STUDIES ON CHRONIC RESPIRATORY DISEASE OF CHICKENS II. ISOLATION OF A VIRUS*

J. E. FAHEY** J. F. CRAWLEY**

INTRODUCTION

The causative agent of chronic respiratory disease (CRD) was ascribed originally to a virus by Delaplane and Stuart in 1943 (1) on the basis that no bacteria could be demonstrated in infective material propagated in chick embryos. The etiological agent has been variously described as a psittacoid-like virus (2) and a rickettsia (3). Recently work on the etiology of this disease has stressed the importance of pleuropneumonia-like organisms (PPLO) (4, 5,6,7) which can be isolated readily from most field cases. However, proof has been lacking as to the ability of PPLO to initiate the entire disease syndrome, commonly referred to as "colds" or "air sac" infection in broilers or adult birds. As a matter of fact it has been the authors' experience that no more than mild respiratory symptoms such as nasal exudate and head shaking can be produced in normal birds by inoculating pure cultures of PPLO isolated from field cases of CRD.

It is the purpose of this paper to describe the isolation and some of the characteristics of an agent which is believed to be a virus and which causes a chronic respiratory disease in chickens.

METHODS

All eggs and chicks employed in this study were from CRD free flocks. This was determined by regular observation of hens, and chicks hatched from the hens. Periodically groups of chicks are raised through the broiler period. No symptoms of CRD have ever been observed in the hens or their progeny.

The material used in this study was obtained from field cases of CRD from Ontario broiler flocks. Egg passages at various levels were represented among the 52 strains mentioned in this work.

Diluents employed in this study are designated A and B. Diluent A consisted of potassium G penicillin 100 u/ml., neomycin 0.5 mgm./ml. and normal horse serum at a 5% level. Diluent B was composed of A plus 0.5 mgms. dihydrostreptomycin/ml.

All chorio-allantoic sac (CAS) fluids harvested in this study were routinely tested for hemagglutination (HA) activity against chicken red blood cells. These fluids were also plated on blood agar plates to check for possible contamination.

The strains employed were stored in the frozen state at -35°C. Aliquots of material were dispensed in ampoules and sealed for this purpose.

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**Connaught Medical Research Laboratories, University of Toronto.

Routine titrations were carried out in 9 day eggs by the CAS route. Generally eggs from a single source were used but eggs from 3 other sources were used periodically for purposes of comparison.

Birds employed in each experiment were housed in separate quarters and were inspected at least three evenings a week. Blood samples were taken every 2-3 weeks. Control birds for each experiment from the same hatch and source were kept in a neutral observation centre.

The hemagglutination-inhibition (H-I) tests for the detection of antibodies to PPLO were performed in essentially the manner previously described (8,9). Organisms were grown in PPLO medium (Difco), buffered at pH 7.62, containing 1% bovine serum fraction A. The test was performed at room temperature since the HA titer of PPLO was found to be practically identical at room temperature as at incubator or cold room temperature. Drilled leucite plates (10) were used for this test. The antigen was titred prior to test to determine suitability. The highest dilution of PPLO causing agglutination of red blood cells was called an HA unit. In the test 8 HA units were added to each serum dilution. Equal volumes of 2% chicken red blood cells, washed three times, were added to each serum-PPLO mixture giving a final dilution of 2 HA units. The sera were diluted by twofold dilutions from 1/5 - 1/160. It was not found necessary to inactive the sera since only an occasional serum was found which inhibited the H-I at a dilution of 1/5. This did not interfere with the interpretation of the test. The serum-PPLO mixtures were incubated together for 15 minutes, prior to addition of red blood cells. Suitable positive or negative controls were included in each test.

RESULTS

Material from 52 strains, mostly yolk sac (YS) egg passages were inoculated into eggs via the YS route with and without streptomycin, i.e. in diluent A and B. This experiment was designed to determine the number of strains of the "agent" showing resistance to this antibiotic, since field results with streptomycin were not producing the desired effect. It was felt that acquired antibiotic resistance might explain this fact.

The growth characteristics of 4 of the 52 strains which grew in presence of streptomycin are presented in Table 1. It can be seen that these strains were characterized by early deaths following either YS or CAS inoculation. The fact that there were specific deaths in the presence of streptomycin suggested that the agents were either not PPLO or a strain of PPLO which had become resistant to streptomycin. However, the characteristic early deaths via the CAS route in the presence of streptomycin suggested that PPLO were not involved. All CAS fluids were tested for HA and found negative as were further passages of these CAS fluids.

The CAS fluid was plated on blood agar plates and found sterile after 48

hours of observation. Samples of fluid were incubated in PPLO media along with known PPLO strains and carried for 3 subcultures. Although the medium supported growth of these known PPLO strains there was no success in cultivating a PPLO from any of the CAS fluids harvested. This experiment was repeated 3 times with CAS fluid from various passage levels of different strains.

A single strain (#51) was chosen for detailed laboratory investigation. This strain was passaged 3 times by the CAS route with the result that a high titre (Log LD_{50}-7.84) preparation was obtained. The mortality pattern of this

TABLE 1

ISOLATION PROCEDURE FOLLOWED IN OBTAINING FIRST 4 STRAINS OF VIRUS

Inoculum		Number of eggs per group	Day after inoculation (deaths)													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Strain 9	A	6		5												
	B	6		1*	4*					1						
14	A	6			1	2			1	1						
	B	6			1*	1*			1							2
33	A	6			1								1			2
	B	6										3*				
51	A	15		3	7	1						1				
	B	15		6*	8*											
YS inoculation																

*Transferred as follows:

Strain 9	YS	10		4	6											
	CAS	10		7	2											
14	YS	10	1	6	1	1	1									
	CAS	10		4	5	1										
33	YS	10	3	5		1										
	CAS	10	1		1						4					
51	YS	10	1	6	3											
	CAS	10		3	1	6										

Diluent B used in all second transfers.

strain grown in the CAS in 9 day eggs is shown in Table II. The nature of the dosage response can be seen from these figures. The early deaths occurring with low dilutions of this strain are typical since other strains were also observed to produce specific deaths in 18-36 hours.

Titration of a single lot in YS, CAS and on chorio-allantoic membrane (CAM) are presented in Table III. It can be seen that the Log LD_{50} of the material inoculated by the YS route is essentially the same as for the CAS route, whereas the CAM titer, although not accurately estimated, lies in the same range. It should be noted that an estimate of the infectivity on CAM is different since the estimate is based upon presence or absence of pock-like lesions.

The lesions produced on the chorio-allantoic membrane are presented in Figure 1. These lesions which appear as pocks are small whitish areas with an irregular margin. The membranes are usually thickened with areas of greyish diffuse opacity. At low dilutions the pocks are not discrete but appear as a large mass of necrotic tissue and the membranes are very fragile.

TABLE II

MORTALITY PATTERN OF STRAIN #51 IN 9 DAYS FERTILE EMBRYOS (CAS ROUTE)

Dilution of virus*	Day after inoculation											
	1	2	3	4	5	6	7	8	9	10	11	12
−1	4	2	4	7	3							
−2	4	2	2	12								
−3			4	9	2	5						
−4				4	6	4	4	2				
−5				4	2	4	4	2	3			
−6				4	1	5	4	2				
−7						4	3	4	1			
−8							6	2	1			
−9								2	1	1	2	
−10									2	1		1
−11												
Diluent control							1					

*The virus was suspended in a diluent containing antibiotics adjusted so that each egg received 0.5 mgms. streptomycin and 0.25 mgms. terramycin. Twenty eggs were used per dilution.

Log LD50 = 7.84 (Probit method).

TABLE III

TITRATION OF STRAIN #51 IN EGGS BY YS, CAS AND CAM ROUTES

Route of inoculation	Log LD50
Yolk sac	−6.71
CAS	−7.18
CAM	Pock-like lesions at −6.00 but not observed at −7.00

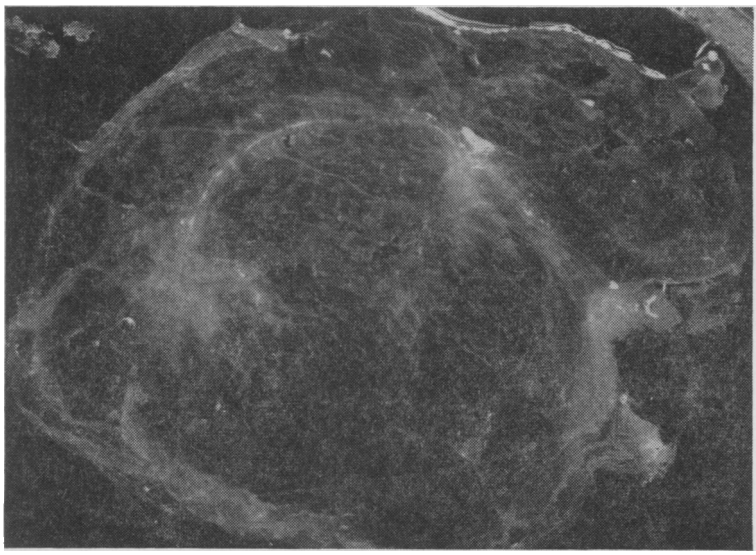

Figure 1

Pock-like lesions on choric - allantoic membranes caused by virus isolated from cases of CRD.

Embryos dying following inoculation with the virus presented the typical appearance of eggs dying from infection with the "CRD agent." (1) The embryos were hemorrhagic, the earlier deaths presenting a picture of extreme gross hemorrhage in the embryo with little change in the YS or CAM. Those deaths occurring at 4 days or later showed hemorrhagic or congested YS, thickened CAM with diffuse opacity and occasionally foci of small pock-like lesions. The embryos always exhibited some degree of hemorrhage and congestion.

This agent was tested for sensitivity to various antibiotics, three of which have been reported to be active against the CRD agent (11). The virus was tested at 2 dilutions and 3 levels of antibiotics were employed. These results are presented in Table IV and reveal that the antibiotics had no effect on prevention of mortality or prolongation of life of the embryo.

The agent was filtered through Seitz EK filter pads and a sintered glass

ultrafine (U.F.) with little loss in titre (Table V). It should be noted that comparable filtration studies on filterability of broth grown PPLO revealed that the filters retained the PPLO. This was proven by the failure to obtain growth of PPLO from filtrates using known strains as controls.

Neutralization tests against known Newcastle disease (ND) and infectious bronchitis (IB) immune sera were performed. This was done by adding undiluted sera to serial dilutions of virus ranging from undiluted to 10^{-9}. The Log LD_{50}'s of the 4 groups are shown in Table VI. There was no effect observed on the average day of death or on potency of the virus.

The virus was shown to be stable for at least 68 days at cold room temperatures (4°C.) in the form of CAS fluid sealed in ampoules. Samples were removed periodically and titrated. There was no appreciable loss in infectivity over this period (Table VII). The virus was stable at room temperature for a single day (the longest period tested).

TABLE IV

SENSITIVITY OF STRAIN #51 TO ANTIBIOTICS

Antibiotic	Amount per egg	Dilution of Virus			
		1/200		1(20,000	
		Mortality/10	Mean DD*	Mortality/10	Mean DD
None (virus controls)		10	12.0	6	15.1
Streptomycin	0.5mg.	10	13.3	5	17.8
	0.05mg.	10	12.7	5	14.6
	0.005mg.	10	12.8	6	15.3
Aureomycin.....	0.25mg.	10	12:3	4	14.5
	0.025mg.	10	12.5	6	14.5
	0.0025mg.	10	12.3	5	17.0
Terramycin	0.25mg.	10	12.7	6	14.5
	0.025mg.	10	11.8	5	15.4
	0.0025mg.	10	11.9	6	15.8
Neomycin.....	0.5mg.	10	12.6	7	14.4
	0.05mg.	10	12.1	5	16.6
	0.005mg.	10	11.6	5	15.4
Bacitracin.....	10 u	10	12.0	7	15.8
	1 u	10	12.0	7	14.2
	0.1 u	10	12.0	4	16.7
Aerosporin.....	0.25mg.	10	12.1	5	13.8
	0.025mg.	10	12.2	7	15.0
	0.0025mg.	10	12.3	7	14.8

*Mean day of death.

Antibiotic Control

Antibiotic	Amount per egg	Mortality/10
Streptomycin	0.5mg.	1
Aureomycin	0.25mg.	0
Terramycin	0.25mg.	0
Neomycin	0.5mg.	1
Bacitracin	10 units	0
Aerosporin	0.25mg.	0

TABLE V

EFFECT OF FILTRATION ON TITRE OF STRAIN #51

Treatment	Log LD50
Control virus	−7.90
Seitz filtered	−7.00
Sintered-glass U. F.	−7.10

TABLE VI

TITRATION OF STRAIN #51 WITH IB AND ND IMMUNE SERA

Serum	Log LD50	Mean DD −7.00	Mean DD −8.00
Normal horse serum	−7.80	13.8	15.6
IB immune sera	−7.85	14.9	14.5
IB and ND immune serum(1)	−7.92	13.2	15.2
IB and ND immune sera (2) (Standard immune sera)	−7.77	13.6	16.2

Once the above characteristics of the virus had been established, efforts to isolate this agent from field outbreaks were attempted. A further 5 strains were obtained and one of these strains was used for infectivity studies.

A total of 4 orientating experiments were conducted to study the clinical course of CRD and the serological responses. In two experiments the birds were first vaccinated with IB-ND at one day old by the spray method (12). At three

weeks of age when the vaccination "take" was completed the birds were inoculated intranasally with material containing this virus and the virus plus PPLO. Typical cases of CRD were produced in 10-15 days and persisted for 8-10 weeks at which time the birds were sacrificed. During the course of the disease sick birds were killed and isolation attempts made. Both virus and PPLO were isolated from both groups.

The sera of sick birds contained H-I antibodies against PPLO and preliminary investigations indicate that virus neutralizing antibodies are also present. Determination of the virus neutralizing antibodies is dependent upon

TABLE VII

EFFECT OF STORAGE AT 4°C. ON THE TITRE OF STRAIN #51
(9 DAY EGGS — CAS ROUTE)

Time interval (days)	Log LD50
0	7.77
16	7.51
31	7.40
44	7.25
68	7.35

TABLE VIII

MORTALITY PATTERN IN NON-VACCINATED CHICKS FOLLOWING INJECTION

Day after infection	Experiment 1** (86 chicks)†	Experiment 2*** (100 chicks)	Controls (56 chicks)
1	1	2	0
2	5	6	0
3	20	3	0
4*	6	11	0
5	2	4	0
6	1	4	0
7	2	4	0
8	2	3	1
9	1	0	0
10	1	3	0
11	1	0	0
12	1	5	1
13	0	6	0
14	3	8	0
15	0	1	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0
21	0	0	0

*Respiratory symptoms commenced and persisted in surviving birds for 10 weeks. No symptoms observed in controls.
**Virus + PPLO.
***Virus alone.

reduction of the pock-like lesions on the CAM. It should be noted that PPLO could be isolated when H-I antibodies to this agent were present.

The mortality pattern in chicks infected with this virus is presented in Table VIII. The early mortality with a peak at 3-7 days after inoculation is typical of that obtained with chicks hatched from hens infected with CRD (13).

SUMMARY

An agent which appears to be a virus and which is apparently involved in the etiology of chronic respiratory disease has been isolated and some of its characteristics described.

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