SOME OBSERVATIONS ON THE ISOLATION, CULTIVATION AND VARIATION OF SPHEROPHORUS NECROPHORUS ASSOCIATED WITH INFECTIOUS ATROPHIC RHINITIS, LIVER ABSCESSES AND NECROTIC ENTERITIS

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In a recent paper the authors reported the isolation of *Spherophorus necrophorus* (1) from the turbinates and ethmoids of swine affected with infectious atrophic rhinitis. Besides its relation to rhinitis this observation seemed significant in view of the fact that it has become customary to associate this organism with processes involving considerable necrosis. Atrophic rhinitis of swine in the form observed in Ontario is not characterized by marked necrosis. It is considered probable that the pathogenic potentialities of this bacterium have not been fully realized and that greater familiarity with successful methods of isolation and cultivation would facilitate its recovery and identification.

We are indebted to Klieneberger (2) and Dienes (3) for their elucidation of the remarkable variation observed in strains of *Bacteroides*. In a number of reports they have shown that these organisms may possess a growth phase which resembles in many respects that of the pleuropneumonia-like group of organisms. It is generally referred to as L type growth and consists of the production in bacterial cultures of large bodies resembling yeast cells. Each large body arises as a result of the union of granules produced by bacillary forms. The large bodies break up to discharge either bacilli or small granules. Pairs of granules unite to form large bodies again. The L type growth constitutes the continuous production of large bodies and granules without the appearance of bacilli. The L forms of *Bacteroides* strains produce fluffy colonies in Brewer's medium and those studied by Dienes and Weinberger (4) reverted to the bacillary phase on prolonged incubation. The production of L growth can be stimulated in many species by adverse circumstances, e.g., penicillin, homologous antibody, and in some instances anaerobiosis.

In reports (5, 6) and text books dealing with the isolation and cultivation of *S. necrophorus* the colonies observed are usually described as small, round and convex, resembling those of *Haemophilus influenzae*. These colonies have been obtained in small numbers on horse blood agar from cases of necrotic enteritis and bovine liver abscesses. Besides these pin point colonies L type colonies can usually be seen in large numbers from morbid material. They have been observed in cultures from seventy-one cases of atrophic rhinitis. Because of concomitant saprophytic bacteria they are difficult to obtain in pure culture directly from the nose of the pig.

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In order to eliminate saprophytes infectious nasal material was injected into the subcutis of rabbits in the manner described previously (1). When a marked lesion developed the rabbit was destroyed and cultures were made from the subcutis. On anaerobic blood agar the predominant colony was again the minute L type. They were readily detectible with the aid of the dissecting microscope (X75) on blood agar and on serum agar with the lower power of the conventional microscope after removal of the condenser.

A number of colony types have been observed on blood and serum agar. There is the small, round or irregularly shaped, glistening colony referred to frequently in the literature. When culture plates are examined at 24 hours the incipient, small, glistening colonies are readily detectible. However, the great majority of these colonies take on a granular appearance (see Fig. 9) on further incubation as their components are transferred to L variants. At 48 hours many of these colonies lose their original shape and texture and appear as pyramidal projections and pits in the agar. Autolysis appears to have taken place and growth has proceeded into the agar. Very minute L type colonies (see Fig. 9) which have arisen from L elements and not bacilli, can be seen in platings of morbid material as well as from cultures of L forms which have shown no tendency to revert to the bacillus.

Growth of *S. necrophorus* in fluid media was obtained by dropping sections of agar containing colonies into thioglycollate broth containing 10% horse serum. The L growth is lightly turbid, fluffy, and can easily be overlooked when not compared with the medium of an uninoculated tube. Growth can usually be discerned after 24 hours incubation and the peculiar morphological elements are demonstrable in hanging drops of cultures containing a small loopful of Loeffler's methylene blue or in Giemsa-stained smears.

In our work it has been customary to inoculate 6-10 tubes of thioglycollate from each blood agar plate. The largest numbers of early bacillary cultures have been obtained when young colonies, 9-18 hours, have been seeded into thioglycollate. The bacillary phase has appeared in some tubes within 48 hours and in others after incubation for as long as fifteen days. In many instances only L type growth was obtained in more than half of the tubes inoculated. Our observations have been based on the inoculation of several hundred tubes of thioglycollate medium.

The first indication of bacillary growth in fluid thioglycollate was evidenced by the appearance of small polypi (see Fig. 1) in the anaerobic portion of the culture tube. As these polypi increase in numbers and size, small bubbles of gas rise to the surface of the broth. After several subcultures in Brewer's medium growth is more rapid and diffuse, and large amounts of gas are produced. When plated on blood agar the more rapidly growing cultures produce a predominance of the regular, non-L type colony. After seventy-two hours incubation many of the colonies are as large as those of some strains of

Pasteurella multocida. The organisms from these colonies are principally cocci, coccobacilli, short and long rods. Remarkable pleomorphism can be observed from broth cultures and agar colonies at different periods of incubation. The

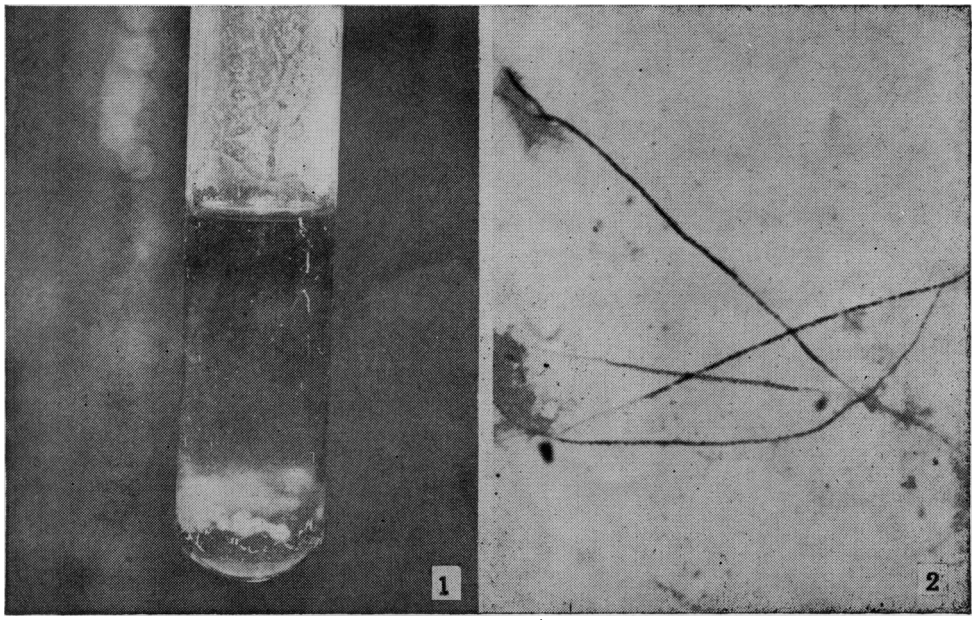

Fig. 1. Growth of *S. necrophorus* in serum enriched Brewer's medium.
Fig. 2. Long filaments displaying dark staining granules. Gram's method, X970.

long filaments shown in Fig. 2 possess grams-positive granules and are characteristic of young bacillary cultures in Brewer's medium.

The morphological elements of the L phase of *S. necrophorus* on solid medium can be demonstrated by special techniques. One of the methods employed for many of the preparations shown in the photomicrographs involved the formalin fixation of colony impressions on coverslips and their subsequent staining with dilute methylene blue or carbol fuchsin, and finally mounting in balsam. All photomicrographs with the exception of Fig. 1, 2 and 10 were prepared from the growth of *S. necrophorus* isolated from a case of necrotic enteritis. Fig. 1, 2 and 10 were from cultures of a strain of *S. necrophorus* isolated from a case of atrophic rhinitis.

Fig. 3 shows part of a colony after six hours incubation. It is small and consists of masses of filaments. The periphery of the colony in Fig. 3 is displayed under higher magnification in Fig. 4. Marked beading is evident in the filaments and numerous large bodies can be seen.

In Fig. 5 a colony is shown after eighteen hours incubation. With the dissecting microscope it appeared as smooth, entire, convex, and glistening. The

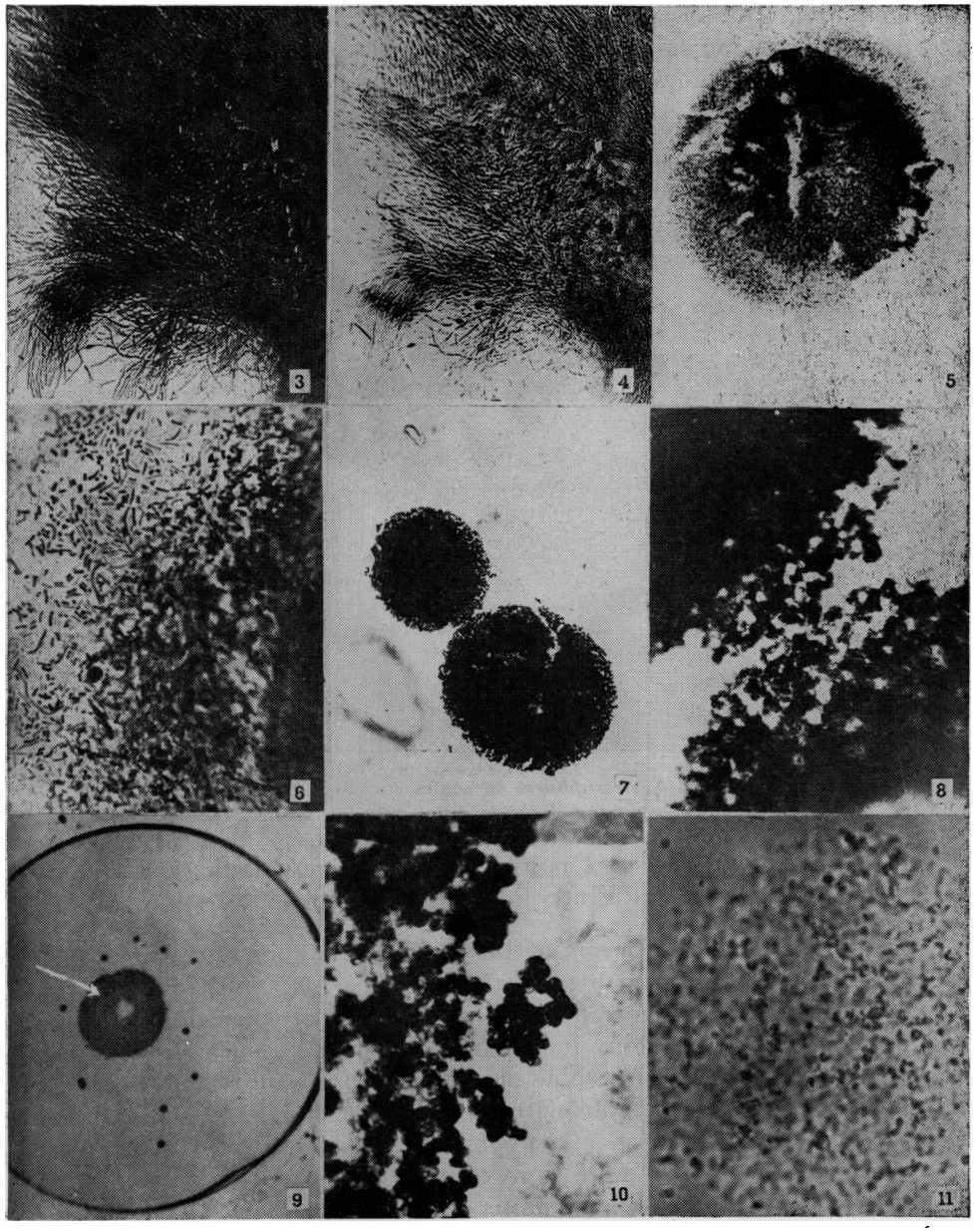

Fig. 3. Part of a young colony consisting of filaments, X200.

Fig. 4. Periphery of the colony shown in Fig. 3. Beading and large bodies are evident, X1200.

Fig. 5. Eighteen hour colony, X200.

Fig. 6. Periphery of colony displayed in Fig. 5. Filaments, large bodies, coccobacillary forms and short rods are evident, X1200.

Fig. 7. L type colonies, X200.

Fig. 8. L type morphological elements, X1200.

Fig. 9. Large colony surrounded by L type colonies. One L type colony within the large colony is indicated by the arrow, X100.

periphery of this colony is exhibited in Fig. 6. Filaments, large bodies, coccobaccillary forms, and short rods are all clearly visible. It is shortly after the stage of development reached in this colony that the surface begins to take on a granular appearance.

L type colonies after five days growth are shown in Fig. 7. They possess a definitely granular appearance and imbed themselves in the medium. When lifted off the agar they leave a small depression. The periphery of both colonies is displayed in Fig. 8 and an abundance of the L type morphological elements can be seen.

A large colony of *S. necrophorus* is shown in Fig. 9 surrounded by several minute L type colonies. The larger one represents the regular colony form generally referred to in the literature. The surfaces of both colony types appear to be granular. The arrow shows an L type colony within the larger colony. In Fig. 10 the periphery of the L colony within the larger colony is shown. The large whitish area represents autolysis in the large colony, while the darker staining portion of the figure consists of the L elements at the periphery of the small L colony.

An eighteen hour L type colony in serum agar is shown in Fig. 11. The microscope is focused near the surface of the medium and considerable growth can be seen out of focus in the agar. The growth consists of L elements of dimensions not completely resolvable at the magnification employed. The description of the technique for this type of stained agar preparation was obtained from Dr. Dienes (7).

DISCUSSION

In our studies on *S. necrophorus* it was evident that there was considerable variation in the capacity of strains from different sources to propagate on artificial media. As a general rule cultures in the bacillary phase could be recovered from lesions in which there was marked tissue damage and necrosis. The greatest difficulty in obtaining the bacillary phase was encountered with material from cases of atrophic rhinitis. The difficulty of obtaning bacillary cultures of *Bacteroides* from certain pahological processes has been referred to by others (8). However, the regular recovery of L forms of *Bacteroides* from pathological processes does not appear to have been reported.

In our work on atrophic rhinitis of swine it was important to determine whether or not the L forms observed were pleuropneumonia-like organisms or the L phase of *S. necrophorus*. That they were in most instances the L phase of *S. necrophorus* is attested by the following: 1. Reversion to bacilli on prolonged incubation and repeated subcultures; 2. Production of small amounts

Fig. 10. Periphery of L type colony within the large colony of Fig. 9. The large whitish area represents autolysis in the large colony, while the darker staining portion consists of L elements at the periphery of the small L type colony, X1500.

Fig. 11. Eighteen hour L type colony in serum agar, X970.

of gas by some L cultures (4); 3. Attempts to recover L forms under anaerobic conditions from other bacteria recovered from diseased turbinates and ethmoids were negative.

Work in progress suggests that atrophic rhinitis of swine cannot be produced with regularity employing only L forms. However, as reported elsewhere (1), rabbit subcutis yielding only *S. necrophorus* and *Pasteurella multocida* have regularly produced characteristic atrophic rhinitis when instilled into the noses of baby pigs.

SUMMARY

The colonial form of *Spherophorus necrophorus* described widely in the literature has been observed infrequently in cultures of morbid material from atrophic rhinitis, bovine liver abscesses, and necrotic enteritis. However, L type colonies and colonies giving rise to L morphological elements have been encountered frequently. Methods for the isolation, recognition and cultivation of *S. necrophorus* are described. The different colonial manifestations and L type variation are illustrated in detail with photomicrographs. *S. necrophorus* in relation to the aetiology of atrophic rhinitis of swine is discussed.

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