TITRATION OF FOWL POX VIRUS AND ANTISERUM

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INTRODUCTION

The purpose of this study was to develop a titration method for fowl pox virus and to conduct neutralization tests against standard samples of this virus with antiserum prepared from previously immunized chickens.

Previous attempts to titrate fowl pox virus by the use of serial dilutions inoculated onto the chorioallantoic membrane and counting the number of lesions generally have been unsuccessful due to the development of secondary lesions and confluent necrosis that result from multiple virus infection. The ideal titration method would be one in which each virus particle produces one single lesion that may be distinguished and counted, such as in the titration method used for bacteriophage.

To avoid the difficulties of secondary lesions and confluent necrotic areas, this study involved titration by a method whereby serial dilutions of virus were inoculated onto the chorioallantoic membranes, and the appearance of one or more lesions of fowl pox was considered evidence of infection. Instead of counting lesions, any embryo showing one or more lesions was scored as positive. The 50 percent end point, or the dilution at which 50 percent of the membranes would have one or more lesions, was calculated. Greater accuracy was attained with commercial vaccine strains by using two-fold, instead of ten-fold, dilutions of virus. A single source of antiserum was used to neutralize three different virus samples and the results were compared.

Inoculation onto the chorioallantoic membrane is a tedious and time-consuming task. For this reason, simpler methods of virus titration were also investigated. Titrations were attempted

by the allantoic cavity route in which death rather than lesions indicated infectivity. Also, hemagglutination tests were conducted with fowl pox and pigeon pox viruses. Precipitin tests were attempted with both these viruses, using commercial vaccine strains.

Since there is little scientific literature on fowl pox virus, the review of literature is more or less comprehensive in order to provide a background of the properties of fowl pox virus and the disease.

REVIEW OF LITERATURE

Fowl pox is a disease complex consisting of lesions or tissue injuries of the skin (pox) and of the mucous membranes (diphtheria). It affects many species of domestic fowl and free-flying wild birds.

It is now known that fowl pox is caused by a virus. From time to time many agents, including bacteria and protozoa, have been indited as the cause of the disease. Bunyea (1942) cited an old book on poultry diseases in which the origin of "diphtheria" was attributed mainly to "improper care and sudden changes of weather." About the beginning of this century it was discovered that the infective agent producing fowl pox was a filterable virus.

Bunyea states that there are two types or strains of the virus known to be infectious for avian species. The more common and important strain causes natural outbreaks in chickens and other barnyard fowl, including turkeys, guinea fowl, pheasants, ducks, geese, and other species. Of less importance is pigeon

pox virus which produces the disease in pigeons. Other species are relatively resistant to pigeon pox virus, but it has produced mild lesions experimentally in chickens, particularly when inoculated into feather follicles. Pigeons are quite resistant to the other type of virus.

According to Bunyea, the virus of fowl pox is very resistant to dessication. This factor permits the disease to spread to susceptible birds many months after the virus has been scattered on the premises of a chicken yard.

A new outbreak usually begins with the occurrence of the typical pox lesions on the face parts of the fowl. The virus may gain entrance where the comb or wattle has been wounded. Certain species of mosquitos may spread the disease by carrying the infectious agent for as long as 27 days after feeding on a fowl, according to Bunyea. It is probable that the virus gain entrance only through broken skin or mucous membranes of the respiratory system.

Fowl pox virus is not known to be infectious for human beings or any species of mammals. However, Seigel (1956) reported the identification of a filterable agent from a human skin papilloma as a strain of avian pox virus resembling canary pox, but he concedes that the pox virus may have arisen as a laboratory contaminant.

Bunyea states that fowls of all species are susceptible to the disease. Large-combed and large-wattled birds seem to acquire pox lesions more often. The diphtheria manifestations affect all breeds equally.

The first symptoms appearing are usually the cutaneous (pox) lesions on the unfeathered part of the body, according to Bunyea. The diphtheric manifestations occur as a deposit on the mucous membranes of the eyes, mouth, or respiratory regions, and are sometimes accompanied by coughing and gasping. Both types of lesions frequently occur in a single outbreak of the disease. For many years the two manifestations were regarded as entirely distinct diseases, but within recent times it has been clearly demonstrated that the two disease symptoms have a common origin.

In describing the pox manifestations of the infection, Bunyea states that the lesions appear three to four days after exposure in the form of grayish pimples or blisters containing a straw-colored fluid which is very virulent. In a few days the blisters may enlarge and run together. After ten to fourteen days the blisters usually begin to darken and form dry, hard scabs resembling warts that cling to the skin another week or longer. Finally the scabs loosen and drop off leaving new and possibly scarred skin beneath.

In the diphtheric form the disease has no definite course but may persist for weeks or months before being terminated by death or recovery. Yellowish or whitish cheesy patches form on the mucous membrane of the toungue, mouth, esophagus and larynx. These patches are very tough and adherent. Similar deposits may occur on the sinuses of the eye, preceded by watering and inflammation. The eyelids eventually become swollen and stick together.

These cheesy patches interfere with the bird's vision, eating and respiration. Weakness, starvation, and suffocation

which result add to the high mortality rate of the diphtheric infection.

Fowl pox virus is not affected by any medicinal treatment known and is inactivated only by certain strong disinfectants. This leaves the poultry man with quarantine and sanitation as the only methods of control.

The fact that fowl that recover from the disease possess a solid immunity for a year or more has been the basis for much experimental work leading to the present methods for vaccination for the prevention of fowl pox. As early as 1910 investigators reported favorable results from the injection into the veins of pox-scab material ground in a physiological salt solution. Bunyea stated that early methods failed to gain wide acceptance owing to the lack of uniformly satisfactory results. However, later studies on immunogenic properties led to the development of more satisfactory vaccines.

Akawawa (1951) prepared a vaccine by collecting the crusts from lesions on the comb eight days after infection and shaking them with phenol solution. After washing the material with saline solution he placed it in a solution of 60 percent glycerine and 0.005 percent phenol. This solution was diluted 1:200 with 0.85 percent saline solution. An inoculum given subcutaneously over the breast muscle caused no systemic reaction or loss in egg production. In four weeks immunity was fully established.

In further studies on immunogenic properties of fowl pox virus Bryan (1949) reported that a vaccine prepared from undesicated, entire chicken embryos infected with fowl pox

suspended in 50 percent buffered glycerol showed 92.9 percent takes. When stored at 14° C. the vaccine was active eight months after preparation; when stored at 5° C. the vaccine was viable for two years. The particular value of this type of vaccine lies in the fact that, since it is not necessary to desicate the virus material, the vaccine can be prepared in a laboratory in a short time and dispensed ready for use.

In regard to the morphology of fowl pox virus several studies have been made with the electron microscope, the results of which are more or less correlative. In an early study, Groupe, et al. (1946) found that the elementary bodies of fowl pox closely resemble those of vaccinia virus, being approximately 250 x 350 millimicrons in size and having a rounded quadrangular shape. They observed that the particles of avian poxes are most frequently attached to one another at their corners and that the characteristically flattened corner observed on many particles probably results from the separation of the particles thus joined.

A further study of the morphological characteristics of the virus with particular reference to internal structure was made by Groupe and Rake (1947). They used the shadow casting technique of Williams and Wyckoff (1945). Heavily infected chorioallantoic membranes were ground with sand and then partially purified by differential centrifugation. The virus pellet was then suspended in distilled water and placed on a collodion film support. After air drying the elementary bodies were shadowed by vaporizing about 20 milligrams of gold about 8 to 10 centimeters from the specimen in a vacuum.

Electron microscopy revealed the elementary bodies to be mostly rectangular in shape, although some rounded forms were observed. The bodies are assumed to be short cylinders, so the rounded forms were explained by the way in which the particles settle on the film. The particles were most frequently joined at their corners by interparticular bridges and not infrequently large, central, mound-like elevations were observed. In addition, numerous strands of taffylike material, apparently under tension, connected many particles with one another; these strands were easily distinguishable from the interparticular bridges. These findings suggested that the particles were coated with a sticky material. The sharply circumscribed piled up mass of elementary bodies were interpreted as representing a Bollinger or inclusion body.

In similar electron microscopic examinations of ultracentrifuged preparations of fowl pox virus, Morgan and coworkers (1954) reported two strikingly different forms of the virus. The intracytoplasmic virus, at one stage of its development, was shown to be composed of a nucleus-like structure, a granular "viroplasm," and a single limiting membrane. However, near the surface of the host cell and in the extracellular space the virus was seen to have a dense, possibly disk-shaped, inner body enclosed by a double limiting membrane. Other viral particles were encountered which were believed to represent transitional forms. The initial sites of development were confined to the cytoplasm.

Fowl pox virus was the first virus to be studied on the chorioallantoic membrane of the chick embryo. Woodruff and

Goodpasture (1931) found that lesions could be readily produced when infective material was placed on the exposed membrane. Burnet (1936) also produced lesions in this manner from several strains. Beaudette and Hudson (1938) experimented with pigeon pox virus on the chorioallantoic membrane and obtained extensive, generalized infection as far as the 19th passage. At this point they dried the virus and found it to give a high percentage of "takes" when used as vaccine.

Practically all reported work with fowl pox virus has made use of the chorioallantoic method. Histologically, fowl pox lesions are probably the simplest of all virus lesions on the egg membrane. There is practically no necrosis of the affected ectodermal cells and consequently little inflammatory reaction.

In an investigation of the possibility that egg passage of the virus altered its infectivity for the embryo or the chicken, Brandly (1935) passed fowl pox through eight series of eggs via the chorioallantoic route. He concluded that the virus was not significantly altered in virulence for the developing eggs or chickens of several ages. These cultivated viruses gave satisfactory "takes" and immunity as compared to the comb-lesion virus from nearly mature chickens.

In further studies of factors which might affect the virulence of egg-propagated fowl pox virus, Brandly (1936) experimented further with successive egg passage and storage. He concluded that the only effect of successive egg passage was the enhancement of the filterability which did not appear to be associated with change in size or character of the Borrell bodies.

Drying in vacuo over $CaCl_2$ and H_2SO_4 at 0 to 8° C. was adopted as the best method for storage of the virus, however, the practice of storing the egg lesion "seed" in the bacteria-free chorioallantoic tissue at 0° C. was used with success during the earlier course of Brandly's work.

In connection with the studies of these viruses in the chorioallantois of the developing chick embryo, Thorning, et al. (1943) have shown the distribution of the virus in other parts of the embryo. In a comparative study of immunogenic properties of virus obtained from various parts of the embryo, their experiments indicated that the virus was present in the embryo proper and yolk as well as the chorioallantois. In the chorioallantois, virus was generally demonstrated in a dilution of 1:640 (the highest employed), while in the yolk and embryo proper virus was demonstrated in dilutions ranging from 1:10 to 1:160 with greater concentration in the yolk.

Literature reveals very few records of attempts to grow fowl pox virus in tissue cultures. Following a more or less characteristic experiment, Bang, et al. (1950) reported survival and multiplication of the virus in roller tube cultures of chick lung fibroblast, but the cells failed to show any inclusions or destruction. The virus grown in cultures of lung epithelium killed the epithelial cells without formation of inclusion bodies. After failing at repeated attempts to develop the typical cytoplasmic inclusions of fowl pox virus in tissue culture by maintaining the virus in chick fibroblast medium for periods exceeding 100 days, Bang made his analysis of the inclusion itself on the

chorioallantoic membrane. He indicated that it consisted of many virus particles buried in a fatty matrix.

As an explanation of why inclusions failed to develop in tissue culture, Bang stated that conditions in the constantly washed tissue culture differ sufficiently from those of the more static chorioallantoic membrane so that the large, fatty structure characteristic of the latter cellular infection does not develop in tissue culture.

Siegel (1956) reported a study of a strain of avian pox virus resembling canary pox whereby at least four passages each on HeLa cells, human amnion, monkey kidney, and rabbit testicular tissue tube cultures failed to show any indications of proliferation. This strain did, however, cause definite cellular degeneration in chick fibroblast cultures, but without the formation of lesions.

It may seem that the failure to find fowl pox inclusions in tissue culture is not significant because of the low yield of virus in these cultures, and it may therefore be assumed that the infected cells in the roller tube do have inclusions but that they are too few to be seen. However, this latter assumption seems quite unlikely, since the typical inclusion is so characteristic and so large that a single one can be seen under a microscope in the midst of a colony of cells.

A study made by Buddingh (1938a) on the intracerebral inoculation of fowl pox virus into young chicks provided several interesting observations. In the first place, he produced an experimental disease characterized by drowsiness followed by

spastic paralyses and convulsions and death on the seventh or eighth day. The pathological lesions were found chiefly in the meninges, perivascular structures, the choroid plexus, paranasal sinuses, mastoid cells, the marrow of the cranial bones and the orbital tissues. In the second place, this method of propagation produced marked changes in the behavior of the virus. One expression of change was a decided increase in the virulence of the virus for epithelial cells. The virus also acquired the property to infect cells of mesodermal origin. In the third place, the virus acquired in this environment the property of altering all types of infected cells regardless of their origin so that they became spherical in shape and detached from one another. This experiment was carried through 14 successive passages. The symptoms and lesions in the chicks inoculated with the 14th passage showed no marked differences from those of the first passage. No enhancement of the changes produced in the virus by the intracerebral environment took place upon repeated passages.

As a follow-up of his above-mentioned study Buddingh (1938b) studied this fowl pox virus variant in the chorioallantoic membrane of the chick embryo and in the skin of baby chicks. This variation obtained by intracerebral passage continued to express itself when propagated away from intracerebral conditions. Environmental factors were obviously the cause of the variation. The modification seemed to be a fixed one as far as Buddingh's experiments were carried. However, he did not specify how many passages were made, and evidence is not sufficient to conclude

this change as a true, fixed mutation or one from which a gradual reversion to the normal strain occurs.

Foley (1944), reporting on the nature of the reinfection response in fowl pox, stated that chickens infected with the virus developed lesions at an accelerated rate upon reinoculation of the virus. The incubation period of reinfection lesions was from one to two days, in contrast to the usual three- to four-day incubation period of primary infection. The reinfection lesions not only developed quickly, but they also disappeared more rapidly, usually by the fifth day. This behavior suggested an allergic reaction which was the conclusion Foley reached after making a number of experiments following the reinfection response in the skin of chickens in order to compare it with well-known instances of allergic response seen in other infections.

It has been shown by Burnet (1936) and others that there were degrees of immunity to fowl pox in the domestic fowl which appeared to be conditioned by the extent of the primary lesion and the time elapsing between primary infection and the secondary application of the virus.

As Foley pointed out, his findings must be considered in the light of these facts and cannot be taken to indicate a type reaction to be expected in all attempts at reinoculation.

A review of the literature on fowl pox virus revealed only a few scientific studies with this virus. Further research in this field may possibly lead to the titration of fowl pox virus and antisera in tissue culture. Recombination studies may be

made in an attempt to produce strains of fowl pox more suited for the preparation of vaccines and various other purposes.

EXPERIMENTAL PROCEDURES

Materials and Methods

Titrations. Viruses used in this experiment consisted of the following:

1. Fowl pox virus from an infected comb of a chicken with the disease, supplied by Dr. West of the Department of Pathology.
2. A commercial vaccine strain of fowl pox virus and a commercial vaccine strain of pigeon pox virus donated by Dr. M. K. Nadel of Research Laboratories, Inc., St. Joseph, Missouri.
3. Commercial vaccine strains of fowl pox and pigeon pox were purchased but failed to produce lesions when inoculated onto the chorioallantois. No further reference to these will be made.

The comb of a chicken infected with fowl pox virus was ground in a mortar with clean, sterile sand and 1 to 2 ml. of saline into a thick paste. The paste was diluted with one part by weight to 9 parts of nutrient broth, which was considered to be the 10^{-1} dilution, and centrifuged for 30 minutes, then filtered through a Seitz filter. The filtrate was placed in the freezer compartment of a household refrigerator to be used as stock.

The stock virus was inoculated in 0.1 ml. aliquots onto the chorioallantoic membrance of one dozen 12-day embryonated eggs

and incubated 5 to 6 days. All embryos dead at the end of the first 24-hour period were regarded as non-viral fatalities and discarded. All eggs were candled twice daily. Dead embryos were opened and inspected for lesions. Also, one living embryo was opened each day. From these observations it was determined that the lesions were more completely formed on the fifth day than at any other time and that this would be the most satisfactory incubation period.

All the membranes with lesions were harvested, ground in a mortar, and the samples were centrifuged and filtered. These viruses were used for the process of titration. Serial ten-fold dilutions were made with 10^{-1} the greatest concentration. Five 12-day embryonated eggs per dilution were inoculated by the chorioallantoic (CAM) route with 0.1 ml. of virus. The eggs were incubated lying flat with the point of inoculation upward, and the eggs were not turned during incubation.

The embryos were again checked twice daily, and the dead ones were examined for the presence of lesions. All remaining embryos were opened after five days. In replicated studies six similar passages and inspections were made.

The commercial vaccine strains of fowl pox and pigeon pox viruses were titrated similarly to the infective fowl pox, except with the greatest concentration used as 1:5 followed by two-fold serial dilutions, since these strains were less virulent than the infected comb strain. Therefore, the ten-fold dilutions were too great stepwise to give a reasonably accurate titer.

Seven passages were made on the chorioallantoic membranes of 12-day embryonated eggs.

In an attempt to titrate fowl pox and pigeon pox viruses by the allantoic cavity route, ten-fold serial dilutions ranging from 10^{-1} to 10^{-12} were inoculated in 0.1 ml. aliquots into the allantoic cavities of 10-day embryonated eggs. At the end of five days all embryos were alive and healthy, except a few killed by trauma. Five additional experiments were made. All of these proved unsuccessful.

Serology. The serological tests made consisted of neutralization tests, hemagglutination tests, and precipitation tests.

Neutralization Tests. Two eight-weeks-old chickens were bled and the normal serum obtained. They were then inoculated intramuscularly twice weekly for three weeks with 0.5 ml. of fowl pox virus obtained from the infected comb of a diseased chicken. Following a ten-day interval both chickens were given a booster shot of the stock virus. After one week the antiserum was collected.

Neutralization tests were conducted in the following manner. The infective virus stock from the chicken comb was prepared in serial dilutions ranging from 10^{-1} to 10^{-10}, and 0.25 ml. of each dilution was mixed with 0.25 ml. of undiluted antiserum. The mixture was allowed to stand for one hour at room temperature. Five 12-day embryonated eggs per dilution were inoculated with 0.1 ml. of the antiserum-virus mixture onto the chorioallantoic membrane. The eggs were candled at 24-hour intervals during the five days following inoculation. Embryos dying during the first 24 hours were discarded as due to non-specific death. At the

end of five days all live embryos were opened and scored for lesions.

In all cases of neutralization tests, the virus sample alone was titered so that the neutralization index (Cunningham, 1954) could be determined.

Similar neutralization tests were conducted with this antiserum and commercial strains of fowl pox and pigeon pox viruses, except that two-fold dilutions were used.

A series of neutralization tests were also conducted on the basis of various time periods with mixtures of antiserum and fowl pox commercial vaccine virus to detect the influence of time on the neutralization ability of the antiserum. The mixtures were allowed to stand at room temperature for periods of 1, 15, 30, and 40 minutes. Ten-fold dilutions ranging from 10^{-1} to 10^{-8} were used. Three 12-day embryonated eggs per dilution were inoculated with 0.1 ml. of the antiserum-virus mixture onto the chorioallantoic membrane. This procedure was repeated with each mixture after the various time periods had elapsed.

Hemagglutination Tests. Hemagglutination tests were made by the addition of 0.25 ml. of red blood cells to serial two-fold dilutions of fowl pox and pigeon pox viruses, ranging from 1:5 to 1:2560. A control tube was maintained.

Precipitation Tests. In conducting precipitation tests the precipitin tubes were filled to 1/2-inch levels with undiluted fowl pox antiserum. Serial two-fold dilutions of fowl pox vaccine strain virus, ranging from 1:5 to 1:5120, were layered onto the antiserum. A control tube was retained. This procedure was

repeated with pigeon pox vaccine strain virus. All tubes were incubated at 37° C. and observed for evidence of precipitation at intervals of 15, 30, 45, and 60 minutes.

Results

Table 1 shows the results of the sixth passage in the titration of infective fowl pox virus from the comb of a chicken with the disease. The titer on each passage was determined to be approximately 10^{-5} which means in this case that the virus at this dilution produced definite lesions on the chorioallantoic membrane of embryonated eggs, whereas no lesions were formed at dilutions of 10^{-6} and above. The 50 percent end point of $10^{-5.25}$ was used in the determination of the neutralization index of the serum sample (Table 4).

Table 2 presents the results of titers on material from the seventh passage of a commercial vaccine strain of fowl pox virus on the chorioallantoic membrane of 12-day embryonated eggs. Titrations were also made on samples from the previous six passages, but the data is not presented because only the seventh passage titer was used in the calculation of the neutralization index. The 50 percent end point of this sample was 1:769 and was the value used in determination of the neutralization index of the serum sample in Table 5.

The infectivity titer for pigeon pox commercial vaccine virus was determined to be 1:2985, as shown by Table 3. It was the

Table 1. Results of titration of infective fowl pox virus obtained from infected comb.

Dilution	Eggs per dil.	Eggs without lesions	Eggs with lesions	Accumulated eggs without lesions	Accumulated eggs with lesions	% of eggs with lesions
10^{-1}	5	0	5	0	23	100
10^{-2}	5	0	5	0	18	100
10^{-3}	5	1	4	1	13	93
10^{-4}	5	0	5	1	9	90
10^{-5}	5	1	4	2	4	67
10^{-6}	5	5	0	7	0	0
10^{-7}	5	5	0	12	0	0

The 50 percent end point (the dilution which produces lesions on 50 percent of the eggs inoculated) equals $10^{-5.25}$, as determined by the Reed and Meunch method as follows:

$$\text{Proportionate distance} = \frac{\text{\% with lesions just greater than 50} - 50}{\text{\% with lesions just greater than 50} - \text{\% with lesions just less than 50\%}}$$

Proportionate distance multiplied by the stepwise difference in exponents and added to the dilution giving a percent of lesions just greater than 50% equals the end point.

Table 2. Results of titration of commercial vaccine fowl pox virus.

Dilution	Eggs per dil.	Eggs without Lesions	Eggs with lesions	Accumulated eggs without lesions	Accumulated eggs with lesions	% of eggs with lesions
1:5	4	0	4	0	37	100
1:10	5	0	5	0	33	100
1:20	5	1	4	1	28	96
1:40	5	1	4	2	24	92
1:80	5	1	4	3	20	87
1:160	5	1	4	4	16	80
1:320	5	2	3	6	12	66
1:640	5	2	3	8	9	53
1:1280	5	3	2	11	6	35
1:2560	5	3	2	14	4	22
1:5120	5	4	1	18	2	10
1:10,240	5	4	1	22	1	4
1:20,480	5	5	0	27	0	0

The 50 percent end point as determined by the Reed and Meunch method equals 1:769, $10^{-2.89}$.

Table 3. Results of titration of commercial vaccine pigeon pox virus.

Dilution	Eggs per dil.	Eggs without lesions	Eggs with lesions	Accumulated eggs without lesions	Accumulated eggs with lesions	% of eggs with lesions
1:5	3	1	2	1	28	97
1:10	3	0	3	1	26	96
1:20	3	0	3	1	23	95
1:40	3	1	2	2	20	91
1:80	3	0	3	2	18	90
1:160	3	0	3	2	15	88
1:320	3	0	3	2	12	86
1:640	3	0	3	2	9	82
1:1280	3	0	3	2	6	75
1:2560	3	0	3	2	3	60
1:5120	3	3	0	5	0	0
1:10,240	3	3	0	8	0	0
1:20,480	3	3	0	11	0	0

The 50% end point as determined by the Reed and Meunch method equals 1:2985, $10^{-3.47}$.

value used for the determination of the neutralization index of the serum sample (Table 6).

Neutralization tests conducted with antiserum and fowl pox virus from an infected chicken comb resulted in the formation of lesions in 4 of 5 eggs inoculated with the virus-antiserum mixture in which the virus was diluted to 10^{-1}. All other dilutions of virus up to 10^{-5} were inhibited from formation of lesions by the antiserum, as shown in Table 4.

A commercial vaccine strain of fowl pox virus mixed with antiserum produced lesions in all three eggs inoculated in which dilution of 1:5, 1:10, 1:20, 1:40 and 1:80 were used. In subsequent dilutions the viruses were neutralized by the antiserum, as shown in Table 5.

Table 4. Results of neutralization tests with infective fowl pox virus and antiserum.

Dilution	Eggs per dil.	Eggs without lesions	Eggs with lesions	Accumulated eggs without lesions	Accumulated eggs with lesions	% of eggs with lesions
10^{-1}	5	1	4	1	4	80
10^{-2}	5	5	0	6	0	0
10^{-3}	5	5	0	11	0	0
10^{-4}	5	5	0	16	0	0
10^{-5}	5	5	0	21	0	0

The 50% end point as determined by the Reed and Meunch method equals $10^{-1.37}$. The neutralization index as determined by subtracting the exponent of the 50% neutralization end point from the exponent of the 50% titration end point ($10^{-5.25}$) equals 3.88 (Cunningham, 1954).

Table 5. Results of neutralization tests with commercial vaccine strain fowl pox virus and antiserum.

Dilution	Eggs per dil.	Eggs without lesions	Eggs with lesions	Accumulated eggs without lesions	Accumulated eggs with lesions	% of eggs with lesions
1:5	5	0	5	0	18	100
1:10	5	1	4	1	13	93
1:20	5	1	4	2	9	80
1:40	5	2	3	4	5	55
1:80	5	3	2	7	2	22
1:160	5	5	0	12	0	0
1:320	5	5	0	17	0	0
1:640	5	5	0	22	0	0
1:1280	5	5	0	27	0	0
1:2560	5	5	0	32	0	0
1:5120	5	5	0	37	0	0
1:10,240	5	5	0	42	0	0
1:20,480	5	5	0	47	0	0

The 50% end point as determined by the Reed and Meunch method equals 1:46 or $10^{-1.66}$. The neutralization index as determined by Cunningham's method equals 1.23 (2.89 minus 1.66).

Commercial vaccine pigeon pox virus mixed with antiserum produced lesions in all three eggs inoculated in which dilutions of 1:5, 1:10, 1:20 and 1:40 were employed, but the virus was neutralized in dilutions upward from 1:40, as shown in Table 6. This was to be expected due to the lower infectivity titer of this strain.

Table 6. Results of neutralization tests with commercial vaccine strain pigeon pox virus and antiserum.

Dilutions	Eggs per dil.	Eggs without lesions	Eggs with lesions	Accumulated eggs without lesions	Accumulated eggs with lesions	% of eggs with lesions
1:5	5	1	4	1	16	94
1:10	5	1	4	2	12	85
1:20	5	0	5	2	8	80
1:40	5	2	3	4	3	43
1:80	5	5	0	9	0	0
1:160	5	5	0	14	0	0
1:320	5	5	0	19	0	0
1:640	5	5	0	24	0	0
1:1280	5	5	0	29	0	0
1:2560	5	5	0	34	0	0
1:5120	5	5	0	39	0	0
1:10,240	5	5	0	44	0	0
1:20,480	5	5	0	49	0	0

The 50% end point as determined by the Reed and Meunch method equals 1:36 or $10^{-1.56}$. The neutralization index as determined by Cunningham's method equals 1.91 (3.47 minus 1.56).

The results of neutralization tests in which the virus and antiserum mixtures were allowed to stand for various time periods are shown in Table 7. Due to a shortage of eggs the control in this experiment was not carried out at the time of this writing but will be conducted in the near future. Nevertheless it may be seen from Table 7 that neutralization progresses with time but

that a plateau is not reached even after 30 to 40 minutes.

Table 7. Results of time neutralization tests with fowl pox commercial vaccine strain virus.

Dilution	Eggs per dilution	Eggs with lesions 1 min.	15 min.	30 min.	40 min.
10^{-1}	3	3	3	2	1
10^{-2}	3	3	2	1	0
10^{-3}	3	3	2	0	0
10^{-4}	3	3	3	0	0
10^{-5}	3	0	0	0	0
10^{-6}	3	0	0	0	0
10^{-7}	3	0	0	0	0
10^{-8}	3	0	0	0	0

A brief comparison of the neutralization indices obtained shows a wide variation which may be explained by the antigenic specificity of the three different samples of viruses used.

Table 8 shows the result of hemagglutination tests for both strains of viruses. Partial hemagglutination was observed in dilutions ranging from undiluted to 1:10. The titer was derived at 1:10.

Table 8. Results of hemagglutination tests of vaccine strains of fowl pox and pigeon pox viruses.

Tube												
1	2	3	4	5	6	7	8	9	10	11	12	
Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560		C
+	+	+	-	-	-	-	-	-	-	-	-	

Precipitin tests conducted with both fowl pox and pigeon pox viruses were negative with no precipitate formed at the junction of the virus and the antiserum.

DISCUSSION

Titration of fowl pox virus by the production of lesions on the CAM seemed to be satisfactory though somewhat laborious due to the time required for inoculation and the difficulty in detecting lesions. The process of detecting lesions may be accomplished more precisely by the removal of the chorioallantoic membrane and placing it in a petri dish with a small amount of saline and viewing it against a dark background under a microscope.

There is no standard fowl pox diagnostic antigen available similar to commercial antigens available for Newcastle disease virus and other viruses. In this study, material originating from the infected comb or commercial vaccine preparations were used for the neutralization tests. In all cases, however, the virus material used for titration had been passed through chick embryos for several passages. In comparing neutralization indices obtained for the three different viruses used, vast differences were obtained even though the same antiserum was used. This may be explained on the basis that these three viruses were antigenically non-identical (the virus from the infected comb was antigenically more specific for the antiserum used). Also, possibly there are particles in the commercial vaccine preparation which do not produce lesions but neutralize the antiserum. Thus, part of the antiserum neutralization would be dissipated on these particles and a lower index would be obtained.

In conducting neutralization experiments a good source of antigen is of the most importance. From the results obtained, it may be seen that it is far better to use the virus material

from an infected chicken than to use a commercial vaccine, even though the latter may contain live virus. In the light of this experiment, a standard neutralization test for fowl pox virus should include, in addition to a good source of antigen from an infected chicken, antiserum prepared from previously immune chickens to be used for control, two-fold dilutions of antigen and at least a one-hour neutralization period before inoculation.

This experiment indicated that the ten-fold serial dilutions of the highly virulent fowl pox virus from the infected chicken comb gave a reasonably accurate infectivity titer. Two-fold serial dilutions used for the less virulent commercial vaccine viruses also gave an accurate, although much lower, titer. The method of scoring the presence or absence of lesions, rather than attempting to count lesions was considered to be accurate, especially since an absolute count of lesions was not possible.

An attempt to reduce the labor involved by using a simpler procedure of injecting serial dilutions of the virus into embryonated eggs by the allantoic cavity route proved unsatisfactory due to failure of the virus to kill the embryos. The question arose as to whether the allantoic cavity inoculation, which is a standard titration method for Newcastle disease virus and other viruses, could be used for fowl pox. This method failed completely in this experiment.

It is the author's intention to carry this investigation further in an attempt to titrate fowl pox virus and antiserum in tissue culture. There is no standard method of titration and neutralization of fowl pox virus. The virus used may vary widely

in infectivity range, as indicated by this experiment. It is desirable to obtain an effective quantitative measurement for titration of fowl pox virus in order to use antiserum for neutralization of the virus.

SUMMARY

A virulent strain of fowl pox virus from the comb of an infected chicken and commercial vaccine strains of fowl pox and pigeon pox viruses were successfully titrated on the chorio-allantoic membrane of embryonated hen eggs. The method used was to score the presence or absence of lesions at the various dilutions, rather than attempting to count the lesions, a procedure which is complicated by the development of secondary lesions and confluent necrosis resulting from multiple virus infection.

For the more virulent strain from the diseased comb, serial ten-fold dilutions were used. The 50 percent end point (meaning the dilution producing lesions of 50 percent of the eggs inoculated) was determined as $10^{-5.25}$. Two-fold serial dilutions were made of the less virulent commercial vaccine strains of fowl pox and pigeon pox. The 50 percent end points were determined by seven passages and were found to be 1:769 and 1:2985, respectively, indicating that the ability of pigeon pox to infect was lower than that of the fowl pox virus.

Attempts to titrate these viruses by the use of the allantoic cavity proved unsuccessful due to the failure of the viruses to kill the embryos.

Serological tests, including neutralization, hemagglutination, and precipitation, were also conducted with these viruses. Of these, only the neutralization test gave positive results.

In the light of these experiments, a standard neutralization test for fowl pox virus should include a source of antigen preferably from an infected chicken, antiserum prepared from previously immune chickens for use in controls and at least a one-hour neutralization period before inoculation.

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TITRATION OF FOWL POX VIRUS AND ANTISERUM

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Previous attempts to titrate fowl pox virus have generally made use of the method of counting the lesions formed by serial dilutions of the virus inoculated onto the chorioallantoic membrane of embryonated eggs. This method is often unsuccessful due to the development of secondary lesions and confluent necrosis resulting from multiple virus infection. When membranes with such lesions are examined, it is difficult for the observer to record a numerical value of the lesions present.

The purpose of this study was first to titrate fowl pox by a method whereby serial dilutions of the virus were inoculated onto chorioallantoic membranes and to score the appearance of one or more lesions as evidence of infection. The 50 percent titration end point (the dilution which would produce lesions on 50 percent of the eggs inoculated) was then calculated. Once a satisfactory titration method was established, it was used to conduct neutralization tests using a single antiserum sample prepared by injecting the infective fowl pox virus into two young chickens.

Three different strains of virus were used in the neutralization tests. In one case, an infective strain of fowl pox virus from the comb of a diseased chicken was titrated as the control. The titer derived was $10^{-5.25}$, which means that the virus at this dilution would be expected to produce definite lesions on the chorioallantoic membrane of 50 percent of embryonated eggs. Upon titration of this virus plus the antiserum sample, the 50 percent end point was $10^{-1.37}$, resulting in a

neutralization index of 3.88 (determined by the method of C. H. Cunningham, 1954). This neutralization index was calculated by subtracting the exponent of the titer of the antiserum-virus sample (1.37) from that of the virus alone (5.25). In a second case, a commercial vaccine strain of fowl pox virus was used in the titration. The neutralization index was determined as 1.23. In a third case, a commercial vaccine strain of pigeon pox virus was titrated. The neutralization index was derived as 1.91. The varying neutralization indices could be explained on the basis that these three viruses were antigenically nonidentical, even though all strains has been grown on chick embryos.

An attempt to substitute a titration method in embryos by inoculation of virus into the allantoic cavity was unsuccessful.

Hemagglutination tests were performed with both strains of commercial vaccine viruses. Partial hemagglutination in each case was observed in dilutions ranging from undiluted to 1:10; a hemagglutination titer of 1:10 is insufficient to be considered as positive. Precipitin tests conducted with both fowl pox and pigeon pox viruses were negative with no precipitate formed at the junction of the virus and the antiserum.

The results of this study indicate that observation of lesions on the chorioallantoic membrane was the only satisfactory titration method of those attempted. Because it is difficult, by methods used previously, to specify a numerical value for the lesions present, it is suggested that a 50 percent titration end point may be calculated by scoring membranes as

either positive or negative for the presence of lesions. It is further suggested that the virus strain used in the neutralization test be carefully selected so that a maximum index will result in testing positive serum. In this study, such a virus strain was found by isolation from the comb of a natural case of fowl pox.