CULTURAL REQUIREMENTS OF THE FOWL-CORYZA BACILLUS

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DeBlieck (1932), Nelson (1933), Eliot and Lewis (1934), Delaplane and Stuart (1934) and Schalm and Beach (1934) have reported the isolation from chickens affected with infectious coryza on farms in Holland, New Jersey, Maryland, Rhode Island and California, respectively, of a hemophilic bacillus with which a coryza of short duration can regularly be produced in susceptible chickens. DeBlieck pointed out its similarity to the influenza bacillus of man, both in its morphology and cultural requirements, and proposed the name Bacillus haemoglobinophilus-coryzae-gallinarum. Eliot and Lewis proposed the binomial Hemophilus gallinarum. Delaplane has expressed his willingness to accept this latter name, but Nelson (1935) expressed his opinion that there is as yet insufficient knowledge concerning this organism to warrant such a definite classification.

The fowl-coryza bacillus is a small, Gram-negative, non-motile rod which is highly pleomorphic and frequently develops into irregularly staining filaments, often of remarkable length. On two occasions branching of these filaments was observed. The bacillus shows a marked tendency to stain more heavily at the poles than in the center, and frequently a deeply staining granule is observed at each pole, while the remaining portion takes the stain less and less as the center is approached. In cultures more than twenty-four to forty-eight hours old, the bacteria usually are swollen, stain faintly and finally become fragmented so that only shapeless masses are found. This degeneration has been observed in cultures only eighteen hours old, but this is exceptional. The phenomenon has been noted on all media on which growth has been obtained.
Davis (1917, 1921), Thjotta and Avery (1921), Fildes (1921) (1922), and Rivers and Poole (1921) have shown that *Hemophilus influenzae*, the type species of the genus *Hemophilus*, requires for its growth two accessory factors in addition to a nutrient medium. These factors were likened to vitamins A and B by Davis, and later were designated as X and V factors by Thjotta and Avery. The X factor is heat stable, the V factor resists boiling for five minutes but is destroyed by autoclaving. Hemoglobin is the principal source of the X factor, although it is present to some extent in certain vegetable tissues, especially potatoes. The V factor is more widely distributed in nature, being produced by nearly all species of bacteria and present in perhaps all fresh animal and vegetable tissues. Blood is an excellent source of both factors, the X factor being confined to the red blood cells and the V factor present in both the red cells and the serum.

**CULTURE MEDIA AND SOURCES OF X AND V FACTORS**

*Boiled blood agar.* (Levinthal’s medium, 1918.) This contains both the X and the V factors and was prepared as follows: Defibrinated blood was added to melted nutrient agar in sufficient quantity to produce a concentration of 8 per cent. The mixture was then boiled for several minutes and filtered through sufficient thicknesses of sterile cheesecloth to remove the coagulated particles. The cultures used in the experiments reported in this paper had been grown for many generations on this medium, which, when properly prepared, is transparent and free of blood pigments. Since much of the hemoglobin is removed in the preparation of boiled blood agar, it should contain less of the X factor than fresh blood agar consisting of 8 per cent defibrinated blood in an agar base made with beef extract. This is an important consideration, for Davis (1907) has shown that the amount of X factor required for growth of hemophilic bacilli is very small, and Thjotta and Avery (1921) that enough of the X factor to support growth of a hemophilic organism may be carried over in making transplants from a medium containing an abundance of this factor to one deficient in it.

1 Horse blood used exclusively. All media adjusted to pH 7.4.
On boiled blood agar, the colonies are convex, smooth, clear to slightly opaque and glistening, and vary in diameter from 0.05 mm. or less to 0.6 mm., while on fresh blood agar, the colonies are smaller, rarely exceeding 0.3 mm. in diameter, less convex, more opaque, dull to slightly glistening and show a marked tendency to undergo rough transformation. While the reason for the difference in the size and character of the colonies of the organism on the two media is not clear, it might be that fresh blood contains a factor which has an inhibiting influence on the growth of the organism and which is destroyed by heat, and, therefore, not present in boiled blood agar.

*Autoclaved blood agar.* This contains only the X factor, and was prepared in the same manner as boiled blood agar except that it was autoclaved at 15 pounds for thirty minutes.

*Autoclaved blood extract.* This contains only the X factor, and was prepared according to the method of Rivers and Poole (1921) as follows: A clot from 500 cc. of blood was infused with 200 cc. of 0.85 per cent saline solution, boiled, filtered through cheese-cloth, tubed and autoclaved at 15 pounds for thirty minutes.

*Serum from defibrinated blood.* This contains both the X and the V factors. Freshly-drawn blood was defibrinated, centrifuged at a high speed for twenty minutes, and the supernatant serum removed to sterile test tubes and stored in the refrigerator. The defibrination procedure breaks up some of the red cells and thus liberates some of the X factor into the serum.

*Serum from clotted blood.* This contains the V factor only. Blood was allowed to clot in sterile tubes, and then centrifuged at a high speed for twenty minutes. The supernatant serum was transferred to sterile tubes and stored in the refrigerator.

*Washed red blood cells.* This contains both the X and the V factors. The blood cells remaining in the centrifuge tube, after removal of the serum in preparation of serum from defibrinated blood, were washed three times by resuspending them in saline and centrifuging. After the third washing, the cells were suspended in a volume of saline equal to that of the serum, and stored in the refrigerator.
GROWTH OF THE FOWL-CORYZA BACILLUS ON BOILED OR FRESH BLOOD AGAR PLATES OR SLANTS INCUBATED AEROBICALLY, IN AN EXCESS OF CARBON DIOXIDE, OR WITH THE PLATES AND TUBES SEALED WITH CLAY OR PARAFFIN

DeBlieck, Delaplane and Stuart, and Eliot and Lewis have reported no difficulty in obtaining growth of the fowl-coryza bacillus under aerobic conditions. Nelson, however, stated that he was unable to grow his organism on open plates but succeeded very well when the plates were sealed with clay. Schalm and Beach reported failure to cultivate the organism either aerobically or on sealed plates, but obtained good growth when the cultures were placed in an atmosphere of 10 per cent carbon dioxide. The writers have since investigated this problem further with cultures obtained from Nelson and from Delaplane and with California strains of the fowl-coryza bacillus. Regardless of source or strain, the organism colonized not at all or very meagerly on open plates or slants incubated aerobically, but satisfactory growth was obtained on sealed plates or slants or on unsealed plates or slants in sealed jars containing ordinary air or 10 per cent carbon dioxide. The growth of the bacillus on the sealed plates or slants might be explained on the basis of Rockwell's (1927, 1928) theory that carbon dioxide is the principal source of the carbon required by bacteria for multiplication; that the carbon dioxide produced through the metabolic activities of bacteria may be used by them as a source of carbon; and that when the carbon dioxide produced through metabolic activity is not allowed to escape from culture plates or tubes the concentration of it in the contained air would be increased and the growth of certain species of bacteria thereby favored. In the experiments presented herein all cultures were incubated in sealed jars containing 10 per cent carbon dioxide.

CULTURES USED IN THE EXPERIMENTS

Twelve different cultures of the fowl-coryza bacillus, representing a New Jersey strain, a Rhode Island strain and six California strains, were employed. These cultures varied in their period of cultivation on artificial media which ranged from 4 to 157
transfers made every 4 days. All cultures were maintained on boiled blood agar slants with broth at the base. The growth was washed from the slant into the broth and a 2 mm. loopful was transferred to the test medium. In one experiment, single colonies were transferred from a boiled blood agar plate to the test medium.

**TABLE 1**

*Growth of the fowl-coryza bacillus on a medium containing only the X factor compared with its growth on a medium containing both the X and the V factors*

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>FOUR-DAY TRANSFER NUMBER</th>
<th>GROWTH AFTER SEVENTY-TWO HOURS INCUBATION*</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Boiled blood agar slant (X and V factors)</td>
<td>Autooclaved blood agar slant plus 1 cc. horse serum (X and V factors)</td>
<td>Autooclaved blood agar slant plus 1 cc. plain broth (X factor)</td>
<td></td>
</tr>
<tr>
<td>Calif. 1</td>
<td>114th</td>
<td>+++</td>
<td>++</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Calif. 3</td>
<td>70th</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Calif. 1</td>
<td>54th</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Calif. 4</td>
<td>54th</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Rhode Island N 37</td>
<td>20th</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Calif. 4</td>
<td>18th</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Rhode Island N 37</td>
<td>18th</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Calif. 6</td>
<td>6th</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*+++= Good growth.
+++ = Fair growth.
+ = Slight growth.
0 = No growth.

**GROWTH OF THE FOWL-CORYZA BACILLUS ON A MEDIUM DEVOID OF BOTH X AND V FACTORS**

Seven cultures, representing 1 New Jersey, 1 Rhode Island and 4 California strains, were employed. Transfers were made to plain nutrient agar which contained neither of the two factors and to boiled blood agar slants which supplied both factors. These cultures were incubated at 37°C. for ninety-six hours. The plain agar failed to support growth of any of the cultures, but a very good growth of all cultures was obtained on the boiled blood agar controls.
GROWTH OF THE FOWL-CORYZA BACILLUS ON A MEDIUM CONTAINING X FACTOR ONLY

The cultures and media shown in table 1 were employed. It is seen that the bacillus failed to grow on autoclaved blood agar.

TABLE 2

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>FOUR-DAY TRANSFER NUMBER</th>
<th>GROWTH AFTER SEVENTY-TWO HOURS INCUBATION*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calif. 3</td>
<td>104th</td>
<td>++</td>
</tr>
<tr>
<td>Calif. 6</td>
<td>54th</td>
<td>+++</td>
</tr>
<tr>
<td>Rhode Island N 37</td>
<td>50th</td>
<td>+++</td>
</tr>
<tr>
<td>New Jersey B 5504</td>
<td>40th</td>
<td>+</td>
</tr>
<tr>
<td>Calif. 5</td>
<td>4th</td>
<td>+</td>
</tr>
</tbody>
</table>

* The relative degree of growth was ascertained by streaking a 2 mm. loopful of each culture on a boiled blood agar plate which was incubated at 37°C. for 48 hours.

+++ = 100 or more colonies.
++ = 50 to 100 colonies.
+ = 25 to 50 colonies.
0 = No growth.

plus broth which contained the X factor only, but when, instead of broth, serum, to supply the V factor, was added, colonies developed which were of microscopic size, flat and dull, and largest near the serum at the base of the slant. On the boiled blood agar controls, the colonies were smooth, convex, slightly opaque and glistening, and varied from 0.05 mm. or less to 0.4 mm. in diameter.
GROWTH OF THE FOWL-CORYZA BACILLUS IN A FLUID MEDIUM CONTAINING X FACTOR ONLY, V FACTOR ONLY, AND BOTH FACTORS

The cultures and media to supply the X and V factors alone or in combination, which were used in this experiment, are given in table 2.

No macroscopic change occurred in any of the culture tubes; so, to obtain evidence of bacterial growth, a loopful of each culture was streaked on a boiled blood agar plate which was incubated at 37°C for forty-eight hours. All cultures failed to develop in plain broth or in plain broth plus autoclaved blood extract (X factor only) or in plain broth plus serum from clotted blood (V factor) but in the media containing both X and V factors, growth of all strains was obtained. It is seen, however, that the medium containing washed red cells supported growth less effectively than that containing serum from defibrinated blood or that containing serum from clotted blood plus autoclaved blood extract, and that in the case of a recently isolated California strain 5, no growth occurred in the medium containing washed red blood cells. A possible explanation of this difference is that much of the X and the V factors remained within the cells instead of being dispersed through the broth. The comparatively weak growth obtained in any instance indicates that none of the media were favorable to the organism, but the results, nevertheless, show that both the X and the V factors must be present before the bacillus can develop.

GROWTH OF THE FOWL-CORYZA BACILLUS ON PLATES CONTAINING X FACTOR FROM BLOOD AND V FACTOR FROM A BACTERIAL COLONY

The sources of the X and the V factors and the cultures employed in this experiment are shown in table 3.

A single colony of the fowl-coryza bacillus was streaked on each plate of test medium, and, to provide the V factor to some of them, the center of the seeded area was inoculated with Serratia marcescens.

No growth appeared on the media containing the X or the V
factor alone but colonies invariably developed when both of these factors were present. The colonies of the fowl-coryza bacillus on the autoclaved blood agar plates having a *Serratia marcescens* colony varied in size and shape according to their distance from this V-factor-producing colony, those within 0.5 cm. of it being smooth, convex and clear to slightly opaque and from 0.15 to 0.25 mm. in diameter; those at a distance of 1 cm. being of the same appearance but not exceeding 0.1 mm. in diameter, and those from 1.5 to 2.0 cm. distant being microscopic in size, flat, and often dull and rough.

**TABLE 3**

*Growth of the fowl-coryza bacillus on plates containing X factor from blood and V factor from a bacterial colony*

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>FOUR-DAY TRANSFER NUMBER</th>
<th>GROWTH AFTER FORTY-EIGHT HOURS INCUBATION*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calif. 1</td>
<td>112th</td>
<td>+++</td>
</tr>
<tr>
<td>Rhode Island N 37</td>
<td>79th</td>
<td>+++</td>
</tr>
<tr>
<td>Calif. 6</td>
<td>77th</td>
<td>+++</td>
</tr>
<tr>
<td>New Jersey B 5504</td>
<td>65th</td>
<td>+++</td>
</tr>
<tr>
<td>Calif. 1</td>
<td>24th</td>
<td>+++</td>
</tr>
<tr>
<td>Calif. 8</td>
<td>14th</td>
<td>+++</td>
</tr>
</tbody>
</table>

*+++ = Good growth over entire seeded area.  
+ = Growth only in the immediate vicinity of the *S. marcescens* colony.  
0 = No growth.

This experiment clearly demonstrates the necessity of both the X and the V factors for growth of the fowl-coryza bacillus on an artificial medium. It also shows, as the previous experiments have done, that even after prolonged cultivation on culture media, the organism still required the presence of the two factors for growth.

**DISCUSSION AND SUMMARY**

Experiments are reported in which an effort was made to determine whether the fowl-coryza bacillus requires the presence of
both the X and the V factors for growth on or in an artificial medium. Twelve different cultures, representing 1 New Jersey, 1 Rhode Island and 6 California strains, were studied. These cultures varied in their period of cultivation on artificial media for from 4 to 157 transfers made every four days.

It was found that, regardless of strain or age, all cultures of the fowl-coryza bacillus required the presence of both the X and the V factors for growth on or in an artificial medium. On this basis, the fowl-coryza bacillus is to be classed in the genus Hemophilus. The name Bacillus haemoglobinophilus-coryzae-gallinarum, suggested by deBlieck, describes the organism satisfactorily, but, because of its length, is awkward and contrary to the rules of nomenclature. The binomial Hemophilus gallinarum, proposed by Eliot and Lewis, seems better suited for this organism.

REFERENCES

Davis, D. J. 1907 Jour. Infect. Dis., 4, 73.