Comparison of Agar-Gel Precipitin Responses among Strains of Fowl Adenovirus Using Antigens Prepared from Chorioallantoic Membranes and Chicken Kidney Cell Cultures

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(Received 17 October 1994/Accepted 10 January 1995)

ABSTRACT. Agar-gel precipitin responses obtained for serologically different strains of fowl adenovirus (FAV) in tests using antigens prepared from FAV-infected chorioallantoic membranes (CAM antigen) and chicken kidney cell cultures (CKC antigen) were compared. Findings showed that both types of antigens exhibited less sensitivity to heterologous than to homologous antisera and that quantitative differences in sensitivity were present between serotypes. CAM antigens were more sensitive than CKC antigens to heterologous antisera. Polyvalent CAM antigens containing 2 or 3 antigens increased sensitivity in testing of field serum samples, resulting in a higher rate of detection.—**KEY WORDS:** agar-gel precipitation, fowl adenovirus, serotype.

J. Vet. Med. Sci. 57(2): 327–330, 1995

Fowl adenoviruses (FAVs) have been isolated world wide from many chickens with respiratory disease or inclusion body hepatitis and from clinically normal chickens [5–7]. They are sometimes present as a secondary pathogen in association with other viruses such as chicken anemia virus and infectious bursal disease virus [6, 7]. They have also been found to include many serotypes [1, 3, 4, 8].

The agar-gel precipitin (AGP) test has been used to detect antibody to FAV because it is simple, inexpensive and capable of detecting an antigen common to all FAVs relatively quickly. For the AGP test, antigens are prepared from chorioallantoic membrane of chicken embryonating eggs (CAM antigen) or from chicken kidney cell cultures (CKC antigen) infected with FAV [5].

In the present study, the AGP cross-reaction using CAM and CKC antigens was used to test laboratory and field serum samples with serologically heterologous FAV strains in order to determine whether each serotype possessed the group antigen to the same degree.

MATERIALS AND METHODS

Viruses: Representative FAV strains of Ote (serotype-1), SR48 (–2), SR49 (–3), KR5 (–4), TR22 (–5), CR119 (–6), YR36 (–7), TR59 (–8), and TR630 (–8) described by Kawamura *et al.* [4] and the strain S-PL1 (–2) isolated by Takase *et al.* [10] were used. The serotypes cited here were those proposed by McFerran and Adair [7].

CKC antigens: CKC cultures were prepared from 2- to 4-week-old specific-pathogen-free (SPF) White Leghorn chickens obtained from the Chemo-Sero-Therapeutic Research Institute, and incubated stationally in Roux bottles (10 × 13 cm) as previously described [9]. On the 4th day of incubation, CKC cultures were inoculated with 10^{6.0} TCID₅₀/bottle of each FAV strain and incubated with maintenance medium containing no bovine serum. The infected culture fluids were harvested at 48 to 72 hr

post-inoculation (PI) when the cytopathic effect (CPE) was observed in about 75% or more of the cultured cells. The fluids were after removal of cell debris concentrated to about 1:100 with 50% saturated ammonium sulfate as described [5] for use as AGP antigen.

CAM antigens: SPF embryonated eggs of 10 days old were each inoculated with 10^{6.5} TCID₅₀/egg of one of the FAV strains tested via the CAM and incubated. CAMs were harvested 1 to 5 days PI, homogenized without diluent, frozen and thawed twice, and centrifuged at 2,000 g for 15 min. The supernatant was used as AGP antigen.

Antisera and field sera: Ten SPF chickens of 3 weeks old were each orally inoculated with 10^{6.0} TCID₅₀/bird of one of the FAV strains, Ote, SR48, YR36, TR630 and S-PL1. They were re-inoculated 2 weeks after the first inoculation in the same manner, and were bled 2 weeks after the second inoculation to obtain antiserum. The 10 sera were used individually. Ten SPF chicken serum specimens obtained by bleeding at 150 days of age were also used. In addition, 320 samples of field serum which had been collected for serodiagnosis from a variety of poultry farms in 1993 at the authors' Institute were tested.

AGP test: The agar gel used in the test included 1.0% Bacto agar (Difco), 9.0% sodium chloride, 0.01% Na₃N, 1.15% Na₂HPO₄ and 0.2% KH₂PO₄. The melted gel was poured onto a glass slide and solidified. Wells 3 mm in diameter were cut at 3-mm intervals and contained 50 µl of each antigen or antiserum. The reaction was performed in a moisture box at 37°C overnight and then at 22°C for 2 days, and precipitin lines were observed daily macroscopically with reverse lighting.

Titers of antigen or antiserum: Antigen titer was determined by testing antigen against positive serum at serial double dilutions prepared with a physiologically buffered solution. Titer of antiserum was determined similarly against positive antigen which had been adjusted to contain 4 units of precipitin titer. These titers are expressed as reciprocals of the highest dilutions forming a

Table 1. Precipitin titers of FAV-infected CAMs at various times of harvest

Virus strain	Harvest time (days after inoculation)				
	1	2	3	4	5
Ote	— ^{a)}	2	4	4	2
SR48	—	8	8	4	4
YR36	8	16	16	8	8
TR630	2	2	4	2	2
S-PL1	—	8	16	8	8
PBS	—	—	—	—	—

a) Reciprocals of the highest dilution forming a recognizable precipitin line to homologous antiserum. "—" indicates negative with non-diluted antigen.

recognizable precipitin line.

RESULTS

CAM antigen harvest time: The best harvest time to produce antigen was determined using 5 strains of FAV inoculated onto CAM. FAV-infected CAMs displayed gross lesions characterized by necrosis and swelling from the one day PI. These lesions were most severe at 5 days PI. The antigen was detected from the one or two days PI to 5 days PI with titers of 1:2 to 1:16 (Table 1). Although these titers differed depending on FAV strain and harvest time, the highest titer for each strain was obtained at 2 or 3 days PI.

Sero-positivity numbers in experimentally infected birds: Testing was performed for AGP antibodies to CAM and CKC antigens in 10 birds orally infected with Ote, SR48, YR36 or S-PL1 strain, and 10 SPF serum samples (Table 2). The largest number of positive sera was obtained when the homologous antigen was used. When heterologous antigens were used, CAM antigens exhibited greater sensitivity than CKC antigens yielding a larger number of positive sera, as shown in "Total" in Table 2.

Cross-reaction in AGP test: Cross-reaction between homologous or heterologous antisera was investigated using CAM and CKC antigens. For this test, antisera homologously positive to each of the FAV strains with high antibody titer were selected beforehand from among immune sera (listed in Table 2) and adjusted to contain 4 units antibody titer before use.

The results showed that antibody titers varied depending on the antigen (Table 3). When CAM antigens were used, anti-Ote serum was negative to 6 heterologous antigens, but the other antisera reacted to all antigens. When CKC antigens were used, no reaction was observed by anti-Ote, -SR48 and -TR630 to totals of 9, 1 and 2 antigens, respectively. "Titer points" was thus higher for CAM antigens than for CKC antigens.

Rate of positivity for FAV in field serum: A total of 320 samples of field serum were tested on AGP with the 5 CAM antigens Ote, SR48, YR36, TR630 and S-PL1 (Table 4). The rates of positivity ranged by antigen from 42.2% (Ote antigen) to 46.5% (TR630 antigen). The

Table 2. Detection of antibodies to FAV strains in orally infected chickens on AGP test using homologous and heterologous antigens

Antigen	Ote	Chickens were infected with:				Total
		SR48	YR36	S-PL1	SPF	
CAM antigen						
Ote	8 ^{a)}	4	3	5	—	20
SR48	4	9	5	8	—	26
YR36	3	4	9	7	—	23
S-PL1	2	7	6	10	—	25
TR630	3	4	5	8	—	20
SR49	—	4	3	2	—	9
CR119	2	5	6	7	—	20
TR22	6	7	7	8	—	28
KR5	6	5	7	8	—	26
TR59	4	3	3	6	—	16
Total	38	52	54	69	—	213
CKC antigen						
Ote	7	1	2	1	—	11
SR48	2	6	3	8	—	19
YR36	2	4	10	3	—	19
S-PL1	2	2	2	10	—	16
TR630	2	1	5	5	—	13
SR49	—	2	4	4	—	10
CR119	3	3	5	5	—	16
TR22	2	2	2	5	—	11
KR5	—	2	2	4	—	8
TR59	3	3	4	5	—	15
Total	23	26	39	50	—	138

a) Number of sero-positive birds out of 10 orally inoculated. "—" indicates zero.

Table 3. Antibody titers for 5 representative antisera to homologous and heterologous antigens in AGP cross-reaction tests

Antigen	Ote	SR48	Antiserum		S-PL1	Titer points ^{a)}
		YR36	TR630			
CAM antigen						
Ote	4 ^{b)}	2	4	2	4	16
SR48	1	4	2	1	4	12
YR36	1	4	4	2	4	15
TR630	—	2	4	4	4	14
S-PL1	1	4	4	2	4	15
SR49	—	2	2	1	2	7
CR119	—	2	4	2	4	12
TR22	—	2	2	1	2	7
KR5	—	1	2	1	2	6
TR59	—	1	2	1	4	8
Titer points ^{a)}	7	24	30	13	34	112
CKC antigen						
Ote	4	1	2	1	1	9
SR48	—	4	4	1	4	13
YR36	—	1	4	1	1	7
TR630	—	1	4	4	4	13
S-PL1	—	4	4	—	4	12
SR49	—	1	4	1	4	10
CR119	—	1	2	1	4	8
TR22	—	1	4	—	2	7
KR5	—	—	2	1	1	4
TR59	—	1	4	2	4	11
Titer points ^{a)}	4	15	34	12	31	94

a) Total of titers in each column.

b) Reciprocals of the highest serum dilution forming a recognizable precipitin line: "—" indicates negative with original samples adjusted to contain 4 units antibody titer.

Table 4. Prevalence of antibodies in field serum samples on AGP test using 5 CAM antigens

Chicken flock	No. of serum samples tested	CAM antigen				
		Ote	SR48	YR36	TR630	S-PL1
Layer	108	49 ^{a)}	53	56	58	50
Broiler	182	81	86	83	86	90
Breeder	30	5	4	4	5	4
Total	320	135	143	143	149	144
(%)		(42.2)	(44.7)	(44.7)	(46.5)	(45.0)

a) Number of positive serum samples.

number of serum samples reacting with any one of 5 antigens was 175 (54.7%), including 5 (16.7%) breeders, 72 (66.7%) layers and 98 (53.8%) broilers. Of the 175 positive samples, 115 samples reacted to all 5 antigens, 15 to 4, 14 to 3, 9 to 2, and 22 to only one antigen.

The AGP antibody titers were determined for 14 field serum samples which were positive to all 5 antigens (Table 5). Many of these samples did not show the same titers to each FAV antigen used. In serum No. 9 and 13, there was four-fold difference between the lowest and highest titers.

The results of AGP reaction using mono-, bi-, and trivalent antigens with field sera are summarized (Table 6). The trivalent antigens reacted with larger number of sera than the mono- and bivalent antigens. The highest

Table 5. Antibody titers to 5 CAM antigens for 14 field serum samples

Serum No.	CAM antigen				
	Ote	SR48	YR36	TR630	S-PL1
1	1 ^{a)}	1	1	1	1
2	1	1	1	1	1
3	1	2	2	1	1
4	1	2	1	1	1
5	1	1	1	1	1
6	1	2	2	2	2
7	1	1	1	1	1
8	8	4	4	4	4
9	4	1	4	4	4
10	2	1	1	1	1
11	1	1	1	1	1
12	4	4	4	2	4
13	2	4	4	4	8
14	1	1	1	1	1

a) Reciprocals of the highest dilution forming a recognizable precipitin line.

Table 6. Prevalence of antibodies in field serum samples in AGP testing using mono-, bi- or trivalent CAM antigen

Monovalent		CAM antigen Bivalent		Trivalent	
TR ^{a)}	149(46.5) ^{b)}	SR/TR	165(51.5)	SR/TR/Ote	172(53.7)
SPL	144(45.0)	YR/TR	164(51.2)	YR/TR/SR	171(53.4)
SR	143(44.7)	SR/YR	160(50.0)	SR/SPL/TR	169(52.8)
YR	143(44.7)	SPL/TR	160(50.0)	Ote/YR/TR	168(52.5)
Ote	135(42.2)	Ote/YR	158(49.4)	Ote/YR/SR	165(51.5)
		Ote/TR	158(49.4)	Ote/TR/SPL	164(51.2)
		Ote/SR	157(49.0)	SPL/YR/TR	164(51.2)
		SR/SPL	154(48.1)	SR/SPL/Ote	161(50.3)
		Ote/SPL	153(47.8)	SR/SPL/YR	159(49.7)
		SPL/YR	152(47.5)	SPL/YR/Ote	158(49.3)

a) CAM antigens: TR=TR630, SPL=S-PL1, SR=SR48, YR=YR36, and Ote strains.

b) Number of positive (%) samples out of 320.

rates of positivity were 46.5%, 51.5% and 53.7% with monovalent (TR630), bivalent (SR48/TR630) and trivalent (SR48/TR630/Ote) antigens, respectively.

DISCUSSION

Although CAM [5] and CKC antigens [2, 4, 5] have commonly been used in the AGP test for FAV infection, production of CAM antigens is easier than that of CKC antigens, and requires neither long period for preparation nor special apparatus. This study showed that the optimal harvest time of CAM for use as antigen was 2 or 3 days PI, though this figure seemed to vary, probably depended on the strain and titer of FAV inoculated. Our study also suggests that CAM antigens exhibit greater sensitivity than CKC antigens to heterologous antisera, as shown in Tables 3, 4 and 5. The reason for this difference is not clear. However, use of CAM antigen appears to be preferable in the AGP test for FAV infection.

Kawamura *et al.* [4] demonstrated quantitative differences between eight serotypes in results of complement fixation tests with CKC antigens. However, similar findings have not yet been demonstrated for the AGP test. We demonstrated the existence of the quantitative differences among serotypes of FAV in the present study with CAM antigens. Recently, Cowen [2] also reported that some antigens failed to react with certain heterologous antisera in the AGP test, and demonstrated that the monovalent FAV antigen could not be used to detect much more precipitin to heterologous FAV strains, and that use of trivalent antigen markedly improved this insufficient detection.

In the present study, monovalent antigen of Ote strain, which has been used as the most representative strain of FAV by many workers in Japan, reacted with the least number of field sera despite strong reaction with its own antiserum. Similar findings were reported by Cowen [2], who described that the chicken embryo lethal orphan (CELO) virus, which is of the same serotype as the Ote strain, was the least sensitive of the three antigens tested. In 1964, when Kawamura *et al.* [4] isolated and classified 110 FAVs, Ote strain belonged to the serogroup then most frequently isolated. The distribution of serotypes of FAV in the field may recently have changed.

Given the findings of the present and previous studies [2, 4], polyvalent FAV antigens prepared from CAM appear preferable to monovalent antigens for use in AGP test because of their higher sensitivity in detection of antibodies in serum samples.

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