The dissociation of Pasteurella mastitidis
by Peter H Matisheck

A THESIS Subnitted to the Graduate Committee in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology
Montana State University

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The iridescent and white opaque groups produced a small amount of hemolysis on blood agar, but no toxin was found.

No significant differences in carbohydrate utilization between variants were found. The reactions were weak, and somewhat variable. There was no gas production.

The capsule of the encapsulated variants (the iridescent and white opaque groups) was destroyed rapidly by heat, phenol and acid in the medium. The iridescent and white opaque groups were highly pathogenic for sheep and nice, and the blue-grey was low.

Cross agglutination reactions showed considerable group specificity. Possible antigen complex was postulated to account for this. The blue-grey variant was highly antigenic, and showed little group specificity.

This variant shows promise in the development of a good vaccine, and suitable diagnostic test.

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A THESIS

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ABSTRACT

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THE DISSOCIATION OF PASTEURELLA MASTITIDIS (MEISSNER & SCHOOP) HAUDUROY ET AL.

INTRODUCTION

The organism, Pasteurella mastitidis, was first described by Dammann & Freese in 1907, and was known as the Bacillus of Dammann & Freese. In 1932 it was identified with outbreaks of mastitic infections of sheep in Germany, by Meissner & Schoop, who called it Bacterium mastitidis. In the same year Haupt described it as Bacterium ovinum. Hauduroy's Dictionnaire des Bacteries Pathogenes pour l'Homme, les Animaux et les Plantes (1937) lists it as Pasteurella mastitidis Dammann & Freese; and Bergey's Manual (1939) lists it as Pasteurella mastitidis (Meissner & Schoop) Hauduroy et al.

Marsh, in 1932, isolated this organism from mastitic infections enzootic in sheep in Montana. He was the first to report this organism in the United States, identifying it with the Bacillus of Dammann & Freese, and describing it as a Pasteurella. A number of cultures have been studied at this laboratory, and a high degree of variability noted, indicating a need for dissociation studies. This work was begun in the hope that a highly antigenic and serologically non-specific variant, suitable for immunization and serological diagnosis, might be found.

REVIEW OF LITERATURE

The earliest dissociation work in this genus was reported by Manninger (1919) who described an unencapsulated, avirulent, and highly immunogenic variant of the fowl cholera organism. De Kruif (1922), working with a rabbit septicemia *Pasteurella*, described a virulent "D", and an avirulent, rough "G" type. Webster and Burn (1926), working with *Bacterium lepisepticum*, found the same types, and an intermediate "I", and a relatively stable mucoid "M" type. Anderson, Coombes & Mallick (1929-30) found the same variant types in *Bacterium avisepticus*, but used the modern "S" and "R" terminology to replace De Kruif's "D" and "G" types.

Hughes (1930), working with *P. avicida* in relation to the epidemiology of fowl cholera, described fluorescent, blue, and intermediate variants. The fluorescent type was highly virulent, and definitely associated with epidemic fowl cholera. It was stable on blood agar, but on infusion agar it dissociated to the blue type. The blue variant showed no fluorescence, and had no virulence. It was associated with endemic cholera. This variant was stable on solid media, and Hughes believed it to be similar to the "G" form of *P. lepiseptica*. The intermediate type varied in its characteristics from the fluorescent to the blue types, and its virulence varied accordingly. This came from only one outbreak during epidemic and post-epidemic periods. It was very stable. With agglutinnin absorption tests all three types proved to be closely related. Although no capsule could be demonstrated on the fluorescent variant, its inagglutinability with homologous antiserum was believed due to the protection of the capsule.

Priestly (1936) showed that the virulent variants of this organism were encapsulated, and those cultures having greater capsule width were more virulent. The capsule was destroyed almost at once at boiling temperature and disappeared when incubation continued beyond 24 hours at 37 C.

Mørch and Krugh-Lund (1931), and Ochi (1931 and 1933), found variations in hemorrhagic septicemia pasteurellae similar to those described by Hughes. Cornelius (1931), working with P. suiseptica, found a similar change, but used the terminology "I" for the fluorescent, and "A" for the blue variant. The direction of dissociation was like that found by Hughes.

Brigham and Rettger (1935), in a study of the genus Pasteurella, found "S", "R", and "I" variants. Rosenbusch & Merchant (1939) studied the hemorrhagic septicemia pasteurellae. They found "S", "M", and "R" forms, and mention intermediate forms. However, their work was primarily from the standpoint of classification. The variants were not well described, although they were correlated to some extent with biochemical properties. The permanent variants differed in biochemical properties, and immunological behavior. A cyclic behavior in biochemical properties was noted, which the authors did not believe originated with permanent phases.

There have been no reports in the literature relating to the variants of Pasteurella mastitidis. Jasmin, in unpublished work done at this laboratory, found three types of colonies. One was a smooth, translucent colony showing chains of organisms, which he designated as "R" or "IR". This variant had few or no capsules, and was avirulent. Another type, designated "S", showed a bluish-green iridescence, with a high degree of encapsulation and virulence. A third type was a dense colony, very granular with irregular edges and very friable. Encapsulation and pathogenicity were variable.

MATERIALS AND METHODS

The standard culture medium used throughout this investigation is designated as VRL medium. It is a modification of the "hormone" medium described by Huntoon (1918). He avoided filtration in order to retain the growth factors. The VRL medium used in this study requires filtration and therefore does not meet Huntoon's criterian of a "hormone" medium. Pasteurella mastitidis grows well on this medium, and it has the added advantages of being clear and lightly colored. The different types of colonies are well differentiated. The medium is made up as follows:

Beef (round steak)....................	500 g
Distilled water.......................	1000 ml
Bacto-Peptone.........................	10 g
Sodium chloride.......................	5 g
Eggs..................................	2

All fat and connective tissue are cut away, and the meat is ground fine in a meat grinder. The meat, peptone, sodium chloride, and the egg yolks are added to the water and heated with constant stirring to 68 C, no higher. Normal NaOH is added until the medium is slightly alkaline to litmus paper. The pH at this point is about 7.6. Stir in the whites of the eggs beaten up in a little water. Heat in an Erlenmeyer flask in an Arnold sterilizer for an hour. Remove and separate the clot from the sides of the flask with a glass rod. Heat again for an hour. Pour the liquid from the clot and filter. This constitutes the VRL broth used in this investigation. Agar is added to this to give a final concentration of 1.2 per cent agar. The agar is dissolved by autoclaving and the precipitate filtered out. The final reaction should be about 7.2. For use, sterile normal horse serum is added to the cool, liquid agar to give a final concentration of 10 per cent serum. This constitutes the VRLS agar. For VRL agar the serum is omitted.

Beef extract agar is the standard medium used in bacteriological work, with the exception that it contains only 1.2 per cent agar. Beef extract broth is identical with that used in standard bacteriological procedure.

For sugar fermentation reactions, VRL broth was used. To this was added brom cresol purple to give a final concentration of 0.0016 per cent. The broth was adjusted with a glass electrode potentiometer to give a final pH of 7.0. The sugars in 15 per cent solution in distilled water were autoclave sterilized for 15 minutes at 15 pounds pressure. This sterile solution was added aseptically to the indicator broth to give a final concentration of 1.0 per cent sugar.

For the study of variants streak plates were made so that about half the plate showed well isolated colonies. The agar in these plates was no more than 4 mm thick. The colonies were studied and picking was done using a binocular dissecting microscope. The light source was a small adjustable lamp using a 6 volt bulb, connected to the 110 volt line through a transformer. This lamp had a condensing lens that formed a beam of parallel light rays. The correct angle of the light beam to the surface of the agar was found to be about 45 degrees.

The capsule stain used throughout this study was that of Jasmin (1945). His technique consists of mixing a loop of culture with a mixture composed of 10 per cent serum in distilled water or 0.85 per cent salt solution to which 0.5 to 1.0 per cent phenol was added. This was done on a clean glass slide. After the mixture had dried the slide was dipped into absolute methyl alcohol and flamed. The slide could then be stained by the gram technique or by any of the common bacteriological stains. It was found that the film of coagulated serum could be well stained with dilute carbol fuchsin.

EXPERIMENTAL

Isolation and Description of Variants

All of the variants used in this study were isolated from a single culture. This culture (2459) was isolated from the udder of an ewe with a fatal case of mastitis. A 24 hour VRLS agar plate showed colonies with iridescence, and some of a white opaque type. A beef extract agar plate showed the same types, with the white opaque type predominant.

The standard medium for colony study used throughout this study was VRLS agar. It showed maximum growth, and the greatest amount of colony differentiation. Dissociation was produced by growing the original culture in VRL broth plus 10 per cent serum at 37 C for a maximum of 2 months. At various intervals VRLS agar plates were made from the dissociated broth cultures, and the variants were isolated from these. They were stabilized by picking selected colonies daily for a minimum of 2 months. The encapsulated variants were picked for greatest encapsulation, using a capsule stain.

The following descriptions refer to colonies on VRLS agar streak plates, pH about 7.2, and incubated for 24 hours at 37 C. These are the stable variants. Several other types were observed, but they were not sufficiently stable for cultural work.

White opaque:

The colonies are generally large and white opaque with a slight yellowish iridescence at the edge. There may be some wrinkling. On prolonged incubation, the center becomes more opaque, and the edge tends to become transparent. All iridescence is lost. The organisms are large and many have squared ends. They occur in short chains, pairs and singly. Their average dimensions are 1.0 by 0.7 micron. From 10 to 30 per cent of the organisms are encapsulated. These capsules are light and irregular.

Wrinkled:

The colonies are large with some yellow iridescence, opaque, and have a wrinkled surface. The wrinkling is not constant, and is lost on continued incubation. Like those of the white opaque variant these organisms are large and sometimes square ended, but they form longer chains. The dimensions are the same as for the white opaque organisms. From 40 to 60 per cent are encapsulated. The capsule width is about 0.3 micron.

Iridescent:

The colonies are smooth and small, with a yellow iridescence. After 24 hours they lose their iridescence at the center. The organisms are medium in size, ovoid, regular, and occur singly and in pairs. They measure 0.9 by 0.7 micron. From 60 to 100 per cent of the organisms show heavy capsules. The capsule width is about 0.27 micron.

Blue iridescent:

The colonies are smooth, small, and highly blue iridescent. After 24 hours they become blue-grey at the center, and more dense, with iridescence only at the edge. The organisms are small and rod shaped, and occur singly and in pairs. They average about 0.9 by 0.5 micron. From 90 to 100 per cent of the organisms have heavy capsules. The capsule width is about 0.28 micron.

Blue-grey:

These colonies are smooth, small, and somewhat granular, with a blue-grey color. On continued incubation the center becomes white and more granular. The organisms are small and rod shaped, and occur singly and in pairs with no capsules. They average about 0.9 by 0.6 micron.

Blue:

These colonies are small, smooth and blue, with slight granulation. They remain blue on continued incubation, but large colonies become white at the center. The organisms are small and rod shaped, occuring singly and in pairs. From 0 to 10 per cent of the organisms have light capsules. Their average dimensions are about 0.9 by 0.6 micron.

Brittle colonies occur in all these variants. They are opaque with irregular surfaces and edges. On sub-culturing they revert to the parent type in which they were found. When grown on media with a pH below 7.1, they tended to increase in number. Growth on 2 per cent agar media also increased the proportion of brittle colonies. They could not be stabilized.

The variants described above were also isolated from a number of typical cultures of <u>Pasteurella mastitidis</u>, indicating that they are common to this species. Strains recently isolated from acute cases of mastitis contained the encapsulated forms predominantly. In strains from chronic cases the types found were generally non-encapsulated.

Recently isolated cultures held in storage are not stable. After several months the predominant types are no longer identical with those of the original culture. Strain 1500 carried on agar for several years consisted entirely of blue colonies. It was very stable. A culture in VRL broth plus 10 per cent serum stored at 37 C for two months, showed no other variants.

Influence of Environment on Dissociation

The dissociative changes in various media at different temperatures of growth and storage were determined. Two tubes of VRL broth, plus 10 per cent serum, were inoculated with encapsulated organisms from the original culture. These were stored at 37 C. Three VRLS agar slants, three VRL agar slants, three slants of beef extract agar plus 10 per cent serum, and three beef extract agar slants were inoculated as above. One culture of each of these was stored at 37 C, one culture of each at room temperature, and the third culture of each was incubated for 24 hours at 37 C, and stored at 4 C. Streak plates were made from these cultures on VRLS agar, and beef extract agar at various intervals up to 58 days, or until the culture died. The cultures held at 37 C, and those in the media more favorable for growth, i.e. the VRL media, dissociated more rapidly, and showed predominantly the non-iridescent forms. With less favorable media, i.e. the beef extract media, and at the lower temperatures, dissociation was less rapid, and the cultures remained predominantly in the iridescent and encapsulated forms. During this time no one type became stabilized. In comparisons between VRLS agar and beef extract agar as plating media, the beef extract agar showed more iridescent and encapsulated forms than the VRLS agar.

Growth in Various Media

The growth characteristics of the six variants in broth were determined. Tubes of VRL broth, plus 10 per cent serum, were inoculated in duplicate and incubated at 37 C for 6 days. The pH of the broth was 7.3. The results are tabulated in Table I. This experiment was duplicated using beef extract broth plus 10 per cent serum. The pH of the medium was 7.4. The same conditions were maintained. The results are shown in Table II. From growth characteristics in these two media, three general groups are evident: first

Table I

Growth characteristics in VRL broth plus 10 per cent serum.

	7 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
White opaque	cloudy	sediment cloudy	cloudy sediment	cloudy sediment	no change
Wrinkled	cloudy	sediment cloudy	cloudy sediment	cloudy sediment	no change
Iridescent	cloudy	cloudy	cloudy	cloudy sediment	no change
Blue irid.	cloudy	cloudy	cloudy	cloudy sediment	no change
Blue-grey	clear	pellicle filaments	ring sediment	sediment	no change
Blue	clear	pellicle filaments	pellicle sediment	pellicle sediment	no change

At 144 hours all tubes were clear.

Table II

Growth characteristics in beef extract broth plus 10 per cent serum.

	7 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
White opaque	cloudy	cloudy	pellicle sediment	pellicle sediment	no change
Wrinkled	cloudy	cloudy	pellicle cloudy	sediment	no change
Iridescent	cloudy	pellicle sediment	pellicle sediment	ring sediment	no change
Blue irid.	cloudy	cloudy	pellicle cloudy	cloudy sediment	no change
Blue-grey	floc.	pellicle filaments	pellicle sediment	sediment	no change
Blue	floc.	pellicle sediment	pellicle sediment	sediment	no change

At 144 hours all tubes were clear.

the white opaque and wrinkled; second, the iridescent and blue iridescent; and third, the blue-grey and blue. These three groups have previously been indicated by colony characteristics, and cell morphology.

After the above 24 cultures had been incubated for 144 hours, dissociation was studied by plating each culture on VRLS agar. The variants found and their approximate percentages are as follows:

<u>VRL Broth</u>	<u>Beef extract Broth</u>
White opaque	White opaque
Wrinkled	Wrinkled no change
Blue iridescent (98%) & iridescent (2%)	Blue iridescent (60%) & blue-grey (40%)
Iridescent (98%) & blue-grey (2%)	Iridescent (70%) & blue-grey (30%)
Blue-grey no change	Blue-grey
Blue	Blue

It can be seen that all these variants, with the exception of the blue iridescent gave rise to brittle colonies. The blue iridescent variants dissociated to the iridescent variant, and both dissociated to the blue-grey variant. These dissociative changes have been found to be typical of the blue iridescent and iridescent variants. The reverse does not occur under these conditions.

The Effect of Different Media on Colony Characteristics

Colony characteristics of a representative member of each of the three groups were studied on VRLS agar, and beef extract agar. The white opaque, iridescent, and blue-grey variants were grown for 24 hours on VRLS agar, and from that streaked on plates of beef extract agar, and grown for 24 hours. They were finally transferred back to VRLS agar. A second set consisted of two serial transfers on VRLS agar, and served as standards for comparison. All plates were incubated at 37 C, and each colony type was checked for cell morphology with a capsule stain.

The iridescent, and blue-grey variants did not change on one transfer to beef extract agar. On beef extract agar the white opaque variant produced colonies resembling in every characteristic the iridescent. However, this change was not permanent, for on transferring back to VRLS agar, the colonies reverted to the white opaque type.

The white opaque and iridescent variants were more extensively studied on the following 6 media: 1, VRLS agar; 2, beef extract agar with 10 per cent serum; 3, beef extract agar with 20 per cent serum; 4, VRL agar with 5 per cent defibrinated rabbit's blood; 5, medium A; and 6, medium B.

Medium A was composed of:

casamino acids	0.4 g
yeast extract	0.4 g
peptone	0.3 g
proteose-peptone	0.3 g
tryptose	0.3 g
neopeptone	0.3 g
tryptone	0.3 g
sodium chloride	3.0 g
meat extract	0.3 g
distilled water	100 ml
phosphate buffer pH 7.6	2.0 ml
1.2 per cent agar &	
10 per cent serum	

Medium B was composed of :

casamino acids	0.5 g
yeast extract	0.5 g
phosphate buffer pH 7.6	1.0 ml
distilled water	100 ml
1.2 per cent agar plus	
10 per cent serum	

Typical white opaque and iridescent colonies on VRLS agar were plated on each medium and serially transferred on that medium for three transfers. The fourth transfer was made to VRLS agar plates. Plates were incubated for 24 hours at 37 C between transfers. Readings were made after 24 hours incubation and the colonies were checked for cell morphology with a capsule stain. The order of transfer and colony types found are shown in Tables III and IV.

Again the white opaque resembled the iridescent variant on the beef extract media, and on these media the iridescent lost some iridescence and some encapsulation. On media A and B the white opaque variant did not show increased iridescence. In three transfers the variants did not basically

Table III

Colony characteristics of the white opaque variant on various media.

Typical white opaque colonies on VRLS agar were transferred to each of the following:

	1	2	3	4	5	6
1st	typical	smaller & iridescent	smaller & iridescent	typical	more transparent & grey	very small wrinkled & more opaque
2nd	typical	same as above	same as above	same as above	same as above	same as above
3rd	typical	same as above	same as above	small zone of hemolysis	same as above	larger
4th	1	1	1	1	1	1

All colonies were typical white opaque

Table IV

Colony characteristics of the iridescent variant on various media.

Typical iridescent colonies on VRLS agar were transferred to each of the following:

	1	2	3	4	5	6
1st	typical	more iridescent	more iridescent	typical	wrinkled less irid.	small less irid.
	'	'	'	'	'	'
2nd	same as above	same as above	same as above	same as above	same as above	same as above
	'	'	'	'	'	'
3rd	same as above	same as above	same as above	slight hemolysis	same as above	same as above
	'	'	'	'	'	'
4th	1	1	1	1	1	1

All colonies were typical iridescent

change as is shown by the typical colonies on VRLS agar at the end of the series. Both of these variants, when grown for several transfers on blood agar, acquire the ability to produce hemolysis. Hemolysis is more pronounced with the white opaque variant than with the iridescent. The blue and blue-grey variants were grown on blood agar plates, and after four transfers did not show hemolysis. This indicates a basic difference between the encapsulated and non-encapsulated variants.

Toxin Production

Some evidence had been obtained from guinea pigs and mice which indicated the presence of an exotoxin. Several attempts were made to demonstrate a soluble toxin. Each variant was grown on VRLS agar for 24 hours, then washed off and suspended in distilled water, and killed at 55 C for a half hour. A second set was prepared in the same manner, except that the organisms were suspended in physiological saline. These suspensions were incubated for periods up to 35 days. At various intervals the suspensions were centrifuged, and the bacteria-free supernate injected intraperitoneally into mice in doses as high as 0.5 ml. In none of these suspensions was there any evidence of a soluble toxin. Suspensions of living organisms treated in the same manner also gave negative results. A 24 hour VRL broth plus 1 per cent glucose culture, when freed of organisms by centrifuging, and injected into mice, also gave no indication of toxin.

The conditions under which these cultures have been grown differ markedly from those encountered in natural infection, especially regarding CO_2 and O_2 tension. Also the medium may lack certain nutrient factors required for toxin production.

Pathogenicity

A week after isolation the original strain was tested for pathogenicity. A 6-hour culture killed a mouse injected intraperitoneally with a 0.10 ml dose, and 0.50 ml killed a guinea pig. Six-hour cultures of the blue iridescent, and of the white opaque variants were suspended in physiological saline and inoculated into 2 ewes. This was done by swabbing the opening of the teat canal with the culture suspension. Both variants produced severe mastitis. At this time the blue-grey variant also produced severe mastitis. One year later, after stabilization of the variants, both the iridescent and white opaque still produced severe mastitis in ewes. The blue variant produced moderate mastitis, and the blue-grey, mild to none.

After stabilization of the variants more pathogenicity tests were done on mice and guinea pigs. Six to 7-hour VRLS agar cultures were used, and the animals injected intraperitoneally. The cultures were made on VRLS agar slants in standard 15 mm tubes, and the growth in each was suspended in 1.0 ml sterile saline. Death, in most cases, occured in less than 24 hours. The results are summarized in Table V.

Table V.

Pathogenicity of Variants

Culture	Animal		mld
parent culture	mouse		0.05-0.10 ml
	g.p.		0.25-0.50 ml
white opaque	mouse		0.05 ml
	g.p.	less than	0.25 ml
wrinkled	mouse		0.05 ml
	g.p.	less than	0.25 ml
blue iridescent	mouse		0.05 ml
	g.p.	less than	0.25 ml
iridescent	mouse		0.10 ml
	g.p.		0.25-0.50 ml
blue-grey	mouse		0.15-0.25 ml
	g.p.		0.50 ml

The white opaque, wrinkled, and blue iridescent variants were about equally pathogenic, although the blue iridescent variant did not produce death as quickly as the first two. The pathogenicity of the iridescent variant was somewhat lower. The least pathogenic of these variants was the blue-grey. This general order agreed with the results obtained with ewes. The value of pathogenicity tests using mice or guinea pigs is doubtful since in sheep the route and type of infection is entirely different. These tests, however, do give information on the relative order of pathogenicity of the different variants.

Agglutination Reactions

In cross agglutination tests between different strains of Pasteurella mastitidis at this laboratory there was found to be a great lack of homogeneity. It was thought that this lack of homogeneity was due to the predominance of different variants in different strains. In order to determine the agglutinnin specificity of the variants, cross agglutination tests were done.

Preparation of Antisera.

Rabbits were injected in duplicate, intravenously, at 5-day intervals, starting with 0.05 ml killed culture, and doubled until 2.0 ml was reached. Live cultures were then injected i.v., starting with a 0.10 ml dose and doubled until 2.0 ml was reached. This dose was repeated, and two more doses of 4.0 ml and 8.0 ml were given. For the killed cultures the 16-18 hour growth from VRLS agar slant in a standard 15 mm tube was suspended in 1 ml sterile saline after discarding the supernatant fluid. This suspension was killed at 55 C for 30 minutes. The live cultures were obtained in the same way; except that they were not heated. About 10 days after the last injection the rabbits were bled, and the serum obtained. Some of the sera were preserved with

1 per cent chloroform and some with 1/10,000 merthiolate.

<u>Preparation of Antigens</u>

Just before the antigens were prepared, the variants used were picked several times to insure purity of the cultures. The parent culture was taken directly from a lyophilized tube. Single colonies of each culture were picked to VRLS agar slants. After 7 hours at 37 C the condensation fluid was discarded, and the growth suspended in sterile saline. The growth from one slant was inoculated into a 16 oz. prescription bottle containing 1.5 per cent VRLS agar. After 18-20 hours at 37 C the growth was washed off with 40 ml sterile saline plus 0.5 per cent phenol. The organisms were killed at 55 C for 1 hour, then washed three times in the centrifuge. The growth was suspended in phenolated saline, and the suspension was adjusted to 6 cm on the Gates nephelometer. These antigens were used for the agglutination tests.

The lowest dilution of antiserum used in these tests was 1-25. The dilution was doubled in each successive tube. The reactions were read after 48 hours incubation at 37 C. The endpoint was taken as the highest dilution of anti-serum at which complete agglutination took place. The titres obtained are only approximate. The cross agglutination reactions between the variants are summarized in Table VI.

Table VI

Cross agglutination reactions between the six variants and the parent culture.

Antigens

Sera	White opaque	Wrink.	Blue	Blue-grey	Blue irid.	Irid.	Parent Culture
Parent Culture	25	50	50	1600	3200	6400	12,000
White opaque	200	200	100	200	100	200	400
Wrinkled	400	800	200	200	200	800	400
Blue	800	1600	800	800	25	800	1600
Blue-grey	200	200	200	3200	3200	1600	3200
Blue irid.	50	50	50	400	800	3200	1600
Irid.	25	50	25	100	100	400	400

The above titres are the greatest dilution at which complete agglutination occurs after 48 hours incubation at 37 C.