STUDIES ON CHRONIC RESPIRATORY DISEASE OF CHICKENS IV. A HEMAGGLUTINATION INHIBITION DIAGNOSTIC TEST[2]

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The preceding papers in this series have drawn attention to the following facts: (a) that chronic respiratory disease (CRD) is present in Canadian poultry flocks (1); (b) that a pleuro-pneumonia-like organism (PPLO) which is associated with the disease is egg transmitted (2); and (c) that a second agent which possesses the characteristics of a virus can be isolated from many field cases (3). The need of a serological test to unravel some of the mysteries of this disease is self evident.

Diagnosis of CRD has to date been dependent upon isolation of the so-called 'CRD agent', either in fertile hens' eggs or in fluid media. Inoculation of material from suspected cases either into chickens or the infraorbital sinuses of turkeys has been a common diagnostic procedure. Generally a consideration of the clinical nature of the outbreak, coupled with the internal appearance of the birds at autopsy, has provided presumptive evidence of the presence of CRD.

A serological approach to the diagnosis of CRD is suggested by the fact that avian PPLO are able to agglutinate fowl red blood cells (rbc) and that this reaction can be inhibited by sera from birds infected with this agent (4,5). While this does not suggest that PPLO are involved directly in the etiology of CRD, it is evident to those engaged in work on this disease, that PPLO are almost constantly associated with this condition. It is the purpose of this paper to present data concerning the use of a hemagglutination inhibition (HI) test, utilizing PPLO as antigen, in the study of CRD.

METHODS

The methods of isolation of PPLO from chickens have been previously described (3). The turkey strains of PPLO employed in this study were isolated in the same manner except that material from infected sinuses was used.

Bacto phenol red broth plus 1.0% bovine serum fraction A was used for propagation of the PPLO strains. The above media, which was used for preparation of the antigen, was buffered at pH 7.65 with 0.15 M phosphate and contained 0.1% maltose.

After isolation of the organisms the strains were transferred weekly 4 or 5 times in broth after which period hemagglutination (HA) activity could be regularly demonstrated. Following these transfers 40 ml. of the test tube cultures

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were transfered to 400 ml. of the PPLO media in Erlenmeyer flasks. The cultures were incubated at 37°C and tested daily for HA activity, which was generally noted by the 3rd day and reached a maximum titer by the 7th-10th day. The HA titer was taken as the highest dilution of PPLO antigen which caused complete agglutination of 1% chicken rbc. This titer seldom exceeded a dilution of 1/16.

After the growth pattern of the strain had been established, 20 ml. of the flask culture were tansferred weekly to 400 ml. of fresh media and tested daily as described above.

The chicken rbc which were used were obtained the day of the tests and were washed three times in physiological saline. Suitable cell suspensions were also prepared in saline.

Sera to be tested were diluted by 2 fold dilutions from 1/5 to 1/80 for routine titrations of field sera. In the case of sera from experimental birds the dilutions were carried from 1/5 to 1/640. A suitable positive control (standard immune serum) was included in each test. The tests were performed in drilled leucite plates (6).

To each dilution of sera was added an equal volume of the PPLO antigen. The serum PPLO mixtures were allowed to stand for 15 minutes at room temperature after which time chicken rbc were added so as to give a final red cell concentration of 1% and a PPLO antigen concentration of 4 HA units. The use of 4 HA units was adopted since this resulted in an endpoint which was much clearer and easier to read, although 2 HA units could be used. The results were read 1 hour after addition of the rbc and are recorded as the highest dilution of serum causing complete inhibition of agglutination.

The birds employed in the infection experiments were obtained from a White Rock supply flock in which no symptoms of CRD had ever been observed. Sera from the breeders were tested periodically for PPLO HI antibodies and found negative in all tests. Several thousand birds from this flock have been raised through the broiler stage without any evidence of CRD or development of PPLO HI antibodies.

RESULTS

Before the HI test could be used routinely it was necessary to standardize the procedure.

The use of chicken rbc was convenient but turkey rbc were agglutinated equally well by all strains. A final concentration of 1% rbc in the test was shown to provide the most reproducible results. At concentrations of rbc below 1% and particularly below 0.5% the titers tended to be erratic and at concentrations greater than 1% it was difficult to demonstrate agglutination by the PPLO.

A time of 15 minutes was established as a satisfactory time for the serum dilutions to stand with the PPLO at room temperature prior to the addition of the rbc. An interval less than 15 minutes tended to give slightly lower titers but after 15 minutes there was apparently no further neutralization of the PPLO by the serum dilutions. The tests could be read after one hour at room temperature since in this period the cells which were not agglutinated would settle completely into a small compact button. The agglutination of the cells by the PPLO is of a more permanent nature than that which occurs in the presence of Newcastle disease virus since such agglutination does not tend to break apart and 'fall in'. For this reason the tests could still be read after 24 hours with no change in titer.

It was found that identical titers would be obtained using saline buffered over a range of pH from 6.20 to 8.30 as diluent for the sera. For routine use unbuffered saline at a pH of 6.2 to 6.4 was adopted.

The choice of a suitable test strain for use with sera from a great variety of sources was a problem of importance. To determine if the avian PPLO were antigenically homogeneous with respect to the HI test, 6 strains were employed, 4 of these originated from chickens and two from turkeys. The turkey strains have been described previously in a study of the HI reaction in turkeys infected with infectious sinusitis (7). It was found that any serum which was positive using one strain was likewise positive with the other 5 strains and had an identical titer with each strain. Conversely sera which were negative with one strain were negative with all the others. Since the strains appeared antigenically homogeneous, strain D, which was originally isolated from turkeys was used routinely in these investigations. This strain has now been passaged 18 times in broth and has become stabilized with regards to growth and HA activity.

The HI test was used to follow the serological course of CRD in groups of chickens inoculated with untreated suspensions of lungs and tracheae from infected birds. This 'strain' of CRD has been passaged 6 times in birds under controlled laboratory conditions and produces symptoms in birds in 5-10 days. It contains both a PPLO and the virus previously described. A group of 150 11-day old White Rock chicks were inoculated by the intranasal route with 2 drops of a 25% tissue suspension. The birds were bled approximately once a week and the sera titrated immediately. After primary testing the sera were frozen and after 12 weeks all sera were re-tested at one time to determine the effect of test to test variations. The results of this test are presented in Table I.

All inoculated birds developed respiratory symptoms within one week after inoculation but the control group, which was housed in separate quarters and which was not inoculated, remained symtom-free and negative to the HI test. The respiratory symptoms persisted in the inoculated birds for about 8 weeks. After this time all respiratory symptoms stopped of their own

accord although PPLO could still be isolated from these birds and the PPLO HI titers remained at the same high level.

It should be noted that the HI titers first became positive in a few birds 9 days after infection and by 29 days all birds had a positive HI titer. There were only minor variations between the results of the final test and the individual weekly tests which indicates the reproducibility of the results. The pooled sera were obtained by mixing aliquot samples from each of the 10 sera taken weekly. The agreement between the theoretical and actual mean is of interest.

TABLE I

DEVELOPMENT OF HI TITRES TO PPLO IN BIRDS ARTIFICIALLY INFECTED WITH CRD

CLINICAL NOTES	AGE (Days)	HI TITERS*										TITER OF ALIQUOT POOL
		CONTROLS	INFECTED GROUP									
		<5	<5	5	10	20	40	80	160	320	640	
150 White Rock chicks+200 control started	1	10	10									<5
Chicks inoculated with bird passage material intranasally	11	10	10									<5
Resp. Symptoms in all inoculated birds	20	10	8	2								<5
Resp. Symptoms present	26	10	6	1	3							5
" "	34	10	6	2	2							5
" "	40	10		1	1	2	4	2				40
" "	47	10				2	5	3				80
" "	54	10				1	5	4				80
" "	61	10				1	3	3	2	1		80
Lessening of resp. symptoms	68	10				1	1	3	4	1		80
Birds symptom free	75	10					1	5	2	1	1	160
" " "	82						2	4	3	1		160
" " "	89	10					3	3	3	1		160

*Expressed as the reciprocals of the highest serum dilution causing complete inhibition of agglutination. Titers of <5 are considered to be negative.

In Table II are presented the results of an experiment conducted over a 4 month period. The birds were infected by the intranasal route when 16

TABLE II

PPLO HI ANTIBODIES IN BIRDS INFECTED WITH CRD AT 16 DAYS OF AGE AND CHALLENGED AT 93 DAYS OF AGE

CLINICAL NOTES	AGE (Days)	NO. OF SERA	HI TITERS								TITER OF ALIQUOT POOL
			∠5	5	10	20	40	80	160	320	
42 chicks started....	1	10	10								∠5
Inoculated intranasally with bird passaged material..	16	10	10								∠5
Severe respiratory symptoms from 20 to 88 days.....	44	21		2	5	7	5	2			20
	88	21			1	8	8	4			40
Re-inoculation.....	93	10			1	5	2	2			40
No. resp. symptoms..	100	6				2		4			80
" "	107	6					5		1		80
" "	114	6					4	2			40
" "	121	6					1	4		1	80
" "	128	6				1	2	2	1		80
" "	135	6					2	3	1		80

days of age and respiratory symptoms commenced 5 days later. These respiratory symptoms consisted of sneezing, coughing, shaking of the head and were accompanied by continuous running of the eyes and nostrils. Such symptoms persisted more or less unabated for about 8 weeks, after which time the severity of the symptoms noticeably lessened and gradually disappeared. PPLO were isolated when the birds were 93 days old, that is, approximately one week after all respiratory symptoms had disappeared. At this time these birds were again inoculated intranasally with bird passaged material containing the two agents and at the same time a group of 149 day old normal chicks were likewise inoculated with this material. The development of HI antibodies in this latter group of chicks is shown in Table III. These chicks served as infection controls as well as providing further information as to the nature of the response to infection with CRD. It can be seen from both these experiments that all birds which were infected became positive to the HI test in less than one month after infection.

The 13 week old birds described in Table II which had recovered clinically from CRD and which were re-exposed did not develop any sign of respiratory illness during a 6 week observation period. There was no apparent in-

crease in the HI titers following re-exposure. However, the day old chicks (Table III) infected at the same time did succumb to a severe case of CRD and experienced a 60% mortality.

TABLE III

DEVELOPMENT OF PPLO HI ANTIBODIES IN CHICKS INFECTED ARTIFICIALLY AT ONE DAY OF AGE WITH BIRD PASSAGED CRD

CLINICAL NOTES	AGE (Days)	NO. OF SERA	HI TITERS							TITER OF ALIQUOT POOL
			∠5	5	10	20	40	80	160	
149 chicks infected intranasally.....	1	10	10							<5
Respiratory symptoms present.	5	10	10							∠5
Resp. symptoms plus mortality.....	13	10	3	2	3	2				5
" "	19	10		6	2	1	1			10
" "	26	10			1	2	4	3		40
" "	33	10				2	4	4		40
Resp. symptoms present.....	40	10					4	4	2	80
" "	47	10					1	6	3	80

The results of using the HI test to conduct a preliminary serum survey on flocks in Ontario and British Columbia are presented in Table IV. The sera from British Columbia were obtained from birds during 1953 and the first 2 months of 1954. With about 10 exceptions, all were from adult laying flocks. The sera from Ontario were all obtained in the first two months of 1954, and are classified according to the age of the birds. In each case the result cited is based on a single serum sample from a flock. Where more than one serum was available from a single flock the result cited is that of a pooled sample. It is evident that there is a high incidence of antibodies in the laying flocks and older broilers. Although the number of flocks tested is small, the estimated incidence is considered conservative in view of the method of sampling

DISCUSSION

It appears that the PPLO HI test, using a single strain of PPLO as antigen, can be used either for following the course of infection in birds afflicted with CRD or for diagnostic work. This statement does not necessarily imply that PPLO are the 'cause' of CRD. The work presented here supports the view that PPLO are involved in the pathogenesis of CRD but not necessarily as the primary etiological agent. If such a test as the one proposed in this paper is to be used routinely it would appear necessary to establish exactly the role

TABLE IV

INCIDENCE OF PPLO HI ANTIBODIES IN ONTARIO AND BRITISH COLUMBIA POULTRY FLOCKS (Single sample test)

PROVINCE	AGE GROUPS	No. FLOCKS TESTED	HI TITERS						% POSITIVE
			<5	5	10	20	40	80 and >80	
Ontario....	BROILERS—								
	0—3 weeks	15	15						0
	3—6 weeks	5	3			1		1	40.0
	6—9 weeks	14	6			3	2	3	57.1
	9—12 weeks	72	22	3	11	8	11	17	69.4
	HENS	35	14	2	2	4	6	7	60.0
B.C.....	HENS	179	58	3	16	37	36	29	67.6
	TOTAL.....	320	118	8	29	53	55	57	63.1

of PPLO in this disease. If these organisms are a constant factor in CRD, then certainly the PPLO HI test will be of value in diagnostic and survey work.

The fact that the HI titers develop simultaneously with the disease in three separate experiments provides some evidence for the validity of the test. However, in each case, bird passaged material, which contained both the PPLO and a virus was used to infect the birds. In each experiment all the birds infected became positive in less than 4 weeks and in the case of chicks infected at one day of age positive titers in all birds were observed 19 days after infection. In two experiments it was shown that some chicks developed recognizable titers within one week. It would appear that approximately eight weeks are required before maximum HI titers are reached.

While it is true that the 13 week old recovered birds did not succumb to challenge. these same birds did have high titers against PPLO while they still had the disease. An important point is established however in showing that birds with chronic respiratory disease do recover clinically and that they are not immediately susceptible to a clinical recurrence of the disease. It has been shown by other workers that recovery and subsequent resistance to challenge occurs and that recovered birds are still carriers of the 'CRD agent' (8). The present data supports the observation that recovered or symptom-free birds are still carriers of PPLO. This suggests the possible mechanism of egg transmission through persistance of the PPLO in recovered birds. However, it

would be impossible to conclude from the above data that PPLO HI antibodies are a measure of immunity.

Preliminary observations on the incidence of PPLO HI antibodies in Canadian poultry flocks would seem to indicate a widespread infection. While case histories were not available for all flocks tested it can be stated that where clinical CRD was known to be present in a flock for more than one month, all such flocks were positive according to this test. The absence of PPLO HI antibodies in the younger age group of broilers would be expected from the experimental results. Under field conditions it would be very unlikely that all the birds in a flock would be infected at one day of age, and hence one could not conclude, on the basis of a negative HI test, that CRD was not present.

The major value of this test would appear to lie in the ability to determine the presence or absence of CRD in laying flocks, although interpretation of titers must be tempered with good judgment until further results are available as to the persistence of antibodies following infection. At this time the results should be interpreted only when coupled with serological tests for infectious bronchitis and Newcastle disease.

SUMMARY

(1) A pleuropneumonia-like organism (PPLO), isolated from a turkey, has been used as an antigen in a hemagglutination inhibition (HI) test for recognition of infection with this agent.

(2) The PPLO HI antibodies arise between the second and third week after infection and reach their peak 6-8 weeks later.

(3) The incidence of PPLO HI antibodies in 320 flocks of various ages from Ontario and British Columbia was 63%.

(4) In two experiments, PPLO were isolated following the cessation of the clinical signs of CRD.

(5) Birds recovering naturally from CRD are resistant to challenge with bird passaged material immediately following recovery.

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BOOK REVIEW

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THE PHARMACEUTICAL PRESS, LONDON, W. C. 1, 1953.
xxiii + 737 pp., 45s. (plus 1s. postage).

This is one of the most significant contributions of recent years to veterinary literature. It is the veterinary equivalent of the well known B. P. codex and it arises from proposals by the Royal College of Veterinary Surgeons and the British Veterinary Association made some four years ago that such a volume should be compiled. The Pharmaceutical Society appointed a committee in 1950 and the present book is the result of its deliberations. It is compiled in three parts with a series of appendices. The first part — 412 pages — contains the monographs on drugs, chemicals and related substances, described alphabetically and detailing not only the constituents, actions, uses and doses, but the accepted standards — many of them identical with those of the British Pharmacopoeia. Part II. deals with serums and vaccines, with similar monographs on individual products, while Part III. is the Formulary with full details, formulae and standards of preparations. The appendices deal mostly with tests, assays and similar things. The volume concludes with a therapeutic and pharmacological index, which is a guide to the "action and uses" statements of the first two parts; there is also a complete general index.

While perhaps not quite so indispensible in places where the British Pharmacopoeia is not in common use, as it will be in Great Britain, there is no doubt whatever, that this work will be invaluable to every veterinarian in every English-speaking country. Veterinary Pharmacology has now been placed on a firm and well-defined basis.