of necrosis were observed on the liver and spleen. These lesions were also suggestive of tularemia, and a tentative diagnosis of that disease was made. Cultures from this guinea pig gave pure cultures of the organism under consideration.

One of the mice died on the fourth day post-inoculation and two more on the sixth day. White foci were noted on the spleen of one mouse, and cultures were obtained from the tissues of both mice that died 6 days after injection. The cultures were similar to those isolated from the guinea pig.

Suspensions of the bacterium failed on repeated tests to agglutinate in specific anti-tularensis serum, and tissues of animals which died following injection with the original water sample, as well as tissues from serially infected animals, gave negative Ascoli tests (*2*) with the same serum.

The failure of the serologic tests to identify the organism as *P. tularensis* was puzzling since the time of death of test animals and the gross

Dr. Larson is the director, Mr. Wicht is a laboratory technician, and Dr. Jellison is the parasitologist, Rocky Mountain Laboratory, National Microbiological Institute, Public Health Service.

lesions were suggestive of tularemia; also, the growth of the organism on glucose-cystine-blood agar and the appearance of the organism from culture and tissue smears, when stained with carbolthionin, greatly resembled *P. tularensis*.

Materials and Methods

The methods used in identification of this organism are largely those recommended in the Manual of Methods for Pure Culture Study of Bacteria (*3*). However, some variations have been necessary, since the organism does not grow on certain of the media which are ordinarily employed for biochemical tests. Routinely, glucose-cystine-blood agar was used for maintenance of the organism. The proteose-peptone broth of Snyder and his co-workers (*4*) was employed as the liquid medium. For fermentation tests, filter-sterilized carbohydrates and polyhydroxyalcohols were added in 2-percent concentration, and bromthymol blue was added as indicator. Experimental animals were from stock maintained at the Rocky Mountain Laboratory. Embryonated eggs were inoculated after incubation at 37° C. for 7 days.

Ascoli tests were performed, using bacterial or tissue suspensions treated with ether, according to methods previously described (*2*).

Organisms used in this study included the bacterium isolated from water from Ogden Bay, *P. tularensis* (strains 453 and 454, which were isolated from streams in the Bitterroot Valley, Mont.), as well as *Pasteurella pestis*, *Pasteurella multocida*, and *Pasteurella pseudotuberculosis*, which are maintained at this laboratory.

Results

The newly isolated bacteria under study are nonmotile, gram-negative rods, which do not form spores. There is some tendency toward bipolar staining, but this is not marked and does not serve as a distinguishing feature. No flagellae are observed. No definite capsule is apparent in preparations from artificial media stained with carbolthionin, Giemsa, or Wayson's stains, although clear areas are observed around cells in smears from tissues of infected animals. In smears prepared from spleens of guinea pigs and stained with carbolthionin, the organisms are ovoid to coccoid, measure 0.28μ in length and 0.20 to 0.28μ in width, and are arranged singly and in small clusters. When grown in liquid media they are 0.7μ in width and 1.7μ in length, and are predominantly short, thick rods with slightly bulging sides. They are arranged singly or in pairs. A few coccoid forms are present. If the bacteria are grown in broth containing 3 percent NaCl, the organisms are found to be pleomorphic, and rods as long as 4.2μ are noted. When grown on solid media, the organisms measure 0.47μ to 0.94μ in length and 0.47μ in width. These examinations were made by phase microscopy with organisms grown at 37° C. for 24 or 48 hours. Broth cultures were examined for motility after incubation for 18, 24, and 48 hours at 25° C. and 37° C., by both light- and dark-field microscopy.

On primary isolation, the culture grows well on glucose-cystine-blood agar, but not on nutrient agar (Difco). Cultures made directly from animal tissues by smearing liver or spleen upon the surface of horsemeat-infusion agar do not grow except in the immediate vicinity of small pieces of tissue that adhere to the medium. After 11 serial passages in guinea pigs, *P. novicida* still could not be isolated on media lacking added cystine, yeast extract, or whole blood. Plating of serial dilutions on the surface of various media indicates that glucose-cystine-blood agar, glucose-cystine agar, blood agar, or yeast-extract agar are about equally suitable media. No growth occurred in plain horsemeat-infusion agar, with or without addition of dextrose, even when as many as 50,000 organisms were present in the inoculum.

On glucose-cystine-blood agar, well-separated colonies may attain 8 mm. in diameter after 72 hours' incubation. They are gray with a definite blue cast, smooth, slightly elevated, glistening, amorphous, and have entire edges. The colonies are butyrous or viscid and are easily emulsified in water to form a homogeneous suspension. Colonies average about 4 mm. in diameter on blood agar but otherwise resemble those described above. On glucose-cystine agar, they average 6 to 7 mm. in diameter and are translucent. On yeast-extract agar, the colonies are about 3 mm. in diameter, clear, convex, glistening, and with smooth edges. Growth

is moderate in 24 hours at 37° C. on slanted media, and isolated colonies have characteristics similar to those described for each of the above media. The growth is filiform and tends to pile up at the edges. There is no hemolysis on blood agar.

In deep agar shakes of media containing yeast extract or cystine incubated for 8 days at room temperature at 30° C. and 37° C., the organisms grow on the surface and to a depth not exceeding 0.7 cm. below the surface. The surface colonies approximate 5 mm. in diameter whereas those growing within the agar are 1 mm. or less.

In the fluid medium employed (*4*), growth is abundant, producing a moderately uniform turbidity. There is no pellicle or surface growth. A slight deposit develops, which may be disintegrated by shaking. No growth can be seen on raw potato.

Fermentation

The first isolate of *P. novicida* was found to ferment dextrose, sucrose, levulose, and mannose, with production of acid but no gas. A culture isolated from the 11th passage in guinea pigs was tested in Snyder's medium containing 2-percent concentrations of the various substrates and bromthymol blue to determine the fermentative reactions of the organism. The medium was dispensed in 25-ml. volumes in 100-ml. flasks and incubated at 37° C.

Two cultures of *P. tularensis* (isolates 453 and 454) were studied under identical circumstances. Each flask of medium was inoculated with 1 ml. of suspension of organisms grown in Snyder's medium for 24 hours at 37° C. Flasks of Snyder's medium were included as uninoculated controls and flasks containing indicator but no added substrate served as inoculated controls. The color changes were noted and the pH determined, with the aid of a pH meter, 4 and 14 days after incubation. The results of pH determinations are presented in table 1.

Additional carbohydrates and polyhydroxy-alcohols not included in the table were tested. These were inulin, rhamnose, trehalose, sorbitol, arabinose, adonitol, dextrin, melizitose, salicin, inositol, xylose, lactose, esculin, raffinose, galactose, mannitol, and dulcitol. The final pH attained by media containing these substances was 7.3 to 7.6 for *P. tularensis* isolate 453, 7.4 to 7.8 for *P. tularensis* isolate 454, and 7.4 to 7.7 for *P. novicida*. Fermentation of sucrose by *P. novicida* serves to differentiate it from *P. tularensis*.

Table 1. Comparative fermentation studies on Pasteurella tularensis and Pasteurella novicida

Substrate	pH reactions of media after 4 and 14 days' incubation with—					
	P. tularensis (453)		*P. tularensis* (454)		*P. novicida*	
	4 days	14 days	4 days	14 days	4 days	14 days
Sucrose	7.6	7.5	7.6	7.6	5.6	5.5
Dextrose	6.9	6.5	6.9	6.8	5.7	5.6
Levulose	6.6	5.8	6.5	6.2	5.7	5.2
Mannose	6.9	6.7	6.9	6.6	5.8	5.5
Glycerol	7.6	7.6	7.5	7.6	7.3	6.9
Maltose	7.6	7.5	7.6	7.6	7.7	7.6
Inoculated control	7.5	7.5	7.5	7.6	7.7	7.7

The effect of fermentable sugars upon the growth of the organism was determined. Cultures were made in Snyder's medium containing dextrose, sucrose, maltose, and lactose, all with bromthymol blue as indicator. After 3 days' incubation at 37° C., when the color of the medium indicated that fermentation of dextrose and sucrose had taken place, serial tenfold dilutions were made of the bacterial suspensions, and counts were made on glucose-cystine-blood agar. The bacterial counts of each of the media were in very close agreement, being 25×10^8 and 27×10^8 for media containing dextrose and sucrose, respectively, and 24×10^8 and 19×10^8 for those with maltose and lactose, respectively. Thus, the presence of fermentable sugars had no effect on the growth of *P. novicida*.

Biochemical Reactions

The following biochemical reactions were observed: nitrates not reduced to nitrites; indol negative by Kovac's method; H_2S positive by lead acetate paper strips; ammonia not produced; methylene blue reduced, catalase positive; gelatin contained no added cystine supported growth but was not liquefied; litmus milk unchanged; methyl red and Voges-Proskauer negative, although growth was manifest

in the medium; no growth on McConkey's medium.

The resistance of the organism, comparable to that of *P. tularensis*, is not great. Suspensions containing 4×10^8 bacteria in saline were killed in 10 minutes by exposure to a temperature of 60° C. and in 20 minutes by exposure to 1 percent phenol. Cultures of *P. novicida* and *P. tularensis* (453 and 454) were grown on glucose-cystine-blood agar at temperatures of 25°, 32°, 37°, and 41° C. Three subcultures were made during an observation period of 16 days. None of the strains of organisms was affected by exposure to the limits of temperature selected since there was confluent growth on each of the agar slants at the termination of the experiment.

No evidence for the presence of a soluble toxin or for the filterability of the organism was found. A culture of the organism was grown in Snyder's medium at 37° C. for 48 hours and filtered through a sintered glass filter (grade UF). The first aliquot of 10 ml. was discarded. The remainder of the filtrate was collected and tested for sterility. No growth occurred on glucose-cystine-blood agar plates or on Snyder's medium. Mice and guinea pigs inoculated intraperitoneally with 0.1 and 5.0 ml., respectively, of this filtrate were not infected.

Pathogenicity

P. novicida is pathogenic for a wide range of experimental animals. The pathogenicity for white mice and guinea pigs is shown in table 2. As few as 50 organisms injected subcutaneously into mice caused death, whereas as few as 5 organisms administered intraperitoneally caused death in 3 of 4 mice. Small numbers of organisms were also shown to be lethal for guinea pigs by intraperitoneal injection. Hamsters were as susceptible as mice and guinea pigs and showed more distinctive gross lesions. Titrations of cultures and tissues from infected animals or embryonated eggs demonstrated that suspensions containing two bacteria, as determined by growth on glucose-cystine-blood agar plates, were capable of producing lethal infections in 7-day-old embryonated eggs. The disease produced in embryos was fatal within 2 to 7 days.

There was considerable resistance to infection in rabbits, white rats, and pigeons, and large numbers of organisms were needed to produce signs of illness and death. Six million organisms caused death within 4 days of three pigeons inoculated intramuscularly, but 6×10^5 organisms failed to produce illness in a similar group of birds. Rabbits infected with as few as 3×10^4 organisms succumbed if inoculated either intraperitoneally or subcutaneously, but some lived as long as 25 days. White rats were susceptible to large doses of organisms administered intraperitoneally (3×10^7) but not to similar amounts given subcutaneously.

There were no pathognomonic lesions in embryonated eggs examined after death of the embryo, but hemorrhages and congestion were commonly noted. Mice inoculated subcutaneously showed congestion of the subcutaneous tissues, hemorrhages at the site of inoculation, with enlargement and congestion of the local lymph nodes, hemorrhages and congestion of

Table 2. Mortality among groups of white mice and guinea pigs inoculated with a suspension of spleen from a guinea pig dying during the 10th serial passage of Pasteurella novicida in guinea pigs

Animal and route of inoculation [1]	Dilution of spleen suspension [2]	Amount injected (ml.)	Approximate number of organisms	Results [3]
White mouse				
IP	10^{-1}	0.2	5,000	4/4
IP	10^{-2}	.2	500	4/4
IP	10^{-3}	.2	50	4/4
IP	10^{-4}	.2	5	3/4
SQ	10^{-1}	.2	5,000	4/4
SQ	10^{-2}	.2	500	3/4
SQ	10^{-3}	.2	50	4/4
SQ	10^{-4}	.2	5	0/4
IM	10^{-1}	.1	2,500	4/5
IC	10^{-1}	.03	*Ca* 800	5/5
Guinea pig				
IP	10^{-1}	0.2	5,000	4/4
IP	10^{-2}	.2	500	4/4
IP	10^{-3}	.2	50	4/4
IP	10^{-4}	.2	5	0/4
SQ	10^{-1}	.2	5,000	4/4
SQ	10^{-2}	.2	500	4/4
SQ	10^{-3}	.2	50	4/4
SQ	10^{-4}	.2	5	2/4

[1] IP, intraperitoneal; SQ, subcutaneous; IM, intramuscular; IC, intracerebral.
[2] No deaths among animals inoculated with 10^{-5}, 10^{-6}, or 10^{-7} dilutions.
[3] Numerator, number died; denominator, number inoculated.

the lungs, and enlargement of the spleen, with multiple small foci of necrosis. There were no gross lesions in the liver. The subcutaneous tissue of guinea pigs was hemorrhagic and congested, the local lymph nodes were enlarged and hemorrhagic, the spleen was enlarged, containing many small, raised gray foci of necrosis and was usually covered with a gray exudate. The liver contained few to many foci of necrosis. The lungs were congested and hemorrhagic and exhibited foci of necrosis in many instances. The lesions noted in rabbits, white rats, and hamsters were essentially similar to those noted in white mice and guinea pigs. In one rabbit, surviving for 25 days after infection, many large caseous areas of necrosis were found in the lungs.

Serologic Reactions

Agglutination and precipitin tests were performed with serums from rabbits immunized by repeated intravenous inoculation of formalin-killed suspensions of *P. tularensis*, *P. novicida*, *P. pestis*, *P. pseudotuberculosis*, and *P. multocida*. A formalin-killed suspension of each of the organisms was employed for agglutination tests. Precipitin tests were done with the supernatant fluid obtained after suspensions of the organisms in saline had been treated with two volumes of ether, and the aqueous phase centrifuged and harvested (*2*). The tests were incubated at 37° C. for 4 hours and placed in the refrigerator overnight before being read the following morning.

It was found that formalin-killed suspensions of *P. novicida* reacted in the agglutination test with serum prepared against the homologous organism but not with serums prepared against the heterologous organisms. Similarly, the immune serum prepared against *P. novicida* agglutinated suspensions of *P. novicida* to a titer of 1:320, *P. tularensis* and *P. pseudotuberculosis* to titers of 1:10 only and failed to react with *P. pestis* and *P. multocida*.

The several serums were tested for the presence of precipitins against *P. novicida*. The *P. novicida* antigen employed failed to react with serums from rabbits immunized with *P. pseudotuberculosis* and *P. multocida*, but reacted with undiluted serums specific for *P. pestis* and *P. tularensis* and to a titer of 1:32 against homologous serum. The anti-novicida serum precipitated with antigens derived from *P. pestis* and *P. tularensis* in dilution of 1:4, from *P. pseudotuberculosis* to a dilution of 1:2, from *P. novicida* to a titer of 1:32, and failed to precipitate antigen from *P. multocida*. When dilutions of antigen prepared from *P. novicida* were tested against whole serums in the precipitin test, the antigen did not react with serums specific for *P. multocida* and *P. pseudotuberculosis* but reacted at dilutions of 1:4 with serums against *P. pestis* and *P. tularensis* and at a dilution of 1:64 against the homologous serum. In general, the serums from rabbits immunized against formalin-killed antigens gave specific reactions in both agglutination and precipitin tests.

Precipitin tests were performed with serums from rabbits immunized with suspensions of *P. novicida* and *P. tularensis* killed: (*a*) by heating at 60° C. for 30 minutes, (*b*) by addition of 2 volumes of ether, and (*c*) by 0.2 percent formalin or 0.5 percent phenol. Antigens were obtained by extraction with ether. The results given in table 3 indicate that the antibodies produced are relatively specific for each bacterial species and do not appear to be significantly varied by the type of treatment afforded the antigen employed to immunize the animals.

Table 3. Results of precipitin tests with serums of rabbits sensitized with variously treated antigens

Type and serum No.	Method of killing	Titer of reaction with antigen from—	
		P. novicida	*P. tularensis*
P. novicida			
9650	Ether	1:32	1:4
9651	do	1:32	1:1
9652	Heat	1:128	1:4
9654	Formalin	1:64	1:2
9655	do	1:128	1:2
9657	Phenol	1:128	1:2
P. tularensis			
9658	Ether	1:8	1:128
9662	Formalin	1:4	1:128
9665	Phenol	1:2	1:64

Discussion

The organism described has certain characteristics that indicate a relation to the organisms contained in the family Parvobacteriaceae, but it cannot be associated directly with any of the various tribes within the family. Morphologically, it has considerable resemblance to *P. tularensis*, being similar in microscopic appearance, in showing considerable dependence upon growth factors in blood, yeast extract, or cystine, and in characteristic colonial growth on glucose-cystine-blood agar. The lesions produced in experimental animals and the wide range of hosts susceptible to infection increase the apparent resemblance. Both may be found in natural waters of the western States. They differ markedly, however, in immunological characteristics and may be distinguished by either agglutination or precipitin tests. In addition, *P. novicida* ferments sucrose, whereas *P. tularensis* does not.

There has been considerable discussion as to whether or not the etiological agent of tularemia is justifiably included in the genus *Pasteurella*. At present, it would appear best to leave it in the genus *Bacterium* until its taxonomic position is clarified. We would prefer to place the newly isolated organism in the genus *Bacterium* for the same reason. However, the genus name *Bacterium* is a "rejected generic name" by recent (1954) action of the International Committee on Bacteriological Nomenclature (*5*) so it is not available. The only alternative to the use of *Pasteurella* would be to establish a new genus name for the two organisms. This we are not prepared to do at this time.

Further examination of natural waters in the western United States, including Ogden Bay, has not resulted in additional isolations of *P. novicida*. Many cultural studies of the laboratory animals maintained at the Rocky Mountain Laboratory have not revealed the organism to be present in the animal colonies, justifying the conclusion that the isolation of *P. novicida* from guinea pigs and mice inoculated with water represented a valid isolation from the water sample involved.

Summary

A micro-organism highly pathogenic for mice, hamsters, guinea pigs, and rabbits has been isolated from a water sample collected in Ogden Bay, Utah. In gross appearance of cultures, microscopic appearance, and pathogenicity, it closely resembles *Pasteurella tularensis*. It may be distinguished, however, from *P. tularensis* by fermentation studies and by serologic tests. The organism is described and the name *Pasteurella novicida* sp. nov. is proposed.

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