SWINE INFLUENZA

III. Filtration Experiments and Etiology

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McBryde, Niles, and Moskey (1) made five attempts to pass the etiological agent of swine influenza through small Berkefeld or Mandler filters but were unable to reproduce the disease by dropping such filtrates into the nostrils of normal hogs. In two of these experiments unfiltered material failed to produce the disease in their control animals. Although these experiments are too few in number to be conclusive, they indicate that the causative agent of swine influenza is not a filtrable virus.

EXPERIMENTAL

Since the studies in filtration reported by McBryde and his coworkers were not conclusive, the question of the filtrability of the etiological agent of swine influenza has been reconsidered. The results of ten filtration experiments with infectious material from the two strains of the disease obtained in 1928 and the two secured in 1930 (2) were inconstant and confusing. In these preliminary experiments the writer was not then cognizant of the possible etiological relationship existing between *H. influenzae suis* (3) and a filtrable agent to be described.

Material for filtration was prepared as follows.

Diseased lung and bronchial lymph nodes were minced with sterile scissors and added to bronchial exudate. This mixture was ground with sand in a mortar. When it had been reduced to a pasty and fairly homogeneous consistency, a 10 to 20 per cent suspension was made by gradually adding sterile distilled water or infusion broth (pH 7.3). It was then shaken with glass beads in a flask for 10 to 15 minutes and centrifuged. The supernatant fluid was removed by pipette and if more than moderately turbid it was centrifuged again. 24 hour bouillon cultures

of *B. prodigiosus* were used to test the efficiency of all filters, and filtrates cultured in 1 cc. amounts on plain agar slants containing defibrinated blood were incubated at 37°C. for 48 to 72 hours before examination for growth.

Swine receiving filtered material were placed in carefully sterilized isolation units where isolation precautions were taken. In certain experiments the control animal receiving unfiltered material and those receiving sterile filtrates were from the same source or even from the same litter.

Of the ten preliminary filtration experiments, three were at the time interpreted as negative, while in the remaining seven some evidence was obtained that the injected filtrate had contained an infectious agent. The disease induced by this filtrable infectious agent, however, was definitely not typical swine influenza and will be referred to hereafter as "filtrate disease."

Clinically the filtrate disease was much milder than swine influenza. In most cases there was no elevation in temperature, while in a few a fever temperature for 1 day was observed. This was at marked variance with the 4 to 6 day fevers seen in typical swine influenza. The usual symptoms shown by filtrate-inoculated swine were a moderate and transient apathy, some diminution in appetite for a period not exceeding 3 days, occasionally a slight cough, and, as in typical swine influenza (2), a moderate or quite marked leucopenia. The extreme prostration so common in swine influenzal infections was not seen. In some instances the disease was so mild that it almost escaped recognition altogether. On this account and in the light of experiments to be outlined later in this paper, it seems possible that in the three preliminary experiments considered as negative, infections were actually produced but so mild in character that they escaped recognition.

The lesions exhibited at autopsy were similar in kind but different in extent, as a rule, from those encountered in typical uncomplicated swine influenza (2). The cervical and bronchial lymph nodes were moderately enlarged and edematous and there was usually a scant to moderate amount of thick, tenacious mucous exudate in some of the smaller bronchi. The amount of pulmonary atelectasis exhibited by filtrate-infected swine varied from a scant amount in one or two of the upper lobes of the lung to an amount as extensive as that shown by mild cases of swine influenza. In all cases, however, the clinical picture was that of the filtrate disease.

It is significant that the filtrate disease was highly contagious and that the incubation period, like that of swine influenza, was about 4 days. In animals infected by contact with filtrate-infected swine the disease was clinically and pathologically identical to that induced by direct inoculation with the filtrate. In experiments to test the con-

tagiousness of the filtrate disease, the normal animals were placed in the pens with inoculated swine after the appearance of symptoms of disease. This was usually 2 days following inoculation.

The filtration experiments just outlined indicated that infectious material from experimental cases of swine influenza contains an agent capable of passage through Berkefeld filters V and N and possessing pathogenic properties when administered intranasally.

H. influenzae suis, which was constantly encountered in culturing the respiratory tracts of animals with typical swine influenzal infections (3), was not found in similar cultures from animals with the mild filtrate disease.

Anaerobic cultures of seven filtrates of swine influenza infectious material in blood broth and in 5 per cent serum bouillon over sterile rabbit kidney have failed to show growth. Four of the seven filtrates thus cultured were tested by intranasal inoculation into swine and all were found capable of inducing the mild filtrate disease.

Intranasal Inoculations with Mixtures of the Filtrable Agent and H. influenzae suis

Since the only constant difference bacteriologically between the mild disease induced by the filtrable agent and typical spontaneous or experimental swine influenza lies in the absence of *H. influenzae suis* in the filtrate-infected swine, the combination of the organism and the filtrable agent may be essential for the production of the natural disease. Experiments were conducted in which swine were inoculated intranasally with cultures of *H. influenzae suis*, which had been under cultivation for a long time (over 2 years in most instances), mixed with Berkefeld filtrates of infectious material from experimental cases of swine influenza.

In these experiments the isolation and filtration practice outlined above was followed. The cultures of *H. influenzae suis* used were grown in defibrinated horse blood at the bases of plain agar slants in most instances for 24 hours. The undiluted blood culture was used in the inoculations and in all experiments the culture injected alone was identical with that mixed with filtrate before injection. The Berkefeld filtrate mixed with cultures of *H. influenzae suis* was identical with that injected alone in individual experiments. With the excep-

TABL

Effect of Inoculating Swine with Mixtures of I

Experiment No.	Infectous material from swine No.	Swine inoculated No.	Inoculated intranasally with	Clinical picture
1	860	859	10 cc. Berkefeld N filtrate	Mild filtrate disease
	Strain 14 (1930)	861	8 " " " " + 2 cc. culture HIS*	Typical and severe influenza
	In infusion broth	871	10 cc. unfiltered suspension	Typical influenza
2	872	875	4 " Berkefeld N filtrate	Mild filtrate disease
	Strain 15 (1930)	874	4 " " " " + 2.5 cc. culture HIS	Typical influenza
	In infusion broth	873	2.5 cc. culture HIS in 4 cc. infusion broth	No illness
		876	4 " unfiltered suspension	Typical influenza
3	878	894	7 " Berkefeld N filtrate + 2 cc. sterile horse blood	Mild filtrate disease
	Strain 15 (1930)	897	Infected by contact with Swine 894	" " "
	In distilled water	892	7 cc. Berkefeld N filtrate + 2 cc. culture HIS	Typical influenza
		896	Infected by contact with Swine 892	Very severe influenza
		893	2 cc. culture HIS in 7 cc. distilled water	No illness
		895	5 " unfiltered suspension mixed with 10 cc. normal swine serum	Typical influenza
4	907	911	4 cc. Berkefeld N filtrate	Mild filtrate disease
	Strain 15 (1930)	910	4 " " " " + 2 cc. culture HIS	" influenza
	In infusion broth	915	Infected by contact with Swine 910	" "
		912	2 cc. culture HIS in 4 cc. infusion broth	No illness
		913	4 " unfiltered suspension	Mild influenza
5	918	919	8 " Berkefeld N filtrate**	" filtrate disease
	Strain 15 (1930)	920	8 " " " "	" " "
		921	8 " " " "	" " "
	In infusion broth	923	8 " " " " + 2 cc. culture HIS	Typical and severe influenza
		922	2 cc. culture HIS in 8 cc. infusion broth	No illness
		951	8 " unfiltered suspension	Typical influenza

* HIS = *H. influenzae suis.*

** *B. prodigiosus* present in filtrate. *H. influenzae suis,* however, could not be demonstrated. All

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Filtrable Agent and H. influenzae suis

Autopsy findings	*H. influenzae suis* in		Remarks
	Lung	Bronchial exudate or scrapings	
Very few	Absent	Absent	Illness extremely mild
Influenzal pneumonia	Pure culture	Pure culture	More severe disease than control (871)
Typical	" "	Mixed "	Control of unfiltered suspension
Very few	Absent	Absent	Illness extremely mild
Typical	Pure culture	Mixed culture	Disease about same severity as control (876)
Negative	Absent	" "	Control of culture alone
Typical	Mixed culture	Pure "	" " unfiltered suspension
Few	Absent	Absent	Scarcely recognizable illness
Not autopsied			" " "
Typical	Mixed culture	Pure culture	Same severity as disease in control (895)
Influenzal pneumonia	Pure "	Mixed "	Moribund when killed
Negative	Absent	" "	Control of culture alone
Typical	Mixed culture	Not cultured	" " unfiltered suspension
Few	Sterile	Sterile	Scarcely recognizable illness
Typical	"	Mixed culture	Same type of disease as control (913)
"	Pure culture	" "	" " " " " " (913)
Negative	Absent	Pure "	Control of culture alone
Typical but few	"	" "	" " unfiltered suspension
Not autopsied			Scarcely recognizable illness
" "			" " "
" "			" " "
Typical	Pure culture	" "	More severe than disease of control (951)
Not autopsied			Control of culture alone
Typical	" "	" "	" " unfiltered suspension

other filtrates recorded were sterile.

tion of the first experiment, all animals used in individual experiments were from the same source and in most instances litter mates. The results of these experiments are recorded in Table I.

All eight of the swine infected by inoculation with Berkefeld filtered infectious material or by contact with filtrate-infected swine developed only a mild disease. In some instances it was so slight as almost to escape recognition. None of the animals exhibited a febrile reaction. In 1 to 3 days after inoculation they appeared listless and apathetic for 2 or 3 days and there was some diminution in appetite. Those coming to autopsy showed enlarged and edematous cervical and bronchial lymph nodes, a small amount of tenacious mucous exudate in some of the smaller bronchi, and a scant scattered type of pulmonary atelectasis of one or more of the upper lobes of the lung.

The swine which were inoculated intranasally with pure cultures of *H. influenzae suis* were completely negative both clinically and at autopsy.

All the swine which received mixtures of the filtrable agent and *H. influenzae suis* developed a disease that was typical both clinically and at autopsy of swine influenza (4). Of the seven hogs infected either by direct inoculation with the filtrate-culture mixture or by contact with swine so infected, three developed typical swine influenza, and the disease was of about the same severity as that which developed in the control animals inoculated with unfiltered infectious material. Two others had a mild influenza, but in this instance the disease which developed in the control animal was also atypically mild. The remaining two swine developed exceptionally severe swine influenza and at autopsy both exhibited typical pneumonia. These two animals showed a more severe type of infection than did their controls infected by unfiltered material.

Swine recorded in Table I as "infected by contact" were not placed in the pens with the animals to whose disease they were to be exposed until the onset of illness in the inoculated animals.

Immunity Experiments

During the fall and winter months when swine influenza occurs, individual herds of hogs are occasionally reported to have swine influenza two or even three times during one season. In such instances

the outbreaks follow one another at short intervals. The disease is very uncommon in animals (brood sows and boars) over 1 year of age on farms which suffered losses the previous year. This indicates the acquisition of immunity from the attack of the preceding year.

Our experience with the experimental disease indicates that the majority of animals are made immune by one attack of the disease. We have repeatedly placed freshly infected swine in the same units with convalescent animals without producing a second attack of the disease in the convalescent animal. This is in marked contrast to our experience with previously unexposed animals.

While the failure to develop the disease by contact is an indication of some degree of immunity, it is a less severe test than submitting the animals to actual reinoculation with infectious material. This has been done with six animals convalescent from typical swine influenza. These swine were inoculated intranasally with material, the infectiousness of which was controlled by parallel inoculation of a normal animal. The reinoculations were made at intervals varying from 15 to 24 days following the animal's first infection and 8 to 16 days after symptoms had disappeared. Of these animals, four were not reinfected, one developed a typical attack of swine influenza, and one developed a very abortive type.

One sample of convalescent serum has been tested for its neutralizing capacity.

The animal which furnished this serum, Swine 806, had undergone a typical, severe attack of swine influenza. After recovery it was exposed to four typical cases of the disease in sequence, remaining in the same pen with the infected swine from the time of inoculation until they were killed on the 4th day of disease. No illness resulted from any of the exposures. The serum was obtained 40 days after the initial inoculation and 16 days after the last exposure to a case of the disease. Strain 14 was the source of infectious material used either directly or by contact. The serum was tested against Strain 15 material as follows.

Swine 879 was inoculated intranasally with 5 cc. of Strain 15 material mixed with 10 cc. of convalescent Serum 806. The mixture stood at room temperature for 3 hours before use. No evidence of illness developed in this animal during a 10 day observation period. The control, Swine 895, inoculated intranasally with 5 cc. of the same infectious material mixed in the same manner with 10 cc. of normal swine serum, developed a typical swine influenza which was at autopsy found to be characteristic and extensive. Swine 879 after an observation period

of 10 days was injected intranasally with 10 cc. of Strain 15 material. After a slightly prolonged latent period the animal developed typical swine influenza with a temperature reaching 41.5°C. At autopsy the disease was typical and extensive.

In this instance, then, serum of a hog that had undergone a typical swine influenza and that was demonstrably immune to reinfection by contact neutralized swine influenza material. No demonstrable immunity developed in the animal receiving the neutral mixture.

Storage of Infectious Material

Experiments to test the keeping qualities of the agents of swine influenza have been complicated by differences in the period of survival of the two components. Pieces of atelectatic lung and bronchial lymph nodes from one experimentally infected swine were stored for 15 to 33 days and from another swine for 15 and 41 days in 50 per cent glycerol before testing them for infectivity. They have been found capable of inducing only the mild filtrate disease typical in its course and at autopsy. With one exception *H. influenzae suis* has not been demonstrable in cultures from the respiratory tract of swine infected with this material. With infectious material frozen and dried by Swift's method (5) the disease induced by stored material was somewhat different. Material that had been stored for 34 days proved capable of inducing only the filtrate type of disease when inoculated into two susceptible swine and *H. influenzae suis* was not found in the respiratory tracts of these two animals at autopsy. However, another tube of this same material tested after 54 days' storage proved capable of inducing typical and rather severe swine influenza in which at autopsy *H. influenzae suis* was found in both the bronchial exudate and the atelectatic lung. It appears that the swine influenza virus is capable of storage in a dried state or in glycerol for at least 54 or 41 days, respectively, but that the bacterial component of the mixture is less resistant to such storage. The irregularity in the results obtained with dried infectious material may have been due to faulty freezing or drying of the particular tubes of dried material tested after 34 days' storage, for it is difficult to understand why *H. influenzae suis* should not survive freezing and drying. If it were desirable to preserve both factors the virus could be maintained in a dried state or in glycerol, while the organism could be kept under cultivation on artificial media and the two mixed before inoculation.

DISCUSSION

In a series of preliminary experiments to determine the pathogenic properties of bacteriologically sterile Berkefeld filtrates of infectious material from experimental cases of swine influenza, it has been possible in most cases to induce a definite but mild illness by the intranasal inoculation of swine with such filtrates. The disease thus induced has been transmissible by contact without altering its clinical or pathological characteristics. The possibility was thus eliminated that the filtrate disease is the result of inoculation with a toxic bacterial or tissue end-product or aggressin incapable of self-propagation in series. The observation that the illness which developed in animals exposed to cases of the filtrate disease does not differ in its clinical or pathological characteristics from that resulting from direct inoculation with the filtrable agent indicates that the mildness of the filtrate disease is not due to dilution of the inciting agent during filtration. Since the filtrate-induced disease has consistently been at variance with typical swine influenza, it was obvious that the disease induced by a filter-passing virus could not rightly be considered swine influenza. The impression gained after consideration of a series of these mild infections was that the disease both clinically and pathologically represented natural swine influenza in an incomplete form.

In the preceding paper (3) it was shown that a hemophilic bacillus, *H. influenzae suis*, was constantly demonstrable in the respiratory tracts of swine ill with influenza. It has been consistently absent from the respiratory tracts of swine ill with the filtrate disease. To test the possibility that swine influenza is the result of the two agents acting together, swine were inoculated intranasally with mixtures of the filtrable agent and *H. influenzae suis*. A disease typical of swine influenza in all clinical and pathological respects and indistinguishable from that induced by unfiltered infectious material resulted in all instances. Control animals receiving cultures of *H. influenzae suis* alone developed no evidence of illness and swine receiving the filtrable agent alone developed the mild filtrate disease. It seems permissible to interpret these experiments as indicating that swine influenza is due to a filtrable virus and *H. influenzae suis* acting together. Their mode of action is unknown although two possibilities are obvious:

The first possibility is that the pathological activities of the virus are such as to create a portal of entry for *H. influenzae suis* and to furnish a favorable medium in which it can multiply. Under such an assumption the virus serves merely as an entering wedge for the organism and the latter determines the clinical picture and pathology. There can be little doubt from the data presented in this and the preceding paper (3) that, in the presence of the swine influenza virus, *H. influenzae suis* possesses invasive powers which it completely lacks when administered alone.

The second possibility is that the virus is the important component in contributing to the pathology and perhaps also to the symptoms characterizing the clinical picture, and that *H. influenzae suis* increases to a marked degree the pathogenic properties of the virus and hence the severity of the resulting disease. In this respect, the influence of *H. influenzae suis* on the pathogenic properties of the swine influenza virus suggests the effect of certain tissue extracts on various viruses pointed out first by Duran-Reynals (6) and later amplified by Hoffman (7).

Whatever the relation of the virus and the organism in respect to the disease, the data presented indicate that they act together.

The hypothesis is not new that a disease may be induced by a bacterium and an invisible agent, not readily demonstrable alone. It applies most directly to diseases in which the suspected bacterial agent, while readily and uniformly isolated from cases of the disease, either is completely incapable of reproducing the infection or very rapidly loses its ability to do so under conditions of artificial cultivation. It is possible that such organisms do not become non-pathogenic because of rapid loss of virulence but because of the absence of an invisible inciting agent. The proven association of organisms in swine influenza furnishes a tangible and experimentally reproducible justification of this hypothesis.

A question which naturally arises is whether any organism other than *H. influenzae suis* can induce swine influenza in conjunction with the virus. In twenty-one infections of swine with virus alone none of the organisms comprising the normal bacterial flora of the respiratory tract have been capable of producing swine influenza. Also, the constant presence of *H. influenzae suis* in experimental infections

induced by eight strains of swine influenza collected during three separate epizootics would seem sufficient to indicate that, if it is not the only organism capable of completing the etiological complex, it is at least the predominating one for the region from which our original material has been obtained.

It has been of interest to find in several of the fatal influenzal pneumonias that no organism other than *H. influenzae suis* could be cultivated from the pneumonic lungs, heart blood, or pleural and pericardial exudates. It is believed that in these cases, probably because of factors in the host itself, *H. influenzae suis* alone, or in conjunction with the virus, was endowed with invasive and pathogenic characters which in ordinary non-fatal influenzal infections it did not possess.

Since swine are subject to another filtrable virus disease, hog cholera, it has been essential to ascertain that the causative agent of swine influenza is not the hog cholera virus in a disguised or uncommon form. Our evidence indicating that the swine influenza virus is distinct from that of hog cholera may be summarized briefly as follows. Immunization against hog cholera affords no protection against swine influenza, while conversely animals recovered from swine influenza are still susceptible to hog cholera. The administration of hog cholera virus by way of the respiratory tract results in typical hog cholera with no tendency to produce pulmonary lesions suggestive of swine influenza. Convalescent swine influenza serum capable of completely neutralizing swine influenza virus does not neutralize hog cholera virus.

The clinical picture of swine influenza, characterized by fever, anorexia, extreme prostration, leucopenia, and evidence of respiratory involvement and of muscular tenderness, is strikingly suggestive of human epidemic influenza. The onset is sudden, the course short, and convalescence usually uneventful. Death, when it occurs, is the result of an edematous type of pneumonia. The pathology of non-fatal swine influenza, characterized as it is by an exudative bronchitis and extensive pulmonary atelectasis, cannot be compared with the findings in non-fatal human influenzal infections because of our lack of knowledge of the latter. Probably the most significant similarity concerns the predominant bacterium encountered in the two conditions; *H. influenzae suis* is indistinguishable morphologically and culturally from *H. influenzae*. The frequency with which *H. influenzae*

has been encountered in careful bacteriological studies of human influenza parallels the frequency of occurrence of *H. influenzae suis* in swine influenza, and, as in the case of the latter organism, has suggested an etiological significance. Without drawing analogy too far, the irregularity in the outcome of the filtration experiments reported, especially by French and English investigators, in attempting to determine whether a filtrable virus causes human influenza, is very similar to the experience of the writer in the early filtration experiments with swine influenza. The preliminary obstacles encountered in studying the nature of the etiological factors in swine influenza have had much in common with those met by investigators of human influenza. A careful investigation would seem warranted of the possibility that Pfeiffer's bacillus and a filtrable agent act in concert to cause influenza in man.

SUMMARY AND CONCLUSIONS

1. It has been possible to demonstrate, in Berkefeld filtrates of infectious material from experimental cases of swine influenza, a virus which when administered intranasally to susceptible swine induced a mild, usually afebrile illness of short duration. The changes in the respiratory tract resembled those in swine influenza but were usually much less extensive. When the filtrable virus was mixed with pure cultures of *H. influenzae suis* and administered to swine a disease identical clinically and pathologically with swine influenza was induced. The data presented indicate that the filtrable virus of swine influenza and *H. influenzae suis* act in concert to produce swine influenza and that neither alone is capable of inducing the disease.

2. One attack of swine influenza usually renders an animal immune to reinfection. Blood serum from an animal made immune in this way neutralizes infectious material from swine influenza *in vitro*, as shown by the failure of the mixture to produce disease in a susceptible animal.

3. The virus can be stored in a dried state or in glycerol for several weeks at least. In one instance dried material apparently retained both the virus and *H. influenzae suis* in viable form for a period of 54 days.

4. Fatal cases of experimental swine influenza have been observed

in which *H. influenzae suis* was the only organism that could be cultivated from the respiratory tract.

5. Attention has been called to some features of marked similarity between epizootic swine influenza and epidemic influenza in man.

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