Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Res. vet. Sci. 1971, **12**, 565-569

Isolation of Viruses from 94 Flocks of Fowls with Respiratory Disease

J. B. McFerran, W. A. M. Gordon, S. M. Taylor and P. J. McParland

Veterinary Research Laboratories, Stormont, Belfast BT4 3SD

SUMMARY. Virological studies were made on 94 flocks of fowls suffering from respiratory disease. Infectious bronchitis virus was detected in 50 flocks by egg inoculation and using tissue culture techniques adenovirus was detected in 41, reovirus in 3 and mycoplasmas in 4 flocks. In addition small 20 nm. particles associated with adenoviruses were seen on 6 occasions and it is believed that they may be adeno-associated parvoviruses.

Direct examination of allantoic fluid or tissue cultures using the electron microscope was found to be a quick and efficient method of virus and mycoplasma identification. In addition it demonstrated strains of infectious bronchitis virus which failed to cause dwarfing and allowed the detection of parvoviruses.

DURING THE LAST two years respiratory disease in poultry has become a problem of major importance in Northern Ireland. The initial investigation of these outbreaks indicated that infection with infectious bronchitis (IB) virus was the probable cause of at least some of them; however other agents were also isolated from the respiratory tract of infected fowls. In order to investigate the distribution and relative importance of viruses in respiratory infections, from November 1968 to August 1969 a variety of tissues were taken for virus isolation from 94 flocks of fowls with respiratory trouble. The relative value of egg and tissue culture inoculation as a means of detecting viruses was also assessed.

MATERIALS AND METHODS

Specimens

A total of 94 flocks was studied varying in age from 1 week to 52 weeks. The flocks included broiler, replacement pullet and laying fowls. Material was normally taken from several freshly killed fowls and each type of tissue was pooled and made into an approximately 10% suspension in nutrient broth containing 1,000 units of penicillin and 1,000 µg. of streptomycin per ml. The suspension was centrifuged at 3,000 rev./min. for 30 min. and the supernatant fluid was used either immediately or stored at − 70°C.

Egg Inoculation

Embryonated eggs were obtained from a specific pathogen free (SPF) breeding flock maintained at the laboratory for some years. 0·2 ml. from each specimen was inoculated into the allantoic cavity of 3 ten-day-old eggs. These were incubated for 48 hr., after which the allantoic fluid was collected, pooled and inoculated into 3 more eggs. After a further 48 hr., the allantoic fluid was pooled again and divided into 2 aliquots. One aliquot was inoculated into 4 ten-day-old eggs which were incubated for 6 days and then examined for evidence of dwarfing of the embryo. The other aliquot was used for electron microscopy.

Chick Kidney Cultures

Kidneys from 2- to 6-week-old SPF chicks were trypsinised and the dispersed cells were suspended in Eagle's medium containing 10% calf serum and distributed into tubes. When the cells had grown the medium was changed to Earle's lactalbumin solution with 2% foetal calf serum. 0·2 ml. of specimen supernatant was then inoculated into each of 2 tubes and the cultures examined for 6 to 8 days to detect a cytopathic effect (CPE). If no CPE was observed, the tubes were frozen and thawed, pooled and 2 further tubes were inoculated. If these showed no CPE after 6 to 8 days, the specimens were normally considered to be negative.

Electron Microscopy

Allantoic fluid was applied directly to formvar-carbon grids, dried, and then stained with 4% sodium phosphotungstate pH 7·0.

In the case of cell cultures, the supernatant fluid was removed from the tube and replaced by 2 drops of distilled water. This was frozen and thawed and applied to a grid. If cultures were in an advanced state of degeneration with many cells in suspension,

the remaining cells were suspended by scraping, centrifuged and resuspended in two drops of water. This was then frozen and thawed, applied to a grid and stained with phosphotungstate.

Virus Identification

All viruses isolated in tissue culture were identified by electron microscopy of negatively stained material. Most IB isolates were identified using an electron microscope but in some cases this was dispensed with when an isolate produced dwarfing of chick embryos typical of that caused by IB viruses (Anon., 1963). A representative sample of isolates was titrated against antisera prepared in SPF birds to a Massachusetts strain of IB virus using a standard method (Anon., 1963).

RESULTS

Clinical Observations

In most outbreaks of respiratory disease, the first sign noticed by the owners was a high-pitched croak, detectable only when the birds were not disturbed. This was quickly followed by more marked respiratory sounds and in some cases nasal discharge. Conjunctivitis with associated photophobia, sufficiently severe to stop the birds eating, was also observed frequently.

In laying flocks as well as respiratory signs, which were often slight, egg production fell usually by 30 to 40% but falls of up to 77% were recorded. Furthermore, 3 to 5 weeks after infection the proportion of downgraded eggs was high—up to 90% in extreme cases—due to the production of abnormally small eggs, the presence of hair cracks and shells of poor quality.

Agents Isolated from Flocks with Respiratory Disease

One or more viruses were recovered from at least one specimen from 79 flocks out of the 94 examined. The results are summarized in Table I.

TABLE I
VIRUSES ISOLATED FROM 94 POULTRY FLOCKS WITH RESPIRATORY ILLNESS

Organism isolated	No. of flocks
Infectious bronchitis virus	28
Infectious bronchitis virus + adenovirus*	18
Infectious bronchitis virus + reovirus	3
Infectious bronchitis virus + mycoplasma	1
Adenovirus*	26
Adenovirus + mycoplasma	3
No. viruses isolated	15

* Six of these isolates also contained particles morphologically indistinguishable from parvoviruses (Fig. 5).

Detection of IB Virus in Eggs

On several occasions in the initial investigations, viruses were not isolated from flocks which, on clinical grounds, were almost certainly infected with IB virus. These failures sometimes occurred, even when birds were obtained during the acute stages of an outbreak. In these cases, allantoic fluid from inoculated eggs was applied directly to grids, negatively stained and examined in an electron microscope. Virus was detected using this method (Fig. 1) even though the third passage material failed to cause dwarfing. However, when particles were detected by electron microscopy dwarfing of the embryo invariably occurred after 3 to 5 further passages of the virus. In view of these results, allantoic fluid was routinely examined by negative staining before any specimen was regarded as negative. This increased the efficiency of detection of the virus by about 15%. All the isolates examined by serum neutralization proved to be of the Massachusetts type, although some viruses were better neutralized than others.

Relative Efficiency of Various Tissues for the Isolation of IB Virus

Tissues from several birds in each flock were pooled before inoculation into eggs. The material taken varied to some extent depending on the clinical signs and *post-mortem* examination. IB virus was ultimately detected in 50 flocks using lung, trachea, pharynx, cloaca or conjunctiva. The relative efficiency of virus isolation from these organs is shown in Table II. It should be noted that any single specimen was inadequate to ensure virus isolation even

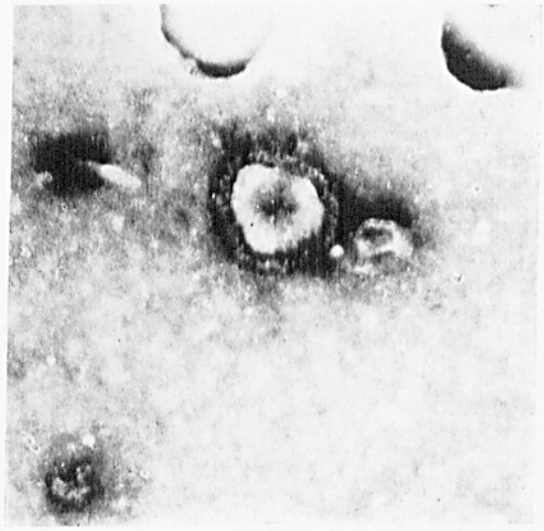

FIG. 1. Infectious bronchitis virus (coronavirus). Direct examination of allantoic fluid. × 100,000.

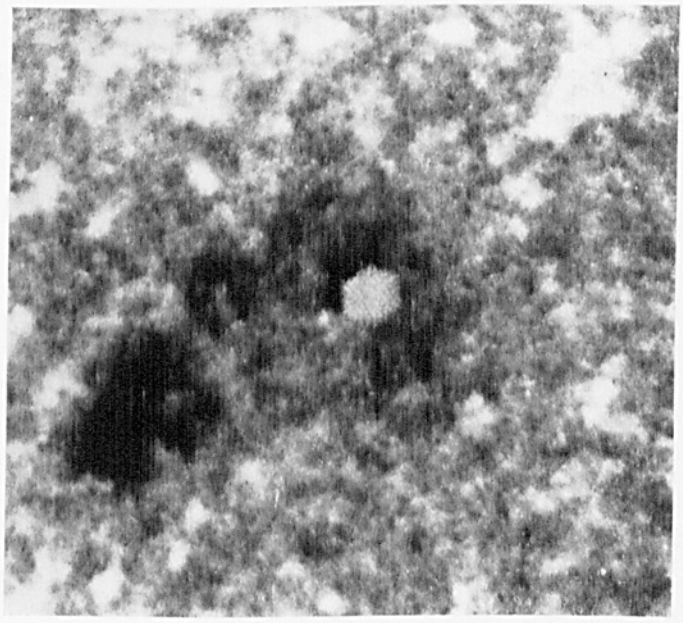

FIG. 2. Adenovirus. × 100,000.

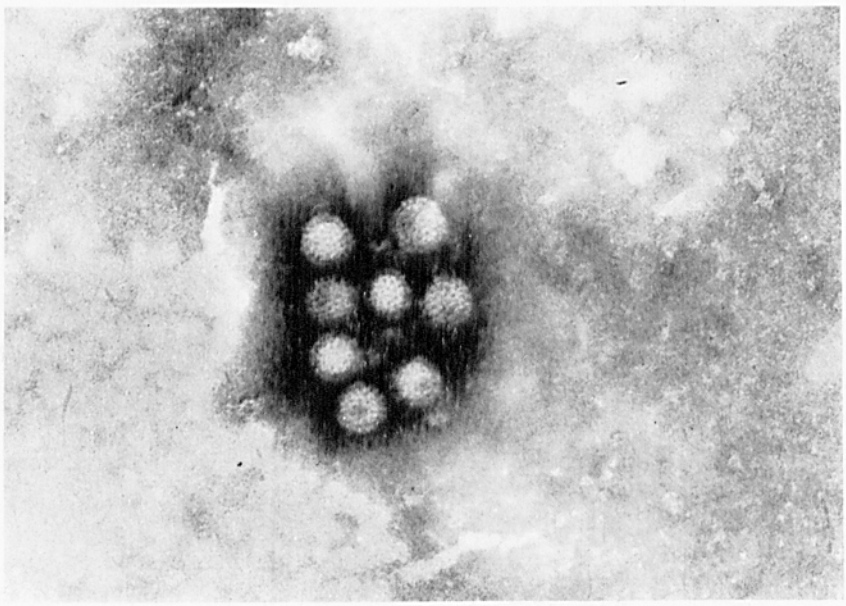

FIG. 3. Reovirus. × 100,000.

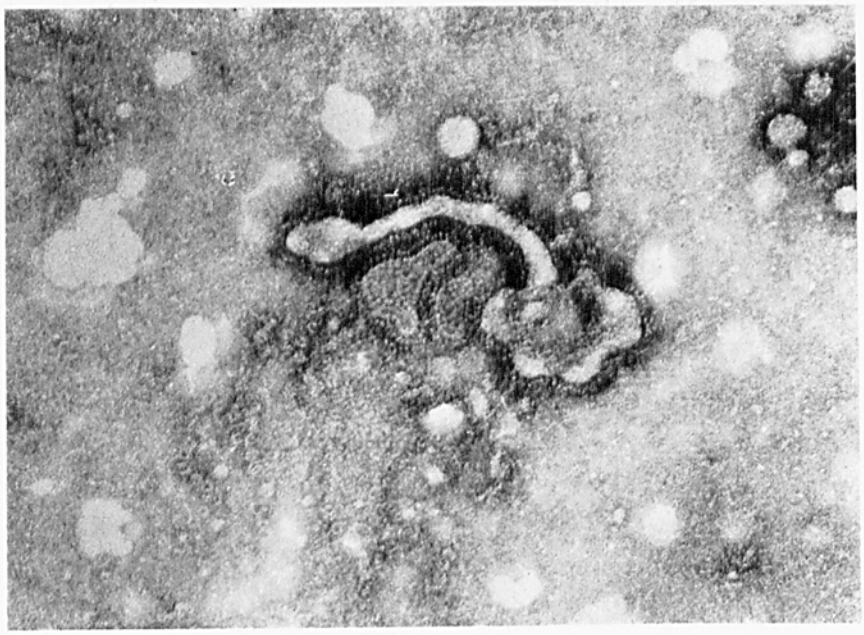

FIG. 4. Mycoplasma. × 100,000.

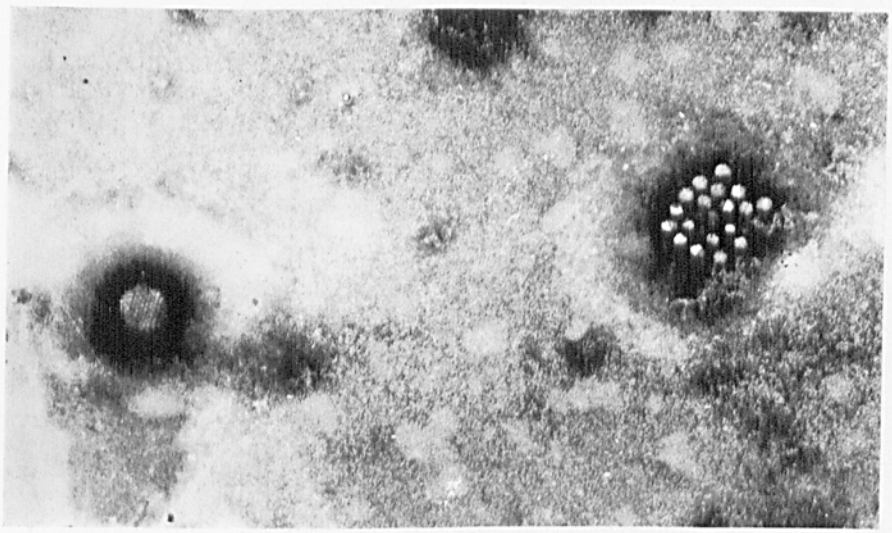

FIG. 5. Adenovirus and small 20 nm. particles (probably parvoviruses). × 100,000.

TABLE II

INFECTIOUS BRONCHITIS VIRUS DISTRIBUTION IN THE ORGANS OF FOWLS

	Numbers		
	Positive	Tested	%
Lung	35	44	80
Trachea	32	43	74
Pharynx	21	31	67
Caeca and contents	5	14	35
Conjunctiva	3	17	17

This table refers to 44 flocks naturally infected with IB virus as indicated by virus isolation from one or more organs.

when a pool of this tissue was taken from a flock known to be infected with IB virus.

Agents Isolated in Chick Kidney Cultures

Fifty-six% of positive specimens gave a CPE on the first pass, 40% on the second pass and only 4% on the third pass. It was therefore considered that in practice only one 'blind' passage was justified. These recovery rates are similar to those of Clemmer (1964) who found that 65% of his isolates of adenovirus were made on first passage.

Agents belonging to at least two virus groups were detected in tissue culture: adeno- and reoviruses. They were identified by negative staining electron microscopy (Figs. 2 and 3). Reoviruses were isolated from faeces on 3 occasions. They did not give a clearly defined CPE but their presence was established unequivocally by electron microscopy (Fig. 3).

An attempt was made to find out if there was any association between the frequently isolated adenoviruses and clinical respiratory disease. Various tissues were taken from sick birds both with and without apparent respiratory disease. The results are shown in Table III. Although adenoviruses were recovered from the respiratory tract and conjunctiva, it should be noted that the highest recovery rate was from faeces (Table IV).

In addition to adenoviruses and reoviruses, two other groups of agents were detected when

TABLE III

ADENOVIRUS ISOLATIONS FROM GROUPS OF FOWLS WITH AND WITHOUT RESPIRATORY DISEASE

	Number of flocks		
	Tested	Positive	%
With respiratory disease	94	41	44
Without respiratory disease	74	11	15

tissue cultures were examined in the electron microscope: mycoplasmas (4 isolates) and viruses associated with adenoviruses (6 isolates). The mycoplasmas (Fig. 4) were isolated even in the presence of antibiotics. They have not yet been examined in detail but are reported here because some of them caused a CPE similar to that caused by viruses. The agents seen in association with adenoviruses were spherical (Fig. 5) and measured 20 nm. in diameter. They appeared to belong to the parvovirus group and they have not been propagated in the absence of adenoviruses.

Relative Value of Eggs and Tissue Culture for the Detection of Viruses

In general egg inoculation detected only IB virus whereas adeno- and reoviruses were only isolated in tissue culture. Occasionally specimens known to contain IB virus produced a poorly defined CPE in kidney cell cultures, but serial propagation of the virus did not produce a clearly defined CPE.

Adenoviruses seen in allantoic fluid examined in the electron microscope were detected by egg inoculation on only one occasion. This low detection rate was probably due to the short period of incubation allowed during the first two 'blind' passages.

DISCUSSION

The efficient isolation of IB, adeno- and reoviruses from the domestic fowl requires the use of both egg inoculation and chick kidney tissue cultures. However, even though the

TABLE IV

ADENOVIRUS DISTRIBUTION IN THE ORGANS OF FOWL FROM FLOCKS, WITH OR WITHOUT RESPIRATORY DISEASE, FROM WHICH ADENOVIRUSES WERE ISOLATED

	With respiratory disease			Without respiratory disease		
	Positive	Numbers Tested	%	Positive	Numbers Tested	%
Conjunctiva	6	24	25	0	5	0
Pharynx	5	27	19	2	8	25
Trachea	3	26	11	1	2	50
Lung	6	27	22	0	2	0
Caeca and contents	41	43	94	11	11	100

This table refers to 43 flocks with and 11 without respiratory symptoms naturally infected with adenovirus as indicated by virus isolation from one or more organs.

systems used were adequate to propagate the viruses, difficulties arose in detecting their presence: thus not all IB viruses caused dwarfing at the third passage and at least one 'blind' passage of tissue culture material was necessary to detect adenoviruses efficiently. Electron microscopy of negatively stained material proved invaluable to overcome these difficulties and also as a means of virus identification. Using this technique IB viruses, which initially failed to cause dwarfing of chick embryos, were detected, and agents causing dwarfing could be identified unequivocally as coronaviruses without resorting to serological tests. However routine examination of allantoic fluid by the electron microscope is time consuming and therefore a method employing fluorescent antibody staining of allantoic cells was subsequently developed but not used in this study (Clarke, McFerran & Gay, to be published). Negative contrast was even more valuable for the identification of agents isolated in tissue culture and adeno- and reoviruses could be quickly identified even though the reoviruses gave a poor CPE; parvoviruses, which would otherwise have gone undetected, were seen on 6 occasions.

Phosphotungstate staining was also found to be useful for identifying mycoplasmas, although the value of this approach has recently been questioned by Wolanski & Maramorosch (1970). Their work, however, was apparently limited to mycoplasmas without surface projections whereas all the mycoplasmas recognized by negative staining in this series had such projections (Fig. 4). All the mycoplasmas detected in tissue culture subsequently formed colonies when plated onto solid medium and it is felt to be essential that all mycoplasma-like agents should be tested routinely for ability to grow on inanimate medium before a firm diagnosis is made.

Although IB virus was recovered from about a third of the flocks studied, adenoviruses were recovered from another third of the flocks, thus suggesting, in view of the higher recovery rate of adenoviruses from birds with respiratory disease, that adenoviruses are important in the aetiology of respiratory disease and associated with falls in egg production. This finding is in agreement with the serological studies of Flir (1969) and Monreal (1968) who both showed that adenoviruses, especially in association with either mycoplasma or infectious bronchitis could cause disease. Berry (1969) also demonstrated experimentally that adenoviruses alone could cause a 10% fall in egg production and that in association with either infectious bronchitis or mycoplasma the fall could be even more severe.

The reoviruses probably played no real part in the clinical picture. The isolations of mycoplasma were unexpected because streptomycin (1,000 μg./ml.) was present in the tissue culture

media. In retrospect we regret that it was not practical to attempt the isolation of mycoplasma by conventional techniques.

As preliminary work suggests that they cannot be grown in the absence of adenoviruses, the 6 isolates of small spherical agents measuring 20 nm. in diameter seen on electron microscopic examination could represent the first record of adeno-associated viruses isolated from fowl. Dutta & Pomeroy (1967) described small particles 20–24 nm. in size seen when studying the quail bronchitis virus. They suggested that these represented the cores of adenoviruses but we believe they may be parvoviruses.

Received for publication, February 22nd, 1971

REFERENCES

ANON. (1963) 'Methods for the Examination of Poultry Biologics'. 2nd ed., National Academy of Sciences, National Research Council, Washington, D.C. Publication 1038.

BERRY, D. M. (1969) *Vet. Rec.* **84,** 397

CLEMMER, D. I. (1964) *J. infect. Dis.* **114,** 386

DUTTA, S. K. & POMEROY, B. S. (1967) *Am. J. vet. Res.* **28,** 296

FLIR, K. (1969) *Dt. tierärztl. Wschr.* **76,** 7

MONREAL, G. (1968) *Zentbl. VetMed.* **15B,** 685

WOLANSKI, B. & MARAMOROSCH, K. (1970) *Virology,* **42,** 319