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# Detection of avian infectious bronchitis viral infection using in situ hybridization and recombinant DNA

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ABSTRACT

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A recombinant DNA probe with specificity for the 3′ end of genomic RNA from the Ark 99 strain of infectious bronchitis virus (IBV) was found to hybridize with extracted RNA of three strains with the Ark serotype, as well as the Mass41, Holl52, Gray, JMK, Conn, Fla and SE17 strains of IBV. Viral infection was detected in the cytoplasm of chicken embryo kidney cells inoculated with Mass41, Ark99, SE17 or two recent field isolates of IBV using in situ cytohybridization and a biotinylated probe. In vivo infections were detected in individual cells of tracheas and lungs 2,4, and 6 days after inoculation of chicks with Mass41 and Ark99. In situ hybridization of Ark99 infected tissue sections using $^{32}$P-dATP labelled probe indicated that more viral replication was present in the trachea on day 4 than either days 2 or 6; whereas more viral RNA was found in the lungs on day 6 than days 2 or 4 after inoculation.

## INTRODUCTION

Avian infectious bronchitis virus (IBV), the prototype virus of the Coronaviridae family, has a positive-stranded RNA genome and three major structural protein components: the club-shaped spikes on the outside of virion, the matrix protein and the nucleocapsid protein (Sidell et al., 1983). Infection with IBV causes a highly contagious, respiratory disease in chickens with high mortality in young chicks (Hofstad, 1984). In addition to the upper and lower respiratory tract, IBV also affects the genital and urinary tracts and has been isolated from many tissues, including the lung, trachea, cecal tonsils, kidneys, Harderian (paraocular) glands and spleen (Hofstad, 1984). In laying flocks, IBV infection can cause complete cessation in egg production or production

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of thin-shelled, rough and misshapen eggs, and in broilers, infection can result in decreases in feed consumption (Hofstad, 1984).

Rapid diagnostic procedures are especially critical for control of respiratory diseases. Currently, the most common procedure for IBV diagnosis depends on amplification of virus after inoculation of the sample into the allantoic sac of embryonating chicken eggs (ECE). Confirmation of IBV infection is then made on the basis of such procedures as electron microscopy, and serum neutralization and hemagglutination assays (Hopkins, 1974; Darbyshire et al., 1979; King and Hopkins, 1983). These processes are time consuming, expensive, and require highly skilled technicians.

We have used an in situ cytohybridization procedure with recombinant DNA to detect bluetongue viral infection in the developing chicken embryo (Wang et al., 1988). In situ hybridization was used in the present study to detect IBV infection in cell culture and tissues taken from infected chicks. The recombinant DNA probe used was specific for the 3′ end of the IBV genome and was shown to hybridize by dot blot analysis to extracted RNA from all IBV strains examined.

## MATERIALS AND METHODS

### *Viruses*

The following strains were obtained from Dr. R.W. Winterfield at Purdue University; Ark99, Conn, JMK, Florida (Fla), Gray, Aust T, Mass41 and SE17 (Winterfield and Hitchner, 1962; Cumming, 1963; Winterfield et al., 1964, 1971; Johnson et al., 1973; Hopkins, 1974; Darbyshire et al., 1979). Dr. J.K. Rosenberger (University of Delaware) provided us with ArkDPI-11 (11th egg passaged) and -75 (75th egg passaged) strains. The Holl52 isolate was a commercial vaccine strain obtained from the University of Delaware. Gray and Aust T are highly virulent nephropathogenic strains. The Newcastle disease virus (NDV) strain used was B1. Each of the viruses was purified in our laboratory by three series of terminal dilutions in specific pathogen free (SPF) embryonating chick embryos before using as inoculum.

### *Viral inoculation and tissue preparation*

Chicken embryo kidney cells (CEK) were prepared from 20-day-old ECE and cultured in Eagle's minimal essential medium (EMEM) with 5% fetal calf serum and 5% bovine allantoic fluid (SA500 from Cell Culture Laboratories, Cleveland, OH) on glass coverslips. The CEK were inoculated with approximately $10^7$ egg infective doses$_{50}$ ($EID_{50}$) of either Mass41 of ArkDPI-75, viral stock per ml of EMEM or with only EMEM. After incubating at 37°C for 24 h, the coverslips were fixed with 3 volumes of 95% ethanol and 1 volume of glacial acetic acid.

One-week-old chicks hatched from SPF eggs were inoculated intranasally

with approximately $2 \times 10^6$ $EID_{50}$ of Mass41 or Ark99 virus. Each group of chicks maintained in isolated facilities was inoculated with a single strain of IBV or NDV or sham inoculated with allantoic fluid. On days 2,4 and 6 after inoculation, three chicks were sacrificed from each group and the tissues from the tracheas, and lungs were removed and frozen in methyl cellulose (OCT, Miles Scientific, Naperville, IL) at $-70°C$. Eight micron sections were made with a Lipshaw cryotome and placed on pretreated glass slides (Haase et al., 1984; Wang et al., 1988). Sections were treated with 4% paraformaldehyde and dehydrated with ethanol (Wang et al., 1988).

*DNA probe preparation*

The recombinant DNA probe, T68, which is specific for the 3′ 1350 bases of Ark99 genomic RNA (manuscript in preparation), was inserted into the Pst 1 site of the pUC9 plasmid and propagated in *Escherichia coli* JM 109 cells. The IBV specific insert was excised with Pst 1 and separated from the plasmid by agarose electrophoresis before nick-translating with a mixture of biotinylated dUTP and dATP, unlabelled dCTP and dGTP and $^3$H-dATP which was used to quantitate incorporation (Berger and Kimmel, 1987; Wang et al., 1988). Random priming with synthesized oligonucleotides and Klenow enzyme was used to make $^{32}$P-dATP labelled probe (Boehringer Mannheim, Indianapolis, IN).

*Hybridization assays*

Dot blot hybridization was done with RNA from virus propagated in ECE (Kafatos et al., 1979; Maniatis et al., 1982). The allantoic fluid was harvested and clarified, and the virus precipitated with a final concentration of 10% polyethyleneglycol-6000 in 2.3% sodium chloride overnight at 4°C, banded on a 30 to 50% glycerol-potassium tartrate gradient and concentrated by ultracentrifugation. Virus was treated with proteinase K and sodium dodecyl sulfate (SDS), and the RNA was extracted with phenol and 0.3 M sodium acetate, pH 5, and concentrated with ethanol (Niesters et al., 1986). A modification of the dot blot procedure described by Kafatos et al. (1979) and Schleicher & Schuell, Inc. (Keene, NH) was used. Briefly, the RNA was pretreated with formaldehyde, formamide and sodium phosphate buffer (PBS). After heating to 65°C for 10 min, SSC (0.15 M sodium chloride, 15 mM sodium citrate, pH 7) was added to a 15×final concentration. Four-fold serial dilutions of RNA starting with an equivalent of 1 μg of RNA were blotted onto nitrocellulose, pretreated with 20×SSC, (Maniatis et al., 1982) with a Bio-Dot Apparatus (Bio-Rad, Richmond, CA) manifold. After baking at 80°C under a vacuum for 2 h, the samples were prehybridized for 4 h at 37°C in 50% formamide, 5×Denhardt's solution, 0.1% SDS, 150 μg/μl calf thymus DNA and 5×SSC (Maniatis et al., 1982) and hybridized for 24 h at 37°C in the prehybridization solution with $10^6$ cpm/ml of probe labeled by nick-

translation with $^{32}$-alpha dATP (Maniatis et al., 1982; Berger and Kimmel, 1987). The blot was washed with 2×SSC and 0.1% SDS twice at room temperature and twice at 37°C, and once at 56°C and twice with 0.2× SSC and 0.1% SDS at 56°C. After drying at 56°C, the blot was autoradiographed.

The in situ cytohybridization procedure used was similar to that previously described (Singer et al., 1986; Wang et al., 1988). Both in vivo chick tissues and cultured cells were rinsed with PBS before prehybridizing for 1 h in 2×SSC, 50% formamide, 10×Denhardt's, 1 mg/ml salmon sperm DNA, and 1 mg/ml extracted chicken embryo RNA. The prehybridization solution was replaced with 30 to 40 μl of hybridization solution consisting of 50% formamide, 2×SSC, 5×Denhardt's solution, 10% dextran sulfate, 100 ng/ml salmon sperm DNA, 1 mg/ml whole chicken embryo RNA and 20–50 ng/slide biotinylated cDNA. After covering with silicon treated coverslips, the slides were heated for 5 min at 80°C, and hybridization was allowed to proceed overnight at 37°C. Post-hybridization, washing and visualization were performed as described for the Enzo kit (Enzo Bio-Chem, New York, NY). Positive hybridization was detected by the reaction of streptavidin-horseradish peroxidase, 3-amino-9-ethylcarbazole (Enzo Bio-Chem, New York, NY) and hydrogen peroxide, or with fluorescein labelled streptavidin (5 Prime→3 Prime, Paoli, PA). The nuclei of cells from the enzyme-treated slides were counter-stained with a 0.25% methyl green in 30 mM sodium acetate pH4.8. Positive hybridization was indicated by areas with dark red–brown grains surrounding blue–green nuclei.

Tissues used for in situ whole tissue hybridization were prepared as described for the cytohybridization assay; however, the probe was labelled by nick-translation with $^{32}$P-dATP, and hybridization was detected by autoradiography. Slides treated with RNase were first washed twice in 2×SSC and incubated in a solution of 100 μg RNase A (preheated at 80°C for 30 min) in 1 ml of 2×SSC for 30 min at 37°C. Two additional washes in 2×SSC then preceeded the hybridization procedure as described above.

## RESULTS

### *Dot blot hybridizations*

The hybridization specificity of T68, a recombinant DNA probe complementary to the 3′ end of genomic RNA of the IBV Ark99 strain, was initially examined by dot blot hybridization (Fig. 1). All 10 strains of IBV with eight distinct serotypes were found to hybridize with the probe although the Gray RNA appeared to hybridize to a lesser extent than the other strains.

### *Hybridization of infected cultured cells*

Positive hybridization with the T68 probe and Mass41 infected cells is shown in Fig. 2b. The positive signal was the result of RNA hybridization

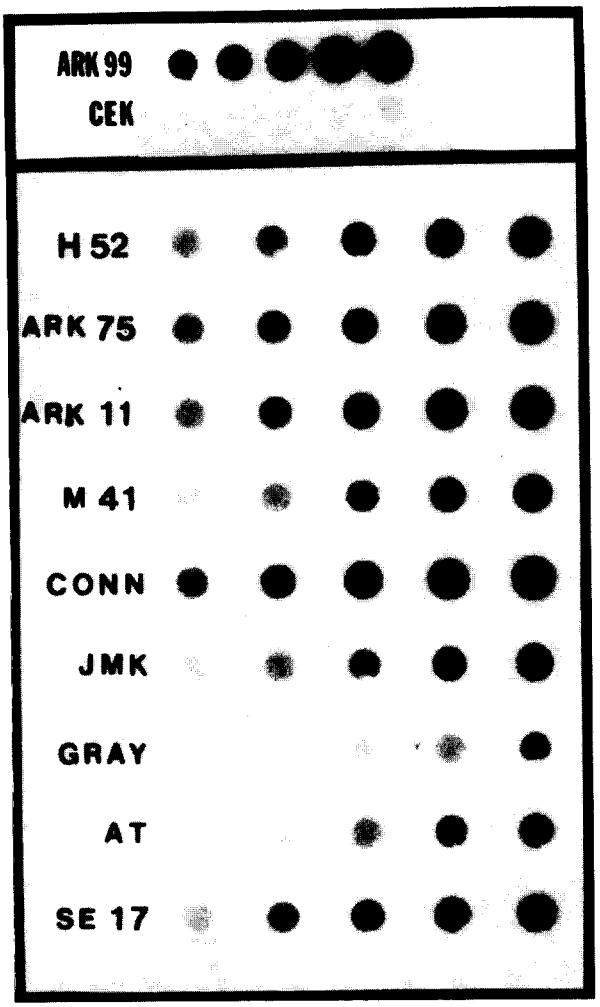


Fig. 1. Dot blot hybridizations of [alpha$^{32}$P]dATP-labelled insert T68 with RNA from uninfected chick embryos or avian IBV strains Ark99, H52 (Holl52), Ark 75 (ArkDPI-75), Ark 11 (ArkDPI-11), M41 (Mass41), Conn, JMK, Gray, AT (AustT), and SE17. Fourfold serial increases in concentration of RNA are shown with the greatest concentration on the right of 1 $\mu$g of RNA per dot.

since infected cells treated with RNase prior to reacting with the probe failed to produce a positive signal (Table 1). Similar hybridization was also detected in the cytoplasm of CEK infected with Mass41, Ark99, SE17 and two isolates collected from field infected chicks during an outbreak of IBV (Table 1). The absence of hybridization in uninfected cell controls (Fig. 2a) and controls infected with NDV indicated that the hybridization was specific for cells infected with IBV. Controls in which only probe was eliminated or in

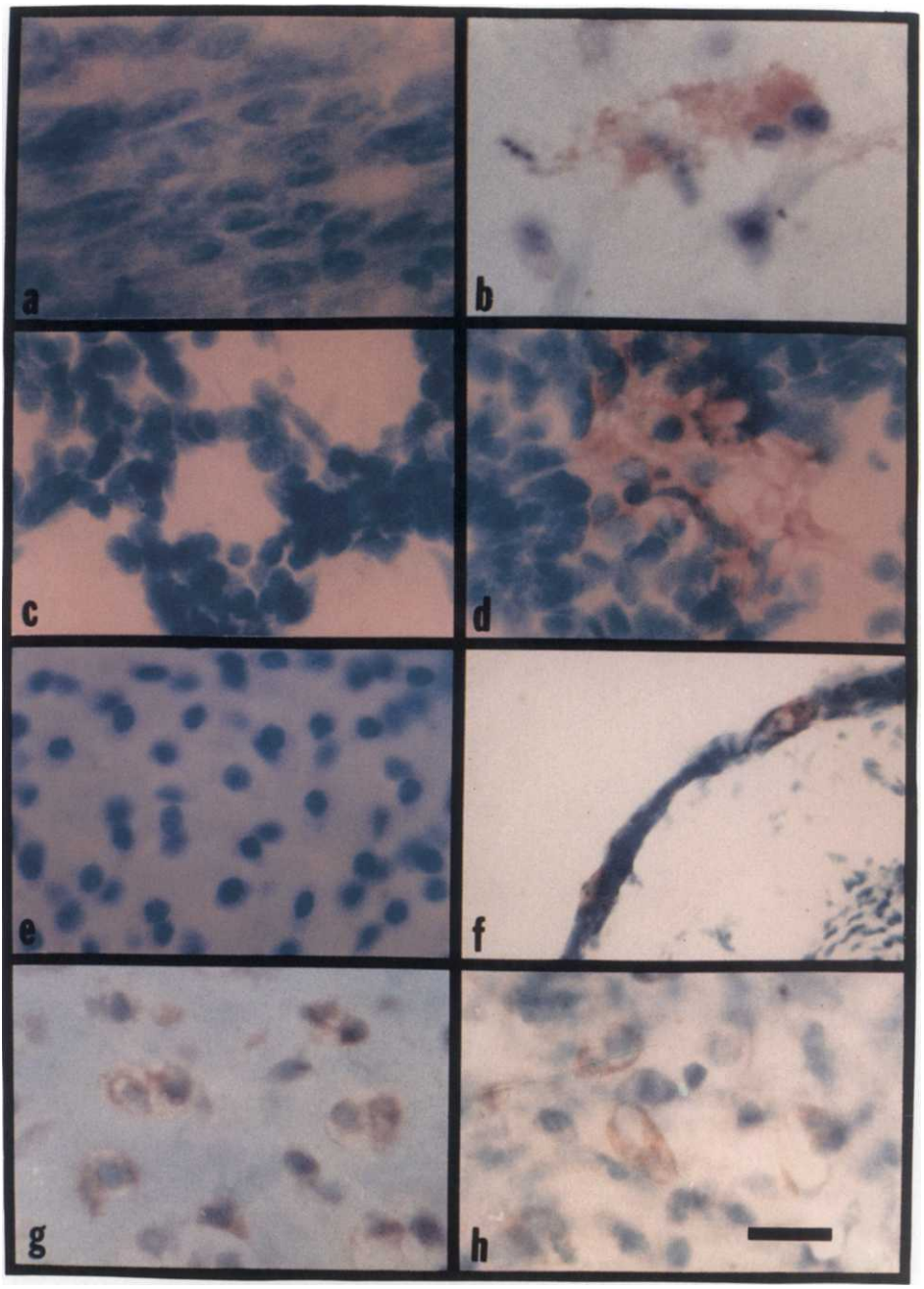


Fig. 2. In situ cytohybridization with biotinylated T68 nick-translated probe and enzyme-linked indicator (for (a) through (e), (g) and (h) the bar = 8 $\mu$m; for (f) the bar = 20 $\mu$m): (a) and (b) represent CEK, uninfected and infected with SE17, respectively. Chick tissue sections are shown in (c) through (h). Lung sections are shown in (c), uninfected and (d), infected with Mass41; cartilage regions of the trachea (e), from an uninfected chick (g), from a chick infected with Mass41 and (h), from a necropsy section of a naturally infected chick. The epithelial lining of a chick trachea experimentally infected with Mass41 is shown in (f).

TABLE 1

In situ cytohybridization using the T68 probe specific for the 3′ end of Ark genomic RNA

| | Mass41 | ArkDPI | SE17 | Field isolate 1 | Field isolate 2 | NDV | Uninfected | RNase treated[1] |
|---|---|---|---|---|---|---|---|---|
| Trachea | + | + | ND[2] | ND | ND | – | – | – |
| Lung | + | + | ND | ND | ND | – | – | – |
| CEK | + | + | + | + | + | – | – | – |

[1]Mass41 or Ark DPI infected specimens were pretreated with RNase A; +, positive and –, negative.
[2]Not done.

which streptavidin was eliminated also were negative for the enzyme complex signal.

*Detection of viral replication in chick tissues*

Chicks infected with Ark99 were sacrified 2, 4 and 6 days post-inoculation (p.i.). Tracheas and lungs from three chicks were used for each day p.i. In situ hybridizations of whole tissues examined by autoradiography using a $^{32}$P-dATP labelled probe indicated that replication of Ark99 could be found in sections of tracheas and lungs taken on all 3 days examined (Fig. 3). The autoradiographs also suggested that the greatest amount of viral RNA on the days examined was present in the tracheas on day 4 p.i. and in the lungs on day 6 p.i.

The in situ cytohybridization assay using biotinylated probe detected viral

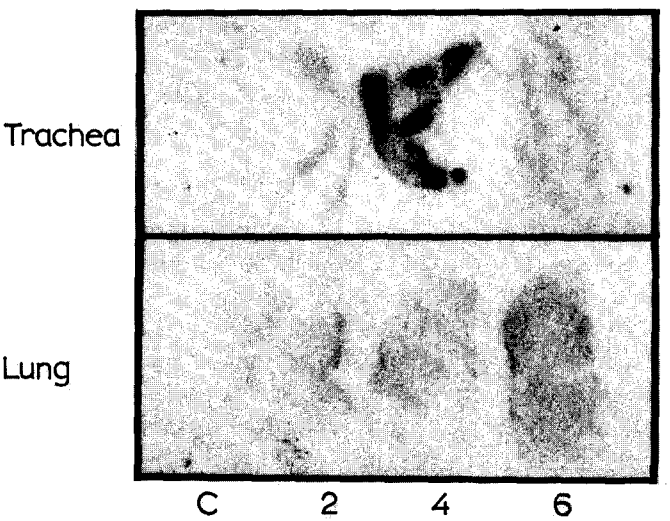


Fig. 3. Autoradiograph of trachea and lung sections from uninfected control chicks (C) and tissues from chicks infected with Ark99 on days 2, 4, or 6 before collecting tissues.

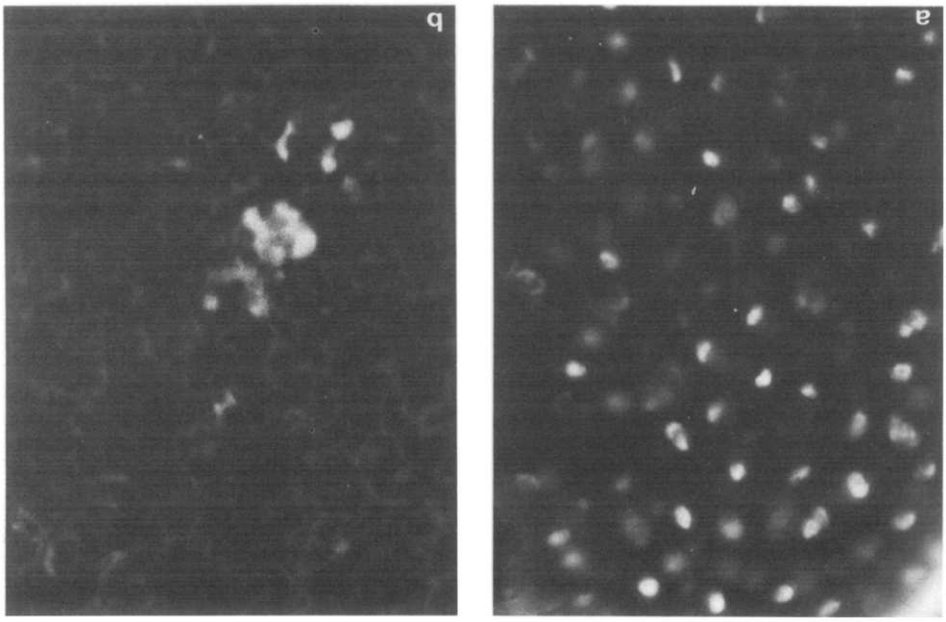


Fig. 4. In situ hybridization using fluorescein-labelled streptavidin as an indicator of infection in; (a), trachea and (b), lung tissues from chicks infected with Mass41.

replication in individual cells of the trachea and lungs of chicks infected with Mass41 or Ark99 on days 2, 4 and 6 after infection (Table 1). The absence of hybridization in tissues from uninfected chicks and the elimination of the signal with RNase pretreatment again established the specificity of the signal and the involvement of RNA in the signal, respectively. Although less viral RNA appeared to be present on day 2 than 4 or 6, it was difficult to objectively quantitate the degree of infectivity with the cytohybridization assay using the enzyme-linked detection procedure.

Positive hybridization of a section of trachea and lung taken from a chick infected with Ark99 is shown in Fig. 2d, 2f and 2g, along with negative controls of tissues from infected chicks pretreated with RNase and tissues from uninfected chicks shown in Fig. 2c and 2e (Table 1).

In the trachea, viral RNA was concentrated primarily in the chondrocytes of the cartilage although viral replication could also be seen in epithelial cells. Cells in the lungs supporting viral infectivity appeared to be mostly epithelial-like. As with the infected cultured cells, the hybridization signals in both the lung and trachea could be eliminated in tissues treated with RNase prior to adding the probe (Table 1). The specificity of the hybridization in the chondrocytes was also confirmed by the absence of the signal in these cells in a total of ten controls from uninfected chicks and three from NDV infected

chicks. The in situ hybridization procedure was also used to detect viral replication in the trachea of a chick necropsied as a result of an outbreak of infection with IBV (Table 1). Positive hybridization in the chondrocytes of the trachea collected from an infected chick during an outbreak of IBV infection (Fig. 2h) was identical to that seen in experimentally infected birds. Similar hybridization could be seen in tissues, including chondrocytes using fluorescein-conjugated streptavidin rather than enzyme-linked streptavidin-substrate complexes (Fig. 4). Infectivity from triplicate samples of chicks infected with either Mass41 or Ark99 were similar.

## DISCUSSION

The T68 recombinant DNA probe, specific for the 3′ end of the Ark99 genome, was found to be group specific, hybridizing to extracted RNA from a number of distinct strains of IBV. The antigenic diversity of this group of viruses has been demonstrated by neutralization assays, and Western blot assays (Hopkins, 1974; Darbyshire et al., 1979; Sneed et al., 1989). They represent diverse pathotypes; attenuated and virulent strains, and the known nephropathogenic strains, Gray and AustT. The broad specificity of this probe was predictable since it contains the majority of the coding sequences for the nucleocapsid protein which has been shown by Western blot analysis to be antigenically highly conserved among these same strains (Boursnell et al., 1984; Sneed et al., 1989). Viral replication could also be detected in the in situ cytohybridization assays with the T68 probe in cultured cells infected with the Mass41, Ark99 and SE17 strains, and in tissues of chicks experimentally infected with Mass41 and Ark99. Mass41, Ark99 and SE17 also represent genetically diverse viruses since antigenic differences have been detected by neutralization and hemagglutination assays which depend on differences in the peplomer proteins (Darbyshire et al., 1979; King and Hopkins, 1983; Cavanagh et al., 1984; Mockett et al., 1984) and by Western blot analysis which identified differences in the membrane proteins (Sneed et al., 1989). In addition to reacting with all strains examined, the practical value of the assay was demonstrated by the ability for the probe to detect viral replication in naturally infected chick trachea.

The in situ hybridization procedure, using the T68 probe, not only has potential as a group-specific procedure for diagnosing IBV, but also as an experimental technique for studying viral pathogenesis. Viral replication could be detected as early as 2 days after infection in both the trachea and lungs. Virus has been routinely isolated from these tissues and ciliary activity and immunofluorescent assays have been used to demonstrate virus in the trachea (Braune and Gentry, 1965; Darbyshire et al., 1979). The cytohybridization assay identified viral RNA in epithelial cells of tracheas from chicks infected

with either Mass41 or Ark99, but in addition, larger numbers of chondrocytes in the trachea were found to support viral replication. Since chondrocytes from the infected trachea taken during an outbreak of IBV were similarly infected, the involvement of these cells in the cartilage would appear to be a common feature of IBV infection. In the lungs, IBV infection appeared to be located in epithelial cells.

Although viral infectivity was examined on only 3 days p.i. viral replication could be demonstrated as early as 2 days p.i. in both the trachea and lungs. The greatest concentration of virus in the trachea was seen on day 4 and in the lung on day 6, that is, the last day examined. Since the trachea would presumably be exposed to virus before the lung in birds exposed after nasal inoculation, viral replication may be expected to be established in the trachea first. We are now extending this work by examining infectivity before 2 days and after 6 days p.i. to determine the early events in virus–host interaction and the persistence of virus not only in the trachea and lungs, but also in additional tissues, including the cecal tonsils and kidneys.

The broad specificity and practical application of the in situ cytohybridization technique using enzyme detection systems rather than radioactive isotopes can be adapted for use by minimally trained personnel for direct rapid diagnosis of necropsy tissue in the face of outbreaks requiring immediate mobilization of control measures. We are currently developing tissue blot assays which will further simplify these procedures for use in facilities without equipment for sectioning tissues.

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