THE ISOLATION AND STUDY OF SPHAEROPHORUS NECROPHORUS FROM BOVINE LIVER ABSCESSES

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Abscessed bovine livers are condemned for human consumption and are made into tankage. Over the nation about five per cent of all beef livers are abscessed (1). Loss from the condemnation of the infected livers has been estimated by federal agencies at several million dollars a year (2).

The abscesses start as very small necrotic areas, which grow until they may be over an inch in diameter (3), and then, in the course of about 180 days, regress and leave insignificant scar tissue. Most of the abscesses project into the surface of the livers and some fairly large ones protrude on both the dorsal and ventral surfaces of the lobe in which they are located. The smaller ones are spherical and the larger are oblate spheroids.

The developed abscesses are surrounded by a tough connective tissue capsule, and the entire abscess may be dissected from the surrounding tissue with little or no loss of contents, and the abscesses are readily stored in the frozen state after being dissected out. The center of the abscess shows purulent necrosis and liquefaction. One of the abscesses, which we have kept at about forty degrees below zero, for over two years, was cut along its diameter, and we are still growing the causative bacteria from the abscess.

Many workers have complained of considerable difficulty in isolating and maintaining the bacteria which are isolated from these abscesses. As we have developed methods in our laboratories which seem reasonably simple and reliable, we take this opportunity to describe our procedures and results.

Bits of the frozen abscess are chipped off with a sterile scalpel and collected in a sterile sheet-aluminum boat until about half a gram of material has been obtained. We generally undertake to include in the sample bits of both necrotic center and outer capsule of the abscess. The sample is weighed and removed to a Ten Broek tissue grinder of about seven ml capacity, where it is ground with nine volumes of a suitable liquid culture medium until it is all homogenized excepting a small bit of white, fibrous material. The resulting homogenate is regarded as being approximately a 1:10 dilution of the original abscess.

One ml of the homogenate is added to nine milliliters of liquid medium in a screw-capped 15 x 125 mm test tube, and careful mix-

ing results in a 1:100 dilution of the original abscess. Serial decimal dilutions are then prepared out to one part in ten billion in the screw-capped tubes. In order to minimize the inclusion of oxygen in the medium, mixing, while thorough, is done as gently as possible. The dilution series is placed in the thirty-seven degree incubator for observation.

In twenty-four hours, the first members of the dilution series have developed strong turbidity and have evolved a great deal of gas. The cultures have the foul odor which is characteristic of the growth of this bacterium. By forty-eight hours, individual masses of growth, which might be called colonies, appear in the depths of the other tubes in the series. These colonies appear from the very first as elongated masses of growth, like those shown in the illustration, only much smaller. During continued incubation, the colonies continue to increase in both length and diameter, and after the fourth day may show diffuse growth at their upper ends. A certain amount of gas may be evolved in these tubes without upsetting the structure of the culture nor the integrity of the individual colonies.

The higher dilutions of the series finally contain, during the second or third day, one to a few discrete areas of growth similar to the two illustrated. Because of the agar in the medium, the tubes may be handled, and even transported some distance with care, without causing the loss of the individuality of the colonies. Of course, one may desire to work with colonies which have not been exposed to the risk of mixing with other colonies in the same tube, in which case several replicates of the higher dilutions of the series may be prepared, with reasonable certainty that single colonies will appear in several of the tubes.

The choice of culture medium should be discussed at this point, as none of the conventional anaerobic media which we have tried has been entirely satisfactory. The feasability of the formation of the colonies in the liquid media depends upon the fact that many of the liquid anaerobic media have in them a fraction of a per cent of agar, to hinder convection and to help to confine dissolved oxygen to the upper portions of the tube. The growths which are initiated in such media, especially from non-motile bacteria, tend to remain in the place where they originate. These masses of growth are generally elongated, and tend to grow from the depths upwards. The result is the linear colonies illustrated.

Difco's thioglycollate medium was the most obvious first attempt, and occasional good results were obtained. The growth was very unpredictable, however, and it appeared, on the one hand, that freshly-autoclaved medium contained some unknown toxic substance which tended to become less effective with the passage of a few days' time, and on the other, that as the medium aged, it took

up oxygen to the extent that it soon became unsuitable for the propagation of anaerobes.

In freshly autoclaved thioglycollate medium, growth could usually be initiated only from such large inocula that the individual colonies could not be seen, whereas in media aged a few days, a third to a half of the tubes containing small inocula would show growth. These would appear in a random manner throughout the dilution series.

Diluting the thioglycollate medium with brain-heart infusion with a little agar added somewhat alleviates the difficulties so that more reliable results may be obtained, but the same faults still persist. Brain-heart infusion with a little agar added used alone fails under ordinary handling to provide an environment sufficiently anaerobic.

Difco A-C Medium, which contains ascorbic acid to provide the reducing environment required for anaerobic growth, shows no toxicity towards the organisms, but it contains too much agar, so that it is difficult to make dilution series in the medium, and also the reducing effectiveness of the ascorbic acid is rather short-lived. A combination consisting of three parts of the A-C medium and one part of N.I.H. thioglycollate medium provides the ideal material for primary isolation from the abscesses, for determining numbers of organisms, or for serial maintenance of the cultures. As the N.I.H. thioglycollate medium contains no agar, it can be mixed with the A-C medium to reduce its viscosity. Meanwhile, the larger volume of the A-C medium seems to eliminate completely the irregular toxic effect of the thioglycollate. The mixture of the two media result in a product which seems not to take up atmospheric oxygen as rapidly as does the A-C medium, whose useful life after autoclaving is rather short.

The medium finally chosen for the primary isolation of this fastidious organism, then, is best prepared by making up 750 ml of Difco A-C medium according to the instructions on the container, and 250 ml of N.I.H. thioglycollate, and mixing the two together. The mixture is then distributed in 9 ml quantities in 15 x 125 mm screw-capped tubes. The caps are screwed down tightly and the medium is autoclaved in the usual manner.

The illustration shows how it is possible to obtain individual colonies of the Sphaerophorus in the semi-liquid culture media. One may reach into the depths of the culture medium in the test tube by means of a sterile 1 ml pipette and a rubber bulb, and remove one of the colonies for sub-culture. Again, the viscosity of the medium allows the removal of all of one colony without disturbing a near-by colony in the same medium. It is possible to separate two or more different colonies by this means for sub-culture into flasks.

Sub-culture is accomplished in ordinary brain-heart infusion, with no added agar or added reducing agents, in a 125 ml Florence flask. Several of the flasks are charged with 100 ml each of the medium, and are capped with aluminum foil and autoclaved in the standard way. While the autoclave and the flasks are still very hot, one flask is filled to within less than a centimeter of the top of the neck by pouring hot medium from another flask. The foil cap is replaced and the spherical part of the flask is immersed in cold water until testing with the hand shows that the medium is cooled down to forty degrees centigrade or less. The foil cap is again removed, and the colony drawn from the test tube of special anaerobic medium is pipetted into the depths of the freshly cooled medium in the Florence flask.

The foil is replaced again and the flasks are placed in the thirty-seven degree incubator. The accumulation of atmospheric oxygen in the fundus of the flask is sufficiently slow, because of the small neck, the foil cap, and possible reducing substances in the medium, so that the anaerobe has a chance to grow until it can adjust the oxidation-reduction potential of the medium to its own needs by means of its own metabolites.

In about 24 hours, growth is well established in flasks inoculated as described. Gas and a floccular sediment are produced. In another few hours, the growth will be diffused throughout the flask, and very turbid, as though containing several billion bacteria per ml of medium. The evolution of gas provides an oxygen seal and probably helps to maintain anaerobosis. This evolution of gas continues during the increase in turbidity of the medium and for a while after growth seems to have stopped.

Flasks of any desired size can be inoculated as described, and as large a quantity of the organisms may be grown as is desired, for chemical fractionation, for use as antigen, or for sub-culture to other media.

In the early stages, the floccular growth consists mostly of long filaments. When maximum turbidity (hence, presumably, maximum number of viable organisms) is attained, the filaments tend to fragment into rods of variable length. If the bacteria are used at this stage for agglutination tests with sera from various cattle, it can be shown that the several isolates from a single abscess may be immunologically distinct. This poses interesting questions requiring further study.

Tube agglutinations may be set up easily, using serial dilutions of appropriate sera in physiological saline, and the culture just as it is in the flask, as the antigen. It is also possible to dye the bacteria, while in the flask, by adding hematoxylin, followed by a trace of ferric chloride. The resulting purple bacteria may be spun

out in a centrifuge, washed once in distilled water, and resuspended in saline. The resulting purple antigen may be used in milk for a ring test similar to the screening test for brucellosis used in dairy herds. The perfection, comparison, and evaluation of these two tests are still in progress.

We wish also to mention briefly the work we have done in adapting the *Sphaerophorus necrophorus* to serial passage in the laboratory white mouse. We found, first, that intraperitoneal inoculation of cultures either from the tubes or from the flasks results in multiple internal abscesses, with some tendency towards progressive necrosis at the site of inoculation, with adhesions between the abscesses of the visceral organs and the abdominal wall, and occasional perforation of the abdominal wall. Careful serial passage of tissue homogenates from the lesions in the mouse has resulted in strains which tend, when given in small inocula, to induce abscess formation in the livers of the mice. Unfortunately, this does not afford an accurate model for the pathogenesis in cattle, as the liver abscess in the mouse tend not to be self-limiting, and appear to lack the connective tissue capsule found in the bovine liver. A visceral abscess may grow by direct extension until it causes the death of the mouse by destroying a vital organ.

Interesting results have followed the inoculation of the bacteria intracerebrally in the mouse. Serial passage of the brain abscess homogenates has resulted in a strain of the *S. necrophorus* which

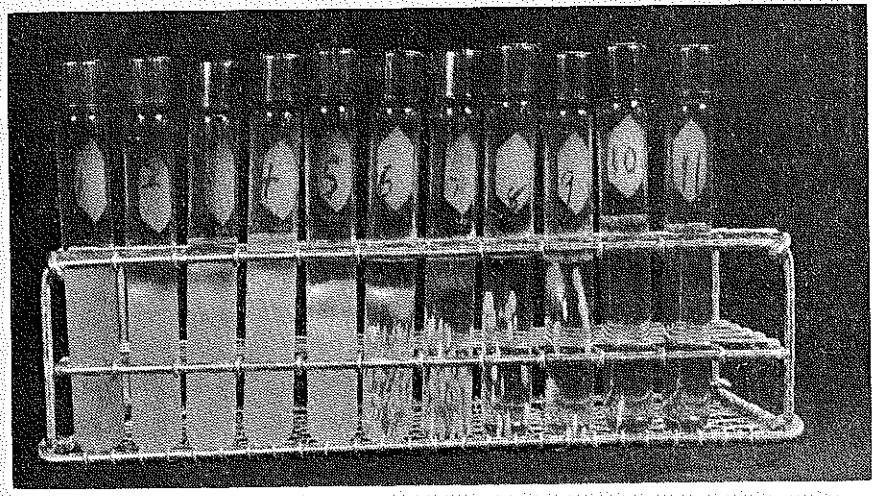

Figure 1. Serial decimal dilutions of liver abscess in one of the special media described in the text. Tube 10 shows a single colony, giving some idea of the numbers of *S. necrophorus* present in the lesions.

will kill when about twenty thousand of the bacteria are inoculated. This compares very favorably with the large number of cells of other pathogens, which are the lethal dose following intracerebral inoculation in the mouse. Many virulent strains of pneumococci require larger inocula than this for intraperitoneal inoculation.

It is difficult to say, at this time, what lines of approach may finally elucidate the problems relating to the pathogenesis and ecology of *S. necrophorus* infection in cattle, but the methods of study which we have developed seem to be promising means for attacking these problems.

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