EPIZOOTIOLOGY OF AN ISOLATE OF INFECTIOUS BRONCHITIS VIRUS AND THE PATHOGENICITY IN GENETICALLY DIFFERENT CHICKEN EMBRYOS

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ABSTRACT

EPIZOOTIOLOGY OF AN ISOLATE OF INFECTIOUS BRONCHITIS VIRUS AND THE PATHOGENICITY IN GENETICALLY DIFFERENT CHICKEN EMBRYOS

by Harvey Graham Purchase

A mild strain of infectious bronchitis virus was isolated from chickens with respiratory symptoms at the U. S. Regional Poultry Research Laboratory. Confirmation of identity was based on symptoms, histopathology, fluorescent antibody, lesions in chicken embryos and lack of lesions on the chorioallantoic membrane, hemagglutination with trypsin-treated virus, and lack of hemagglutination inhibition. Using serum-virus neutralization tests in embryos, the virus was more closely related to the Iowa 97 serotype of Hofstad (1958) than to any of the other six serotypes described in the literature.

The epizootiology indicates that the virus is maintained in the nonisolated breeding stock as an inapparent infection. Periodically there is a break in the quarantine and the disease occurs in the isolated area in more mature birds.

The 23rd chicken embryo passage of virus can be used to immunize immature chickens to prevent a possible drop in egg production if the birds are exposed during the laying period.

The candling method of determing positive responses was almost as accurate as examining the embryos for lesions. There was some correlation between the constant serum--decreasing virus and the constant virus--decreasing serum methods of determining antibody. The latter method is useful for screening large numbers of sera, is less expensive, and is of sufficient accuracy for epizootiologic studies.

Passage in the allantoic sac of embryonating chicken eggs increased the pathogenicity of the virus for line 7 embryos significantly but the pathogenicity for line 6 embryos remained unchanged. Inoculation of embryos from a diallel cross mating of inbred lines 6, 7 and 151 demonstrated that line 7 embryos die significantly later than line 6 embryos. Like 151 is intermediate between these two. Some matings had significantly more or less mortality than would be expected from a consideration of the lines themselves. The reasons for this are obscure. The difference between reciprocal crosses is significant at 7 days postinoculation. The effect of the line 7 male on delaying the mortality is greater than that of the female. This may be due to one or more sex-linked genes for delaying mortality in line 7 or to a maternal effect in line 7 females causing earlier mortality.

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By

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INTRODUCTION

Infectious bronchitis (IB) was first detected at the U.S.D.A., Regional Poultry Research Laboratory (RPRL) in 1955 by virus isolation and serum neutralization tests performed by Dr. C. H. Cunningham, Department of Microbiology and Public Health, Michigan State University. A diagnosis of Newcastle disease (ND) was made by Dr. C. F. Hall, Department of Veterinary Pathology, Michigan State University in 1957 on the basis of a serum hemagglutination inhibition (HI) titer. Since then there have been periodic occurrences of respiratory symptoms and several laboratory diagnoses of IB and ND have been made. Respiratory symptoms were often noticed in 2-week-old chicks being inoculated with avian tumor viruses. Sometimes they occurred among the line 15 isolated (15I) breeding stock and occasionally among chickens in other isolated areas. In these cases signs of the disease were often noticed in more mature birds.

A mild respiratory disease occurred in January 1963, in birds on a leukosis experiment. A positive HI test was obtained with serum of 2

birds submitted to Dr. J. P. Newman, Department of Microbiology and Public Health, Michigan State University, on January 28, 1963 but virus was not isolated. An additional 6 samples submitted on January 30 were negative.

The purpose of the present study was to isolate and identify the agent (designated Regional Poultry Laboratory infectious bronchitis virus (RPL-IBV) causing the respiratory disease, to study the source and spread of infection and to institute prophylactic measures. Every consideration had to be given to the nature of the other research being conducted at the laboratory.

LITERATURE REVIEW

Etiological Agent

Infectious bronchitis is an acute, highly infectious and sometimes fatal viral disease of chickens. It is widespread throughout the world (Hofstad, 1959; Kawakubo et al., 1961). The virus is 80-120 mμ in diameter and is related to the influenza group (Cunningham, 1957; Hofstad, 1959; Berry et al., 1964; Tevethia, 1964).

Incubation and Symptoms

The incubation period is usually 18-36 hours but may be as long as 15 days depending on the strain of virus, dose, passage level and amount of inoculum (Raggi and Bankowski, 1956). The characteristic symptoms are a nasal discharge, watery eyes, gasping, rales, coughing and sneezing. The mortality may be high. In laying flocks there is a sudden decline in egg production and the shells may be misshapen, rough or soft.

The duration of the disease is usually 1-2 weeks but this depends on the environmental conditions and secondary complications such as chronic respiratory disease (Cunningham, 1957; Hofstad, 1959).

Pathology

Post mortem findings include a serous or catarhal tracheitis and catarrhal or fibrinous aerocystitis. There is often a focal pneumonia localized around the bronchi. In chicks there is usually a catarrhal rhinitis, sinusitis and conjunctivitis.

Histologically there is a non-purulent catarrhal tracheitis, bronchitis and broncheolitis. Inclusion bodies have not been reported. The microscopic changes have been regarded by some authors as pathognomonic (Jungherr et al., 1956). Other authors consider they are only useful for susceptibility studies (Hofstad, 1945a). The temporal changes after artificial exposure were described by Jungherr et al. (1956).

Epizootiology

The epizootiology of the disease is not well understood. The chicken is the natural host and, as far as is known, no other avian species are affected (Van Roekel, 1955). Air-borne transmission is probably the most common route in nature (Hofstad, 1959). The virus can be easily transmitted artificially and the intratracheal route is most commonly employed (Cunningham, 1957).

The virus can be isolated readily from lung and tracheal material taken throughout the respiratory phases of the disease (Hofstad, 1947; Fabricant, 1949; Fabricant, 1950; Fabricant and Levine, 1951; Kawakubo et al., 1958) and from eggs laid 2 to 43 days after infection (Fabricant and Levine, 1951). Delaplane and Stuart (1941) showed that chickens could carry the virus for at least 8 weeks. Chicks can transmit the virus as long as 35 days after the appearance of symptoms but only if infected within the first few weeks of life (Hofstad, 1945b; 1947).

Virus Isolation and Passage.

Infectious bronchitis virus can be propagated readily in embryonating chicken eggs. The more usual route of inoculation is via the allantoic cavity but other routes can be used (Cunningham and Jones, 1953; Kawakubo et al., 1958).

Characteristic lesions in chicken embryos which are employed as diagnostic criteria have been described by many workers (Loomis et al., 1950; Hofstad, 1956b; Hofstad, 1959). Embryos may be dwarfed as much as one-half and are "curled" into a firm compact ball with the feet compressed over the head. There is less amniotic

fluid and the allanto-amnionic membrane is often thickened. Living embryos are sluggish in their movements. Edema, necrosis of the kidneys and a persistent mesonephros containing urates are often found. Hemorrhage and necrosis of the liver, pneumonia, and dermal petechiation have also been reported. Embryo mortality is not a constant finding (Cunningham, 1957). More than one serial passage may be necessary before a diagnosis can be made (Fabricant, 1949).

Serial passage of the original (O) isolate in embryonating eggs is accompanied by an increase in virulence of the virus for embryos and development of the derivative (D) or egg adapted phase. Concurrently there is usually a decrease in virulence for chickens and a loss of antigenicity and immunogenicity (Delaplane and Stuart, 1941; Larose and Van Roekel, 1961), though this is not always the case (Kawakubo et al., 1961). The O phase is more thermostable than the D phase (Singh, 1957; Singh, 1960). The embryo-adapted virus is specifically neutralized by anti-IB serum (Fabricant, 1951).

Hemagglutination

The virus does not agglutinate chicken erythrocytes unless modified with trypsin (Corbo and Cunningham, 1959; Muldoon, 1960). Specific inhibition of hemagglutination (HA) has not been demonstrated. Anti-IB sera do not have HI titers of greater than 160 against Newcastle disease virus (NDV) (Fabricant, 1950).

Immunity

Neutralizing antibodies are first detectable about 2 weeks after infection, they can be reliably demonstrated at 3 weeks and their titer continues to rise until about 5 weeks after infection (Fabricant, 1951; Page and Cunningham, 1962). A serum-virus neutralization index (NI) greater than 2.0 is indicative of previous exposure to IB (Cunningham, 1951; Fabricant, 1951). The serum antibody level of day-old chicks is almost identical with that of their respective dams but this maternal antibody declines rapidly until it is lost by 4 weeks of age (Jungherr and Terrell, 1948). Even chicks with a high maternal antibody level are not necessarily protected from showing symptoms when challenged during the first week after hatching (Hofstad and Kenzy, 1950).

Diagnosis

A diagnosis of IB is dependent on the demonstration of the following:

1. A typical history and symptoms
2. Characteristic histo-pathologic changes
3. Positive fluorescence with a fluorescein labelled anti-IB serum but not with other labelled antisera.
4. Lack of HA except with modified virus
5. A negative HI titer using Newcastle disease virus antigen
6. Virus isolation and the demonstration of typical lesions in embryos
7. A NI greater than 2.0 against an isolate of IB
8. Lack of neutralization of other viruses

Serology

Jungherr et al. (1956) reported that an isolate from Connecticut (Conn) differed from an isolate from Massachusetts (Mass). These were the only two sero-types recognized until 1958 when Iowa 97 and Iowa 609 were shown to be serologically different from the Mass and Conn types (Hofstad, 1958). Then two additional and unrelated isolates, Holte and Grey, were reported by Winterfield and Hitchner

(1962). Yates and Fry (1957) and DuBose and Grumbles (1959) showed the chicken embryo lethal orphan virus (CELO) to be similar to the quail bronchitis virus described by Olson (1950).

Hitchner et al. (1964) showed conclusively that the above viruses could be grouped into 6 groups or serotypes using cross serum-virus neutralization tests. According to the information presently available at least 28 isolates have been classified according to serotype (Table 1).

There is still some confusion as to the importance of these serotypes and the exact relationship of one type to another since there are weak cross reactions. Immunization with one virus will in many cases afford protection against challenge with another virus of a different serotype even though there are no demonstrable antibodies to the challenge virus. This has lead to the feeling that a cellular and not a humoral immunity is responsible for the protection (Hofstad, 1961; Hitchner et al., 1964). The differences among serotypes and immunologically different strains needs additional research.

TABLE 1. Six serotypes of IB and related viruses with isolates falling under each serotype

Serotype	Virus Isolates	Reference
Mass	Massachusetts	Jungherr et al. 1956
	No. 82828	Jungherr et al. 1956
	IBV 41	IB Virus Repository*
	Beaudette	Beaudette & Hudson 1937
	No. 66579	Jungherr et al. 1956
	Strain 20	Hofstad 1956a
	IBV 42	IB Virus Repository
	A 36896 (IB D G 5)	Jungherr et al. 1956
	Iowa 33	Hofstad 1956a
	Iowa 76, 104, 132, 314, 346, 433, 581, 619, 630, 661	Hofstad 1958
	Iowa 704, 726, 727, 730, 758	Hofstad 1961
Conn	Connecticut	Jungherr et al. 1956
	No. A 5968	Jungherr et al. 1956
	L-2	Hofstad 1961
	IBV 46	IB Virus Repository
Iowa 97	Iowa 97	Hofstad 1958
Iowa 609	Iowa 609	Hofstad 1958
Gray & Holte	Gray	Winterfield & Hitchner 1962
	JMK	Winterfield et al. 1964
	Holte	Winterfield & Hitchner 1962
CELO	Chicken Embryo Lethal Orphan (CELO)	Yates & Fry 1957
	Quail Bronchitis Virus (QBV)	Olson 1950
	1750	Hitchner et al. 1964

* Department of Microbiology & Public Health, Michigan State University, East Lansing, Michigan.

Genetic Studies

Fowls differ genetically in their ability to resist invasion by many parasites including viruses, bacteria, fungi, protozoa, and helminths. Genetic differences have been demonstrated between breeds, strains of the same breed and even between families (Waters and Burmester, 1963; Hutt, 1958).

Numerous factors have an influence on the expression of the genetic resistance. Genetic differences must be distinguished from physiological effects, e.g. maternal antibody. By studying the reciprocal crosses of the resistant and susceptible lines, genetic resistance to coccidiosis (Rosenberg et al., 1954), pullorum disease (Roberts and Card, 1935), and ND (Reta et al., 1963) has been demonstrated. The effect of the sire was greater than that of the dam suggesting possible sex linkage but the differences were not statistically significant. The decrease in the weight of the body and the visceral organs due to vaccination with ND is different for different strains of birds (Francis et al., 1964).

In many instances animals highly resistant to one disease are susceptible to another. In very few instances has the actual mode of inheritance to disease been demonstrated. In mice the

gene for resistance to some arboviruses is dominant and autosomal, whereas in mouse hepatitis the gene for susceptibility seems to be dominant. The susceptibility to hepatitis is of a cellular nature since results similar to the above were obtained in cell culture (Goodman and Koprowski, 1962; Kantoch et al., 1963).

In fowls the mode of inheritance has been established for resistance to Rous sarcoma virus (Waters and Burmester, 1961; Crittenden et al., 1963) and erythroblastosis virus (Waters and Burmester, 1963). In both instances inheritance is by a single autosomal gene. In the former susceptibility is dominant whereas in the latter resistance is dominant. Line 7 is homozygous for resistance and Lines 6 and 15I homozygous for susceptibility to Rous sarcoma virus (Crittenden, 1965). The resistance in Line 7 has been proven to be a cellular phenomenon since it occurs in embryo cell culture, embryos and hatched chicks (Crittenden et al., 1963).

MATERIALS AND METHODS

Chickens and Eggs.

All eggs and chickens were produced at the RPRL from one or more of the six highly inbred lines of Single Comb White Leghorns. Birds from this closed flock were considered to be free of pathogenic pleuropneumonia-like organisms (PPLO) since lack of symptoms and repeated serologic and cultural tests have failed to reveal evidence of PPLO infection. All birds were maintained on commercial feed with a coccidiostat and were placed in semi-isolated pens on corn-cob litter.

Eggs were collected and saved for two weeks before setting and were maintained at 15°C. They were incubated at 37.5°C in a forced draft, thermal and humidity controlled incubator* with trays that mechanically turned the eggs approximately once every two hours.

Line 15I chickens are highly susceptible to neoplasms caused by viruses of the avian leukosis complex and have been reared in strict isolation and inbred for 26 years. This line has a very low incidence of natural lymphomatosis and is free of

*James Manufacturing Company, Model 252B

agents having "resistance inducing factor" (RIF) activity (Rubin, 1960).

Any vaccine used in these birds would have to be RIF-free. Therefore all virus isolation, passage of the virus and preparation of stock virus were done in RIF-free line 15I eggs. As the supply was limited, eggs from line 6 hens mated with line 15I cockerels were used for all serum neutralization tests.

Allantoic Sac Inoculation

The eggs were candled on the 9th or 10th day of incubation. A mark was made in an area distant from the embryo and amniotic cavity, on the shell over the air cell about 2-3 mm above the base of the air cell. Care was taken not to locate the mark directly over a blood vessel. After cleansing with 70% alcohol a hole was punched at the site using a dentist's probe sharpened to a pyramidal point. By rotating the probe the hole could be enlarged. The punch was dipped in 70% alcohol and flamed after punching every 30 eggs. The inoculum was deposited in the allantoic sac using a separate 26 gauge $\frac{1}{4}$ inch needle, and 1 cc disposable tuberculin syringe for each lot. The hole was sealed with adhesive and sealing compound.*

*Prince Company-cellulose nitrate base.

Chorio-allantoic Membrane Inoculation

Uncandled 10-day incubated eggs were cleansed over the air cell with 70% alcohol and a hole was punched through the shell and enlarged to allow free air flow. The egg was then candled and if infertile, cracked, or if the embryo was dead it was discarded. A rubber tube was attached to the housing of the candler. Oral suction on the tube drew air from around the light source. When an egg was placed with the opened air cell against the opening of the candler, a negative pressure was created within the air cell. An area, not directly over the embryo, but having a fairly good blood supply, was chosen. It was disinfected with alcohol and a small hole was then punched between two blood vessels. The punch was rotated gently so as to enlarge the hole through the shell and then pierce the shell membrane. Simultaneously suction was applied to the original air cell and as soon as the shell membrane was pierced the chorio-allantoic membrane (CAM) dropped. Suction was continued until the original air cell was obliterated. The punch was dipped in 70% alcohol and flamed between each egg. With practice 200 eggs per hour could be handled in this way and losses due to faulty dropping of the

CAM amounted to 1 to 2%. The inoculum was deposited on the CAM with a ¼ inch 26 gauge needle and 1 ml tuberculin syringe. The holes were then sealed with adhesive compound taking care to allow the adhesive over the artificial air cell to dry before sealing the hole in the original air cell. The eggs were either placed upright in incubator trays and not allowed to turn, or were placed in egg flats and stacked 2 to 3 flats deep.

Diluent

In all experiments, unless otherwise stated, the diluent was either Simm's balanced salt solution with 2% horse serum or cell culture medium 199 with 2 to 3% calf serum. In either case 100 to 500 units potassium penicillin G and 0.1 to 0.5 mgm dihydrostreptomycin sulfate were added per ml.

Virus Isolation

A bird with acute symptoms was killed. The trachea and bronchi, from the larynx to the beginning of the bronchioles, were removed aseptically and then cut in half longitudinally.

For virus isolation one-half the trachea was weighed to the nearest 0.1 g and then cut into pieces no larger than 5 mm in diameter. The pieces were placed in a "Ten-broek" grinder, kept in crushed

ice, to which enough cold diluent without antibiotics was added to make a 20% weight/volume extract of trachea. After grinding periodically until there were no visible pieces of trachea, the homogenate was centrifuged at about 1000 X g in a refrigerated centrifuge* at 2°C for 30 minutes. The clear supernatant fluid was removed and 1.5 ml portions were placed in soft glass ampoules kept on ice. The ampoules were flame-sealed and stored at -75°C. At the time of use, 2 ampoules were thawed rapidly under cold running water at about 20°C. To one was added 1 ml of diluent with antibiotics while the second was left untreated. Both preparations remained on ice for 10 to 15 minutes by which time the eggs were ready for inoculation. Six eggs were used for each lot and 0.2 ml was inoculated into the allantoic sac of each egg. All eggs were candled daily and dead embryos were examined for lesions.

Passage of the Virus

On the third or fourth day after inoculation the allantoic fluid was harvested from two embryos taken at random. The embryos were alive or may have died within the previous 24 hours. The

*International Equipment Company, Model PR-2

allantoic fluid was pooled and immediately injected into other eggs without freezing. Aseptic techniques were used throughout.

Preparation of Virus Stocks

Virus, 0.2 ml, was inoculated into the allantoic sac of each of a group of 10-day-old embryonating eggs. After 3 days incubation, the allantoic fluid from each egg was harvested and pooled in a flask kept in crushed ice. The pool was then divided into 10 ml portions in soft glass ampoules in ice, sealed and stored at -75°C. The vials were periodically subdivided into smaller portions suitable for individual experiments. In this case they were thawed rapidly by vigorous agitation under cold running water at about 20°C. As the last piece of ice melted the ampoule was placed in a container of crushed ice, the top was cracked open and 1.5 ml portions were transferred to smaller ampoules also in the ice bath. These were then sealed and refrozen.

Before use and as close as possible to the time of inoculation, an ampoule was thawed rapidly as above. The allantoic fluid was then centrifuged at about 1000 X g for 30 minutes at 2°C. All the precipitate which had appeared on freezing and thawing was sedimented by this treatment. Only the clear supernatant fluid was used.

Hemagglutination

The 7th and 10th passages of RPL-IBV were inoculated into the allantoic sacs of 10-day-old embryonating eggs. On the 3rd and 6th days post-inoculation, allantoic fluid was harvested from one or two living embryos and the remaining eggs were incubated to determine the effects of the virus (Table 2).

The allantoic fluids were frozen, thawed and then centrifuged at about 1000 X g for 30 minutes at 2°C. Part of each sample was treated with trypsin by adding 0.25 ml of 1% trypsin (1:250*) to 0.5 ml of the supernatant fluid. The mixture was incubated at 48°C for 30 minutes and then at 56°C for 10 minutes. Egg white trypsin inhibitor, 0.25 ml, was then added and the mixture was agitated intermittently and incubated at room temperature for one hour.

Six ml of fresh chicken blood was collected in 0.6 ml of 2.5% potassium citrate. The mixture was centrifuged at about 1000 X g in a clinical centrifuge** and the plasma removed. The erythrocytes were washed six times in Hemagglutination

*Nutritional Biochemicals Corporation

**International Clinical Centrifuge

TABLE 2. Source of virus for trypsin treatment and hemagglutination

Virus Passage	No. Eggs	Harvested 3rd day p.i.*	Harvested 6th day p.i.	By 10th day p.i. No. dead	By 10th day p.i. No. with lesions
7	18	2		13	14
10	12	1	1	10	10
Uninoc.	8	1	1	0	0

* post inoculation.

buffer* and resuspended in the buffer, 0.5% cells by volume.

Two-fold dilutions of the untreated virus and of the trypsinized virus (T) were made in buffer. An equal quantity of washed erythrocytes was added to each tube (thus giving a $\frac{1}{4}$ dilution of virus in the first dilution tube). The mixtures were then incubated at room temperature for 2 hours and observed for agglutination. They were graded and recorded from - (no agglutination) to ++++ (full agglutination). The end-point was the highest dilution with a ++ or greater agglutination.

Hemagglutination-Inhibition

Sera were collected from 21 infected birds 3 weeks after the initial respiratory disease. Two-fold dilutions of the sera were titrated against 10 HA units of killed USDA Newcastle disease virus antigen by the method described by Cunningham (1963).

A known positive (accession 2709) and a known negative (accession 1339) serum obtained from Dr. J. P. Newman, Department of Microbiology, Michigan State University, were used as controls.

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*Baltimore Biological Laboratory Ltd.

Pathology

All birds dying at the RPRL are routinely necropsied. Where necessary, samples of affected organs are fixed in 10% formalin buffered with calcium carbonate. Sections are stained with hematoxylin and eosin for microscopic examination.

Fluorescent Antibody

The half of the trachea not used for virus isolation was used for fluorescent antibody studies. Using a scalpel, the mucous membrane near the bifurcation of the trachea was scraped several times. The mucus and dislodged cells were spread thinly onto 3 glass slides. They were allowed to dry at room temperature for 30 minutes and then placed in a humidity chamber. Two drops of fluorescein-labelled antiserum against NDV, infectious laryngotracheitis virus (ILT) and IB virus obtained from M. O. Braune, Pennsylvania State University, were placed onto each respective slide and they were allowed to incubate for 30 minutes at room temperature. The slides were then blotted dry, placed in a rack and submerged in continuously agitated phosphate buffered saline (pH 7.2) for 30 minutes. The slides were then mounted using a 9:1 glycerine:buffer mixture and examined under a

Leitz fluorescence microscope. The brightness and specificity of fluorescence were graded from - to ++++.

Serum-virus Neutralization Tests.

The purposes of using neutralization tests in these studies are the following (Cunningham, 1963):

1. To identify the virus and show its relation to other strains of IBV.

2. To show the presence of circulating antibodies as a measure of immunity after inoculation.

3. To compare the constant serum with the constant virus method of antibody assay.

4. To study the epizootiology of the virus among the RPRL flock.

The constant serum-decreasing virus method and the constant virus-decreasing serum method were used. The former was employed for the titration of virus and antibodies and the demonstration of serologic relationships between viruses. It requires large numbers of eggs and more complicated dilution procedures but it gives accurate titers of the sera (Page and Cunningham, 1962) and the virus is titrated simultaneously. The latter method is most efficient for the screening of large numbers of samples for the presence of antibody because fewer

eggs are used for each serum. The amount of virus used depends on a previous titration of that virus stock. However, by having adequate controls, one can determine the reliability of the results.

Constant Serum-decreasing Virus Method

The method outlined by Cunningham was used with the following modifications:

1. All sera were inactivated at 56°C for 30 minutes.

2. A mixer* was used to mix the contents of a tube rather than repeated aspiration and expulsion by pipette.

3. The prepared dilutions of virus consisted of 10 parts of each tenfold dilution of virus to which was added 8 parts of diluent.

Serum, 0.2 ml, was placed in each of the serum tubes and 0.2 ml of diluent in the virus control tube. To each tube was added 1.8 ml of the prepared tenfold dilutions of virus. This gave a final serum dilution of 1/10. Thus this is equivalent to adding equal quantities of the respective tenfold dilution of virus to a 1/5 dilution of serum.

4. The inoculum was 0.2 ml per egg. This

*Vortex Jr. Mixer, Scientific Industries Incorporated.

represents 0.1 ml of serum and 0.1 ml of the respective dilution virus.

Constant Virus-decreasing Serum Method

A method similar to that of Fontaine et al. (1963) was followed.

1. Each serum, 0.2 ml, was placed in a tube and inactivated at 56°C for 30 minutes. The tubes containing serum were then placed in an iced bath.

2. The virus was diluted to contain 10^3 EID_{50} per ml and placed in an iced bath. To every 10 parts of this virus was added a further 8 parts of cold diluent and then 1.8 ml of the final dilution was added to each tube using a sterile "Cornwall" continuous pipetting apparatus.* Using the above method there were 100 EID_{50} of virus in each 0.2 ml of inoculum and a final dilution of 1/10 of serum.

3. Diluent, 0.2 ml, was placed in the first control tube and 1.8 ml in each of another 5 tubes. To the first tube was added 1.8 ml of the final dilution of virus described above. Four tenfold dilutions were made by transferring 0.2 ml from tube to tube using separate pipettes and mixing

*Beckton, Dickinson and Co.

between each transfer. The last tube was left with diluent alone.

4. The tubes were incubated at room temperature (25-30°C) for 30 minutes and then replaced in an iced bath until inoculation. The inoculum was 0.2 ml into each of 5 or 6 eggs for each lot.

5. In some cases dilutions of the serum were prepared before being added to the tubes.

6. The positive virus responses were detected by candling alone (Table 3).

Calculation of Titers

The Reed and Meunch (1938) method of calculating 50% endpoints was used wherever applicable.

Virus titers are expressed as the positive logarithm to base 10 ($\log_{10}$) of the number of 50% egg infective doses (EID_{50}) per ml of original allantoic fluid, e.g., allantoic fluid with $10^{6.5}$ EID_{50} per ml would have a titer of 6.5.

Virus doses are expressed as the $\log_{10}$ of the final dilution plus the $\log_{10}$ of the inoculation factor, e.g., if 0.2 ml of a 10^{-3} dilution is inoculated the dose is -3.699. They represent the $\log_{10}$ of the fraction of 1 ml of original allantoic fluid inoculated.

TABLE 3. Criteria for constant virus screen method

No. of eggs per lot	Positive Virus Responses		
	Antibody positive	Doubtful	Antibody negative
4	1	2 or 3	4
5	1	2, 3 or 4	5
6	1 or 2	3 or 4	5 or 6

In the calculation of serum titers it was assumed that one antibody molecule is responsible for neutralization of one virus particle, i.e. the neutralization of virus by serum is a first order reaction (Hirst, 1959). This is an arbitrary assumption and does not always hold true. Thus the neutralization index (NI) of the serum using either the constant serum or constant virus method is:

$$\frac{\text{The amount of virus neutralized in } EID_{50}}{\text{Serum dilution}}$$

or

the $\log_{10}$ of amount of virus neutralized minus the $\log_{10}$ serum dilution.

Immunization

The 8th and the 23rd passages of RPL-IBV were used in immunization trials. At least 10^4 EID_{50} were given to each chicken. The intratracheal route was used except where the 6-day-old chicks were reimmunized. In this case the chickens were given one drop on the conjunctiva and two drops intranasally.

Collection of Serum

When small quantities of serum (1 to 5 ml) were needed, birds were bled from the heart with

a "Peel-a-way"* syringe and either a 20 guage $1\frac{1}{2}$ inch or a 22 guage 1 inch needle depending on the size of the chicken.

When birds were to be exsanguinated, blood was collected from the heart using an 18 guage $1\frac{1}{2}$ inch needle on one end of a piece of polythene tubing. To the other end of the tubing was attached a shorter needle which was inserted through a rubber sleeve stopper on a 100 ml prescription bottle. A second piece of polythene tubing attached to another needle was also inserted through the stopper. Negative pressure was obtained in the bottle by oral suction on the second tubing. A piece of glass tubing packed with cotton was used as a mouthpiece and filter on the end of the tubing. The bottle and tubes were sterilized before use. A separate apparatus was used for each bird.

All blood samples were allowed to clot for 1 to 2 hours at room temperature and then the clot was loosened from the glass by shaking. It remained at room temperature overnight. The mixture of serum and erythrocytes removed from the clot was centrifuged at about 1000 X g for 10 minutes and the clear supernatant serum was placed in plastic ampoules**and stored at -25°C.

*Scientific Products.
**Insemikit Company.

During collection and processing of the sera aseptic technique was used. No difficulty was encountered with contamination.

Studies on the Genetic Resistance of Embryos to RPL-IBV

Over the past 26 years several lines of White Leghorns have been maintained at the RPRL. They have been inbred to the point where the coefficient of inbreeding as measured by Wright's standard (F) is greater than 95% (Waters and Burmester, 1961; Waters and Burmester, 1963). This indicates a very high degree of homozygosis. Of the many lines maintained, lines 6, 7, and 15I have been studied most extensively. These lines of chickens vary in their response to different avian tumor viruses (Burmester et al., 1960). The purpose of the following experiments was to determine whether or not there was any genetic difference in response to RPL-IBV between the different lines.

Experiment I

Lines 6 and 7 were chosen for a pilot experiment because of the availability of eggs and the great differences between the lines in all respects studied previously. Six eggs were used per lot of line 7 and five eggs per lot of line 6 according to the availability of the eggs.

Dilutions from 10^0 to 10^{-8} of each of the 8th and 23rd passages of RPL-IBV were prepared and 0.1 ml of each dilution was injected into the allantoic sac of each egg. Each egg in the tenth lot was inoculated with 0.1 ml of diluent alone. The eggs were identified inconspicuously by lot number and were placed randomly in the incubator trays. The eggs were candled daily until the 10th day after inoculation. The number of embryos dead in each lot was recorded and the date of death was recorded on each egg. The eggs with dead embryos were removed each day and stored at 4°C. The spaces in the trays left when eggs were removed were filled using eggs taken from a row at one end of the tray. In this way an additional randomization of the eggs of each lot occurred. On the 10th day the total number of eggs in each lot was rechecked before discarding the eggs.

Experiment II

Experiment I yielded significant results and a second, more extensive diallel cross experiment was performed. Matings of pure lines 6, 7, and 15I, their crosses and reciprocal crosses were prepared. For each of the 9 possible combinations there were

two pens containing 15 to 20 hens and two cockerels each as follows:

		Males (2 per pen).		
		6	7	15I
Females	6	6X6	7X6*	15IX6
(15-20	7	6X7	7X7	15IX7
per pen).	15I	6X15I	7X15I	15IX15I

Ten eggs were used per lot and care was taken to insure that each lot contained the same number of eggs from each of the 2 pens supplying each pure line or cross. Only the 23rd passage of RPL-IBV was used. Details of dilution, inoculation and candling were the same as in the previous experiment.

Statistical Analysis

In Experiment I, different numbers of eggs were used for line 6 and 7 and titration of the virus in these eggs yielded similar endpoints. The only difference observed was between latent periods before death of the embryos. Line 6 embryos died earlier than line 7. A statistical analysis was performed using the method of Mantel and Haenzel (1959) since this takes into account the number of

*In any cross e.g. 7X6 the male is always given first.

embryos at risk on each day (i.e. includes latent period and titer) and is not influenced by the number of embryos in each lot.

In Experiment II where there was a three-way diallel cross setup, the effects of the crosses and reciprocal crosses were examined as well as the effect of the lines. The number of eggs in each lot was nearly constant. Only those embryos dying within 24 hours after inoculation affected the number per lot. The number of embryos dead before a certain date reflects the latent period, i.e. the shorter the latent period the greater the number of deaths before the selected time and the fewer the deaths afterward. In this experiment the number of embryos dying during the first 5 or 7 days was compared using the method outlined by Griffing for diallel cross systems (Griffing, 1956a and 1956b).

RESULTS

Symptoms and Pathology

On September 19, 1963 severe respiratory symptoms were observed in chickens in house B_6 (Fig. 1).

1. Immediately on entry into the vestibule of the pen almost continuous coughing and sneezing sounds could be heard with no difficulty. There were approximately 100 ten-week-old birds in the pen.

2. On entry into the pen many of the birds were coughing and sneezing and some were gasping and appeared cyanotic, especially after being handled. Rales could often be heard.

3. Clinically there was a pronounced conjunctivitis, but no nasal discharge, even when pressure was applied to the infra-orbital sinuses.

4. There was less than 4% mortality but the morbidity was estimated at 90-100%. The birds that died during the acute and convalescent stages had a catarrhal tracheitis over the entire length of the trachea with an increase in mucous exudate in the lumen. No changes were seen in the air sacs.

5. On September 20, 1963 the only other pen of birds in the same building had the same symptoms.

Fewer birds were so acutely affected.

6. About a week after the first symptoms were observed all birds appeared to have recovered. In one pen, one bird gasped for many weeks afterward. It was thought that the bird had an unrelated respiratory or circulatory lesion.

7. These birds were not yet laying. However, when the same disease occurred elsewhere on the premises, there was a sudden severe drop in egg production which did not return to normal for over one month. No misshapen eggs were layed.

A tentative diagnosis of infectious bronchitis was made and material was taken from one bird of this group for virus isolation.

Histopathology

The few birds that died during this initial outbreak and during the vaccination experiments had typical histopathological changes associated with IB. These included:

1. A slightly thickened mucosa with edema and congestion. Sometimes there was disruption of the mucosa and loss of cilia.

2. A marked infiltration of lymphocytes and macrophages in the sub-mucosa. Occasionally heterophils were also present.

3. Inclusion bodies were not found.

Virus Isolation and Passage

In the initial isolation, all seven embryos inoculated with the tracheal extract alone died within 24 hours due to bacterial contamination. However, of the 8 inoculated with the extract to which antibiotic had been added, 2 embryos had died by the fourth day. At this time 2 embryos were taken as donor material for passage. By the seventh day after inoculation, 2 more had died. The last 2 died on the ninth day after inoculation and had the following lesions:

1. They were obviously dwarfed or stunted. This could be seen on candling.

2. The embryos were curled and held in a firm compact ball by the closely adherant and thickened amniotic membrane.

3. The down was underdeveloped, i.e., clubbed.

4. On necropsy, the embryos had a severe generalized congestion of the liver and lungs and an accumulation of urates in the mesonephrons and ureters.

The above signs are typical of those encountered when embryos are inoculated with IB virus in low egg passage.

The mortality pattern in all passages of the virus is shown in Table 4. Between the 14th and 18th passages an attempt was made to "clone" a more rapidly growing and more lethal virus. For each passage the more stunted embryos were taken for harvest and the allantoic fluid was diluted between 10^{-1} and 10^{-3}. Passages 19 through 23 gave a more uniform mortality than previous passages.

Effect of Passage

To confirm the differences between the 8th and 23rd passages noted above these two preparations were used for inoculation of line 6 and line 7 embryos as described under "Studies on Genetic Resistance to RPL-IBV." In the cumulative mortality graph (Fig. 3), the 23rd passage of virus gave consistently earlier mortality than the 8th passage when assayed in line 7 eggs. When analyzed statistically by the method of Mantel and Haenzel (1959), the difference is significant at P=0.05 when the virus was assayed in line 7 (Table 16). For line 6 embryos, there was no difference between the two virus preparations.

The titer of the 23rd passage of virus was slightly higher than that of the 8th passage (Table 16). This effect has also been included in the statistical analysis.

TABLE 4. Mortality pattern on isolation and passage of RPL-IBV every 3-4 days in 10 day old Line 15I embryos

Passage No.	Dilution	No. dead/ Total	No. lesions/ Total
1.	10^{-1} dilution	6/6	4/6
2.	Undiluted	3/6	5/6
3.	"	3/5	4/5
4.	"	2/3	3/3
5.	"	All used as donors	
6.	"	10/14	12/14
7.	"	19/19	19/19
8.*	"	13/16	14/16
9.	"	4/4	
10.	"	6/6	
11.	"	10/10	10/10
12.	"	11/12	
13.	"	8/10	
14.	10^{-3} dilution	17/18	
15.	10^{-2} "	15/18	
16.	10^{-2} "	17/25	
17.	10^{-3} "	7/11	
18.	10^{-1} "	16/16	
19.	Undiluted	4/4	
20.	"	14/14	
21.	"	All used as donors	
22.	"	13/13	
23.*	"	7/7	

* Virus stock prepared.

Fifteen embryo passages of the virus and some selection has produced a preparation which can kill embryos sooner i.e. it can partially overcome the factor in line 7 embryos which delays the mortality.

Propagation and Storage

For the preparation of a virus pool, ten-day-old embryos were inoculated with decimal dilutions of the 23rd passage of virus. Ten embryos were used for each lot. The allantoic fluid was harvested after 3 days, pooled by lot and later titrated. The highest virus yield came from embryos inoculated with a 3.7 and a 4.7 log dose of virus (Table 5). With higher doses of virus there was a decrease in titer. This pehnomenon was described by Von Magnus (1954) and reviewed by Hirst (1959) for influenza virus where the input multiplicity was very high.

RPL-IBV decreased 0.8 logs in activity during 6.5 weeks at -75°C (Table 6).

Candling Methods

By the 8th passage it became evident that almost all dead embryos had typical lesions of IB. The number of positive cases was not appreciably increased by examining the embryos that lived until the 20th day of incubation. In addition some of

TABLE 5. Propagation of virus in the allantoic sac

Dose inoculated	-0.7	-1.7	-2.7	-3.7	-4.7	-5.7
Yield of virus	7.0*	7.0	7.0	7.6	7.6	7.5

* log titer.

TABLE 6. Loss of virus on storage at -75° C

Date of titration	12/17/63	1/3/64	1/11/64	1/31/64
No. of weeks stored	0	2.5	3.5	6.5
Titer of virus	7.6	7.1	6.9	6.8

these survivors were so stunted that the stunting was easily visible on candling and they would certainly have died before hatching. The following arbitrary criterion was employed. Any embryos definitely stunted and lacking motility on candling at the 10th day after inoculation were included with those that died between the 1st and 10th days and were considered as positive responses. A similar method of reading responses was used by Hitchner et al. (1964) and gives results equivalent to the embryo inspection technique used by earlier workers.

To test the accuracy of the method of scoring positive responses one of the routine constant serum-decreasing virus neutralization tests was utilized. All eggs were candled and the positive responses were recorded. Each egg was opened and the living and dead embryos were examined. The results are presented in Table 7. Some embryos died without lesions and some embryos had lesions that were not detected by candling alone. The overall agreement was good so the method using candling alone was used for all subsequent experiments.

Identification of the Virus

1. Symptoms in affected birds.

TABLE 7. Comparison of endpoints of a serum-virus neutralization using different methods of reading positive virus responses

Method of reading	Virus alone	Serum 1 (Neg)	Serum 2 (Neg)	Serum 3 (Neg)	Serum 4 (Pos)
Lesions alone	7.5	7.7	7.3	6.6	3.2
Dead and lesions	7.7	7.7	7.8	6.8	3.9
Candling	7.7	7.7	7.5	6.8	3.6

TABLE 8. Fluorescent antibody results on tracheal scrapings of two birds with signs of IB

Trachea from:	Fluorescent antiserum	Results	Remarks
Bird 1	IB	+++	Clear specific
	ILT	+	Some non-specific
	NDV	-	Negative
Bird 2	IB	+++	Clear specific
	ILT	-	Negative
	NDV	-	Negative

2. Fluorescent antibody. Half of the trachea used for virus isolation (Table 8, Bird 1) and the trachea of a second bird that died 2 days later (Table 8, Bird 2) were used for fluorescent antibody studies (which were positive for IB).

3. Lesions in embryos.

4. CAM inoculation. The 23rd passage of virus was used to inoculate 26 eggs on the CAM. The absence of pocks on the 8th day post inoculation eliminated the possibility that ILT or fowl pox were present in the inoculum.

5. Hemagglutination. The virus did not cause direct HA. Trypsin treated virus harvested on the 3rd day from both the 7th and 10th passages agglutinated chicken erythrocytes which is a characteristic of IB virus (Corbo and Cunningham, 1959; Cunningham, 1960). Uninfected allantoic fluid treated with trypsin did not cause HA (Table 9).

In the 7th passage, third day harvest, there was no agglutination at 1/4 and only 2+ at 1/8. This appears to be a "prozone" phenomenon which was not described by Corbo and Cunningham (1959).

6. Hemagglutination inhibition. Serum from one of the 2 birds submitted to Dr. Newman on January 28, 1963 had an HI titer of 1280. However,

TABLE 9. Hemagglutination using trypsin treated RPL-IBV

Virus Source	Treatment	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
7-3*		-**	-	-	-				
10-3		-	-	-	-				
10-6		-	-	-	-		not done		
0-3		-	-	-	-				
0-6		-	-	-	-				
7-3	T	-	2+	3+	4+	3+	+	-	-
10-3	T	4+	4+	4+	4+	4+	3+	+	-
10-6	T	-	-	-	-	-	-	-	-
0-3	T	-	-	-	-	-	-	-	-
0-6	T	-	-	-	-	-	-	-	-

Titer of 7-3 is 1:64 and of 10-3 is 1:128.
* 7-3. Inoculum was 7th passage of RPL-IBV. Harvested on the 3rd day post inoculation.
** - No agglutination.
+ 25%
2+ 50%
3+ 75%
4+ 100% agglutination.

an additional 6 samples submitted later were negative. There was no HI in 21 additional samples (Table 10). This was indicative that the birds had not been infected with ND. No explanation could be found for the one positive serum.

7. Serum-virus neutralization tests. On the basis of the results of neutralization tests by both the constant serum and the constant virus methods (Table 11), the following conclusions can be drawn:

a. RPL-IBV was not neutralized by hyperimmune sera against ND, ILT, or avian encephalomyelitis viruses.

b. The antiserum against "gallus adeno-like" (GAL) virus (Sharpless and Jungherr, 1961; Sharpless et al., 1961) prepared at the RPRL had antibody activity against this agent. On the other hand, the antiserum prepared in New York had no activity even though it had a high titer against GAL. Thus RPL-IBV does not cross-neutralize with GAL virus and the presence of activity in the above serum indicated inadvertent infection with RPL-IBV. At the time the antiserum was prepared (1958), RPL-IBV had not been recognized.

TABLE 10. HI titration of sera from survivors of RPL-IBV infection using 10 HA units of killed NDV (USDA)

(A) Serum titration

Serum no.	Serum dilution									
	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
1.	+*	+	+	+	+	+	+	+	+	+
2.	+	+	+	+	+	+	+	+	+	+
3.	+	+	+	+	+	+	+	+	+	+
4.	+	+	+	+	+	+	+	+	+	+
5.	+	+	+	+	+	+	+	+	+	+
6.	+	+	+	+	+	+	+	+	+	+
7.	+	+	+	+	+	+	+	+	+	+
8.	+	+	+	+	+	+	+	+	+	+
9.	+	+	+	+	+	+	+	+	+	+
10.	+	+	+	+	+	+	+	+	+	+
11.	+	+	+	+	+	+	+	+	+	+
12.	+	+	+	+	+	+	+	+	+	+
13.	+	+	+	+	+	+	+	+	+	+
14.	+	+	+	+	+	+	+	+	+	+
15.	+	+	+	+	+	+	+	+	+	+
16.	+	+	+	+	+	+	+	+	+	+
17.	+	+	+	+	+	+	+	+	+	+
18.	+	+	+	+	+	+	+	+	+	+
19.	+	+	+	+	+	+	+	+	+	+
20.	+	+	+	+	+	+	+	+	+	+
21.	+	+	+	+	+	+	+	+	+	+
1339(-)	+	+	+	+	+	+	+	+	+	+
2709(+)	-	-	-	-	-	-	-	-	-	+
Saline	-	-								

(B) Antigen titration

Antigen dilution										
2	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
+	+	+	+	+	+	-	-	-	-

* + Greater than 50% agglutination.
- Less than 50% agglutination.

TABLE II. Neutralization of RPL-IBV by sera from various sources

Source	Host	Serum against	No. of Sera	Constant Virus	Constant Serum NI
NADL*	Turkey	NDV	2	-	
NADL	Chicken	NDV	2	-	
NADL	Chicken	LT	2	-	
RPRL	Chicken	GAL	1	+	
NY	Chicken	GAL	1	-	
CONN	Chicken	AE	3	-	
NADL	Chicken	Mass IBV	2	-	
ISU	Chicken	Mass IBV (726)	1	-	1.2
MSU	Chicken	Mass IBV (41)	1	-	<1.8
MSU	Chicken	Mass IBV (41)	1	-	<1.5
NADL	Chicken	Conn IBV	1	-	
MSU	Chicken	Conn IBV (46)	1	-	<1.5
ISU	Chicken	IBV 609	1	-	<1.0
ISU	Chicken	IBV 97	2	±	2.3
RPRL	Chicken	RPL-IBV	1	+	5.6
RPRL	Chicken	RPL-IBV	1	+	4.9
RPRL	Chicken	RPL-IBV	1	+	4.3
RPRL	Chicken	Negative	2	-	<1.8
NADL	Chicken	Negative	1	-	

* NADL - National Animal Diseases Laboratory, Ames, Iowa.
RPRL - Regional Poultry Research Laboratory, East Lansing, Mich.
NY - American Cyanamid, Pearl River, New York.
CONN - University of Connecticut, Storrs, Conn.
ISU - Iowa State University, Ames, Iowa.
GAL - Gallus Adeno-like virus.
AE - Avian Encephalomyelitis.

c. RPL-IBV was neutralized by antiserum against Mass IBV (IBV 41 or Iowa 726), Conn IBV (IBV 46), or Iowa 609 isolate (Hofstad, 1958).

d. RPL-IBV was neutralized by antiserum against Iowa 97 isolate (Hofstad, 1958) and by homologous antiserum. Thus RPL-IBV is most closely related to Iowa 97.

Constant Serum versus Constant Virus

To compare these two methods sera from 6 immune and 5 non-immune birds were tested in parallel against the same virus preparation by both methods (Table 12). Even though the titers did not exactly agree, all the positive sera were positive by both tests and all the negative sera were negative by both tests.

Because the constant virus method was less expensive, requiring fewer eggs per serum tested, it was used for the majority of the screening tests.

Epizootiology

Using the constant virus method, 184 sera were tested for antibody against RPL-IBV (Table 13). The layout of the buildings and isolation areas at the RPRL is given in Figure 1. The results from

TABLE 12. Constant serum versus constant virus titrations*

	Serum No.	Status	Constant Serum	Constant Virus
Test I	3143	Immunized	5.7**	4.5
	3186	"	4.9	5.1
	3137	"	4.3	3.5
	3105	Not immunized	<1.8	<3.1
	3104	"	<1.8	<3.1
Test II	3474	Immunized	2.3	3.0
	3185	"	4.8	3.3
	3187	"	3.6	3.0
	3472	Not immunized	<1.0	<2.5
	3473	"	1.2	<2.5
	3190	"	<1.0	<2.5

* RPL-IBV was used for immunization and as antigen in both the constant serum and constant virus neutralization tests.

** Log_{10} neutralization index (NI).

TABLE 13. Screen for antibodies to RPL-IBV (Sera collected between Sept. 63 and May 64)

Source	Age	No. Tested	% Pos.
W_2	6 weeks	19	0
W_3	Adult	25	100
W_3-E_3*	6 days	10	90
W_3-E_3*	2 weeks	6	100
W_4	Adult	23	100
W_6	Adult	25	88
W_7	9 weeks	8	50
B_1	2 months	18	6
B_5	4 months	6	20
B_5	2 months	6	0
B_6**	7 months	6	100
B_7	4 months	12	0
E_3	1 month	6	0
E_3	3 months	9	0
E_4	2 months	5	0

* Dams located in W_3 but chicks reared in E_3.

** Isolate of RPL-IBV came from these birds.

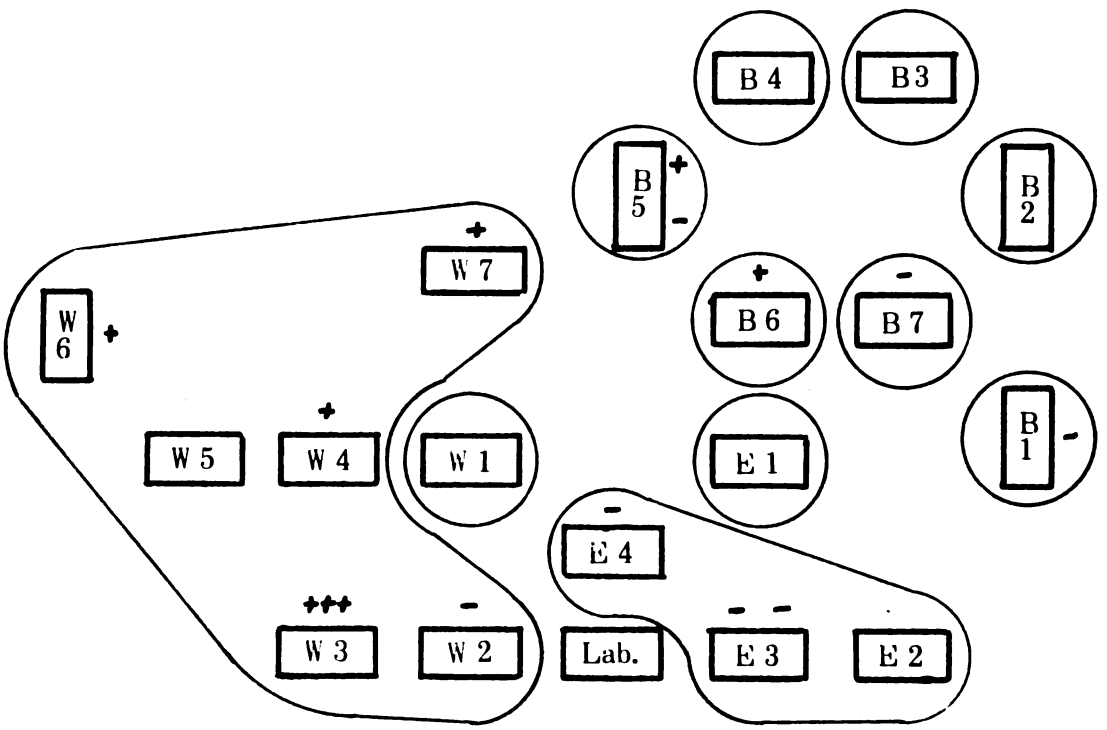

FIG. 1. Diagram of the buildings and isolation areas at the Regional Poultry Research Laboratory, East Lansing, Michigan. The individual quarantine areas have been outlined.

Table 13 are also recorded in this figure. Where 20% or more of the samples from a house were positive then that group of birds was assumed to have been infected and the results are recorded as + on Figure 1. When there was less than 20% positive samples the results were recorded as -.

The west (W) area contains all the breeding stock and birds not kept in isolation. The only other breeding stock is located in the east area (E_1). All the W houses except W_2 were positive. W_2 is the brooder house and the chicks were young and had not yet developed antibody. There was antibody in these same chicks about 1 week after being moved to W_7 indicating the previous presence of the virus in the brooder house. Tests on the E area were negative. The chickens in house B_6 all had antibody. In B_5 the 2 groups were in opposite ends of the building and separated by empty pens. It is quite possible that the virus failed to spread from the 4-month-old to the 2-month-old chicks. The one bird with antibody in B_1 cannot be explained.

The results above indicate that RPL-IBV infection is common in birds in the W area and usually occurs as a very mild symptomless disease.

The C area appears to be free of infection, however there are sporadic occurrences in the B area. Quarantine procedures appear to be partially effective since infection has not spread throughout the farm.

Development of a Vaccine

In order to evaluate the virus as a live vaccine the 8th and 23rd passages of the virus were inoculated into chickens of various ages (Table 14).

In 6-day-old chicks with maternal antibody there were no respiratory signs after vaccination. The antibody response was also low but could be re-stimulated by a second vaccination.

With 8 and 12-week-old birds some mild respiratory symptoms were observed. Six of the 32 8-week-old birds died. All birds in both age groups had an antibody response.

There were no symptoms in the 16-week-old birds, however, the antibody response was not as great as in the younger birds. The 8th passage of virus elicited a slightly greater antibody response than the 23rd passage both in the inoculated and contact birds. On the basis of antibody response and clinical signs, contact exposure was greater in the 8-week-old than in the 16-week-old birds.

TABLE 14. Neutralization of RPL-IBV by sera from birds vaccinated by various routes with different passage levels of the homologous virus

Age	Route	Virus Passage	No. Vac.	Pre-Vac.	Post-Vac.	Age Revac.	Post-Vac.
6 dys.	i.t.*	23	9	100**	66**	7 wks.	100
8 wks.	i.t.	8***	8	0	100		
	Contact	8	8	0	100		
	i.t.	23***	8	20	100		
	Contact	23	8	0	100		
12 wks.	i.t.	8	3	0	100		
	i.t.	23	3	0	100		
16 wks.	i.t.	8	8	0	63		
	Contact	8	8	0	25		
	i.t.	23	8	0	50		
	Contact	23	8	0	14		

* Intratracheal.

** Percent antibody positive (constant virus screen at a neutralization index of about 2.5 logs).

*** A combination of vaccination and cold weather caused a mortality of three birds in each of these lots. These were the only lots having obvious symptoms.

The 23rd passage of RPL-IBV was used as the antigen in all serum-virus neutralization tests.

All chicks hatched from dams in the W area probably have a very high maternal antibody level. Vaccination is not fully effective until this maternal antibody level decreases at about 4 weeks of age. The 23rd passage of virus could be used as a vaccine anytime thereafter but slight respiratory signs may result. If it is necessary to produce immunity without respiratory signs then vaccination before 3 weeks of age, while the chicks have maternal antibody, and revaccination 3 to 4 weeks later will result in a good immune response. The above experiments also indicate that if half the birds in a group are vaccinated, all will become infected and develop antibodies.

Genetic Resistance of Embryos to RPL-IBV

Experiment I.

The experimental design and the number of embryos that died in each lot on each day after inoculation with the virus are presented in Table 15. Line 6 embryos died earlier than those in the corresponding lots in line 7. In the latter case the greatest mortality occurred on the 9th day post inoculation. In Figure 2 the mortality in the 6 lots that received the greatest amount of virus

TABLE 15. Titration of RPL-IBV in line 6 and line 7 embryos

		Line 6										Line 7									
		8th Passage of RPL-IBV																			
Dose		-1	-2	-3	-4	-5	-6	-7	-8	-9	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	0
Days post inoculation	1																				
	2																		1		
	3				1*	1						1									
	4		2	2		1															
	5		1		1																
	6		1				2														
	7					1		1					2			1		1			1
	8		1	1	1	1	1					1					1				
	9			2	2		2	1	1			3	4	4	4	4	2	2			
	10					1		1		1	1	1		2	2	1	2	1			2
Response			5/5	5/5	5/5	5/5	5/5	3/5	1/5	1/5	0/5	6/6	6/6	6/6	6/6	6/6	5/6	4/6	1/6	0/6	1/6

		23rd Passage of RPL-IBV																			
Days post inoculation	1																				
	2																				
	3	2	1		1																
	4	2		4											1	1					
	5		2		1	1		3													
	6	1	2			1	1							1		1			1		
	7				2	1						3				1		1			
	8											1	1	1	2	1	1	1			
	9			1	1	1	3	2				1	4	4		2	5	3	1		
	10						1		1			1	1		3						
Response		5/5	5/5	5/5	5/5	4/5	5/5	5/5	1/5	0/5	0/3	6/6	6/6	6/6	6/6	6/6	6/6	5/6	2/6	0/6	0/6

* One line 6 embryo died on the 3rd day after a -4 log dose of the 8th passage of RPL-IBV was inoculated into the allantoic sac, etc.

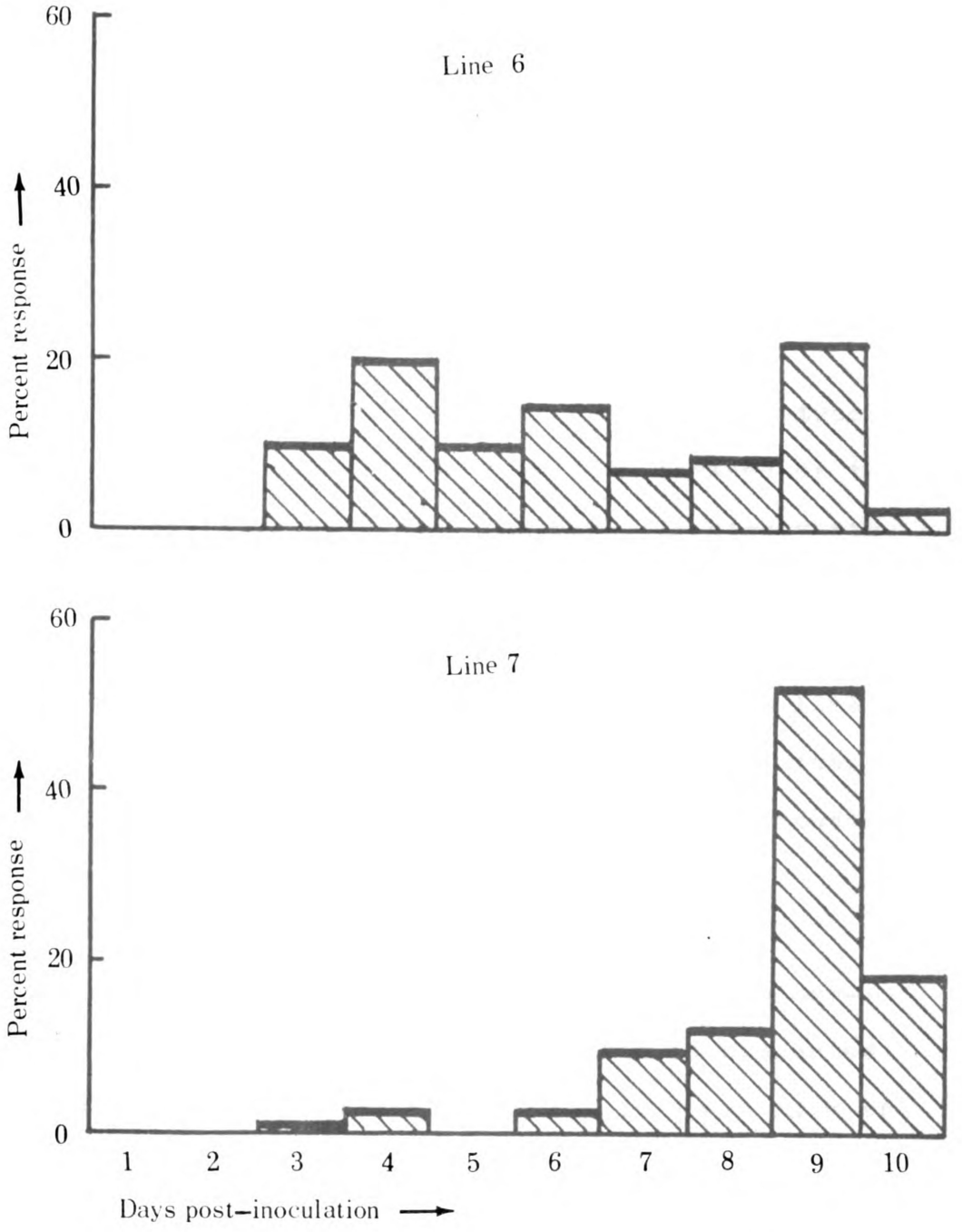

FIG. 2. Percent response of embryos of two inbred lines of chickens to RPL–IBV (8th and 23rd passages combined).

of each passage has been combined and plotted against the days after inoculation. When the cumulative mortality in these lots is plotted against the days post inoculation (Fig. 3), the curve for the mean of the virus preparations in line 6 embryos is straighter than in line 7 embryos which is concave upwards.

The X^2 indicates that the difference between lines 6 and 7, using either the 8th passage or the 23rd passage or combining the data, is highly significant (Table 16).

The 23rd passage of virus killed embryos earlier than the 8th passage, especially in line 7 (Fig. 3), and also had a slightly higher titer (Table 16). These differences have been discussed under "Effect of Passage of the Virus."

Experiment II

The experimental design and the embryo mortality on each day after inoculation is presented in Table 17. The daily mortality in the 6 lots of embryos inoculated with the highest doses of virus has been pooled and is presented in Fig. 4. Embryos of line 6X6 died earlier and those of line 7X7 died later. Line 15IX15I was intermediate between these two, some dying early and some late.

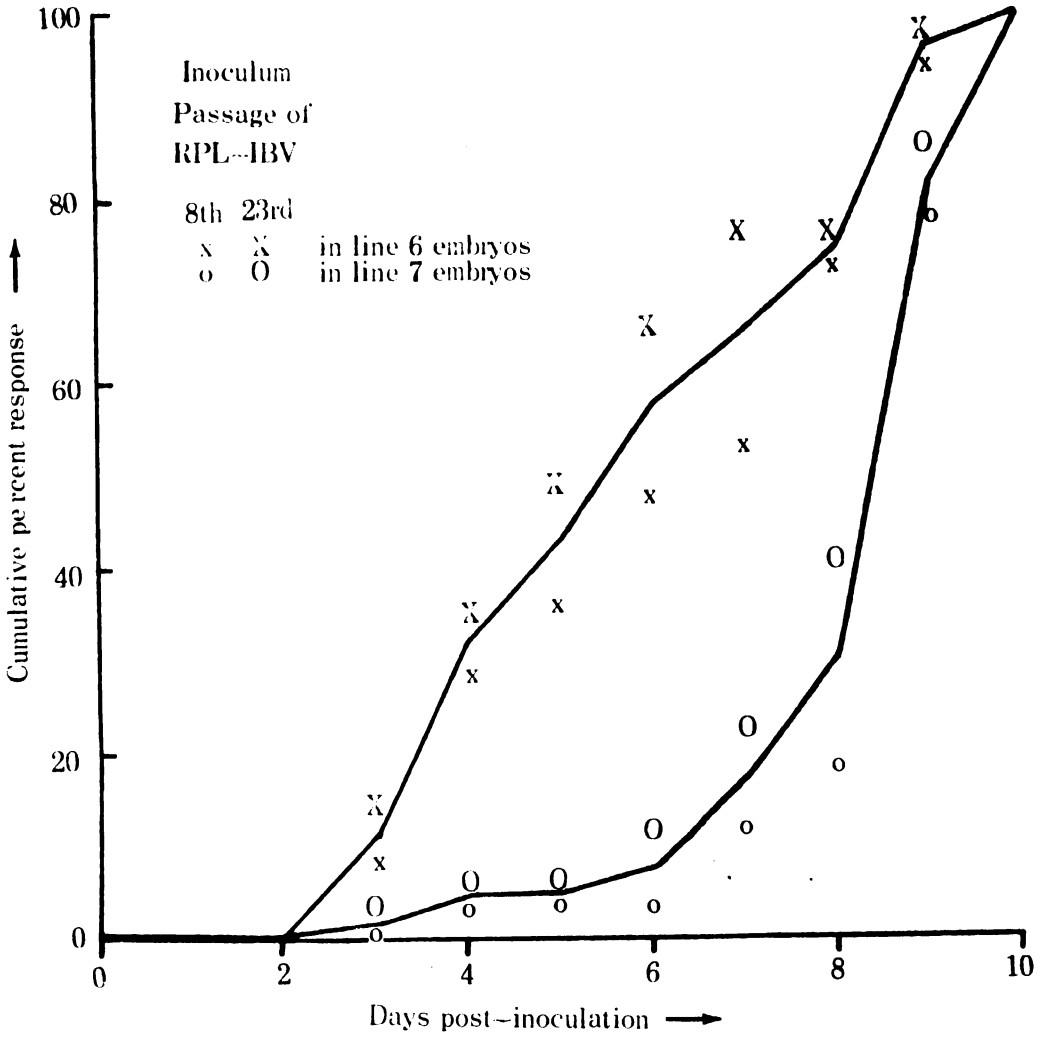

FIG. 3. Cumulative percent response of two inbred lines of chickens to two passage levels of RPL-IBV.

TABLE 16. Titers and statistical analysis of the RPL-IBV titrations in experiment 1

(A) Titers of RPL-IBV		
Virus	Line 6	Line 7
8th passage	7.5	7.2
23rd passage	7.6	7.6

(B) x^2 for the difference between line 6 and line 7		
8th passage	15.94*	Data Combined 28.76
23rd passage	11.81	

(C) x^2 for the difference between the 8th and 23rd passages of virus	
Line 6	0.06
Line 7	5.28

* One degree of freedom in every case.

At $p_{.01}$ $x^2 = 6.64$

At $p_{.05}$ $x^2 = 3.84$

TABLE 17. Titration of RPL-IBV in diallel cross matings

Days Post Inoculation	Line 6 x 6										Line 7 x 6										Line 151 x 6									
Dose	-1	-2	-3	-4	-5	-6	-7	-8	-9	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	0
1							1						1				1				1							1		1
2	4*	2	3	2	1						3	2		3							7		1	1						
3	1	3	3	2	1	1					1	1										4	3	1						
4	4	1	3	4	1						1	1											3	1	1	1				
5				1	2	4	2														1	1	1		1	1				1
6		2			3	1	1									1					1	1		1		2				
7	1	1				2					1			2	3	1						1	1	1	1		1			
8		1		1	2						1	2	2	2	1							1	1	1	4	2				
9						1					2	2	5	2	5	2						2		4	2	2	2			1
10			1			1	2	2			1	2	2	1	1	6	1			1				1		1				
Response	10/10	10/10	10/10	10/10	10/10	10/10	5/9	2/10	0/10	0/10	10/10	10/10	9/9	10/10	10/10	10/10	1/9	0/10	0/10	1/10	9/9	10/10	10/10	10/10	10/10	8/10	4/10	0/9	0/10	2/9

TABLE 17. (Continued)

	Line 6 x 7										Line 7 x 7										Line 151 x 7									
Dose	-1	-2	-3	-4	-5	-6	-7	-8	-9	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	0
1			1	1	1				1					2			2	1					1	1				1		
2	3										1		1	1	1				1		1	2				2	1			
3			2								1		1	1	1						1	1								
4	1	2	2	1		1					1			1								1	1	1	3					
5		2	1	3							1	2	2			1					1		1							
6	3	1				1	1				1													1		1				
7	1	1	2	2		1						1		2	1						3	3	4	3	3					
8	1	1	2		5	2					1	1	2		3	2	1				2	3	2	1	2	1	1			
9	1	2		2	2	2	3				2	4	2	2	1	3	2				2			2	2	2				
10		1		1	2	3					2	2	1	1	3	4	3							1		3	3			
Response	10/10	10/10	9/9	9/9	9/9	10/10	4/10	0/10	0/9	0/10	10/10	10/10	9/10	8/8	10/10	10/10	6/8	0/9	1/10	0/10	10/10	10/10	8/9	9/9	10/10	9/10	4/10	0/9	0/9	0/10

Days Post Inoculation

TABLE 17. (Continued)

	Line 6 x 15I										Line 7 x 15I										Line 15I x 15I									
Dose	-1	-2	-3	-4	-5	-6	-7	-8	-9	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	0
Days Post Inoculation 1		1									1																			
2	4	2	1	1							1	1									3	4	1	1	1					
3	1	2		1																		2	1	1		1				
4			3	4	1						1	1			1						1	2	3			1				
5	1				3			1				1									1	1		2						
6	2	3	1	1	3	2						1	1									1								
7		1	2	2	1	1					1	1		1							2		1							
8		1	2	1	1	2					4	1	5	3	2	1					1		2	3	3	1	2			
9	2		1		1	4	3				1	1	4	5	6	5					2		2	1	5	1				
10										1	1	3			1	2		1						2	1	4	1	1			
Response	10/10	9/9	10/10	10/10	10/10	9/10	3/10	1/10	1/10	0/10	9/9	10/10	10/10	9/10	10/10	8/10	0/10	1/10	0/10	0/10	10/10	10/10	10/10	10/10	10/10	8/10	3/10	1/10	0/10	0/10

* Four line 6 x 6 embryos died on the second day after a -1 log dose of the 23rd passage of RPL-IBV was inoculated into the allantoic sac.

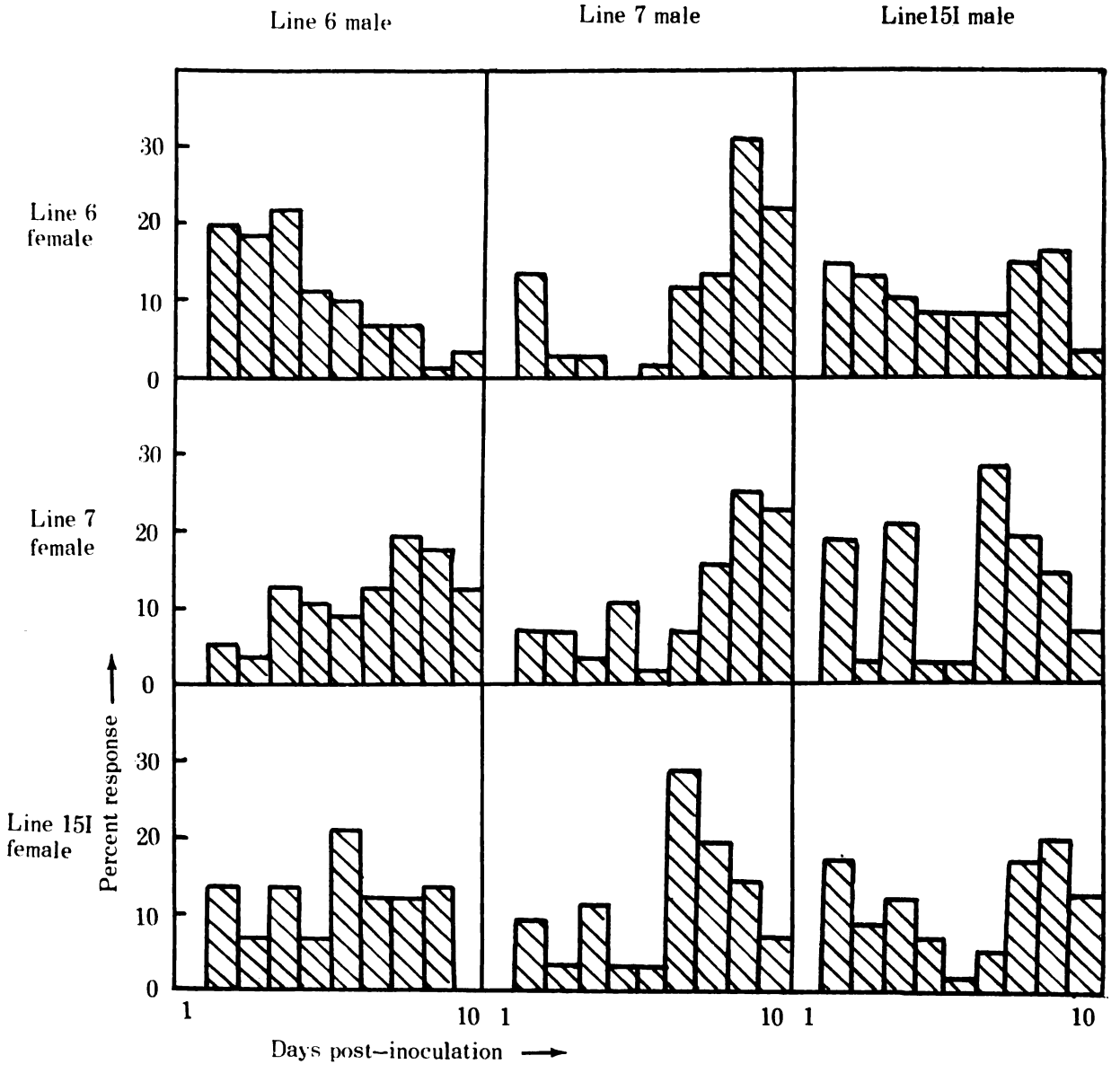

FIG. 4. Percent response of embryos of diallel cross matings to RPL–IBV.

The cumulative mortality figures are shown in Fig. 5. In each of the 9 graphs an arbitrary point has been placed in the same position relative to the ordinate and abscissa in each graph. Wherever line 7 is one of the parents of the embryos, the curve passes beneath this point and in all other cases it passes over this point. In the former the embryos died later than in the latter.

Statistical analysis by the method of Griffing (1956) yielded the information in Table 18. The difference between the crosses (i.e. between each of the 9 different matings) is significant both at the 5th and at the 7th day post inoculation. The most obvious difference was between the 6X6 and 7X6 matings (Fig. 5). In the former the graph is concave downward whereas in the latter it is concave upwards.

The effect of dose was significant as was expected. The higher the dose of virus, the shorter the latent period. The titers of RPL-IBV calculated from mortality to the 10th day post inoculation, are similar in all crosses (Table 19).

The general combining ability (GCA) is a measure of the differences between the lines of chickens, irrespective of influences of other lines

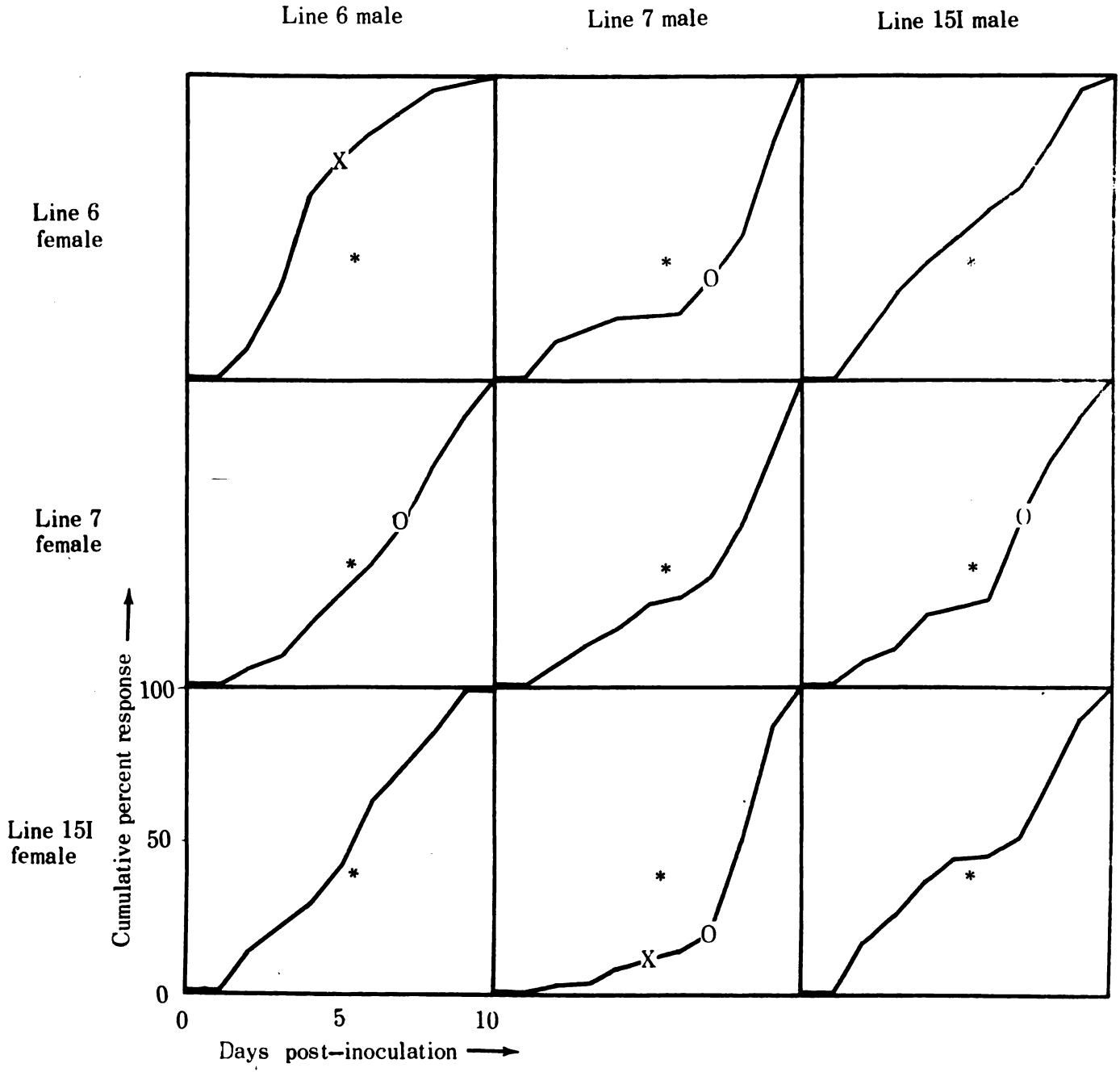

FIG. 5. Cumulative percent response of embryos of diallel cross matings to RPL-IBV. For the symbols *, X and O see the text.

TABLE 18. Results of statistical analysis of diallel cross matings

	Sources	Degrees of Freedom	F
5th day	Crosses	8	7.80**
	Lots (doses)	5	8.11**
	Lots x crosses	40	<1.00
	GCA	2	19.73**
	SCA	3	5.95**
	Reciprocal	3	1.69
7th day	Crosses	8	9.15**
	Lots (doses)	5	8.32**
	Lots x crosses	40	<1.0
	GCA	2	22.6**
	SCA	3	2.8
	Reciprocal	3	6.5**

** Significant at P=0.01.
GCA = General combining ability.
SCA = Specific combining ability.

TABLE 19. Titers of RPL-IBV in embryos of diallel cross matings

(A) To the 10th day post inoculation

		Sire		
		Line 6	Line 7	Line 15I
Dam	Line 6	7.2	6.6	6.7
	Line 7	6.8	6.8	6.8
	Line 15I	6.8	6.4	6.7

(B) To the 7th day post inoculation

		Sire		
		Line 6	Line 7	Line 15I
Dam	Line 6	6.3	2.4	4.4
	Line 7	3.9	2.5	4.1
	Line 15I	5.2	1.7	3.5

to which they may be mated. The GCA is highly significant at both the 5th and 7th days post inoculation. The difference is due to the effect of line 7. There was delayed mortality in every mating in which either the dam or the sire or both were line 7 (Fig. 5). The same trend can be seen for line 7 in the LD50 titers to the 7th day post inoculation (Table 19).

The specific combining ability (SCA) is a measure of whether there are cases in which certain combinations (i.e. matings) cause relatively more or less mortality than would be expected on the basis of average performance of the lines involved. For the mortality to the 5th day post inoculation, the SCA is significant, probably due to the effects of lines 6X6 and 7X15I as shown by an X in Fig. 5. The former had more and the latter less mortality than would be expected from a consideration of the effect of the lines in other crosses.

For mortality to the 7th day post inoculation, the effect of the reciprocal crosses is significant. This can be most clearly seen in the 6X7 and 7X6 reciprocal crosses and also in the 15IX7 and 7X15I crosses (shown by an O in Fig. 5).

In both of these combinations when the female was line 7, the mortality was earlier than when the male was line 7. Both in the cumulative mortality (Fig. 5) and in the titer to the 7th day post inoculation (Table 19), the line 7 male had a greater effect in delaying mortality than the female. This could be due to a maternal factor in the line 7 females, making their embryos more susceptible or it could be due to sex linkage of the gene for delayed mortality.

DISCUSSION AND CONCLUSION

Identity of the Respiratory Disease

Infectious bronchitis virus was isolated from chickens with respiratory symptoms at the RPRL. The virus is closely related to the Iowa 97 isolate of Hofstad. The infection has probably been present in the flock at the RPRL since the first outbreak in 1955. It was certainly present when antisera were prepared against GAL virus in 1958. The original source of the virus remains unknown. The mode of entry to the laboratory is obscure since rigid isolation and quarantine precautions on all equipment and personnel have been maintained since 1939 when the laboratory was first used.

Passage of the Virus

Passage of the virus 15 times in the allantoic sac of embryonating eggs produced a significant effect in the pathogenicity of the virus for embryonating eggs. It is quite probable that the differences would have been much greater had virus in passages earlier than the 8th been used for comparison. The differences between the 8th and 23rd passages were a combination of

1. A slight increase in titer and
2. An earlier mortality.

The shorter latent period is undoubtedly the most significant effect. In chickens there is in addition a slight decrease in the immune response of vaccinated and contact birds. A similar decrease in immunogenicity has been described by Delaplane and Stuart (1941) and Larose and Van Roekel (1961).

Titration and Neutralization Procedures

The diagnosis of positive responses by candling alone, as described by Hitchner et al., 1964, requires a little practice. Once mastered it is reasonably accurate and saves so much time that it appears well worth using for routine assays. When used in conjunction with the constant virus method for screening serum samples, epizootiologic studies are greatly facilitated. Both expense and time are reduced considerably.

Epizootiology

RPL-IBV is a mild strain of IB virus and is probably maintained from year to year as an inapparent infection in the non-isolated birds at the RPRL. Factors contributing to its maintenance are:

1. Continuous hatching all the year round. There are always young susceptible chickens available.

2. Movement of birds and caretakers between buildings.

3. The large population and high density of birds in this area.

4. The placing of more than one generation of birds in each house.

When chicks are infected, they are more likely to become carriers and spread the virus than if infected when older (Hofstad, 1947). Respiratory symptoms are sometimes present in very young chicks and it is probably some of them that continue to harbor the virus for prolonged periods. Immunological tolerance and a "carrier state" have been described in chicks infected with other viruses at a very early age (Rubin, 1962). If chicks are infected after complete immunological competence is attained, the chicks recover, develop antibodies and do not carry the virus. The continuous infection of susceptible chicks predisposes to the carrier state and maintenance of the virus within the population. Only the more severe outbreaks are noticed.

The W breeding areas present a continuous hazard to the isolated areas. Breaks in isolation are facilitated by the following:

1. There is direct contact between the caretaker and the environment of the birds. There is no exchange of caretakers between the more isolated E and B areas and the W area although they do congregate at lunch time. Caretakers must wash hands and change clothing on entry into each isolated area. However, entry into these areas must be frequent e.g. 5 times daily when trapnesting. It is interesting to note that 5 of the 6 outbreaks of IBV recorded in W_1 and E_1 (the areas in strictest quarantine) between 1955 and 1963, occurred between September and February and trapnesting is practiced between September and April. There would be a great improvement in isolation if the caretakers could be kept out of the environment of the chickens e.g. maintain chickens in isolators and handle them with gloves.

2. Certain equipment must be used near all areas even though it does not enter the buildings. This includes the feed delivery wagon and the manure disposal equipment. Cross contamination is possible and this is a weakness almost impossible to overcome when such large numbers of

chickens are involved

3. The prevailing wind is usually westerly. However, since there is more than 100 feet between any two buildings the wind probably does not play an important role in spread to the isolated areas.

Even though the highly contagious IB virus can cross the quarantine barriers, the isolation procedures used at the RPRL have been effective and satisfactory for the less contagious oncogenic viruses. More elaborate facilities will have to be used when working with the more contagious leukosis viruses which appear to be as infectious as IB virus.

Vaccination

The 23rd passage of RPL-IBV appears to be an excellent immunizing agent for birds 4 weeks to 12 weeks of age. In younger birds revaccination may be necessary if they possess maternal antibody. Immunity was not obtained in all older birds. In a program for the elimination of IB it would probably be wiser to vaccinate birds older than 4 to 6 weeks of age so that fewer of them are likely to become carriers. An elimination program should include an initial vaccination of all birds, a season where there is no hatching, and strict

isolation between generations of birds.

Genetic Studies

Embryos of line 7 die later than embryos of other lines of chickens. This immediately poses several problems. Is the late mortality actually an indication of resistance to infection? At present it seems unlikely because the 10-day post inoculation titers of the virus in each of the lines were so similar. If line 7 had been more resistant to infection by the virus then one would have expected fewer deaths and a lower titer of RPL-IBV in line 7. This was not the case.

How is the mortality delayed? Some of the factors involved could be:

1. The properties of the allantoic fluid into which the virus is injected.

2. Differences in the speed of adsorption, penetration, synthesis and spread of the virus through the embryo.

3. Resistance of the embryo to the mechanism of lethality of the virus or an increase in tolerance to the virus e.g. more efficient detoxification of metabolites, more rapid cellular repair or greater cellular capacity for nucleic acid and protein synthesis without disruption of cellular metabolism.

The mechanisms of resistance to Rous sarcoma virus (Crittenden et al., 1963) and mouse hepatitis virus (Kantock et al., 1963) are cellular in nature. They affect cells in culture as well as the animal in vivo. If this were shown for resistance to IB in line 7 the practical importance immediately becomes evident. The mechanisms of resistance to virus diseases require further study. The present results, although not conclusive, fit the model that the gene for delayed mortality is a dominant or incompletely dominant gene which has a very high frequency in line 7 and a low frequency in line 6. It may be sex linked or maternally influenced. It is possible that additional genetic influences could be found in other lines of chickens.

Even though embryos of inbred lines of chickens respond differently to RPL-IBV it does not follow that similar effects would be found with other strains of IB. The effects of different strains of the virus and different lines of chickens need further study.

Virus isolation and titration experiments using eggs from chickens similar to line 7 could be very misleading. This would be aggravated if

the mortality was recorded at 7 days post inoculation instead of at 10 days. The results could be even more confusing if eggs came from more than one source.

SUMMARY

1. A strain of infectious bronchitis (IB) was isolated from birds with respiratory symptoms at the Regional Poultry Research Laboratory.

2. It was identified as IB based on symptoms, histopathology, fluorescent antibody, lesions in embryos and lack of lesions on the chorio-allantoic membrane (CAM), hemagglutination (HA) with altered virus, and lack of hemagglutination inhibition (HI). Using serum-virus neutralization tests in embryos it was found to be most closely related to the Iowa 97 strain of Hofstad (1958).

3. The virus lost 63% of its activity on storage for 6.5 weeks at -75°C.

4. Passage of the virus in the allantoic sac of embryonating eggs increased the virulence for line 7 embryos significantly but the pathogenicity for line 6 embryos remained unchanged.

5. The candling method of determining positive responses was as accurate as examining the embryos for lesions.

6. There was some correlation between the constant serum and the constant virus methods of determining antibody. The latter method is very useful for

screening large numbers of sera and is much less expensive. It is of sufficient accuracy for epizootiologic studies.

7. The epizootiology indicates that the virus is maintained in the non-isolated breeding stock as an inapparent infection. Periodically there is a break in the quarantine and the disease occurs in the isolated area in more mature birds.

8. The 23rd passage of virus can be used as a vaccine for the immunization of immature chickens to prevent a decrease in egg production if the birds are exposed during the laying period.

9. Line 7 embryos die significantly later than line 6 embryos when infected with RPL-IBV. Line 15I is intermediate between these two.

10. The difference between reciprocal crosses is significant at 7 days post inoculation. The effect of the line 7 male on delaying the mortality is greater than that of the female. This may be due to one or more sex-linked genes for delaying mortality in line 7 or to a maternal effect in line 7 females causing earlier mortality.

11. Some matings of inbred lines had significantly earlier mortality than would be expected from a consideration of the lines themselves. The reasons for this are unknown.

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