A BACTERIOLOGICAL STUDY OF INFECTIOUS LARYNGOTRACHEITIS OF CHICKENS[1]

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Since its first recognition in 1924 as a distinct disease of chickens, infectious laryngotracheitis, more commonly termed infectious bronchitis, has assumed a position of major economic importance to the poultry industry of the United States and Canada. It is not known to have occurred elsewhere in the world.

From 1924 to 1926 studies of the disease were reported by Gwatkin (1) in Canada, May and Tittsler (2) in Rhode Island, Eriksen (3) in Missouri, Hinshaw (4) in Kansas, and Beach (5) in California. By these studies the infectious and distinct nature of the disease was established. The causative agent was found to be contained in the exudate which accumulates in the larynx and trachea of infected chickens but its presence in other organs was not demonstrated. Transmission to susceptible chickens was readily accomplished by intralaryngeal or intratracheal inoculation with exudate from the larynx and trachea of infected fowls. Attempts to produce the disease by subcutaneous, intravenous, intramuscular, or intraperitoneal injection of tracheal exudate, however, yielded either entirely negative or questionable results. Efforts by ordinary bacteriological procedures to isolate from diseased chickens any species of bacteria with which the disease could be reproduced were uniformly unsuccessful. Similar results from studies of the disease were reported by Kernohan (6) in 1930. He also reported inability to produce disease with material after passage through Seitz, Berkefeld W, Chamberland F, or Mandler filters.

For the studies reported in this paper chickens were infected by means of intratracheal injections with one or another of four strains of the causative agent of the disease. The original infective material

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consisted of exudate from the trachea of diseased fowls from two farms in New Jersey and from two farms in California. It was not used for the experiments until it had been passed at least twice through chickens in which it produced characteristic and uncomplicated symptoms and lesions. The manifestations of disease in the experimental fowls were uniform for this reason, and the interpretation of the results of the experiments was not confused by the occurrence of an intercurrent infection. The sole source of chickens for the experiments was an inbred strain of Rhode Island Reds that had been neither affected with nor exposed to any infectious disease for several generations. Through the cooperation of Mr. Raymond Ring a supply of chickens from this source was continuously available.

The extremely contagious nature of the disease made it unusually difficult to avoid accidental cross-infection among the experimental animals. For this reason, the means that were successfully employed in preventing such cross-infection are worthy of brief description.

The metal cages used were sterilized by boiling. The caged chickens were kept in isolation units unconnected with each other, each having its own equipment of utensils and of overalls, rubber gloves, and rubber overshoes. These were invariably worn by anyone who entered a unit, and the persons entering were limited to the writer, and occasionally his assistant. A unit and its equipment were never used a second time without thorough cleaning and sterilization with very hot water, procedures found through several years' usage to be adequate to prevent the transfer of infection. Chickens inoculated with different types of material were never placed together. Groups of chickens inoculated with material of the same type or with graded doses of the same material were, however, kept in a single unit, each chicken in a separate cage. In such instances the data were discarded if the time of the first indications of disease made it possible that later cases resulted from cross-infection from the first cases. In the early experiments a cage containing one or more normal fowls was placed in the same unit with each lot of inoculated chickens to provide a control on the presence of virus in the unit. This precaution was discontinued when it became evident that it was unnecessary.

Bacteriological Studies

Films and cultures were made from the laryngeal and tracheal exudates and mucosa, spleens, and livers of chickens that had died of the disease or been chloroformed after definite symptoms had developed.

The films were usually stained with methylene blue for 24 hours and with Giemsa. In some instances fresh preparations were examined by dark-field illumination. The culture media included chicken- and horse-blood agar plates, chicken- and horse-blood agar slants, chocolate agar slants, chicken- and horse-blood broth, coagulated serum slants, serum agar slants, and cooked beef heart. The last mentioned medium was the only one used in the endeavor to demonstrate anaerobic bacteria. Tubed media were usually inoculated in pairs, one sealed with wax. All plate cultures were incubated for not less than 48 hours. They were examined both with the unaided eye and microscopically. No tube culture was discarded as negative until after from 10 to 20 days' incubation and after microscopic examination of stained preparations of liquid media and of the liquid at the base of the slant surface of solid media. In the majority of instances all of the types of media listed were employed. By these procedures examinations have been made of tracheal exudates of 61 chickens, of the spleens of 67 chickens, and of the livers of 48 chickens.

Results of Examinations of Tracheal Exudates.—In twenty-four instances no bacteria were observed in the stained films of the exudate. The films of tracheal exudate from thirty-seven chickens contained varying numbers of cocci and rod-shaped organisms of varying size but there was no form that could be regarded as predominant. No bacteria were seen in dark-field examinations of fresh preparations unless they were also present in stained preparations of the same material. Spirochetes, which Gibbs (7) reported to have been present in the larynx and trachea of half of forty-two chickens with infectious laryngotracheitis which he examined, were not observed.

The failure previously mentioned of a number of investigators to isolate any organism that could be regarded as the causative agent of laryngotracheitis made it evident that if the disease were due to any bacterium it was not one that could be readily cultivated. For this reason, in the examination of cultures from the tracheal exudate, attention was given only to minute or slowly developing colonies. The results obtained in cultures of the tracheal exudate of the 61 chickens were as follows:

In thirty-two instances (52.4 per cent) the bacterial growth in all cultures was so heavy and profuse that pure culture isolation was not attempted. In view of the exposed location of the exudate and of the findings in many of the films such results were to be expected.

In seventeen instances (27.8 per cent) it was possible to pick discrete,

minute colonies and therefrom to secure pure cultures. One of these proved to be a small Gram-positive coccus, one a small Gram-negative rod of the pasteurella type, and fifteen were diphtheroids. These last may be similar to the organism reported by Graham (8) as present in laryngotracheitis lesions and found by him to possess considerable pathogenicity for chickens. He stated, however, that this organism was not regarded as a primary cause of laryngotracheitis.

In twelve instances (19.6 per cent) all media inoculated with tracheal exudate remained sterile. It was rather surprising to find bacteria absent from the tracheal exudate in so many cases.

Results of Examinations of Spleens and Livers.—No bacteria were observed in the stained films or in fresh preparations examined by dark-field illumination from any of the spleens and livers. The cultures from 64 spleens and 45 livers were negative. Growths of a diphtheroid type of organism were obtained in the cultures from two spleens and two livers; of a coccus from one spleen; and of a streptococcus from one liver.

Pathogenicity of the Strains Isolated.—Since, as will be shown later, it was found possible to produce laryngotracheitis by inoculation with material that was bacteria-free, the only effort to determine the pathogenicity of the strains isolated was one series of injections with a saline suspension of a mixture of ten strains. In this trial one chicken was injected with the suspension by the subcutaneous, one by the intravenous, one by the intramuscular, and one by the intratracheal method. The results were entirely negative.

Causative Agent Present in Spleen and Liver

In attempts to find the causative agent in tissues that would be more satisfactory for bacteriological examination than tracheal exudate and also to throw some light on the distribution of the virus in the body, chickens were injected by the intratracheal method with saline or broth suspensions of the spleens and livers of infected fowls. In some instances the spleen or liver of a single chicken was used and in others the organs of two or more were pooled. All tissue suspensions were carefully searched for bacteria by cultural methods as previously described.

The causative agent of laryngotracheitis was demonstrated in

eighteen of the thirty spleen emulsions and two of the six liver emulsions by the appearance of typical symptoms and lesions of laryngotracheitis in the fowls on the 3rd, 4th, or 5th day following injection. Bacteria were not found in any of the tissue emulsions which were infective. The incubation period was from 24 to 48 hours longer than that following inoculation with tracheal exudate, which was regularly 2 or 3 days; but the character of the disease was no less severe.

The adequate control of environmental conditions and the uniformity of the results made it certain that the disease which developed in the chickens following the injections of emulsions of spleen or liver resulted from the infective properties of the material injected. Furthermore, in one experiment the inoculation of fowls separately with emulsions of fresh and of desiccated portions of the same spleen gave identical results, that is to say, laryngotracheitis after an incubation period of 4 days. In four experiments, in each of which two chickens were injected with a suspension of spleen tissue, but one of each two became diseased. In two other experiments in which graded dilutions of spleen tissue were injected the causative agent was not demonstrable in dilutions higher than 1:10 and 1:50 respectively. These results show that the causative agent is much less concentrated in spleen tissue than in tracheal exudate, with which infection of fowls has regularly been accomplished with dilutions as high as 1:100,000 or 1:1,000,000. The fact that both negative and positive results were obtained with spleen tissue secured from fowls on the 2nd, 3rd, 4th, and 5th days after they had been inoculated, indicates that the time between inoculation of the fowls and removal of the spleens is not a determining factor of the presence of demonstrable amounts of virus in these organs.

The results of these experiments with spleen and liver tissue, together with the absence of lesions in any organs except the respiratory tract, indicate that the presence of the causative agent in the liver or spleen does not imply any real involvement of these organs. It seems probable that injury to the walls of the blood vessels of the larynx and trachea permits the entrance of the etiological agent into the blood stream and its distribution. Further evidence that laryngotracheitis is not accompanied by a general organic or systemic involvement is provided by the fact that no thermal reaction of significance

occurred in the experimentally infected chickens. In illustration of this the temperature records of three chickens which are representative of many are given in Table I.

TABLE I

Temperature Record of Fowls after Inoculation with Laryngotracheitis

Chicken No.	Day				
	1	2	3	4	5
1740	42.2*	42.4	42.2**	42.2***	
1824	42.0*	42.4**	42.1	41.9	42.6***
1845	41.8*	41.8**	42.1	42.3	42.1***

* Inoculated.

** First symptoms.

*** Marked symptoms. Chloroformed.

Host Specificity of the Disease

The transmission of laryngotracheitis to birds other than chickens was attempted to determine, first, to what extent other species, particularly the wild species and domesticated but free-flying species such as pigeons, might be susceptible and therefore of importance as agencies in the spread of the infection, and second, if there were some bird host less susceptible than the chicken, by passage through which the virulence of the causative agent might be so modified that it could be utilized for the immunization of chickens.

In these experiments 5 sparrows, 1 crow, 3 doves, 1 starling, 9 pigeons, and 15 ducks were used. The wild birds were trapped on the Institute grounds; the pigeons were secured from a source that was known to be free from disease and from all contact with chickens; the ducks were hatched and reared at the Institute. The injections were entirely by the intratracheal method. The virulence of the inoculum was always demonstrated by the inoculation of susceptible control chickens.

These experiments resulted in complete failure to transmit the infection to any of the birds except the control chickens. When the initial injection into pigeons failed, an endeavor was made to enhance the virulence for these birds by serial passage. This

likewise failed. Furthermore, by the intratracheal injection into fowls of the scrapings of the tracheal mucosa of pigeons that were chloroformed on the 5th, 8th, or 10th days after injection it was determined that the causative agent had not survived in the tracheas of the pigeons. These findings are not in accord with Kernohan's (6) report of the observance of spontaneous infection with laryngotracheitis in sparrows and pigeons on infected poultry farms.

Attempts have been made to transmit laryngotracheitis to rabbits, guinea pigs, and white rats, by intratracheal, intracerebral, and intravenous injections, and to one pig by intratracheal injection of tracheal exudate taken from diseased fowls. The presence of the causal factor in the material injected was demonstrated in all instances by its ability to infect control chickens. None of the animals proved susceptible to the disease.

SUMMARY AND CONCLUSIONS

1. The causative agent of infectious laryngotracheitis of chickens was found to be present in bacteriologically sterile tracheal exudate, spleens, and livers of diseased fowls.

2. The causative agent was present regularly in the tracheal exudate, in the spleens of about 60 per cent, and in the livers of about 30 per cent of chickens with active laryngotracheitis infection.

3. Suspensions of the spleen and liver were less effective in inducing the disease than those made from the tracheal exudate. This finding, together with the absence of pathological changes in the spleens and livers would seem to indicate that they are not actively involved but that the causative agent is carried to them by way of the blood.

4. The disease could, in our experience, be produced only in chickens Domesticated ducks and several wild and free-flying species of birds, including sparrows, crows, starlings, doves, and pigeons were found to be refractory, and so too were rabbits, guinea pigs, white rats, and one pig that was tested.

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