STUDIES ON MODIFIED COMPLEMENT FIXATION OF FOWL POX IN CHICKEN

Yun Seong Jeon
Dept. of Veterinary Science, College of Agriculture

SUMMARY

From these studies eighteen different experimental results were obtained and are summarized here.

1. The methodology of the modified complement fixation test for avian pox virus has been described.
2. The distribution and properties of CAM antigen resulted from the infected chicken embryo were studied.
3. The antibody responces and strain specificity of avian pox viruses were studied.

INTRODUCTION

A specific demonstration, by means of a practical serological method, of fowl pox antigen-antibody complex of chicken has been nceessiated in the various purposes such as the measurement of immune responce of chicken for the vaccination, demonstration of strains specificity and serological diagnosis of fowl pox.

Although virus neutralization has been developed for the purpose of the antibody detection on the chorioallantoic membrane of embryonating eggs (1), the antibody is frequently so low in titer that it can not be detected in the sera of chickens immune to challenge. Furthermore, the method has not found wide acceptance for routine titrations due to difficulties in execution and interpretation (2).

It has been shown that the conventional complement fixation test is unable

to detect antigen antibody complex when chicken antiserum is heated (3). The indirect complement fixation test, accordingly has been adapted for detection of fowl pox antibody in chicken (4).

However, the indirect complement fixation test has some disadvantages. It requires positive serum from a heterologous species and better stability of antigen is needed due to the prolonged time of reaction.

A preliminary study of the modified complement fixation made possible to detect the specific antigena-ntibody complex of fowl pox in chicken. Although the test requires the chicken factor, it minimizes the disadvantages found in the serum neutralization and indirect complement fixation test (5).

The purpose of this study is to describe a modified complement fixation on the emphasis of methodology of the test, antigen analysis, requirement of the chicken factor and raise of antibody in the experimentally infected chickens and cross reactions among a pigeon and fowl viruses.

1. This investigation was supported by research grant from the Graduate School of Seoul National University.

MATERIALS AND METHODS

DILUENT: The diluent was veronal-NaCl buffered solution, containing 0.145 M NaCl and 0.005 M veronal buffer at pH 7.2 with 0.00015 M Ca++ as calcium chloride dihydrate, and 0.0005 M Mg++ as magnesium chloride hexahydrate (6).

SHEEP ERYTHROCYTES: To prevent coagulation of blood and for its preservation Modified Alsever's solution was used (7). In the final step of preparation of the solution, it was sterilized by employing filteration procedure instead of autoclaving. The solution was immediately used or stored at 2°C. for the future use. The stock sheep cells were washed more than 3 times with veronal-NaCl buffered solution by centrifugation at 1,000 r.p.m. for 10 minutes. A 2 per cent suspension of erythrocytes was made and used.

HEMOLYSIN AND TITRATION: Hemolysin was made by inoculation of rabbits with boiled sheep erythrocytestroma (8). Analysis of antigenic nitrogen, by using micro-Kjeldahl method, was substituted with turbidometric method. The antigenic concentration was made to be equal to the first tube of McFalend's turbidometric tube. Hemolysin made from rabbits was heat inactivated at 56°C. for 30 minutes and stored at —60°C. For sensitization of the cells 2.5 units were used. (5).

GUINEA PIG COMPLEMENT AND TITRATION: The serum of guinea pig was harvested and pooled. Aliquots of 1.0 ml. were dispensed and stored at —60°C. The complement was titrated on two successive days or until stability was obtained and thereafter used for to months without further titration. In all subsequent titration two exact units of the guinea pig complement were employed. (5).

CHICKEN FACTOR AND TITRATION: For the preparation of chicken factor, a pooled of not lipemic serum was obtained from young and fowl pox unimmune chickens. The normal serum was used immediately or poured in test tube before storing at —60°C. It was used for at least 3 months after bleeding.

In the preparation of ChC'l, the dilution method (10) was applied. Heat stable fraction (ChH) of the normal chicken serum was prepared by using the same or different batches of the serum by heating at 56°C. for 30 minutes. The fraction was used immediately or stored at —60°C. until to be used.

Chicken factor was prepared by mixing ChC'l and ChH, in which the ratio of two components to be 2.5. The quantity of ChC'l was based on the amount of normal serum used but not the precipitate. In other words the factor which has the ratio 2.5 was prepared by precipitation of 2.5 ml. normal chicken serum and the precipitate was disolved with ChH up to 1.0 ml. Dissolivng the 2 components was made by agitation with a glass rode for 15 to 20 minutes. The residual flocculent material was removed by centrifugation for 5 minutes at 2,000 r.p.m.

The preliminary dilution shows the various concentrations of the concentrated factor in veronal buffer to be tested. This procedure is required because even

when the ratio of the ChC'l/ChH is 2.5, 0.01 ml. may give an anticomplementary effect. However, the dilution of the factor with veronal buffer gives the same degree of fixation but no anticomplementary effect. The first section shows the effect on the antigen of these concentrations. This procedure is required because unfixed antigen shows more anticomplementary tendency which may bring about false positive reaction. The second section shows the effect of the chicken factor in the presence of an immune complex. The titration of the above factor in the presence of antigen-antibody complex is a control of the factor after the various dilutions with veronal buffer solution.

In the first chicken factor titration of the experiment, arbitrarily diluted antigen (1/4 to 1/5 diluted) and antiserum (1/16 diluted) were used and the best quantity, in 0.01 ml., of the factor was determined. The preliminarily titrated factor was then used in the determination of the antigen and antiserum units. The factor was then retitrated using 2 units of antigen and antiserum as determined above and the factor ratio was adjusted accordingly. Several diferent lots of antigen were used. The above procedures were carried out to set up an original protocol yeilding a know antigen, antibody. Henceforth a new supply of factor could now be titrated. In this case, the second to the strongest concentration that causes fixation without being anticomplementary was considered 1 unit of chicken factor. This concentration was incorporated in aliquots of 0.01 ml. and added to test system and all controls. The chicken factor was used on the same day of preparation or of thawing from frozen status. (5).

VIRUS STRAINS: Two different virus of avian pox, namely pigeon pox virus of Minnesota strain and fowl pox virus of 2755 strain were employed. The Minnesota and 2755 strains of virus were obtained from Dr. Siccardi, University of Minnesota and Dr. Hanson, University of Illinois, respectively. The stock virus materials as an infected chorioallantoic membrane of embryonating eggs were stored by suspending in 50 per cent phosphate buffered saline glycerine at 2°C. or without suspending at —60°C. for future use.

ANTIGEN AND TITRATION: The original virus materials were hydrated in 10 per cent with 0.1 M phosphate buffered saline (pH 7.2), containing 2,000

units crystalline penicillin G and 2,000 micrograms dihydrostreptomycin per ml. of the solution. The hydrated virus materials were incubated for 30 minutes at room temperature and the supernate was obtained by centrifugation at 2,000 r.p.m. for 10 minutes. This was used to the antigen preparation. The supernate in amount of 0.2 ml was inoculated 9 to 12 day-old chicken embryo via the chorioallantoic membrane. The eggs were further incubated for 3 to 5 days and harvested the infected CAM where a typical lesion was formed.

In the preparation of antigen, the harvested CAM was immediately washed with a chilled physiological saline before storing at —40°C. until to use. The antigenic material was thawed and water of the CAM was partially removed on the filter paper. The CAM was then weighed and made 20% suspension by using Hank's BSS or 0.1 M phosphate buffered saline solution. A small amount of abrasive may not incorporated in the preparation of the viral suspension. The suspension was centrifuged at 3,000 r.p.m. for 10 minutes and the supernate was subjected to the antigen titration. In the antigen titration, more than 2 different substituted with 1/16 diluted negative chicken serum. An optimum quantity of antigen was established in which no anticomplementary effect on the negative serum group but gives a solid fixation on the positive serum group. Addition of the reagents was followed chicken factor, serum, antigen and guinea pig complement in that order before the primary incubation.

ANTISERUM AND TITRATION: In the preparation of a standard antiserum against 2755 strain of fowl pox virus and against Minnesota strain of pigeon pox virus, 10 chickens of 6 weeks old were employed. Before the immunization the chickens were tested whether chickens have antibody or not. Fowl pox virus was inoculated in the wing web by means of sticking method. On the other hand, the pigeon virus was inoculated in feather follicle of leg by means of brush. The chickens were booster injected for 3 times at one week internal. After weeks of the last virus inoculation, the chickens were killed and the individual serum was harvested and stored at —20°C. after 56°C. 30 minutes heat inactivation.

The antiserum was used to the antigen and chicken factor titration.

EXPERIMENTAL RESULTS

1. EFFECT OF CHICKEN SERUM AND FACTOR ON COMPLEMENT FIXATION.

It has been known that the chicken immune complex in an unheated fresh state of antiserum, can fix guinea pig complement but it is unable if the antiserum is heat inactivated. However, the supplementation with a fresh unheated serum which is obtained from the normal chicken bring about the fixation of complement to the heated antibody-antigen complex. The degree of the fixation is enhanced if the system is supplemented with a chicken factor in which a concentrated chicken C'1 dissolved in chicken heated and zymosan treated serum (ChHz).

In accordance with the above facts, the requirement of chicken serums and factor in fowl pox system was tested in this experiment. The antigen-antibody complex is consisted with 2755 strain of fowl pox virus and 1/16 diluted 2 units of 2755 antiserum prepared in young chickens. The antigen was prepared by using the virus infected CAM harvested at 4th day of virus inoculation. It was emulsified with BSS described previously. Normal serum was obtained from 6 young chickens and the absence of a specific antibody was tested before use as a normal serum or factor.

The results were illustrated in Table I. The results indicate the following:

1) Fresh antiserum-antigen complex of fowl pox virus can fix guinea pig complement in a direct complement fixation.
2) Heat inactivation of the antiserum was unable to fix guinea pig complement even in case of normal serum supplementation.
3) Supplementation of chicken factor to the heated antiserum-antigen system was able to fix guinea pig complement and the degree of the fixation was enhanced.

2. DISTRIBUTION OF CF ANTIGENS IN CHICKEN EMBRYO

In these studies, distribution of antigenic material in the allantoic, amnionic, embryo tissue and chorioallantoic membrane (CAM) resulting from CAM inoculation of virus was analyzed.

The same lot of 20 embryonating eggs divided into control and test groups, received 0.2 ml. of the per cent seed virus of 2755 strain at the 8 days of age. After 3~4 days incubation at 37°C., fluids and tissues were harvested only from the live embryos that were chilled for 4 to 6 hours at 2°C. The whole embryo antigen was made as described in the CAM antigen. In one day, the antigenic materials were titrated as illustrated in Table II.

Throughout the experiments the following results were obtained:

1) The allantoic and amnionic fluid antigens prepared via CAM inoculation of the virus had equally negative antigenicity.
2) The embryo tissue antigen showed neither antigenicity nor specific fixa-
3) The CAM antiegn showed a specific fixation.

3. EFFECTS OF CHEMICALS ON CAM ANTIGEN

In the previous experiments the CAM was proved as an unique antigen which caused a specific fixation. However, the antigen showed a tendency of anti-complementary effect unless it is prepared and titrated under a cautious circumstance. Therefore, in these studies, it was attempted that the elimination of the anticomplementary effect by treating the CAM with either of acetone or ethyl ether. As controls, two different buffered solutions, namely 0.1 M phosphate buffered saline and Hank's balanced salt solution were employed in the extraction negative and positive serum was used. In case of antigen control, the diluent was of antigen.

A 4 groups of 5 grams of the antigenic CAM were separately ground in a mortar. The first 2 groups were treated with either of acetone or ethyl ether at room temperature for 3 times. After treatment the residual solvent was evaporated and the antigenic material was suspended in Hank's BSS to be 20 per cent solution. Other procedures for the first 2 groups and the latters were followed as described previously.

The results were illustrated in Table III. Throughout the experiments the following results were obtained:

1) Both of the acetone and ethyl ether treated CAM antigens were unable to act as antigens.

2) Hank's BSS showed better result than that of BSS in the extraction of antigenic components from the CAM.

4. THERMAL STABILITY OF CAM ANTIGEN

In these studies, the thermal stability of the CAM antigen was tested. By employing the same lot of chicken embryos and strain of virus, the antigenic CAM was obtained. It was immediately titrated or exposed at the various temperature and time of storing. In the antigen titration, a 20 per cent suspension of the CAM in either of Hank's or PBS solution was made and titrated without delay.

Heat inactivation of the antigen at 56°C. was accomplished at the same day of the virus harvesting and a 20 per cent suspension of the CAM in either of Hank's or PBS solution was made and titrated without delay. The suspension was poured in a screw caped test tube and subjected to 56°C. water bath for 30 and 60 minutes.

In the case of glycerinated antigen, CAM, without grinding, was suspended in a 50 per cent phosphate buffered glycerine until to titrate. The temperature effect on the CAM antigenicity was illustrated in Table IV.

Throughout the experiments the following results were obtained:

1) The antigenic component became inactivated when heated at 56°C. for 30 minutes.

3) The glycerinated CAM stored at 2°C. for 4 days in a suspension was able to acts as an antigen, it had a tendency to be anticomplementary.

5. EFFECT OF QUANTITY OF REAGENTS

It was noted that, in the titration of antibody under the presence of 2 exact units of guinea pig complement and 2 per cent, 2 units sensitized erythrocytes, the quantity of antigen and antiserum effects on the degree of the fixation. In other

words, all of each reagent in amount of 0.1 ml. caused anticomplementary effect even the quantity and quality of the chicken factor were regulated. However, 0.2 ml. and 0.3 ml. systems brought about a specific reaction in case of positive system.

The detailed procedures and results were illustrated in Table V. The results of the experiments indicate the following:

1) All of each reagent in amount of 0.1 ml. caused a non specific fixation under the regulated condition.

2) All of each reagent in amount of 0.2 and 0.3 ml. brought about the specific fixation under the regulated condition.

6. STUDIES OF CF ANTIBODY FORMATION IN ARTIFICIALLY INFECTED CHICKENS

The formation of CF antibody in the artificially infected chickens was studied. The experimental chickens were procured and tested for CF antibody titers one week prior to experimental infection. Thirty-nine, 4 week old, chickens were divided into test and control groups. The former was further divided into 4 different group and exposed to 2755 strain of fowl pox virus via wing web route. At the 1st week post infection, 10 ml. of blood from each bird of the 1st week group was obtained. Then at 1 week intervals, blood samples were collected for 4 weeks. The same group chickens were never bled thereafter. The serum was harvested, and heated at 56°C, for 30 minutes and the antibody was titrated as described previously. The results were illustrated in Table VI. The results of the experiments indicate the following:

1) On the first week postinfection, no CF antibody was demonstrated in the bood. However, at the second week, the antibody titer began to appear.

2) On the 3rd week of post infection a peak CF titer was observed and this was maintained for more than 4th week.

7. CROSS REACTION OF 2755 ANTIGEN WITH FOWL AND PIGEON POX ANTISERA

A possible strain specificity between fowl and pigeon pox virus was studied. Antigen used in these experiments was prepared as described previously. In the preparation of antisera, 5 chickens of 3 week old were used to each strain of the viruses. The pigeon pox antisera, namely anti-Minnesota, anti-Kitasato and anti-Nakano were prepared by the feather follicle method.

On the other hand, antiserum specific to An-Yang strain of fowl pox virus was prepared by the wing web method.

At the 3rd week of post infection the blood samples were collected and sera were titrated after 56°C. for 30 minutes heat inactivation. Results were illustrated in Table VII. Throughout the experiments the results indicate the following.

1) The 2755 antigen of fowl pox virus showed a specific fixation if the antigen is reacted with 2755 antiserum or An-Yang antiserum of fowl pox virus.

2) No cross reactions between 2755 antigen and pigeon pox antisera were observed.

DISCUSSION

Supplementation of normal chicken serum or chicken factor in the detection of chicken antibody-antigen complex by means of a direct complement fixation test was first described (10), and developed (5).

A series of the studies of a modified complement fixation test on the various antigen-antibody systems the authors demonstrated that the system require not only chicken C'1 but heat and zymosan stable chicken complement (11). Such discovery brought abought the possible detection of chicken antibody-antigen complexes, by means of the modified complement fixation, such as IB, ND, FP or PP, and Celo virus.

The fundamentals of the modified complement fixation test, in this paper, were applied to fowl and pigeon pox viruses which had no serological test has been available.

Supplementation of chicken factor to the heated antiserum-antigen system was able to fix guinea pig complement and the degree of the fixation was enhanced. However, the fixation could not observed in the case of the supplementation of normal chicken serum. This is annlogous to that of ND and IB systems. This may be due to an impurity or inadequate extraction of the antigenic component from the infected CAM. Such inadequacy of the antigen also may explains the false reaction in the case of 0.1 ml. system of the reagents.

From the view of the thermal stability test of the CAM antigen, an effective antigen appears to be a heat labile component. Distribution of antigenic material in chicken embryo resulting from CAM inoculation of virus may indicate the CAM is the most reliable source of the antigen in the modified complement fixation test of avian pox virus systems.

Finally, it is desirable to solve the most potent method of antingen extration which may give a minimum anticomplementary effect and a maximum antigenicity.

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國文抄錄

닭에 있어서의 鷄痘에 대한 改良補體結合反應에 關한 硏究

全　　允　　成 <서울大學校農科大學副敎授>

닭의 血淸으로 구성되는 抗血淸과 이에 특이한 抗原과의 結合物에는 기니픽의 補體가 結合되지 못해서 直接補體結合反應이 불가능하다. 따라서 이 연구에서는 改良補體結合反應으로 아직 실현되지 못한 鷄痘의 補體結合反應을 가능케 하였다. 이 연구에서 얻은 실험성적은 다음과 같다.

(1) 鷄痘의 改良補體結合反應에 관한 方法을 발전시켰다.

(2) 鷄痘毒에 感染된 鷄胎兒의 漿尿膜으로 만든 抗原의 性狀을 밝혔다.

(3) 鷄痘 및 鳩痘毒의 毒株別 特異性과 닭에 있어서의 抗體形成과정을 밝혔다.

Table I. Effects of Heating Chicken Normal Serum and Factor on the Fixation of Guinea Pig Complement

Types of Supplement	Amount of Supplement	Types of Serum(1/16)	Antigen Dilution 1/4	1/5	1/6	1/7	1/8
No Supplement but fresh		P-Serum	4	4	3	0	0
		N-Serum	0	0	0	0	0
No supplement but heated		P-Serum	0	0	0	0	0
		N-Serum	0	0	0	0	0
Ch-S	0.01ml.	P-Serum	0	0	0	0	0
		N-Serum	0	0	0	0	0
Ch-S	0.02ml.	P-Serum	2	2	2	2	2
		N-Serum	2	2	2	2	2
Ch-S	0.03ml.	P-Serum	3	3	3	3	3
		N-Serum	3	3	3	3	3
Ch-S	0.04ml.	P-Serum	4	4	4	4	4
		N-Serum	4	4	4	4	4
Ch -F	1U, 0.01ml.	P-Serum	4	4	4	3	3
		N-Serum	0	0	0	0	0

Ch-S: Normal Chicken Serum

Table II. Distribution of Antigen Prepared from Chicken Embryo

Types of Antigen	Types of Serum(1/16)	Undil.	Antigen Dilution 1/2	1/3	1/4	1/5	1/6
Allantoic	P-Serum	0	0	0	0	0	0
fluid antigen	N-Serum	0	0	0	0	0	0
Amnionic	P-Serun	0	0	0	0	0	0
fluid antigen	N-Serum	0	0	0	0	0	0
Whole embryo	P-Serum	4	4	4	4	3	2
tissue antigen	N-Serum	4	4	4	3	3	2
Chorioallantoic	P-Serum	4	4	4	4	4	4
membrane antigen	N-Serum	4	4	1	0	0	0

Table III. Effects of Chemicals on CAM Antigen

Types of Antigen	Types of Serum(1/16)	Antigen Dilution 1/2	1/3	1/4	1/5	1/6
Acetone treated	P-Serum	0	0	0	0	0
antigen	N-Serum	0	0	0	0	0
Ethyl ether	P-Serum	2	2	2	0	0
treated antigen	N-Serum	1	1	1	0	0
0.1M PBS	P-Serum	4	4	4	4	0
extracted antigen	N-Serum	4	4	3	0	0
Hanks' BSS	P-Serum	4	4	4	4	3
extracted antigen	N-Serum	1	1	0	0	0

Table IV. Heat Stability of CAM Antigen

Types of CAM Antigens	Types of Serum(1/16)	Antigen Dilution 1/2	1/3	1/4	1/5	1/6	1/7
Frozen CAM stored at -20°c for 1 month	P-Serum	4	4	4	4	4	4
	N-Serum	4	4	2	0	0	0
Refrigerated(2°C.) PBS suspended, stored 4 days	P-Serum	4	4	4	4	4	4
	N-Serum	4	4	4	2	0	0
Refrigerated(2°C.) glycerinated, stored 1 month	N-Serum	4	4	4	3	3	0
	P-Serum	4	4	0	0	0	0
Fresh control antigen	P-Serum	4	4	4	4	4	4
	N-Serum	4	4	1	0	0	0

56°C. 30 minutes heated antigen	P-Serum	0	0	0	0	0	0
	N-Serum	0	0	0	0	0	0
56°C. 60 minutes heated antigen	P-Serum	0	0	0	0	0	0
	N-Serum	0	0	0	0	0	0

Table V. Effect of Quantity of Reagents

System	Amount of Ch-F	Types of Serum	Ch- F Dilution 10/10	10/15	10/20	10/25	10/30
0.1 ml. system							
Serum 0.1ml.							
Antigen 0.1 ml.		P-Serum	4	4	4	4	4
GpC' 0.1ml.							
EA 0.1ml.	0.005 and 0.01 ml.	N-Serum	4	4	4	4	4
0.2 ml. system							
Serum 0.2 ml.							
Antigen 0.2 ml.		P-Serum	4	4	4	4	5
GpC' 0.2 ml.							
EA 0.2 ml.	0.01 ml.	N-Serum	1	0	0	0	0
0.3 ml. system							
Serum 0.3 ml.							
Antigen 0.3 ml.		P-Serum	4	4	0	0	0
GpC' 0.3 ml							
EA 0.3 ml.	0.01 ml.	N-Serum	0	0	0	0	0

Table VI. Raise of CF Antibody Titers in Experimentally Infected Chickens

Week of Post Infection	No. Of Serum	CF Titers for Test Group	CF Titers for Control Group
	1	<8	—
	2	<8	—
	3	<8	—
1 week	4	<8	—
	5	<8	—
	6	<8	—
	1	<8	<8
	2	<8	<8
	3	16	<8
2 weeks	4	16	<8
	5	<8	<8
	6	16	<8
	1	64	<8
	2	32	<8
	3	64	<8
3 weeks	4	64	<8
	5	16	<8
	6	died	
	1	64	<8
	2	64	<8
	3	32	<8
4 weeks	4	64	<8
	5	died	—
	6	died	—

Table VII. Cross Reaction of 2755 Antigen to Fowl and Pigeon Pox Antisera

Antigen-Antibody Systems	CF Titers
2755 antigen plus 2755 antiserum	64
2755 antigen plus AnYang antiserum	16
2755 antigen plus Minnesota antiserum	<8
2755 antigen plus Kitasato antiserum	<8
2755 antigen plus Nakano antiserum	<8