A PLEOMORPHIC BACILLUS FROM PNEUMONIC LUNGS OF CALVES SIMULATING ACTINOMYCES.

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PLATES 25 TO 28.

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During investigations upon the diseases of young calves, a mild epidemic of pneumonia in calves over 4 weeks old was encountered in the winter of 1917. A few scattering cases occurred during the remainder of the same year. Only one case occurred in the winter of 1918. The character of the lesions in these cases was the same and suggested some one underlying etiological agent.

The amount of lung tissue involved varied considerably from case to case. In all at least one of the smaller lobes (cephalic, ventral, azygos) was affected. Usually all were pneumonic. More rarely, in addition, the adjacent portions of the large caudal lobes were involved. The distribution was with few exceptions symmetrical. The trachea and bronchi usually contained soft, opaque, whitish masses embedded in mucus. When one of the affected lobes was cut across, a pearly white, thick, mucoid mass slowly oozed out of the cut ends of the small bronchioles within the diseased region. The affected lobes were slightly or considerably larger than in the normal collapsed state. The tissue was bright reddish and permeated with grayish 1 to 2 mm. foci, closely set. The texture of these did not differ appreciably from the rest of the tissue. Sections of the diseased lobes indicated a suppurative bronchopneumonia, with some fibrin in the most recently invaded tissue.

This brief description applies to the recent acute stage, such as was found in animals killed when dyspnea was marked. Older stages of the disease with a considerable change of the picture given above were found in several calves, 3 or more months of age. In these, circum-

scribed portions of lung tissue had become encapsuled abscesses. Leaving a more thorough description of this and other types of pneumonia for a later communication, we will consider here only one kind of microorganism encountered whose claim to be the primary or exclusive etiological factor need not be insisted on until more cases have been studied with the culture methods thus far successful in bringing it to the surface.

The organism to be described possesses for the bacteriologist an interest quite apart from its etiological relation to the disease process associated with it. Owing to the peculiar morphological and cultural characters, a description is best begun with the most striking stage which is its growth at about the 3rd day in tubes containing coagulated blood serum and sealed with sealing wax.

The method of sealing tubes is an outgrowth of the Nowack method of cultivating *B. abortus* in a sealed jar with cultures of *B. subtilis*. In work done in 1911[1] it was found that the Nowack procedure could be simplified by directly connecting the tube containing *B. subtilis* with the culture of *B. abortus* by means of a rubber tube or by simply sealing the culture tube. This latter method has proved uniformly satisfactory in cultivating *B. abortus*.

If bits of the involved lung tissue about 3 to 4 mm. in diameter are placed in the condensation water of coagulated horse serum and the tube is sealed with sealing wax, there will be noticed after several days minute, whitish flocculi in the condensation water best seen by transmitted light when the tube is tilted or shaken. The flocculi vary from mere specks to 1 mm. in diameter according to age and the special characters of the culture medium (Fig. 6).

Under a low power one of these bodies appears as shown in Fig. 1. An irregularly spherical mass is made up on the periphery of club-shaped, expanded, roundish, or pear-shaped bodies. Similar bodies appear when the focus is raised. Evidently the entire flocculus is made up of radiating filaments ending in clubs. The filaments are usually obscured by the clubs. Besides the latter, the flake contains

[1] Smith, T., and Fabyan, M., Ueber die pathogene Wirkung des *Bacillus abortus* Bang, *Centr. Bakteriol., 1te Abt., Orig.*, 1912, lxi, 549. Fabyan, M., A contribution to the pathogenesis of *B. abortus*, Bang.—II, *J. Med. Research*, 1912, xxvi, 441.

minute, irregular masses of crystals, shown as black objects in Fig. 1. The crystals are very minute and probably bunches of acicular bodies. Under a high power the clubs appear as slightly refringent bodies with a sharply defined limiting membrane (Fig. 2). Not infrequently in small flocculi cylindrical filaments not enlarged at the ends are seen growing out among the clubs. The latter are about 10 microns in diameter. Within certain terminal bodies a second and even a third membrane is visible. The terminal filaments and clubs are flexible and are gently moved to and fro in currents under the cover-slip. The structure of the more central parts of the flocculus cannot be made out owing to the density of the peripheral zone. When crushed the body becomes a mass of detritus in which occasionally filaments, about 2 microns broad, are seen as parallel strands very faintly outlined.

The impression gained at first from a study of the unstained flocculi in the fluid in which they grow is that the culture consists of a filamentous organism whose frequent branching produces the roundish flake. If flocculi 2 to 4 days old are allowed to dry undisturbed upon cover-slips, then heated and stained in the usual way with some aniline dye, such as alkaline methylene blue, for several hours and decolorized in 0.01 per cent of acetic acid to remove the dye from the background of the specimen, it will be seen that the spot covered by a flake is unstained. Its interior is, however, occupied by a tangle of very fine, segmented filaments made up of bacilli. There is no evidence of branching (Fig. 4). The bacilli measure from 0.4 to 0.5 microns in diameter. They stain much as the common bacteria do in alkaline methylene blue and diluted carbolfuchsin, but are decolorized according to Gram. As stated, the flocculi themselves fail to take any stain and appear white when the background of the film is still tinted with the stain.

The above type of culture on horse serum has been maintained in one case for many generations. The growth was materially improved when about 1 cc. of calf serum water was added to the condensation water present. All the tubes have been sealed. Multiplication was not evident in open tubes. In subcultures, after several weeks either at 37°C. or at room temperature, the slanted surface may or may not become covered with very minute, elevated, pointed-like colonies

which, on microscopic examination, have the same characters as the flocculi in the condensation water. All serial cultures have been obtained by transferring several loops or a drop of the condensation water to a fresh tube and not from the surface colonies. When the culture medium is not very favorable, the flocculi remain very small and the clubs become large and even spherical in outline, or else the flakes become quite large.

After 3 or 4 days changes begin to set it. One of the most important is shown by staining. The flocculi now begin to take up some of the stain and the material of which they are made up has a striated or lamellated appearance, as if some substance had been deposited within the membrane. Peculiar, bizarre forms are the result. The nearest objects to which they may be compared are the myelinic or so called Buhl's bodies seen in fresh unstained sputum originating from the alveolar epithelium of the lungs. At this stage the stained film no longer gives the clear picture of the contents of the flake. Bacteria are no longer detected in most of the flakes. They have either been dissolved or are no longer accessible to the stain or else are obscured by the absorbed dye in some of the clubs. To see the chains of bacilli within the flakes it is therefore necessary to stain early vigorous stages of growth.

After some months of cultivation, with transfer intervals of about 5 days, in horse serum tubes, or rather in the condensation water of such tubes, certain changes have been observed in some substrains which permanently affect the substance enveloping the bacilli and which are presumably degenerative in character.

The clubs of the flocculi become surrounded with a layer of fine granules and eventually concealed by them. The granules are slightly refringent up to 1 micron in diameter and very variable in size. They become detached from the flakes and form small groups which collect at the surface of the condensation water as a white, creamy layer. The appearance and physical characters suggest some fatty substance. Osmic vapor stains them feebly brownish. Scharlach R placed on a dried film is absorbed by the material which becomes well tinted. Similarly, a dried film stained with the fat dye in the usual way for sections and mounted in water shows imbibition of the dye. However, similar effects are obtainable with alkaline methylene blue and the specificity of the fat stain remains questionable.

Cultures upon slanted agar were obtained in two ways, (*a*) from lung tissue direct and (*b*) from the horse serum tubes.

(*a*) Growth upon slant agar tubes to which particles of diseased lung tissue had been added and the tube sealed with sealing wax occurred quite regularly. In some cases a film covered the agar surface just above the tissue in the condensation water. On others besides this growth, scattering 1 to 2 mm. yellowish colonies appeared on the slope. The colonies are roundish, slightly raised masses.

When examined under a high power fresh the colony was found to be composed of roundish, ring-like, rather faint bodies about 2 microns diameter (Fig. 5). On the periphery or near the center of the body is a very minute refringent speck. The appearance suggests a spore within some material of low refringence. Staining dried films did not give more information. The disc-like bodies became more strongly outlined by a feeble peripheral stain but no staining equivalent to that of ordinary bacteria was obtained.

Subcultures of such colonies under the same conditions failed to multiply. Tentatively the explanation was that the organisms sporulated and failed to germinate in a second tube. Transfers to other media also failed.

(*b*) After the serum cultures had become established so that multiplication occurred regularly in subcultures, transfers to slanted agar were frequently made. These were only occasionally successful and from such transfers series of agar cultures were maintained. The medium contained a bit of guinea pig spleen and some calf serum water added to the condensation water on hand. These tubes also were kept sealed. The colonies were very minute, drop-like, discrete, or else fairly large, *i.e.* 1 to 1.5 mm. diameter, or else both minute and large colonies appeared on the same slanted surface (Fig. 7). A smooth continuous layer of growth usually started from the condensation water and extended 5 to 10 mm. upwards on the slant. The substance of the large colonies was more or less coherent since the colony could be moved along or picked up with a wire *in toto*. In films made from such colonies, which were in this as well as in former series of a straw color, the growth was found always to be bacilli with no indications of a capsular substance. The bacilli were lying close together. There was slight variation among the bacilli, both as to width and length and staining capacity, from the same colony (Fig. 8).

After 1 or 2 weeks growth on agar slants, the condensation water either remained clear, or else a thin, creamy layer formed at the surface. No flocculi appeared in it at any time. In the meantime changes went on in the growth, leading to the disappearance of the bacillus type and the appearance of the coccus type as found in original agar cultures from diseased lung tissue (Fig. 5). This occurred after 7 days and the process was completed in about 10 days (Figs. 9 and 10).

The coccus type, therefore, must represent a definite stage in the growth cycle of this microorganism. Does it represent a minute organism enclosed in a feebly staining capsule, and if so, is this minute body an endospore or an arthrospore? No definite answers can be given to these queries at present from the morphological standpoint, owing to the minuteness of the bodies. In one case in a fresh, unstained film, what appeared to be endospores were found both at the ends and in the course of the filaments. These bodies were markedly refringent and very slightly bulged the walls of the rods outward. Further culture studies may throw some light on the nature and function of these bodies.

In addition to the horse serum and agar media described, a variety of culture media was tried without success. Either the multiplication was feeble or else absent altogether. Among the media tried were ordinary slanted agar, milk and potato tubes, sealed and unsealed; bouillon plus bits of tissue, covered with paraffin oil; ascitic fluid plus tissue and paraffin oil; and fermentation tubes of bouillon plus tissue. Calf serum water by itself produced only a very feeble growth.

Evidently the multiplication depends upon certain definite substances in culture media in a given, slightly reduced, oxygen tension. Prolonged cultivation may in time bring the organism to multiply in the ordinary culture media, but this stage has not been reached after 5 months of continuous cultivation on special media.

Naturally, in meeting the peculiar morphological entities in cultures, one would at first regard them as contaminations, and this assumption, as stated above, retarded the work of isolating and identifying the microorganism for some time. When bits of animal tissues are used both for purposes of inoculation and for stimulating growth the

question of mixed cultures must be kept constantly in sight. In the work before us, the culture tubes containing agar plus guinea pig spleen had been in the incubator from 2 to 4 weeks, sealed, and then in room temperature for another period of 1 to 2 months before they were used. A chance for any contaminated tube to slip through unnoticed may be regarded as almost negligible. When bits of diseased lung tissue were inoculated—and this is probably the only way for securing growth of the organism—the results were very uniform. When the organism was present alone, all cultures contained the same growth. When other bacteria were associated they were usually present in all cultures and suppressed the growth of the former. This was then recognizable for a short time in the condensation water by its peculiar growth forms.

Inoculations of relatively large quantities of culture material into the peritoneal cavity of mice, guinea pigs, and rabbits have not produced anything beyond slight local changes. In several guinea pigs and one rabbit one, two, and three doses were given, separated by intervals of weeks in order to develop any susceptibility to subsequent inoculations. No multiplication was evident beyond slight local peritoneal opacities and thickenings, except in one rabbit which received three separate intraperitoneal injections and was chloroformed 16 days after the last injection. A small, encapsulated abscess about the size of a pea was found attached to a coil of the colon. This was removed and crushed and portions of the contents were added to tubes of solidified horse serum. From this the *Actinomyces*-like organism was recovered and successfully subcultured.

This organism has been identified microscopically in cultures from ten cases of pneumonia having common anatomical and histological characters. Owing to the confusing pleomorphism in different culture media and at different ages of the same culture, it was not identified in the three earliest cases and the cultures regarded as mixtures. The notes describing the forms seen were, however, sufficient to make a diagnosis possible later on. In the subsequent cases the peculiarities of the serum cultures aroused attention but they were not at once identified with the original agar cultures from the same tissues. The organisms in the serum tubes from three cases were kept alive through several generations but owing to interruptions in the work and the

apparently feeble vitality of the organism they were lost. Finally cultures from one of the last cases have been maintained through many generations. Unfortunately the supply of material which was abundant in the winter of 1917 became scarce soon after, so that the successful methods for continuous cultivation over long periods could be used on only one case.

Relationship of the Pleomorphic Bacillus to Bacteria Already Described.

The suggestion which occurs to one seeing this bacillus for the first time is that it is related to or identical with one or another of the *Actinomyces* types cultivated by Bostroem, Wolff and Israel, J. H. Wright, and others. This suggestion is soon dispelled. The organism is a minute bacillus, growing in chains, the chains held together by intercellular substance. There is no branching and the Gram stain is negative. The peculiar *Actinomyces* type of growth obtained suggests the possibility, however, that when the proper culture medium is found the ray fungus itself will be cultivated with clubs as it occurs in animal tissues. The rare and uncertain appearance of clubs in *Actinomyces* cultures has led observers to infer that the clubs may at least in part be formed by the host tissues. The observations upon this bacillus suggest the compromise theory that the clubs are formed by the bacillus and later impregnated with tissue fluids. The absence of any capacity for holding dyes on the part of the clubs in the cultures of the bacillus and the relative ease with which acid dyes are held by the clubs of the *Actinomyces* grains as obtained from pus tends to support this view.

In looking over the literature to find some types which might be assimilated to the described bacillus, the writer found a publication by Lignières and Spitz[2] which seems to supply the missing link. These authors describe an affection which prevailed as an epizootic in Argentina in 1900 and 1901. It was at first regarded as true actinomycosis but the failure to stain the filaments in pus with accepted methods and negative cultures on potato induced Lignières to undertake an investigation.

The disease assumes various forms. (1) As an abscess of the subcutaneous tissue of the throat. The abscess develops very slowly and is associated with

[2] Lignières, J., and Spitz, J., Actinobacillose, *Rev. Soc. méd. arg.*, 1902, x,, 105 9 plates.

little pain. It eventually ruptures and discharges a very thick, sticky, pasty pus. Abscesses may also appear over the parotid gland, the superior maxillary bone, at the base of the ear. The disease has been found to occur in many other parts of the body with involvement of the regional lymph nodes. (2) As an affection of the tongue which is clinically identical with the affection known as wooden tongue (*langue de bois*), as an affection of the pharynx, the parotid gland, the mammary gland, and as localizations in the lungs leading to lesions resembling tuberculosis. Hepatization may also occur. Lesions of the bones were rarely found. They were located in the superior and inferior maxillary bones and gave rise to appearances formerly described as osteosarcoma. That form described first as cold abscess of the neck makes fully 80 per cent of all cases; localization in the tongue 5 per cent.

Lignières found in the pus, either directly or after treatment with caustic potash and centrifuging, radiating masses of clubs which did not show any mycelium and failed to stain according to Gram. Acid dyes, like picric acid, gave a distinct coloration to the clubs.

When pus from closed abscesses is ground up and tubes of agar-agar inoculated with it, growth is evident at 37°C. within 24 hours. The organism may appear in early cultures as a bacillus, later like a diplococcus or a streptobacillus, and in old cultures as bizarre involution forms. It multiplies in plain, glycerol and sugar bouillon, giving the latter a plainly acid reaction. In gelatin the growth is very feeble. Liquefaction does not occur. On agar the colonies, if crowded, are small, translucent, bluish; larger, opaque when scarce. Activity of growth increases with successive transfers and may become like and nearly as abundant as that of the typhoid bacillus. Milk is regarded as a good medium but coagulation does not occur.

Lignières' strain was of a relatively high virulence. A culture on agar injected into the peritoneal cavity of guinea pigs killed in 12 to 24 hours, the lesions being those of an acute peritonitis. Rabbits were far less susceptible. In the mouse a subcutaneous inoculation produced only a transient induration. In cattle a local abscess was always produced after subcutaneous inoculation.

The organism of Lignières and Spitz presents many features that are like those of the bacillus described. The fundamental one is that an *Actinomyces*-like organism gives rise to a bacillus in cultures whose pleomorphism agrees well with that found by the writer on agar slants. The differences between the two organisms are, however, numerous. Lignières' bacillus grew well on ordinary media and in open tubes. The one described here could not have been cultivated in this way. Lignières found the *Actinomyces*-like characters in pus from tissues but failed to reproduce them in cultures. The reverse is the experience of the writer who found the organism in the lungs in the form of very fine bacilli. Lignières traced his organism in a

variety of lesions in adult cows, whereas the bacillus here described was associated with a very characteristic and extensive broncho-pneumonia of young calves.

On the whole, the writer is inclined to regard Lignières' bacillus as identical with his own with such variations as are likely to appear within the boundaries of any species or strain, widely separated geographically and attacking animals of different age periods.

The name given by Lignières and Spitz to their bacillus—actino-bacillus—involves the establishment of a new genus. This is hardly justifiable when we consider that the characters are given to the group by a variable and disappearing physiological factor analogous to a capsule or capsular material. The writer suggests a new species name—*Bacillus actinoides*—for the culture here described and leaves it to future investigations to determine whether it is specifically identical with Lignières' actino-bacillus or not.

Following the publication of Lignières and Spitz, Nocard[3] was able to confirm their results on cases of wooden tongue of cattle in France. Higgins[4] described four cases of "actino-bacillosis" in cattle (tumor of the region of the pharynx, abscess of parotid, disease of the tongue, and a growth on the jaw). Neither of these authors found any difficulty in isolating and cultivating the bacillus. Neither obtained any *Actinomyces* forms in their cultures. According to Higgins, guinea pigs inoculated into the peritoneum die of a generalized infection in 19 to 31 days. Rabbits are also susceptible and a generalized eruption follows intraperitoneal inoculation.

Bearing upon the peculiar culture forms of this bacillus a somewhat analogous instance of the dependence on special media may be referred to here. *Leuconostoc mesenteriodes*, a micrococcus causing injurious fermentations in sugar refineries, was studied by Liesenberg and Zopf[5]

[3] Nocard, E., Les maladies microbiennes des animaux, Paris, 3rd edition, 1903, ii, 374, footnote.

[4] Higgins, Actino-bacillosis, *Bull. No. 1, Dept. Agric., Health of Animals Branch, Dominion of Canada*, 1904.

[5] Liesenberg, C., and Zopf, W., Ueber den sogenannten Froschlaichpilz (Leuconostoc) der europäischen Rübensucker- und der javanischen Rohrzuckerfabriken, Beitr. Physiol. u. Morphol. niederer Organismen, 1892, pt. i; abstracted in *Centr. Bakteriol.*, 1892, xii. 659.

who found that while enormous, gelatinous capsules are developed in media containing sugars, these fail to appear in media not containing dextrose or saccharose. In these it appears like an ordinary streptococcus. This measures 0.9 to 1.2 microns in diameter, whereas the capsules may attain a diameter of 6, 10, or even 20 microns.

In the case of the bacillus here described the formation of the flocculi with terminal clubs is evidently dependent on some substance in blood serum which survives the coagulating temperature of 70–75°C.

SUMMARY AND CONCLUSIONS.

A bacillus was found associated in pure culture with an extensive lobar bronchopneumonia in calves. It occurs in the exudate as a minute bacillus in small groups. In cultures it appears in three forms: as a bacillus, as a coccus-like endospore or arthrospore, and as a conglomerate *Actinomyces*-like flake or colony with peripheral clubs. The bacillar and coccoid forms occur on agar, the *Actinomyces* form in the condensation water of coagulated serum (horse). The coccoid form is probably a spore state, the minute refringent spore being contained in a roundish, unstainable mass representing either the remnants of bacillar substance or some capsular material. The somewhat striking similarities between this organism and *Actinomyces* are expressed by the massed growth with terminal clubs, the bacillar and coccoid stages, all of which are characteristic of *Actinomyces*.

Sealing the tubes is essential for multiplication. Cultures must be renewed within a few days, otherwise multiplication fails. The substance which forms the bulk of the radiate flocculi is probably of capsular nature, greatly overproduced in serum tubes and scarce or absent on agar. Its nature is unknown.

The organism is not appreciably pathogenic when injected into certain small laboratory animals.

EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. A flake or colony with peripheral clubs from the condensation water of a horse serum culture. The blackish specks on the colony are masses of acicular crystals. The flake is flattened out between slide and cover-glass. × 120.

FIG. 2. The margin of a flake in condensation water flattened gently between slide and cover-glass. × 1,000.

PLATE 26.

FIG. 3. A flake or colony dried on a slide and stained in alkaline methylene blue. The flake is feebly outlined. The stained masses are bacilli. Thirteenth transfer on horse serum, 2 days old. × 115.

FIG. 4. Margin of a flake from the same culture as the one from which the flake in Fig. 3 was taken. The bacilli appear in chains. The dark bodies are masses of bacilli. × 1,000.

FIG. 5. Colony from an original agar culture from the lungs as described in the text. × 1,000. The film is unstained and spread between slide and cover-glass in fluid from the culture. Coccoid bodies containing each a highly refringent granule.

PLATE 27.

FIG. 6. Sealed culture on horse serum showing the flocculi or colonies in suspension in the condensation water. Some have become attached to the sides of the tube as a result of shaking the tube.

FIG. 7. Sealed culture on an agar slant containing in the condensation water a piece of guinea pig spleen and some calf serum water. Culture 7 days old. After 5 months of artificial cultivation (about thirty transfers).

FIG. 8. Film stained in alkaline methylene blue from an agar slant plus guinea pig spleen about 48 hours old. Note only rod forms. Some of these are more intensely stained and a trifle thicker than the great mass. × 1,000.

PLATE 28.

FIG. 9. Film from a culture on the same kind of medium as given for Figs. 7 and 8. 6 days old. Alkaline methylene blue. Among rod forms are roundish coccus-like, feebly stained bodies. × 1,000.

FIG. 10. Film from the same culture stained several hours in diluted carbolfuchsin. The roundish forms of Fig. 9 now appear as deeply stained, smaller bodies suggesting endospores. × 1,000.

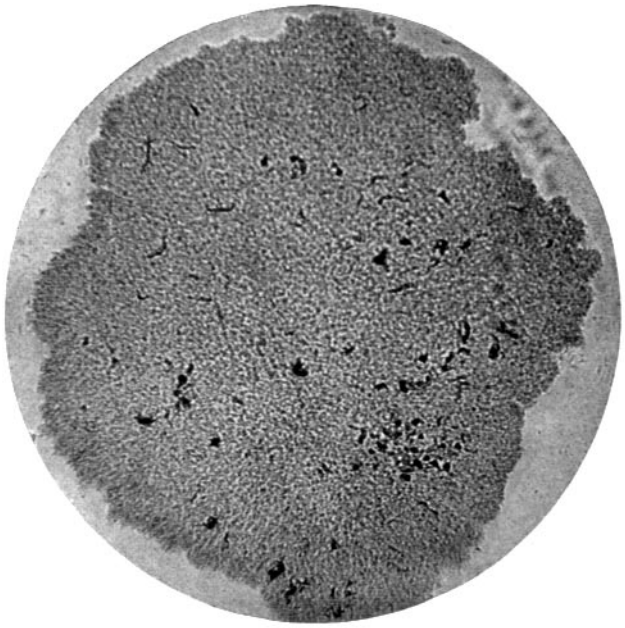

FIG. 1.

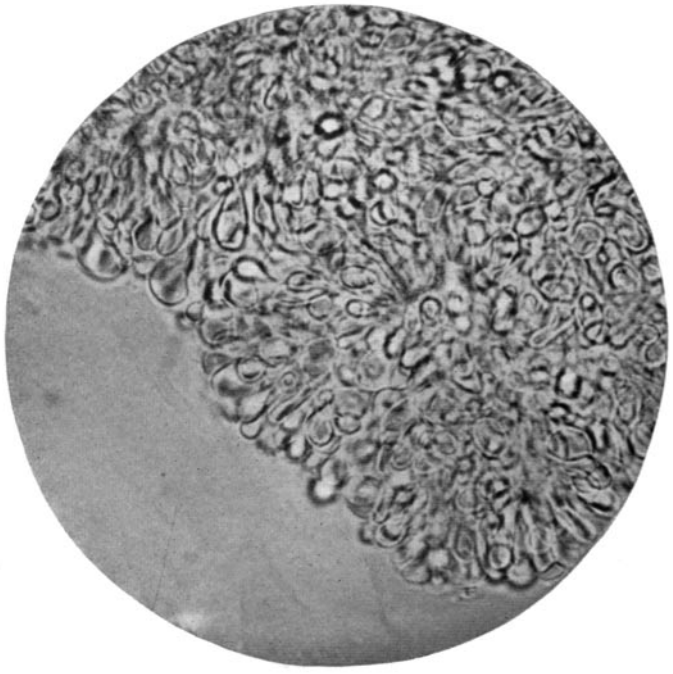

FIG. 2.

(Smith: Pleomorphic bacillus from pneumonic lungs.)

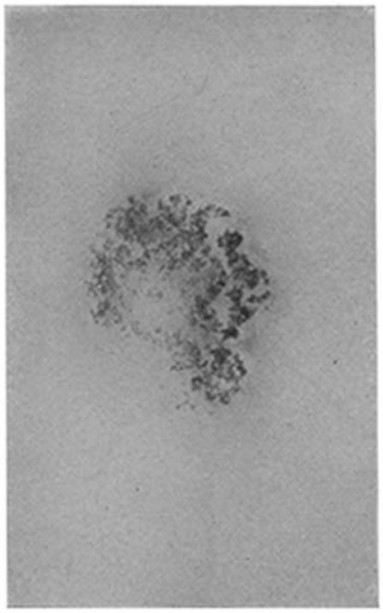

FIG. 3.

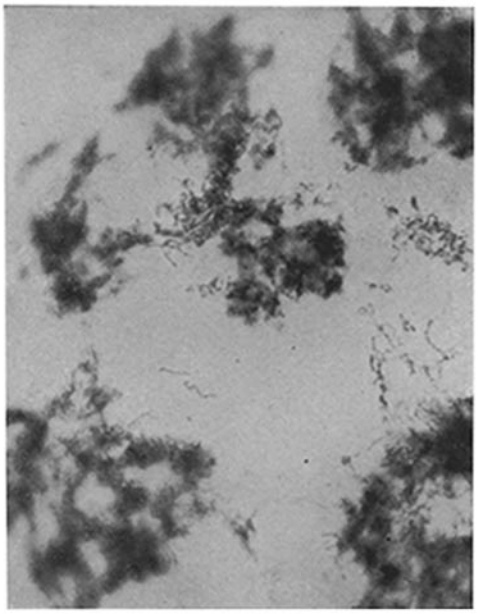

FIG. 4.

FIG. 5.

(Smith: Pleomorphic bacillus from pneumonic lungs.)

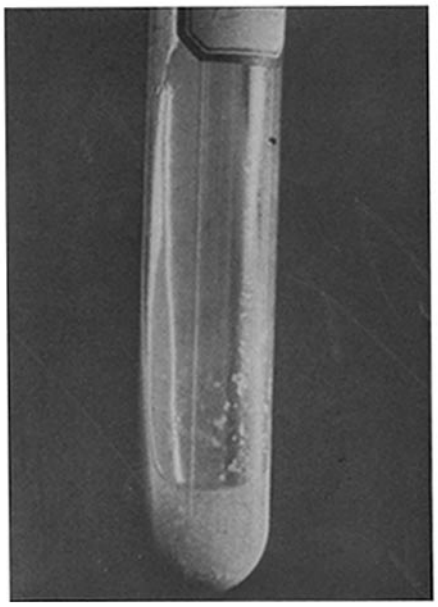

FIG. 6.

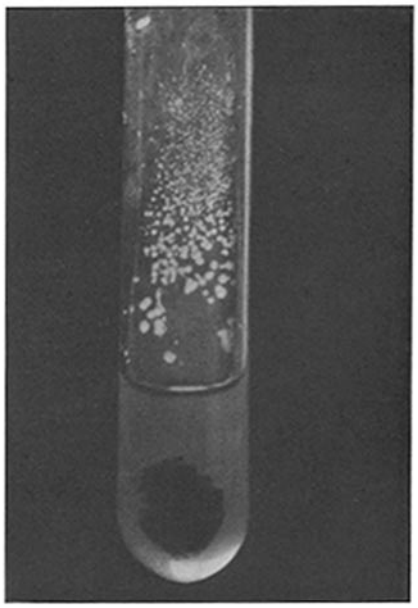

FIG. 7.

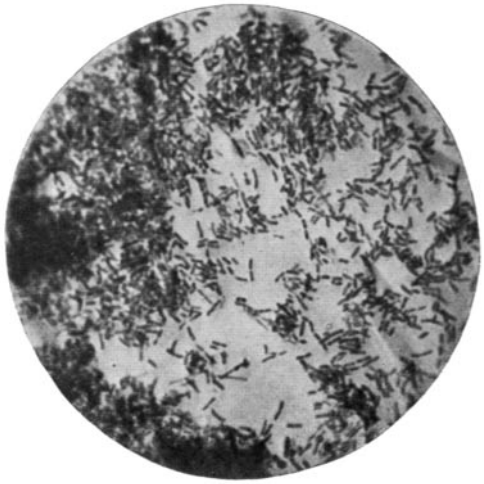

FIG. 8.

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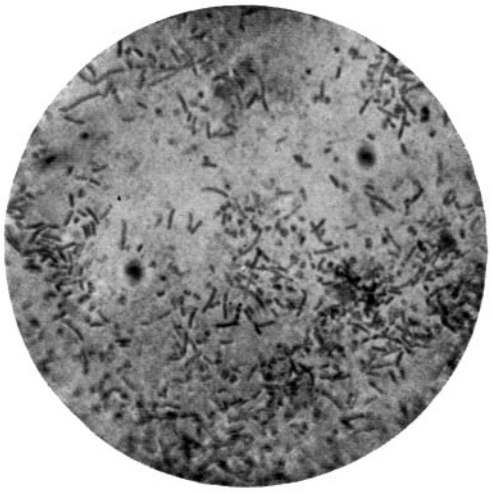

FIG. 9.

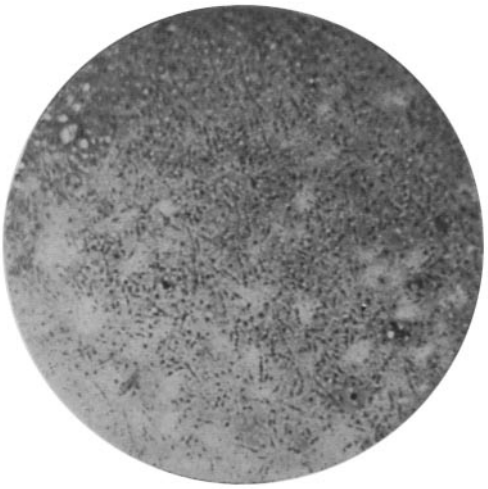

FIG. 10.

(Smith: Pleomorphic bacillus from pneumonic lungs.)