SWINE INFLUENZA

II. A Hemophilic Bacillus from the Respiratory Tract of Infected Swine

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PLATE 35

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Murray (1), McBryde, Niles, and Moskey (2), and recently Fulton (3) have attempted to discover the nature of the inciting agent of swine influenza. Murray described a small Gram-negative coccus as the cause of the disease. By inoculating normal swine intravenously with relatively small doses of pure cultures of this organism, he induced a disease which he believed was clinically and pathologically swine influenza. McBryde, Niles, and Moskey failed to obtain Murray's micrococcus in their cultures. They isolated only two types of organisms from cases of the disease with any degree of frequency, a pleomorphic Gram-positive bacillus and *Bacillus suisepticus*. Neither of these organisms produced the disease when injected into susceptible swine. Fulton recently confirmed the presence of McBryde's pleomorphic Gram-positive bacillus in the respiratory tracts of swine with influenza and, with very freshly isolated cultures, reproduced the disease experimentally. However, cultures that had been transferred only three times or original cultures that had been kept on ice for as short a period as 2 weeks were incapable of inducing influenza when administered intranasally to normal swine.

Spray (4) using slaughter house material cultured the respiratory tracts of a large number of normal swine and of those showing pneumonia. From both classes he frequently isolated an inulin-fermenting streptococcus and *B. suisepticus*. These organisms were encountered more often from pneumonic than from normal lungs; the inulin-fermenting streptococcus was frequently present in pure culture from the pneumonic areas. Since Spray did not differentiate infectious pneumonia, hemorrhagic septicemia, and influenza, his findings are not of great value.

EXPERIMENTAL

Bacteriological studies have been conducted upon a large number of swine experimentally infected with swine influenza from the 1928, 1929, and 1930 epizootics (5), and upon a small number of spontaneous field cases of the disease from the mild 1929 epizootic. In addition,

thirty-five swine not suffering from influenza have served as a control group. These animals were either normal, or infected with hog cholera or spontaneous pneumonia. They were from local sources similar to those of the animals used in the study of experimental swine influenza.

In suspected cases of influenza, primary cultures were made from atelectatic or pneumonic lung, bronchial exudate, spleen, and, in certain cases where indicated, from other organs, on plain agar slants (pH 7.3 to 7.6), to the condensation fluid of which 0.5 to 1 cc. of sterile defibrinated horse or swine blood had been added. Cultures of heart blood were made on plain agar slants inoculated by pipette with approximately 1 cc. of blood. In some cases primary cultures were also made on blood and chocolate agar plates. Similar cultures were made from the control animals on the same kinds of media.

The suspected organism was very similar to, if not identical with, *H. influenzae*. The first culture was obtained from the heart blood of our first experimental case of the disease. This blood was apparently sterile when streaked on plain and blood agar plates. In approximately 1 cc. amounts it was added to the condensation fluid of about twenty plain agar slants which were incubated for 2 days. At this time the slants appeared sterile. The blood was not hemolyzed and there was no evidence of growth. However, films prepared from the bloody condensation fluid of these slants, when stained with methylene blue and examined microscopically, revealed the presence of organisms in moderate numbers. These were predominantly thin, curved bacilli which varied considerably in length. Some were less than 1μ long while other apparently single individuals sometimes approached 4μ in length. Some threads of continuous protoplasm extended completely across the field. Other shorter threads appeared as tangled masses enmeshing small curved bacillary forms and both large and small coccoidal forms. Attempts to obtain subcultures on plain agar, infused blood agar, inspissated serum, or in bouillon or Smith-Noguchi medium were unsuccessful. Growth could be continued, however, in sterile defibrinated swine or horse blood at the bases of plain agar slants and fair growth reaching a maximum in 48 hours occurred on chocolate agar.

This organism has been found in the respiratory tracts of all swine experimentally infected with influenza that have come to autopsy within 7 days of the onset of fever; it has been recovered from the respiratory tracts of six spontaneous cases of the disease from five different herds (all of the spontaneous cases which were examined); and it has been found in the original material used in establishing the eight strains of the disease which have been studied experimentally (5). In the case of all eight strains the organism has been recovered from the respiratory tract of the first experimentally infected animal

TABLE I

The Incidence of H. influenzae (Variety suis) in Experimental and Spontaneous Swine Influenza

Strain	No. of swine cultured	Material cultured	*H. influenzae suis*				
			Present	Absent	Per cent positive	Present in pure culture	Per cent present in pure culture
1 (1928)	33	Lung	28	4	87	11	34
		Bronchial exudate	17	6	74	8	35
		Heart blood	6	20	23	5	19
2 (1928)	9	Lung	7	2	78	4	44
		Bronchial exudate	8	1	89	3	33
		Heart blood	0	9	0	0	0
5, 6, 7, and 10 (1929) combined	7	Lung	7	0	100	5	71
		Bronchial exudate	6	0	100	2	33
		Heart blood	0	7	0	0	0
Field cases (1929)	6	Lung	5	1	83	1	17
		Bronchial exudate	6	0	100	2	33
		Heart blood	2	4	33	0	0
14 (1930)	8	Lung	6	2	75	5	63
		Bronchial exudate	8	0	100	2	25
		Heart blood	2	5	29	2	29
15 (1930)	25	Lung	23	2	92	15	60
		Bronchial exudate	24	0	100	9	38
		Heart blood	8	17	32	8	32

TABLE II

The Incidence of Hemophilus influenzae (Variety suis) in a Control Group of Animals

Group	Material cultured	*H. influenzae suis*		
		Present	Absent	Percentage
Swine experimentally infected with hog cholera—16	Lung	0	16	0
	Bronchial mucus	0	13	0
Normal swine—8	Lung	0	8	0
	Bronchial mucus	0	8	0
Swine dying of or showing pneumonia at autopsy—11	Lung	0	11	0
	Bronchial mucus	0	8	0
Iowa swine suffering from a non-influenzal respiratory disease—4	Lung	0	4	0
	Bronchial mucus	0	4	0

in each series. In these groups of cases it has frequently been present in pure culture. Because of its growth requirements, porcine origin, and morphological and cultural characteristics, to be discussed later, the name *Hemophilus influenzae* (variety *suis*) is suggested for this organism. It will be referred to hereafter as *H. influenzae suis*. The incidence of *H. influenzae suis* in influenza-sick swine killed within 7 days after inoculation is recorded in Table I. In addition to the cultures recorded in Table I, it has been recovered from the pleura or pericardium whenever these have contained exudate, and from the spleens of most fatal cases. In two cases which developed otitis media during the course of their experimental disease it was the predominant organism in the middle ear exudate. It has not been encountered in the swine serving as bacteriological controls, as indicated in Table II.

Cultural and Morphological Characters of H. influenzae suis

Morphologically and in the character of its growth on media *H. influenzae suis* resembles *H. influenzae* very closely.

Media to sustain growth must contain both factors V and X (6). Hematin prepared by the method of Anson and Mirsky (7) and supplied by them was used as the source of factor X, while factor V was furnished either by an actively growing culture of a non-hemolytic streptococcus (spotted on the hematin-containing plate) or by a sterile Berkefeld filtrate of an old bouillon culture of this streptococcus added to hematin-containing agar.

Chocolate agar is the best solid medium and heated blood bouillon the most satisfactory fluid medium for the growth of *H. influenzae suis*. Cultures of the organism that have been well established grow feebly and as very minute colonies on blood agar. In mixed culture on blood agar luxuriant growth of *H. influenzae suis* colonies occurs about the colonies of the contaminating organism. On chocolate agar well established cultures reach a maximum in between 24 and 48 hours at 37°C. The colonies on this medium vary from less than 1 mm. to more than 2 mm. in diameter, are circular, grayish, semitranslucent, flattened, and have a sharply contoured edge. Films prepared from chocolate agar cultures contain three distinct forms. Examined after 24 hours' growth the predominant form is a small thin bacillus varying from less than 0.5μ to 2μ in length and approximately 0.2μ in thickness (Fig. 1). A few longer, curved, thread-like forms appear also. 48 hour cultures contain more of the long thread-like types and in some cultures these form clumps of tangled masses of organisms (Fig. 2). Small coccoidal organisms are found at 48 hours. Cultures examined at 3 days and thereafter reveal

increasing numbers of the coccoidal forms. In blood bouillon or heated blood bouillon 24 hour cultures are found to be composed largely of small, thin, straight or slightly curved rods. A few coccoidal forms are present and these usually in clumps of from six to thirty or more organisms, seldom singly. At 48 hours almost the entire culture is composed of clumps of small cocci (Fig. 3). In fluid media the long thread-like forms are not regularly encountered. "Giant" coccoidal and large club and comma shaped forms are numerous in some cultures (Fig. 4).

The capacity of films of cultures from either solid or fluid media to absorb the usual dyes decreases rapidly with the age of the culture. Loeffler's alkaline methylene blue, which is the most satisfactory, imparts no more than a faint bluish gray color to organisms from cultures 3 or more days old. By phenolizing the methylene blue and steaming it on the film a slightly more intense color is obtained.

The organism is relatively inert as to growth and fails to act on various substances used for differentiating bacteria. In blood bouillon, with brom-cresol purple as indicator, definite growth occurs. There is, however, no demonstrable action on dextrose, lactose, saccharose, dulcitol, mannitol, glycerol, inulin, or arabinose. Some fading of the indicator occurred in all tubes. All cultures were observed for 7 days. None of the cultures examined produced indol or hydrogen sulfid. Litmus milk containing blood fostered but slight growth and was unchanged. Nitrates were reduced to nitrites by all cultures examined. No growth could be obtained with well established cultures on plain or glycerin agar, Dorset's egg medium, potato, coagulated blood serum, or in gelatin. The organism is non-hemolytic, non-motile, and Gram-negative.

As may be evident from the preceding description, there is no real basis for differentiation between *H. influenzae suis* and non-indol-producing strains of *H. influenzae*. If a culture of *H. influenzae suis* were isolated from a human throat, no doubt would be raised as to its identity with *H. influenzae*. Since primary cultures of *H. influenzae suis* grow somewhat more feebly on heated blood agar than do primary cultures of *H. influenzae*, such a hypothetical culture would perhaps be considered more difficult to establish than the typical Pfeiffer bacillus.

For the primary isolation of *H. influenzae suis*, the plain agar slant containing from 0.5 to 1 cc. of sterile defibrinated horse blood at the base is the medium of choice. In this medium the organism grows in the blood at the base of the slant and the pure culture on inspection appears sterile. Examination of stained films of the bloody condensation fluid, however, readily reveals the organism in characteristic form. It is felt that for the isolation of *H. influenzae suis* this medium is superior to heated blood agar. It has been the practice not to examine

TABLE III

Direct Agglutination

Culture strains	Agglutinating sera																																		
	H. influenzae suis																												*H. influenzae* BIW							
	14							451							459							660													
	Dilutions																																		
	1:50	1:100	1:200	1:400	1:600	1:800	1:1200	1:50	1:100	1:200	1:400	1:600	1:800	1:1200	1:50	1:100	1:200	1:400	1:600	1:800	1:1200	1:50	1:100	1:200	1:400	1:600	1:800	1:1200	1:50	1:100	1:200	1:400	1:600	1:800	1:1200	Control
H. influenzae suis																																				
11-T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
652	C*	C	+	+	—	—	—	C	C	C	C	+	—	—	C	C	C	C	+	—	—	C	C	C	C	+	—	—	C	C	C	C	+	—	—	—
451	—	—	—	—	—	—	—	C	C	C	C	+	x	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	C	C	+	—	—	—	—	—
459	—	—	—	—	—	—	—	+	x	—	—	—	—	—	C	C	C	+	—	—	—	—	—	—	—	—	—	—	C	+	x	—	—	—	—	—
660	C	C	C	+	—	—	—	C	C	C	C	+	—	—	C	C	C	C	+	+	—	C	C	C	C	+	x	—	+	C	C	+	—	—	—	—
11-I	x	+	—	—	—	—	—	C	C	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—·	—	—	—	—
15	C	C	+	—	—	—	—	+	+	x	—	—	—	—	C	C	+	—	—	—	—	C	+	—	—	—	—	—	C	C	+	—	—	—	—	—
14	C	C	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	C	C	+	—	—	—	—	C	C	+	—	—	—	—	—
H. influenzae																																				
WF	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
K	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	x	+	—	—	—	—	—	—
S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	C	C	C	+	—	—	—	—
14	C	C	+	—	—	—	—	C	+	+	—	—	—	—	+	—	—	—	—	—	—	+	x	—	—	—	—	—	C	C	C	C	C	C	C	—
52	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	C	C	+	—	—	—	—	—
BIW	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	C	C	C	C	C	C	C	—

* C = complete agglutination; +, strong agglutination; x, weak to trace agglutination.

primary cultures until after 48 hours' incubation, since this length of time is required for the development of the curved and tangled thread-like forms which are characteristic and identifiable even in mixed culture. Cultures are viable in this medium for 2 weeks, but stock cultures have been transferred at weekly intervals and kept in the incubator.

Serological Relationship between Strains of H. influenzae suis and of H. influenzae

It was realized that, because of the great serological diversity of strains of *H. influenzae*, attempts to demonstrate any serological relationship between *H. influenzae suis* and *H. influenzae* would be inconclusive. However, the near identity of the two organisms both culturally and morphologically made it seem of interest to compare the reciprocal agglutinability of a few strains of each organism. The cultures of *H. influenzae* and the agglutinating serum were generously provided by Dr. Olga R. Povitzky of the laboratories of the New York Department of Health. The *H. influenzae* serum was of very high titer whereas the sera prepared with cultures of *H. influenzae suis* were of relatively low titers. All agglutination tests were conducted at 55°C. and readings were made after 2 hours at this temperature and refrigeration overnight. Cultures of *H. influenzae suis* tested were from widely separated sources and no two cultures were from the same strain of the disease (5). The results of these direct agglutination tests are given in Table III.

The data given in Table III indicate only one possibility that any two of the *H. influenzae suis* cultures are serologically identical. By use of the agglutinin absorption test it was possible to demonstrate that Cultures 652 and 660 were serologically identical. Conversely, by means of the agglutinin absorption test, it was possible to demonstrate that cultures of *H. influenzae suis* 451, 459, 652, and 660 which were agglutinated, but not to titer, by *H. influenzae* BIW were capable of removing no agglutinin for *H. influenzae* BIW.

Pathogenicity of H. influenzae suis for Laboratory Animals

As a rule *H. influenzae suis* is non-pathogenic for rabbits and can be administered intravenously to them in doses of 2 cc. of a heavy suspen-

sion of growth from chocolate agar without inducing any observable evidence of illness. However, such injections occasionally prove fatal in from 3 to 6 hours after injection.

One culture, when injected intraperitoneally, has been found to kill

TABLE IV

The Effect of Inoculating Swine with Pure Cultures of H. influenzae suis

Swine No.	Route of inoculation	Culture used			Clinical evidence of illness	Autopsy findings
		No.	Time since isolation	No. of transfers on media		
507	i.n.*	451	18 days	4	Suggestive of influenza	Suggestive of influenza
515	i.v.**	451	18 "	4	Moribund in 3 days	No respiratory pathology
739	"	451	17 mos.	86	Negative aside from transient temperture elevation	Not autopsied
591	i.n.	Mixture of 1928 cultures	All over 3 mos.	All 15 or more	Negative	Negative
590	"	"	All over 2 mos.	All 10 or more	"	"
588	"	11-T	12 days	6	"	"
648	"	15	16 "	5	"	"
651	"	660	17 "	5	"	"
826	"	791 + 794	14 and 17 days	7 and 8	Death in 2 days	Pneumonia
873	"	451 + 459	Over 2 yrs.	125 and 113	Negative	Negative
893	"	451 + 459	" 2 "	126 and 114	"	"
904	"	896	9 days	7	"	"
912	"	451	Over 2 yrs.	131	"	"
917	"	905	16 days	7	"	"
922	"	451	Over 2 yrs.	132	"	"

* i.n. = intranasally.

** i.v. = intravenously.

guinea pigs in from 18 to 24 hours. Two other cultures are completely non-pathogenic for guinea pigs.

About 50 per cent of the white mice injected intraperitoneally with various strains of *H. influenzae suis* have died. The organism is non-pathogenic when injected intraperitoneally into white rats.

Pathogenicity of H. influenzae suis for Swine

In an attempt to determine the etiological relationship of the organism to swine influenza, thirteen swine were inoculated intranasally with cultures of *H. influenzae suis* from various sources and under cultivation for varying periods of time on artificial media. Two swine received the organism intravenously. The cultures used in these experiments were grown in defibrinated blood at the bases of plain agar slants and the usual dose administered was 2 cc. of this bloody condensation fluid. The results of these experiments are recorded in Table IV.

The data presented in Table IV indicate that *H. influenzae suis* was completely non-pathogenic for eleven of the thirteen swine to which it was administered intranasally. Given intravenously, a relatively recently isolated culture induced a severe illness. However, at autopsy no disease of the respiratory tract suggestive of swine influenza was encountered. There was an acute glomerular nephritis. An older culture given intravenously to another animal proved non-pathogenic.

Swine 507 and 826 are noteworthy in that both animals became ill after intranasal inoculation with *H. influenzae suis* and, in both, the picture presented could readily have been confused with swine influenza. Swine 507 was infected during the early part of the 1st year's work with the disease and at the time was considered to be a fairly typical although mild case of swine influenza. Since *H. influenzae suis* was obtained in pure primary culture from both the bronchial exudate and lung of this animal at autopsy, Koch's postulates had apparently been fulfilled for the organism, and the writers at first believed that it was the inciting agent of the disease. Subsequent work has indicated the mistaken nature of this view but no certain and acceptable explanation of this one experiment, provided Swine 507 actually had influenza, can be offered. It may be that the four transfers on artificial media to which the organism was subjected before its use in inoculating Swine 507 were insufficient to remove mechanically any accompanying virus. Swine 826 died 2 days after inoculation with *H. influenzae suis* and at autopsy presented pulmonary lesions very like those observed in fatal cases of swine influenza. The true pneumonia was limited to the cephalic lobes and, as in fatal swine influenza, the caudal lobes presented an intense bloody edema. *H. influenzae suis* was recovered in pure culture from the kidney, heart blood, and spleen, and was obtained in mixed culture from the lung and bronchial exudate. The disease which developed in Swine 826, however, was recognized as definitely different from swine influenza in that it was not contagious. A normal susceptible

hog placed in the same unit soon after Swine 826 had been inoculated developed no disease. This is markedly inconsistent with the highly contagious nature of swine influenza. A possible explanation of the fatal pneumonia in Swine 826 is that this animal had been taken from its mother at birth, deprived of colostrum, and fed an artificial diet. The influence of such treatment on the course of an influenzal infection will be discussed more fully later.

The experiments recorded in Table IV seem sufficient to demonstrate that *H. influenzae suis* alone is not capable of inducing swine influenza even though it may under certain conditions be pathogenic for swine.

SUMMARY

1. A hemophilic bacillus has been regularly obtained in culture from the respiratory tract of a series of swine experimentally infected with swine influenza and from a small number of spontaneous field cases of the disease. It has not been observed in respiratory tract cultures from a group of swine free from influenza.

2. The cultural and morphological characters of the organism have been described and the name *Hemophilus influenzae* (variety *suis*) suggested. The organism exhibits marked serological diversity, since only two out of eight strains studied were serologically identical. It is usually non-pathogenic for rabbits and white rats, and irregularly pathogenic for white mice. One strain of the organism was pathogenic for guinea pigs while two others were not.

3. Eleven out of thirteen attempts to induce symptoms of disease in swine by intranasal inoculation with pure cultures of *H. influenzae suis* were entirely negative. The remaining two attempts which suggested a positive result have been discussed.

4. Attention has been called to the marked similarity which exists between non-indol-producing strains of *H. influenzae* and *H. influenzae suis*.

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EXPLANATION OF PLATE 35

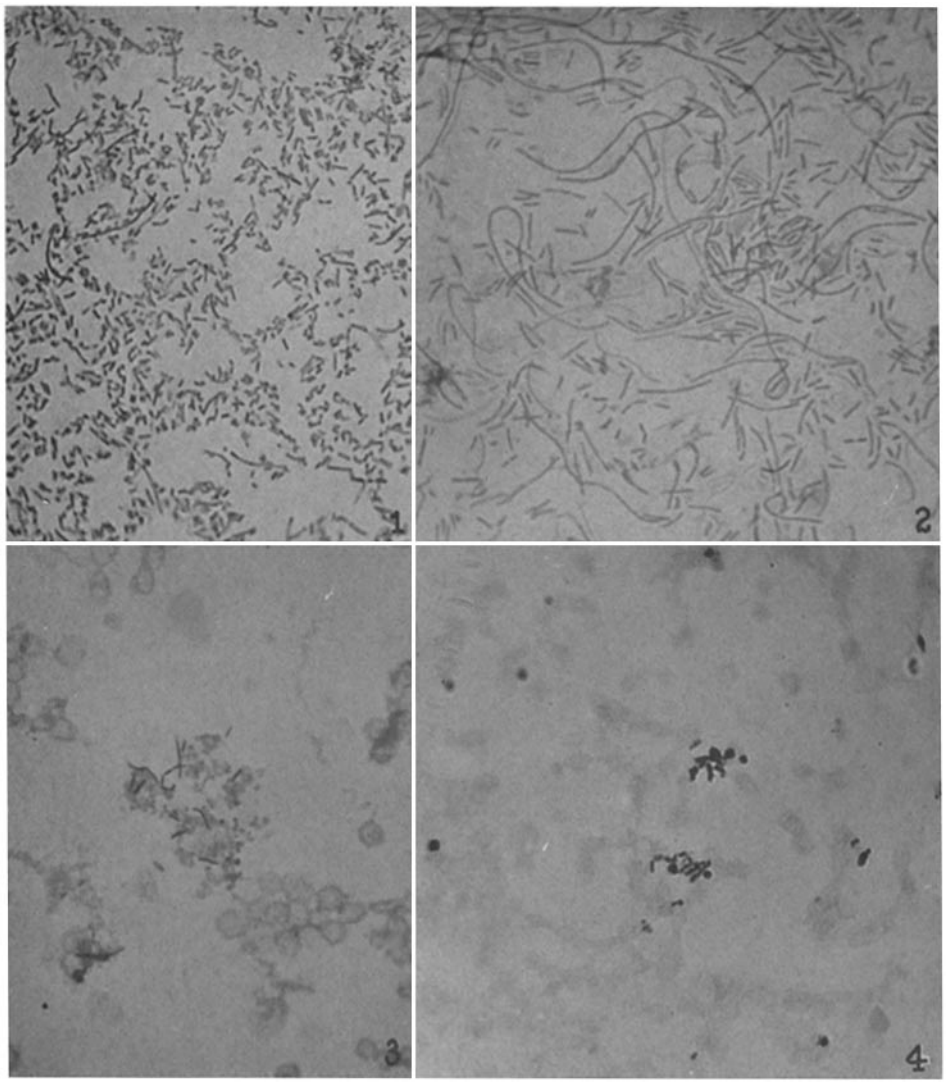
Films from cultures of *H. influenzae suis*. × 905.

FIG. 1. 24 hour chocolate agar culture of Strain 660 showing predominantly small bacillary forms. Alkaline methylene blue.

FIG. 2. 48 hour chocolate agar culture of Strain 913 showing predominantly long thread-like forms. Dilute carbolfuchsin.

FIG. 3. 72 hour blood broth culture of Strain 913 showing clumps of small coccoidal and bacillary forms. Dilute carbolfuchsin.

FIG. 4. 5 day blood broth culture of Strain 459 showing clumps of "giant" coccoidal and club-shaped forms. Alkaline methylene blue.



(Lewis and Shope: Swine influenza. II)