APPLICATIONS OF IMMUNOFLUORESCENCE IN VETERINARY VIRAL DIAGNOSIS

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ABSTRACT

This paper describes some applications of immunofluorescence in the laboratory diagnosis of veterinary virus infections. These include (1) the direct examination of clinical material for virus antigens, a procedure particularly useful for rapid diagnosis of Aujeszky's disease and bovine respiratory disease (2) the recognition of the growth of 'difficult' and/or non-cytopathogenic viruses eg avian infectious bronchitis virus in eggs and BVD virus and rotaviruses in cell cultures (3) the detection of viral antibodies by indirect immunofluorescence and (4) the recognition of new viruses eg an entero-like virus associated with runting in broiler chickens.

INTRODUCTION

This paper describes the principle applications of immunofluorescence in virus diagnosis in this laboratory. These fall into 4 main categories (a) direct examination of clinical material for virus antigens, (b) recognition of the growth of 'difficult' and/or non-cytopathogenic viruses in various culture systems, (c) detection of viral antibodies by indirect immunofluorescence and (d) recognition of new viruses.

MATERIALS AND METHODS

Conjugation of antisera

Antisera for direct immunofluorescence were conjugated with fluorescein isothiocyanate isomer 1 (FITC) (Sigma Chemical Company Ltd., London).

Ten ml amounts of sera were cooled in an ice bath and the globulin precipitated by slow addition of cold, saturated ammonium sulphate to a final concentration of 50% for mammalian antisera and 33% for avian antisera. The mixture was stirred for 15 mins and centrifuged for 10 mins at 4°C at 4,000 g. The supernatants were discarded and the precipitated globulins resuspended in 10 ml 33% or 50% saturated ammonium sulphate solution in 0.15 M saline. These were centrifuged as before, the supernatants discarded and the precipitates resuspended in 10 ml 0.15 M saline. These solutions were dialysed against 100 volumes of 0.15 M saline for 2 days at 4°C. Following dialysis, the globulin solutions were centrifuged at 3,000 g for 10 mins and the total protein content estimated using a UV spectrophoto-

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meter at 280 nm (O.D. 1.0 = 1.0 mg protein/ml). FITC was added to the globulin solutions at a ratio of 3.5 mg FITC to 100 mg protein. The fluorescein powder was dissolved in 1.5 ml (15% total volume) of carbonate-bicarbonate buffer, pH 9.0 (3.7 g sodium bicarbonate and 0.6 g sodium carbonate in 100 ml water) and added slowly to the chilled globulins. The mixture was incubated at 4°C for 18 hours with gentle stirring.

Six g Sephadex G25 was taken for each 10 ml labelled serum and swollen in phosphate-buffered saline (0.01 M phosphate, 0.15 NaCl, pH 7.2) (PBS). A column was poured with the Sephadex and the labelled serum added and eluted with PBS. The first peak was collected.

One g DEAE-cellulose was taken for each ml of conjugate and equilibrated in PBS. A column was poured, the labelled globulin was added and eluted with PBS. All the elutate which was green in colour was collected in small aliquots and tested for potency and specificity. The positive fractions were pooled, dispensed in 1 ml amounts and stored at -70°C.

Absorption of conjugated antisera

All conjugates used for direct immunofluorescence were absorbed with tissue homogenates. In general, homogenates used for absorption were homologous with the tissue being examined and all conjugates were also absorbed with homogenised bovine lymph nodes. Material for homogenates was taken from animals which were demonstrated to be free of the virus under test and processed in the following manner. The tissue was washed in PBS, cut into small pieces and washed in PBS until free of blood. The tissue fragments were homogenised in an equal volume of PBS, frozen at -70°C, thawed and centrifuged at 3,000 g for 20 mins. The cell deposit was resuspended in PBS and centrifuged as before. This was repeated until the supernatant was clear. After the final centrifugation the supernatant was decanted and the homogenised tissue deposit resuspended in a small volume of PBS to make a workable slurry. This was dispensed in 10 ml amounts and stored at -20°C. For absorption, equal volumes of homogenate and conjugate were mixed for 24 hours at 4°C. The mixture was centrifuged at 5,000 g for 30 mins at 4°C and the supernatant harvested. The absorption procedure was repeated and the final supernatant fluid passed through a 0.45 μ Millipore filter, dispensed in 1 ml amounts and frozen at -70°C.

Procedures for preparation and immunofluorescent staining of tissues

Samples of nasal mucus were collected using a portable suction

apparatus and shaken with glass beads in 10 ml amounts of PBS. They were decanted and centrifuged at 2,000 g for 10 mins and the supernatant discarded. The cell pellets were resuspended in 1 ml PBS and smears made on clean, degreased microscope slides. These were air dried at room temperature and fixed in acetone for 10 mins at room temperature (McNulty et al., 1983).

Impression smears from necropsy material were made as soon after death as possible and a number of different sites were taken from the tissues under test. These tissues were cut using a sterile scalpel blade and the clean freshly cut surfaces used to make the smears on degreased, glass microscope slides. Smears were dried and fixed in acetone as above.

Tissues for cryostat sections were placed in isopentane, chilled in a liquid nitrogen bath, transferred to a freezing cryostat and sections 4 μ thick cut. These were fixed in acetone for 30 mins at -20^{o}C.

Cell cultures for immunofluorescence were grown on coverslips and fixed in acetone for 10 mins at room temperature.

Following fixation, tissues were stained for 1 hour at 37^{o}C with FITC conjugates, washed in several changes of PBS for at least 30 mins, mounted in buffered glycerol, and examined using incident ultra-violet illumination.

Fluorescent microscope

All instrumentation was supplied by E Leitz (Instruments) Ltd., 48 Park Street, Luton. This comprised the following:

1. Ortholux 2 microscope stand.
2. Pleomopak 2.2 fluorescence illuminator.
3. Filter block 12-T-Auramine, FITC, FDA.
4. Stabilised starter unit XBO75/HBO100.
5. Lamphouse 100Z with HBO100 mercury vapour burner.
6. Fluorite oil X40/1.30 objective.
7. Periplan GF X10 eyepieces.
8. Supression filters. EGG455-1 EGG4751.

RESULTS AND DISCUSSION

Direct examination of clinical material

Direct immunofluorescent examination of clinical material has been more widely used in medical (Gardner and McQuillin, 1980) than veterinary virology. Its main advantage is speed. It avoids the necessity of isolating

the virus before identifying it, and combines, in one step, the dual objectives of virus detection and identification. The main problem with immunofluorescence is that interpretation of staining is sometimes rather subjective and calls for considerable experience.

Direct immunofluorescence can be carried out on impression smears of various tissues made on glass microscope slides or on cryostat sections. The latter method has traditionally been the more widely used. Results with cryostat sections are generally easier to interpret as the pattern of staining and the precise location of fluorescing cells within a piece of tissue can usually be established. Obviously, however, staining of impression smears is quicker and less laborious. A practical approach is to examine material first by impression smears and to confirm positives and doubtfuls by cryostat sections. Whatever method is used, a specific, high titre primary antiserum is essential. Antisera which give little or no non-specific staining with cultured cells may produce some non-specific staining with clinical material, particularly if large numbers of inflammatory cells are present. This non-specific staining can usually be removed by absorption with tissue homogenates. Because most commercially available FITC-labelled antispecies immunoglobulins produce non-specific staining with clinical material, we prefer to use the direct rather than the indirect method of immunofluorescent staining.

Virus infections for which direct immunofluorescence is routinely used for diagnosis in this laboratory are listed in Table I.

TABLE 1 Routine applications of direct immunofluorescence on clinical material at Stormont.

Species	Specimens	Viruses
Bovine	Nasal mucus	Respiratory syncytial
	Lung*	Parainfluenza type 3
		Corona
		BVD
		IBR
	Foetal tissues*	BVD
Porcine	Brain, pharynx	Aujeszky's disease
	Foetal tissue*	Parvo
Fowl	Bursa	IBD
	Trachea*	ILT
		ND

* Cryostat sections preferable

Immunofluorescence has proved particularly useful for rapid diagnosis of Aujeszky's disease in pigs (Fig. 1). Allan et al. (1984a) compared the

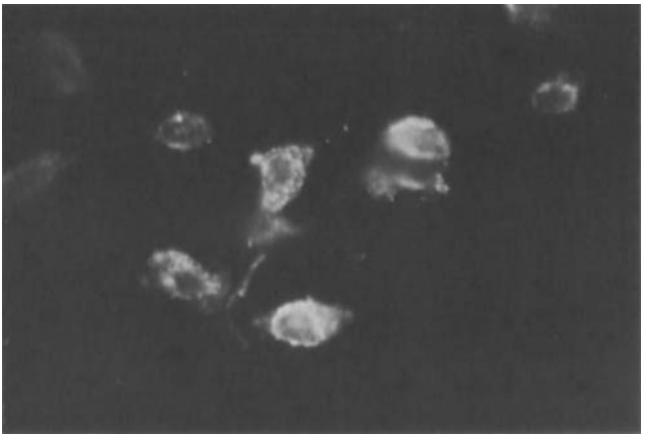

Fig. 1 Aujeszky's disease virus antigens in brain smear of young pig which died with nervous signs.

sensitivity of immunofluorescence and virus isolation for detecting Aujeszky's disease virus in experimentally infected pigs. Virus isolation from pharynx was more successful than from brain specimens, reflecting the higher titres of virus in the pharynx. Immunofluorescent staining of pharyngeal impression smears was slightly more sensitive than virus isolation from pharynx in that immunofluorescence remained positive when circulating antibody prevented virus isolation. In contrast, immunofluorescent staining of impression smears of anterior and mid-cerebrum and medulla oblongata was slightly less sensitive than virus isolation from the same material. With 166 specimens of brain and pharynx from 78 suspected field cases of Aujeszky's disease, there was 98% agreement between the results obtained by virus isolation and immunofluorescent staining of pharyngeal and brain impression smears. Apart from its speed and sensitivity, immunofluorescence has the advantage that it can still detect virus antigen in material in which virus infectivity has been inactivated by post-mortem autolysis.

Another useful application for direct immunofluorescent staining of clinical material has been in the diagnosis of bovine respiratory disease, particularly indoor calf pneumonia. The technique is particularly suitable here because some of the viruses involved eg respiratory syncytial virus (RSV) and coronavirus, are extremely difficult to isolate in cell cultures.

Furthermore, most isolates of bovine virus diarrhoea/mucosal disease (BVD) virus are non-cytopathogenic and immunofluorescent staining of infected cell cultures is necessary to demonstrate these. Mixed virus infections are sometimes associated with indoor calf pneumonia. These are easier to detect by direct immunofluorescence than by virus isolation. When virus isolation is used, slow growing viruses are usually eclipsed by rapidly growing viruses and may be missed. Epithelial cells present in samples of nasal mucus are examined for viral antigens by staining with a battery of conjugated antisera (McNulty et al., 1983). This works well with all the viruses listed in Table 1 (Fig. 2), except for BVD virus, where the low

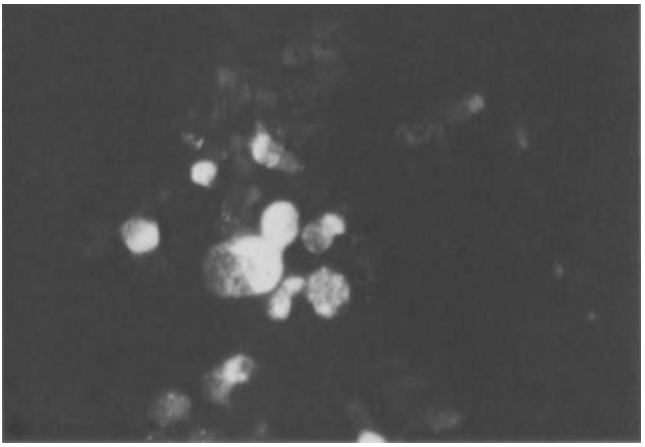

Fig. 2 Respiratory syncytial virus antigens in epithelial cells from nasal mucus of a calf with respiratory disease.

concentration of viral antigen and the diffuse pattern of staining can make interpretation very difficult. Impression smears and cryostat sections of lung tissue (Fig. 3) can be examined when necropsy material is available. In a study of calves experimentally infected with RSV, the results of immunofluorescent staining of lung impression smears were in complete agreement with those obtained using cryostat sections (McNulty et al., 1983).

Direct immunofluorescence on clinical specimens also has applications in avian diagnostic virology. Diagnosis of infectious bursal disease (IBD) by virus isolation is complicated by the fact that very few strains of IBD virus grow in cell cultures and not all strains grow in eggs. The only reliable method of IBD virus isolation is by inoculation of specific pathogen free chickens, which is obviously inconvenient and time consuming. Direct immunofluorescent staining of bursal impression smears provide an easy,

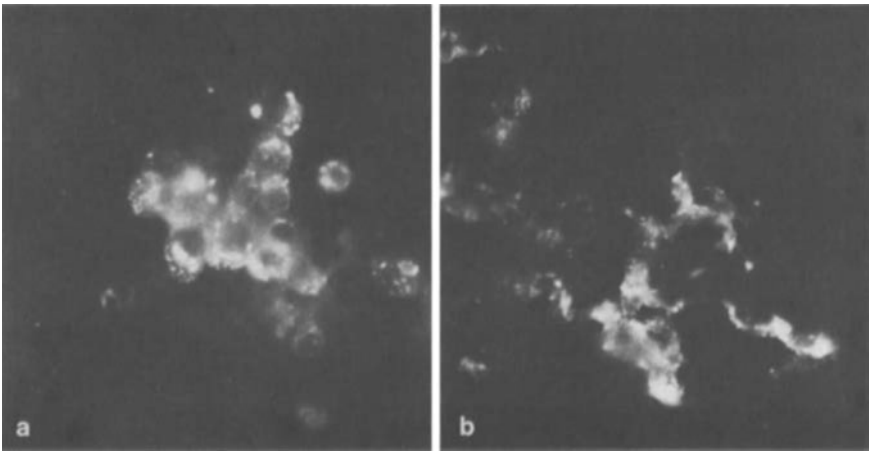

Fig. 3 Respiratory syncytial virus antigens in (a) impression smear and (b) cryostat section of pneumonic calf lung.

rapid and sensitive means of detecting the virus (Allan et al., 1984b). Newcastle disease virus and infectious laryngo-tracheitis virus are not difficult to isolate in either cell cultures or eggs. However these viruses cause diseases which are notifiable in Northern Ireland. Rapid diagnosis is important because it enables control measures to be put into effect as soon as possible. This is critical in controlling the spread of Newcastle disease, in which large amounts of virus become airborne and can be carried for miles on the wind. Although less sensitive than virus isolation, immunofluorescent staining of cryostat sections of trachea can be used for rapid diagnosis of both these diseases. Fluorescence occurs in the tracheal epithelial cells.

Recognition of the growth of 'difficult'/non-cytopathogenic viruses in various culture systems

Immunofluorescent staining of allantoic cells has been used in this laboratory for over a decade for isolating and detecting growth of avian infectious bronchitis (IB) virus in eggs (Fig. 4). Immunofluorescence has several advantages over the standard embryo dwarfing test as a criterion for the detection of IB virus. It is a specific test whereas dwarfing is not. It can be carried out in half the time required for dwarfing and fewer eggs are required. Furthermore, immunofluorescence is more sensitive because many isolates of IB virus require 4 to 8 embryo passages before they produce dwarfing. In contrast, only 2 embryo passages are generally

required for diagnosis by immunofluorescence (Clarke et al., 1972).

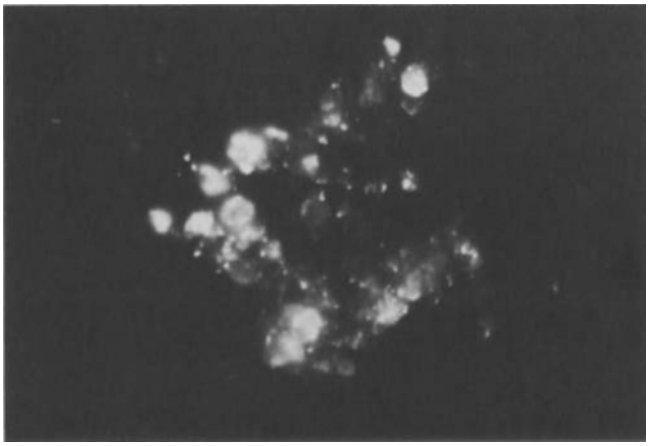

Fig. 4 Avian infectious bronchitis antigens in allantoic cells of eggs inoculated with tracheal material from pullets with respiratory disease.

While some bovine respiratory viruses eg RSV and coronavirus are difficult to isolate in cell cultures, isolation attempts in bovine tracheal organ cultures are often successful. Neither RSV nor coronavirus causes ciliastasis. However growth of these viruses in tracheal organ cultures can be monitored by immunofluorescent staining of cells harvested from the culture media (Fig. 5).

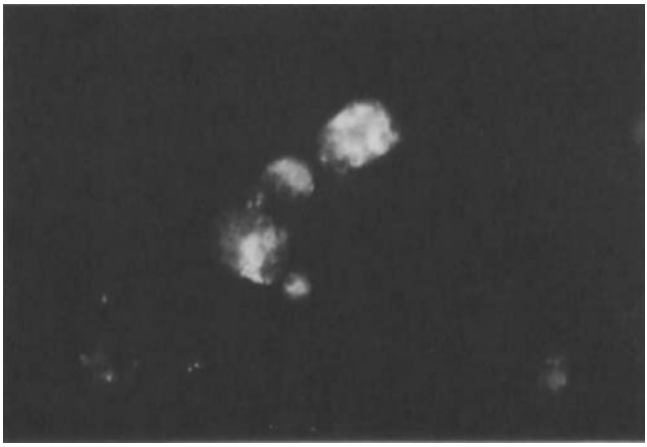

Fig. 5 Coronavirus antigens in cells harvested from bovine tracheal organ culture inoculated with lung material from calves with respiratory disease.

BVD virus is one of the commonest bovine viruses isolated in this

laboratory. This virus is also a frequent contaminant of foetal bovine serum and hence also of many cell cultures. As mentioned above, most isolates of BVD virus are non-cytopathogenic. Immunofluorescent staining of susceptible cell cultures inoculated with suspect material is the best way of detecting these viruses. Immunofluorescence due to BVD virus is characteristically diffuse and often dull (Fig. 6). The choice of objective

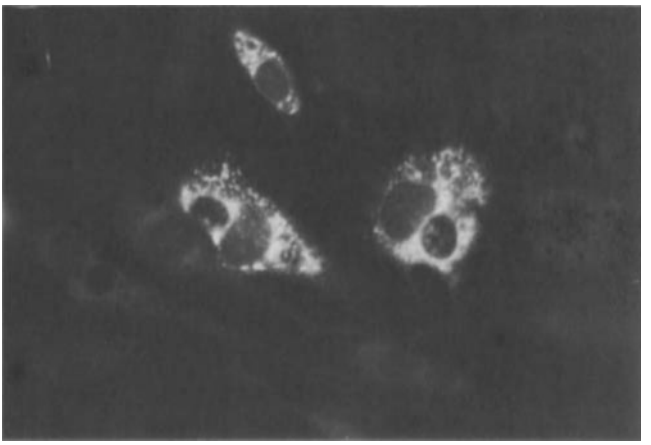

Fig. 6 BVD virus antigens in foetal bovine lung cells.

lens is critical here. BVD virus immunofluorescence detected with a X40 lens with a numerical aperture of 1.30 is not always visible using a X40 lens with a numerical aperture of 0.65.

Rotaviruses have proved difficult to adapt to growth in cell cultures using conventional techniques. However, by treating faecal specimens containing rotavirus with trypsin, some, but not all rotaviruses can be grown serially in cell cultures. Immunofluorescence has proved invaluable in monitoring the growth of these isolates (Fig. 7), most of which are non-cytopathogenic. Immunofluorescence has also been used to recognise isolates of atypical rotavirus ie those which lack the conventional rotavirus group antigen (McNulty et al., 1981).

Detection of viral antibodies by indirect immunofluorescence

Viral antibodies can be rapidly and fairly simply demonstrated by indirect immunofluorescence. The viral antigen is reacted with the serum under test and counterstained with the appropriate FITC-conjugated anti-species immunoglobulin. In general, this test detects antibodies directed against both type and group antigens of a particular virus. The broad

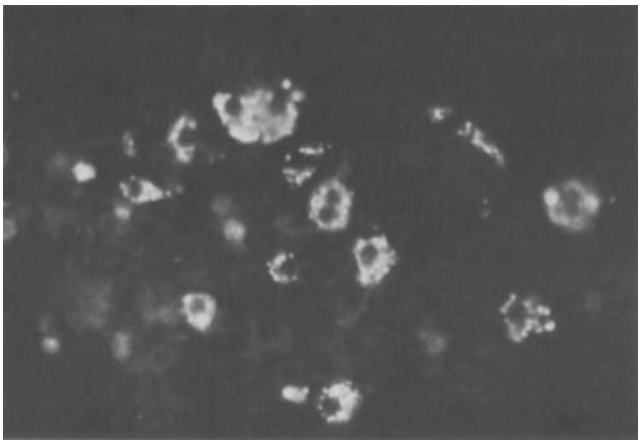

Fig. 7 Rotavirus antigens in chick embryo liver cells inoculated with faecal material from 3 week old broiler chickens.

specificity afforded by reaction with group antigens makes indirect immunofluorescence very useful for survey purposes. Furthermore, indirect immunofluorescence is sensitive eg a microtitre indirect immunofluorescence test detected antibodies to adenovirus in 70.8% of 595 avain field sera, whereas the standard agar gel immunodiffusion test detected antibodies in only 52.6% of these sera (Adair et al., 1980). Similarly, indirect immunofluorescence has been shown to be more sensitive than the serum neutralisation test for detecting antibodies to infectious laryngo-tracheitis virus and the haemagglutination inhibition tests for bovine parainfluenza virus type 3 and Egg Drop Syndrome adenovirus (B M Adair, personal communication).

Indirect immunofluorescence provides a convenient method for detecting antibodies to non-cytopathogenic viruses eg rotaviruses (McNulty et al., 1979, 1984a). Cryostat sections of infected tissues can also be used as a source of viral antigens, so the method can also be used for viruses which have not been adapted to growth in cell cultures.

Another application of indirect immunofluorescence is to look for evidence of infection in animal species from which a particular virus has not yet been isolated. Thus, although neither coronaviruses nor pneumoviruses have been isolated from sheep, we have found antibody reacting with bovine enteric coronavirus and bovine RSV in sheep sera. The staining pattern obtained with sera from 'heterologous' animal species may differ from that seen with sera from the 'homologous' species. For example, most ovine sera do not react with the 'flecks' of viral antigen on and outside the

plasma membranes of cells infected with bovine RSV, but stain only the cytoplasmic inclusions. This suggests that the pneumovirus which infects sheep shares a nucleocapsid antigen with bovine RSV, but has different surface antigens.

Recognition of new viruses

The way in which immunofluorescence has been used in the recognition of previously unknown virus infections is illustrated below.

During investigation of outbreaks of infectious runting in broiler chickens, small round viruses resembling enteroviruses were detected by electron microscopy in the faeces of birds from affected flocks during the first week of life. However attempts to isolate these viruses in cell cultures were unsuccessful. This suggested that these viruses might be bacteriophages rather than animal viruses. In an attempt to answer this question, the small round viruses were partially purified from infected faeces and an antiserum to them was prepared in chickens and conjugated with FITC. This conjugate was then used to look for small round virus antigens in chickens orally inoculated with faecal suspensions containing the virus. Specific immunofluorescence was observed in the villous epithelial cells of the small intestine (Fig. 8). The specificity of this immunofluorescence was

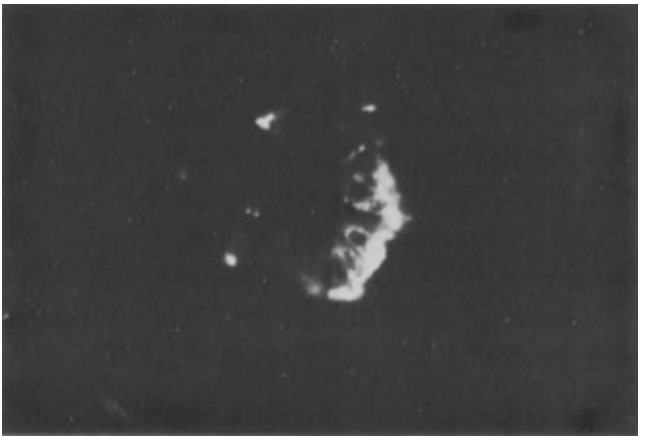

Fig. 8 Immunofluorescence in villous epithelial cells of jejunum of broiler chicken orally inoculated with an enterovirus-like agent associated with the runting syndrome.

confirmed by thin section electron microscopy, which revealed crystalline aggregates of enterovirus-like particles in the cytoplasm of enterocytes. Further characterisation of the small round virus indicates that it appears

to be a previously unknown chicken enterovirus (McNulty et al., 1984b).

The detection in sheep of antibodies reacting with bovine enteric coronavirus antigens has been described above. A FITC-conjugated antiserum to bovine enteric coronavirus has been used to look for coronavirus antigens in necropsy material from sheep. So far, only a small number of ovine carcases has been available for examination. However, immunofluorescence has been detected in the cytoplasm of bronchiolar epithelial cells of a lamb with pneumonia. This provides further evidence for coronavirus infection in sheep.

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