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Characterization of Avian Adenovirus Associated Virus

I. Isolation and Identification of Avian Adenovirus Associated Virus

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Introduction

The avian adenovirus associated virus(AAAV) is a small, defective, single stranded DNA virus of the parvovirus group.

The virus was observed in a suspension of quail bronchitis virus(QBV) by electron microscopy, but was regarded as subunits of the mature particle by Dutta and Pomeroy.[3] Later, it was suggested that these small particles were distinct entities and morphologically similar to the primate adenovirus associated virus by Hoggan in 1971.[6] McFerran *et al.*[10] observed 20nm particles associated with avian adenoviruses isolated from chicken flocks.

Yates, *et al.*[15] isolated and purified AAAV, and studied some of its serological, biological and ultrastructural properties. They demonstrated that the virus was antigenically different from the primate adenovirus associated viruses by immunoelectronmicroscopy. The virus propagated in chicken embryo and chicken kidney cell culture coinfected with a proper amount of "helper" virus, however, pathogenicity or cytopathic effect was not observed. Contamination of avian adenovirus stocks and field isolates with AAAV was determined by serology and electron microscopy by many workers.[4,16,17] however, the identification of the virus is still limited because of technical difficulties.

This study was undertaken to identify and characterize AAAV by means of serological methods.

Materials and Methods

Propagation and Purification of AAAV: AAAV from Olson's quail bronchitis virus (QBV) was propagated in 11-day-old chicken embryos by chorioallantoic inoculation. Infected aminoallantoic fluid (AAF) was harvested when the embryos began to die, usually four or five days post inoculation.

The AAAV purification technique was a modification of that of Yates *et al.*[15] In short, infected AAF was clarified by centrifugation at $8,000 \times g$ for 20 minutes. The clear supernatant was then layered on 5ml of a 42% CsCl solution in tris buffer (pH 8.0) contained in an SW 25 rotor tube. After centrifuged at $50,000 \times g$ for 2 hours(Spinco ultracentrifuge) the fluid above the band and cushion was removed, and additional clarified AAF was added. Centrifugation was repeated at $50,000 \times g$ for 2 hours.

After two or three cycles of this partial purification process, the opaque band formed at the CsCl cushion was harvested through the side of the tube. The specific gravity of the virus suspension was determined using a refractometer(Baush & Lomb), and adjusted to 1.38 gm/cm^3 by adding crystalline CsCl (ICN Pharmaceutical).

The virus suspension was then placed in a SW 39 rotor tube and centrifuged at $100,000 \times g$ for 44 hours. Several visible bands were formed. Fractions were obtained by piercing the bottom of the tube with a 22 gauge hypodermic needle. A total of 24, four drop fractions were obtained.

The density of each fraction was determined before storage at $-20°C$. Later, fractions including the visible bands were collected through the side of the tube. Five fractions were collected by this method.

Preparation of AAAV Stock: The virus fraction with a density of 1.42gm/cm^3 was dialysed against excess PBS (pH 7.2), and diluted to make a 10% suspension in Hank's balanced salt solution (HBSS) containing 1% heat inactivated normal rabbit serum.

This suspension was further clarified by serial filtration through 220, 100 and 50nm membrane filters (Millipore) premoistened with HBSS containing 10% heat inactivated normal rabbit serum. The filtrate was divided into small aliquots and kept at −70°C.

Preparation of Rabbit Anti-AAAV Hyperimmune Serum: The preparation followed the methods described by Yates *et al.*[15] with minor modifications. Rabbit anti-CELO immune serum was added to AAAV stock and reacted overnight in the cold. The resulting precipitate was removed by low speed centrifugation. The clear supernatant was emulsified with an equal amount of Freund's complete adjuvant (Difco). Two ml of the emulsion was injected intramuscularly into a New Zealand white rabbit at two or three locations. Two weeks later, 1 ml of the purified virus suspension was inoculated intravenously, as a booster.

The trial bleedings were made two weeks after the booster inoculation, and antibody activities were determined by the ID test. When the antibody titer reached a desirable level, blood was collected by heart puncture. Serum was harvested and kept at−20°C until use.

Serological Test for AAAV

(1) Immuno-diffusion (ID) test: A 3% solution of Ion agar #2(Oxoid) was prepared in distilled water by boiling. After melting, 7 ml of stock agar was dispensed into a 20ml test tube stoppered and refrigerated.

In the preparation of agar gel plates, the stock agar was melted and to it was added an equal amount of barbital buffer(pH 8.2, μ0.005) containing 0.01% sodium azide. The resulting 14 ml of the agar solution was poured onto a $3^1/_4 \times 4''$ glass plate(Kodak). Wells were cut in the solidified agar using templates (Gelman or Colab) and the agar blocks were removed by using an aspirator. Capillary tubes (Sherwood) were used for filling the wells with reagents. Over or under filling was carefully avoided. In some studies, two additional fillings were made before the wells dried.

After filling with reagents, the agar plates were kept for one week in a moisture chamber at room temperature. When precipitation lines appeared, readings were made and recorded. The plates were frequently photographed using an illuminating device.

For future reference, the fully developed agar plate was immersed in saline for two days to remove all the unreacted reagents and then put in distilled water for another two days. After drying at 37°C, the plates were stained with Amido Black 10B (0.2% solution in mixture of 5 parts water; 4 parts methanol; and 1 part acetic acid) for 15 min and decolorized with 0.5% acetic acid solution until clear precipitin lines appeared.

When chicken sera were used, barbital buffer containing an additional 16% NaCl was added in preparing the agar.

(2) Preparation of AAAV Antigen for ID Test: Fractions having densities of 1.38 and 1.42gm/cm^3 were pooled. One tenth ml of rabbit anti-CELO immune serum was added to the one ml of virus suspension and allowed to react overnight at 4°C. After low speed centrifugation, the supernatant was collected and used as the AAAV antigen in the ID test. The optimum concentration of AAAV antigen was determined by testing against a constant amount of anti-AAAV rabbit immune serum.

(3) Complement Fixation Test: The test followed the procedure described by McCormick *et al.*[9] with slight modifications. Rabbit anti-AAAV immune serum was heated at 56°C for 30 minutes and absorbed with acetone dried normal AAF powder and sheep red blood cells. A checkerboard titration was carried out to determine the antibody unit with reference AAAV antigen. Four units of antibody was selected for the determination of AAAV antigen.

The complement fixation test was carried out using microtitration V plates(Cooke Engineering). The test system consisted of 2 unis of complement, 2 units of hemolysin and a 2.5% suspension of sheep red blood cells. Primary and secondary incubations were for 60 and 30 min at 37°C, respectively, with occasional shakings.

Antigen titers were recorded as reciprocals of the highest antigen dilution showing more than 50% fixation. Appropriate controls were included in each test.

Serological Tests for Avian Adenovirus: Rabbit

anti-CELO immune serum was used for the determination of avian adenovirus common group antigen[2] by the CF and the ID tests following the methods described above.

Hemagglutination (HA) activity of type 1 avian adenovirus[16] was determined using a rat red blood cell suspension in microtitration U plate as described by Anderson *et al.*[1] In short, two fold dilutions of QBV fractions were allowed to react with an equal amount of a 1% suspension of rat red blood cells at 37°C. PBS (pH 7.2) containing 1% normal rabbit serum, previously heated at 56°C for 30 min was used for dilution of the reagents.

Preparation of Avian Adenovirus Cultures: Nine avian adenoviruses used in this study were from departmental stocks maintained by Dr. V. J. Yates, University of Rhode Island, Kingston, R.I. These were Irish strain 75, 58, 340, 764, 695 and 506,[11] QBV,[12] CELO,[13] and Tipton strain.[5]

The avian adenovirusese were propagated in chicken embryo kidney (CEK) cell cultures as described by Yates *et al.*[14] When cytopathic effects (CPE) were apparent, the cells were frozen. After two or three cycles of freeze and thaw, infected cell culture fluids were pooled and concentrated about 10 times by dialysis against Carbowax (Union Carbide).

The presence of avian adenovirus common group antigen and AAAV antigen in concentrated cell culture fluids was determined by ID and CF tests.

Serological Specificity of Anti-AAAV and AAAV Antigen: Four different avian viruses and their antisera were used to determine the specificity of the AAAV antigen and its antiserum by the ID test.

The names and sources of the viruses are as follows:

Marek's disease virus antigen and antiserum, Dr. B. W. Calnek, New York Veterinary College, Ithaca, N.Y.; Infectious tenosynovitis virus antigen and antiserum, Dr. L. van den Heide, Univ. of Connecticut, Storrs, Conn; Infectious bursal disease virus antigen and antiserum, Cobb research farm, Milton, N.H.; and CELO virus antigen and antiserum prepared in this laboratory.

Electron Microscopic Studies: Fractions of QBV, obtained by isopycnic centrifugation, were dialysed against excess PBS (pH 7.2), stained with 1% phosphotungstic acid (pH 7.2)[7] and viewed using a transmission electron microcope (RCA-EMU-36).

Determination of AAAV Infectivity: Various ten fold dilutions of QBV fractions obtained by isopycnic centrifugations were used with "helper" CELO virus (10^5 PFU) to infect sets of four, 11-day-old chicken embryos via the chrioallantoic sac route. Infected AAF was harvested five days later, and the level of AAAV antigen was determined by the CF test. The mean AAAV CF antigen induction (CFI_{50}) titer was determined by the method described by Yates *et al.*[15]

Results

Serological, Biological and Morphological Characteristics of QBV Fractions Obtained by Isopycnic Centrifugation: After isopycnic centrifugation of QBV using CsCl, five opaque bands were observed as shown in Fig. 1.

The virus fractions collected from the bottom of

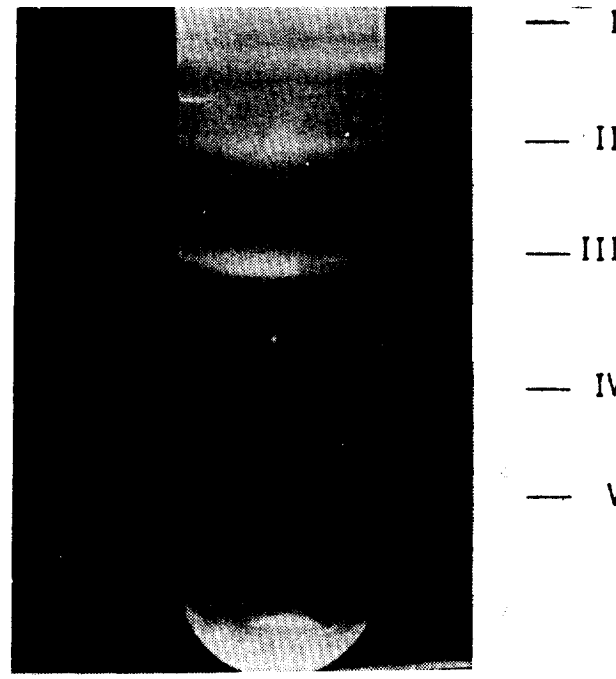

Fig. 1. Isopycnic centrifugation of QBV in CsCl. Following isopycnic centrifugation of QBV, 5 bands were visible. The densities of these bands were as follows: Band I, 1.29gm/cm^3; band II, 1.31gm/cm^3; band III, 1.34gm/cm^3, band IV, 1.38gm/cm^3 and band V, 1.42gm/cm^3.

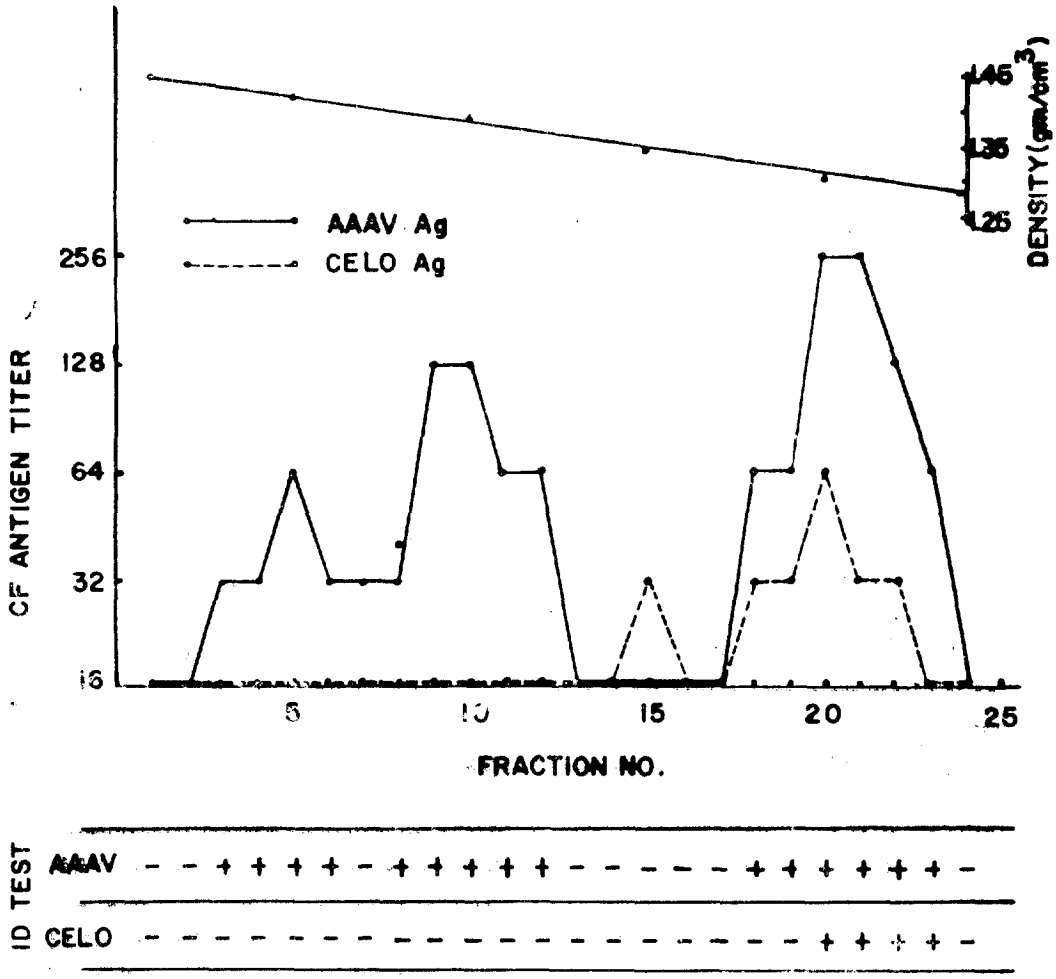

Fig. 2. Detection of avian adenovirus common group antigen (CELO) and AAAV antigen in QBV following isopycnic centrifugation. The antigens of avian adenovirus and avian adenovirus associated virus were detected by the complement fixation (CF) test and immuno-diffusion (ID) test. Numbers 1 to 24 represent different fractions collected from the bottom of the tube to the top.

the tube were assayed for AAAV and avian adenovirus common group antigen by the CF and ID tests. As shown in Fig. 2, AAAV CF antigen was detected in fractions of densities 1.42, 1.38 and 1.31gm/cm^3 AAAV antigen was also detected by ID test in fractions showing a positive CF reaction(Fig. 3). The ID test results correlated well to those of the CF test in specificity for detecting AAAV antigen.

Avian adenovirus group antigen appeared in fractions with densities of 1.34 and 1.31gm/cm^3 by the CF test. With ID test, faint precipitation lines were observed only in fractions with a density of 1.31 gm/cm^3 and lighter (Fig. 2, Fig. 3).

Fractions representing each visible band were obtained through the side of the tube. These were assayed for viral antigens and viral infectivity. They were also stained and observed using the electron microscope.

As shown in Table 1, AAAV antigen was detebted by CF test in all fractions. An immuno-diffusion reaction was also observed in all fractions except fraction III (1.34gm/cm^3). The precipitation lines were thicker in lighter fractions(1.31 and 1.29gm/cm^3) than in fractions of 1.42 and 1.38gm/cm^3. Avian adenoviral antigen appeared in lighter fractions (1.34, 1.31 and 1.29 gm/cm^3) by the CF test,

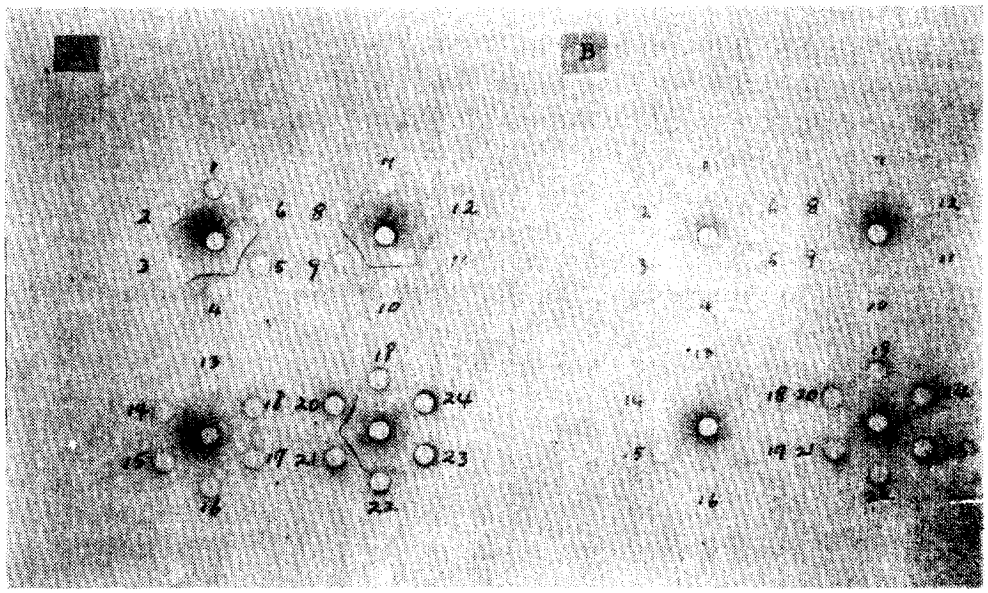

Fig. 3. Immuno-diffusion reaction of QBV following isopycnic centrifugation with anti-AAAV and anti-CELO immune sera. Numbers 1 to 24 represent the different fractions collected from the bottom of the tube to the top. Fig. 3A shows the distribution of AAAV antigen. Central well contains anti-AAAV immune serum. Fig. 3B shows the distribution of avian adenovirus common group antigen. Central well contains anti-CELO immune serum.

Table 1. Characterization of 5 Fractions of QBV Following Isopycnic Centrifugation

Fraction	Density[a] gm/cm³	HA[b]	CF Test[c]		ID Test[d]		Infectivity[e] CPU(log¹⁰)
			CELO	AAAV	CELO	AAAV	
I	1.29	16	64	64	+	##	4.0
II	1.31	—[f]	32	128	+	##	4.0
III	1.34	32	16	16	—	—	5.5
IV	1.38	—	—	64	—	#	9.0
V	1.42	—	—	32	—	+	9.0

a. In CsCl
b. Hemagglutination activity with rat RBC
c. CF antigen titer
d. In the immuno-diffusion test, the intensity of the precipitating line was graded as (+), (#), (##); (+), being the least intense line and (##), being the most intense line; (−) indicates no precipitating line.
e. Complment fixation antigen induction unit.
f. No reaction at 1:8 antigen dilution.

althoughfaint precipitationlines occurred in fractions with adensity of 1.31 and 1.29gm/cm³.

AAAV infectivity occurred mostly in the fractions with densities of 1.42 and 1.38gm/cm³, even though serological reactions were stronger in lighter fractions.

Electron micrographs showed virus particles with typical morphologies (Fig. 4). The heaviest fraction with a density of 1.42gm/cm³ consisted of only complete AAAV particles (Fig. 4-V). The less heavy fraction(1.38gm/cm³) was heterogenous with particles having complete and incomplete viral morphologies (Fig. 4-IV). The light fractions (1.31 and 1.29gm/cm³) contained both avian adenovirus

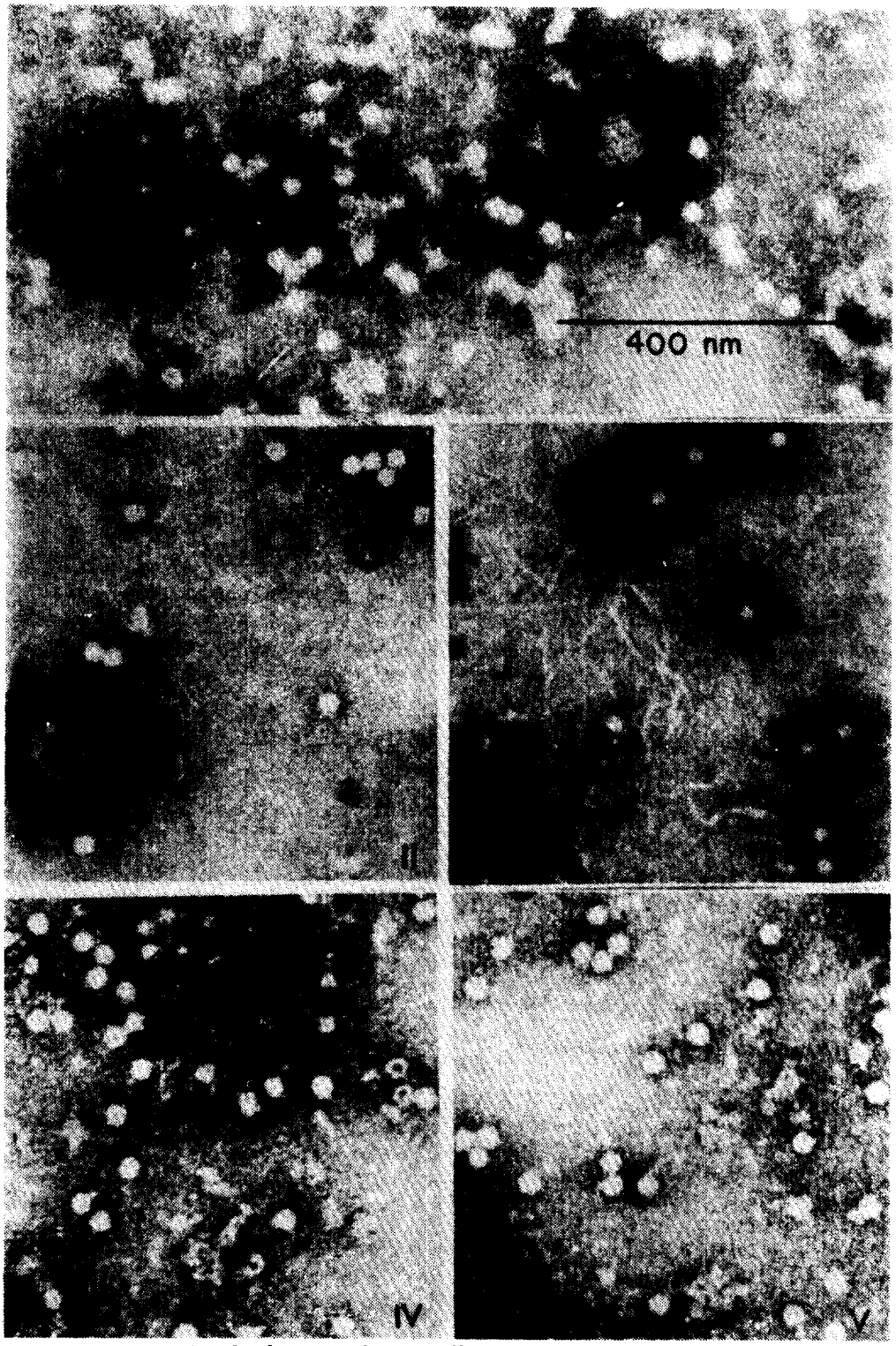

Fig. 4. Electron micrographs of 5 fractions of QBV Following isopycnic centrifugation. I represents the top visible band with a density of 1.29gm/cm^3, II, the second band with a density of 1.31gm/cm^3, III, the third band with a density of 1.34gm/cm^3, IV, the fourth band with a density of 1.38gm/cm^3 and V, the bottom band with a density of 1.42gm/cm^3.

and AAAV particles(Fig. 4-I, II). The fraction with a density of 1.34gm/cm^3 was particularly interesting, as avian adenovirus particles were not observed, but fiber-like structures were eminent (Fig. 4-III).

HA activity appeared in fractions with a density of 1.34 and 1.29gm/cm^3.

Specificity of Serological Reactions between AAAV and Anti-AAAV Rabbit Hyperimmune Serum: In previous studies, it was observed that anti-AAAV rabbit immune serum reacted specifically to AAAV antigen in fractions of QBV obtained by isopycnic centrifugation procedures. The specificity of the reaction was further studied by carrying out serological tests with known antigens and antisera most often used for the serological diagnosis of avian viral disease.

As presented in Fig. 5, anti-AAAV rabbit serum reacted only to AAAV antigen, and did not react with CELO, MD, IT and IBA antigens. Since AAAV antigen was isolated from QBV which was serologically identical to CELO virus, it was concluded that anti-AAAV was highly specific to AAAV.

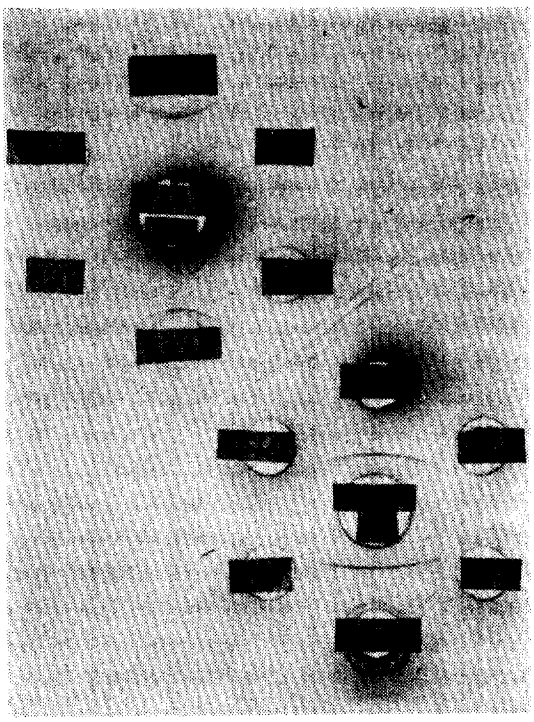

Fig. 5. Immuno-diffusion reaction of AAAV antigen and its antiserum against 4 avian viruses. The top central well contains anti-AAAV immune serum; the lower central well contains the purified AAAV antigen. The 4 avian viruses were Marek's disease virus (MDV); infectious tenosynovitis virus (ITV); infectious bursal agent (IBV) and chicken-embryo-lethal-orphan (CELO virus).

Purified AAAV antigen was also tested for purity with known antisera. As shown in Fig. 5, AAAV antigen only reacted with the homologous antiserum. No reaction occurred with other antisera.

Contamination of Avian Adenovirus Stocks with AAAV: As shown in Fig. 6, all the avian adenovirus shared acommon antigen. Of 9 avian adenoviruses, QBV, 75 and 58 reacted with anti-AAAV.

Contamination of avian adenoviruses with AAAV were also determined by the CF test. Adenoviruses, QBV, 75 and 58 contained AAAV antigen but no reaction was observed in CELO and Tipton virus (Table 2).

Table 2. Detection of AAAV in Avian Adenoviruses by CF test

Virus	AAAV Antigen Titer[f]
CELO[a]	—[g]
QBV[b]	16
75C[c]	16
58[d]	32
Tip[e]	—

a. CELO, chicken-embryo-lethal orphan virus
b. QBV, quail bronchitis virus
c. 75, Irish strain
d. 58, Irish strain
e. Tipton virus
f. CF antigen titer
g. No reaction at 1 : 4 antigen dilution

Discussion

In this study, attempts were made to isolate and purify AAAV from an adenovirus, QBV. This was accomplished by isopycnic centrifugation. The purity of the AAAV was demonstrated by electron microscopy and serological reaction.

In earlier studies by the authors, the isolation of a pure culture of AAAV was not possible by isopycnic centrifugation. The fraction with a density of 1.42 gm/m^3 inevitably contained some avian adenovirus antigen and had to be neutralized by its anti-serum. The reason for this is not clear. Similar observations were recently made by Mayer *et al.*[8]. They reported

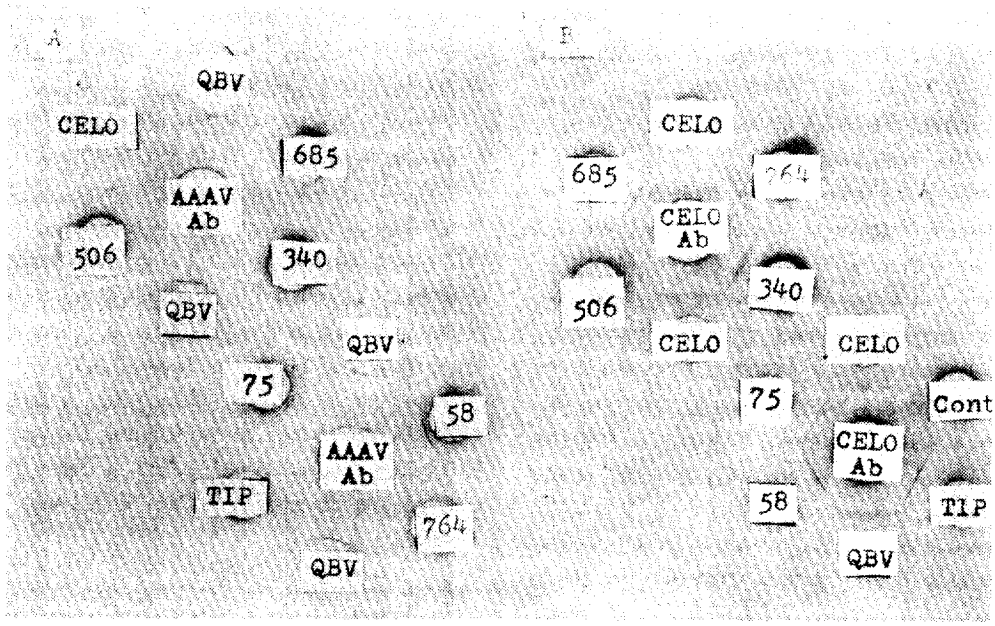

Fig. 6. Immuno-diffusion reaction of nine avian adenoviruses with Anti-AAAV and anti-CELO immune sera. Fig. 6A: The upper and lower central wells contain AAAV immune serum. Fig. 6B: The upper and lower central wells contain CELO immune serum. Nine avian adenoviruses were quail bronchitis virus (QBV); Chicken-embryo-lethal-orphan (CELO) virus, 506, 685, 340, 75 and 58, Irish strains; Tipton (Tip) virus. Uninoculatedtissue culture fluid was used as the control (cont).

that anti-adeno satellite virus rabbit immune serum reacted strongly with homologous virus as well as with the adenovirus used as "helper." But this serum did not react with other adenovirus serotypes which could afford good "helper" function for satellite virus. They suggested that the purified virus preparation contained some helper virus component adopted for the replication of the defective virus.

The contamination of AAAV in avian adenovirus stocks was demonstrated by the ID and CF tests. These results were in good agreement with those of El Mishad *et al.*[4], although different serological methods were used.

AAAV and avian adenoviral common group antigens were distributed widely in the fractions obtained by isopycnic centrifugation. AAAV infectivity mostly appeared in fractions of densities of 1.42 and 1.38gm/cm^3 (Table 1), but the stronger serological reactivities were observed in the lighter fractions.

This observation indicated that AAAV particles in QBV fractions were heterogenous. But they were equally reactive in serological tests, since the antigenic determinant is present on the viral coat proteins. Electron microscopic observation supported the above suggestion. The lighter fraction(1.38gm/cm^3) showed typical AAAV particles consisting of complete and incomplete virus, while the heavier fraction(1.42gm/cm^3) showed only complete virus(Fig. 4). From this observation, it is suggested that the fract ion at a density of 1.4gm/cm^3 could be used for AAAV stock. However, fractions of densities of 1.38 and 1.42gm/cm^3 were free from any adenovirus contamination and equally reactive in the serologi cal test regardless of the heterogenous properties. These two fractions, when pooled and treated with anti-CELO virus rabbit immune serum, could be used as AAAV antigen in the immuno-diffusion test.

Conclusion

The avian adenovirus associated virus (AAAV) was isolated from the avian adenovirus Quail bronchitis virus (QBV) by isopycnic centrifugation. Fractions having a density of 1.38 or 1.42gm/cm^{3}c

contained the purest AAAV. This was shown by electron microscopy and serological reactions.

The fraction of QBV with a density of 1.42gm/cm^3 was used for the production of anti-AAAV in rabbit. The purified AAAV antigen for serological tests was prepared by collecting fractions of QBV having densities of 1.38 and 1.42gm/cm^3 and by treating with anti-CELO rabbit immune serum.

AAAV and its antiserum showed no cross serological reactions with common viruses in chickens. These viruses were: Marek's disease virus, infectious bursal disease virus, infectious tenosynovitis virus and CELO virus, an adenovirus.

Among nine strains of avian adenoviruses, three showed cross serological reactions with anti-AAAV. These three avian adenoviruses were QBV, Irish strain 75 and 58.

It is evident that AAAV antigen and its antiserum is specific in reaction and can be applicable for the detection of AAAV antibodies and for the identification of AAAV.

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Avian Adenovirus Associated Virus (AAAV)의 특성에 관한 연구

Ⅰ. AAAV의 분리 및 동정

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抄　　錄

매추리 유래 가금 adenovirus인 QBV로부터 CsCl을 사용한 균질밀도분배원심분리법으로 AAAV의 분리를 시도하였으며, 분리된 바이러스의 순수성을 전자현미경 및 혈청학적인 방법을 통하여 증명하였으며 순수 분리된 AAAV를 항원으로 사용한 혈청반응을 개발하였다.

즉, CsCl의 밀도 1.38 및 1.42gm/cm³의 분획들은 순수한 AAAV이었으며 1.42gm/cm³의 분획은 토끼에서 양성혈청제조용 면역원으로 1.38 및 1.42gm/cm³의 분획은 혈청반응용 표준항원으로 사용하였다.

AAAV 표준항원 및 가토 양성혈청계는 MDV, IBDV, ITV 및 "helper"인 가금 adenovirus와의 교차반응이 없었으며 혈청학적인 특이성이 인정되었다. 또한 상기 혈청학적 반응을 이용하여 AAAV의 동정은 물론 AAAV에 대한 닭의 항체검출을 가능하게 하였다.