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The Use of Chicken Tracheal Organ Cultures for the Isolation and Assay of Avian Infectious Bronchitis Virus

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With 1 Figure

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Summary

A study has been made of the use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis (AIB) virus from both naturally and experimentally infected chickens. Six strains of AIB virus were investigated, 3 of which had been isolated from natural outbreaks of disease. Two of the virus isolations from the outbreaks of AIB were made directly into tracheal organ cultures without passage in embryonated eggs.

Organ cultures prepared from 20-day-old embryos were used since they were found to be somewhat more sensitive in virus assay than those derived from chickens of up to 31 days of age. Ciliostasis, which was used as the marker of infectivity, was complete by 3 days after inoculation with each strain of virus examined.

Virus could be isolated from both respiratory and non-respiratory tissue in tracheal organ cultures and these cultures were found to be at least as sensitive as 9-day-old embryonated eggs in detecting AIB virus either in pathological material or in serial dilutions. When virus was assayed in both systems, the titres were very similar. It is considered, therefore, that chicken embryo tracheal organ cultures offer a reliable alternative system to embryonated eggs for studying AIB virus.

Introduction

The development of techniques for the maintenance of organ cultures of ciliated epithelium has led to their widespread use for the isolation and growth studies of various respiratory tract viral pathogens (11). TYRRELL and BYNOE (17) found that organ cultures of human foetal trachea were more efficient than conventional monolayer cultures for the primary isolation of certain viruses associated with respiratory disease in man. Furthermore, MCINTOSH *et al.* (15) were able to isolate coronaviruses of human origin from nasopharyngeal washings in organ

cultures of human embryo trachea, although such viruses could not be demonstrated when conventional tissue culture techniques were employed.

The method at present used for the isolation of avian infectious bronchitis (AIB) virus from infected chickens is by serial passage in embryonated chicken eggs. Several passages are usually required before characteristic signs of infection can be observed in the embryo and this could accordingly be an undesirable feature since such repeated passages may well alter the virulence or antigenic character of the virus.

Up to the present time, attempts to isolate AIB virus from pathological material directly in conventional monolayer tissue cultures of various types have proved unsuccessful.

Chicken tracheal organ cultures have been shown to be susceptible to infection with AIB virus and may be employed as an accurate assay system if ciliostasis is used as the criterion of infection (12, 4, 13, 2, 7). Furthermore, JOHNSON and NEWMAN (13) found that tracheal explants provided a better assay system than embryonated chicken eggs because the results were more reproducible.

Chicken tracheal organ cultures do not appear to have been used for making primary isolations of AIB virus from pathological material although GEILHAUSEN *et al.* (9) isolated AIB virus from tracheal organ cultures prepared from experimentally infected chickens. In the present paper, the results are presented of a study of the use of tracheal explants from chick embryos or young chickens for the isolation and subsequent study of the replication of both field and laboratory strains of AIB virus.

Materials and Methods

Viruses

The Massachusetts (Strain IBV-41) and Connecticut (Strain IBV-46) serotypes (embryo adapted), were supplied by Dr. C. H. Cunningham, Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan, U.S.A. The H120 Noblis vaccine strain (Massachusetts type) was obtained from Intervet Laboratories Ltd.[1], and used as the 121st embryo passage material. The VF70/888 strain was supplied by Dr. J. B. McFerran, Ministry of Agriculture, Veterinary Research Laboratories, Stormont, Belfast, Northern Ireland and used after 9 passages in embryonated eggs. Two strains (HV-2, HV-10) had been isolated in this laboratory from field outbreaks of respiratory disease in chickens and had not been passaged in embryonated eggs.

Stocks of embryo-propagated virus were prepared by the inoculation of AIB virus into the allantoic cavity of 9-day-old specific pathogen free (SPF) embryonated eggs. The allantoic fluids were collected 48 to 72 hours later, pooled and stored at —20° C. Organ culture-propagated virus was prepared by harvesting the fluid medium from chicken tracheal organ cultures 72 hours after inoculation with virus and stored at —60° C after being first diluted 1:2 (v/v) with 0.2 per cent gelatin in phosphate buffered saline (PBS), pH = 7.2, containing 4.5 per cent glucose.

Preparation of Tracheal Organ Cultures

Tracheal explants were prepared after the method of CHERRY and TAYLOR-ROBINSON (3) from 20-day-old chicken embryos or from chicks of 1, 10, 17, 24 or

[1] Intervet, Bar Hill, Cambridgeshire.

31 days of age obtained from a flock SPF for a selection of viruses including AIB and for Mycoplasma spp. Approximately 17 rings were made from each embryo trachea and up to 60 rings from each chicken trachea. The rings were placed individually in 16 × 125 mm tubes and 1 ml Eagle's minimum essential medium (MEM) added, containing penicillin (50 units/ml) and streptomycin (50 μg/ml), and were rolled (8 revolutions/hour) at 37° C throughout (10). The explants were always used within 5 days of preparation and each was checked microscopically for cilial vigour prior to inoculation.

Passage of Virus in Organ Culture

Groups of 5 embryo tracheal explants were drained, 0.1 ml of virus inoculum added and adsorption allowed to proceed for 1 hour at 37° C with the cultures incubated stationary. The inoculum was removed and the explants were then either washed 3 times in PBS or not washed, as detailed under individual experiments. Maintenance medium (0.5 ml Eagle's MEM) was added and rolling continued at 37° C. Cultures were examined microscopically daily thereafter for evidence of complete ciliostasis and passaged after 72 hours, 3 passages being given before a specimen was considered to be free of virus. Fluids harvested from organ cultures were buffered as above and stored at —60° C.

Assay of Virus in Organ Culture

Serial 10-fold dilutions of virus were preapred in Eagle's MEM, and 0.1 ml of each dilution was inoculated into each of 5 drained embryo tracheal explants and virus allowed to adsorb, after which the explants were overlayed with maintenance medium. Cultures were examined microscopically for up to 7 days for evidence of complete ciliostasis to determine the end points. These were calculated according to the method of Reed and Muench (16) and expressed as the fifty per cent ciliostatic dose (CD_{50}) per ml.

Preparation and Use of Chick Kidney Cultures

Primary cultures of chick kidney (CK) were prepraed as monolayers in tubes from 2 to 5 week-old chicks by the method of Dulbecco and Vogt (8) as modified by Youngner (18). The growth medium was Eagle's MEM containing 10 per cent tryptose phosphate broth, 5 per cent unheated calf serum and antibiotics.

Prior to inoculation the tubes were washed 3 times with PBS and the drained sheets were each inoculated with 0.1 ml of virus. After adsorption for 1 hour at 37° C, 0.5 ml of maintenance medium, consisting of a Hanks'-Earle's balanced salt solution (1:1) supplemented with 0.5 per cent lactalbumin hydrolysate and antibiotics, was added. The cultures were examined for cytopathic effect (CPE) for up to 3 days before being harvested and passaged, up to 4 passages being given before a specimen was considered to be negative.

Isolation and Assay of Virus in Embryonated Chicken Eggs

In attempts to isolate AIB virus from chicken specimens, groups of eight 9-day-old SPF embryonated eggs were inoculated (0.1 ml) via the allantoic cavity. Allantoic fluid from half the surviving embryos was harvested after 48 to 72 hours for further passage, the remaining embryos being incubated at 37.5° C for 7 days when mortality and embryo stunting, characteristic of AIB virus infection, were recorded. Up to 3 serial passages were made before a sample was considered to be negative.

Titrations of AIB virus in 9-day-old embryonated eggs were performed according to the standard procedure (1); end-points were calculated by the method of Reed and Muench (16) and expressed as the fifty per cent egg infectious dose (EID_{50}) per ml.

Neutralization Tests

Neutralization tests to identify virus isolations as being AIB virus were performed by the standard procedure (1) in either 9-day-old embryonated eggs or in embryo tracheal organ cultures. Specific antisera were produced by the intratracheal inoculation (0.2 ml) of SPF chickens kept in isolation and were used at a dilution of 1:10.

Passage of Virus in Chickens

Groups of SPF chickens, kept in isolation, were inoculated (0.1 ml) intratracheally with AIB virus. Three days later the chickens were killed and the lungs and trachea removed aseptically from each, pooled, and a suspension (1:10 w/v) prepared in nutrient broth. This was subjected to low speed centrifugation and the supernatant fluid was used to inoculate further chickens, embryonated eggs, CK cultures or embryo tracheal organ cultures. Specimens were stored at —20° C until required.

Other types of tissue were collected from chickens at various intervals after infection with the H120 strain and prepared for examination in a similar manner.

Results

Comparison of Tracheal Organ Cultures Prepared from Chickens of Different Ages for Susceptibility to Virus Infection

The H120 and IBV-46 strains of AIB virus propagated by embryo passage and the HV-10 organ culture propagated strain were assayed simultaneously in embryo tracheal organ cultures and in tracheal explants prepared from chickens ranging in age from one to 31 days. The results are shown in Table 1 and represent the mean titres ($\log_{10}$) of at least 3 replicate titrations in cultures from each age of chicken. In the case of each strain of virus, organ cultures prepared from the 20-day-old embryos were as suceptible in most instances as were those prepared from chickens of different ages.

Table 1. *Comparison of tracheal organ cultures prepared from chickens of different ages for virus assay*

	Mean titre ($\log_{10}$ CD_{50}/ml) in tracheal cultures from					
	20-day	Chicken (age in days)				
Virus strain	embryo	1	10	17	24	31
H120	5.8	5.4	5.0	4.9	5.2	5.5
IBV-46	6.8	5.9	5.8	6.3	6.9	6.3
HV-10	4.3	4.2	3.6	NT	3.5	4.3

NT Not tested

Rate of Onset of Ciliostasis Following Virus Inoculation

Comparisons were made of the rates at which 3 strains (H120, IBV-46 and HV-10) of AIB virus caused ciliostasis. Five tubes were inoculated with 50 CD_{50} of a virus strain and the decrease in ciliary motility over the whole circumference of each ring was estimated at short intervals. The results of at least 3 sets of observations, done without prior knowledge of the inocula, show that with the H120 and HV-10 strains the rates of onset of ciliostasis were very similar (see Fig. 1), but this rate was much more rapid initially with the IBV-46 strain. In the case of all 3 strains, 80 per cent of cilia became static within 50 hours after inoculation; but, with strains IBV-46 and HV-10, ciliostasis was not complete until approximately 70 hours after inoculation. In the latter instances, a few tufts of cilia continued to beat after the majority had become static.

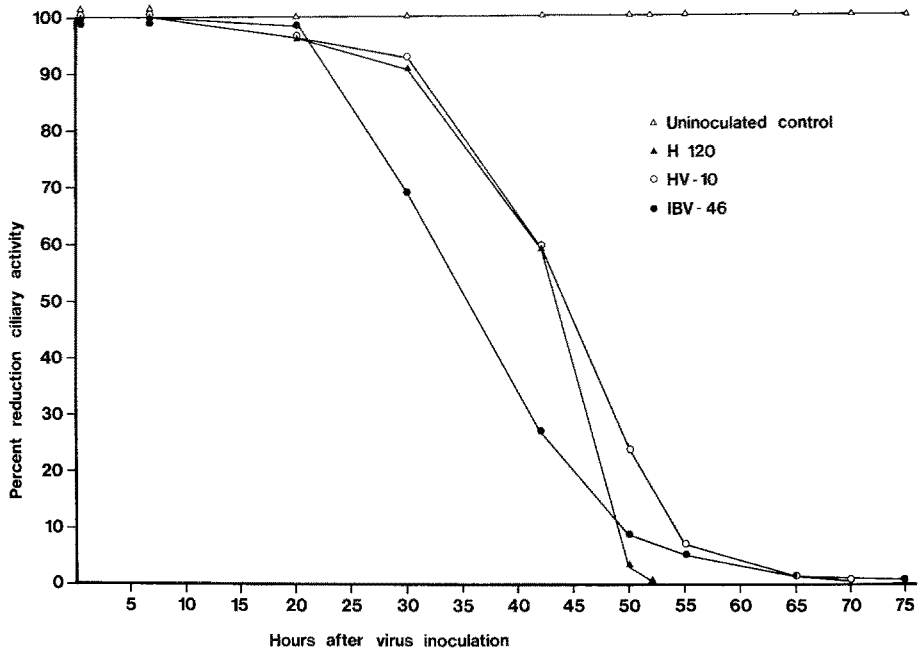

Fig. 1. Ciliary activity of chicken embryo tracheal organ cultures inoculated with different strains of virus

Isolation of Virus from Outbreaks of Respiratory Disease

Lungs and tracheae collected from chickens involved in 2 natural outbreaks of respiratory disease were used to prepare suspensions which were then passaged in embryonated chicken eggs, tracheal organ cultures and CK cultures to compare the efficiency of the 3 systems for virus isolation. Both viruses, designated HV-2 and HV-10 respectively, were isolated in tracheal explants on the first passage on the basis of ciliostasis; one strain (HV-2) was also isolated in embryonated eggs after one passage, while the other (HV-10) required 3 embryo passages. Neither strain caused a CPE in CK cultures after 4 passages. Neutralization tests confirmed that both viruses were AIB virus.

Recovery of Virus from the Respiratory Tract of Experimentally Inoculated Chickens

Four strains of AIB virus (HV-2, HV-10, VF 70/888 and IBV-41) were used; strain HV-2 after 5 organ culture passages, HV-10 as first organ culture passage fluid, VF 70/888 after 9 passages in embryonated eggs and IBV-41 as high embryo passaged fluid. In an attempt to readapt the virus, each strain was passaged serially 4 times in chickens and, after the final passage, reisolation of virus was then attempted in each of the 3 systems. As a control, uninfected allantoic fluid received 4 similar serial passages in chickens and virus reisolation attempts were made.

The results, presented in Table 2, show that all 4 strains of virus were recovered in organ cultures on the first passage and at least 3 of them in embryonated eggs

after one passage. The HV-10 strain, in effect, required 3 embryo passages before being reisolated definitively since only inconclusive embryo stunting occurred on earlier passages. No virus was recovered from any specimen after 4 passages in CK culture. The control specimen yielded no virus throughout.

To demonstrate that replication of each virus strain had occurred in the organ cultures, 4 serial passages were made. At each passage the inoculum was diluted 1:10 in Eagle's MEM, allowed to adsorb for 1 hour, then removed and the cultures washed 3 times with PBS. A comparison of the virus infectivity assays of the harvests from the 4th organ culture passages with those of the original lung homogenates show that, in each instance, there was no significant difference in titre over the 4 passages (Table 3).

Table 2. *Comparison of tracheal organ cultures and embryonated eggs for virus recovery from the respiratory tracts of experimentally inoculated chickens*

Passage number	Serial passage of virus strains in embryonated eggs (E) or tracheal organ cultures (OC)									
in E or OC	IBV-41		VF 70/888		HV-2		HV-10		Control	
	E	OC	E	OC	E	OC	E	OC	E	OC
1	5[a]	4	4	5	5	3	NT[b]	4	0	0
2	5	5	5	5	5	4	3	4	0	0
3	5	5	NT	4	NT	5	5	4	0	0
4	NT	5	NT	5	NT	5	NT	5	0	0

[a] Number affected / 5 inoculated
[b] Not tested

Table 3. *Virus replication on passage in tracheal organ cultures*

Virus strain	Titre ($\log_{10}$ CD_{50}/ml) in		Result of 4th organ culture passage inoculated into chickens	
	Lung homogenate	4th organ culture passage fluid	Respiratory signs[a]	Antibody production
IBV-41	3.3	3.0	+	7/8[b]
VF 70/888	3.3	3.2	+	4/8
HV-2	4.2	3.5	+	6/7
HV-10	4.2	4.1	+	5/7

[a] 3 days after inoculation
[b] Number of sera positive / Number examined

When the harvest of the 4th organ culture passage of each strain was inoculated into groups of 7—8 chickens, respiratory signs were observed within 3 days. Sera collected from each group 21 days after inoculation were examined for antibodies to the homologous strain by neutralization tests in 9-day-old embryonated eggs. In the case of each strain, the majority of chickens had produced significant levels of antibody (Table 3).

The fluid harvested from each organ culture passage was inoculated as a lateral passage into embryonated eggs and CK cultures. Embryo mortality, sometimes

accompanied by characteristic embryo deformities, was found after only one such passage with 3 of the strains, but the 2nd and 3rd organ culture passages of the remaining strain (HV-10) required 2 or 3 embryo passages respectively before virus could be recovered. One strain (VF 70/888) was given 16 serial passages in tracheal organ cultures at limiting dilution without loss of titre or lethality for embryos; this strain failed to produce a CPE in CK cultures at any passage level.

No CPE was observed when the organ culture harvests of each of the strains were given 3 lateral passages in CK cultures.

Recovery of Virus from Tissues of Experimentally Inoculated Chickens

Attempts were made to recover the H120 strain of AIB virus from lung, spleen, kidney, ovary and caecal tonsil of each of at least 4 groups of experimentally inoculated chickens in both embryonated eggs and tracheal organ cultures. Virus was recovered from samples of all the tissues on either the first or second passage with equal facility in the two systems and confirmed as AIB virus by neutralization tests in tracheal organ cultures.

Comparison of Tracheal Organ Cultures and Embryonated Eggs for Susceptibility to Virus Infection

To compare the susceptibility of embryonated eggs and tracheal organ cultures for the detection of AIB virus, attempts were made to recover the virus in both systems from a series of seven 5-fold dilutions of each of the 4 strains of virus which had previously been subjected to 4 serial passages in chickens. Reisolations were attempted in duplicate and the results are summarized in Table 4.

In the case of the egg-adapted strain (IBV-41), virus recovery was comparable in both systems. With the low embryo-passaged strain (VF 70/888) as well as one field strain (HV-2), the tracheal organ cultures were more sensitive than embryonated eggs. In the case of the remaining field strain (HV-10), the tracheal organ cultures were markedly superior in demonstrating the presence of virus.

Table 4. *Comparison of tracheal organ cultures and embryonated eggs for virus recovery from serial dilutions*

Virus strain	Exp. No.	Number of passages required to detect virus in serial dilutions (log_{10}) 0.7 E	0.7 OC	1.4 E	1.4 OC	2.1 E	2.1 OC	2.8 E	2.8 OC	3.5 E	3.5 OC	4.2 E	4.2 OC	4.9 E	4.9 OC
IBV-41	1	1	1	1	1	1	1	2	–[a]	–	–	2	–	–	–
	2	1	1	1	1	1	1	1	1	1	2	–	–	–	–
VF 70/888	1	1	1	2	1	2	1	–	1	–	–	–	–	–	–
	2	2	1	2	1	2	1	2	1	3	1	–	–	–	–
HV-2	1	1	1	1	1	1	1	2	1	2	2	3	1	–	–
	2	1	1	1	1	1	–	–	2	–	–	–	–	–	–
HV-10	1	2	1	3	1	3	1	–	1	–	1	–	–	–	–
	2	3	1	3	1	2	1	3	1	3	2	–	–	–	–

[a] No virus isolated
E Embryonated eggs
OC Tracheal organ cultures

Comparison of Tracheal Organ Cultures and Embryonated Eggs for the Assay of Virus

A minimum of 3 replicate assays of 2 egg adapted strains of AIB virus (H120 and IBV-46) and the 4 strains subjected to 4 serial passages in chickens were performed simultaneously in both tracheal organ cultures and embryonated eggs. The results, presented in Table 5, show that the titres obtained with 5 of the virus strains were very similar in the 2 assay systems. The mean titre for the remaining strain (HV-10) was $\log_{10}$ 4.2 CD_{50}/ml in tracheal organ cultures and $\log_{10}$ <1.0 EID_{50}/ml in embryonated eggs. Assays in tracheal explants gave highly reproducible results with more clearly defined end points than were found with assays performed in embryonated eggs.

Table 5. *Comparison of virus assays in tracheal organ cultures and embryonated eggs*

	Mean virus titre ($\log_{10}$ ID_{50}/ml) in	
Virus strain	Tracheal explants	Embryonated eggs
H120	6.4	6.5
IBV-46	6.9	7.1
IBV-41	3.7	4.0
VF70/888	3.6	3.3
HV-2	3.8	3.5
HV-10	4.2	<1.0

Discussion

The results of the titrations of 3 strains of AIB virus yielded titres which were at least as high in the tracheal explants prepared from 20-day-old embryos as in explants from young chickens and all strains grew well in the embryo tracheal cultures whether they were adapted to embryos or not. This, together with the fact that embryo tracheal explants are easier to prepare, have a lower failure rate following preparation and are more convenient to observe *in toto* microscopically, led to their sole use throughout the subsequent work.

COLWELL and LUKERT (4), using tracheal explants prepared from 4-week-old chickens, found that 3—4 days were required for the complete exfoliation of ciliated cells following infection by the Massachusetts or Connecticut serotypes of AIB and furthermore stated that the Iowa 97 and Beaudette strains required up to 13 days to cause complete ciliostasis. In the present work, no difference was found in the time required for the 2 strains of the Massachusetts type to cause complete ciliostasis and, although the Connecticut strain had a more rapid initial effect, ciliostasis was complete in all cases by approximately 70 hours. It appears, therefore, that while the average time for AIB virus to cause complete ciliostasis is approximately 3—4 days, some strains may require a longer period.

Virus was recovered from experimentally inoculated chickens consistently on the first passage in organ cultures whereas it occasionally required 2 to 3 passages before being demonstrated conclusively in embryonated eggs. In the attempts to readapt the virus strains to chickens, the 4 serial passages might have been insufficient and it is possible that these strains behaved as atypical field strains

of AIB virus. Although only 2 field strains (HV-2, HV-10) were available for study these gave similar results. Replication of the virus in organ culture was shown to occur with no loss of capacity to kill or cause dwarfing of chicken embryos after at least 4 serial passages and in the case of one strain (VF70/888) after 16 such passages.

The finding that AIB virus could not be reisolated from respiratory tissues of infected chickens in CK cultures confirms the findings of various workers including KAWAMURA *et al.* (14) and CUNNINGHAM (5). They have suggested that adaptation of the virus to embryonated eggs was necessary before it became cytopathogenic for CK cultures. In the present work, virus passaged up to 16 times in tracheal explants did not produce a CPE in CK cultures.

The titres of all the strains of AIB virus examined were highly reproducible in tracheal explants. When compared in embryonated eggs and in organ cultures, with the exception of strain HV-10 which did not grow in embryonated eggs, all strains attained similar titres in both systems. Probit analysis showed that the lines produced by data obtained in the 2 systems were parallel throughout, indicating that the same parameters were being measured in both instances. This comparability of titres is at variance with the results of BUTLER *et al.* (2) who found that titres in embryo tracheal explants were consistently lower than in embryonated eggs by a factor of $\log_{10}$ 2.0—3.0 ID_{50}/ml.

The interpretation of the quantal response of tracheal explants is less subjective than that obtained with embryonated eggs where, in addition to noting embryo mortality, it is also frequently necessary to take the characteristic embryo dwarfing into consideration when calculating end points. Tracheal explants have the added advantage that ciliostasis is complete within 3 days after inoculation whereas embryonated eggs require incubation for 7 days before any embryo deformities can be assessed. Although ciliostasis results from infection with other agents, neutralization tests can be used to distinguish AIB virus.

The fact that the HV-10 strain reached a titre of approximately $\log_{10}$ 4.0 CD_{50}/ml in tracheal explants but had no effect on embryonated eggs suggests that this strain might be regarded as a field strain. The other virus isolation from a field specimen (HV-2), readily killed embryonated eggs on the first passage, suggesting that it was of vaccinal origin. During attempts to reisolate AIB virus from serial dilutions the use of tracheal explants proved to be markedly superior for the reisolation of the HV-10 strain implying that such explants would detect field strains of AIB virus more readily than would embryonated eggs.

These results indicate that tracheal organ cultures offer a reliable alternative to embryonated eggs for the isolation and assay of AIB virus, although the existence of non-ciliostatic strains of AIB virus cannot be excluded. CUNNINGHAM (6) has stated that AIB virus must first be isolated in and adapted to embryonated eggs before it will replicate in tracheal explants, but the results presented here suggest that this is unnecessary. The considerable advantage in the use of tracheal explants is the facility to propagate AIB virus in a system other than embryonated eggs, thereby eliminating the inherent disadvantage of repeated passaging in the latter system which may alter the antigenic character of the virus. It should now be possible to use organ cultures to make serological comparisons between strains of AIB virus.

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