Plastic Multiwell Plates to Assay Avian Infectious Bronchitis Virus in Organ Cultures of Chicken Embryo Trachea

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Simple assay systems for infectivity titrations of avian infectious bronchitis virus (IBV) in chicken embryo trachea organ cultures (OC) were developed using plastic multiplate wells with one tracheal ring per well; these assays appeared to be much more satisfactory than the conventional rolled-tube method. The medium, 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered Eagle minimal essential medium was not changed during observation. A medium containing 0.4% bovine serum albumin did not influence the virus yield, but did stabilize virus viability during storage. Reproducibility of results obtained in the OC system was confirmed by performing replicate titrations of the Beaudette strain with three different passage histories. The mean virus titers in the OC were lower than those in chicken embryos, depending on the IBV passage histories. The time required for ciliostasis was related not only to the concentration of virus, but also to the IBV passage history. Application of OC techniques for the constant serum-variable virus neutralization test gave low neutralization indexes with excellent reproducibility as compared with those obtained in the chicken embryo assay system. Also, the slopes of neutralization curves obtained by assays in OC were less steep than those seen in the chicken embryo system.

Organ cultures (OC) of chick or chicken embryo trachea (CET) have been used for studies of avian infectious bronchitis virus (IBV) (5-9, 12, 15, 16).

OC can be used to quantitatively measure virus, and it gives reproducible and sensitive results (9, 15, 16). Infectivity titrations of virus in OC have been conducted mainly by the rolled-tube method (5, 6, 8, 9, 13).

We describe here the development of simple and reproducible IBV assay systems in OC which are similar to conventional cell culture assays in multiwell plates. This technique can also be used for the constant serum-variable virus neutralization test.

MATERIALS AND METHODS

Tracheal OC. Tracheal rings were prepared from specific-pathogen-free (SPF) chicken embryos according to established procedures (6, 13). Unless otherwise indicated, the medium consisted of autoclavable Eagle minimal essential medium (MEM) (Nissui Seiyaku Ltd., Osaka) supplemented with an additional 0.1% glucose (final concentration, 0.2%), 300 U of penicillin per ml, 300 μ g of streptomycin per ml, 5 μ g of amphotericin B per ml, and 0.05 M HEPES (*N*-2-hydroxyethyl-*N'*-2-ethanesulfonic acid) buffer (Flow Laboratories, Inc., Rockville, Md.). The pH was adjusted to 7.2 to 7.5 with 1 N NaOH. Preliminary experiments showed that increasing the HEPES concentration from 0.025 to 0.075 M had no effect on the maintenance of OC or

multiplication of IBV; therefore 0.05 M HEPES was routinely used. Groups of ten tracheal rings were placed in plastic dishes (15 by 60 mm; Falcon Plastics, Los Angeles, Calif.) containing 3 ml of the medium, and the dishes were placed in a humidified 37°C incubator in an atmosphere of 5% CO₂ in 95% air. On the following day, the rings were washed by pipetting to remove the cellular debris from the lumen of the rings and to prevent the outgrowth of dedifferentiated cells. The grade of ciliary activity, as described by Schiff and Shefner (19), was observed microscopically with directly transmitted light. Each tracheal ring with 100% ciliary activity and without any cellular debris in its lumen was placed into a well in a plastic multiwell plate (FB-16-24-TC; 24 wells/plate; Linbro Scientific Co., Inc., New Haven, Conn.), using a Komagome pipette. These OC were used for the experiments described without a change of medium. These were the standard culture conditions for the routine assay of IBV infectivity and for the neutralization test. To observe the effect of medium with bovine serum albumin (BSA, fraction V; Armour Pharmaceutical Co., Chicago, Ill.) in the experiment described in Fig. 2, the standard conditions were compared with a "preconditioned" agar method similar to that described by Stalheim and Gallagher (20). Rolled-tube OC was made from the same batches of tracheal rings according to a procedure described by Cherry and Taylor-Robinson (6). The tube cultures were rotated at 0.18 rpm at 37°C.

In an experiment aimed at obtaining a higher infectivity titer of OC-adapted virus, the standard and the preconditioned agar methods were used with plastic

petri dishes (10 by 35 mm), each containing 10 rings, with 1.5 ml of 0.05 M HEPES-buffered Eagle MEM with or without 0.4% BSA.

Virus. The Beaudette strain (IBV-42), supplied by H. Kawamura, Poultry Disease Laboratory, National Institute of Animal Health, Gifu, Japan, at the 69th passage level in chicken embryos (E_{69}), was passaged two or three times in SPF chicken embryos (E_{71} or E_{72}) in our laboratory. Cloning three times and subsequent adaptation of the strain were also done seven or eight times in SPF chicken embryo kidney (CEK) cell cultures (E_{71} CEK₁₀ or E_{71} CEK₁₁), followed by passage twice in SPF chicken embryos (E_{71} CEK₁₀ E_2) or 30 times in OC (E_{71} CEK₁₀CET₃₀). To avoid large aggregates of virus particles, all three viruses (E_{72} , E_{71} CEK₁₁, and E_{71} CEK₁₀CET₃₀) used in the comparative titrations and neutralization tests were filtered (22) through a 0.45- μ m filter membrane (Millipore Corp., Bedford, Mass.), and BSA was added (3) for the E_{71} CEK₁₁ and E_{11} CEK₁₀CET₃₀ viruses. Viruses were stored at -50°C in small volumes.

Virus titration. To titrate IBV in OC in multiwell plates, 0.1 ml of virus diluted in 10-fold steps in 0.05 M HEPES-buffered Eagle MEM was inoculated into each of four wells. After adsorption for 1 h at 25°C , the inoculum was removed by aspiration, and 1 ml of prewarmed fresh medium was added, based upon the results shown in Fig. 1. The plates were placed in a humidified 37°C incubator in a 5% CO_2 atmosphere. The infectivity titers of the viruses were based on daily microscopic observation of ciliary activity and deterioration of the ciliated epithelium (50% ciliostasis dose [CLD₅₀]) for 5 to 7 days postinoculation, or upon lethality of culture fluids taken after the last observation for 9- to 10-day chicken embryos (50% OC infective dose [OCID₅₀]).

For titration in chicken embryos (50% embryo lethal dose [ELD₅₀]), five embryos per dilution were inoculated as described elsewhere (11, 18). Fifty percent end points were calculated by the method of Behrens-Kärber.

Neutralization test. Standard neutralization tests were performed by the constant serum-variable virus method in chicken embryos and OC (16, 18). Antisera were prepared in 9-week-old SPF chickens by inoculation twice with E_{71} CEK₁₀ E_2 virus or once with E_{72} virus. These viruses had been inactivated with beta-propiolactone and adsorbed onto $\text{Al}(\text{OH})_3$ adjuvant. The sera from inoculated or uninoculated chickens were collected, pooled, and stored at -50°C until use. Serial 10-fold dilutions of virus or a 1:2 dilution of antiserum was prepared in 0.01 M HEPES-buffered M-20 (10) containing 0.1% BSA. The neutralization index (NI) was determined by subtracting the log₁₀ titer of the virus in the presence of antiserum from the log₁₀ titer of the virus in the 0.01 M HEPES-buffered M-20 (18).

Reference viruses were uncloned (E_{72}) and cloned (E_{71} CEK₁₁ and E_{71} CEK₁₀CET₃₀) in SPF CEK monolayers according to the recommendation of Cunningham (11).

RESULTS

Maintenance of OC. Various volumes of the medium were tried to determine the best one for

maintaining strong ciliary activity in the multiwell-plate OC without changing the medium (Fig. 1). A volume of 0.2 ml of medium was at a level just below that which covered the tissue, and ciliary activity could not be maintained when a smaller volume was used. Some rings in rolled tubes had ciliary activity for as long as those in the multiwell plates, but the rolled-tube cultures were generally less satisfactory than the plate cultures maintained with each ring with 1 ml of medium for at least 7 days. We found that the preconditioned agar method was suitable when the medium was supplemented with 0.4% BSA (Fig. 2). The standard OC in medium supplemented with BSA did not maintain the strong ciliary activity of the tracheal rings for long, due to outgrowth of dedifferentiated cells (fibroblast-like) from the base of the rings, which interfered with ciliary activity.

Histology. In tracheas maintained in standard plastic multiwell plates, the cellular mor-

FIG. 1. Effect of volume of the medium on ciliary activity of CET organ culture maintained in plastic multiwell plates and rolled tubes. Twenty-four rings were used for each experiment.

FIG. 2. Effect of culture conditions on ciliary activity of CET organ culture maintained in plastic multiwell plates. Twenty rings were used for each experiment.

phology of uninfected tissue showed evidence of some change from pseudostratified ciliated epithelium to simple cuboidal epithelium after 7 days of incubation, but otherwise the cells appeared normal. Tracheal tissues infected with IBV showed evidence of damage to the epithelial layer with pyknosis and vacuolization of nuclei, and almost all of the cells appeared to have lost their cilia, as reported previously (12).

Time required for ciliostasis. We determined the time required for ciliostasis as compared with that for embryo lethality by using diluted inocula of the Beaudette strain with three different passage histories. Complete ciliostasis was observed within 5 days even when $-0.2 \log \text{CLD}_{50}$ of E_{72} virus was inoculated into the plastic multiwell plates (Fig. 3). However, end-point determinations of $E_{71}\text{CEK}_{10}\text{CET}_{30}$ and $E_{71}\text{CEK}_{11}$ viruses required 2 more days of incubation. The end point of E_{72} virus determined at 5 days was higher for OCID_{50} than for CLD_{50} .

Reproducibility. Reproducibility of results

obtained in the titration systems was determined as CLD_{50} on different days with the three stock viruses, including the OC-passaged virus. In the multiwell-plate OC system, the inherent errors of E_{72} , $E_{71}\text{CEK}_{11}$, and $E_{71}\text{CEK}_{10}\text{CET}_{30}$ shown by the standard error of the mean CLD_{50} were ± 0.08 , ± 0.12 , and $\pm 0.09 \log_{10}$, respectively (Table 1). One standard deviation of 10 replicates was $\pm 0.27 \log_{10}$ for E_{72} , $\pm 0.39 \log_{10}$ for $E_{71}\text{CEK}_{11}$, and $\pm 0.29 \log_{10}$ for $E_{71}\text{CEK}_{10}\text{CET}_{30}$. The majority (26/30) of the mean titers of CLD_{50} in these tests were within 0.5 $\log_{10}$ deviation, the highest deviation being 0.75 $\log_{10}$. The test deviations were all small, and the OC test seems to be as reproducible as the one using chicken embryos. End points assayed in chicken embryos of E_{72} and $E_{71}\text{CEK}_{11}$ viruses could be recorded within 4 days after inoculation with the lowest dilution of virus, whereas embryos inoculated with $E_{11}\text{CEK}_{10}\text{CET}_{30}$ grown in OC showed low mortality during the first 4 days of observation (Fig. 4).

Neutralization test. In the constant serum-

FIG. 3. Effect of concentration of virus on time required for ciliostasis in plastic multiwell plates inoculated with the Beaudette strain of IBV of three different passage histories. Pooled results of 10 repetitive titrations are shown.

variable virus test with OC-passaged virus (E₇₁CEK₁₀CET₃₀), a high titer of virus infectivity was necessary to obtain a neutralization end point with high-titered antiserum, but virus production in the standard OC was much less than that in chicken embryos and cell cultures. We

tried to determine the optimal time for harvesting virus by defining the growth curve and examined whether BSA added to the culture medium would give higher virus titers, as reported by some investigators (19, 21). The peak titers of virus were attained with both culture methods

TABLE 1. Infectivity titrations of the Beaudette strain of IBV of three different passage histories^a

Virus	Passage history	Mean infectivity (log ₁₀) ± SE ^b		
		OC		Chicken embryo, ELD ₅₀ /0.1 ml
		CLD ₅₀ /0.1 ml	OCID ₅₀ /0.1 ml ^c	
Chicken embryo adapted	E ₇₂ ^{d-}	4.80 ± 0.08	4.88 ± 0.08	6.90 ± 0.12
Chicken embryo kidney cell culture-adapted	E ₇₁ CEK ₁₁ ^{e-}	3.40 ± 0.12	2.72 ± 0.15	5.17 ± 0.30
Chicken embryo trachea OC adapted	E ₇₁ CEK ₁₀ CET ₃₀ ^{e-}	3.70 ± 0.09	3.35 ± 0.06	4.32 ± 0.07

^a Results of 10 titrations.
^b SE, Standard error.
^c Determined by the evidence of specific death of chicken embryos inoculated with culture fluid from each well.
^d End point was determined at 5 days.
^e End point was determined at 7 days.

FIG. 4. Effect of concentration of virus on time required for lethality of chicken embryos inoculated with different passage levels of the Beaudette strain of IBV. Pooled results of 10 repetitive titrations are shown.

at 32 h after infection, just before ciliostasis began to be noticeable. The virus propagated by the preconditioned agar OC method in plastic petri dishes had about the same titer as that propagated by the standard OC method (Table 2). Virus grown with medium supplemented with BSA was more thermostable when stored at -50°C (Table 3).

The OC method for detecting IBV-neutralizing antibody was comparable with the classical method using chicken embryos as the assay system.

In tests with reference virus (E_{72}) and antisera

with low or high neutralizing antibody levels or normal serum (Table 4), replicate assays in OC and chicken embryo systems showed that the mean NIs of both antisera assayed in OC were lower than those assayed in chicken embryos, but the reproducibility of NIs assayed in the OC system was as good as that assayed in the embryo system. Compared with neutralizing antibody, nonantibody inhibitor of normal chicken serum was slightly more reactive with IBV in the OC than in the embryo assay. The slopes of neutralization curves determined for $\text{E}_{71}\text{CEK}_{11}$, $\text{E}_{71}\text{CET}_{30}$, and E_{72} viruses in the chicken embryo

TABLE 2. Effect of BSA on multiplication of OC-adapted virus 32 h postinfection

Culture method	Supplement of 0.4% BSA	Type of culture	Expt no.	Ciliostasis (%) at 32 h postinfection	Virus titer ($\log_{10}$)	
					ELD ₅₀ /0.1 ml	CLD ₅₀ /0.1 ml
Standard	Yes	Trachea ^a	1	0	3.3	2.0
			2	15	4.7	3.6
		Medium	1	0	4.5	3.1
	No	Trachea ^b	1	0	3.5	1.6
			2	0	5.0	3.8
		Medium	1	0	4.6	3.2
Preconditioned agar	Yes	Trachea ^a	1	0	4.0	2.3
			2	5	4.7	3.3
		Medium	1	0	4.7	2.9
	No	Trachea ^b	1	0	3.3	1.6
			2	15	4.8	3.6
		Medium	1	0	4.5	3.0

^a Fifty percent emulsion was made with 0.05 M HEPES-buffered Eagle MEM + 0.4% BSA.

^b Fifty percent emulsion was made with 0.05 M HEPES-buffered Eagle MEM.

TABLE 3. Effect of BSA on thermostability of CET-passaged Beaudette strain IBV stored frozen at -50°C

Virus	Supplement of BSA	Before freezing	Titer							
			After freezing (month)							
			0	1	2	3	4	5	6	7
$\text{E}_{71}\text{CEK}_{10}\text{CET}_{14}$	No	2.0 ^a	1.4	1.2	1.0	0.6	0.4	ND ^b	ND	<0.1
$\text{E}_{71}\text{CEK}_{10}\text{CET}_{30}$	Yes	3.0	2.9	3.3	ND	ND	ND	3.3	ND	ND

^a Expressed as $-\log_{10}$ CLD₅₀/0.1 ml.

^b ND, Not done.

TABLE 4. Reproducibility of neutralization tests in OC and chicken embryos

Assay system	Reference virus	Serum	NI								Mean $\pm$ SE ^a
			1 ^b	2	3	4	5	6	7	8	
OC	E_{72}	Anti- E_{72}	0.7 ^a	1.2	1.0	1.0	1.3				1.04 $\pm$ 0.10
	E_{72}	Anti- $\text{E}_{71}\text{CEK}_{10}\text{E}_2$	3.0	2.7	2.7	2.7	2.7	2.7	2.5	2.7	2.71 $\pm$ 0.05
	E_{72}	Normal	0.5	0.7	0.7	0.9	1.2	0.5	0.5	0.7	0.71 $\pm$ 0.09
Chicken embryo	E_{72}	Anti- E_{72}	2.7	2.2	2.7	2.9	2.0				2.50 $\pm$ 0.17
	E_{72}	Anti- $\text{E}_{71}\text{CEK}_{10}\text{E}_2$	4.1	4.1	4.5	4.1	4.7	4.7	4.5	4.1	4.35 $\pm$ 0.09
	E_{72}	Normal	0	0.4	0.5	0.3	0.7	0.7	0.5	0.5	0.45 $\pm$ 0.08

^a SE, Standard error.

^b Experiment number.

system were about -1.86 , -1.27 , and -1.27 , respectively, whereas those determined in the OC system were -1.80 , -0.89 , and -0.63 , respectively (Fig. 5).

DISCUSSION

In maintaining OC, our rolled-tube techniques could not maintain complete ciliary activity as long as has been reported by some other investigators (6), perhaps because there was no change of medium (6) or BSA supplement (2). However, with the plastic multiwell plates, complete ciliary activity of OC could be maintained for at least 7 days postincubation with 0.05 M HEPES-buffered Eagle MEM (Fig. 1 and 2).

Rotating the cultures without a change of medium seemed to accelerate their aging and thus might reduce virus recovery and increase damage to ciliated epithelium. However, when the medium is changed frequently and is unlikely to become markedly hypertonic, it still seems to be harmful to cell metabolism (21).

BSA added to the medium has been reported to give high titers of influenza B (21) and Sendai viruses (19). However, its addition did not increase the IBV titers, although its presence stabilized virus viability and might possibly prevent aggregation of virus particles after storage for 5 months at -50°C . Results in Table 2 clearly show that the preconditioned agar method with addition of BSA to the medium did not influence

the state of virus-producing cells, but did improve prolonged ciliary activity of the CET epithelium as compared with the standard method with BSA (Fig. 2).

Determination of infectivity end points based on the appearance of complete ciliostasis after infection was influenced by the passage history of IBV and the quantity of virus inoculated. Beaudette strains of unknown passage history reportedly required 5 (6), 13 (7), or 8 to 14 days (16); a strain with 450 passages in eggs showed an end point within 3 to 4 days of observation (5). These discrepancies may be related to different passage levels of the strain. We observed complete ciliostasis with E_{72} virus within 5 days, whereas that with $E_{71}\text{CEK}_{10}\text{CET}_{30}$ and $E_{72}\text{CEK}_{11}$ required 7 days when the minimum quantity of these viruses, estimated by CLD_{50} , was inoculated (Fig. 3). In addition, the spread of ciliostasis was directly related to the quantity of virus inoculated into the culture.

When the assay was performed in chicken embryos, embryo-passaged (E_{72}) and CEK-passaged ($E_{71}\text{CEK}_{11}$), virus titers were 2.1 and 1.8 $\log_{10}$ higher than those assayed in OC (Table 1); these findings agree with those of earlier studies (5, 8). OC-passaged ($E_{71}\text{CEK}_{10}\text{CET}_{30}$) virus showed a diminished capacity to kill chicken embryos during 3 days of incubation, whereas embryo- and CEK-passaged viruses killed almost all embryos (Fig. 4), and $E_{71}\text{CEK}_{10}\text{CET}_{30}$ virus titers assayed in chicken embryos were

FIG. 5. Slopes of neutralization with chicken anti- $E_{71}\text{CEK}_9E_2$ virus serum assayed with the Beaudette strain of virus with different passage histories in OC (-----) and chicken embryos (—).

only 0.6 log₁₀ higher than those assayed in OC (CLD₅₀). These findings partially agree with the observations of Johnson and Newman (16), who found that IBV passaged serially in either chicken embryos or OC had higher titers when assayed in the homologous system.

Generally, OC might be a better assay system, especially for IBV not adapted to embryos, as reported by other investigators (8, 9, 15). Our results from the OC titrations with IBV adapted to embryos and other hosts were as reproducible as those from titrations in chicken embryos. Although disadvantages of plastic-plate methods have been pointed out by Cherry and Taylor-Robinson (6), our procedure for OC infectivity titrations in the multiwell plate was less laborious and time consuming than the rolled-tube method. Thus, the multiwell-plate technique has advantages, especially for large-scale OC neutralization of IBV, due to its simplicity, rapidity, and uniform sensitivity.

The most critical factors for the neutralization test are the indicator system and the reference virus. The chicken embryo is the most sensitive indicator (11, 17), but for maximum accuracy, quantitative scoring of infected embryos may require narrower dilution increments and more embryos within the critical range of virus infectivity (1).

The embryo-adapted Beaudette strain employed in this study has been used extensively as a reference virus, although its original antigenic and immunogenic properties have been greatly modified due to numerous passages in chicken embryo (11). Our results with the OC method for detecting IBV-neutralizing antibody were comparable to those of Lukert (17), who showed that the slope of neutralization with plaque reduction was not steeper than that with the embryo method. The OC method was less sensitive than methods using chicken embryos, and the slopes of the neutralization curves, -1.86 to -1.27 in chicken embryos and -1.80 to -0.63 in OC, differed for viruses with different passage histories (Fig. 5). Bülow reported that the slope of the neutralization curve obtained with chicken embryos was about -2 (4). The slope was influenced by host susceptibility, the route of inoculation, and the amount of virus used (14). The most susceptible host showed the steepest slope, and the less susceptible ones gave more gradual slopes (14). NIs may not be affected by the initial dilution of serum when assayed by the OC method, in accord with the finding of Lukert (17) that a virus-serum mixture may have a more stable union in cell culture system than in chicken embryos where greater dilution (1:100) of the mixture results in more dissociation of the virus-antibody complex.

Evaluation of the OC technique, for virus isolation from chickens experimentally infected with the OC-adapted Beaudette strain, which is noninfectious to embryo, is in progress. Cook et al. (9) reported that the OC method does not require extensive adaptation of the field virus in order to obtain clear signs of infection.

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