# RESPIRATORY SYNCYTIAL VIRUS AND PARAINFLUENZA TYPE 3 VIRUS INFECTIONS IN CATTLE

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## SUMMARY

Respiratory diseases are economically important in the U.K. Those which affect young, immature, housed calves have been difficult to define in aetiological terms despite many years of research. Many viruses, bacteria and mycoplasmas have been associated with these diseases but in recent years the pathogenic roles of respiratory syncytial virus and parainfluenza type 3 virus have demanded particular attention. The aim of this thesis was to investigate the part played by these two viruses in the respiratory diseases of cattle with particular reference to the pathogenesis, diagnosis and differential diagnosis of these two infections in naturally occurring outbreaks of disease. These primary aims were to be accompanied by attempts to produce viral infection in experimental calves using isolates of the viruses obtained during the field investigations.

A multidisciplinary investigation into 22 outbreaks of acute respiratory disease in immature cattle was carried out using clinical, microbiological and pathological techniques. Eight outbreaks were detected in which there was unequivocal evidence of infection with either RS virus or PI3 virus. In addition 16 individual cases of RS viral infection and one case of PI3 viral infection were detected in material referred from Veterinary Investigation Centres in England.

Common clinical findings in RS viral cases were hyperpnoea, tachypnoea and, in severe cases, dyspnoea. On pathological examination there was pneumonia involving the cranial parts of the lung lobes and, in fatal cases, severe interstitial emphysema with the formation of gas-filled bullae in the caudal lobes. Microscopically syncytium formation in the epithelia of the bronchioles, the alveoli and, very occasionally, the bronchi was found. Virus-infected cells sometimes contained eosinophilic intracytoplasmic inclusion bodies. Three new strains of RS virus were recovered during these investigations.

Relatively few individual cases of PI3 viral infection were discovered, however, in these there was also inclusion body formation in the cytoplasm of respiratory epithelial cells and, occasionally, epithelial syncytia were present. Secondary bacterial infection was common with Pasteurella multocida and Pasteurella haemolytica types A1 and A2 involved.

Due to the similar nature of the specific pathological changes due to RS viral and PI3 viral infections it was found necessary to develop a technique which could distinguish between these two infections in the lung. Immunofluorescent staining of viral antigens in either acetone-fixed or formalin-fixed sections of pneumonic lung was developed for this. The detection of antigens in lung which was been fixed for conventional histopathology was a significant advance on previous techniques as it enabled the relationship between the viral antigens and the histopathological changes to be assessed more fully.

Experimental infections were carried out using RS virus and PI3 virus. Unfortunately it was not possible to use the isolates of RS virus which had been recovered during the field investigations. Using an RS virus isolate obtained from a another laboratory calves were successfully infected. Combined infection with the cattle lungworm Dictyocaulus viviparus was attempted in order to potentiate the effects of the viral infection. Although viral infection was established there was no unequivocal evidence that this was increased in severity by concomitant D.viviparus infection.

Two groups of calves, one aged two weeks and the other four months, were infected with a recent field isolate of PI3 virus. In both groups viral infection was associated with clinical signs of respiratory disease and pathological lesions, specifically recognisable as being due to PI3 viral infection, were produced in the lower respiratory tract.

SECTION ONE

# GENERAL INTRODUCTION

# REVIEW OF THE LITERATURE ON RESPIRATORY SYNCYTIAL VIRUS AND PARAINFLUENZA TYPE 3 VIRUS INFECTIONS IN CATTLE

## GENERAL INTRODUCTION

Respiratory diseases have troubled the cattle industry for many years. The most spectacular losses are those due to mortality, however, significant costs may also be incurred during the aftermath of a respiratory disease incident due to loss of productive efficiency and the cost of veterinary attention. Of the respiratory diseases which affect cattle in the U.K. some are well defined clinically and pathologically e.g. parasitic bronchitis, severe infectious bovine rhinotracheitis and the pneumonias of adult cattle (Jarrett and others, 1957a; Wiseman and others, 1979; Breeze and others, 1975). However, the respiratory diseases which veterinarians in general practice most frequently encounter, that is those in housed calves which have never been at grass, have been difficult to define properly on aetiological grounds and despite years of research are still incompletely understood. Consequently, treatment is at best symptomatic and prophyllaxis empirical. A variety of viruses, bacteria and mycoplasmas have been implicated in these diseases and difficulties have arisen in identifying specific infections and in assessing their significance in relation to the pathological changes in the lung.

The particular role of viruses in causing these diseases has long been the subject of controversy. In early experiments lesions were produced in the lungs of calves by inoculating bacteria-free filtrates of pneumonic lung (Lamont and Kerr, 1939) and at that time filter-passing agents were assumed to be viruses. Similar experiments, with similar results, were performed by other workers (Jennings and Glover, 1952). In the 1950's researchers at the Glasgow Veterinary School published a series of papers describing the clinical and pathological features of some respiratory diseases of calves and Jarrett (1954) suggested a classification of these pneumonias on morphological grounds. By analogy with pneumonias in other species two entities were thought to be associated with viral infection. The first, characterised by peribronchial and peribronchiolar accumulation of lymphocytic cells and given the name "cuffing pneumonia", was

compared with "virus pneumonia" of pigs and "grey lung disease" of mice. Although these latter two conditions were generally thought to be of viral origin Jarrett pointed out that this had not been specifically proven and highlighted the danger of inferring aetiology purely from the morphological appearance of the lesions. The second pneumonia, this time characterised by epithelial changes, was compared to two diseases of proven viral aetiology. In these cases the outstanding features were that the alveoli, alveolar ducts and terminal bronchioles were lined with a single layer of low cuboidal epithelium in which syncytia were formed by focal proliferation. Epithelial cells in the alveoli, the bronchioles and the bronchi contained eosinophilic intracytoplasmic inclusion bodies. These changes strongly resembled those in distemper virus pneumonia in dogs and measles giant cell pneumonia in man and Jarrett suggested that a related virus might be present in the bovine. The condition was named "inclusion body pneumonia" and subsequently was recognised elsewhere in the U.K. (Baxter, 1960). It was also recognised that bacterial infection of a pneumonia of this type could obscure the original epithelial changes giving the lesion a different appearance.

Since the advent of convenient tissue culture techniques and of methods of localising viral antigens in pneumonic lung much effort has been devoted to the study of viruses in the respiratory diseases of housed calves. The aim of this thesis is to apply some of these techniques to the study of the calf pneumonias, with particular reference to the viruses now thought to be involved in the syndrome of "inclusion body pneumonia" namely respiratory syncytial (RS) virus and parainfluenza type 3 (PI3) virus. Whilst most attention has been directed at the calves themselves it should not be forgotten that older animals, including adults, may be important in the epidemiology of the viruses which are associated with these respiratory diseases.

## REVIEW OF THE LITERATURE ON RESPIRATORY SYNCYTIAL VIRUS AND PARAINFLUENZA TYPE 3 VIRUS INFECTIONS IN CATTLE

In view of the extensive literature concerning these two viruses they will be considered separately with a final section on dual infections.

### RESPIRATORY SYNCYTIAL VIRUS

Respiratory syncytial virus is a member of the genus pneumovirus in the family Paramyxoviridae (Fenner, 1976). It was first isolated from a throat swab taken from one of a group of chimpanzees during an outbreak of upper respiratory tract disease (Morris and others, 1956) and initially was named chimpanzee coryza agent (CCA). Experimental exposure of chimpanzees to this virus, even those with serum complement fixing (CF) antibodies, resulted in infection and was associated with clinical signs of respiratory disease. Of greater interest, however, was the fact that one of the attendants handling the infected chimpanzees developed an upper respiratory tract illness indistinguishable from some "common colds". Viral isolation was attempted from a throat swab but was unsuccessful, however, a significant (ie four-fold or greater) rise in titre of CF activity to CCA was demonstrated between a serum sample taken during the acute phase of his illness and one taken three weeks later. From this finding it appeared that humans were susceptible to infection with this or a related virus and could develop clinical symptoms. Attempts to show an association between infection with CCA and respiratory disease in a wider population were inconclusive although some patients had high titres of CF activity in their serum.

In 1957 a virus serologically related to CCA was recovered from throat swabs taken from two infants with lower respiratory tract disease (Chanock and others, 1957) and sera from both patients become positive for CF antibody to the virus during the convalescent phase of the illness. The virus produced multinucleated syncytia in tissue culture cells and was shown to be

antigenically distinct from the other viruses and microbiological agents which were known to cause respiratory disease at that time. In a later paper the association of the newly discovered virus with respiratory disease was studied (Chanock and Finberg, 1957) and although the results were again inconclusive infection in patients with respiratory disease was found to be more common than in those without. In view of the site of isolation of the virus and its typical syncytial cytopathology in tissue culture it was proposed that it be re-named respiratory syncytial virus.

Since this early work many studies have been carried out on the role of RS virus in human respiratory disease (Beem and others 1960; Holzel and others, 1965) and it is now recognised as the major serious lower respiratory tract pathogen in infants under six months of age and one of the many causes of the "common cold" in adults. Important points which have arisen from human research are that components of the immune system may play a role in the pathogenesis of the disease (Chanock and others, 1970), that infection may take place in the presence of serum antibody and that re-infection is probably frequent but may not be associated with the severe clinical disease seen in some primary infections. Whether or not persistent or latent infection occurs in the individual is not yet clear and the virus is thought to survive in the population by repeat, possibly inapparent, infection of partially immune adults (Henderson and others, 1979).

The first indirect evidence that RS virus or a serologically related agent could infect cattle came from the observation that some calf sera, when used as a constituent of tissue culture medium, could inhibit the formation of syncytia by human strains of the virus (Taylor-Robinson and Doggett, 1963). Later this finding was investigated further to determine whether or not the inhibitory factor was specific antibody (Doggett and others, 1968). After comparing its properties with those of other inhibitors known to occur in bovine sera it was concluded that it was more akin to antibody, however, the authors were not prepared to state categorically that it was antibody until a virus serologically

related to RS virus had been isolated from cattle. They also assayed paired sera from 33 cattle with respiratory disease of unknown aetiology and found significant rises in titre of RS inhibitor in three calves. This work provided very strong circumstantial evidence that RS virus, or a serologically related agent, could infect cattle and suggested that it might play a role in some cases of respiratory disease.

Proof of the existence of RS virus in cattle was finally achieved in 1970 when a virus, serologically related but not identical to human RS virus, was recovered from the conjunctivo-nasal secretions of two adult cattle, from two separate farms each with an acute outbreak of respiratory disease (Paccaud and Jacquier, 1970). The virus was isolated in foetal bovine kidney cells and grew slowly at 37°C with one sub-passage being necessary before cytopathic changes were seen. Even after several passages in tissue culture the yield of virus was low and was not altered significantly by incubation at a lower temperature or an increased oxygen tension more representative of the nasal passages. Foetal bovine kidney and foetal bovine lung cells were found to be equally sensitive to the virus and were thought to be suitable for its further study. When stained with haemotoxylin and eosin (H&E) the syncytia induced by the replicating virus were seen to contain eosinophilic intracytoplasmic inclusion bodies. Virus infected cells and viral antigens did not haemadsorb or haemagglutinate guinea pig erythrocytes and this served to distinguish the new virus from PI3 virus.

The relationship between viral infection and clinical respiratory disease on the two farms from which the virus was recovered seemed clear. Virus was detected by isolation in both groups of animals during the acute phase of the disease and seroconversion occurred between acute and convalescent sera in most of the animals sampled. Only animals less than seven years old were affected and those cattle over two years old were most severely ill. The initial clinical signs were dramatic with sudden onset of pyrexia, nasal discharge and "signs of bronchopneumonia".

Response to treatment with cortisone and antibiotics was apparently good although the more severely affected animals took some time to recover. None of the people associated with the cattle had signs of respiratory disease. In a limited study on the distribution of antibody to bovine RS virus in a wider population it was found that 30% of cattle sera tested were positive. Sera from horses were negative.

Although the authors considered their investigations to have shown an association between bovine RS viral infection and respiratory disease they felt that its causal role could not be proven until experimental infections had been carried out. However, their paper stimulated interest in the virus and over the next five years reports appeared from various parts of the world on bovine RS virus and its association with respiratory disease.

Workers in Japan reported an epizootic of acute respiratory disease associated with RS viral infection which had apparently swept through the islands of that country between October 1968 and April 1969 (Inaba and others, 1970; Inaba and others, 1972). Clinical signs were dramatic with a sudden onset of pyrexia, nasal discharge, lachrymation and respiratory distress in groups of animals. Over 43,000 cases were diagnosed on clinical grounds but this was confirmed by viral isolation and serology in only three affected herds. In these herds there was a significant number of seroconversions to bovine RS virus but not to other respiratory viruses. However, when examining sera from clinically diagnosed cases in other parts of Japan it was found that although the seroconversion rate to bovine RS virus was high (62% of 271 paired sera) there were also significant numbers of seroconversions to bovine adenovirus type 7 and, or PI3 virus in some areas. The case fatality rate was low (126,0.23%) but where post-mortem examinations had been carried out the features mentioned were "hepatisation" of ventral parts of cranial lung lobes, "oedema in the lungs" and emphysema. Experimental infection of a six month old calf with a strain of bovine RS virus isolated during the epizootic produced mild clinical signs similar to those in the

majority of natural cases. There was fever, anorexia, depression, serous nasal discharge and lachrymation. Virus was recovered from nasal secretions between days five and 10 after infection and the titre of virus produced reached $10^{3.5}$ $TCID_{50}$ per ml of nasal secretion. Neutralising antibody appeared in the serum by day 14 after infection.

In the USA bovine RS virus was recovered from the nasal secretions of calves with acute respiratory disease (Smith and others, 1975) and was implicated by the same authors in another outbreak of respiratory disease on the basis of serological findings. Of five calves which were experimentally infected with the virus clinical disease was noted only in those with pre-existing serum antibody. Bovine RS virus was also recovered from cattle with respiratory disease in other parts of the world e.g. Belgium (Wellemans and others, 1970), Norway (Odegaard and Krogsrud, 1977), Hungary (Koves and Bartha, 1975) and Czechoslovakia (Pospisil and others, 1978). The Norwegian paper described a rapidly spreading acute respiratory disease characterised clinically by pyrexia, forced respiration, coughing, nasal discharge and conjunctivitis. In some cases there was extensive subcutaneous emphysema. Deaths were rare but when they occurred post-mortem examination revealed epithelial damage in the trachea and the bronchi with haemorrhages, bronchopneumonia and marked interstitial emphysema. Parainfluenza type 3 virus was also isolated from two groups of animals with this condition but as there was no concomitant seroconversion to this virus its presence was regarded as incidental. A nasal swab from one outbreak yielded bovine RS virus and examination of sera from "most" cases showed seroconversion to this virus but not to other viruses. In view of the acute nature of the disease the authors considered that this probably represented the first introduction of the virus into Norway. Similar clinical and post-mortem findings were found in the Hungarian report where the virus was recovered from nasal secretions and also from a lymph node of an animal which had been slaughtered, although precisely which lymph node was not specified. On clinical grounds these authors suspected that a

similar syndrome could be caused by PI3 virus and this observation highlighted an area of potential controversy and difficulty in differential diagnosis. In Holland bovine RS virus was considered to be the cause of a condition known as "Pinkengriep" which literally translated, means yearling influenza (Holzhauer and van Nieuwstadt, 1976). Classically this affected yearling cattle after being housed following their first grazing season and was characterised by sudden onset tachypnoea, hyperpnoea and varying degrees of respiratory distress. Fatal cases had pneumonia involving the cranial parts of the lung lobes and severe interstitial emphysema. In some outbreaks there was also infection with PI3 virus but this was considered to be an incidental finding.

Edington and Jacobs (1970) drew the attention of veterinary practitioners to the probable existence of bovine RS virus in cattle in the U.K. Later, they were able to report the isolation of the virus from nasal swabs collected during an investigation into an "early" acute outbreak of respiratory disease in a group of 14, four months old calves (Jacobs and Edington, 1971). In this incident the clinical signs were sudden onset of serous nasal discharge, pyrexia and tachypnoea. There were no deaths. Paired sera from four of five animals sampled showed seroconversion to bovine RS virus but not to PI3 virus. A gnotobiotic calf was successfully infected with the viral isolate and showed a marked biphasic pyrexia. In a later paper by the same authors the experimental work was greatly extended (Jacobs and Edington, 1975). Gnotobiotic, colostrum-deprived and conventional calves were used and all three types were successfully infected with the virus, although not consistently. Some showed a clinical response, albeit mild, and virus was recovered from nasal secretions between days four and 10 after infection, and from necropsy samples taken from various levels in the respiratory tract up to day 10. No isolations were made from non-respiratory tissues although a wide range were sampled. Infection was possible in calves with pre-existing serum antibody but, unlike Smith and others (1975), they found no evidence of exacerbation of disease in these animals. Neither was the clinical severity of disease

affected by the dose of virus given, by its passage level in tissue culture nor by the cell type in which it was grown. Serum neutralising antibody appeared, in previously sero-negative calves, between eight and ten days after infection. Calves were killed at intervals after infection and although few macroscopic lesions were found at post-mortem examination there were microscopic changes in the epithelium and lamina propria of the respiratory mucosae. Focal areas of degeneration and necrosis of epithelial cells were found in the nasal passages, the trachea and the bronchi. In some cases neutrophils were seen in the epithelium and in the lamina propria. Multinucleated syncytia were occasionally seen in the lumen of bronchioles although the origins of these cells was not clear. Alveolar lesions, other than collapse and congestion, were rarely mentioned. In one calf, killed eight days after infection, intracytoplasmic inclusion bodies were seen in degenerating and in apparently healthy epithelial cells in the nasal mucosa. At the same time virus was recovered from a sample of nasal mucosa, however, virus was also recovered from the tracheal and the bronchial mucosa at this time although no inclusion bodies were seen at these sites.

The authors concluded that bovine RS viral infection was only one of possibly several factors which interacted to produce naturally occurring calf respiratory disease in the field. This was in view of the mild nature of disease caused by experimental infection of calves and because the pathological changes which were induced were not the same as, or as extensive as, those in naturally occurring respiratory disease.

One experiment performed by these authors was interesting from a comparative point of view. A gnotobiotic calf was successfully infected with a human strain of RS virus. A mild respiratory illness resulted and the virus was recovered from nasal secretions. This led to the suggestion that the human could act as a reservoir of infection for the bovine and vice versa. The bovine isolates with which Jacobs and Edington were working showed differing abilities to grow in cells of human origin. Whereas

three isolates did not grow in cells derived from a human cervical carcinoma one isolate did. Paccaud and Jacquier (1970) had differentiated bovine and human strains of RS virus by the inability of the former to grow in human cells. Whether this was true for all strains was now less clear.

The experimental work of Jacobs & Edington was largely confirmed by Mohanty and others (1975) working in the USA. In one of their calves, killed 14 days after infection, virus was isolated from the lung and multinucleated giant cells were present in the alveoli. The origin of these cells, however, was not clear and they did not contain inclusion bodies. Nevertheless, the authors considered these cells to be "characteristic of bovine RS virus multiplication". The same authors also studied the effect on calves of re-exposure to bovine RS virus (Mohanty and others, 1976). Calves challenged intra-nasally with the virus five weeks after an initial intra-nasal dose of virus were protected from clinical disease although three out of five became infected. The duration of viral shedding in nasal secretions was reduced and protection appeared to be related to the presence of antibody in nasal secretions. This gave hope that vaccination against disease due to bovine RS viral infection was feasible.

In addition to investigating its role in respiratory disease several workers had studied the physio-chemical properties of bovine RS virus (Ito and others, 1973; Inaba and others, 1973; Smith and others, 1975; Cash and others, 1977; Lehmkuhl and others, 1979). It was found to be a highly pleomorphic enveloped virus with a diameter ranging from 80 to 450nm (mean 200nm). On the outer surface of the envelope were club-shaped projections measuring 13-17nm long by 4-7nm wide and with their centres 7-9nm apart. The envelope itself was 7-15nm thick and enclosed herringbone-shaped strands of nucleocapsid of diameter 11-15nm. Its bouyant density in caesium chloride was 1.225g.cm$^{-3}$. The virus matured by budding from altered cell membrane and its genome was probably composed of RNA, as there was no inhibition of viral replication by 5-iodo-2-deoxyuridine.

Using polyacrylamide gel electrophoresis bovine RS virus was found to contain at least six polypeptides, four major and two minor, when grown in foetal bovine kidney cells. Two of the major polypeptides were glycosylated (molecular weights 41K and 33K) and two were non-glycosylated (41K and 27K). The minor polypeptides (25K and 10K) were non-glycosylated. Two isolates of virus, recovered three years apart, had identical polypeptide profiles. Human RS virus produced a very similar polypeptide profile to bovine RS virus when grown in foetal bovine kidney cells except that there was an additional non-glycosylated major polypeptide (molecular weight 38K). An analogous polypeptide was detected in bovine RS virus preparations produced in Madin and Darby bovine kidney cells. No functions were ascribed to any of the polypeptides.

In its physical properties bovine RS virus was sensitive to trypsin, was inactivated by chloroform, ether and 0.1% sodium deoxycholate and was also inactivated by low pH. At 56°C the virus titre declined very rapidly but was more stable at 4°C and very stable at -80°C. Cycles of rapid freezing and thawing had little effect on viral infectivity. Extensive attempts to demonstrate haemadsorbing or haemagglutinating activity were unsuccessful. The lack of these activities along with the nucleocapsid diameter and serological characteristics distinguish bovine RS virus from other members of the Paramyxoviridae.

Diagnosis of infection with bovine RS virus has presented problems, largely due to its lability and poor growth in tissue culture (Wellemans, 1977). Rapid development of antibody in infected animals was thought to neutralise infectious virus in nasal secretions and lung tissue even though the appearance of neutralising antibody in serum was, in fact, slow (Wellemans and Leunen,1975). Mohanty and others (1976) emphasised the importance of inoculating cell cultures soon after sampling and preferably without freezing and thawing the sample. Jacobs and Edington (1975) thought it vital to use fully susceptible cells and preferred bovine testicle cells, however, isolates have been

recovered in a wide range of cell types e.g. foetal calf kidney (Paccaud and Jacquier, 1970; Inaba and others, 1970) secondary calf lung (Jacobs and Edington, 1975) and bovine turbinate (Odegaard and Krogsrud, 1977). Using different temperatures of incubation (37°C and 33°C) and high and low oxygen tensions has not provided clear evidence as to which culture conditions are best (Rosenquist, 1974). Thomas and Stott (1981) used immunofluorescent staining of cells in nasal secretions to detect viral antigen. The results were comparable with virus isolation in experimentally infected calves, however, problems with non-specific staining detracted from the value of the test. They used a similar technique on frozen sections of pneumonic lung from calves which had died in natural outbreaks of disease and considered that this technique, in combination with serology, to be the methods of choice for field investigations. However, immunofluorescence staining of lung tissue is only applicable when dead animals are available. Wellmans (1977) found that diagnosis could be based on serum samples taken in the initial stages of the disease if 50% or more of the affected animals had CF antibody. Most serological investigations, however, have been based on paired sera, one being taken during the initial stages of the outbreak and another three to four weeks later.

At this stage, although much work had been done, several important questions remained to be answered. Most investigators had no doubt that bovine RS viral infection was associated with respiratory disease although the exact nature of the association was unclear. Some natural infections had been associated with severe clinical signs and even death, however, most infections were mild and non-fatal. Most of the deaths were thought to be due to secondary bacterial infection but other cases were bacteriologically sterile (Holzhauer, 1978) and Wellemans (1977) suggested that hypersensitivity reactions could play a part in the pathogenesis of the disease. The association of the virus with disease was based largely on evidence gained from the investigation of outbreaks of disease, although few specific outbreaks had been reported in detail. A temporal association had been shown between

infection with the virus and the presence of clinical respiratory disease. It was pointed out, however, that the significance of these observations was difficult to interpret as the distribution of the virus in healthy animals was largely unknown (Stott and others, 1978). By routinely sampling animals passing through a beef progeny testing station these authors showed that a significantly greater number of bovine RS viral infections were detected during outbreaks of respiratory disease than at other times. An interesting finding was that infection could be detected on more than one occasion in the same animal. It was suggested that this might represent either re-infection or, possibly, persistent infection in a proportion of individuals. Persistent infection was suggested as the source of virus in an outbreak of respiratory disease in a group of three months old calves which had been isolated from other cattle at 10 days of age (Thomas and others, 1980). Indirect or direct infection via people attending the calves was thought unlikely but was not specifically ruled out. In Canada reactivation of a "latent" infection due to the "stress" of movement was suggested as the source of virus in one disease outbreak (El-zhary and others, 1982). In-vitro experiments have shown that persistent infection may be established in cultures of bovine cells (Stott and others, 1977), however, this phenomenon has not yet been shown to occur in-vivo.

More recent work in the U.K. has strengthened the association of RS virus with respiratory disease in different parts of the country. In Northern Ireland a comprehensive investigation into 34 outbreaks of indoor calf pneumonia, occurring during the winter, revealed bovine RS viral activity in 11 on serological evidence (Bryson and others, 1978a; 1978b). Four of these outbreaks had a characteristic clinical presentation with dyspnoea being a prominent sign (Bryson and others, 1979a). Similar gross and microscopic pathological features were recorded in six animals with dyspnoea which had either died or been slaughtered. These comprised pneumonia involving the cranial parts of the lung lobes and severe interstitial emphysema with bulla formation in the caudal lobes. Histologically there was bronchiolitis and

alveolitis with epithelial syncytia containing eosinophilic intracytoplasmic inclusion bodies. Parainfluenza type 3 virus was isolated from all of the lungs examined and in three of the four outbreaks a significant proportion of the in-contact calves seroconverted to this virus. In three outbreaks there was seroconversion to bovine RS virus and in the fourth the initial serum titres were high. This syndrome resembled cases of acute respiratory disease associated with bovine RS viral infection which had been reported from the Netherlands (Holzhauer and van Nieuwstadt, 1976) and which had been given the name "Pinkengriep". Here, infection with PI3 virus was regarded as an incidental finding in such cases (Holzhauer, 1978). However, Bryson and his colleagues were more cautious and concluded that the relative importance of these two viruses in producing the severe lung damage noted in their cases was unclear.

In Scotland an outbreak of acute fatal pneumonia in housed suckled calves was attributed to bovine RS viral infection alone (Pirie and others, 1981a). The gross and histopathological features of animals which died or were slaughtererd during the first week of the outbreak were similar to those described in Northern Ireland, however, no evidence of infection with PI3 virus was found. Fifteen of 19 calves sampled had rising titres of antibody to RS virus and nine of these were four-fold or greater. No viruses were isolated, but the samples had been stored at -70°C before being tested. Six weeks after the acute phase seven calves, some of which had been seriously ill, were obtained for pathological and microbiological examination. In some of these animals there were moderately extensive pneumonic lesions but no interstitial emphysema. Microscopically the lesions were either bronchiolitis obliterans or a lymphocytic bronchiolitis as seen in "cuffing pneumonia" (Jarrett and others, 1953). It was not clear, however, whether these lesions represented sequelae to the viral infection or the development of a separate pneumonia.

The publication of this paper led to a controversy which probably characterises the differing views held on calf respiratory

disease. Pritchard and Edwards (1981) objected to the outbreak apparently being attributed solely to one micro-organism without apparently taking into account other possible factors such as "stress" and intercurrent infection. They also disagreed that bovine RS virus per se could cause fatal respiratory disease on the grounds that experimental infections were mild and that the survival rate from natural infection was high. Their view was that bovine RS virus was but one factor in a "multifactorial causal web" and could not be considered to be a cause of respiratory disease on its own. Pirie and others (1981b) replied saying that whilst other factors may have contributed to the outbreak these should not be overemphasised as, on the basis of their investigations, the primary lesions which resulted in the pneumonia, and therefore the disease, were due to infection of pulmonary cells with bovine RS virus and in that sense it could be called the cause of the disease. This did not imply that the pathogenesis of this infection was completely understood.

Recently extensive pneumonia and severe clinical respiratory disease has been produced by the experimental infection of young calves with bovine RS virus (Bryson and others, 1982). In one of the calves there was interstitial emphsysema with bulla formation in the caudal lung lobes. The authors concluded that this experiment proved that at least certain strains of bovine RS virus are per se important respiratory tract pathogens in young cattle.

There seems no doubt that bovine RS virus can be an important respiratory pathogen in calves in the U.K. although the epidemiology and pathogenesis of the infection are not fully understood and the scale of the problem which it causes nationwide is not known. Its interactions with other micro-organisms involved in respiratory disease have not yet been fully explored nor have the sequelae to viral infection.

PARAINFLUENZA TYPE 3 VIRUS

In 1959 in the USA a virus was isolated from the nasal mucus

of two calves with an acute respiratory disease, known colloquially as "shipping fever" (Reisinger and others, 1959). In tissue culture it produced syncytia which on staining with H&E contained eosinophilic intracytoplasmic and intranuclear inclusion bodies. Infected cells adsorbed guinea pig erythrocytes and preparations of viral antigens agglutinated guinea pig, bovine and human type 'O' erythrocytes. Serologically the virus was identical to human strains of PI3 virus and was considered to be a bovine strain of this virus. It was designated strain SF4 and antibody to the virus was assayed using a haemagglutination-inhibition (HAI) test. Non-specific inhibitors of haemagglutination were found in some bovine sera and these were removed or reduced by pre-treatment of the sera with receptor destroying-enzyme (RDE) derived from Vibrio cholerae.

At this time the group of respiratory diseases to which most attention was being devoted in the USA was the "shipping fever" complex, a condition associated with a variety of aetiological factors and agents the sum total of which led to an acute respiratory disease which could be fatal. At post-mortem examination of animals which had died there was often a fibrinous pneumonia and pleurisy associated with infection with Pasteurella sp, particularly P.haemolytica. Shipping fever cases were often associated with movement of cattle into feedlots. Experimental infection of susceptible calves with Pasteurella sp. failed to reproduce severe disease consistently and this led to the suggestion that other agents could be involved. Following the isolation of PI3 virus from clinical cases of this acute respiratory disease intensive efforts were made to investigate the part which PI3 virus might play in respiratory diseases of the bovine. Experimental infections of susceptible calves with North American strains of the virus, when successful, only produced a mild clinical response (Reisinger and others, 1959; Gale and King, 1961). In view of the association of natural respiratory disease with conditions thought to be "stressful" to animals; e.g. movement over long distances, sudden changes in environmental temperature, deprivation of food and water and mixing with other

animals; attempts were made to carry out experimental infections with PI3 virus and Pasteurella spp. under conditions of "stress". Hamdy and others (1963) subjected calves to a 70°F temperature change from 104°F down to 34°F and found that infection with PI3 virus and Pasteurella spp. under these conditions produced more severe disease than when the animals were not "stressed". The results of many similar experiments led workers in the USA to believe that PI3 viral infection per se was not a serious clinical problem and that severe respiratory disease was only produced when several adverse factors such as "stress" and infection with other micro-organisms were combined.

In the UK PI3 virus was first recovered from the tonsils of two of an apparently healthy group of calves which were being screened for cytopathogenic agents (Dawson and Cruickshank, 1963). The strain of virus isolated, designated T1 strain, was serologically indistinguishable from the American SF4 strain and produced syncytia with eosinophilic intracytoplasmic and intranuclear inclusion bodies in infected cell cultures. Cells of bovine, porcine and human origin were susceptible to viral infection. Four other calves in the group had rising HAI antibody titres to the virus. No respiratory disease was noticed in these calves but paired sera from some natural outbreaks of acute respiratory disease showed seroconversion to the virus.

The physical and chemical properties of the virus were studied (Dawson, 1964) and it was found to measure approximately 80-130nm diameter with some smaller particles 40nm diameter. The envelope of the virus was 15nm thick and its external surface was studded with projecting spikes. The internal material was a helical structure with a hollow core and an external diameter of about 127nm. The virus was sensitive to ether. Infected tissue culture cells adsorbed guinea pig erythrocytes and preparations of viral antigens agglutinated guinea pig, bovine and fowl erythrocytes. More recent work by Shibuta and others (1979) found that bovine PI3 virus had a bouyant density of $1.197 g.cm^{-3}$ and was structurally composed of three proteins (P1, P2 and P3) and two

glycoproteins (GP1 and GP2). The molecular weights of these were 79K (P1), 68K (P2), 35K (P3), 69K (GP1) and 55K (GP2). By analogy with Sendai virus GP2 was thought to be responsible for cell fusion.

Dawson (1963) was able to show that the substance in bovine serum which inhibited haemagglutination by PI3 virus was in fact antibody and found no significant levels of non-specific inhibitor in any of the sera tested. This was at variance with the results of Reisinger and others (1959) but in agreement with those of Bakos and Dinter (1960)and Ketler and others (1961). Using the HAI test sera from 2000 cattle of various ages in England and Wales were screened for antibody to PI3 virus (Dawson and Darbyshire, 1964). A total of 1672 (83.6%) had a titre of 32 or greater. This high proportion of positives agreed with surveys in the USA (Abinanti and others, 1961; Kramer amd others, 1963). Antibody titre and frequency of positivity increased with age suggesting continued re-exposure to infection. No differences were noted in the number of antibody-positive animals from different areas of the country although there were differences in geometric mean titre. The authors concluded that infection was widespread in England and Wales, although at this time only one isolate had been recovered. In view of the mild nature of the experimental disease reported by other workers they suggested that natural infections would be difficult to detect clinically. They considered the virus to be most important as a predisposing factor to serious respiratory tract damage in disease outbreaks and therefore that the period of viral shedding would probably be over by the time the disease was thought serious enough to be investigated.

This view was supported by the results of their own experimental infections using the T1 strain of PI3 virus (Dawson and others, 1964; Dawson and others, 1965). In one experiment virus was given by intra-nasal and intra-tracheal inoculation to each of six calves. Two calves, with serum HAI antibody titres greater than 32, did not respond clinically or serologically whereas four colostrum-deprived calves, with no detectable serum

HAI antibody, had mild signs of respiratory disease and responded serologically. The calves became pyrexic with coughing and a mucoid nasal discharge. Virus was recovered from their nasal secretions for up to 12 days after infection. The virus was also isolated from the blood of three calves between three and five days after infection. Those animals which were allowed to live beyond day six responded serologically. At post-mortem examination, on days four and seven after infection, lesions were seen only in the lungs with considerable areas of consolidation in the cranial lobes, the middle lobe and in the cranial parts of the caudal lobes. Microscopically there was bronchiolitis and alveolitis with proliferation and necrosis of epithelial cells. Eosinophilic intracytoplasmic inclusion bodies were seen in bronchiolar and alveolar epithelial cells and there was infiltration of the alveoli with polymorphonuclear and mononuclear cells. The pathological changes seen at this stage were similar to those described in "inclusion body pneumonia" (Jarrett, 1954). Additional features at day seven were syncytia in the alveolar spaces and eosinophilic intranuclear inclusion bodies in a few bronchiolar and alveolar epithelial cells. At this time the cellular infiltrate was less marked and consisted mainly of macrophages.

In spite of the extensive lung pathology produced by the T1 strain in some of the experimental calves the clinical signs of respiratory disease had been mild suggesting that other factors needed to be present before severe disease was produced. However, other workers in the U.K. reported an outbreak of respiratory disease in housed calves in which there were fatalities, apparently due to the effects of PI3 virus alone (Betts and others, 1964). The calves were bought-in as a group and five died over a period of three weeks. The disease spread to other bought-in calves on the same farm which had milder clinical signs. Antibiotic treatment apparently had no significant beneficial effect on the course of the disease with the most severely affected calves dying within seven to 21 days of first showing clinical signs. At post mortem examination there was extensive lung consolidation in the cranial and middle lobes and cranio-ventral parts of the caudal lobes. On

sectioning the lung there was evidence of necrosis and abscessation. Microscopically the principal changes were acute inflammation with evidence of epithelial damage. Bronchiolar and alveolar epithelia were hyperplastic with focal areas of necrosis. Some epithelial syncytia were present in bronchioles and alveoli and a few of these contained eosinophilic intracytoplasmic inclusion bodies. Parainfluenza type 3 virus was isolated from the lungs of two dead calves and Actinobacillus actinoides was also isolated from two calves.

One isolate of PI3 virus, designated J121 strain, was used to infect hysterotomy-derived colostrum-deprived calves (Omar and others, 1966). The type of experimental calf, titre of virus and route of inoculation were similar to those used by Dawson and others (1965), however, the clinical response seemed to be more severe with a markedly increased respiratory rate and effort. Calves inoculated with PI3 virus and A.actinoides had no more severe disease than those receiving virus alone. At five days after infection the histological changes in the lung were similar to those produced by Dawson and others (1965). However, epithelial syncytium formation was more marked. Syncytia were thought to result from fusion of epithelial cells, as opposed to proliferation. The presence of virus in the lung, as determined by virus isolation, correlated with the presence of inclusion bodies and epithelial syncytia. At seven days post infection these features were no longer present and virus could not be recovered. Macrophage syncytia were present at day seven but these were not attached to the epithelium and were morphologically distinct from epithelial syncytia. The conclusion drawn from these experiments was that the J121 strain could be an important pathogen per se, other factors not being necessary to cause marked clinical disease.

Reports of respiratory disease attributed to PI3 virus also came from other parts of the world. In Canada pathological findings similar to those already described were reported in two calves and PI3 viral infection was confirmed by the detection of

viral antigen in formal-saline fixed lung by immunofluorescence (Jolly & Ditchfield, 1965). In the USA a pneumo-enteritis syndrome in a 36 hour old calf was associated with PI3 viral infection and experimental evidence was cited that the virus could cause diarrhoea (Hamdy, 1966). In New Zealand the virus was associated with an upper respiratory tract disease in a group of 18 months old bulls (Oliver and others, 1976). Virus was recovered from the stomach contents of an aborted foetus in the USA (Sattar and others, 1965) and was implicated as a cause of abortion after experimental foetal infection (Sattar and others, 1967; Swift, 1973). In a survey of material received at a diagnostic laboratory in Denmark PI3 virus was recovered from two of 50 pneumonic calf lungs (Bitsch and others, 1976). In other parts of Europe PI3 viral infections were recorded in outbreaks of respiratory disease although the significance of these findings was thought to be unclear and the virus was not thought to be a pathogen of major importance (Wellemans, 1977; Holzhauer, 1978).

In the U.K. the controversy as to the role of PI3 virus in calf respiratory disease continued. Serological evidence implicated the virus in an outbreak of respiratory disease in which the most significant feature was a gradual increase in the frequency of coughing over a period of four weeks (Dawson and others, 1966). Other respiratory pathogens, Mycoplasma bovis and Pasteurella sp. were also present and could have influenced the severity of the disease. Gourlay and others (1970) were unable to record any isolations of virus from 65 pneumonic lungs, nor were they able to demonstrate inclusion bodies by histopathological techniques. A few years later Thomas (1973) investigated 27 outbreaks of "virus pneumonia" and concluded that he could find no significant levels of viral activity in any. Parainfluenza type 3 virus was isolated from nasopharyngeal swabs taken in five of the outbreaks and in one incident five of six animals sampled showed seroconversion to the virus, however, it was felt that these findings could have been coincidental and not necessarily related to the disease present. No lesions suggestive of viral infection were found in the lungs of nine animals which had died during the

outbreaks, nor in seven which had acute lower respiratory tract disease and were slaughtered before treatment. Bacteria were isolated in large numbers from pneumonic lung and most outbreaks of disease appeared to respond to antibiotic therapy. It was felt that the weight of evidence was against the role of viruses, including PI3, in acute lower respiratory tract disease.

As a result of an intensive epidemiological survey of respiratory viral infections occurring in groups of calves, aged between 10 days and 18 months old, passing through a beef progeny testing station it was found that PI3 viral infection was significantly associated with respiratory disease (Stott and others, 1980). In the authors experience, however, experimental infections with this virus were asymptomatic (Thomas and others, 1977) although they acknowledged that this may have been due to inappropriate experimental conditions and did not feel it should be taken as strong evidence against the causal role of the virus. They suggested that a more appropriate test of pathogenicity would be to vaccinate calves against the virus following which one would expect a reduction in the incidence of respiratory disease. Using an inactivated vaccine, at a dose regime which had been shown to protect a high proportion of calves against experimental infection (Probert and others, 1978), a vaccination experiment was carried out on the same beef progeny testing station over two winters. Although the frequency of infection with PI3 virus was reduced in the vaccinates as compared with control calves there was no significant reduction in the incidence of respiratory disease. (Stott and others, 1978).

Allan and others (1978) described some features of a natural infection with PI3 virus in a group of 25 calves. Virus was isolated from the lungs of four calves at post mortem examination and eosinophilic intracytoplasmic inclusion bodies were found in the bronchial, the bronchiolar and the alveolar epithelium of two of them. Gross lung lesions in the virus-positive calves were limited in extent and were present in the cranial and the middle lobes only. Microscopically there was bronchiolitis and alveolitis

but syncytia were not seen. Inclusion bodies seen by staining with H&E corresponded to viral nucleocapsid as visualised in the electron microscope. Clinical signs of respiratory disease were mild and sporadic over the period of observation. Despite the fact that PI3 virus appeared to be present in the group for at least 56 days only two animals showed a significant increase in serum HAI antibody titre.

In a detailed multidisciplinary investigation into 47 outbreaks of respiratory disease in housed calves in Northern Ireland, PI3 viral infection was detected in 11 outbreaks (Bryson and others, 1978a; 1978b). In only five of these was there seroconversion to the virus. In some calves obtained for pathological examination there was histological evidence of viral infection with eosinophilic inclusion bodies in epithelial cells and alveolar epithelial hyperplasia. Four outbreaks had evidence of dual infection with PI3 virus and RS virus and were described separately (Bryson and others, 1979a).

As a follow up to these investigations 13, two to four weeks old colostrum deprived calves were infected with one of the strains of PI3 virus (Bryson and others, 1979b). Moderately severe clinical respiratory disease and pneumonic lesions were produced in most animals. Inoculation was via the intranasal route but a significant change from previous experiments was that repeated doses were given over four days. This, the authors felt, reproduced natural exposure more accurately than a single inoculation of virus. A low passage strain of virus was used to minimise the chances of attenuation by repeated growth in tissue culture. The development of the pulmonary lesions was followed and viral damage to the respiratory epithelium and its subsequent repair, were described; epithelial syncytia were present between days seven and 10 after infection but were never numerous.

The evidence that PI3 virus is a serious pathogen in the U.K. has long been regarded as controversial. Early work by Dawson and others, (1965) suggested that it was a predisposing factor in

outbreaks of respiratory disease yet Omar and others (1966) regarded it as an important pathogen per se. More recently epidemiological evidence showed an association between infection and respiratory disease (Stott and others, 1978) yet vaccination of animals against PI3 viral infection did not reduce the overall incidence of respiratory disease on one farm examined (Stott and others, 1978). Bryson and others (1979b) considered it an important pathogen in Northern Ireland on the basis of field and experimental evidence.

## DUAL INFECTIONS WITH RS VIRUS AND PI3 VIRUS

Combined activity of RS virus and PI3 virus in groups of animals has been reported, however, it has not always been clear whether individual animals have been simultaneously infected with both viruses. In Nothern Ireland four outbreaks of severe respiratory disease, in which a prominent clinical finding was dyspnoea, were associated with dual infection (Bryson and others, 1979a). Parainfluenza type 3 virus was isolated from the lungs of dyspnoeic calves a..d RS viral infection of in-contact animals was demonstrated serologically. Striking pathological findings were epithelial syncytia, alveolar epithelial hyperplasia and severe interstitial emphysema. Similar clinico-pathological features were attributed to RS viral infection alone, without PI3 involvment, in an outbreak of respiratory disease in Scotland (Pirie and others, 1981a). In the Netherlands similar outbreaks were ascribed to RS viral infection and parainfluenza type 3 viral infection, if present, was regarded as an incidental finding (Holzhauer, 1978). Recently dual infections were described by Thomas and others (1982) although few details of clinico-pathological findings were given.

Differential diagnosis of RS viral and PI3 viral infection may present difficulties. Respiratory syncytial virus is labile and grows slowly in tissue culture (Wellemans, 1977) whereas PI3 virus can grow rapidly. Detection of RS viral infection by viral isolation is insensitive. Serological techniques are preferable but as the serological response is rapid (Wellemans, 1977) paired

samples taken three to four weeks apart may not define accurately the period of time during which the virus was actually present in tissues, nor does it localise the virus in the respiratory tract. The histopathological features of infection with both viruses are similar although intranuclear inclusion bodies have not been recorded in RS viral infection and numerous large epithelial syncytia are thought to be more a feature of RS viral infection than infection with PI3 virus (Pirie, 1978).

Whether or not these viruses interact to produce a more severe respiratory illness than that caused by either agent alone is not clear and dual experimental infections have not been recorded.

SECTION TWO

# MATERIALS AND METHODS

## TISSUE CULTURE TECHNIQUES

## MICROBIOLOGICAL TECHNIQUES

## SEROLOGICAL TECHNIQUES

## PATHOLOGICAL TECHNIQUES

## DETECTION OF VIRAL ANTIGENS IN TISSUES

## FIELD INVESTIGATION OF RESPIRATORY DISEASE OUTBREAKS

## EXPERIMENTAL ANIMALS

In this section the general techniques used in the thesis are described. Specialised or new techniques will be detailed more fully in the appropriate chapter.

## TISSUE CULTURE TECHNIQUES

### PREPARATION OF CELLS FROM FOETUSES

Fresh bovine foetuses, at four to eight months gestation, were obtained from a local slaughterhouse and were used to prepare cultures of foetal bovine lung (FBL) or foetal bovine kidney (FBK) cells. Using sterile instruments the lungs and kidneys were removed from the foetus and were transferred to the laboratory in sterile containers. Pieces of the caudal lung lobes or kidney cortex were minced finely in a conical flask. The tissue was rinsed repeatedly with sterile phosphate buffered saline (PBS) pH 7.2 until the effluent was free of visible blood or serum. Excess PBS was carefully drained from the tissue fragments and freshly prepared trypsin solution (0.25% powdered pig trypsin in Hanks' balanced salt solution, pH 7.8) was added to make a 10% suspension. This was stirred gently for 45 minutes at 37°C. The contents of the flask were then allowed to settle, the supernatant discarded and fresh trypsin solution added. Incubation, with stirring, was continued for a further 18 hours at 4°C. The suspension of cells thus obtained was filtered through a double layer of sterile gauze to remove large fragments and the filtrate was centrifuged at 250g for 20 minutes at 4°C. After discarding the supernatant the cell pellet was suspended in 10ml Eagle's medium containing 2.5% foetal calf serum (EFC2.5) and was re-centrifuged at 250g for 20 minutes at 4°C. Again the supernatant was discarded and the cell pellet was resuspended in a further 10ml EFC2.5 and was centrifuged in a graduated tube at 250g for 15 minutes at 4°C. The final cell pellet was diluted 1 in 10 (volume/volume) in Eagle's medium containing 10% foetal calf serum (EFC10) and distributed into glass tissue culture flasks at 20ml cell suspension/80cm$^2$ growth area. The flasks were gassed with 5% $CO_2$ in air and were incubated at 37°C for three days

without being disturbed. After this time the unattached cells were removed by rinsing the cell sheet with EFC2.5. Fresh EFC10 was then added and incubation was continued at 37°C until the cells had formed a complete monolayer. The medium was then replaced with EFC2.5 and the cells were held at 37°C until used for virus isolation.

SUB-PASSAGING CELLS

Cells were sub-passaged at approximately weekly intervals. The medium was decanted and the monolayer was rinsed twice with 20ml 0.02% ethylene diaminotetracetic acid in PBS (EDTA) then dispersed by incubation with 0.01% trypsin solution in EDTA. The cells were resuspended in EFC10 and were divided between three or four sterile tissue culture flasks. Each flask was gassed with 5% $CO_2$ in air and was incubated at 37°C until the cells were confluent. At this point the cells were either used for virus isolation or were maintained at 37°C in EFC2.5

Periodically samples of medium and cells were checked for contamination with mycoplasmas and bovine virus diarrhoea (BVD) virus. Mycoplasmas were isolated using the procedures described by Pirie and Allan (1975). Bovine virus diarrhoea viral antigens were detected using an indirect immunofluorescence technique.

African green monkey kidney cells, strain BS-C-1, were obtained regularly from the Medical Research Council Institute of Virology, Glasgow. They were grown in 2 litre rolling bottles at 37°C and when confluent were maintained at 31°C. The sub-passaging procedure was the same as for FBL and FBK cells except that it was carried out twice weekly.

PREPARATION OF MULTIWELL PLATES

Multiwell plates (Costar, U.S.A. or Linbro, U.K.) were used for (i) virus isolation (ii) identification of virus isolates (iii) titration of virus stocks and (iv) RS viral serology. The plates

were prepared by seeding each well with either $2.0 \times 10^5$ (FBL,FBK) or $1.0 \times 10^5$ (BS-C-1) cells in 0.5ml EFC10. Monolayers were formed after 24 hours at 37°C in a humidified $CO_2$ incubator. For sub-passaging, monolayers were dispersed as for conventional tissue culture.

## MICROBIOLOGICAL TECHNIQUES

### ISOLATION OF VIRUSES

Samples for virus isolation were taken from calves involved in field outbreaks of respiratory disease and from calves experimentally infected with RS virus or PI3 virus. Nasopharyngeal swabs were taken from live animals and samples of respiratory tract tissues were taken from animals which had either died or been slaughtered. They were processed as follows.

#### Nasopharyngeal swabs

Nasopharyngeal swabs (Exogen, Glasgow) taken from calves were transported to the laboratory in a bijou bottle containing 2ml virus transport medium (VTM, see Appendix 1). Samples were kept at 4°C during transport and on arrival at the laboratory were either stored at -70°C or were processed immediately for virus isolation. Nasopharyngeal secretions were washed from the swab by repeatedly rinsing in the VTM using a fine-bore Pasteur pipette. The suspended secretions, which contained respiratory epithelial cells, were used as inoculum without further treatment. Unused inoculum was stored at -70°C.

#### Tissues

Samples were taken routinely from the nasal mucosa, the tracheal mucosa, the bronchial lymph node and from the cranial, the middle and the caudal lobes of the right lung. Using sterile instuments approximately 1g tissue was placed in a universal bottle containing 10ml VTM at 4°C. Samples were then either stored at -70°C or were immediately prepared for virus isolation as follows. The tissue was minced finely using scissors, transferred to a sterile plastic bag and disrupted further in a stomacher (Colworth Instuments, U.K.) for one minute. The tissue suspension was then poured back into the universal bottle and was centrifuged at 650g for five minutes at 4°C to sediment the tissue fragments. The

supernatant was used as inoculum. Unused inoculum was stored at -70°C.

Inoculation of Cells

Monolayers of FBL and FBK cells were prepared in separate multiwell plates, as described previously. Approximately 0.2ml inoculum from each sample was added to each of four wells of FBL cells and four of FBK cells. At the same time stock isolates of RS virus, PI3 virus and BVD virus were inoculated onto both cell types in order to check their susceptibility to these viruses. Adsorption proceeded for one hour at either 37°C (FBL) or 31°C (FBK). The inoculum was then removed and 0.5ml EFC2.5 with extra antibiotics (EFC2.5XAB, see Appendix 1) was added to each well. Incubation was continued at the appropriate temperature for 24 hours then the wells were examined for cellular changes and bacterial and fungal contamination. Any changes were noted and the medium was replaced with fresh EFC2.5XAB. The plates were then examined daily and the medium changed every three days using EFC2.5.

Foetal bovine kidney cells were maintained at 31°C for three weeks. If no cytopathic changes had occurred by this time the cells were checked for evidence of infection with RS virus, PI3 virus and BVD virus by methods described later.

The viral isolation procedure using FBL cells is outlined in Table 1. Foetal bovine lung cells were maintained at 37°C and were sub-passaged after seven days if no cytopathic changes had developed. Cells from all four wells of each sample were dispersed as described previously and pooled in 4ml EFC10. Half of the cell suspension was stored at -70°C and the remainder was distributed equally between four wells of a new multiwell plate. If no cytopathic effect (cpe) had developed after a further seven days two wells from each sample were again sub-passaged and the remaining two examined for evidence of infection with RS virus, PI3

virus and BVD virus. If no cpe developed in the sub-passaged cells after a further seven days incubation then the samples were considered to be negative for the commonly isolated respiratory viruses and were discarded. If a cpe did develop, at any stage, the detached cells and medium were inoculated onto fresh cells in ten-fold dilutions up to 1 in 1 000.

## EXAMINATION OF TISSUE CULTURE CELLS FOR PI3 VIRUS, RS VIRUS AND BVD VIRUS

### Haemadsorption for PI3 virus

Medium from the cells was removed and the cell sheet was gently rinsed twice with cold Eagle's medium containing 0.5% foetal calf serum (EFC0.5). Approximately 0.5ml of a 1% suspension of washed guinea pig erythrocytes in cold EFC0.5 was added to each well to be tested and the plate was placed at 4°C for 30 minutes. After this time the cell sheet was rinsed three times with cold EFC0.5 to remove unattached erythrocytes and then observed for haemadsorption using an inverted microscope (Leitz, U.K). Positive wells were noted and the corresponding sub-passaged cells were observed closely for cytopathic changes.

### Immunofluorescence for RS virus and BVD virus

After haemadsorption for PI3 virus the cells were fixed in situ in the multiwell plate using methanol at -20°C for 20 minutes. The fixative was then discarded and the cells washed with five changes of PBS. Hyperimmune antisera to bovine RS virus and BVD virus, raised by infecting gnotobiotic calves, were generously provided by Dr.J.Stott, Institute for Research on Animal Diseases, Compton, U.K. They were applied simultaneously to each well and the plate was rocked for 45 minutes at room temperature in a humidified atmosphere. After further washes in PBS, rabbit antiserum to bovine IgG, conjugated with fluorescein isothiocyanate (RABIgG-FITC; Miles Laboratories, UK) was added to each well and the plate was rocked for a further 45 minutes at room temperature.

Finally, unbound conjugate was washed off with five changes of PBS and the cells were examined for the presence of RS viral and BVD viral antigens using a fluorescence microscope (Leitz orthoplan with epifluorescence).

Cells showing evidence of infection with either virus were noted and the corresponding sub-passaged cells were observed closely for cytopathic changes.

Identification of Isolates

Virus isolates were identified initially by the character of cytopathic changes, immunofluorescence and haemadsorption. Confirmation of identity was by neutralisation with specific hyperimmune sera.

Bacterial and Fungal Contamination of Cell Cultures

Bacterial contamination was an infrequent problem using the virus isolation methods described. Fungal contamination occurred infrequently from tissue samples but was more common from nasopharyngeal swabs. This was overcome by increasing the concentration of amphotericin B in the VTM during the initial three days of tissue culture. Any contamination which did develop was treated promptly by removing the fungal colony, before sporulation, using a fine-bore Pasteur pipette. The cell sheet was then washed twice with EFC0.5 and the medium was replaced using EFC2.5XAB. Persistent problems in single wells were treated by adding 0.5ml 5M sodium hydroxide. Intractable problems involving a large proportion of the wells on a plate were terminated, after a minimum of seven days, by checking for PI3 virus, RS virus and BVD virus by the methods described before discarding the plate. If necessary the stored original inoculum for the sample was filtered through a 220nm filter (Millipore) and virus isolation was attempted from the filtrate.

ISOLATION OF BACTERIA AND MYCOPLASMAS

Bacteria and mycoplasmas were isolated from nasopharyngeal swabs and from respiratory tract tissues using the methods described by Pirie and Allan (1975). Only those bacteria and mycoplasmas which are commonly regarded as potential bovine respiratory tract pathogens are reported. The relevant bacteria are Pasteurella haemolytica types A1 and A2, Pasteurella multocida, Haemophilus somnus, Corynebacterium pyogenes, Streptococcus pneumoniae and Staphylococcus aureus (Pritchard and others, 1979; Wiseman and Pirie, 1979). The mycoplasmas are Mycoplasma dispar, Mycoplasma agalactiae var bovis and Ureaplasma sp. (Pirie and Allan, 1975; Howard and others, 1976; Gourlay and others, 1976).

## SEROLOGICAL TECHNIQUES

Antibodies to RS virus were titrated using a serum neutralisation test in which the end-point was determined by plaque reduction. Parainfluenza type 3 virus antibodies were assayed using a micro-haemagglutination inhibition (HAI) test except in one outbreak of respiratory disease (outbreak C). In this incident a serum neutralisation test, similar to that used for RS virus, was used. The challenge virus was a bovine strain of PI3. In addition sera were titrated for antibodies to other viruses at the Central Veterinary Laboratory, Weybridge, using the following tests:-

Bovine virus diarrhoea virus - serum neutralisation test using the NADL reference strain of virus.
Adenovirus type A- agar gel diffusion test (Darbyshire and Pereira, 1964) using human adenovirus type 5.
Adenovirus type B- agar gel diffusion test using bovine adenovirus type 4
Reovirus type 1 - HAI test (Rosen, 1960) using the Weybridge 26 strain of reovirus type 1
Reovirus type 2 - HAI test using a human strain of reovirus type 2
Infectious bovine rhinotracheitis virus - serum neutralisation test (Dawson and Darbyshire, 1964) using the Oxford strain of IBR virus.

### SERUM NEUTRALISATION TEST FOR ANTIBODY TO RS VIRUS

Serum samples were inactivated at 56°C for 30 minutes then diluted 1 in 10 with EFCO.5. Two-fold dilutions from 1 in 10 to 1 in 10 240 were prepared in 0.1ml volumes. Approximately 400 plaque forming uinits of challenge RS virus (RSN-2 strain of human RS virus; Faulkner and others, 1976) in 0.1ml EFCO.5 was added to each serum dilution and the virus-serum mixtures were held at 4°C for 18 hours. Known positive and negative serum controls were titrated simultaneously. Using a calibrated pipette (Gilson Instruments, U.K.) 0.05ml of each serum-virus mixture was inoculated onto each of two monolayers of BS-C-1 cells in multiwell tissue culture plates. A period of 60 minutes at 37°C was allowed

for adsorption of virus during which time the plates were rocked every 15 minutes to distribute the inoculum evenly over the cell sheet and prevent dessication. After adsorption the inoculum was removed and the cells were overlaid with 1ml of EFC2.5 containing 0.9% agar. The plates were incubated at 37°C for three to five days until plaques were discernible in the virus control wells. The cells were fixed by layering 1ml of a 1% solution of glutaraldehyde in PBS onto the agar. After three hours at room temperature the agar was flicked off and the cell sheet was stained with Giemsa solution. Plaques were counted using a low-power dissecting microscope. The average of the number of plaques between the two wells of each serum dilution was plotted against the reciprocal of the serum dilution. The serum dilution giving a 50% reduction in plaque count, as compared with no-serum control wells, was determined graphically.

Anti-RS virus neutralising titres were determined using the RSN-2 strain of human RS virus as this gave plaques which were more distinct and appeared more quickly than those produced by bovine strains of RS virus. Antibody titres were similar whether titrated using bovine or human strains of RS virus.

MICRO-HAEMAGGLUTINATION INHIBITION TEST FOR ANTIBODY TO PI3 VIRUS

Preparation of antigen

Parainfluenza type 3 virus, isolated from the lungs of a pneumonic calf, was grown in FBK cells. When maximun cpe had developed virus was harvested by freezing and thawing the cells and medium. The harvest was clarified by centrifugation at 10 000g for 30 minutes at 4°C and the supernatant was stored at -70°C in aliquots. Two random aliquots were thawed and serial two-fold dilutions were made, in 0.025ml amounts, in duplicate in a 'U' bottom microtitre plate (Cookes, USA). To each dilution 0.05ml of a 0.5% suspension of washed guinea pig erythrocytes was added and the plate was held at 4°C for two hours. The haemagglutination titre of the antigen was taken as the highest dilution at which

there was complete haemagglutination. This dilution was considered to represent one haemagglutinating (HA) unit of antigen. Each serum dilution in the HAI test was challenged with four HA units of PI3 viral antigen.

Preparation of Serum

Sera were inactivated at 56°C for 30 minutes and non-specific inhibitors of haemagglutination were removed by absorption with kaolin. 0.1ml inactivated serum was diluted in 0.4ml PBS and to this was added 0.5ml of a 25% suspension of acid-washed kaolin in borate saline (Flow Laboratories, U.K.). The mixture was held at room temperature for 20 minutes and shaken at intervals. The kaolin was then deposited by centrifugation at 1000g for 10 minutes. The supernatant, which represented a 1 in 10 dilution of the original serum was removed and to it was added 0.1ml of packed guinea pig erythrocytes. The resultant suspension was shaken periodically whilst standing at 4°C for one hour. This step removed non-specific agglutinins of guinea pig erythrocytes. Finally, the erythrocytes were deposited by centrifugation at 400g for 10 minutes and the supernatant was used in the HAI test.

Testing the serum

Serial two-fold dilutions of serum, from 1 in 10 to 1 in 10 240, were prepared in microtitre plates. Four HA units of PI3 viral antigen were added to each serum dilution and the plate was incubated at room temperature for one hour. Controls with no serum, a positive serum and a negative serum were included in the test as were erythrocyte and antigen titration controls. Finally, two volumes of a 0.5% suspension of washed guinea pig erythrocytes were added to each well and the plate was placed at 4°C overnight. The titre of each serum was taken as the lowest dilution at which there was complete inhibition of haemagglutination. Sera were titrated in duplicate.

EXAMINATIONS FOR ANTIBODY TO BACTERIA AND MYCOPLASMAS

Antibody to Pasteurella haemolytica types A1 and A2 and Pasteurella multocida was titrated using an indirect haemagglutination test (Shreeve and others, 1972). The level of antibody to Mycoplasma bovis was determined using an indirect immunofluorescence test (Pirie and Allan, 1975).

## PATHOLOGICAL TECHNIQUES

In this section the techniques used in the post-mortem examination of field and experimental respiratory disease cases are described. In addition the processing of tissue for histopathological examination is briefly outlined.

### POST-MORTEM EXAMINATION

Animals were stunned using a captive bolt pistol and were exsanguinated via the anterior vena cava. In some experiments animals were anaesthetised by the intravenous injection of sodium pentobartitone and, before being hoisted for exsanguination, the oesophagus was ligated to prevent the regurgitation of rumen contents.

After exsanguination the larynx, the trachea and the contents of the thorax were removed for pathological examination. The heart, oesophagus and the aorta were trimmed away and the external features of the lungs, trachea and pleura were recorded. Samples from the appropriate sites were taken for microbiological examination. Following this the trachea was opened longitudinally, continuing into the left and right caudal bronchi. Each lung lobe was sectioned at various levels and the appearance was noted. Samples for histopathological examination were taken from the cranial part of the right cranial lobe of the lung, right middle lobe and both cranial and caudal parts of the right caudal lobe. Samples were also taken from the lobar bronchi of the right cranial and right caudal lobes. Further material was taken from the nasal mucosa, the trachea and a bronchial lymph node. If morphologically different lesions were present in the left lung lobes then these were also sampled.

### PROCESSING OF SAMPLES

A part of each sample taken was immediately snap-frozen by immersion in liquid nitrogen for the preparation of frozen tissue

sections. The remainder of the tissue was fixed for conventional histopathology. All samples for paraffin embedding were fixed in 10% buffered neutral formalin and, where appropriate, adjacent pieces of tissue were also fixed in corrosive formol.

After fixing for 24-48 hours the tissues were trimmed and processed in a series involving dehydration in alcohol and clearing in chloroform. Afterwards they were vacuum embedded in paraffin wax. Sections were cut on a microtome (Leitz,U.K) at approximately 5μ thickness and were stained routinely with haematoxylin and eosin (H&E). Other stains were used to demonstrate specific features e.g. phloxine tartrazine (PTI) for inclusion bodies and Unna Pappenheim for plasma cells.

## DETECTION OF VIRAL ANTIGENS IN TISSUES

An indirect immunofluorescence technique was used to detect viral antigens in frozen tissue and in tissue processed for conventional histopathology. Frozen tissue sections were stained for RS, PI3 and BVD viral antigens whereas conventionally processed sections were stained only for RS and PI3 viral antigens. The antisera were raised in gnotobiotic calves as mentioned previously.

### FROZEN TISSUE SECTIONS

The frozen tissue was mounted, without thawing, on a cold cryotome chuck using OCT compound (Miles Laboratories, U.K.). Sections 5μ thick were cut on a cryotome (Slee Instruments, U.K.), placed on grease-free microscope slides and fixed in pure acetone for 10 minutes at 4°C. After washing in three changes of PBS the sections were ready to be stained for viral antigens.

### CONVENTIONAL HISTOPATHOLOGICAL SECTIONS

The technique used for the detection of viral antigens in conventional histopathological sections is described in Section Three.

### IMMUNOFLUORESCENT STAINING

Sections were overlaid with the appropriate dilution of viral or control antiserum and were incubated for 30 minutes at 37°C in a humidified chamber. The sections were then rinsed three times in PBS and RABIgG-FITC was applied to each section. After incubation for 30 minutes at 37°C the conjugate was rinsed off with PBS and the sections were counterstained with 0.1% Evan's blue for five minutes at room temperature. Excess counterstain was washed off with several changes of PBS followed by a final rinse in running tap water.

EXAMINATION OF SECTIONS AND CONTROLS

The sections were mounted under coverslips using 90% glycerol in PBS. Examination for viral antigens was carried out using a fluorescence microscope. Ultraviolet light was produced by an Hb200 mercury vapour bulb and passed through a GG455 barrier filter. Photographs were taken using Afga 50L, or Kodak Ektachrome EPY-136-50.

Controls for each section included antisera to heterologous viruses or fluorescein conjugate alone. Reference blocks of tissue, known to be positive or negative for the appropriate viral antigens, were stained simultaneously. Positive staining was confirmed by blocking with antisera to the appropriate virus raised in a different species.

## FIELD INVESTIGATION OF RESPIRATORY DISEASE OUTBREAKS

During the winters of 1980-1981 and 1981-1982, in collaboration with the department of Veterinary Medicine, outbreaks of acute respiratory disease occurring in housed immature cattle were investigated. Incidents were referred by veterinary practitioners in the west of Scotland and in addition to this some material was received from farms in the north of England via the Ministry of Agriculture, Fisheries and Foods Veterinary Investigation Centre at Penrith in Cumbria.

### INITIAL VISIT TO OUTBREAK OF RESPIRATORY DISEASE

With the permission and co-operation of the farmer and his veterinary surgeon each farm with an outbreak of acute respiratory disease was visited by members of the department of Veterinary Medicine. In the majority of cases this visit took place within one to three days of the farmer first becoming aware of a problem. A full history was taken and the animals involved were examined clinically. Nasopharyngeal swabs and blood samples were taken from all the animals in the affected group; where this was not practicable at least half the animals were sampled. The carcases of any animals which had died were collected and taken to the Veterinary School for pathological and microbiological examination. In addition, where possible, animals suffering from respiratory disease were bought for further investigation. It was initially decided to buy up to five animals from any particular respiratory disease incident but, of course, this depended on the farmer's willingness to sell. In dairy herds farmers were less willing to sell heifer calves as these were potential herd replacements and, in general, were more willing to sell severely ill animals which were likely to die or become chronic respiratory disease cases. As far as possible animals were bought with varying degrees of illness or which, on the basis of their history where this was reliably known, were at different stages of the disease process.

Animals purchased were subjected to further clinical examination at the Veterinary School and then were slaughtered for pathological and microbiological examination. Samples were taken from various tissues as described previously.

FOLLOW-UP VISITS

Follow-up visits were made at weekly intervals whenever possible, and in every case at four weeks after the initial visit. At every visit the affected animals were examined clinically and after four weeks blood samples were taken from those initially sampled.

## EXPERIMENTAL ANIMALS

### SOURCES OF ANIMALS

Dairy and dairy-cross calves, both male and female, were purchased from farms in the west of Scotland at between two and four months of age. Where possible farms were selected on the basis of freedom from serious respiratory disease and calves were transported direct to the Veterinary School without movement through a market. A small number of very young calves were also obtained and transferred directly to the Veterinary School when 10 days old.

### ACCOMMODATION

Nasopharyngeal swabs for viral isolation and blood samples for serum were taken from all calves on entry to the experimental accommodation. Calves were housed in two loose-boxes which were approximately 50m apart and which shared no common access, services or airspace. Each box measured approximately 5m x 5m with solid walls to roof level and a concrete floor. Access to the outside from the calf area was via a door and in addition there was access to the exterior via a passageway. An experimental preparation area was located in the passageway for the use of all personnel entering the box. Protective clothing and footwear were kept in this area.

Ventilation was through a grill-mesh inlet measuring approximately 0.75m by 0.75m in the door and air was vented via a fan-assisted baffled outlet in the roof. Urine was led away via a central drain in the floor and the animals were bedded on straw. Excess dung was removed daily and at the end of each experiment all waste straw was removed and the floor and walls were hosed down with water and steam cleaned.

Young calves were fed on milk substitute until weaning and then had access to hay, water and concentrates ad libitam.

METHODS OF EXPERIMENTAL INFECTION

Calves were infected either intra-nasally or by a combination of intra-nasal and intra-tracheal routes. Depending on the particular experimental protocol infection was either by a single inoculum or by successive inoculations, morning and afternoon, for three days. In one experiment calves were infected endobronchially.

Intra-nasal infection

For intra-nasal infection a 20ml sterile polypropylene syringe was filled with warm (37°C) virus suspension. With the calf's head restrained and slightly elevated inoculum was slowly instilled, in repeated small volumes, into both nostrils. Provided that the inoculum was warm and was instilled slowly sneezing was kept to a minimum.

Intra-tracheal infection

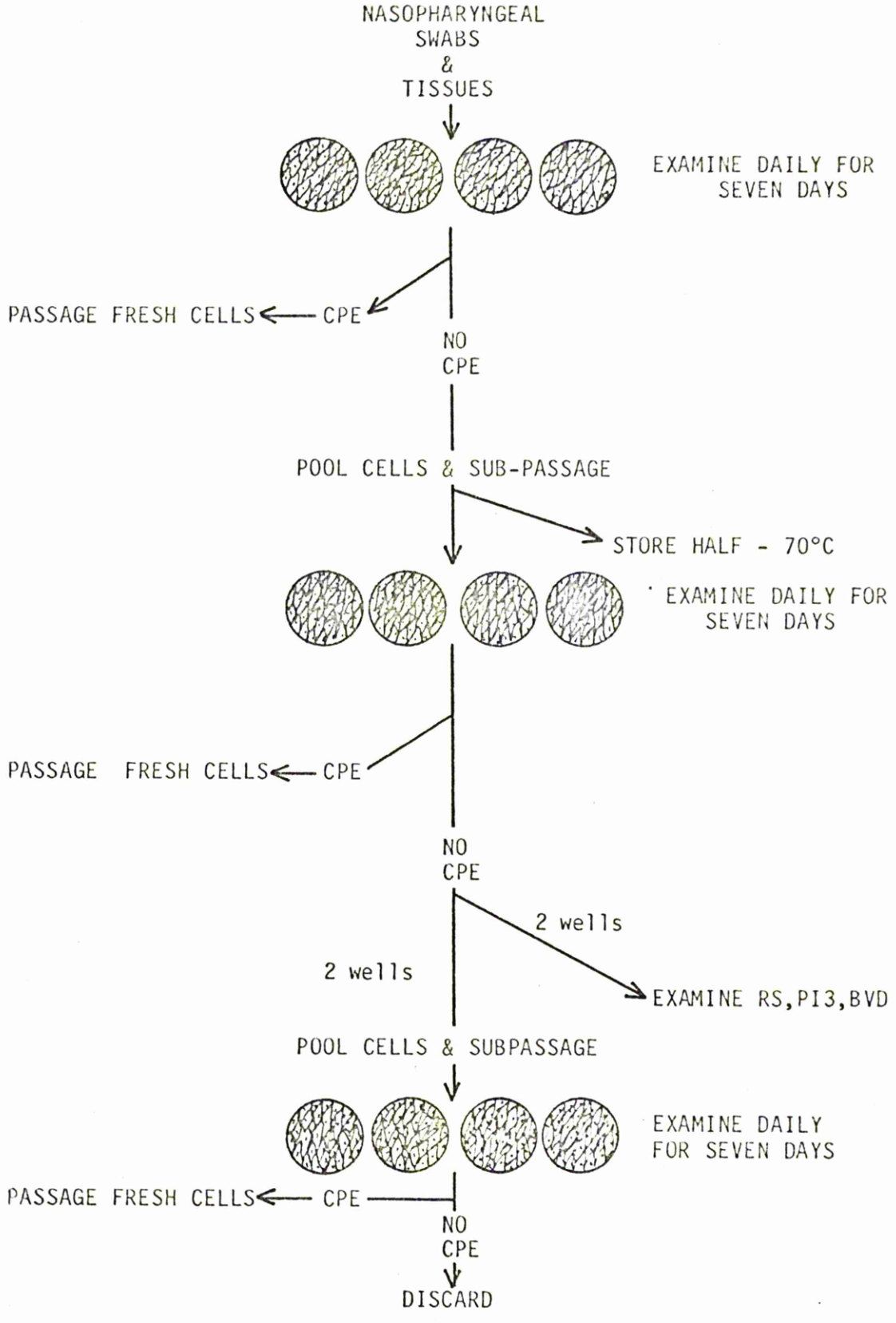
The unsedated calf was restrained standing with head and neck slightly extended. The trachea was grasped, through the skin, approximately 20cm cranial to the thoracic inlet. A 20 gauge needle, attached to a 20ml syringe containing warm (37°C) virus suspension was inserted between adjacent tracheal rings into the lumen of the trachea. Air was drawn into the syringe to check the position of the needle and then the inoculum was slowly injected. Injection was suspended during coughing to prevent the needle becoming displaced. Again, provided the inoculum was warm and was injected slowly, coughing was kept to a minimum.

Endobronchial infection

Calves were sedated by the intravenous injection of 0.5ml Rompun (Bayer,U.K) and were restrained in lateral recumbency on their right side with the head and neck extended. Using a flexible fibreoptic endoscope (Olympus Instruments) a catheter was

introduced into the lobar bronchus of the right caudal lung lobe. Virus suspension was injected via the catheter.

TABLE 1: A flow-diagram of the viral isolation procedure using foetal bovine lung cells



SECTION THREE

# FIELD INVESTIGATIONS

## RESPIRATORY DISEASE INVOLVING RS VIRUS

OUTBREAK A

OUTBREAK B

OUTBREAK C

OUTBREAK D

INDIVIDUAL CASES OF RS VIRAL INFECTION

THE ISOLATION OF RS VIRUS FROM THE NASAL SECRETIONS OF A CALF

## RESPIRATORY DISEASE INVOLVING PI3 VIRUS

OUTBREAK E

OUTBREAK F

OUTBREAK G

OUTBREAK H

INDIVIDUAL CASES OF PI3 VIRAL INFECTION

# INTRODUCTION

The association between viral infection and respiratory disease has often been studied by the investigation of specific disease outbreaks. The most common investigative techniques used have been clinical observation of affected animals, examination of paired sera for a rise in titre of antibody to a particular virus or viruses, the isolation of viruses from the respiratory tract and the pathological examination of animals which have died as a result of the disease.

It can be very difficult to define precisely when an outbreak starts. The clinical signs of disease shown by sick animals result from pathological changes which, in the case of some respiratory diseases, result in turn from infection of the respiratory tract with various micro-organisms. This sequence of events takes time to occur, may vary in severity and will be influenced by many factors. The result is that the point at which a respiratory disease outbreak becomes clinically evident, and therefore the time at which investigative procedures are begun, may be significantly later than the point at which the pathological changes were initiated by the causal organisms. We tend to think of respiratory diseases as being either "acute" or "chronic". These are essentially clinical terms used to indicate the rate of onset and duration of a disease. However, acute has sometimes been used to indicate severity or merely the time at which the disease was first noticed. In serological investigations the acute phase of a disease generally means the stage at which the first blood sample was taken. In viral infections the timing of this sample is crucial if a significant rise in antibody titre to the infecting virus is to be detected and again, the timing depends on when the disease is first noticed. In pathology acute refers to the inflammatory process and what are thought to be its initial recognisable stages. The earliest morphologically detectable events are vascular changes which are seen as congestion and oedema. Shortly following this inflammatory cells such as neutrophils may invade the damaged tissue. It must be recognised

that acute in one sense e.g. clinically, may not necessarily reflect acute in another sense e.g. microbiologically or pathologically. In investigating respiratory disease outbreaks due to infectious agents care should be taken to note the timing of the taking of any samples or of making any observations in relation to the initial stages of the pathogenesis of the disease. Despite the difficulties inherent in this approach it offers the most direct way of studying the association between viruses and disease, given appropriate samples and investigative techniques.

The diagnosis of infection with RS virus has presented many problems in the bovine (Wellemans, 1977). Relatively few diagnoses have been made by viral isolation as the virus is labile and grows poorly in cell culture. Specialised laboratory facilities are needed for these techniques and correct storage and transport of samples must be observed if they are to reach the laboratory in a suitable state for successful viral isolation. Appropriate facilities are not widely available in the UK. The majority of diagnoses have been made on the basis of serological examinations. The conditions under which serum samples should be collected are less stringent, except with respect to timing, than those for viral isolation. The most commonly used serological test is the measurement of serum neutralising antibody. However, the results of these investigations are not available for at least six weeks; four weeks between paired serum samples and two weeks to perform the test. Thus the results may have little relevance to diagnosis "on the farm". Where animals have died pathological examination of lung tissue may achieve a diagnosis, particularly where this is complemented by the detection of viral antigens in the pneumonic lung. Immunofluorescent staining of acetone-fixed frozen sections of lung tissue have commonly been used for this purpose (Wellemans, 1977; Thomas and Stott, 1981; Elazhary and others, 1982; van den Ingh and others, 1982). Whilst frozen tissue sections can afford a convenient method of diagnosis they do have several drawbacks. Specialised equipment is necessary to prepare the sections and these are not widely available. Blocks of tissue are usually small and focal infections may be missed. The loss of morphological

detail incurred during the freezing process can mean that the relationship between viral antigens and the histopathological features are difficult to assess. Finally, storage of material is expensive and may be associated with dessication and destruction of the tissue.

These problems can partly be overcome by using material which has been fixed in conventional histopathological fixatives and embedded in paraffin wax. The facilities for this are more widely available, morphological detail is better preserved and storage is both cheap and effective. There are, however, problems with material processed in this way. The detection of viral antigens in such tissue by immunofluorescence has required specialised methods which have yielded inconsistent results (Fraser, 1964). Recently, however, Huang and others (1975) reported that digestion of de-waxed sections with the enzyme trypsin enhanced the staining of various antigens in formalin-fixed paraffin-embedded tissues. Using this technique Swoveland and Johnson (1979) were able to detect measles viral antigens in human brain, liver and lung. In this thesis a similar technique was developed to detect RS viral and PI3 viral antigens in bovine lung.

METHOD FOR THE DETECTION OF VIRAL ANTIGENS IN PNEUMONIC LUNG

The tissues were fixed in 10% buffered neutral formalin or corrosive formol and were processed for conventional histopathology as described in Section Two. Sections of paraffin-embedded lung, 3 to 5μ thick, were cut and fixed to grease-free microscope slides by heating to 58°C for at least 30 minutes. After de-waxing in three changes of fresh xylene the tissues were rehydrated through graded alcohols and washed thoroughly in running tap water. If the tissue had been fixed in corrosive formol an additional step was required to remove deposits of mercuric salts. The sections were immersed for five minutes in a solution of 5% iodine in 70% methylated spirits and were then decolourised in 2.5% aqueous sodium hypochlorite solution for a few seconds. Following this they were rinsed thoroughly in running tap water.

The sections were then treated with trypsin solution to expose viral antigenic determinants.

Trypsinisation Procedure

Fresh 0.2% trypsin solution was prepared by dissolving 0.2g powdered pig trypsin (Difco) in 100ml de-ionised water. Anhydrous calcium chloride (0.02g) was added and the pH of the solution was adjusted to 8 by the dropwise addition of 0.1M sodium hydroxide solution. Hydrated sections of tissue were incubated in this solution for 30 minutes at 37°C after which they were rinsed thoroughly in running tap water.

Sections prepared by trypsinisation were then stained for RS viral or PI3 viral antigens by the same method as described for frozen tissue sections (see Section Two).

Using the immunofluorescence technique, viral isolation and serological tests 22 outbreaks of respiratory disease in immature cattle in the West of Scotland were investigated in detail. Unequivocal evidence of infection with either RS virus or PI3 virus was detected in eight outbreaks (A to H) and these incidents will be described in this section. Over the same period material was received from outbreaks of respiratory disease which had been referred to Veterinary Investigation Centres in England. Several individual cases of RS viral or PI3 viral infection were detected and these will also be described. They are identified by individual case numbers e.g. case RC1. Photographs relating to respiratory diseases involving RS virus or PI3 virus are presented at the end of the appropriate section.

RESPIRATORY DISEASE INVOLVING RS VIRUS

OUTBREAK A
OUTBREAK B
OUTBREAK C
OUTBREAK D

RESPIRATORY DISEASE INVOLVING RS VIRUS: OUTBREAK A

Introduction

This outbreak occurred in a "closed" dairy herd of 60 Friesian cows which calved to either a dairy bull (by artificial insemination) or a beef bull (hired from neighbours). Friesian heifer calves were reared as herd replacements and other calves were reared for beef. The respiratory disease outbreak occurred during October 1982. At this time the animals on the farm were divided into the following groups (i) adults (ii) calves and (iii) in-calf heifers.

The adult milking cows and dry cows were loose-housed in a cubicle building with access to a self-feed silage face. They had been housed for the winter period during the first week of October but prior to that had been housed at night only for two weeks.

The calves were sub-divided into four groups on an age basis.

Group 1 consisted of 11 calves aged between one day and eight weeks old housed in a converted byre. The solid partitions dividing each stall of the byre were extended over the grip, using wooden rails, to a wooden gate. This made each stall into a pen. One or two calves, of approximately equal ages, were housed on straw in each pen. They had direct contact with neighbours in the immediately adjacent pens over the wooden rails. Milk substitute was fed twice daily from buckets which were washed, but not sterilised, between each feeding. At eight weeks of age calves were weaned and moved into group 2.

Group 2 was five calves aged between eight and ten weeks old. This group was housed in a low-roofed building which had been divided into four pens, each measuring approximately 2.5m by 2.5m, with solid dividing walls 1m high. Three pens were being used, two with two calves in each and one containing one calf. Hay and

concentrates were fed twice daily. From this group calves were moved into group 3 at approximately 10 weeks of age.

Group 3 was 10 calves aged between 10 weeks and six months. This group was housed in a loose-box measuring approximately 8m x 5m and were bedded on straw. The box was open on three sides with a combination of gates and hayracks blocking access to the exterior. Calves were fed hay, straw and concentrates. These calves had not been at grass.

Group 4 was 16 calves aged between six and 12 months. These animals were housed in a building measuring approximately 20m by 6m. The building was open at one end, where there was a tubular steel gate, and for half of one side where there was a feeding rack. At the other end were two stable-type doors, each 1.25m wide and with the top half permanently open. The roof was made of asbestos sheeting with a closed ridge. Hay and concentrates were fed twice daily. This group of animals had been at grass over summer and had been moved indoors at the beginning of October. Two days after housing they were treated with an anthelmintic.

The group of in-calf heifers were at grass at the time of the incident and were not in direct contact with other animals on the farm. A bull, which had been hired from a neighbouring farm at the beginning of October, was running with these animals.

History of Respiratory Disease Outbreak

On 11.10.82 a calf in Group 1 (Calf A11) was noticed to be ill. According to the farmer's wife it was apparently normal at the morning feeding but during the afternoon developed "difficulties with its breathing". Its respiratory rate and depth were raised. Over a period of four to six hours this apparently became worse and the calf was given 7ml oxytetracyline (Pfizer, Terramycin Q50) intramuscularly. Veterinary attention was not sought and the calf recovered.

On 17.10.82 another calf (calf A20) this time in group 2, was noticed to be "off colour" at the morning feeding but seemed to have recovered by the evening. The next morning the calf appeared to be having difficulty breathing yet was bright and able to eat, During the day, however, its condition deteriorated and it appeared to be having difficulty breathing out. Expiratory efforts were accompanied by a grunt and the calf was standing with head and neck extended and its tongue out. Veterinary attention was sought and "pneumonia" was diagnosed. Despite treatment with oxytetracycline and betamethasone the calf died that evening. At this time other calves in Groups 1 and 2 were thought to be ill and all were treated with oxytetracycline and betamethasone.

On 19.10.82 a post-mortem examination was carried out on calf A20 at the local Veterinary Investigation Centre. Significant findings in the lungs were "consolidation" of the cranial lobes, the middle lobe and the cranial parts of the caudal lobes. In addition there was severe interstitial emphysema of all lobes with bulla formation in the caudal lobes. The largest bulla was approximately 6cm in diameter. Small amounts of frothy mucus were present in the trachea and major bronchi. No other significant findings were recorded.

Clinical Findings

First visit: The farm was first visited on 21.10.82. All calves in Group 1 appeared bright and none were anorexic. Three were slightly hyperpnoeic and tachypnoeic. Three calves had a slight serous nasal discharge and two had a slightly elevated rectal temperature (103.5°F). Occasional coughing was noted in the group, particularly from calf A11. On auscultation the only abnormalities detected in Group 1 were in calf A11 which had harsh inspiratory and expiratory sounds. Other calves had harsh sounds but only at the end of inspiration. No adventitious sounds were detected in any calf.

Two of the four calves in Group 2 were slightly dull and had been reluctant to eat that morning. They were also hyperpnoeic and tachypnoeic and one calf had a slight serous nasal discharge. Two calves were slightly pyrexic (103.8°F). All of the calves coughed occasionally. On auscultation two of the four calves had harsh inspiratory and expiratory sounds. No adventitious sounds were detected.

The farmer was not concerned about the health of the calves in Group 3, although they had not "done" as well as he had expected. In view of this, and in spite of the fact that they had never been at grass, they had been treated with an anthelmintic on 20.10.82. There was sporadic coughing in the group at rest. Most of the calves were hyperpnoeic and tachypnoeic.

The farmer was not concerned about the health of the animals in Group 4, however, there was sporadic coughing in the group at rest. No animals were obviously hyperpnoeic or tachypnoeic. They had not been vaccinated against parasitic bronchitis but had been treated with an anthelmintic "several" times whilst at grass and once two days after being housed.

At this visit blood samples and nasopharyngeal swabs were taken from all animals in groups 1 and 2. The other groups were observed but no detailed clinical examination was made, nor were samples taken.

Second visit: A second visit to the farm was made two days later on 23.10.82. At this visit the clinical findings in the animals in Groups 1 and 2 were unchanged.

Since the first visit the farmer had become worried about the health of one of the calves (calf A27) in Group 3. It had been reluctant to feed and seemed to have difficulty breathing on 22.10.82, however, it appeared to respond to treatment with oxytetracycline and betamethasone. On the following day it was dull, dyspnoeic and coughed occasionally. Its rectal temperature

was 101.8°F. On auscultation harsh inspiratory and expiratory sounds were heard in the cranioventral areas of both lung fields. There were no adventitious sounds. One of the other animals in the group (calf A24) was pyrexic (104.8°F), moderately tachypnoeic and had harsh inspiratory and expiratory sounds. The rest of the group were slightly tachypnoeic and hyperpnoeic and coughed occasionally.

The findings in Group 4 were similar to those observed during the first visit. Five animals sampled at random had rectal temperatures within the normal range. The farmer was not worried about the health of the adults.

Blood samples and nasopharyngeal swabs were taken from all animals in Group 3, from 14 animals in Group 4 and from four adults. The farmer was reluctant to have more adults sampled.

On 25.10.82 calf A27, from Group 3, died. Prior to its death it appeared to be having extreme difficulty breathing. It stood with its head and neck extended, its tongue out and was frothing at the mouth. The lungs were obtained for pathological examination.

Third visit: Four weeks after the first visit the farm was revisited and nasopharyngeal swabs and blood samples taken from the animals in Groups 1,2,3 and 4. In addition 11 adults, taken at random, were sampled. Calves born since the last visit were also sampled.

Pathological Findings

Calf A20

Gross: The significant findings at post-mortem examination of calf A20 were consolidation in the cranial parts of all of the lung lobes and severe interstitial emphysema with bulla formation in the caudal lobes.

Microscopic: There was an acute pneumonia with damage to the bronchiolar and the alveolar epithelia. There was oedema and congestion of the alveolar walls and in some areas infiltration of the alveoli with macrophages and neutrophils. There was focal necrosis and hyperplasia of the alveolar epithelium. In most sections there were marked changes affecting the bronchioles. In the epithelium foci of necrosis and of degenerating epithelial cells were apparent. Neutrophils and cellular debris were present in the lumen. Amongst the cellular debris were large syncytia with densely eosinophilic cytoplasm and darkly staining pynknotic nuclei. In these cells the ratio of nuclear mass to cytoplasm was high and the nuclei were generally centrally placed within the cell. At least one of these syncytia contained an eosinophilc intracytoplasmic inclusion body. The peribronchiolar tissues were diffusely infiltrated with small numbers of lymphocytic cells. Most sections had evidence of focal intra-alveolar haemorrhage and interstitial emphysema.

A total of 20 blocks of tissue taken from the right cranial lobe, the middle lobe and the cranial parts of the right caudal lobe showed similar changes, although syncytia were not seen in all sections.

Immunofluorescence: Six of 20 blocks of tissue from the pneumonic areas were found to contain RS viral antigens, but none contained PI3 viral antigens. Respiratory syncytial viral antigens were present in both bronchiolar and alveolar epithelial cells. In some areas the majority of the cells lining an individual alveolus contained antigens whereas in other areas of the same section antigen-positive cells were scanty or absent. No antigens were detected in the alveolar septal cells or in cells free in the alveolar lumen.

In bronchioles viral antigens were present in epithelial cells and in cells free in the lumen. Some antigen positive lumenal cells were multinucleated.

Calf A27

Gross: The significant findings at post-mortem examination of this calf were consolidation in the cranial lobes, the middle lobe and the cranial parts of the caudal lobes and severe interstitial emphysema with bulla formation in the caudal lobes (Fig. 12). A few overinflated non-pneumonic lobules were distributed throughout the posterior part of the left cranial lobe, the cranial part of the left caudal lobe and right middle lobe. The trachea was congested and contained moderate amounts of white frothy mucus.

Microscopic: There was congestion and oedema of the alveolar walls with macrophages and neutrophils in the airspaces. The alveolar epithelium was focally hyperplastic and the septae were thickened with oedema fluid, lymphocytic cells and mononuclear cells. The bronchiolar epithelium was low, flat and the cells were slightly basophilic. In some areas there was early bronchiolitis obliterans and peribronchiolar fibrosis. Diffuse accumulations of lymphocytic cells were present around some bronchioles. There were areas of intra-alveolar haemorrhage and, in some sections, interstitial emphysema. No syncytia or cells with inclusion bodies were seen.

Immunofluorescence: Sections from all lung lobes were negative for RS viral and PI3 viral antigens by immunofluorescence.

## Microbiological Findings

Attempts were made to isolate viruses, bacteria and mycoplasmas from lung tissue taken from calves A20 and A27. No pathogenic organisms were recovered. Nasopharyngeal swabs taken from animals during the outbreak yielded an isolate of RS virus from calf A14.

## Serological Findings

The results of the serological exmainations for antibodies to RS virus and PI3 virus are shown in Tables 2 to 6. In Group 1

none of the calves seroconverted to RS virus although in four the antibody levels did not decline. In Group 2 one calf (A16) seroconverted to RS virus and two had rising antibody titres. In Group 3 five calves seroconverted to RS virus, in Group 4 two animals seroconverted as did one of the adults. There was no evidence of infection with PI3 virus in any of the animals tested. No sera were checked for antibodies to any other viruses.

TABLE 2: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination-inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from the calves in Group 1 in outbreak A.

| Calf number | RS virus 21.10.82 | RS virus 22.11.82 | RS virus CF | PI3 virus 21.10.82 | PI3 virus 22.11.82 | PI3 virus CF |
|---|---|---|---|---|---|---|
| A1 | | 1660 | | | 320 | |
| A2 | | 900 | | | 1280 | |
| A3 | | 900 | | | 320 | |
| A4 | | 3000 | | | 640 | |
| A5 | 140 | 180 | 1.28 | 80 | 80 | 1.00 |
| A6 | 140 | 165 | 1.17 | 80 | 160 | 2.00 |
| A7 | 720 | 500 | 0.69 | 160 | 160 | 1.00 |
| A8 | 630 | 210 | 0.33 | 80 | 80 | 1.00 |
| A9 | 320 | 180 | 0.56 | 160 | 40 | 0.25 |
| A10 | 320 | 330 | 1.03 | 80 | 160 | 2.00 |
| A11 | 1450 | 950 | 0.65 | 640 | 320 | 0.50 |
| A12 | 890 | 210 | 0.23 | 320 | 80 | 0.25 |
| A13 | 1320 | 700 | 0.53 | 160 | 80 | 0.50 |
| A14* | 1780 | 3300 | 1.85 | 320 | 160 | 0.50 |
| A15 | 1100 | 870 | 0.79 | 1280 | 640 | 0.50 |

CF = Conversion Factor

* = RS virus was isolated from a nasopharyngeal swab taken from this calf on 21.10.82.

TABLE 3: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination-inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from the calves in Group 2 in outbreak A.

| Calf number | RS virus 21.10.82 | RS virus 22.11.82 | RS virus CF | PI3 virus 21.10.82 | PI3 virus 22.11.82 | PI3 virus CF |
|---|---|---|---|---|---|---|
| A16 | 10 | 650 | 65.00 | 320 | 320 | 1.00 |
| A17 | 560 | 660 | 1.17 | 320 | 160 | 0.50 |
| A18 | 1260 | 800 | 0.63 | 320 | 160 | 0.50 |
| A19 | 430 | 690 | 1.60 | 160 | 40 | 0.25 |

CF = Conversion Factor

TABLE 4: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination-inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from the calves in Group 3 in outbreak A.

| Calf number | RS virus 23.10.82 | RS virus 22.11.82 | RS virus CF | PI3 virus 23.10.82 | PI3 virus 22.11.82 | PI3 virus CF |
|---|---|---|---|---|---|---|
| A21 | 160 | 640 | 4.00 | 160 | 80 | 0.50 |
| A22 | 1000 | 2300 | 2.30 | 160 | 160 | 1.00 |
| A23 | 400 | 800 | 2.00 | 320 | 160 | 0.50 |
| A24 | 900 | 3800 | 4.22 | 640 | 640 | 1.00 |
| A25 | 400 | 450 | 1.12 | 80 | 80 | 1.00 |
| A26 | 400 | 2000 | 5.00 | 640 | 320 | 0.50 |
| A27 | 3800 | NS | - | 40 | NS | - |
| A28 | 500 | 2100 | 4.20 | 160 | 160 | 1.00 |
| A29 | 500 | 4000 | 8.00 | 320 | 160 | 0.50 |
| A30 | 1380 | 3630 | 2.63 | 160 | 160 | 1.00 |

CF = Conversion Factor

NS = No Sample. This calf died on 25.10.82

TABLE 5: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination-inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from the calves in Group 4 in outbreak A.

| Calf number | RS virus 23.10.82 | RS virus 22.11.82 | RS virus CF | PI3 virus 23.10.82 | PI3 virus 22.11.82 | PI3 virus CF |
|---|---|---|---|---|---|---|
| A31 | 2000 | 2400 | 1.20 | 320 | 80 | 0.25 |
| A32 | 9000 | 13800 | 1.53 | 160 | 320 | 2.00 |
| A33 | 1660 | 6920 | 4.16 | 160 | 80 | 0.50 |
| A34 | 2100 | 2400 | 1.14 | 80 | 80 | 1.00 |
| A35 | 1000 | NS | - | 40 | NS | - |
| A36 | NS | 5000 | - | NS | 40 | - |
| A37 | 1000 | 200 | 0.20 | 160 | 80 | 0.50 |
| A38 | 3200 | 2560 | 0.77 | 320 | 320 | 1.00 |
| A39 | 2000 | 2760 | 1.38 | 160 | 160 | 1.00 |
| A40 | 11500 | 12600 | 1.09 | 160 | 320 | 2.00 |
| A41 | 950 | 3300 | 3.47 | 160 | 160 | 1.00 |
| A42 | NS | 5000 | - | NS | 160 | 1.00 |
| A43 | 4200 | 3800 | 0.90 | 80 | 160 | 2.00 |
| A44 | 520 | 2560 | 4.94 | 80 | 80 | 1.00 |
| A45 | 3800 | 4800 | 1.26 | 80 | 80 | 1.00 |
| A46 | 3800 | 6300 | 1.65 | 160 | 40 | 0.25 |

CF = Conversion Factor

NS = No sample

TABLE 6: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination-inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from the adult cattle in outbreak A.

| | RS virus | | | PI3 virus | | |
|---|---|---|---|---|---|---|
| Adult number | 23.10.82 | 22.11.82 | CF | 23.10.82 | 22.11.82 | CF |
| A47 | NS | 1260 | - | NS | 320 | - |
| A48 | NS | 1000 | - | NS | 320 | - |
| A49 | NS | 3600 | - | NS | 320 | - |
| A50 | 260 | 1250 | 4.86 | 320 | 160 | 0.50 |
| A51 | 1260 | NS | - | 320 | NS | - |
| A52 | NS | 640 | - | NS | 320 | - |
| A53 | NS | 260 | - | NS | 640 | - |
| A54 | NS | 1750 | - | NS | 320 | - |
| A55 | NS | 1660 | - | NS | 640 | - |
| A56 | NS | 5000 | - | NS | 640 | - |
| A57 | 1900 | NS | - | 320 | NS | - |
| A58 | 1660 | NS | - | 160 | NS | - |
| A59 | NS | 640 | - | NS | 640 | - |
| A60 | NS | 800 | - | NS | 80 | - |

CF = Conversion Factor
NS = No sample

## Discussion

On the basis of clinical, pathological, virological and serological findings there is strong evidence that RS virus was the aetiological agent in this outbreak. At the time of the incident RS viral infection was active in Groups 1, 2, 3 and 4 and in the adults. The virus was isolated from the nasal secretions of calf A14 in Group 1 during the acute phase of the outbreak. Calf A20 from Group 2 died in severe respiratory distress and had pathological lesions indicative of RS viral infection. This was confirmed by immunofluorescence. A calf (A16) in direct contact with it seroconverted to RS virus. Calf A27, which also died in severe respiratory distress, had lesions in the respiratory tract epithelium which were suggestive of a prior viral infection and it had a high titre of serum neutralising antibody to RS virus two days before it died. Five of the nine calves in direct contact with it seroconverted to RS virus. There was no evidence of infection with PI3 virus in either dead calf and no pathogenic bacteria or mycoplasmas were isolated from their lungs. Two of the calves (A33 and A44) in Group 4 seroconverted to RS virus as did one of the adults (A50).

The source of infection in this outbreak was not identified conclusively. The farm was a "closed" dairy herd and although cows were occasionally bought none had entered the herd during the previous year. A neighbour's bull had been moved onto the farm in early October 1982 but was running with the in-calf heifers and was not in direct contact with the groups of animals which developed respiratory disease. The farmer's vet had disbudded the calves in Group 1 on 7.11.80 and four days later one of these calves (A11) became ill with respiratory disease. Ten days later, at the first visit, RS virus was present in the nasal secretions of calf A14 which was in direct contact with A11. Calf A14 was showing signs of respiratory disease with coughing, tachypnoea, hyperpnoea and slight dyspnoea. Despite this there was no unequivocal serological evidence of RS viral infection in the rest of the group. None of the calves seroconverted to RS virus although in four calves

(A5, A6, A10 and A14) the antibody titre stayed constant or rose slightly and did not decline as would have been expected if this antibody had been derived from colostrum (Tizard, 1982). Thomas (1973) suggested that maternally derived antibody could mask seroconversion and this could have occurred in the animals in Group 1. Another possible source of viral infection is suggested by the finding that a proportion of individual bovine foetuses had serum antibody to RS virus (Gould and others, 1978). This raises the possibility that in-utero infection of one of the calves in Group 1 could have been responsible for the introduction of virus.

If not from an external source then RS virus may have been present on the farm itself for some time with its presence only coming to light because young susceptible animals had become infected with serious consequences. Infection was present in both Group 4 and the adults without there being severe clinical signs of respiratory disease. There is evidence that older animals such as these are potentially susceptible to infection and disease due to RS virus (Paccaud and Jacquier, 1970; Koves and Bartha, 1975) and therefore that immunity is not related to age per se. In the human secondary and subsequent infections with RS virus are not associated with the severe clinical signs of some primary infections (Beem and others, 1960). This being the case it is possible that Group 4 and the adults were not undergoing their first infection with RS virus and had been exposed before. Stott and others (1980) found that repeat infections with RS virus can occur in the bovine and suggested that persistent infections could also occur. Reactivation of persistent infection has been suggested as the source of virus in two outbreaks of respiratory disease due to RS virus (Thomas and others, 1980; Elazhary and others, 1982). In one incident "stress" was thought to have reactivated a persistent infection with subsequent spread to other susceptible animals in the group. In the present outbreak both Group 4 and the adults had recently been housed and this could constitute a "stress". Alternatively the virus could have

survived in both groups by reinfection of partially immune individuals as is thought to occur in the human (Henderson and others, 1979). On housing the frequency of reinfection may have increased as the animals came in closer and more frequent contact with each other. The titre of virus in the group would rise and could act as a source of infection for other animals on the farm.

How the virus spread from group to group on the farm is not clear. There were no opportunities for direct contact between the animals during the month before the outbreak began. However, in human hospitals indirect transmission of virus between infants by contaminated hands and clothing of nurses and attendants is thought to be a probable means of viral spread (Hall and others, 1980). This could happen during routine procedures on the farm such as dosing and feeding. There was no evidence that the attendants themselves had acute respiratory disease at the time of the incident, although the farmer's father, who helped feed the calves, was a chronic bronchitic. Cats present on the farm were not investigated microbiologically. Although there is evidence that they are susceptible to infection with RS virus (Pringle and Cross, 1978) whether they are susceptible to infection with bovine RS virus is not known.

RESPIRATORY DISEASE INVOLVING RS VIRUS: OUTBREAK B

## Introduction

The farm on which this outbreak occurred was a mixed upland farm with a beef suckler herd and a sheep flock. In addition to the suckler and sheep enterprises batches of young calves, approximately 40 at a time, were bought from a dealer. They were reared on milk substitute from a machine until 10 weeks of age then were weaned. At about four months old they were moved into a slatted-floor house at a separate farm.

## History of Respiratory Disease Outbreak

The batch of calves in which the problem occurred had been "poor doers" throughout the rearing period and had been weaned on 1.12.80. A "few" deaths had occurred over the previous month and had been diagnosed as "pneumonia" by the local Veterinary Investigation Centre. Over the previous week more calves had died and the rest had become ill. All were coughing and reluctant to eat. They were all housed, as a single group, in an open-fronted shed measuring approximately 6m by 16m. Sacks were suspended from the eaves and corrugated iron sheeting was placed over the gates to minimise draughts. On 11.12.80 half of the surviving calves had been moved into another open-fronted shed measuring 10m by 10m. At this stage six animals were purchased for further examination at the Veterinary School. Two arrived dead (calves B1 and B2) and four alive (calves B3, B4, B5 and B6).

## Clinical Findings

First Visit: During the first visit on 13.12.80 there was widespread coughing throughout both groups of calves and a few were pneumonic. Blood samples were taken from 10 calves in each group and nasopharyngeal swabs were taken from half of these. Another two animals were purchased for further examination (calves B7 and B8).

On 15.10.80 a second wave of coughing and pyrexia went through all the calves. They were treated with a combination of short-acting and long-acting tetracyclines and subsequent recovery was uneventful.

Second Visit: At the time of the second visit on 16.1.81 both groups of calves had been moved to the other farm. Occasional spontaneous coughing was present in the group but growth rate and feed comsumption were normal. Repeat blood samples were taken from most of the animals sampled at the first visit.

Both of the farmers attending these calves had respiratory disease around the time of this incident. In one of them, a man 60 years of age, this followed a protracted course and involved a period of hospitalisation. In neither was a specific diagnosis made.

Pathological Findings

Calf B1

This calf was dead on arrival. The carcase was too decomposed to provide any useful and reliable information. The lungs were consolidated and had severe interstitial emphysema.

Calf B2

This animal died of respiratory disease, although exactly when was not clear.

Gross: The pneumonic lesions were very solid and plum coloured. There was severe sub-pleural interstitial emphysema in the caudal lobes and in the right middle lobe with bulla formation in the cranial part of the caudal lobes. Consolidated tissue was mottled in appearance with pale areas and a few small haemorrhages. On cross section it was mainly a dry lesion although in a few places

there was oedema. The mucosae of the trachea and the bronchi were congested.

Microscopic: There was an acute pneumonia with epithelial damage. There was focal necrosis of the alveolar epithelium and macrophages and neutrophils were present in the lumen. In some areas there was focal hyperplasia of the alveolar epithelium. In the bronchiolar epithelium there was focal necrosis and syncytium formation. Some syncytia contained eosinophilic intracytoplasmic inclusion bodies. The peribronchiolar tissues were diffusely infiltrated by small numbers of lymphocytes and, occasionally, plasma cells.

In addition to these lesions there were occasional areas with bronchiolitis obliterans or coagulative necrosis in the cranial lobes. In the caudal lobes there was marked hyaline membrane formation with congestion and oedema of the alveolar walls. There was focal proliferation of the alveolar epithelium with large basophilic cells containing large oval nuclei. No syncytia or inclusion bodies were seen in either the alveolar or the bronchiolar epithelia.

Immunofluorescence: Respiratory syncytial viral antigens were detected by immunofluorescence in the right cranial and middle lobes of the lung. Syncytia and single cells containing antigens were found in the bronchiolar and alveolar epithelium. All tissues were negative for PI3 viral antigens.

Calves B3, B4, B5 and B6

The post-mortem findings in these animals were similar in character but varied in extent.

Gross: There was a severe pneumonia with consolidation of the cranial and the middle lobes and the cranial parts of the caudal lobes. The pneumonic lesions were slightly collapsed with a mottled surface and a few petechial haemorrhages. The bronchial

lymph nodes were enlarged. In addition to these lesions calf B3 had a widespread epithelialising lesion in the caudal lobes. Calf B4 had widespread suppurative lesions in the right cranial and middle lobes. Abscesses, varying in diameter from 0.5cm to 2.0cm were present in the pulmonary parenchyma and there was an overlying fibrinous pleurisy. The lesions in calf B6 were less extensive than in the others.

Microscopic: The reactions in the cranial and the middle lobes and in the cranio-ventral parts of the caudal lobes were similar in type in all of the calves but varied in extent and severity. The most striking and consistent lesions involved the bronchi and the bronchioles. There was bronchitis with infiltration of the lamina propria with lymphocytic cells, the majority of which were plasma cells. Neutrophils were seen in the bronchial epithelium and on its surface. Submucosal blood vessels were congested and the lamina propria was oedematous. The bronchiolar reaction was similar with diffuse early fibrosis in the peribronchiolar tissues. Lymphocytic cells, again largely plasma cells, were present around the bronchioles but not in sufficient numbers to form a "cuff". The bronchiolar epithelium was dysplastic with nuclei at irregular levels. Neutrophils were seen in the epithelium and in the lumen. In some areas there was bronchiolitis obliterans. In the alveoli there were two types of cellular infiltrate. Around the bronchioles the cellular infiltrate was of neutrophils and macrophages, with the former cell type in excess. In other areas the cellular infiltrate was less marked and composed mostly of macrophages. In areas with these infiltrates the alveolar epithelium was hyperplastic but no syncytia or inclusion bodies were seen. Both lymphocytes and plasma cells were seen in the alveolar septae.

These lesions varied in extent and severity between the animals being most extensive in calf B3 and least extensive in calf B6 where there was no involvement of the caudal lobes.

Additional lesions were present in calves B3 and B4. In calf B3, which was dyspnoeic before slaughter, there was widespread diffuse alveolar epithelial hyperplasia with hyaline membrane formation in the caudal lobes. The alveolar walls were congested and oedematous and there were a moderate number of macrophages in the alveoli. In general the bronchioles were not involved in this reaction although there were occasional focally necrotic areas and a few lymphocytic cells in the peribronchiolar tissues. In calf B4 there were foci of suppurative necrosis in the right cranial lobe and the middle lobe. In some areas this process appeared to originate in the centre of a lobule although it was not possible to say with certainty that it was centred on bronchi or bronchioles. Macrophage-type syncytia were occasionally seen free in the alveolar lumen. These did not contain inclusion bodies.

Immunofluorescence: All tissues examined from these calves were negative for RS viral and PI3 viral antigens by immunofluorescence.

Calf B7

This calf was dead on arrival at the Veterinary School.

Gross: There was marked consolidation of the cranial lobes, the middle lobe and the cranial parts of the caudal lobes. The trachea was severely congested and contained copious white frothy mucus. The pneumonic lesions were solid and firm. The interlobular septae were prominent. Fibrin tags were present between the lung lobes and adhering to the parietal pleura.

Microscopic: There were lesions similar to those in calves B3 to B6 but in addition there was a focal necrotising lesion involving alveoli and bronchioles. Adjacent to the lesions the alveoli were congested and filled with oedema fluid. Macrophage-type syncytia were present in the alveoli.

Immunofluorescence: All tissues examined from this calf were negative for RS viral and PI3 viral antigens.

Calf B8

In Calf B8 the pneumonic lesions were less marked. The bronchiolar changes were similar to those in other calves but involved more intensive peribronchiolar lymphocytic infiltration, composed largely of lymphocytes, with early germinal centre formation. This animal also had a necrotising laryngitis. No RS viral or PI3 viral antigens were detected by immunofluorescence.

## Microbiological Findings

The results of microbiological examinations are summarised in Table 7. In addition Mycoplasma bovis was isolated from one nasopharyngeal swab taken during this outbreak.

## Serological Findings

The results of serological examinations for antibodies to RS virus and PI3 virus are detailed in Table 8. Four animals seroconverted to RS virus and one to PI3 virus. In addition there was serological evidence of infection with BVD virus in one calf (B9), with adenovirus type A in two calves (B3 and B4) and with adenovirus type B in three calves (B6, B9 and B10). There was no evidence of infection with reovirus types 1 and 2 or IBR virus. Four calves seroconverted to Pasteurella haemolytica type A1 and three to Mycoplasma bovis.

TABLE 7: The pathogenic viruses, bacteria and mycoplasmas detected in the lungs of the calves in outbreak B.

| Calf number | Viruses | Bacteria | Mycoplasmas |
|---|---|---|---|
| B2 | RS virus | NS | NS |
| B3 | Negative | Negative | Negative |
| B4 | Negative | Negative | Ureaplasma sp. |
| B5 | Negative | Negative | Ureaplasma sp. M.dispar |
| B6 | Negative | Negative | Negative |
| B7 | Negative | S.pneumoniae | Negative |
| B8 | Negative | NS | NS |

NS = No sample

TABLE 8: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination-inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from the calves in outbreak B.

| Calf number | RS virus 13.12.80 | RS virus 16.1.81 | RS virus CF | PI3 virus 13.12.80 | PI3 virus 16.1.81 | PI3 virus CF |
|---|---|---|---|---|---|---|
| B8 | 15 | 60 | 4.00 | 160 | 160 | 1.00 |
| B9* | 85 | 85 | 1.00 | 160 | 320 | 2.00 |
| B10 | 60 | 30 | 0.50 | 320 | 320 | 1.00 |
| B11 | 250 | 70 | 0.28 | 320 | 320 | 1.00 |
| B12 | 45 | 30 | 0.66 | 320 | 160 | 0.50 |
| B13 | 10 | 50 | 5.00 | 160 | 320 | 2.00 |
| B14 | 25 | 25 | 1.00 | 160 | 160 | 1.00 |
| B15 | 20 | 65 | 3.25 | 1280 | 160 | 0.12 |
| B16* | 230 | 310 | 1.34 | 160 | 1280 | 8.00 |
| B18 | 70 | 30 | 0.43 | 320 | 320 | 1.00 |
| B19 | 110 | 830 | 7.54 | 160 | 160 | 1.00 |
| B20 | 60 | 60 | 1.00 | 320 | 320 | 1.00 |
| B21 | 20 | 110 | 5.50 | 320 | 160 | 0.50 |
| B22 | 210 | 100 | 0.47 | 320 | 160 | 0.50 |
| B23 | <10 | 30 | >3.00 | 160 | 80 | 0.50 |
| B24 | 30 | 50 | 1.66 | 1280 | 320 | 0.25 |
| B25 | 130 | 90 | 0.70 | 160 | 160 | 1.00 |

CF = Conversion Factor

* = These calves were moved to the Glasgow University Veterinary School on 13.12.80

## Discussion

This outbreak presented as a marked increase in the frequency and severity of clinical signs of respiratory disease in a group of calves which had had a history of "pneumonia", with deaths, over the previous three months. No precise time of onset could be defined for the recent change in the clinical illness nor was this associated with any obvious managemental or environmental changes. On the basis of histopathological and serological evidence RS virus was a significant pathogen in this incident. Viral antigens were detected by immunofluorescence in the lungs of calf B2 in direct association with the significant acute pathological changes in the epithelia of the bronchioles and the alveoli. Five of 17 calves in direct contact with this calf seroconverted to RS virus. Three others had rising antibody titres, although these could not be considered significant as they were not four-fold or greater rises. No isolates of RS virus were recovered from the material examined despite its presence being detected by immunofluorescence. This reinforces the view that diagnosis of this viral infection by this method is difficult and inefficient from the sort of material usually available from respiratory disease outbreaks (Wellemans, 1977).

Other recognised respiratory pathogens associated with this outbreak, by virtue of their isolation from respiratory tract material, were Mycoplasma bovis, M.dispar, Ureaplasma sp and S.pneumoniae and these could have exacerbated the lesions induced by viral infection. Five calves seroconverted to P.haemolytica type A1, however, this bacterium was not isolated from any of the material examined. The calves had been treated with antibiotics and this may have influenced the isolation of bacteria although recently Gibbs and others (1983) pointed out that antibiotic treatment does not necessarily prevent the isolation of P.haemolytica from either the upper or the lower respiratory tract in animals from naturally occurring outbreaks of respiratory disease. This being the case it is possible that in this particular outbreak the animals may have become infected with this

bacterium after the initial visit and investigations.

Parainfluenza type 3 virus was not associated with this incident. One animal (B16) seroconverted to this virus but this animal was admitted to the Veterinary School on 13.12.80 where it may have been exposed to PI3 virus. There was serological evidence of infection with BVD virus and adenovirus type B in another animal (B9) which had been moved to the Veterinary School and again these results have to be interpreted with caution when considering the original outbreak. Two animals seroconverted to adenovirus type A and two to adenovirus type B but these findings do not specifically localise the infections to the respiratory tract and, in support of this, no virus was isolated from any of the material examined.

A significant finding in all of the animals examined post-mortem was widespread damage to the epithelium of the bronchi, the bronchioles and the alveoli in the cranial parts of the lungs. In only one animal (calf B2) could this be specifically related to RS viral infection. In the others infection with RS virus probably caused damage which was subsequently exacerbated by other organisms. In some animals there was evidence of long standing pulmonary damage with bronchiolitis obliterans and peribronchiolar fibrosis reflecting the long clinical history of respiratory disease. Whether this represented repeated damage by RS virus is not clear.

Both of the farmers attending the calves had undiagnosed respiratory illnesses occurring at the time of the first visit. In one case this involved a prolonged period of hospitalisation. Whether these illnesses were related to those in the calves is not known. There is evidence that human strains of RS virus can infect calves under experimental circumstances (Jacobs and Edington, 1975) although this has not yet been shown to occur under natural conditions. Some strains of bovine RS virus can grow in cells of human origin (Jacobs and Edington, 1975) including those derived from the lungs of human embryos (Matumoto and others, 1974). However, the significance of these findings in relation to the

epidemiology of RS virus in either the human or the bovine is not yet clear.

RESPIRATORY DISEASE INVOLVING RS VIRUS: OUTBREAK C

## Introduction

This outbreak occurred on an upland farm with a herd of 17 Friesian and Ayrshire suckler cows. Home-bred and bought-in calves were multiple-suckled until weaning at approximately three months of age. Calves were housed initially in an open-fronted shed adjacent to the byre housing the cows. Suckling was permitted twice daily and calves had access to a barley/protein mix and hay.

## History of Respiratory Disease Outbreak

Two batches of 14 bought-in calves, one aged approximately six weeks and the other three months, were housed together in the open-fronted shed. Prior to the onset of the incident coughing was noticed in the calves but this was not thought serious enough to warrant veterinary attention. On 25.11.78 the older calves were to be weaned when the farmer noticed one to be "lifting" (i.e. tachypnoeic and hype.pnoeic). This calf was left with the younger ones and the rest were moved to another open-fronted shed 60m away, in which were also 13, six months old animals. A solid brick wall divided the two groups but there was a 10cm gap between it and the roof. The farmer was not concerned about the health of this group of six months old animals and at no time did they become involved in the outbreak.

The first calf to become ill was treated by a veterinary practitioner for three days with a combination of oxytetracycline and betamethasone. Initially its condition improved but on 29.11.78 it relapsed and several other calves, in both the six weeks old and three months old age groups, were noticed to be ill. The practitioner was recalled and the sick calves were treated with antibiotics. On 29.11.78 two calves died, the one which had originally been ill and another from the three months old group.

Clinical Findings

First visit: The farm was first visited on 30.11.78. Two of the three months old calves and three of the six weeks old calves were extremely dull. Many of the younger calves were reluctant to suck. Frequent coughing occurred in both age groups but was much worse in the older animals. Three calves were dyspnoeic and a further four hyperpnoeic. One of the dyspnoeic calves was recumbent. Fourteen calves had a rectal temperature of 103°F or greater and the mean rectal temperature was 103.1±1.15°F. Twenty three calves had respiratory rates in excess of 30/min (mean 48.8± 13/min). Respiratory sounds were harsh but few adventitious sounds were detected. Nasal discharge was not a feature with only one of 26 animals having a purulent nasal discharge. Two live animals (calves C1 and C2), which had first been noticed to be ill on 29.11.78 and one dead animal (Calf C3) were admitted to the Veterinary School. Both live animals were dyspnoeic and very dull. Their respiratory rates were 30 (C1) and 45 (C2) breaths/min and rectal temperatures 101°F and 101.4°F respectively.

On 1.12.78 a further two live calves were admitted (Calves C4 and C5), both of which were dyspnoeic and tachypnoeic. Calf C4 died overnight. Neither were pyrexic.

On 2.12.78 a further three calves (Calves C6, C7 and C8) were admitted, one of which (C6) was extremely dyspnoeic and died during transport.

From 2.12.78 the surviving calves began to improve and over the following six weeks the mean respiratory rate and rectal temperature subsided to normal levels. Coughing still occurred in both age groups but was more prevalent in the older group.

Blood samples were taken from 18 calves (C9 to C27) at this visit.

Second visit: The farm was revisited four weeks later on 28.12.78 and further blood samples were taken from calves C9 to C27. In addition the adult nurse cows (C28 to C44) were blood sampled.

Third visit: On 9.1.79, six weeks after the outbreak had started, a further six animals (Calves C28-C33), some of which had been severely ill during the acute phase at the outbreak were purchased for clinical, microbiological and pathological examination.

Pathological Findings

Calves C1-C3

The pathological findings in the lungs of these animals were similar.

Gross: There was consolidation in the cranial and middle lobes and in the cranio-ventral parts of the caudal lobes. The lesions were deep red or purple in colour and were slightly raised above the surface of the lung. On cross section the lesions were homogeneous and slightly oedematous. Small haemorrhages were present, about 1mm in diameter, in all lobes. In all the lungs there was widespread severe interstitial emphysema with bulla formation in the caudal lobes. The bullae measured up to 10cm in diameter and adjacent lung lobules were collapsed. The trachea was congested with small petechial haemorrhages in the mucosa in one animal. The right lung from calf C1 is illustrated in Fig. 1.

Microscopic: There was bronchitis, bronchiolitis and alveolitis, all with epithelial damage. In the bronchi there was an increase in the number of inflammatory cells in the lamina propria, principally mononuclear cells. Neutrophils were present in the epithelium and there was focal necrosis of epithelial cells. The bronchiolar lesions were similar to those in the bronchi but with more severe epithelial damage. In some bronchioles there was complete necrosis of the epithelium and plugging of the lumen with neutrophils. There was diffuse infiltration of peribronchiolar

tissues with small numbers of lymphocytes and plasma cells. In the alveoli the most striking features were epithelial changes. In some areas there were numerous epithelial syncytia (Fig. 7), some with eosinophilic intracytoplasmic inclusion bodies, and focal necrosis and hyperplasia of the epithelium. The cellular infiltrate was mixed, with macrophages, neutrophils and desquamated epithelial cells. In other areas there was marked epithelial hyperplasia, particularly in calf number 3 which had died as a result of the pulmonary lesions. Alveolar walls were congested and oedematous.

Immunofluorescence: Respiratory syncytial viral antigens were detected in the lungs of these animals. In all cases viral antigens were present in alveolar epithelial cells, both in syncytia (Fig. 9) and in individual cells. No viral antigens were detected in bronchiolar epithelial cells. No PI3 viral antigens were detected.

Calves C4-C8

The findings in these animals were broadly similar although they varied markedly in extent.

Gross: There was consolidation in the cranial and the middle lobes and the cranioventral parts of the caudal lobes. The lesions were deep red or purple and in most animals were slightly collapsed and oedematous. Most animals had widespread severe interstitial emphysema in the cranial and caudal lobes with bulla formation. In C7, although present, the degree of emphysema was slight. Petechial haemorrhages were present in the lungs and trachea of C6 and in the trachea of C4.

Microscopic: The pathological changes affected the bronchi, bronchioles and alveoli with the most striking lesions being present in the epithelium. There was bronchitis with large numbers of mononuclear cells in the lamina propria and neutrophils in the epithelium. The bronchiolar epithelium was dysplastic, with nuclei

at irregular levels in the epithelial layer. Neutrophils were present in the epithelium and in the lumen. In some areas there was early bronchiolitis obliterans. Diffuse accumulations of lymphocytes and plasma cells were present in the peribronchiolar tissues, sometimes forming loose aggregates of cells. In calf C8 peribronchiolar lymphocytic cells were more numerous forming a narrow, regular cuff and there was early germinal centre formation.

The alveolar epithelium was hyperplastic. This change was most marked in calf C6 (Fig. 13) in which a single layer of cuboidal cells with slightly basophilic cytoplasm lined the alveoli. The alveolar airspaces were either collapsed or contained macrophages, neutrophils and oedema fluid. The neutrophilic component was more marked in some areas, especially in C7.

Immunofluorescence: All the tissues examined from these animals were negative for RS viral and PI3 viral antigens by immunofluorescence.

Calves C28-C33

The pathological findings in four of these calves were similar although they varied in extent and degree.

Gross: There were pneumonic lesions confined to the cranial and middle lung lobes. The lesions were slightly collapsed, purple in colour and had a lobular distribution. The surface of the lesion was smooth and homogeneous with occasional white spots. On cross section pneumonic lobules were collapsed and white spots were seen on the surface. Frothy mucopus was present in the larger bronchi.

Microscopic: There was a chronic non-suppurative pneumonia with evidence of epithelial damage and a lymphocytic reaction around the bronchi and the bronchioles. In most sections there was peribronchiolar fibrosis and bronchiolitis obliterans. Accumulations of lymphocytes and a few plasma cells were present

around the bronchi and the bronchioles. In all animals these cells formed cuffs containing lymphoid follicles with germinal centre-like areas. These were most marked in calves C28, C31, C32 and C33 (Fig. 14). In calves C29 and C30 the general reaction was the same as in the others but was less extensive.

In alveolar areas adjacent to bronchi and bronchioles there was thickening of interalveolar septae resulting from an increase in the number of interstitial cells and infiltration with macrophages, lymphocytes and plasma cells. Macrophages were present in the alveolar lumen and there was epithelial hyperplasia in some areas. In others the alveoli were collapsed. Occasional multinucleated macrophages were seen but there were no epithelial syncytia or cells with inclusion bodies.

Immunofluorescence: All the tissues examined from these animals were negative for RS viral and PI3 viral antigens.

Microbiological Findings

The results of the microbiological investigations in calves C1 to C8 are presented in Table 9. No pathogenic viruses or bacteria were detected in calves C28 to C33, however, Mycoplasma dispar was isolated from the lungs of calves C28 and C33.

Serological Findings

The results of serological investigations for RS virus and PI3 virus in this outbreak are presented in Tables 10, 11 and 12. All of the calves examined post-mortem had serum antibody to both RS virus and PI3 virus. Nine of the nineteen calves from which paired sera were obtained seroconverted to RS virus. There was no evidence of infection with PI3 virus. No sera were checked for antibodies to any other viruses.

TABLE 9: The pathogenic viruses, bacteria and mycoplasmas detected in the lungs of calves C1 to C8 in outbreak C

| Calf number | Viruses | Bacteria | Mycoplasmas |
|---|---|---|---|
| C1 | RS virus | Negative | Ureaplasma sp. M.bovis |
| C2 | RS virus | Negative | Negative |
| C3 | RS virus | Negative | Negative |
| C4 | Negative | S.pneumoniae | Ureaplasma sp. |
| C5 | Negative | S.pneumoniae | Negative |
| C6 | Negative | P.multocida | Negative |
| C7 | Negative | Negative | Negative |
| C8 | Negative | Negative | Negative |

TABLE 10: Neutralisation titre to respiratory syncytial (RS) virus and parainfluenza type 3 (PI3) virus in sera from calves C1 to C8 in outbreak C.

| Calf Number | RS virus | PI3 virus |
|---|---|---|
| C1 | 330 | 48 |
| C2 | 1070 | 24 |
| C4 | 145 | 384 |
| C5 | 270 | 768 |
| C6 | 200 | 12 |
| C7 | 440 | 192 |
| C8 | 470 | 192 |

Table 11: Neutralisation titre to respiratory syncytial (RS) virus and parainfluenza type 3 (PI3) virus in paired sera from calves C9 to C27 in outbreak C.

| Calf number | RS virus | | | PI3 virus | | |
|---|---|---|---|---|---|---|
| | 29.11.78 | 28.12.78 | CF | 29.11.78 | 28.12.78 | CF |
| C9 | 10 | 60 | 6.00 | 192 | 192 | 1.00 |
| C10 | 30 | 190 | 6.30 | 48 | 48 | 1.00 |
| C11 | 50 | > 3070 | >61.40 | 768 | 192 | 0.25 |
| C12 | 190 | > 1540 | > 8.10 | 96 | 24 | 0.25 |
| C13 | 240 | 1540 | 6.40 | 192 | 96 | 0.50 |
| C14 | 300 | 6150 | 20.50 | 24 | 12 | 0.50 |
| C15 | 300 | 3070 | 10.20 | 96 | 96 | 1.00 |
| C16 | 380 | 1540 | 4.05 | 96 | 96 | 1.00 |
| C17 | 380 | 1540 | 4.05 | 384 | 96 | 0.25 |
| C18 | 60 | 190 | 3.16 | 96 | 96 | 1.00 |
| C19 | 95 | 155 | 1.63 | 3072 | 3072 | 1.00 |
| C20 | 240 | 480 | 2.00 | 1536 | 192 | 0.13 |
| C21 | 240 | 615 | 2.56 | 96 | 96 | 1.00 |
| C22 | 385 | 385 | 1.00 | 1536 | 1536 | 1.00 |
| C23 | 615 | 1230 | 2.00 | 3072 | 3072 | 1.00 |
| C24 | 770 | 1540 | 2.00 | 12 | 12 | 1.00 |
| C25 | 770 | 770 | 1.00 | 384 | 192 | 0.50 |
| C26 | 770 | 770 | 1.00 | 768 | 192 | 0.25 |
| C27 | 770 | 770 | 1.00 | 1536 | 384 | 0.25 |

CF = Conversion Factor

TABLE 12: Neutralisation titre to respiratory syncytial (RS) virus and parainfluenza type 3 (PI3) virus in sera from the adult cattle in outbreak C.

| Adult Number | RS Virus | PI3 Virus |
|---|---|---|
| C28 | 1250 | 12288 |
| C29 | 3072 | 3072 |
| C30 | 2570 | > 12288 |
| C31 | 3072 | 6144 |
| C32 | 3072 | 12288 |
| C33 | > 6144 | 6144 |
| C34 | > 6144 | 3072 |
| C35 | > 6144 | 12288 |
| C36 | > 6144 | 3072 |
| C37 | > 6144 | 3072 |
| C38 | 3072 | 3072 |
| C39 | 3072 | 3072 |
| C40 | 3072 | 6144 |
| C41 | 2140 | 96 |
| C42 | 1320 | 12288 |
| C43 | > 6144 | 12288 |
| C44 | > 6144 | 384 |

## Discussion

The clinical, pathological and serological investigations carried out on the calves in this outbreak of acute respiratory disease provide strong evidence that RS virus was the aetiological agent. Clinically the significant findings were reduced appetite, pyrexia, tachypnoea, coughing, dyspnoea and death. These findings are similar to those described in natural infections with bovine RS virus by other authors (Wellemans and Leunen, 1975; Holzhauer and van Nieuwstadt, 1976). In the present outbreak severe clinical signs were confined to the calves aged six weeks to three months old. There was no evidence that calves with higher RS virus serum neutralisation titres had more severe disease than other calves as has been suggested in children (Kapikian and others, 1969; Kim and others, 1969). Adult animals, in direct contact with the calves, remained clinically normal throughout the incident although they did have high titres of serum neutralising antibodies against RS virus four weeks after the acute phase of the outbreak. Adult cattle have shown respiratory disease in other incidents (Paccaud and Jacquier, 1970; Odegaard and Krogsrud, 1977). Other calves on the farm, aged approximately six months old and over, also remained unaffected. There was no history of respiratory disease in the people in contact with the calves at any time during the outbreak.

A dramatic and consistent finding at post-mortem in calves C1 to C8 was marked interstitial emphysema, often with the formation of bullae of gas in the caudal lobes. Despite the degree and extent of this finding in some animals there was no evidence of mediastinal or subcutaneous emphysema as has been reported in other cases of RS viral infection (Koves and Bartha, 1975; Odegaard and Krogsrud, 1977). Emphysema was present in all of the dyspnoeic animals. The areas of pulmonary consolidation were confined to the cranial and the middle lobes and the cranial parts of the caudal lobes. In some cases the lesions were less extensive than the severity of the clinical signs would have suggested indicating that pneumonic consolidation *per se* was not wholly responsible for the degree of clinical illness.

A striking feature of the histopathology in calves C1, C2 and C3 was the presence of multinucleated syncytia in the alveolar epithelium. Some syncytia contained eosinophilic intracytoplasmic inclusion bodies. No intranuclear inclusion bodies were seen. Multinucleated syncytia or "giant cells" have been described in experimental RS viral infections (Jacobs and Edington, 1975; Mohanty and others, 1975) and in natural RS viral infections (van den Ingh and others, 1982). In the present outbreak RS virus was confirmed as the cause of the syncytium formation by immunofluorescence. Viral antigens were also detected in many individual single epithelial cells which had not formed syncytia. Some eosinophilic intracytoplasmic inclusion bodies seen in H&E stained sections were shown to be composed of RS viral antigens by immunofluorescence. This is in contrast to the findings of Paccaud and Jacquier (1970) who found that inclusion bodies did not stain positively with fluorescent antiserum prepared from a recovered case of RS viral infection.

In this outbreak immunofluorescence was a specific and more sensitive indicator of viral infection than was the search for syncytia or inclusion bodies. However, all antigen-positive lungs did contain both syncytia and inclusion bodies although they were sometimes difficult to find. The quantity of viral antigen varied throughout the lesions, some sections being markedly positive whilst others, from the same lung, contained few or no antigen-positive cells. This highlights the necessity of taking several blocks of tissue from different sites within the lung and may account for the inability to find syncytia in some field investigations (Elazhary and others, 1982; Thomas and others, 1982). Also of relevance to this point is the fact that no viral antigens were detected in calves C4 to C8. These calves were killed or died later in the outbreak by which time viral antigens may have disappeared from the lungs. In experimental RS viral infections most investigations have failed to isolate virus from the lung beyond 10 or 12 days after infection (Jacobs and Edington, 1975; Bryson and others, 1982). The histopathological changes in these calves were in general similar to those in calves C1 to C3

in that there was widespread epithelial damage in the bronchi, the bronchioles and the alveoli. However, they lacked specific evidence of viral infection.

The source of viral infection in this outbreak was not found. The calves had been housed together since purchase and the only other bovines with which they had been in direct contact were the nurse cows. Whether these latter animals could have been the source of infection could not be ascertained from the samples taken although they did have high serum antibody titres four weeks after the acute phase of the outbreak. Six weeks after the acute phase of the disease no evidence of viral infection could be found in six calves (C28 to C33) by either the isolation of virus or immunofluorescence. The lesions present in four of these calves were "cuffing pneumonia" (Jarrett and others, 1953), a chronic non-suppurative pneumonia with lymphocytic aggregates around the airways. In two of the calves this was associated with *Mycoplasma dispar* infection, an organism previously associated with this condition (Pirie and Allan, 1975). Whether prior viral infection is obligatory for the development of this condition in the bovine is unclear.

Parainfluenza type 3 virus did not appear to play any role in this outbreak unlike the incidents described by Bryson and others (1979a). In their cases the clinical and pathological findings were similar to those described here although PI3 virus was isolated from the lungs.

RESPIRATORY DISEASE INVOLVING RS VIRUS: OUTBREAK D

## Introduction

This outbreak occurred on one of two dairy farms, with Friesian cows, managed as a single business. All home-bred calves were kept and were either used as herd replacements or were fattened up to slaughter weight. Calves were reared in groups of 20 on milk substitute from a "Nursette" machine at one farm until weaning at about seven weeks of age. They were then moved to the other farm in groups of between four and six. Here they were housed in a high, stone-built hay shed, one end of which was part open. In the other end was a high open window which had recently been blocked off to prevent "draughts".

## History of Respiratory Disease Outbreak

In the hay shed were two separate groups of calves (Groups 1 and 2), only Group 1 became involved in the outbreak of respiratory disease. Group 1 were between 14 and 16 weeks of age. Recently there had been an increase in coughing in this group accompanied by tachypnoea and a reduction in appetite. The calves were treated with a combination of penicillin, streptomycin, betamethasone and antihistamine but response to this was poor. There were no deaths.

## Clinical Findings

First visit: The first visit was on 30.12.80. The animals in Group 1 were tachypnoeic but not pyrexic. There was a moderate amount of coughing in the group. On auscultation harsh respiratory sounds were heard with occasional squeaks in the antero-ventral areas of both lung fields. Blood samples and nasopharyngeal swabs were taken from all calves (D1 to D9) in this group.

Second visit: The farm was visited again six days later on 5.1.81. The calves in Group 1 were much improved with only very

occasional coughing and slightly harsh respiratory sounds on auscultation. At this visit there were signs of respiratory disease in a group of 17 younger calves, aged from newly born to seven weeks old, which were housed in a separate building. Two calves were markedly dyspnoeic and the remainder were coughing frequently. According to the farmer these calves were "not doing" and they appeared to be in poor condition. A number of them were being treated with antibiotics. The dyspnoeic animals were not being treated as they did not cough. No samples were taken from this group.

Third visit: The third and final visit was made four weeks after the initial visit on 27.1.81. Repeat blood samples were taken from the Group 1 animals. All of the calves were clinically normal.

Microbiological findings

No viruses were isolated from the nasopharyngeal swabs taken during this outbreak. Pasteurella multocida was recovered from one swab (calf D2). Mycoplasma bovis was isolated from four swabs (calves D2, D5, D6 and D9) and Ureaplasma sp. from five (calves D3, D4, D5, D8 and D9).

Serological Findings

The results of the examination of paired sera from calves D1 to D9 for antibodies to RS virus and PI3 virus are presented in Table 13. Seven of eight animals from which paired sera were obtained seroconverted to RS virus. One calf seroconverted to PI3 virus. There was no serological evidence of infection with BVD virus, adenovirus types A and B, reovirus types 1 and 2 or IBR virus. One calf (D8) seroconverted to P.haemolytica type A1 and three (D1, D5 and D6) to Mycoplasma bovis.

TABLE 13: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination-inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from calves in Group 1 in outbreak D.

| Calf | RS virus | | | PI3 Virus | | |
|---|---|---|---|---|---|---|
| number | 30.12.80 | 29.1.81. | CF | 30.12.80. | 29.1.81. | CF |
| D1 | 40 | 1580 | 39.5 | 10 | 20 | 2.0 |
| D2 | 60 | 1000 | 16.6 | 20 | 20 | 1.0 |
| D3 | <10 | 28 | >2.8 | 20 | 20 | 1.0 |
| D4 | 30 | 500 | 16.6 | 20 | 40 | 2.0 |
| D5 | <10 | 280 | >28.0 | 20 | 20 | 1.0 |
| D6 | <10 | 1580 | >158.0 | 40 | 320 | 8.0 |
| D7 | <10 | 175 | >17.5 | 20 | 20 | 1.0 |
| D8 | 25 | 390 | 15.6 | 20 | 20 | 1.0 |
| D9 | NS | 480 | - | NS | 10 | - |

CF = Conversion Factor

NS = No sample

## Discussion

This was a mild outbreak of acute respiratory disease in which there were no deaths and which was temporally associated with RS viral infection. There was a poor response to antibiotic therapy. The clinical presentation in this particular group of calves was unlike that described in Europe when respiratory distress, or a marked abdominal component in breathing, were prominent clinical signs (Holzhauer and van Nieuwstadt, 1976). The antibody titres to RS virus rose very rapidly between the first and second blood sample. This, and the mild nature of the disease, could suggest that the animals had been exposed to RS virus before. Some of the younger animals on the farm were severely ill with respiratory disease at the time of this incident but unfortunately these were not investigated as the farmer was not particularly worried about them. As some of these animals were dyspnoeic it is possible that they were also infected with RS virus.

Other micro-organisms were also associated with this incident. One calf (D6) seroconverted to PI3 virus although no virus was recovered from its nasopharyngeal swab and no other animals showed serological evidence of infection. Three calves seroconverted to Mycoplasma bovis and this organism was present in the nasopharyngeal secretions of four. Despite these findings the data presented supports the view that the major pathogen in this incident was RS virus. Mycoplasma bovis might have been an additional pathogen although there was no evidence that animals with combined RS viral and M.bovis infection were more severely clinically affected than those with RS virus alone. Ureaplasma sp. were also associated with this outbreak. In the absence of pathological material from the respiratory tract the relative roles played by these micro-organisms in the respiratory disease is difficult to assess.

RESPIRATORY DISEASE INVOLVING RS VIRUS

INDIVIDUAL CASES OF RS VIRAL INFECTION

RS VIRAL INFECTION: CASE RC1

Date 1st April 1982 | Breed Friesian X Charolais

Age Three weeks | Died/Slaughtered Slaughtered

History

This was one of a group of housed, bought-in calves being fed milk substitute at an incorrectly low concentration. The group as a whole were undernourished. This calf was diarrhoeic and emaciated. It was hyperpnoeic and tachypnoeic. Bovine papular stomatitis lesions were present in the mouth.

Pathological Findings

Gross: There was extensive severe pneumonic consolidation of the cranial lobes, the middle lobe and the cranial parts of the caudal lobes, with areas of necrosis scattered throughout. There was no interstitial emphysema.

Microscopic: There was a severe acute exudative pneumonia with epithelial damage and areas of coagulative necrosis. In the non-necrotic areas there were epithelial syncytia, some containing eosinophilic intracytoplasmic inclusion bodies, in the bronchioles and the alveoli. Some of these were necrotic and had detached from the epithelium. There was also focal necrosis and focal hyperplasia of the alveolar epithelium. In the necrotic areas there was complete obliteration of alveolar and bronchiolar architecture in which it was not possible to distinguish remnants of epithelial syncytia.

Immunofluorescence: Respiratory syncytial viral antigens were detected in the bronchiolar and the alveolar epithelia in non-necrotic areas. No PI3 viral antigens were detected.

Microbiological Findings

No samples were available for microbiological examination.

RS VIRAL INFECTION: CASE RC2

| | | | |
|---|---|---|---|
| Date | February 1982 | Breed | Ayrshire |
| Age | Three months | Died/Slaughtered | Slaughtered |

History

This calf was one of a group of 10 housed, weaned bought-in calves. Over a period of four days it developed signs of respiratory disease with coughing, tachypnoea and hyperpnoea. It was never dyspnoeic. Other calves in the group were coughing and were tachypnoeic.

Pathological Findings

Gross: There was collapse and consolidation in the cranial lobes, the middle lobe and the cranial parts of the caudal lobes. The lesions were deep red and slightly raised above the surface of the surrounding normal lung. There was no interstitial emphysema.

Microscopic: There was an acute pneumonia with damage to the bronchial, the bronchiolar and the alveolar epithelia. There were numerous epithelial syncytia in the bronchioles and the alveoli. Some syncytia contained eosinophilic intracytoplasmic inclusion bodies and, occasionally, these features were also present in individual epithelial cells.

Immunofluorescence: Respiratory syncytial viral antigens were widely distributed throughout the bronchial, the bronchiolar and the alveolar epithelia. No PI3 viral antigens were detected.

Microbiological Findings

No samples were available for microbiological examination.

RS VIRAL INFECTION: CASE RC3

| | | | |
|---|---|---|---|
| Date | 21st December 1982 | Breed | Friesian |
| Age | Three months | Died/Slaughtered | Died |

History

This calf was one of a group of 10 home-bred, housed calves. It was involved in an outbreak of acute respiratory disease characterised by coughing, hyperpnoea and tachypnoea. This calf was found dead unexpectedly.

Pathological Findings

Gross: There was moderate pneumonic consolidation of the cranial lobes, the middle lobe and the cranial parts of the caudal lobes. The rest of the lungs were congested and oedematous. There was interstitial emphysema with bulla formation in the caudal lobes.

Microscopic: In the cranial and middle lobes there was an acute pneumonia with epithelial damage. The bronchiolar epithelium was focally necrotic and contained large syncytia, some of which were undergoing degeneration. Syncytia were also present in the alveolar epithelium, but less frequently than in the bronchioles. Similar lesions were present in the cranial parts of the caudal lobes but in the caudal parts there was a diffuse acute proliferative alveolitis with hyaline membrane formation. In these areas there were no syncytia.

Immunofluorescence: Respiratory syncytial viral antigens were present in the bronchiolar and, to a lesser extent, in the alveolar epithelium of the cranial lobes, the middle lobe and the cranial parts of the caudal lobes.

Microbiological Findings

No pathogenic viruses, bacteria or mycoplasmas were isolated from the lungs of this calf.

RS VIRAL INFECTION: CASE RC4

Date October 1982 Breed Not recorded

Age Three months Died/Slaughtered Died

History

This calf was one of a group of home-bred and bought-in calves which were housed together. Respiratory disease had been a problem on the farm for some time, both in home-bred and in bought-in calves. A considerable amount of coughing was regarded as normal and, provided no animals died, treatment was limited to occasional individual animals. This calf was found dead. There was no reliable clinical history available.

Pathological Findings:

Gross: There was extensive pneumonic consolidation of the cranial lobes and middle lobe. Foci of necrosis were scattered throughout the cranial lobes. There was severe interstitial emphysema with bulla formation in the caudal lobes.

Microscopic: There was a severe acute exudative pneumonia with damage to the bronchiolar and the alveolar epithelium and focal areas of necrosis. In addition to this there were chronic lesions in the cranial lobes with peribronchial and peribronchiolar fibrosis and bronchiolitis obliterans. In the middle lobe there was severe congestion and oedema in the alveolar areas but little evidence of epithelial damage. In the bronchiolar epithelium there was focal necrosis and syncytium formation. Eosinophilic intracytoplasmic inclusion bodies were present in individual bronchiolar epithelial cells and in the syncytia. Inclusion bodies, but no syncytia, were present in the bronchial epithelium.

Immunofluorescence: Respiratory syncytial viral antigens were detected in the bronchial and the bronchiolar epithelium in the middle lobe but not in the cranial lobe. All sections were negative for PI3 viral antigens.

Microbiological Findings:

No samples were available for virological examination. Mycoplasma bovis was isolated from the lungs. No pathogenic bacteria were isolated.

RS VIRAL INFECTION: CASE RC5

| | | | |
|---|---|---|---|
| Date | 22nd December 1981 | Breed | Friesian |
| Age | Three months | Died/Slaughtered | Died |

History

This was one of a group of 30 housed calves. An outbreak of respiratory disease occurred in the group characterised by coughing and tachypnoea. This calf became dyspnoeic and died.

Pathological Findings:

Gross: Extensive pneumonic consolidation involving the cranial lobes, the middle lobes and the cranial parts of the caudal lobes. There was severe interstitial emphysema with bulla formation in the caudal lobes.

Microscopic: The striking lesion in all lobes was an acute pneumonia with marked epithelial damage. There was a bronchiolitis with small numbers of lymphocytes and plasma cells in the peribronchiolar tissues. The epithelium was dysplastic or necrotic with cellular debris and inflammatory cells plugging the lumen. The alveolar epithelium was markedly hyperplastic in most sections examined, with a mixture of macrophages and some neutrophils in the lumen. In some sections epithelial syncytia were present in the alveolar epithelium at the periphery of lobules with epithelial hyperplasia. Some syncytia contained eosinophilic intracytoplasmic inclusion bodies. The alveolar areas were severely congested and oedematous and there were foci of intra-alveolar haemorrhage.

Immunofluorescence: Respiratory syncytial viral antigens were detected in the alveolar epithelium, both in syncytia and in single cells. Areas with marked alveolar epithelial hyperplasia were negative for viral antigens. No PI3 viral antigens were detected.

Microbiological Findings

No viruses or pathogenic bacteria were isolated from the lungs of this animal. Mycoplasma bovis was isolated from the cranial lobe of the right lung.

RS VIRAL INFECTION: CASE RC6

Date 25th February 1982 Breed Friesian

Age Three months Died/Slaughtered Died

History

This was a housed, weaned dairy calf in a group with 10 others. The group was involved in an outbreak of acute respiratory disease. Other calves in the group were mildly affected but this one became severely ill, had difficulty in breathing and died.

Pathological Findings

Gross: There was moderate pneumonic consolidation in parts of the cranial lobes, the middle lobe and the cranial parts of the caudal lobes. Pneumonic areas were slightly raised, pink/red in colour and oedematous. There was severe interstitial emphysema with bulla formation in the caudal lobes.

Microscopic: There was congestion and oedema in the alveolar areas with a slight infiltrate of macrophages and a few neutrophils. There were occasional focal areas of epithelial necrosis and of epithelial hyperplasia. The bronchiolar epithelium was swollen with cells protruding into the lumen which was plugged with inflammatory cells. The nuclei of the epithelial cells were large and pale with prominent nucleoli and hyperchromatic nuclear membranes. In some places epithelial cells seemed to overlap slightly but there were few convincing syncytia. No inclusion bodies were seen. In adjacent non-pneumonic areas bronchioles were plugged with inflammatory cells. There was a bronchitis with increased numbers of lymphocytes and plasma cells in the lamina propria. The epithelium was dysplastic but contained no inclusion bodies or syncytia.

Immunofluorescence: Respiratory syncytial viral antigens were detected principally in bronchiolar epithelial cells. Occasional antigen positive cells were present in the alveolar epithelium.

Microbiological Findings

No pathogenic viruses, bacteria or mycoplasmas were isolated from the lungs of this calf.

RS VIRAL INFECTION: CASE RC7

Date 7th December 1982 Breed Limousin

Age Four months Died/Slaughtered Died

History

This was a housed suckler calf, in direct contact with its dam and other calves aged two to six months. The calves were involved in an acute respiratory disease outbreak characterised by coughing and tachypnoea. This calf became dyspnoeic and died over a period of 24 hours.

Pathological Findings

Gross: There was extensive severe pneumonic consolidation of the cranial lobes, the middle lobe and the cranial parts of the caudal lobes. The caudal parts of the caudal lobes were solid and on cross section the surface appeared shiny with oedema fluid exuding from the cut surface. The trachea contained blood stained frothy fluid.

Microscopic: The major features, present in all lobes, were acute pneumonia with damage to the alveolar and bronchiolar epithelium. There was focal necrosis and hyperplasia of the alveolar and the bronchiolar epithelium. In the middle lobe there were, in addition, epithelial syncytia in the alveoli and bronchioles. Some syncytia contained eosinophilic intracytoplasmic inclusion bodies. In the dorsal parts of all lobes and the caudal parts of the caudal lobes there was marked hyaline membrane formation and early proliferation of the alveolar epithelium. In these areas there was little damage to the bronchiolar epithelium and no syncytia or inclusion bodies were present.

Immunofluorescence: Respiratory syncytial viral antigens were detected in the bronchiolar and the alveolar epithelium in the ventral part of the middle lobe. All other areas were negative. No PI3 viral antigens were detected.

Microbiological Findings

No pathogenic viruses, bacteria or mycoplasmas were isolated from the lungs of this calf.

RS VIRAL INFECTION: CASE RC8

Date 15th November, 1982 Breed Friesian cross
Age Four months Died/Slaughtered Died

History

This calf was one of a group of housed, weaned calves. It had been vaccinated, along with the rest of the group, with Tracherine five days prior to becoming ill with respiratory disease. Over a period of 24 hours the calf became dyspnoeic and died.

Pathological Findings

Gross: There was moderate pneumonic consolidation of the cranial lobes and the middle lobe. There was widespread severe interstitial emphysema with bulla formation in the caudal lobes.

Microscopic: There was an acute pneumonia with epithelial damage. Focal necrosis and proliferation of bronchiolar and alveolar epithelium were the most noticeable features. Syncytia, some with eosinophilic intracytoplasmic inclusion bodies, were present in the bronchiolar and the alveolar epithelium (Fig. 3). Macrophages and a few neutrophils were present in alveolar airspaces with the neutrophil component focally intense in some areas. There was a mild bronchitis with epithelial dysplasia andan increased number of lymphocytes and plasma cells in the lamina propria.

Immunofluorescence: Respiratory syncytial viral antigens were present in the bronchiolar and the alveolar syncytia and in single cells at both sites. No PI3 viral antigens were detected.

Microbiological Findings

No pathogenic viruses, bacteria or mycoplasmas were isolated from the lungs of this calf.

RS VIRAL INFECTION: CASE RC9

Date 11th November 1982 Breed Charolais Cross

Age Four months Died/Slaughtered Died

History

This calf was in a suckler herd which had been housed for two weeks. The calves had access to creep feed and were in contact with their dams. There was an outbreak of acute respiratory disease in the calves. This calf became dyspnoeic and, despite extensive antibiotic treatment, died four days after first being noticed to be ill.

Pathological Findings:

Gross: There was extensive pneumonic consolidation of the cranial lobes, the middle lobe and the cranial parts of the caudal lobes. Widespread severe interstitial emphysema with bulla formation in the caudal lobes was a striking feature. The caudal lobes were diffusely solid and heavy and on cross-section oedema fluid exuded from the cut surface.

Microscopic: There was an acute pneumonia in the cranial lobes with damage to the bronchiolar epithelium consisting of focal necrosis and syncytium formation. A few lymphocytes and plasma cells were present in the peribronchiolar tissues. In the lamina propria of the bronchi there were a few plasma cells and occasional loose aggregates of lymphocytes. In the alveoli there was severe congestion and oedema of the walls but few epithelial changes. In the caudal lobes there was focal necrosis of the bronchiolar epithelium, but no syncytium formation. In the alveoli there was widespread hyaline membrane formation (Fig. 11).

Immunofluorescence: Respiratory syncytial viral antigens were detected in the bronchiolar epithelium in the cranial lobe. No viral antigens were detected in other areas or lobes. No PI3 viral antigens were detected.

Microbiological Findings

No pathogenic viruses or bacteria were isolated from the lungs. *Mycoplasma bovis* was isolated from the right cranial lobe.

RS VIRAL INFECTION: CASE RC10

Date 5th November 1980 Breed Friesian Cross

Age Four months Died/Slaughtered Died

History

One of a group of 20, four months old, weaned calves in which there was an outbreak of acute respiratory disease. They were housed in a building with older animals (nine months and 15 months) in adjacent pens. The respiratory disease affected the pen of younger animals only. All were coughing and tachypnoeic. Five calves died, apparently suddenly, over a period of five days. This was the second calf to die.

Pathological Findings

Gross: There was slight pneumonic consolidation in the cranial and the middle lobes. Pneumonic lobules were red and slightly swollen. Intra-lobular haemorrhages were present in the pneumonic and non-pneumonic areas. The consolidated areas were not very extensive, involving only 20% of the cranial lobes and 10% of the middle lobes. Adjacent non-pneumonic areas were overinflated. There was widespread severe interstitial emphysema with bulla formation in both caudal lobes.

Microscopic: There was congestion and oedema in the alveoli but no marked epithelial damage (Fig. 2). In some areas, adjacent to the bronchioles, there was an infiltrate of macrophages and a few neutrophils. There were striking lesions in the bronchiolar epithelium with focal necrosis and the formation of syncytia, some with eosinophilic intracytoplasmic inclusion bodies. There was a mild bronchitis.

Immunofluorescence: Respiratory syncytial viral antigens were detected in the bronchiolar epithelium. No RS viral antigens were detected in alveolar epithelial cells. The sections were negative for PI3 viral antigens.

Microbiological Findings

No pathogenic viruses, bacteria or mycoplasmas were isolated from the lungs of this calf.

RS VIRAL INFECTION: CASE RC11

Date 6th February 1982 Breed Friesian

Age Four months Died/Slaughtered Died

History

This calf was housed in "isolation" at an MMB Bull rearing unit. Eight weeks after arrival at the unit the animal became tachypnoeic and hyperpnoeic. It gradually became dyspnoeic over a period of three days and, despite treatment with antibiotics, died.

Pathological Findings

Gross: There was extensive consolidation in the cranial lobes and the middle lobe. Normal or overinflated lobules were present in the consolidated areas. There was widespread severe interstitial emphysema with bulla formation in the caudal lobes. Pneumonic lobules were present adjacent to the lobar bronchi in the caudal lobes. The trachea was congested with frothy mucus in the lumen.

Microscopic: The principal changes were in the bronchioles with focal necrosis of the epithelium and syncytium formation. In some bronchioles syncytia had detached and become necrotic with degeneration of the nuclei. There was severe congestion and oedema of alveoli with some necrosis and syncytium formation in the alveolar epithelium. Eosinophilic intracytoplasmic inclusion bodies were present in many syncytia. In some areas there were intense focal accumulations of neutrophils in alveoli, in others the infiltrate was composed of roughly equal numbers of macrophages and neutrophils.

Immunofluorescence: Respiratory syncytial viral antigens were detected in the epithelial syncytia and in individual epithelial cells in the alveoli and the bronchioles. No PI3 viral antigens were detected.

Microbiological Findings

No tissues were available for the isolation of viruses or mycoplasmas. The lungs were bacteriologically sterile.

RS VIRAL INFECTION: CASE RC12

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| Date | 20th February 1983 | Breed | Friesian |
| Age | Four months | Died/Slaughtered | Slaughtered |

History

This was one of a group of nine housed calves being used in an experiment. It became moderately ill with respiratory disease and did not respond to antibiotic therapy. It was never dyspnoeic.

Pathological Findings

Gross: There was moderately extensive patchy pneumonic consolidation in the cranial lobes, middle lobe and cranial parts of the caudal lobes. The pneumonic lobules were deep red, slightly swollen and had a smooth, homogeneous surface. At the tips of the lobes the pneumonic lobules were confluent and at the periphery of and dorsal to these areas there were multiple individual pneumonic lobules. The tracheal and bronchial lumina were free of mucopus and there was no marked interstitial emphysema.

Microscopic: The striking feature was the presence of numerous large syncytia in the bronchiolar epithelium, many of which contained one or more eosinophilic intracytoplasmic inclusion bodies. There was necrosis and dysplasia of the bronchiolar epithelium. Lymphocytes were present between the epithelial cells and in close apposition to some syncytia. Cells containing inclusion bodies were present in the bronchial epithelium and there was a bronchitis with increased numbers of lymphocytes and plasma cells in the lamina propria. The alveoli were congested and oedematous. The epithelial cells were swollen and some contained inclusion bodies. Few syncytia were present in the alveoli.

Immunofluorescence: Respiratory syncytial viral antigens were present in virtually all of the bronchial, bronchiolar and alveolar epithelial cells in the pneumonic areas. No PI3 viral antigens were detected.

Microbiological Findings

Respiratory syncytial virus was isolated from the right cranial lobe of the lung.

RS VIRAL INFECTION: CASE RC13

Date 14th November 1982. Breed Frieisan X Hereford

Age Five months Died/Slaughtered Died

History

This was one of a group of 27 calves, aged four to five months, which were housed in direct contact with younger calves one month of age. The calves had been in the same building since five weeks of age and during this time animals had been added to other groups in the building. Over a period of two days three calves became severely ill with respiratory distress and died.

Pathological Findings

Gross: There was extensive consolidation of the cranial lobes and the middle lobe. There was widespread severe interstitial emphysema with bulla formation in caudal lobes. Small haemorrhages were present in the tracheal and the bronchial mucosae. Small quantities of mucopus were present in the airways.

Microscopic: There was a severe acute pneumonia with epithelial damage. Epithelial syncytia, some with eosinophilic intracytoplasmic inclusion bodies were present in the alveolar and the bronchiolar epithelium. Lymphocytes and a few plasma cells were present in peribronchiolar tissues and in the bronchial lamina propria. There was focal necrosis and hyperplasia of the alveolar epithelium. In the caudal part of the caudal lobes there was marked congestion and oedema in the alveoli with hyaline membrane formation. There was focal necrosis of bronchiolar epithelium but no syncytia or inclusion bodies in either bronchioles or alveoli.

Immunofluorescence: Respiratory syncytial viral antigens were detected in bronchial, bronchiolar and alveolar epithelium; both in syncytia and in single cells. No PI3 viral antigens were detected. No viral antigens were detected in the caudal part of the caudal lobes.

Microbiological Findings

No pathogenic viruses, bacteria or mycoplasmas were isolated from the lungs of this calf.

RS VIRAL INFECTION: CASE RC14

Date 29th October 1981 Breed Ayrshire
Age Five months Died/Slaughtered Died

History

A housed, weaned dairy calf which had suffered "bouts of pneumonia" over the previous several months. It suddenly became very dyspnoeic and died.

Pathological Findings

Gross: There was severe consolidation of the cranial lobes, the middle lobe and the cranial parts of the caudal lung lobes. There was widespread severe interstitial emphysema with bulla formation in the caudal lobes. Frothy fluid and floccules of mucopus were present in major bronchi and trachea.

Microscopic: In all pneumonic areas there was a severe acute exudative pneumonia with epithelial damage. Syncytia, some with eosinophilic intracytoplasmic inclusion bodies, were present in bronchiolar and the alveolar epithelium. There were focal areas of epithelial necrosis and of epithelial hyperplasia. Some syncytia had apparently detached from the epithelium. Numerous small cells with very little cytoplasm and densely staining nuclei, presumably lymphocytes, were seen in close apposition to some syncytia. Small numbers of lymphocytes and plasma cells were present in peribronchiolar tissues and in the lamina propria of intra-pulmonary bronchi.

Immunofluorescence: Sections of lung were positive for RS viral antigens. Syncytia and single cells contained viral antigens in both the alveolar (Fig. 8) and the bronchiolar epithelium. Single cells contained antigens in the bronchial epithelium. No PI3 viral antigens were detected.

Microbiological Findings

No samples were available for microbiological examination.

RS VIRAL INFECTION: CASE RC15

Date 17th December 1982 Breed Charolais

Age Eight months Died/Slaughtered Died

History

This animal was involved in an outbreak of acute respiratory disease in a group of 10 bought-in, weaned single-suckled calves which were housed together. All the group were affected with varying degrees of coughing, tachypnoea and dyspnoea. There was no response to antibiotic therapy. This calf died within 48 hours of first being noticed to be ill.

Pathological Findings

Gross: Not recorded

Microscopic: In all the sections of lung examined there was a severe acute pneumonia with epithelial damage. Syncytia were present in the bronchiolar epithelium, some containing eosinophilic intracytoplasmic inclusion bodies. Necrotic syncytia were lying free in the bronchiolar lumen. There was an intense macrophage and neutrophil response in the alveoli with marked congestion and oedema of the alveolar walls. In some areas there was hyaline membrane formation. In all the sections there were varying degrees of lymphocytic cell accumulation around small bronchi, bronchioles and blood vessels.

Immunofluorescence: Respiratory syncytial viral antigens were detected in the bronchiolar and the alveolar epithelium. No PI3 viral antigens were detected.

Microbiological Findings

No samples were available for microbiological examination.

RS VIRAL INFECTION: CASE RC16

Date 30th October 1982 Breed Shorthorn cross

Age Twelve months Died/Slaughtered Died

History

The animal presented with nervous signs - lateral recumbency and nystagmus. It had a rectal temperature of 103.5°F. There were no respiratory signs noted other than harsh inspiratory and expiratory sounds.

Pathological Findings:

Gross: The pathological diagnosis was listeriosis with pneumonia as an incidental finding. There was pneumonia involving the cranial lobes, the middle lobe and the cranial parts of the caudal lobes. The lesions were slightly depressed, dark pink and firm to cut. There was no interstitial emphysema.

Microscopic: The significant features in the lungs were congestion and oedema of the alveoli with epithelial damage in the alveoli, the bronchioles and the bronchi. Syncytia were present in the bronchiolar and, occasionally, in the alveolar epithelium. Some syncytia contained eosinophilic intracytoplasmic inclusion bodies. Inclusion bodies were also present in individual epithelial cells in alveoli, bronchioles and bronchi. There was a mild macrophage response and a few neutrophils were present in the alveolar airspaces. The bronchioles were plugged with inflammatory cells and detached epithelial cells.

Immunofluorescence: Respiratory syncytial viral antigens were present in alveolar, bronchiolar and bronchial epithelium (Fig. 5). The majority of bronchiolar cells were infected but relatively fewer bronchial cells and few alveolar cells. No RS viral antigens were present in the sections of brain which were examined. No PI3 viral antigens were detected in the lungs.

Microbiological Findings:

No samples were available for microbiological examination.

Summary of Findings in RS Viral Cases

A total of 21 individual cases of RS viral infection have been described. Clinical signs which were observed frequently were coughing, tachypnoea, hyperpnoea and severe dyspnoea. Nasal discharge was not a marked feature in most cases. The clinical signs were of sudden onset and, in some cases, animals died suddenly without previously having been noticed to be ill. In all cases there was evidence of respiratory disease in other calves in either direct or indirect contact with the severely affected ones. In some cases there was a history of recurrent respiratory disease on the farm in the previous months or years, either as acute outbreaks or as persistent chronic problems. In other cases this was apparently not so and the respiratory disease incident was thought,by the farmer, to be unusual for the farm. Cases occurred only in housed calves, although some had only recently been housed and others had continual access to the outside. The cases were distributed throughout the winter housing period from October until April but the majority occurred between October and December. Both dairy-type and beef-type calves were affected. Most of the calves which died due to the effects of RS viral infection were between two and six months old, however, there was also one eight months old animal which died. Other calves aged less than two months and over six months were also received but in these cases there were complicating lesions in other organs and the pulmonary lesions were regarded as an incidental finding.

In all cases animals which died were amongst the first in the outbreak to die. Several cases, not described here, were received from later stages of the same outbreaks but the lungs from these did not contain RS viral antigens.

At post-mortem examination the gross findings in those cases which had died or were slaughtered because they had respiratory disease were remarkably similar. These will be described first. Grossly there was pneumonia involving the cranial parts of the lung lobes and widespread severe interstitial emphysema with large

bullae of gas in the caudal lobes. The pneumonic lesions were generally acute and uncomplicated by severe secondary bacterial infection. There was congestion and oedema of the pneumonic areas and the lesions were slightly raised above the level of the normal lung. There was no fibrosis, frank necrosis, fibrinous pleurisy or suppuration. Intra-lobular and sub-pleural haemorrhages were common in both the pneumonic and in the adjacent non-pneumonic areas. Non-pneumonic, overinflated lobules were sometimes scattered throughout the pneumonic areas and were commonly present at their periphery. On cross section the surface of the lesions were smooth, featureless and homogenous with little or no exudate, apart from some frothy mucopus, in the airways. The trachea and the bronchial mucosae were often congested. In a minority of cases there were, in addition to the lesions described, long standing chronic lesions in the cranial lobes with firm, collapsed alveoli, prominent airways surrounded by fibrous tissue and large quantities of mucopus present in the airways.

The extent of the acute lesions was often not thought to be sufficient to be the cause of severe respiratory distress on their own, with less than 20% of the lung volume being consolidated. In other cases, however, there were additional extensive lesions spread diffusely throughout the caudal lobes and dorsal parts of the cranial and middle lobes. In these instances the lungs were uniformly firm and heavy with deep red or red/grey lobules. They were fleshy to cut and the lobules had a smooth, shiny surface from which oedema fluid exuded on pressure.

A striking lesion, common to all cases with severe respiratory disease, was interstitial emphysema. Gas was present in the interlobular septae of all the lobes and had dissected between the lobules to form large bullae up to 10cm in diameter. These were most frequently present in the dorsal part of the caudal lobes but were sometimes present in the dorsal parts of other lobes. Occasionally gas had tracked into the mediastinum and into the mediastinal lymph nodes. The lungs did not collapse on opening the chest and, in some cases, appeared to expand slightly.

In the cases which did not die or were not slaughtered because they had respiratory disease the pneumonic lesions were grossly similar to those described. However, intralobular haemorrhages and interstitial emphysema were less frequent findings and none had lesions in the caudal parts of the caudal lobes. In one case there were areas of coagulative necrosis scattered throughout the pneumonic areas.

Histologically the pneumonic lesions in the cranial and middle lobes and in the cranial parts of the caudal lobes were similar in all of the cases and consisted of acute inflammation and epithelial damage. In some cases there were additional lesions which will be described later. There was bronchitis, a bronchiolitis and an alveolitis, each with epithelial damage, but not necessarily occurring together in all cases. Bronchitis was a consistent feature with congestion and oedema of the bronchial wall and increased numbers of lymphocytes, plasma cells and mononuclear cells in the lamina propria. The epithelium was in most cases dysplastic and hyperplastic. Nuclei were arranged at irregular levels in the epithelial layer and occasionally a few neutrophils and lymphocytes were present between the epithelial cells. Eosinophilic intracytoplasmic inclusion bodies were occasionally present in bronchial epithelial cells and in some cases small epithelial syncytia, with two or three nuclei, were present. In a few cases there were large aggregates of lymphocytic cells in the bronchial wall.

Bronchiolar lesions were a consistent finding in all of the cases although they varied in type and in degree. In most there was epithelial dysplasia and focal epithelial necrosis. There were often epithelial syncytia, usually containing between three and seven nuclei but occasionally up to 12 or 15. The nuclear morphology of the syncytia was characteristic. They were large, pale and oval with peripherally clumped chromatin, hyperchromatic nuclear membranes and prominent nucleoli. In most cases they overlapped each other and were centrally placed within the syncytial cytoplasm. The ratio of nuclei to cytoplasm was high.

Some individual syncytia were seen to be undergoing necrosis and in these the cytoplasm was densely eosinophilic or slightly basophilic and the nuclei were small, dark staining and becoming pynknotic. Eventually the syncytium detached from the epithelium and could be seen free in the bronchiolar lumen amongst the cellular debris. The nuclei finally disintegrated and the cytoplasm became an amorphous mass which eventually fragmented. In some cases the pynknotic nuclei were very obvious under low power examination and gave the first clue that RS viral infection might be present. Viable syncytia were most obvious when they projected from the bronchiolar wall into the lumen. In some cases they were less obvious because they were level with the surrounding epithelial cells. However, they could generally be recognised by the overlapping of the nuclei. Inclusion bodies were also seen in individual bronchiolar epithelial cells and these had nuclei with a similar morphology to those in the syncytia.

In several cases there was complete loss of the normal structure of the bronchiolar epithelium and its replacement with flattened, slightly basophilic cells. This was thought to represent epithelial regeneration following necrosis.

Peribronchiolar changes were a consistent finding in all cases but varied markedly in degree. In the least developed form there were a few lymphocytes and plasma cells distributed evenly around the bronchiole. Occasionally lymphocytes were present between the epithelial cells or in close association with epithelial syncytia. In other cases there were extensive cellular accumulations, principally of lymphocytes, in the peribronchiolar tissues although in only two cases were these sufficiently well developed to form cuffs.

Alveolar changes were, in some cases, less marked than those in the bronchioles and consisted of only congestion and oedema with no significant epithelial damage. In these there was a slight infiltrate of macrophages and a few neutrophils into the alveolar airspaces. In other cases there were marked epithelial changes

with foci of necrosis, foci of epithelial hyperplasia and the formation of epithelial syncytia. The syncytia were, in appearance, similar to those in the bronchioles. They also underwent necrosis and degeneration and could be seen free in the alveolar lumen. In addition, syncytia projecting into the lumen could appear to be free if not sectioned through their epithelial attachment. In this position they could be difficult to distinguish from macrophage syncytia, although these latter cells usually had more abundant cytoplasm and smaller, peripherally placed nuclei. In alveoli containing epithelial syncytia the cellular infiltrate was usually composed of approximately equal proportions of macrophages and neutrophils, although in some cases the neutrophilic component was more intense. Short runs of hyperplastic epithelium were seen in alveolar areas in which there were syncytia. In areas without syncytia this lesion was more extensive.

In a few animals there were additional lesions to those described. These occurred at the periphery of the pneumonic areas and also, in several cases, diffusely throughout the caudal lobes and in the dorsal parts of the cranial and the middle lobes. They consisted of a diffuse acute proliferative alveolitis. The striking changes were severe congestion and oedema of the alveolar walls and hyaline membrane formation. The cellular infiltrate was predominantly composed of macrophages. The alveolar epithelium was hyperplastic, focally in the early stages and diffusely when the lesion was fully developed, with a layer of cuboidal cells with slightly basophilic cytoplasm lining the alveolus. No inclusion bodies or syncytia were present in this lesion although there were occasional binucleate cells in the epithelium. The bronchiolar epithelium was relatively undamaged although there were a few foci of necrosis. The lamina propria of the airways was oedematous and there were a few lymphocytes and plasma cells in the peribronchiolar tissues.

Respiratory syncytial viral antigens were detected in bronchial, bronchiolar and alveolar epithelial cells. Both

syncytia and individual epithelial cells were seen to contain antigens but cells in the lumina of the airways, apart from detached epithelial cells, were negative. Antigens were not demonstrated unequivocally in septal cells, blood vessels or lymphatics. Antigen-positive cells contained intracytoplasmic granules of antigen which varied in size. The larger granules often stained intensely at the edge but lightly in the centre and some of them corresponded to the eosinophilic intracytoplasmic inclusion bodies seen by staining with H&E. No intranuclear antigens were detected. In individual infected cells in the bronchi and the bronchioles viral antigens were present in the cytoplasm between the nucleus and the luminal border of the cell. In syncytia antigens were distributed evenly throughout the cell mass.

Three patterns of antigen distribution were noted within pneumonic lobules and these corresponded to three categories of histopathological appearance. In a proportion of animals antigens were present only in the bronchiolar epithelium, with virtually every cell being infected in most bronchioles. In these cases focal epithelial necrosis and syncytia were seen by conventional staining. Alveolar changes were minimal with congestion, oedema and a few macrophages and neutrophils present in the airspaces but little evidence of epithelial damage and no epithelial syncytia or inclusion bodies. In the second type of change, which was present in most of the animals examined, antigens were present in the bronchiolar, the alveolar and, occasionally, the bronchial epithelia. The histopathological changes were as expected with evidence of viral infection at all three sites. In the third type of change antigens were present primarily in the alveolar epithelium with few or none in the bronchioles or bronchi. In these cases staining with H&E showed epithelial syncytia and eosinophilic intracytoplasmic inclusion bodies in the alveolar epithelium. There was dysplasia or necrosis, often complete, of the bronchiolar epithelium and dysplasia of the bronchial epithelium but no syncytia or inclusion bodies were present at these sites.

Viral antigens were not present in non-pneumonic areas except occasionally in cells in the lumen of bronchi or bronchioles. Areas of diffuse acute proliferative alveolitis were also devoid of demonstrable viral antigens. The number of antigen-positive cells varied from lobule to lobule within the lung and from case to case, although in all cases there were more antigen-positive cells than would have been expected from conventional staining with H&E. In some cases all lobes were positive. In others only a few lobules within one lobe were positive despite there being extensive lung consolidation. Viral antigens were only present in lesions where there was acute inflammation and appeared to be directly related to the earliest phases of acute inflammation.

Other organisms were detected in lungs from cases of RS viral infection but with no consistent pattern of isolation. Pathogenic organisms recovered were Pasteurella multocida, Mycoplasma bovis and Ureaplasma spp. Many cases were free of bacteria and mycoplasmas although treatment with antibiotics may have been responsible for this.

No cases of combined infection with RS virus and PI3 virus were detected.

## Discussion of Findings in RS Viral Cases

There have been few reports dealing in detail with the histopathological features of natural RS viral infection in the bovine. Individual outbreaks of acute respiratory disease due to RS virus have been reported by Pirie and others (1981a) and Elazhary and others (1982). In the former outbreak syncytia and eosinophilic intracytoplasmic inclusion bodies were a marked feature in the alveolar epithelium. However, in the latter neither of these features were present although the lungs of two calves which had died were positive for RS viral antigens by immunofluorescence. A series of eight cases of acute RS viral infection in which the diagnosis was confirmed by immunofluorescence were described by van den Ingh and others (1982). Epithelial syncytia, in either the bronchiolar and/or the alveolar epithelium, were a consistent finding. Thomas and Stott (1981) found "giant cells" to be present in some, but not all, of 11 cases of natural RS viral infection. The origin of these cells was not specified.

Syncytia were present in 20 of the 21 cases of RS viral infection described in the present series although the number and their distribution within the lung varied from case to case. In the extreme cases there could be a few syncytia in only one lobe of the lung or many syncytia in all of the lobes. The number of syncytia did not necessarily relate directly to the extent of the lung consolidation thus errors in sampling could result in their being missed. Once found, syncytia due to viral infection had to be distinguished from those formed from macrophages, although the latter were only rarely found in lungs in which there were RS viral antigens. Differentiation was on attachment to the epithelium, nuclear morphology and position within the cytoplasm and the presence or absence of inclusion bodies. Macrophage syncytia were not derived from epithelium, had small, round, peripherally placed darkly stained nuclei, abundant cytoplasm and did not contain eosinophilic intracytoplasmic inclusion bodies.

In most cases degenerating syncytia were present in the epithelium and these eventually detached to become free in the lumen of either bronchioles or alveoli. Whilst undergoing degeneration and necrosis the cytoplasm became densely eosinophilic or slightly basophilic and the nuclei condensed and became pynknotic. These structures could be an instantly recognisable feature of the lung on low-power microscopical examination making them a useful diagnostic feature.

Another striking feature of RS viral infection is the development of interstital emphysema which can be recognised both grossly and microscopically (Holzhauer and van Nieuwstadt, 1976; Pirie and others, 1981a). This finding has usually been reported in animals which have died as a result of the respiratory disease. When reported in animals which had been slaughtered for post-mortem examination it is obviously not possible to say whether death would otherwise have occurred. In the present series all of the animals which died due to RS viral infection had severe interstitial emphysema at post-mortem examination. In those other cases where RS viral infection was an incidental finding at post-mortem, i.e. the animal was not suffering from a respiratory illness, interstitial emphysema was not present. This feature, therefore, is associated with severe disease due to RS virus. Whether it is the cause of the severity of the disease in every fatal case is not clear.

Interstitial emphysema results from an increase in the resistance of the airways to the flow of air. As the smaller airways such as the bronchioles do not contribute significantly to the resistance to the flow of air in the lung (West, 1977) then the increased airways resistance must result from changes in the larger conducting airways. The effect of this is that the animal has difficulty in breathing and becomes dyspnoeic. The resistance is particularly marked on expiration, leading to overinflation of the alveoli and, in the extreme, to their rupture with leakage of gas into the interstitium (Pirie, 1982). This could then track to the hilus of the lung via peribronchiolar and perivascular tissues and

via the lymphatics. Severe interstitial emphysema may further exacerbate respiratory distress and in severe cases the result could be respiratory failure and death.

Bronchitis, in which the lamina propria was congested and oedematous and contained increased numbers of lymphocytic cells, was a common finding in all cases of RS viral infection. Although few inclusion bodies and very few syncytia were seen in the bronchial epthelium, there was epithelial dysplasia and slight hyperplasia which probably indicated previous viral infection. These changes could have been sufficient to reduce significantly the patency of the bronchial lumen. In addition there could have been spasm of the bronchial musculature during life which would not have been evident post-mortem. Dyspnoea can also result from very extensive pneumonic consolidation. However, in at least some of the cases described the pneumonic consolidation was limited in extent and was not thought sufficient to be the cause of the severe respiratory signs per se. In some cases there was a diffuse epithelial lesion affecting the alveoli in all lobes of the lung. This could lead to respiratory failure.

Using the immunofluorescence technique RS viral antigens were detected in bronchial, bronchiolar and alveolar epithelia. The presence of viral antigens correlated with the presence of acute inflammation. Where the inflammatory process was more advanced, with plasmas cells and lymphocytes being present in significant numbers, there were fewer antigen-positive cells. Antigens were not detected in areas of chronic inflammation or in areas where there was a considerable amount of tissue repair or fibrosis. Thus RS viral infection was directly associated with the early stages of inflammation and, therefore, could be considered a primary pathogen. This view is supported by field and experimental evidence (Holzhauer, 1978; Bryson and others, 1982).

Several authors have commented on the differences in the distribution of RS viral antigens within the lesions in the lungs of naturally infected cases. In most cases both bronchiolar and

alveolar epithelium were positive but in some antigens were restricted to one site or the other (Thomas and Stott, 1981; van den Ingh and others, 1982). The differences were thought to represent stages in the pathogenesis of the viral infection. In the present series of cases similar differences were noted and their relationship to the histopathological changes present indicated the pathogenesis of the infection. In a few cases antigens were present in the bronchiolar epithelium but not in the alveolar epithelium. In these cases there were histopathological changes in the alveoli comprising congestion, oedema and a light infiltrate of neutrophils and macrophages. There was no specific evidence that the alveolar epithelium was infected with RS virus. However, foetal bovine cells infected with bovine strains of RS virus in vitro do not express virus-specified antigens in their cytoplasm until at least 16 hours after infection as judged by immunofluorescence (Rossi and Kiesel, 1977). It is therefore possible that the alveolar epithelium was infected although at an earlier stage than that in the bronchioles.

In most cases viral antigens were present in both the bronchiolar and the alveolar epithelium and there were the appropriate histopathological features to confirm this at both sites. In a few cases viral antigens were present only in the alveolar epithelium. In these cases there was also evidence of damage to the bronchiolar epithelium with necrosis or regeneration and dysplasia. In addition there was a diffuse accumulation of lymphocytic cells in the peribronchiolar tissues. This could indicate that there had been damage to the bronchiolar epithelium by RS virus prior to the alveolar epithelium becoming infected. There was no evidence that the reverse could happen with the alveolar epithelium being infected before the bronchiolar epithelium. Viral antigens were present in the bronchial epithelium of only three animals although there was evidence of epithelial damage at this site in all cases. Of the three positive cases two had been slaughtered and did not, therefore, die of respiratory disease. In the human the bronchioles are considered the primary target for RS viral infection (Aherne and others, 1970).

In the bovine the bronchiolar epithelium is an important site of viral replication although to what extent this contributes to the pathogenesis of the severe disease is not known as the bronchial lesions and, when present, diffuse alveolar epithelial lesions in the caudal lobes are probably more important in the development of dyspnoea.

The microbiological and pathological findings in those animals which died of RS viral infection were similar to those reported by workers in Europe in a condition known as "Pinkengriep" (Holzhauer and van Nieuwstadt, 1976). A similar clinico-pathological syndrome has also been reported from Northern Ireland (Bryson and others, 1979a) where it was associated with combined RS viral and PI3 viral infection. In the present series there was no evidence of PI3 viral infection in these cases as judged by immunofluorescence or, where samples were available, by viral isolation and serology. In four of 12 animals where tissues were available for full microbiological investigation Mycoplasma bovis was isolated from the lung. If there was an association between RS virus and M.bovis which was necessary for the development of "Pinkengriep" then one would have expected the mycoplasma to be isolated more frequently. However, it must be remembered that most of the calves had been treated at least once with broad spectrum antibiotics and this could have affected the isolation of bacteria and mycoplasmas.

## RESPIRATORY DISEASE INVOLVING RS VIRUS

THE ISOLATION OF RS VIRUS FROM THE NASAL SECRETIONS OF A CALF

# THE ISOLATION OF RS VIRUS FROM THE NASAL SECRETIONS OF A CALF

## Introduction

There are very few documented isolations of RS virus from cattle in the U.K. Jacobs and Edington (1971) recovered a strain of the virus after investigating an outbreak of acute respiratory disease in a group of young calves. However, appropriate outbreaks may be few and far between and isolation of virus from field material of this type is difficult and has often been unsuccessful (Wellemans, 1977). An alternative approach is to monitor routinely calves which are kept under managemental conditions in which viral infections are likely to be frequent. Stott and others (1978) monitored a farm which had a high throughput of young, potentially susceptible animals from widely differing sources. Viral infections were common and the survey yielded isolates of most of the common respiratory viruses. For this thesis it was thought necessary to try both potential sources of virus i.e. investigation of outbreaks of respiratory disease and routine monitoring of groups of calves, in order to increase the chances of recovering a strain of RS virus for use in laboratory investigations and in experimental infections.

## Routine Monitoring of Calves on Farm M

Between February and June of 1981 calves on farm M in the West of Scotland were monitored for evidence of viral infections and for clinical signs of respiratory disease. This particular farm was chosen because it had had a history of respiratory problems. There were frequent intakes of young calves bought from many different sources. Although in indirect contact with others, the calves were grouped into manageable numbers for routine clinical and microbiological investigations.

## History and Management on Farm M

Calves born in the South West of England and South Wales

were purchased through a dealer, in batches of 30 to 40, when approximately one month old. On arrival at the farm the calves were split into groups of 10 and were housed in pens in the same building. The groups were not in direct contact with each other but there was indirect contact via the stockmen. They were reared on milk substitute from a machine until weaned at approximately eight weeks of age. In previous batches of calves there had been occasional coughing and individual animals had become ill but there were no severe outbreaks of respiratory disease.

Two groups of calves were selected for monitoring. Group 1 (calves M1 to M10) entered the farm in February 1981 and were sampled four times over the next three months until they went out to grass at the end of April. Group 2 (calves M11 to M20) arrived on the farm in April and were sampled three times over the next two months until they went out to grass at the end of June.

Sampling of Calves

Visits were made to the farm at approximately fortnightly or monthly intervals. At each visit a clinical examination of the calves was made and samples were taken. Nasopharyngeal swabs for viral isolation and blood samples for serum were collected and transferred to the laboratory. Swabs were either processed immediately or stored at -70°C. Serum samples were stored at -20°C until the end of the monitoring period and were then titrated for antibodies to RS, PI3, BVD and IBR viruses.

Clinical Findings on Farm M

During the four months period of observation there was sporadic coughing in both groups of calves but there were no outbreaks of serious respiratory disease.

Microbiological Findings on Farm M

Over the monitoring period one isolate of RS virus was made from a nasopharyngeal swab taken from calf M20 on 20.4.81. On arrival at the laboratory, some two hours after having been taken, the swab was immediately stored at -70°C. Six weeks later it was thawed rapidly at 37°C and an inoculum prepared. Foetal bovine lung and FBK cells were inoculated as described in Section Two.

The cells were checked daily for evidence of cpe but none had developed after seven days incubation in either cell type. The FBL cells were sub-passaged and three days later a single focus of rounded cells appeared in one of the four wells. The medium in this well was changed and the focus slowly enlarged. Two days later small syncytia each containing three to five nuclei were visible at the periphery of the cytopathic focus. Over the next two days the area of cpe and the syncytia enlarged and foci of rounded, refractile cells appeared in other parts of the cell sheet. When the cpe had spread to involve three quarters of the cell sheet an aliquot of medium was removed and transferred to freshly monolayered FBL cells. After four days a similar cpe began to develop in these cells. At no time did cpe appear in the FBK cells and after three weeks they were negative for RS viral antigens by immunofluorescence. Virus-infected cells did not haemadsorb guinea pig erythrocytes and the virus was not neutralised by antisera to PI3 virus, IBR virus, BVD virus or bovine syncytial (BS) virus. It was, however, neutralised by specific antiserum to bovine RS virus. Using the immunofluorescence technique infected cells showed granular and diffuse intracytoplasmic fluorescence with antiserum to bovine RS virus (Fig. 15) but not with antisera to PI3 virus, BVD virus or BS virus. The virus grew well in FBL and FBK cells but not in HEp2 cells. Small syncytia and foci of cell necrosis were produced on BS-C-1 cells.

Serological Findings on Farm M

The results of the serological investigations for RS virus are presented in Table 14. Those for PI3 virus are presented in Table 15. The results for BVD virus are given in Table 16 and for IBR virus in Table 17.

TABLE 14: Neutralisation titre to respiratory syncytial virus in sera from the calves on farm M.

| Calf number | Date of Sampling 4.2.81 | 9.3.81 | 23.3.81 | 20.4.81 | 19.5.81 | 17.6.81 |
|---|---|---|---|---|---|---|
| Group 1 | | | | | | |
| M1 | 912 | 340 | 220 | 100 | | |
| M2 | 360 | 165 | 87 | 60 | | |
| M3 | 85 | 25 | 57 | 300 | | |
| M4 | 470 | 220 | 100 | 170 | | |
| M5 | 160 | 72 | 75 | 30 | | |
| M6 | 160 | 37 | 50 | 37 | | |
| M7 | 100 | 90 | 90 | 95 | | |
| M8 | 21 | 75 | NS | NS | | |
| M9 | 1380 | 620 | 575 | 235 | | |
| M10 | 700 | 270 | 190 | 85 | | |
| Group 2 | | | | | | |
| M11 | | | | 270 | 180 | 160 |
| M12 | | | | 795 | 400 | 420 |
| M13 | | | | 140 | 90 | 75 |
| M14 | | | | 340 | 340 | 210 |
| M15 | | | | 40 | 87 | 87 |
| M16 | | | | 270 | 115 | 290 |
| M17 | | | | 160 | 85 | 28 |
| M18 | | | | 220 | 180 | 110 |
| M19 | | | | 165 | 150 | 83 |
| M20 | | | | 13* | 290 | 300 |

* = RS virus isolated from nasopharyngeal swab

NS = No sample

TABLE 15: Haemagglutination-inhibition titre to parainfluenza type 3 virus in sera from the calves on farm M.

| Calf number | Date of Sampling 4.2.81 | 9.3.81 | 23.3.81 | 20.4.81 | 19.5.81 | 17.6.81 |
|---|---|---|---|---|---|---|
| Group 1 | | | | | | |
| M1 | 320 | 320 | 160 | 160 | | |
| M2 | 10 | 40 | 10 | 10 | | |
| M3 | 40 | 20 | 40 | 320 | | |
| M4 | 160 | 160 | 160 | 640 | | |
| M5 | 320 | 320 | 80 | 10 | | |
| M6 | 80 | 80 | 40 | 40 | | |
| M7 | 20 | 20 | 10 | 40 | | |
| M8 | 10 | 10 | NS | NS | | |
| M9 | 640 | 320 | 320 | 320 | | |
| M10 | 160 | NS | 160 | 160 | | |
| Group 2 | | | | | | |
| M11 | | | | 1280 | 320 | 320 |
| M12 | | | | 1280 | 320 | 320 |
| M13 | | | | 320 | 640 | 160 |
| M14 | | | | 40 | 80 | 160 |
| M15 | | | | 10 | 80 | 2560 |
| M16 | | | | 1280 | 320 | 640 |
| M17 | | | | 10 | 80 | 40 |
| M18 | | | | 80 | 320 | 640 |
| M19 | | | | 320 | 20 | 1280 |
| M20 | | | | 640 | 160 | 640 |

NS = No sample

TABLE 16: Neutralisation titre to bovine virus diarrhoea virus in sera from the calves on farm M.

| Calf number | Date of sampling 4.2.81 | 9.3.81 | 23.3.81 | 20.4.81 | 19.5.81 | 17.6.81 |
|---|---|---|---|---|---|---|
| Group 1 | | | | | | |
| M1 | 3043 | 640 | 452 | 376 | | |
| M2 | 904 | 133 | 80 | 538 | | |
| M3 | <10 | <10 | 2152 | 8605 | | |
| M4 | 160 | 266 | 112 | 4303 | | |
| M5 | 320 | 266 | 266 | 94 | | |
| M6 | <10 | <10 | 224 | 3043 | | |
| M7 | 224 | 28 | 14 | 1280 | | |
| M8 | <10 | <10 | NS | NS | | |
| M9 | 3618 | 266 | 320 | 188 | | |
| M10 | 1809 | 266 | 80 | 188 | | |
| Group 2 | | | | | | |
| M11 | | | | 80 | 14 | <10 |
| M12 | | | | <10 | <10 | <10 |
| M13 | | | | <10 | <10 | <10 |
| M14 | | | | <10 | <10 | <10 |
| M15 | | | | <10 | <10 | <10 |
| M16 | | | | 133 | 188 | 14 |
| M17 | | | | 122 | 14 | 47 |
| M18 | | | | 67 | <10 | <10 |
| M19 | | | | 160 | 67 | <10 |
| M20 | | | | <10 | <10 | <10 |

NS = No sample

TABLE 17: Neutralisation titre to infectious bovine rhinotracheitis virus in sera from calves on farm M.

| Calf number | Date of Sampling 4.2.81 | 9.3.81 | 23.3.81 | 20.4.81 | 19.5.81 | 17.6.81 |
|---|---|---|---|---|---|---|
| Group 1 | | | | | | |
| M1 | - | - | ITT | - | | |
| M2 | - | - | - | - | | |
| M3 | - | - | - | - | | |
| M4 | - | - | - | - | | |
| M5 | 12 | 3 | ITT | 2 | | |
| M6 | - | - | - | - | | |
| M7 | 4 | - | - | - | | |
| M8 | - | - | NS | NS | | |
| M9 | 3 | - | - | 1 | | |
| M10 | ITT | 4 | - | 3 | | |
| Group 2 | | | | | | |
| M11 | | | | - | - | - |
| M12 | | | | - | - | - |
| M13 | | | | - | - | - |
| M14 | | | | - | - | - |
| M15 | | | | - | - | - |
| M16 | | | | 8 | 3 | - |
| M17 | | | | 2 | - | - |
| M18 | | | | - | - | - |
| M19 | | | | - | - | - |
| M20 | | | | - | - | - |

NS = No sample

ITT = Insufficient serum to test

\- = No detectable antibody

## Discussion

In the Group 1 calves there was evidence of infection with RS virus, PI3 virus and BVD virus during the monitoring period. All calves had serum antibody to RS virus in the first sample taken when they were approximately one month old. In most of the calves the level declined over the next 76 days, at a rate similar to that of the natural half-life of bovine IgG in serum (Tizard, 1982). In two calves, however, the pattern of change in antibody titre was different. In calf M7 the titre did not decline over the three months period. This could suggest a persistent antigenic stimulus. However, there is no clear evidence that the calf was infected with RS virus. In calf M3 the antibody titre dropped for the first two months then began to rise. There was a significant rise between 23.3.81 and 20.4.81 indicating RS viral infection. Presumably infection occurred between 9.3.81 and 23.3.81 as the antibody level had begun to rise by the latter date. Infection was not accompanied by severe clinical signs of respiratory disease. However, a mild or inapparent infection could have been missed as clinical examinations were relatively infrequent.

Parainfluenza type 3 viral infection was also present in Group 1. Calf M2 seroconverted between 4.2.81 and 9.3.81. No seroconversions were noted between the next two samples, which were in fact only two weeks apart, but two more calves (M3 and M4) seroconverted between 23.3.81 and 20.4.81. Of interest is that calf M4 showed evidence of infection with both RS virus and PI3 virus at approximately the same time. Despite this it did not suffer severe clinical disease. Surprisingly, despite the calves being in direct contact with each other, PI3 virus appeared to spread slowly throughout the group as judged by serological evidence. Only three calves showed unequivocal evidence of infection over a three months period. However, infection may be present in a group of calves without many showing seroconversion (Allan and others, 1978). Also, Thomas (1973) suggested that

maternal antibody could mask seroconversion. In this instance seroconversion only occurred from low initial levels of antibody (less than 40). Calves with higher initial levels did not seroconvert although in some the level was maintained over four successive samplings (calves M1, M4, M9 and M10) and did not decline at the expected rate. This could indicate that the calves were being challenged with PI3 virus.

Infection with BVD virus first appeared between 9.3.81 and 23.3.81 with two calves becoming infected (M3 and M6). In the succeeding month a further three calves seroconverted. No severe clinical signs were associated with this infection, either respiratory or enteric.

There was evidence of infection with more than one virus in some calves during the period of observation e.g. calves M2 and M7 (PI3 and BVD) and calf M3 (RS, PI3 and BVD). Despite multiple infection severe clinical signs were not noted although sporadic coughing was ever present in the group. Unfortunately, no calves became available for post-mortem examination so the extent and severity of the infections could not be assessed.

In Group 2 there was infection with RS virus and PI3 virus. In this case RS virus was isolated from the nasopharyngeal secretions of calf M20 on 20.4.81 and this calf subsequently seroconverted to the virus. As judged by seroconversions the virus did not spread to the rest of the group. However, in one calf (M16) the antibody level rose two-fold between 19.5.81 and 17.6.81 and in other calves (M11, M12, M15) did not decline at the expected rate. This could be significant in view of the fact that RS virus was being excreted by M20 and that all calves in the group were in direct and continuous contact with each other.

Parainfluenza type 3 virus was also active in the group with some calves showing marked seroconversion (M15 and M19) but no marked clinical signs of respiratory disease.

With so many viral infections being present in both groups one would have expected to isolate virus more frequently, especially in the case of PI3 virus. However, in experimental infections the period of viral shedding in nasal secretions is relatively short, of the order of eight to twelve days, and could have been missed by fortnightly or monthly sampling. In addition the BVD infections could have been primarily enteric rather than respiratory.

One isolate of RS virus was recovered. It grew readily in FBL cells with cytopathic changes first appearing 11 days after inoculating the cells, with one sub-passage. This contrasts with the lengthy period taken to recover some other isolates (Paccaud and Jacquier, 1970; Wellemans and Leunen, 1975). The virus was identified as a bovine strain by its failure to grow in cells of human origin. The swab from which the isolate was recovered had been stored at -70°C for six weeks then thawed rapidly at 37°C immediately prior to the inoculum being prepared. Despite this the virus it contained was still infective. Mohanty and others (1975) stressed the importance of prompt handling of samples without freezing and thawing. Jacobs and Edington (1975) emphasised the use of fully susceptible cells under appropriate conditions of inoculation. Whether more isolates would have been recovered if swabs had been processed immediately after being taken, or if several cell types had been used for isolation, is not clear. Rosenquist (1974) pointed out that despite the multiplicity of culture protocols used for the isolation of bovine RS virus there is no clear evidence as to which is best. The most crucial factor is that there must be virus in the sample.

RESPIRATORY DISEASE INVOLVING RS VIRUS

FIGURES

FIG. 1: Respiratory syncytial viral infection - the right lung from calf C1. There is consolidation in the cranial and the middle lobes and in the cranial part of the caudal lobe. Note the severe interstitial emphysema (blue arrow) and the intralobular haemorrhages (red arrow).

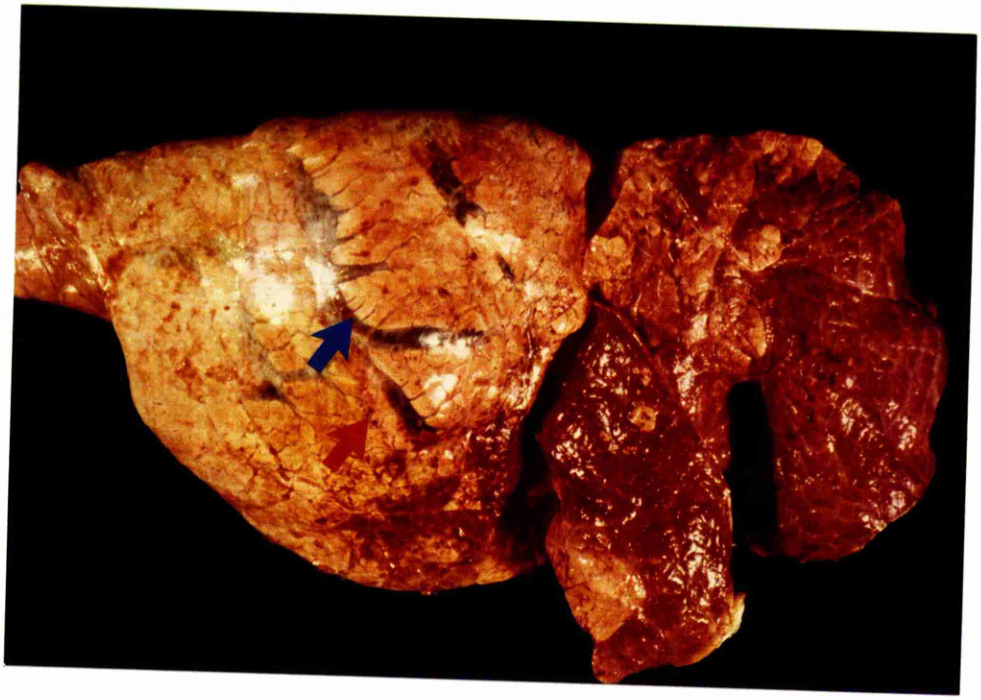
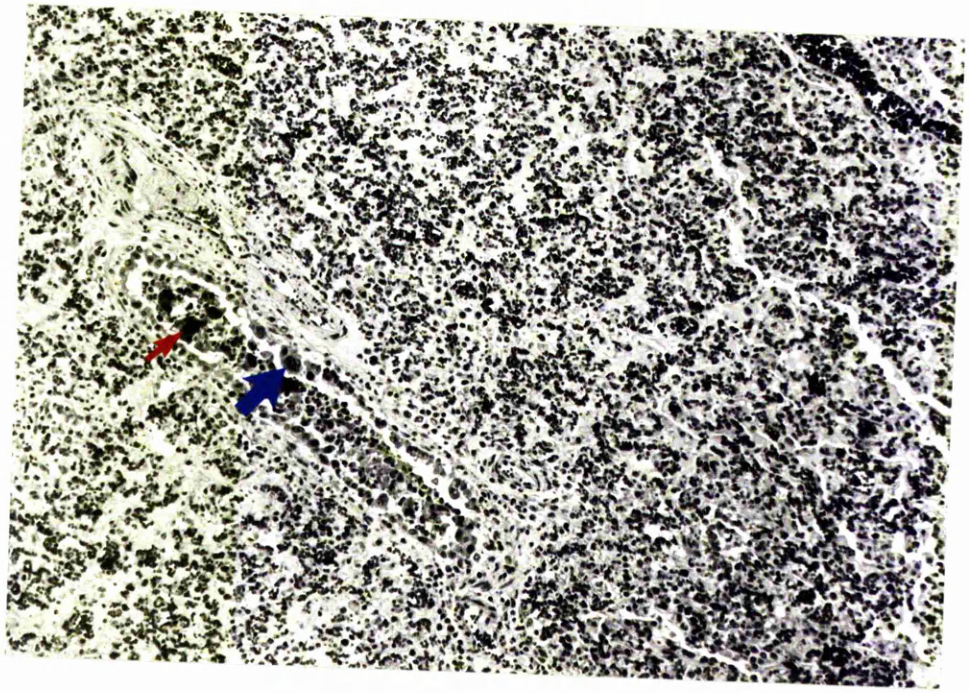


FIG. 2: Bronchiolar epithelium infected with RS virus in case RC10. Syncytia are present in the epithelium (blue arrow) and the remnants of pynknotic nuclei from necrotic syncytia are present in the lumen (red arrow). The alveoli are congested and oedematous but there is no specific evidence of viral infection in the epithelium.

H & E x 100

FIG. 3: Bronchiolar epithelium infected with RS virus in case RC8. Syncytia are present in the epithelium (red arrow) some of which are undergoing necrosis (blue arrow). A detached necrotic syncytium is present in the lumen. The epithelium is flattened in the lower part of the bronchiole.

H & E x 250



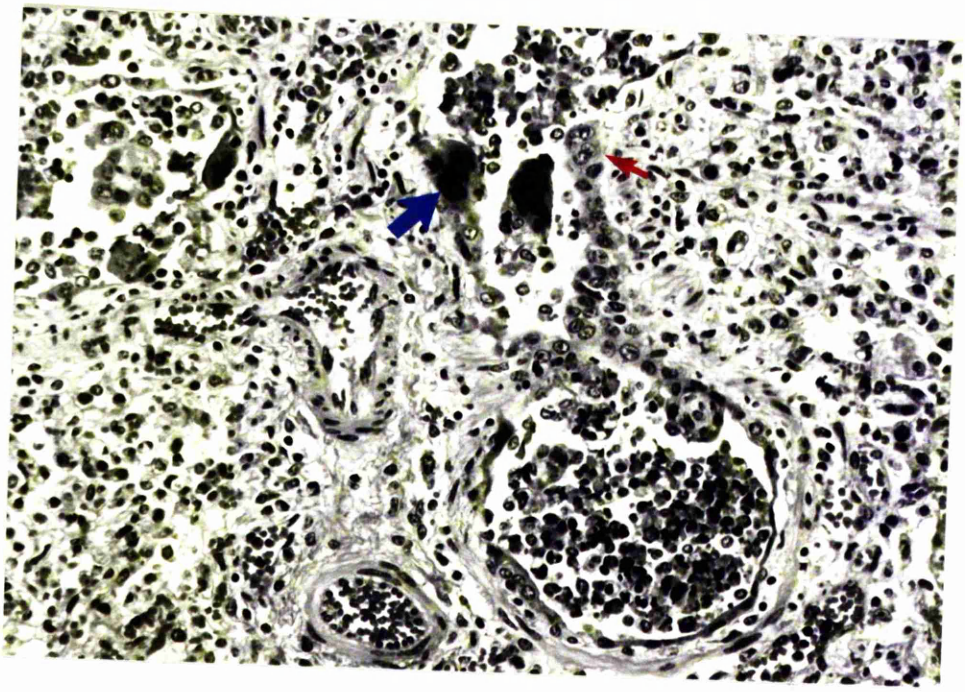
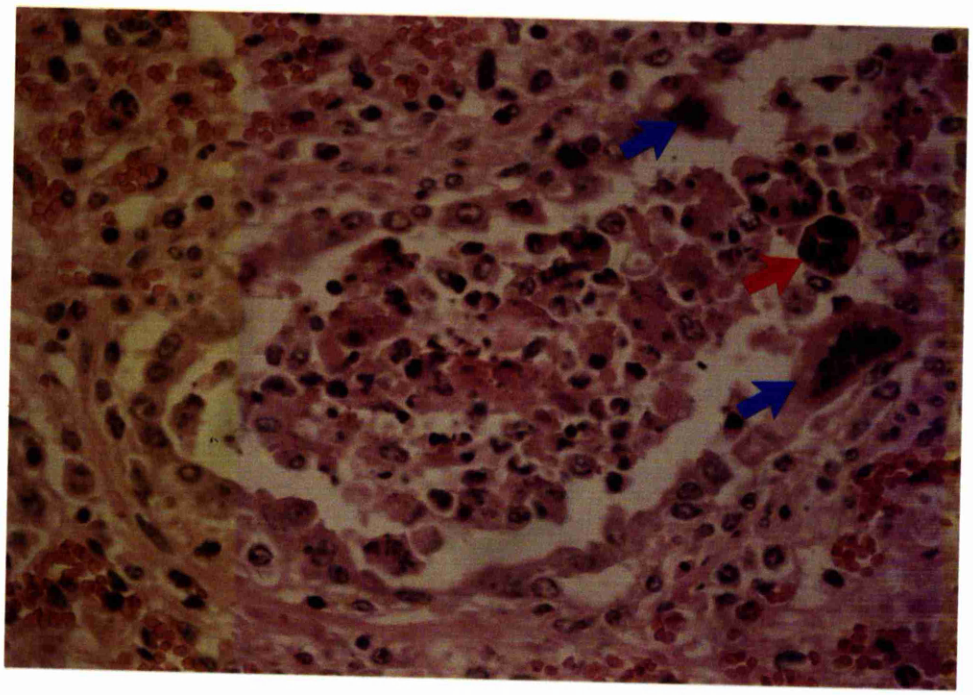


FIG. 4: Bronchiolar epithelium infected with RS virus in case RC10. The epithelium is dysplastic and there is focal necrosis and syncytium formation (blue arrows). Necrotic syncytia are present amongst the cellular debris in the lumen (red arrow).

H & E x 400

FIG. 5: Bronchiolar epithelium infected with RS virus in case RC16. Viral antigens are present in the cytoplasm of the majority of the epithelial cells.

BNF - fixed conventional section, FITC x 250



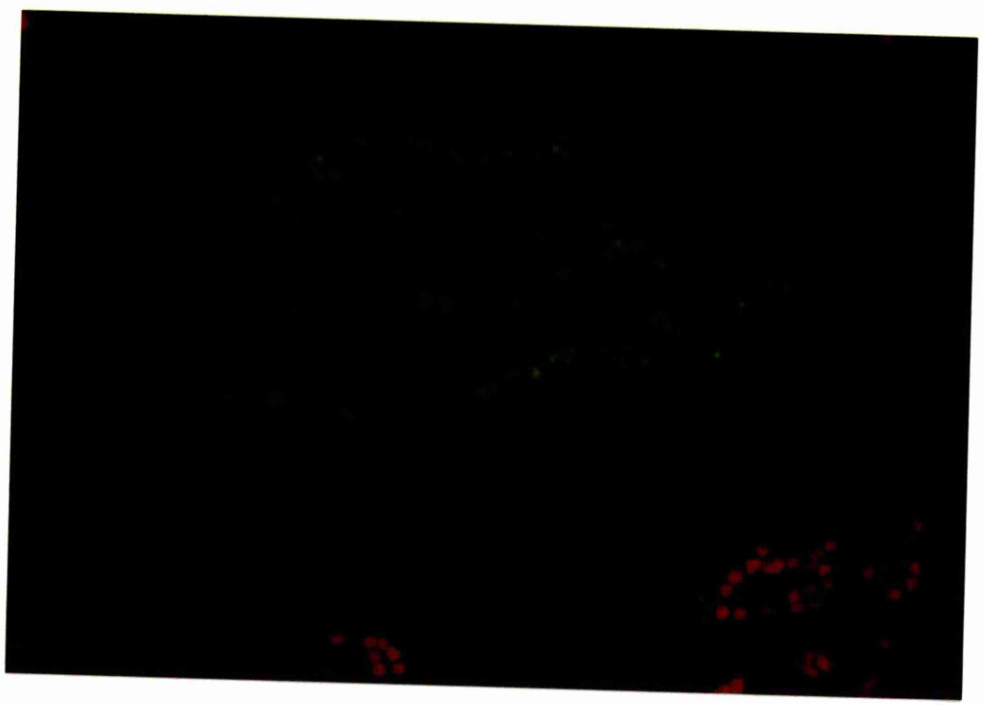
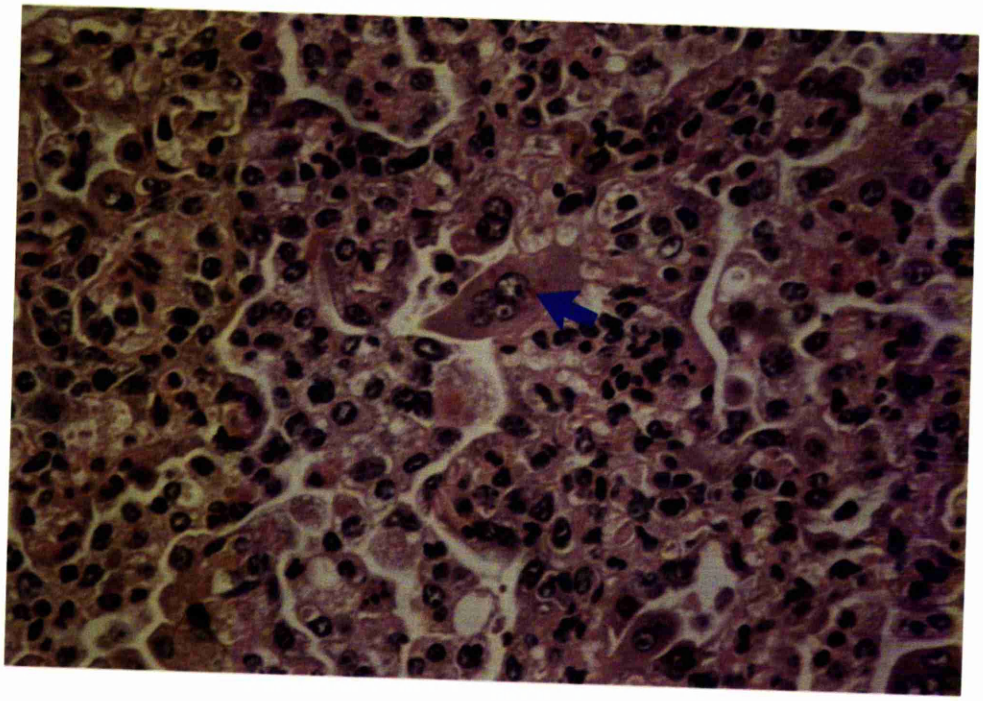


FIG. 6: Alveolar epithelium infected with RS virus in calf C1. Eosinophilic intracytoplasmic inclusion bodies (blue arrow) are present in an epithelial syncytium.

H & E x 400

FIG. 7: Alveolar epithelium infected with RS virus in calf C1. Syncytia are seen projecting from the epithelium into the lumen. Note that the nuclei overlap and are centrally placed within the cytoplasm. They are large, oval and pale staining with hyperchromatic nuclear membranes and prominent nucleoli.

H & E x 400



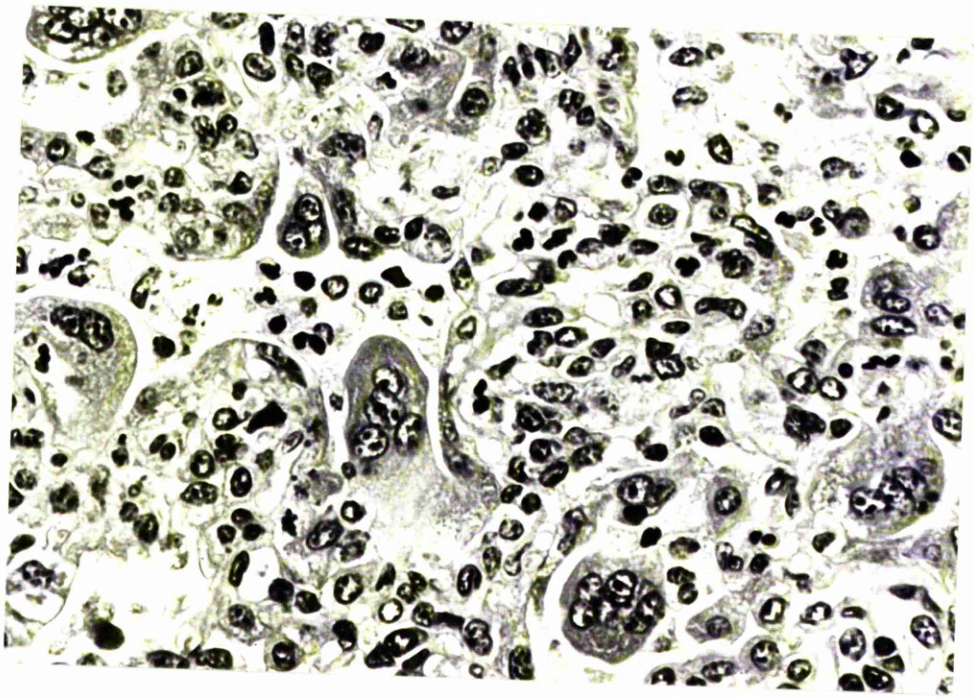
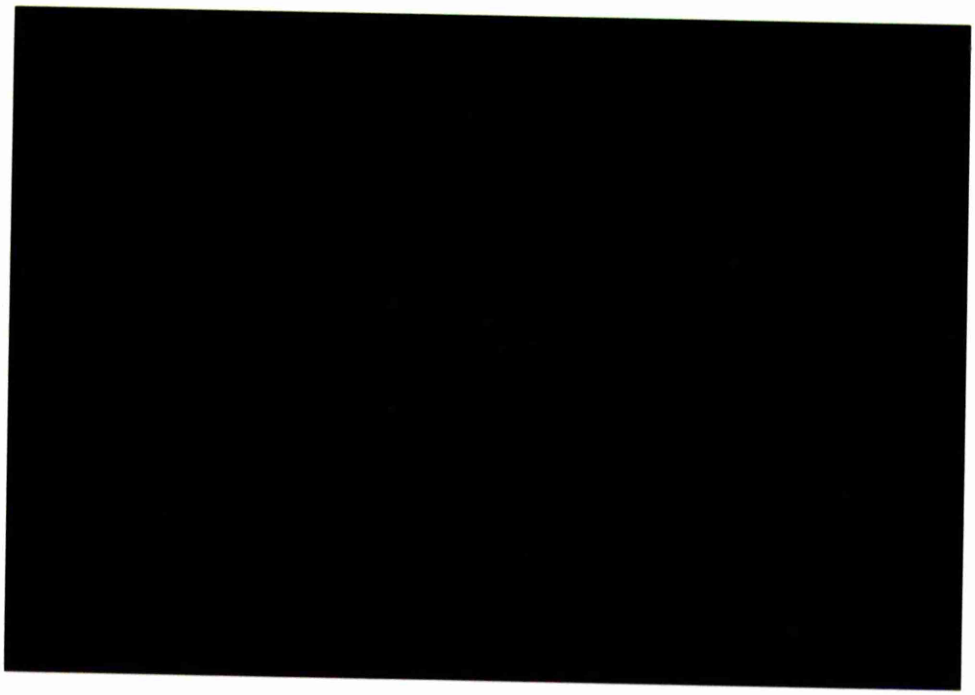


FIG. 8: Alveolar epithelium infected with RS virus in case RC14. Syncytia are seen projecting from the epithelium of several alveoli. Note the variation in size of the granules of viral antigen.

BNF - fixed conventional section, FITC x 250

FIG. 9: An individual RS viral syncytium in an alveolus, projecting from the epithelium into the lumen. Large granules of viral antigen are present in the cytoplasm (red arrow)

BNF - fixed conventional section, FITC x 400



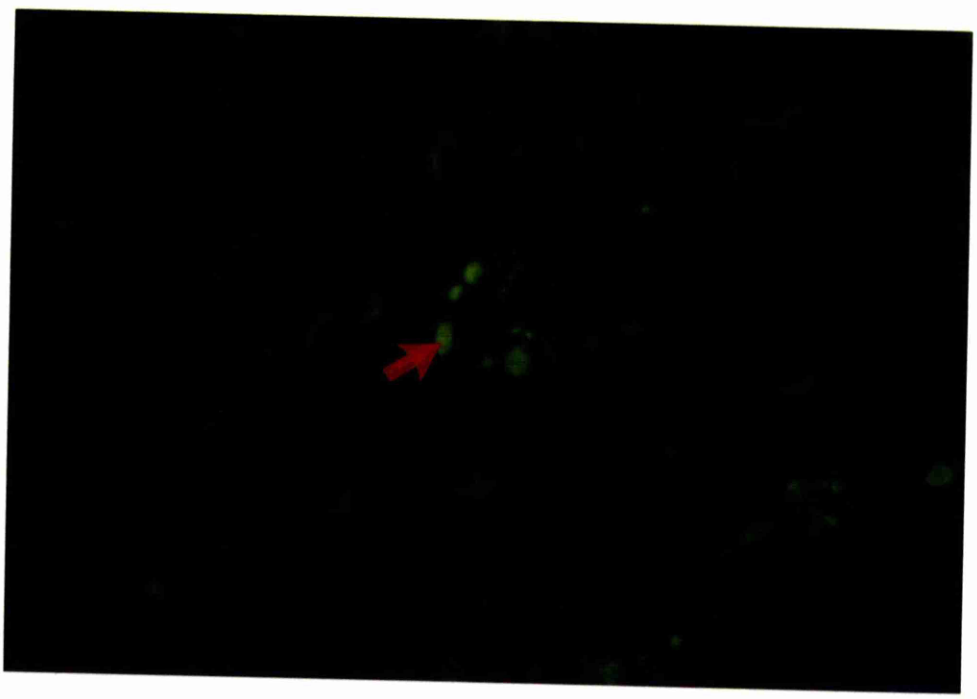
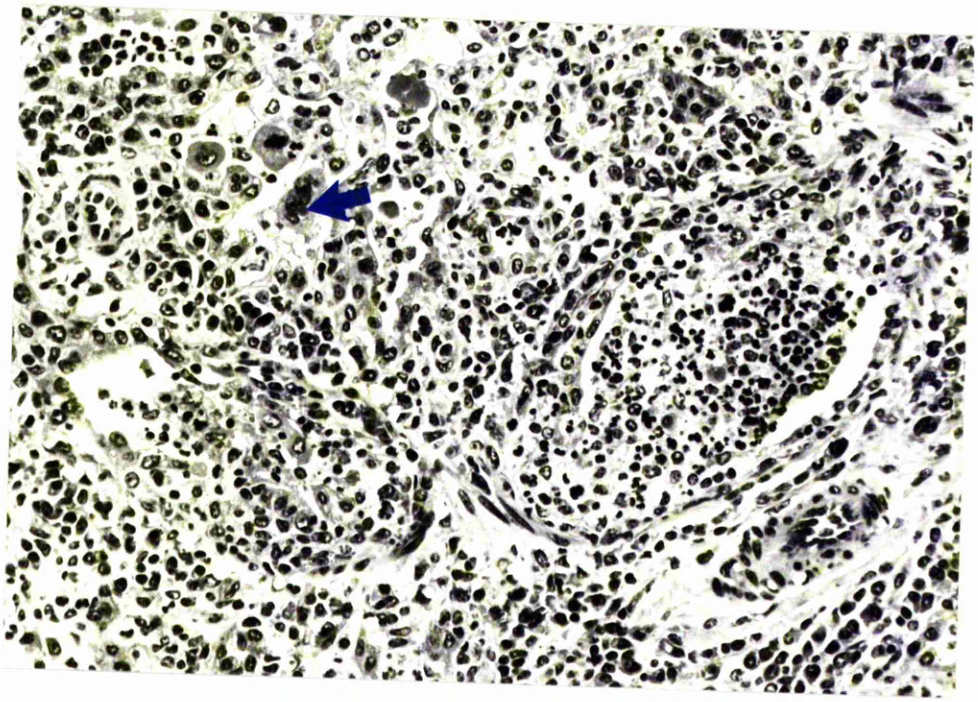


FIG. 10: Alveolar epithelium infected with RS virus in calf C1. An intracytoplasmic inclusion body can be seen in an epithelial syncytium (blue arrow). The epithelium of the adjacent bronchiole is dysplastic with areas of complete necrosis but there is no specific evidence of RS viral infection.

H & E x 250

FIG. 11: A section from the dorsal part of the left caudal lung lobe of case RC9. In the alveoli there is severe congestion and oedema, hyaline membrane formation (blue arrow) and early proliferation of the epithelium. In the bronchiole the epithelium is relatively normal but there is severe oedema of the lamina propria (red arrow). RS viral antigens were not present in this area of the lung but were present in the cranial lobes.

H & E x 250



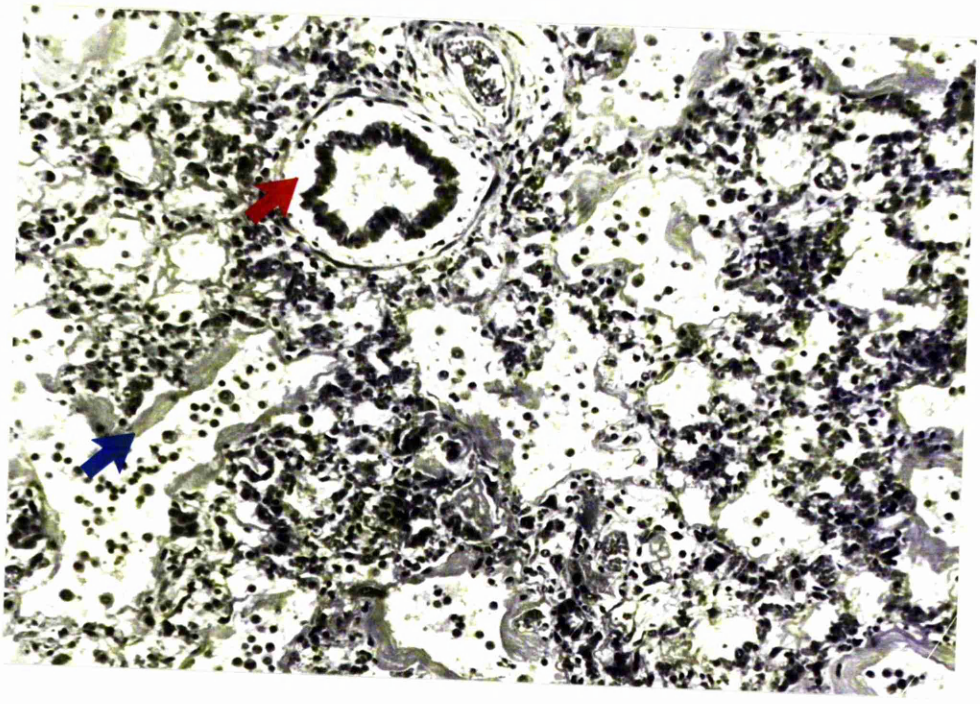


FIG. 12: The lungs of calf A27. There is consolidation in the cranial areas of all lung lobes. Interstitial emphysema is present in the caudal lobes and in the dorsal part of the right cranial lobe (red arrows). Sections from these lungs did not contain RS viral antigens although there was widespread epithelial damage. Five of nine calves in direct contact with this animal seroconverted to RS virus therefore circumstantial evidences incriminates this virus as the cause of the lesions seen here.

FIG. 13: The histopathological appearance of the lesions in the lungs of calf C6. There is dysplasia of the bronchiolar epithelium. Small numbers of lymphocytic cells are present in the peribronchiolar tissues. There is widespread alveolar epithelial hyperplasia (red arrow). This section was negative for RS viral antigens by immunofluorescent staining although this virus was the cause of the respiratory disease outbreak from which this animal came.

H & E x 100

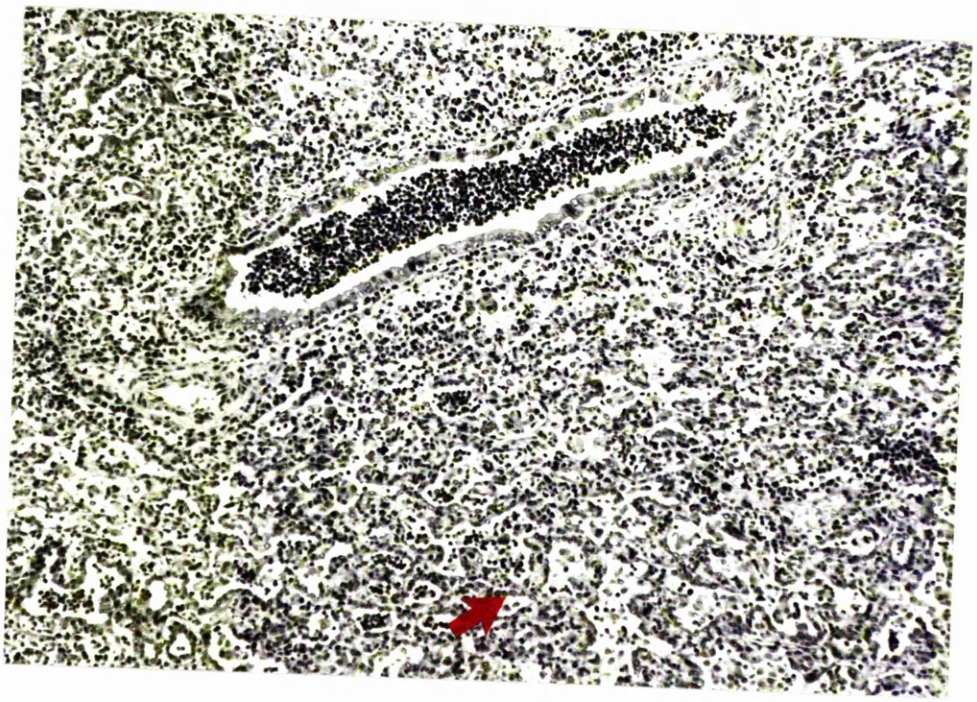


FIG. 14: The histopathological appearance of the lungs of calf C33 six weeks after being involved in an outbreak of respiratory disease caused by RS virus. Note the massive peribronchiolar accumulations of lymphocytic cells and germinal centre formation (red arrow)

H & E x 100

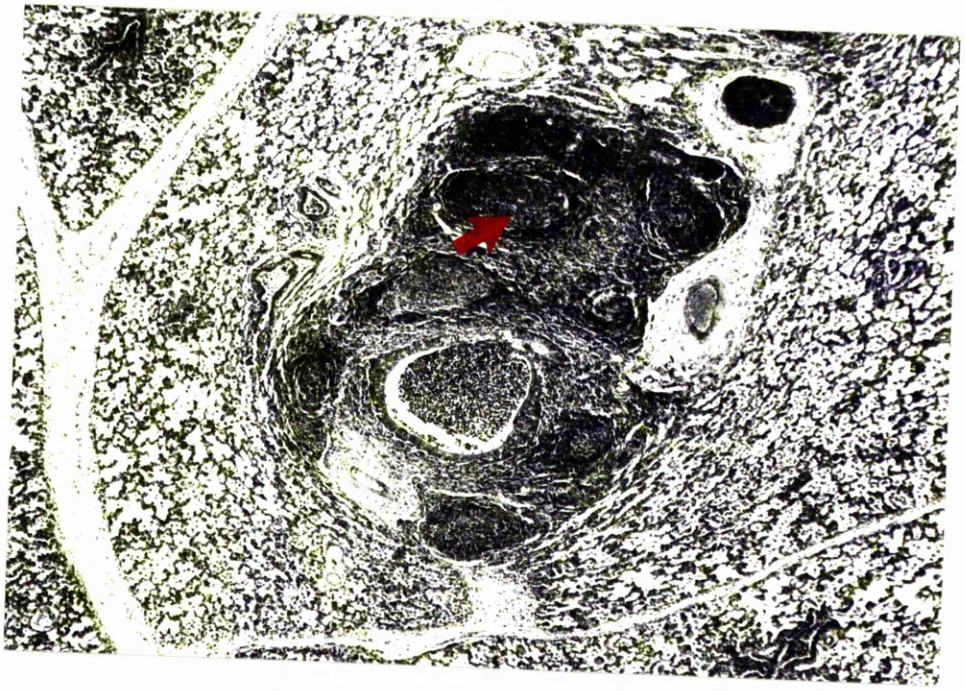
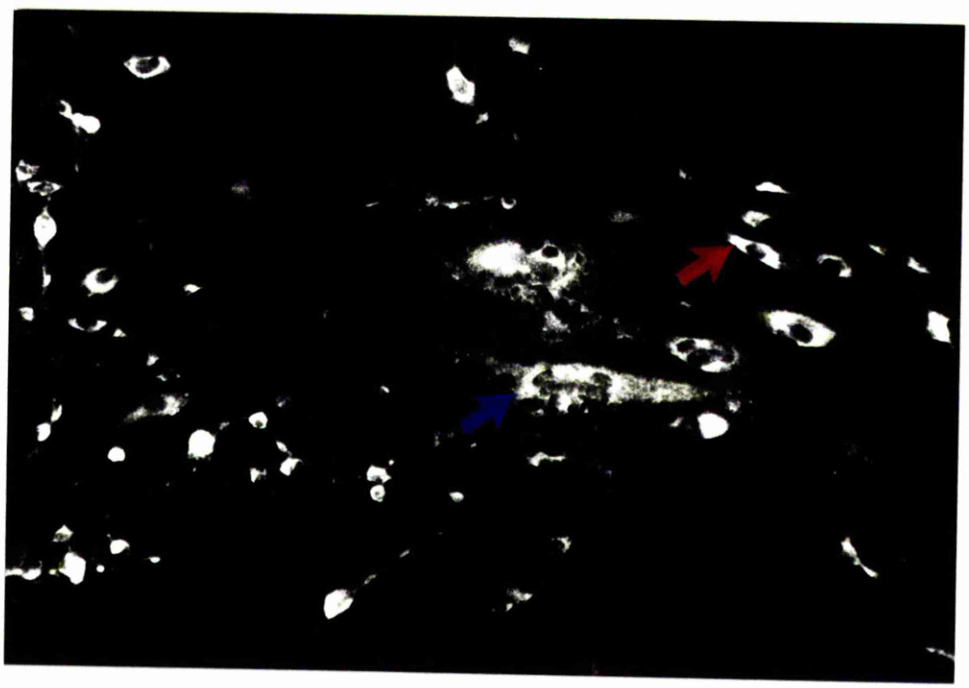


FIG. 15: Immunofluorescence staining of the intracellular antigens produced by the isolate of bovine RS virus from calf M20. Single infected cells (red arrow) and syncytia (blue arrow) are present.

FITC x 200



RESPIRATORY DISEASE INVOLVING PI3 VIRUS

OUTBREAK E

OUTBREAK F

OUTBREAK G

OUTBREAK H

RESPIRATORY DISEASE INVOLVING PI3 VIRUS: OUTBREAK E

## Introduction

This outbreak occurred on a beef rearing unit which bought in weaned single-suckled calves from a local market. The calves were housed on a slatted floor in a building with poor ventilation.

## History of Respiratory Disease Outbreak

The group of calves in which the problem arose were bought three weeks previously at a local market. All came from the same farm. Other groups of animals were bought at the same time but did not become involved in the respiratory disease incident.

The affected group was 10 calves approximately seven to nine months of age. On 4.11.80 the animals were treated with an anthelmintic and at this time one was noticed to be ill. It was dull, tachypnoeic, coughing and had a mucopurulent nasal discharge. By 8.11.80 several more animals were ill with similar clinical signs and all were treated for three days with oxytetracycline administered intra-muscularly. One animal died (E1) and its carcase was obtained for post-mortem examination. Blood samples and nasopharyngeal swabs were taken from the rest of the animals in the group (E2 to E10) on 11.11.80.

The farm was visited again one month later on 4.12.80 in order to take further blood samples. All of the animals were putting on weight and none were ill or coughing.

## Pathological Findings

### Calf E1

Gross: There was oedema and congestion of the tracheal mucosa, especially dorsally, and flecks of purulent exudate were present on

its surface. At the bifurcation of the trachea was a white frothy cast of pus which was blocking the airways. There was no fibrinous pleurisy. There was severe pneumonic consolidation of both cranial lung lobes, the right middle lobe and the cranial parts of the caudal lobes. The solid areas were congested, dark red and on pressing yellowish pasty exudate was expressed from the bronchi. Some lobules were paler in colour and more firm than the rest. There were a few small foci of sub-pleural emphysema.

Microscopic: There was a severe acute exudative pneumonia with the classical changes of severe congestion, oedema, some fibrin, neutrophils and macrophages in the alveoli. There was necrosis of the clumps of exudate cells, some of which looked like "oat" cells, particularly in the bronchioles or alveolar ducts. There was necrosis of the bronchiolar epithelium. Peribronchiolar tissues were oedematous with a few neutrophils in the lamina propria. There was focal necrosis of the alveolar epithelium. No inclusion bodies or epithelial syncytia were noted.

Immunofluorescence: The tissues from this animal were negative for PI3 and RS viral antigens using the indirect immunofluorescence test.

Microbiological Findings

The results of microbiological examination of tissues from calf E1 are presented in Table 18. In addition to these findings PI3 virus was isolated from at least one nasopharyngeal swab taken on 11.11.80. Unfortunately the swabs were pooled for viral isolation so the animal(s) excreting the virus could not be identified. Pasteurella haemolytica type A1 was isolated from four nasopharyngeal swabs (E2, E6, E7 and E8) and Mycoplasma bovis from two (E2 and E8).

Serological Findings

The results of examination of paired sera from calves E2 to

E10 for antibodies to RS virus and PI3 virus are presented in Table 19. Eight animals seroconverted to PI3 virus but none to RS virus. In addition there was serological evidence of infection with adenovirus type B in one animal (E9) and with reovirus type 2 in two animals (E7 and E9). There were no seroconversions to either P.haemolytica type A1 or Mycoplasma bovis.

TABLE 18: The pathogenic viruses, bacteria and mycoplasmas detected in the respiratory tract tissues from calf E1

| Tissue | Viruses | Bacteria | Mycoplasmas |
|---|---|---|---|
| NM | PI3 virus | P.haemolytica A1 | Negative |
| TM | PI3 virus | P.haemolytica A1 | Negative |
| RCR | Negative | P.haemolytica A1 | M.bovis |
| RM | Negative | P.haemolytica A1 | Negative |
| RCD | PI3 virus | P.haemolytica A1 | Negative |

NM = Nasal Mucosa
TM = Tracheal Mucosa
RCR = Right Cranial lung lobe
RM = Right Middle lung lobe
RCD = Right Caudal lung lobe

TABLE 19: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination-inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from the calves in outbreak E.

| Calf number | RS virus 11.11.80 | RS virus 4.12.80 | RS virus CF | PI3 virus 11.11.80 | PI3 virus 4.12.80 | PI3 virus CF |
|---|---|---|---|---|---|---|
| E2 | 1580 | 230 | 0.14 | 10 | 80 | 8.00 |
| E3 | 760 | 180 | 0.23 | 20 | 80 | 4.00 |
| E4 | 330 | 140 | 0.43 | < 10 | 80 | > 8.00 |
| E5 | 160 | 110 | 0.68 | < 10 | 80 | > 8.00 |
| E6 | 690 | 1440 | 2.08 | < 10 | 80 | > 8.00 |
| E7 | 440 | 300 | 0.68 | 80 | 320 | 4.00 |
| E8 | 660 | 600 | 0.91 | < 10 | 640 | > 64.00 |
| E9 | 5500 | 1260 | 0.23 | < 10 | 80 | > 8.00 |
| E10 | 540 | 890 | 1.65 | 80 | 40 | 0.50 |

CF = Conversion Factor

## Discussion

The results of the clinical, pathological and microbiological investigations which were carried out on the animals involved in this outbreak of acute respiratory disease provide strong evidence that the aetiological agents were PI3 virus and Pasteurella haemolytica type A1. Eight of the nine animals seroconverted to PI3 virus and the virus was recovered from at least one nasopharyngeal swab taken during the acute phase of the incident. Parainfluenza type 3 virus was recovered from both the upper and the lower respiratory tract of the dead animal and P.haemolytica type A1 was isolated also. Mycoplasma bovis was also associated with this outbreak, being isolated from two nasopharyngeal swabs and from one lobe of the lungs of the dead animal. The bulk of the evidence, however, points to PI3 virus and P.haemolytica as the major pathogens in this incident.

There was serological evidence of infection with adenovirus type B in one animal and with reovirus type 2 in two animals. Neither of these viruses were isolated from the material examined and there was no histopathological evidence of the intra-nuclear inclusion bodies associated with adenoviral infection.

This outbreak occurred three weeks after the calves had been moved, through a market, onto the farm. All were weaned, single-suckled calves and had originated from the same farm. Other animals were purchased at the same time but none of these became involved in the respiratory disease incident. The source of virus in this incident could not be determined although it is possible that the calves had contracted it during passage through the market. An animal becoming infected with PI3 virus then would have been excreting virus two or three days later. The first animal to become ill did so approximately 10 days later and virological investigations were not carried out for another week. Bearing this in mind, and considering the expected shedding period of PI3 virus in nasal secretions as determined by experimental infection, it is perhaps surprising that there was any specific

evidence of viral infection present at the time when the investigations were made. Another potential source of viral infection could have been other animals on the farm. The affected group had no direct contact with other animals although there was indirect contact via stockmen and other personnel. Another possible source of infection which has not yet been investigated fully is spread by infected humans. There is some, but as yet inconclusive, evidence that bovine strains of PI3 virus can infect man (Ben-ishai and others, 1980). Whether human strains of PI3 can infect the bovine under natural conditions is not yet known.

Despite PI3 virus being isolated from the lungs of the animal which died PI3 viral antigens were not detected by immunofluorescence. Van der Maaten (1969) found isolation to be more sensitive than immunofluorescence for the detection of virus following experimental PI3 viral infection in calves, however, localisation of viral antigens is a more relevant technique for relating viral infection to specific histopathological changes.

RESPIRATORY DISEASE INVOLVING PI3 VIRUS: OUTBREAK F

## Introduction

This outbreak occurred on a beef rearing unit which bought in weaned single-suckled calves and bucket-fed calves from local markets. Respiratory disease had not been a problem in previous years.

## History of Respiratory Disease Outbreak

The group of 10, five to six months old calves in which the problem arose were bought on 21.10.80. On arrival at the farm they were examined by the farmer's veterinary surgeon and, after being kept indoors for 48 hours, were turned out. Two days later (26.10.80) several animals were noticed to be coughing, tachypnoeic and reluctant to feed. All the calves were brought inside to a well ventilated shed and were examined. Clinically affected animals were pyrexic with rectal temperatures of up to 106.0°F. All were markedly tachypnoeic (respiratory rates up to 80/min). Eight were treated with oxytetracycline intramuscularly and two, which were not treated, were purchased by us (F1 and F2).

## Clinical Findings

First visit: The farm was first visited on 29.10.80. Most of the animals were much improved by this time. Several had a mucopurulent nasal discharge and were still tachypnoeic (respiratory rate 50/min). Half of the calves had lesions of infectious bovine keratoconjunctivitis. Blood samples and nasopharyngeal swabs were taken from five calves (F3 to F7).

Second visit: Seven days later on 5.11.80 the farm was visited again. The calves were further improved but there was still occasional coughing.

Third visit: One month after the first visit the farm was visited

again on 27.11.80, further blood samples were taken. At this visit there was virtually no coughing in the group and all were gaining weight.

Pathological Findings

Calf F1

Gross: There were pneumonic lesions in the cranial and middle lobes and in the cranial parts of the left caudal lobe. The lesions were similar in all lobes and were red-purple and slightly collapsed with a smooth homogeneous surface. On cross-section there were excessive amounts of thick creamy mucopus originating from the left cranial lobe. There was a large necrotic area near the retropharyngeal lymph node.

Microscopic: There was a mild rhinitis and tracheitis with neutrophils in the epithelium and plasma cells in the lamina propria. The epithelium was dysplastic with focal areas of necrosis. Some epithelial cells had very clear cytoplasm and many contained large pale oval nuclei with peripherally clumped chromatin, hyperchromatic nuclear membranes and a prominent nucleolus. The lesions in all lung lobes were similar. There was a bronchitis and a bronchiolitis, with epithelial damage and many plasma cells in the lamina propria. Neutrophils were present between cells in the dysplastic epithelium. Many epithelial cells had clear cytoplasm and a large oval nucleus with a prominent nucleolus. In some areas the bronchioles were relatively normal with no marked epithelial changes, apart from cells with clear cytoplasm, and few peribronchiolar changes.

The principal alveolar changes were collapse and focal epithelial hyperplasia. Many epithelial cells had clear cytoplasm which protruded into the alveolar lumen. There was a slight infiltrate of macrophages and neutrophils. In some areas there were aggregates of pale staining lymphocytic cells in the walls of bronchi and large bronchioles with occasional germinal centre

formation. In some places the lymphocytic cells extended round the bronchus or bronchioles to form a sleeve of cells.

Immunofluorescence: Sections of nasal mucosa, tracheal mucosa and pneumonic lung were examined for RS and PI3 viral antigens by immunofluorescence. All were negative.

Calf F2

The changes in this animal were similar to those in calf F1, however, there were certain differences. In parts of the right cranial lung lobe there were bronchioles with early obliterative lesions arising from areas of epithelial loss. The reaction around the bronchioles was slightly different with a more peripherally extensive lymphoid reaction in the peribronchiolar tissues. Again the infiltrating cells were largely plasma cells and a few mononuclear cells.

The most obvious and serious lesions were areas of necrosis. Grossly there were firm, gritty lesions distributed throughout the pneumonic areas. On cross section these were deep red with a friable centre. Microscopically there was coagulative necrosis of areas of the lung parenchyma which were surrounded by neutrophils and macrophages. Adjacent alveoli were involved with congestion and oedema of their walls and fibrin in their lumina.

Immunofluorescence: All respiratory tissues examined were negative for PI3 viral and RS viral antigens by immunofluorescence.

Microbiological Findings

No viruses or pathogenic mycoplasmas were isolated from the lungs of calves F1 and F2. Pasteurella haemolytica type A2 was recovered from the necrotic areas in the lungs of calf F2 and C.pyogenes was recovered from other areas. Mycoplasma bovis was isolated from one nasophayngeal swab taken from calf F4 on 29.10.80

Serological Findings

The results of examination of paired sera from calves F3 to F7 for antibodies to RS virus and PI3 virus are presented in Table 20. Three calves seroconverted to PI3 virus but none to RS virus. There was serological evidence of infection with BVD virus in one calf (F7) although none of the others had antibody in either blood sample. There was no evidence of infection with adenovirus types A and B, reovirus types 1 and 2 or IBR virus.

One calf (F3) seroconverted to Mycoplasma bovis.

TABLE 20: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination – inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from calves in outbreak F.

| Calf Number | RS virus 29.10.80 | RS virus 27.11.80 | RS virus CF | PI3 virus 29.10.80 | PI3 virus 27.11.80 | PI3 virus CF |
|---|---|---|---|---|---|---|
| F3 | 4730 | 910 | 0.19 | 160 | H | - |
| F4 | 1320 | 1820 | 1.38 | 80 | 320 | 4.00 |
| F5 | 4170 | 1320 | 0.32 | 160 | 640 | 4.00 |
| F6 | 5120 | 4790 | 0.93 | 1280 | 160 | 0.12 |
| F7 | 1820 | 720 | 0.39 | 80 | 320 | 4.00 |

H = Serum sample from Calf F3 taken on 27.11.80 haemolysed the guinea pig erythrocytes used in the HAI test despite adsorption with kaolin and guinea pig erythrocytes and heating to 56°C for 30 minutes.

CF = Conversion Factor

## Discussion

The results of the clinical, pathological and microbiological investigations which were carried out on the calves in this outbreak of respiratory disease suggest that PI3 virus and Pasteurella haemolytica type A2 were significant aetiological agents. Three of the four calves which were sampled seroconverted to PI3 virus. Histopathologically there were lesions suggestive of a viral infection in the two calves which were purchased, although these were not specific to PI3 virus and no PI3 viral antigens could be demonstrated by immunofluorescence. All the calves had high titres of serum neutralising antibody to RS virus in the first blood sample but none of the animals seroconverted to the virus and viral antigens were not present in the lungs of the purchased calves. High antibody titres in this age of calf could be due to recent infection although they do not provide specific evidence of this.

Some of the histopathological features in the lungs of the purchased calves, in particular the peribronchiolar reaction, suggest that the lesions had been present for some time although there were also more recent changes such as congestion and oedema. The lesions may, therefore, represent a recent episode of inflammation of several days duration superimposed on a more long standing lesion. The cause of the recent reaction is not absolutely clear although circumstantial evidence would incriminate PI3 virus and possibly P.haemolytica type A2. The cause of the more long standing lesion is even less clear although it could be related to a previous RS viral infection. These findings highlight the difficulty in ascribing causes to certain respiratory disease outbreaks even when they appear to present as an acute condition.

RESPIRATORY DISEASE INVOLVING PI3 VIRUS: OUTBREAK G

## Introduction

This outbreak occurred on a dairy farm which reared its own calves.

## History of Respiratory Disease Outbreak

The group of 11, eight to ten months old calves in which the problem arose were home-bred and had never been at grass. The older calves within the group had been ill with persistent diarrhoea during the summer but no diagnosis had been made.

On 10.11.81 two of the older calves were found showing severe respiratory signs. One died that day (calf G1) and, despite treatment with oxytetracyclines, the other calf (calf G2) also died. The calves were housed in a large building with half open doors at each end. One end of the house opened into a yard adjacent to a silage face where the dry cows congregated.

## Clinical Findings

First visit: The farm was first visited on 12.11.81. There was sporadic coughing in the affected group with slight tachypnoea and hyperpnoea on exercise. No adventitious sounds were heard on auscultation. One of the animals was more severely ill. Another group of younger calves in a completely separate building were coughing, many were hyperpnoeic and tachypnoeic and a few were dyspnoeic. None of this group had died and all apparently responded to treatment with tylosin.

Blood samples and nasopharyngeal swabs were taken only from the group in which calves had died (calves G2 to G11).

Second visit: The farm was visited again approximately one month later on 3.12.81. All calves appeared normal and there were no further respiratory problems.

Pathological Findings

Calf G1

Gross: There were severe pneumonic lesions in all lung lobes. The lungs were heavy and dark red in colour with severe interstitial emphysema. In the caudal lobes there were two large air-filled bullae approximately 6cm in diameter and numerous smaller pockets of air. On cross-section the lung lobules were uniformly dark red apart from several fawn coloured lobules in the caudal lobes. Some lobules contained areas of haemorrhage. Small bronchi were not particularly prominent and contained little exudate. Large bronchi were extremely congested but contained little exudate. The trachea was severely congested.

Microscopic: There was an acute proliferative alveolitis and damage to the bronchiolar epithelium. Striking lesions were congestion of alveolar walls and alveolar epithelial hyperplasia. Hyaline membranes were prominent in some lobules. The alveolar air spaces contained oedema fluid, neutrophils and macrophages. Alveolar epithelial cells had very pink, dense cytoplasm and large hyperchromatic nuclei. Occasional binucleate cells were present but there were no large epithelial syncytia or cells containing inclusion bodies. Mitotic figures were present in the alveolar epithelium but were not numerous. There were occasional areas of intra-alveolar haemorrhage.

The bronchiolar epithelium was dysplastic with irregular cell outlines and nuclei at various levels in the epithelial layer. There was focal necrosis of the epithelial cells but no syncytia or inclusion bodies were seen. In the peribronchiolar tissues were occasional focal aggregates of lymphocytes.

The lamina propria of the intra-pulmonary and lobar bronchi was congested and oedematous and contained increased numbers of lymphocytic cells. Due to post-mortem degeneration specific changes in the epithelium were difficult to recognise.

Immunofluorescence: Sections of trachea, right cranial lobe, right middle lobe and right caudal lobe were examined for RS viral and PI3 viral antigens by immunofluorescence. All were found to be negative.

Calf G2

The gross and microscopic pathological changes in this animal were similar to those in calf G1. Again no RS viral or PI3 viral antigens were detected by immunofluorescence.

Microbiological Findings

No viruses or pathogenic mycoplasmas were detected in the tissues from calves G1 and G2 or in the nasopharyngeal swabs taken on 12.11.81. Pasteurella haemolytica type A2 was detected, by immunofluorescence, in the lungs of calves G1 and G2 and was isolated from two nasopharyngeal swabs.

Serological Findings

The results of the examination of paired sera from calves G3 to G11 for antibodies to RS virus and PI3 virus are presented in Table 21. All nine animals seroconverted to PI3 virus but none to RS virus. There was serological evidence of infection with adenovirus type A in the group with four calves (G1, G3, G4 and G7) seroconverting. There was no evidence of infection with BVD virus, adenovirus type B, reovirus types 1 and 2 or IBR virus. No calves seroconverted to P.haemolytica types A1 and A2 or to Mycoplasma bovis.

TABLE 21: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination-inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from the calves in outbreak G.

| Calf Number | RS virus 12.11.81 | RS virus 3.12.81 | RS virus CF | PI3 virus 12.11.81 | PI3 virus 3.12.81 | PI3 virus CF |
|---|---|---|---|---|---|---|
| G3 | 245 | 450 | 1.83 | 10 | 160 | 16.0 |
| G4 | 1380 | 400 | 0.28 | 10 | 640 | 64.0 |
| G5 | 1000 | 1700 | 1.70 | 10 | 640 | 64.0 |
| G6 | 2340 | 560 | 0.24 | 20 | 640 | 32.0 |
| G7 | 40 | 40 | 1.00 | 20 | 160 | 8.0 |
| G8 | 760 | 1550 | 2.04 | 10 | 320 | 32.0 |
| G9 | 175 | 100 | 0.57 | 10 | 320 | 32.0 |
| G10 | 190 | 510 | 2.70 | 10 | 1280 | 128.0 |
| G11 | 220 | 220 | 1.00 | 10 | 320 | 32.0 |

CF = Conversion Factor

## Discussion

The results of the clinical, pathological and microbiological investigations carried out on the calves in this outbreak suggest that PI3 virus and Pasteurella haemolytica type A2 were the aetiological agents responsible for the acute respiratory disease. All of the nine animals seroconverted to PI3 virus. Pasteurella haemolytica type A2 was recovered from the nasopharyngeal swabs taken from two of the nine animals. This bacterium was not isolated from the lungs of the animals which died. However, in both cases it was detected by immunofluorescence.

Four of the calves seroconverted to adenovirus type A although the virus was not recovered from any of the nasopharyngeal swabs which were taken from the two dead animals. Neither were intranuclear inclusion bodies, suggestive of infection with an adenovirus, demonstrated in the lungs of the dead animals.

In view of th serological evidence of infection with PI3 virus it was surprising that there were no isolations of the virus from either nasopharyngeal swabs or from the tissues of the dead animals. No PI3 viral antigens were detected by immunofluorescence in the dead animals and the histopathological lesions, whilst suggestive of a viral infection, were not specific for PI3 virus. Epithelial syncytia were not detected in either the bronchioles or the alveoli although these features have been described in natural PI3 viral infections (Betts and others, 1964). The serum HAI antibody titres to PI3 virus rose rapidly between the first and the second blood samples but it was not possible to say whether this represented a primary or a secondary exposure to PI3 virus. The calves, at eight to nine months of age, were older than those which normally succumb to respiratory disease when indoors. They had been housed since birth and were not known to have been ill with respiratory disease previously. There was direct contact with adult cattle and these could have been the source of virus in this

outbreak. At the time of the incident younger calves on the farm had respiratory disease although they were not investigated as the farmer was not particularly worried about them. He was more concerned about the older and, therefore , more valuable animals.

There was no evidence of RS viral infection in this outbreak although several calves had high levels of serum neutralising antibody in the first sample. In four animals the titre had risen by the second sample but not by a significant amount. Maternally derived antibody would have been expected to have declined to very low levels by this time. The high initial titres to RS virus could indicate recent infection but there was no specific evidence to confirm this.

RESPIRATORY DISEASE INVOLVING PI3 VIRUS: OUTBREAK H

## Introduction

This outbreak occurred on a dairy farm milking approximately 200 Friesian and Ayrshire cows. All home-bred calves were reared except pure Ayrshire bulls which were sold at one to two weeks of age. Heifer calves were reared as herd replacements and Friesian bull calves were fattened in a barley beef unit.

## History of Respiratory Disease Outbreak

Over the previous few years the number of calves reared on the farm had increased. At the same time pneumonia and coughing in housed calves had worsened. Widespread coughing and sporadic febrile pneumonias had been particularly prevalent in October and November and tended to start in the calves at around six to eight weeks of age. At the time of the outbreak (December) there were 75 to 80 calves at risk. The problem had been tackled by treating individual sick calves with a combination of antibiotics and corticosteroids. Four groups of animals, aged between four and 14 months old, were housed in separate pens in a converted byre. Following a wave of coughing in one of the pens a calf (calf H1), aged eight months old, died. Its carcase was obtained for post-mortem examination. This was the third wave of coughing which this group had experienced, the first being two months previously the second a month ago. The dead calf had been noticed to be ill for 36 hours prior to its death and had been treated with streptomycin and corticosteroids. Coughing appeared to spread from this group to the older animals (12 to 14 months) in the byre and to younger animals in a separate building.

In a separate building were 21, three to four months old calves. These animals were investigated in detail at the first visit to the farm as they were experiencing their first wave of coughing.

## Clinical Findings

First visit: The farm was visited on 10.12.80 and at this time the calves in the group had been coughing for approximately 10 days. Six days prior to our visit one calf had died, two days after the group had been moved into this building. No post-mortem examination was carried out. On 10.12.80 most of the group were tachypnoeic, hyperpnoeic and coughing frequently. Three calves (H2, H3 and H4) were purchased for further examination.

Second visit: The farm was visited again one month later on 8.1.81. The group of calves were much improved but there was still sporadic coughing. Repeat blood samples were taken for serology from the calves sampled at the first visit.

## Pathological Findings

### Calf H1

Gross: The gross post-mortem findings in the lungs of this animal were very striking. There was severe consolidation of the cranial lobes, the middle lobe and the cranial parts of the caudal lobes (Fig. 22). The lesions were slightly collapsed and had a spotted surface. Intralobular haemorrhages were present at the periphery of the lesion. There was severe interstitial emphysema involving principally the caudal lobes but there were no large bullae of gas.

On cross-section the cranial lobes were firm with a speckled surface. There was extensive interlobular emphysema in all lobes with the formation of small interconnecting bullae (2-3cm diameter) in the caudal lobes.

Microscopic: There was a severe acute exudative pneumonia with evidence of damage to the bronchiolar and the alveolar epithelium (Fig. 23). Bronchiolar lesions in the right middle lobe consisted of necrosis of the epithelium with plugging of the lumen with neutrophils and macrophages. In the right cranial lobe there were

similar lesions and, in addition, bronchiolitis obliterans and peribronchiolar fibrosis. Alveolar lesions were principally severe congestion and oedema of the walls and focal epithelial hyperplasia. The airspaces were packed with neutrophils and macrophages. Some small syncytia were also present (Fig. 23), but were not obviously attached to the epithelium. They had densely stained eosinophilic cytoplasm and between two and five peripherally placed nuclei. No intracytoplasmic or intranuclear inclusion bodies were seen in either the bronchiolar or the alveolar epithelium.

Immunofluorescence: Parainfluenza type 3 viral antigens were detected in the syncytia and in occasional single cells in alveolar airspaces. Antigens were also present in alveolar epithelial cells. No RS viral antigens were detected.

Calves H2, H3 and H4

The gross and microscopic pathological changes in these animals were similar although they varied in extent.

Gross: There was consolidation of the cranial and the middle lobes and the cranial parts of the caudal lobes. The surface of the lesion was mottled with pink and pale areas within the lung lobules. At the edges of the lesion in the caudal lobes were small areas of sub-pleural and interlobular emphysema. The pneumonic tissue was slightly collapsed but firm and full. The left lung from calf H2 is illustrated in Fig. 16.

On cross section the lesion was dry with little exudate in the bronchi. Again the surface was mottled but the small bronchi were not particularly prominent.

Microscopic: The lesions were centred on the intrapulmonary bronchi and the bronchioles (Fig. 17). The bronchial epithelium was dysplastic and hyperplastic with collections of neutrophils in spaces within the epithelium (Fig. 18). In some cases these

features seemed to be formed by invagination of the epithelium but in the majority no direct connection to the epithelial surface could be seen. The lamina propria contained an intense infiltrate of plasma cells with a few lymphocytes and neutrophils. The bronchiolar epithelium was dysplastic and hyperplastic with neutrophils present between the cells. There were foci of epithelial necrosis and areas with flattened basophilic cells. The lumen was plugged with an inflammatory exudate of neutrophils, some macrophages and degenerating desquamated epithelial cells. In some sections colonies of bacteria could be seen. The peribronchiolar tissues were thickened with early fibrosis and an infiltrate of mononuclear cells and plasma cells. Occasional loose aggregates of lymphocytic cells were present in the peribronchiolar tissues. In some bronchioles there was early bronchiolitis obliterans with papillary ingrowths of fibroblasts and macrophages into the lumen. There was also early bronchiolectasis.

There were marked alveolar lesions adjacent to the bronchi and the bronchioles. The interlobular septae were thickened with an infiltrate of mononuclear and plasma cells. The alveolar epithelium was focally necrotic and hyperplastic. The cellular infiltrate in alveolar airspaces was composed of neutrophils and macrophages. In some areas the neutrophil component was very intense.

Additional features in calf H2 were eosinophilic intracytoplasmic inclusion bodies in bronchial, bronchiolar (Fig. 19) and, occasionally alveolar epithelial cells. No intranuclear inclusion bodies were present, nor were there any epithelial syncytia.

Immunofluorescence: Parainfluenza type 3 viral antigens were detected in the bronchial (Fig. 20), the bronchiolar and the alveolar epithelium of calf H2, but not in calves H3 and H4. No RS viral antigens were detected in any animal.

## Microbiological Findings

The results of microbiological examinations on the tissues of calves H1, H2, H3 and H4 are presented in Table 22. In addition PI3 virus was isolated from nasopharyngeal swabs from eight calves and these are indicated by asterisks in the serological table (Table 23). Pasteurella multocida was isolated from the nasopharyngeal swabs of six calves but no pathogenic mycoplasmas were recovered.

## Serological Findings

The results of the examination of paired sera for antibody to RS virus and PI3 virus are given in Table 23. One calf (H13) seroconverted to PI3 virus and eleven had rising titres. No calves seroconverted to RS virus and, in fact, most had declining levels of antibody. In addition to this there was serological evidence of infection with adenovirus types A and B in the group of calves, there being five and two seroconversions respectively. There was no evidence of infection with BVD virus, reovirus types 1 and 2 or IBR virus. There were no seroconversions to P.multocida but five calves seroconverted to Mycoplasma bovis.

TABLE 22: The pathogenic viruses, bacteria and mycoplasmas detected in the respiratory tract tissues of the calves in outbreak H.

| Calf Number | Tissue | Viruses | Bacteria | Mycoplasmas |
|---|---|---|---|---|
| H1 | NM | Negative | Negative | Negative |
| | TM | PI3 virus | P.multocida | Negative |
| | L | PI3 virus | P.multocida | Negative |
| H2 | NM | PI3 virus | Negative | Ureaplasma sp. |
| | TM | PI3 virus | P.multocida | Negative |
| | L | PI3 virus | P.multocida | Ureaplasma sp. |
| H3 | NM | PI3 virus | Negative | Negative |
| | TM | PI3 virus | Negative | Negative |
| | L | PI3 virus | Negative | Ureaplasma sp. |
| H4 | NM | Negative | P.multocida | Ureaplasma sp. |
| | TM | PI3 virus | P.multocida | Negative |
| | L | Negative | P.multocida | Ureaplasma sp. |

NM = Nasal Mucosa
TM = Trachea Mucosa
L = Lung

TABLE 23: Neutralisation titre to respiratory syncytial (RS) virus and haemagglutination-inhibition titre to parainfluenza type 3 (PI3) virus in paired sera from the calves in outbreak H.

| Calf number | RS virus 10.12.80 | RS virus 8.1.81 | RS virus CF | PI3 virus 8.12.80 | PI3 virus 8.1.81 | PI3 virus CF |
|---|---|---|---|---|---|---|
| H5 | 330 | 440 | 1.33 | 80 | 160 | 2.00 |
| H6 | 300 | 100 | 0.33 | 80 | 160 | 2.00 |
| H7* | 260 | 220 | 0.84 | 40 | 80 | 2.00 |
| H8* | 300 | 350 | 1.16 | 80 | 160 | 2.00 |
| H9* | 910 | 830 | 0.91 | 40 | 80 | 2.00 |
| H10* | 410 | 550 | 1.34 | 40 | 80 | 2.00 |
| H11 | 200 | 100 | 0.50 | 80 | 80 | 1.00 |
| H12 | 170 | 70 | 0.41 | 40 | 40 | 1.00 |
| H13 | 2690 | 1120 | 0.41 | 40 | 160 | 4.00 |
| H14 | 890 | 440 | 0.49 | 40 | 80 | 2.00 |
| H15* | 520 | 140 | 0.27 | 40 | 80 | 2.00 |
| H16 | 215 | 280 | 1.30 | 40 | 40 | 1.00 |
| H17 | 550 | 190 | 0.34 | 80 | 80 | 1.00 |
| H18 | 1380 | 650 | 0.47 | 40 | 40 | 1.00 |
| H19* | 70 | 60 | 0.85 | 40 | 80 | 2.00 |
| H20 | 3550 | 3000 | 0.84 | 80 | 80 | 1.00 |
| H21* | 260 | 250 | 0.96 | 40 | 80 | 2.00 |
| H22* | 1000 | 550 | 0.55 | 40 | 80 | 2.00 |

CF = Conversion Factor

* = The calves from which PI3 virus was isolated from nasopharyngeal swabs taken on 10.12.80

## Discussion

The results of the clinical, pathological and microbiological investigations on the calves involved in this outbreak of respiratory disease provide strong evidence that PI3 virus was the aetiological agent with secondary infection by Pasteurella multocida. The virus was demonstrated in the lung of the calf which died. It was isolated from eight of 18 nasopharyngeal swabs taken from the group which was examined in detail and was recovered from the respiratory tracts of three of this group at post-mortem. Immunofluorescence demonstrated PI3 viral antigens in direct association with the pneumonic lesions in one calf from the group.

Respiratory disease had been present in the group for 10 days prior to the first visit. This was reflected in the results of the microbiological and pathological investigations. Only one of 18 calves seroconverted to PI3 virus. Antibody titres stayed level or rose by two-fold amounts in the other calves. At the time of the first visit most of the calves had significant titres of antibody to PI3 virus indicating that they had already begun to respond serologically to the viral infection. Virus was recovered more frequently from calves with an initial serum HAI antibody titre of 40 or less (seven isolations from 12 calves, 58%) than from those with a titre greater than 40 (one isolation from six calves, 16%). The former calves were probably at an earlier stage of viral infection.

The lesions in the calves examined post-mortem were very similar and in each case PI3 virus was recovered from the respiratory tract. One calf (H2) had specific histopathological evidence of viral infection and this was confirmed as PI3 virus by immunofluorescence. Despite demonstrating the presence of PI3 virus by isolation and by immunofluorescence, epithelial syncytium formation was not a marked feature of the histopathological findings in any of the calves. Omar and others (1966), in experimental PI3 viral infection, found epithelial syncytia in the

alveoli and the bronchioles at five days after infection but not at seven days. Thus the absence of significant numbers of syncytia in the present cases could have been due to the stage of the infection. However, Bryson and others (1979b) examined the sequential development of lesions in experimental PI3 viral infection over a period of 21 days and concluded that syncytium formation was not a marked feature at any time. It would appear that epithelial syncytia are not necessarily a marked feature of either experimental or natural PI3 viral infections.

Viral antigens, detected by immunofluorescence, were present principally in the cytoplasm of the bronchial and the bronchiolar epithelial cells. No intranuclear antigens were detected. The distribution of viral antigens in the lung corresponded directly to the distribution of the pneumonic lesions. No viral antigens were detected in non-pneumonic areas. In addition to this the presence of antigens corresponded to the presence of the most acute stages of the inflammatory process and where the lesions appeared to be more advanced no viral antigens were detected.

In calf H1, which died of a severe acute exudative pneumonia, PI3 viral antigens were detected in alveolar epithelial cells and in syncytia which were free in the alveolar lumen. By light microscopy the syncytia had abundant cytoplasm and peripherally placed nuclei and resembled macrophage-type syncytia rather than epithelial syncytia. Pasteurella multocida was also isolated from the lungs in this case and the histopathological appearance of the lesions suggested an acute bacterial pneumonia. The bacteriocidal function of the macrophages could have been depressed by infection with PI3 virus as has been suggested by others (Lopez and others, 1976). Secondary infection with P.multocida was probably important in the outbreak as a whole although it is unlikely that it could, per se, have initiated the pathological changes present. The most significant lesions in the earliest stages were present in the bronchiolar and bronchiolar epithelia and these related directly to PI3 viral infection.

Several calves had serological evidence of infection with adenovirus types A or B although the viruses were not isolated from the respiratory tract material examined. Nor were adenoviral intra-nuclear inclusion bodies seen in any of the histopathological sections examined. In view of this their association with this disease incident is probably not significant.

# RESPIRATORY DISEASE INVOLVING PI3 VIRUS

## INDIVIDUAL CASES OF PI3 VIRAL INFECTION

PI3 VIRAL INFECTION: CASE RC17

Date 20th December 1982 Breed Friesian

Age Three weeks Died/Slaughtered Died

History

This was a home-bred dairy calf, housed in direct contact with calves of a similar age. No significant clinical signs had been noticed by the farmer. The calf was found dead one morning.

Pathological Findings

Gross: There was pneumonic consolidation of anterior and posterior parts of the left cranial lobe and of the right middle lobe. The pneumonic areas were red/purple and slightly raised above the level of the normal lung. On cross section the lung lobules were slightly oedematous with small quantities of mucopus in the bronchi. There was no interstitial emphysema.

Microscopic: There was severe congestion and oedema of the alveoli with focal necrosis of the epithelium. The bronchiolar epithelium was also focally necrotic. Epithelial syncytia, many containing one or more eosinophilic intracytoplasmic inclusion bodies, were present in alveoli and bronchioles. Many individual epithelial cells also contained similar inclusion bodies. In the alveoli adjacent to the bronchioles there were macrophages and a few neutrophils. Inclusion bodies were seen in macrophages.

Immunofluorescence: Parainfluenza type 3 viral antigens were detected in the bronchiolar and the alveolar epithelial cells and in cells lying free in lumen. No RS viral antigens were present.

Microbiological Findings

No pathogenic viruses or mycoplasmas were isolated but P.multocida and Staphylococcus sp. were present in the lungs of this calf.

Summary of Findings in PI3 Viral Cases

The investigations yielded only three cases of pneumonia in which PI3 viral antigens were detected. Two animals presented with respiratory disease which was of sudden onset. One animal died due to extensive pneumonic lesions and had widespread severe interstitial emphysema although there were no bullae of gas larger than 1 to 2cm in diameter. The pneumonic lesions involved the cranial and the midde lobes and the cranial parts of the caudal lobes and were firm and slightly collapsed with a mottled or spotted surface.

Histologically there was an acute pneumonia with epithelial damage in the bronchi, the bronchioles and the alveoli. The epithelial changes consisted of hyperplasia and focal necrosis. Neutrophils were present between the epithelial cells and, in one case, in vacuoles within the epithelial layer. Some cells contained eosinophilic intracytoplasmic inclusion bodies but no intranuclear inclusions were seen. Epithelial syncytium formation was a marked feature in only one case where they were present in the alveolar and the bronchiolar epithelia but not in the bronchial epithelium. Most syncytia contained two to four nuclei with occasionally up to eight nuclei. Syncytia and cells containing inclusion bodies had large, oval nuclei with hyperchromatic nuclear membranes and prominent nucleoli. In one case syncytia were free in the alveolar lumen but these were morphologically similar to macrophage syncytia rather than epithelial syncytia.

In all cases there was a lymphocytic reaction in the peribronchiolar tissues. This was composed of both plasma cells and lymphocytes with the former cell type being more numerous. A marked feature in one case was thickening of the peribronchiolar tissues by the proliferation of fibroblasts and infiltration with mononuclear cells. There was also early bronchiolectasis in this case. Superimposed on these changes in all the animals were acute exudative reactions. In all cases Pasteurella multocida was also

isolated from the lung tissue and this could have been responsible for the exudative component.

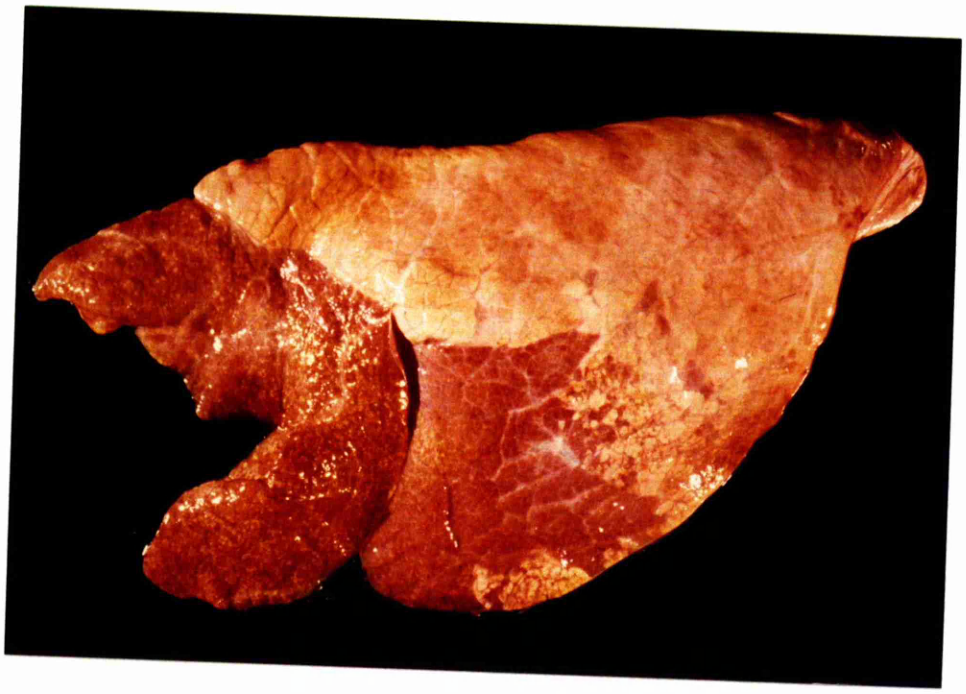
Parainfluenza type 3 viral antigens were detected in bronchial, bronchiolar and alveolar epithelial cells. Specific staining was seen as intracytoplasmic granules of antigen which varied markedly in size and shape. In one case there were many oval and crescentic granules in addition to the more usual round ones. In bronchial and bronchiolar epithelium there was intense staining of the luminal border of the infected cells and viral antigens were present in or on the cilia. Some of the larger granules of antigen corresponded to the inclusion bodies seen by staining with H&E. In all cases more antigen-positive cells were detected than would have been expected from the number of cells containing inclusion bodies which were detected by conventional staining.

FIGURES

FIG. 16: Parainfluenza type 3 viral infection - the left lung from calf H2. There is extensive consolidation in the cranial lobe and the cranial parts of the caudal lobe. There is no marked interstitial emphysema.

FIG. 17: The histopathological appearance of the lesions in calf H2. There is a bronchiolitis, with early bronchiolectasis, and a proliferative peribronchiolar reaction.

H & E x 40



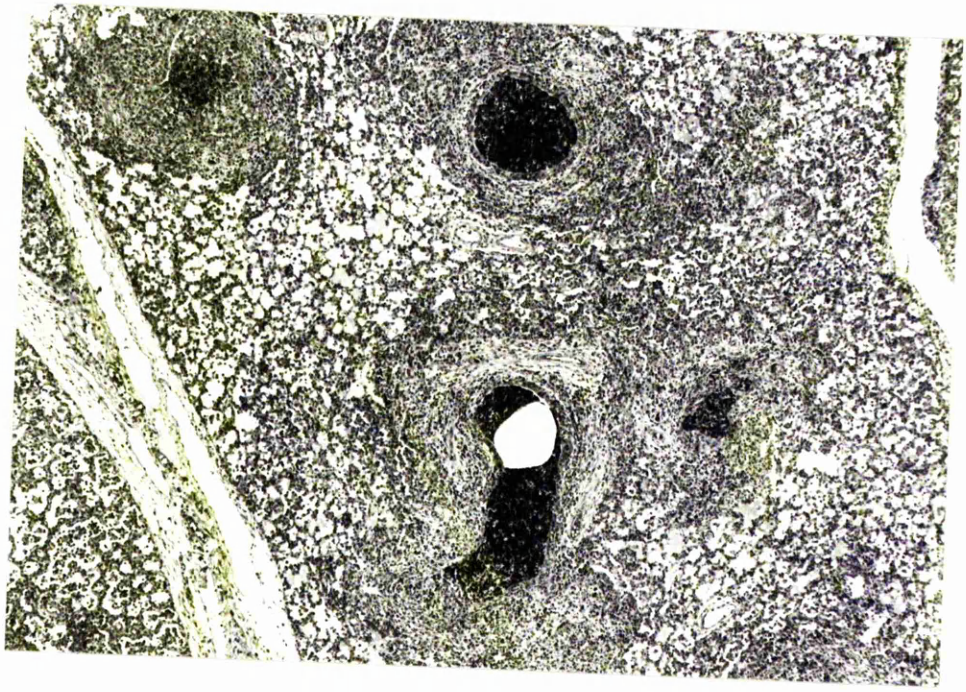
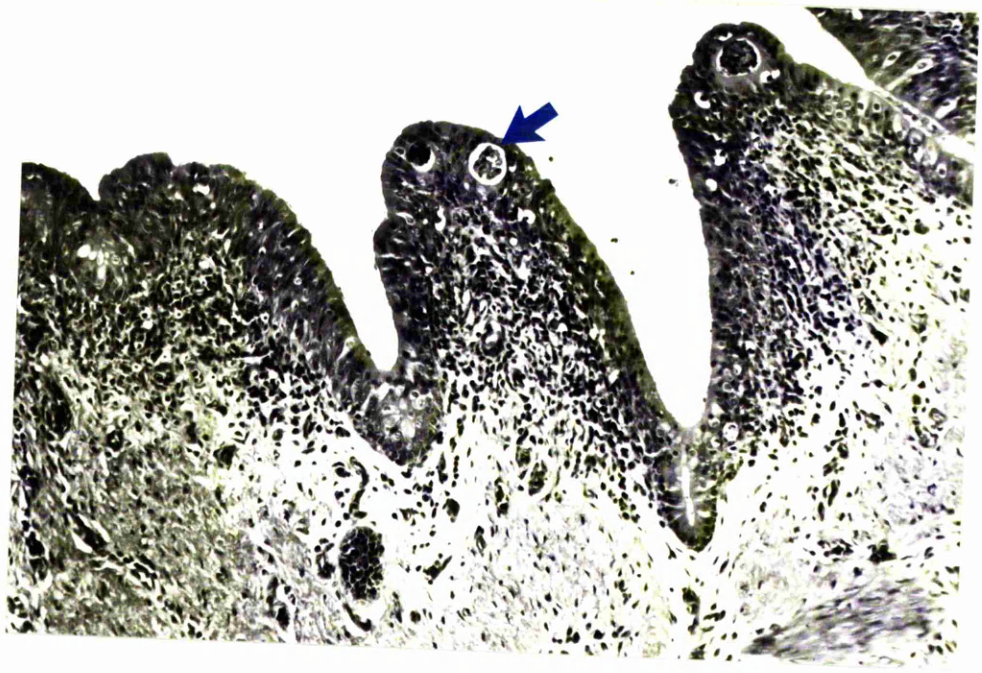


FIG. 18: Bronchial epithelium infected with PI3 virus in calf H2. There are spaces in the epithelium containing cellular debris and neutrophils (blue arrow). The epithelium is dysplastic and hyperplastic. Increased numbers of lymphocytic cells are present in the lamina propria.

H & E x 250

FIG. 19: Bronchiolar epithelium infected with PI3 virus in calf H2. There is dysplasia and hyperplasia of the epithelium and eosinophilic intracytoplasmic inclusion bodies can be seen in some cells (red arrows). Increased numbers of plasma cells and lymphocytes are present in the lamina propria. Neutrophils are present in the epithelium and in the lumen.

H & E x 400



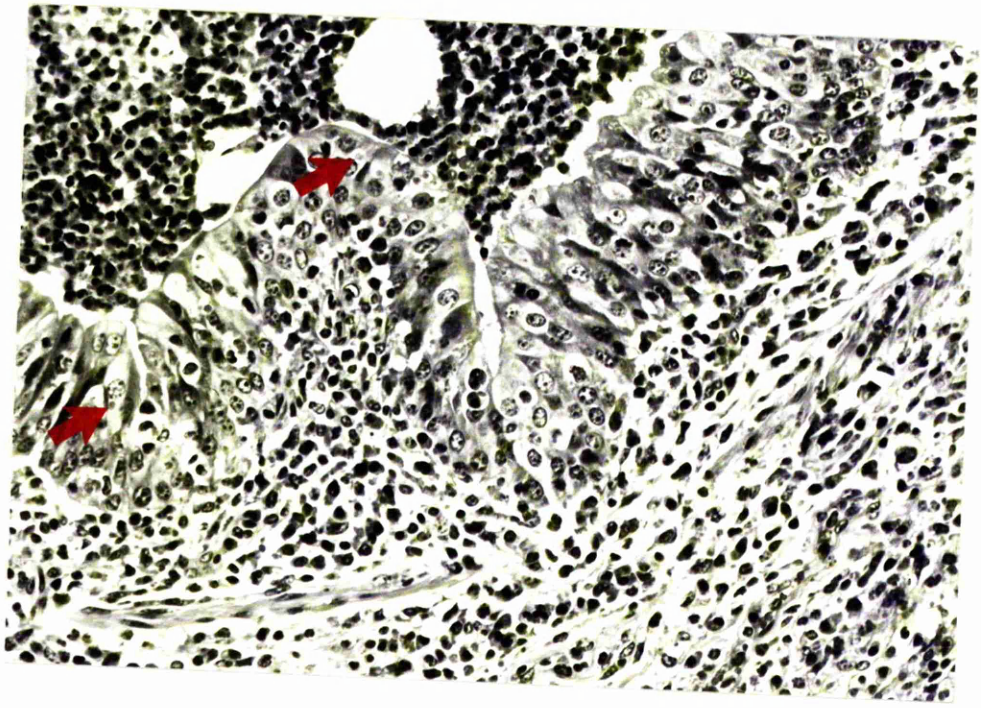


FIG. 20: Bronchial epithelium infected with PI3 virus in calf H2. Viral antigens can be seen in the epithelial cells. Some of the large granules of viral antigen correspond to the eosinophilic intracytoplasmic inclusion bodies seen in sections stained with H & E (blue arrow). In some cells there is intense staining of the cilia on the luminal border (red arrow).

BNF - fixed conventional section, FITC x 400

FIG. 21: An adjacent section to that in Fig. 20 which was stained for RS viral antigens. Note the abscence of specific intracytoplasmic staining

BNF - fixed conventional section, FITC x 400

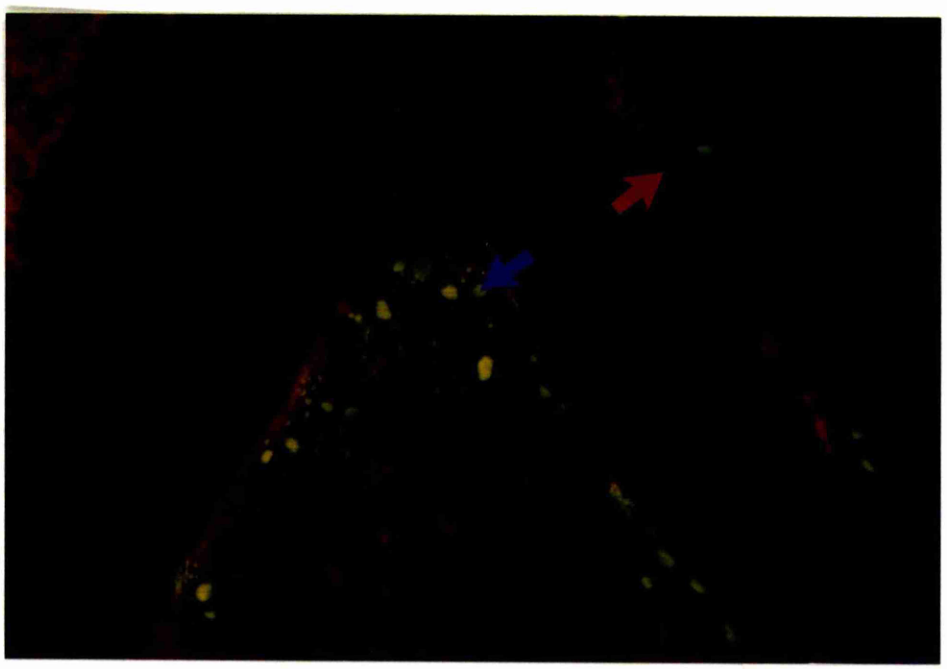
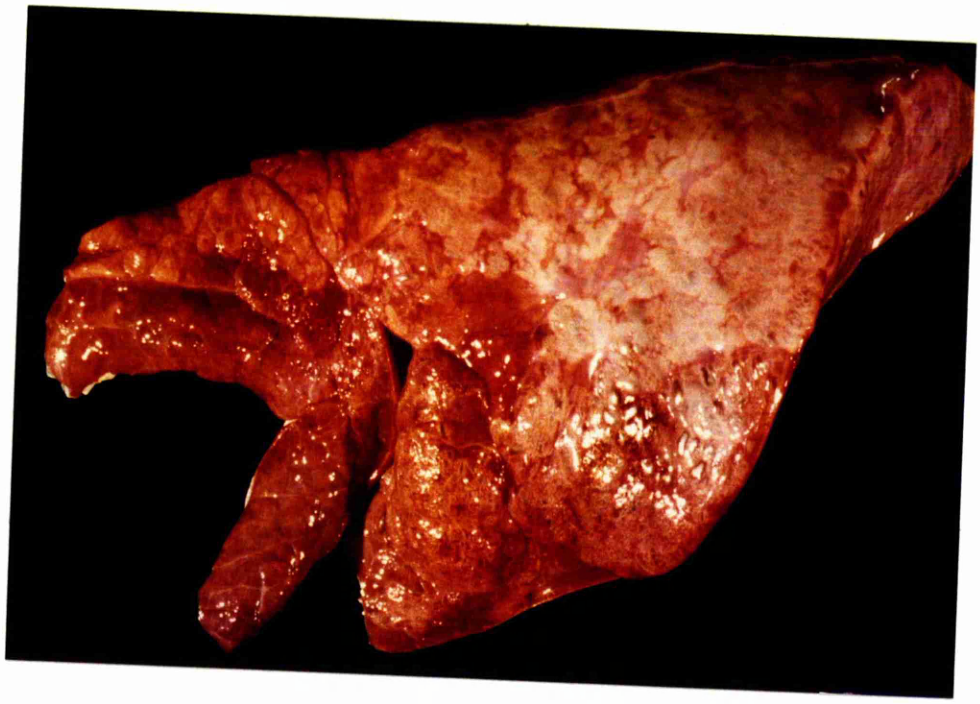
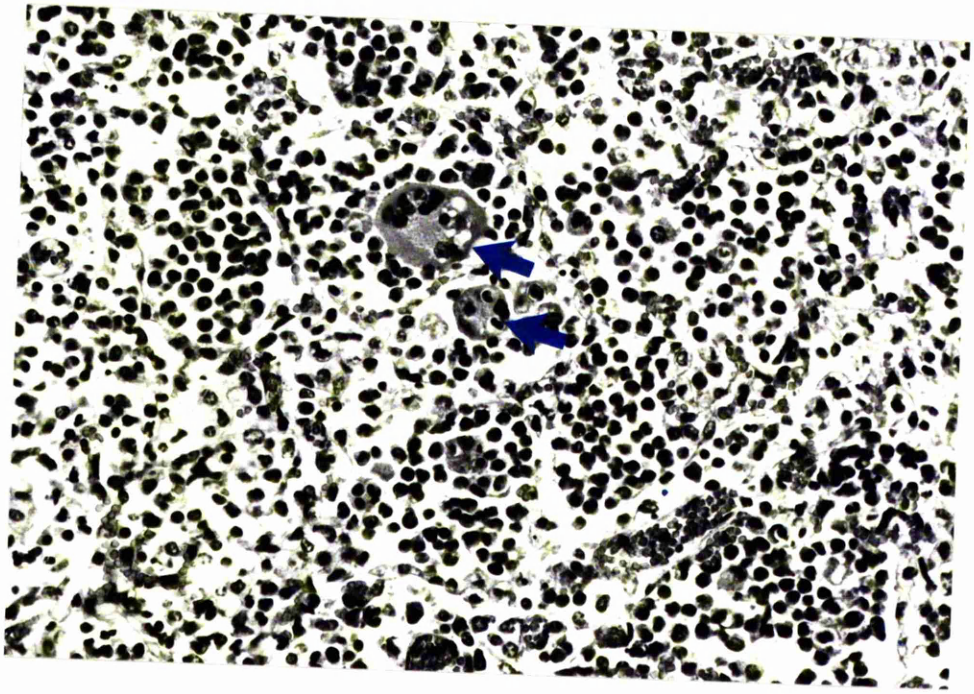


FIG. 22: Parainfluenza type 3 viral infection - the left lung from calf H1. There is consolidation in the cranial lobe and in the cranial part of the caudal lobe. Interstitial emphysema is present in the caudal lobe but there are no large bullae of gas. In addition to PI3 virus, Pasteurella multocida was isolated from the consolidated areas.

FIG. 23: The histopathological appearance of the lesions in the left cranial lung lobe of calf H1. There is a severe acute exudative reaction with large numbers of neutrophils and a few macrophages in the alveolar airspaces. Two syncytia are present, neither of which is obviously attached to the epithelium (blue arrows). They have peripherally placed nuclei and relatively abundant cytoplasm. Cells like these contained PI3 viral antigens.

H & E x 400





SECTION FOUR

# EXPERIMENTAL INVESTIGATIONS

## RS VIRAL INFECTIONS

EXPERIMENT A

EXPERIMENT B

FIGURE

## PI3 VIRAL INFECTIONS

EXPERIMENT C

EXPERIMENT D

FIGURES

## INTRODUCTION

Until recently isolates of RS virus obtained from cattle in the U.K. have produced only mild clinical disease and few pathological lesions when used to infect calves experimentally (Jacobs and Edington, 1975; Thomas and others, 1977). Experimental infections with PI3 virus have produced variable degrees of clinical illness and of pathological lesions. Experiments by Dawson and others (1965) resulted in mild clinical disease yet moderately extensive pulmonary lesions were produced in some animals. Omar and others (1966) were able to produce significant clinical disease and pulmonary lesions using their strain of PI3 virus. Thomas and others (1977) were able to infect animals with PI3 virus but this did not result in significant clinical signs of respiratory disease.

The potential difficulties in producing disease by experimental infections with these viruses were pointed out by Stott and others (1978). Dose and virulence of virus, route of inoculation and susceptibility of the experimental animals were thought to be important variables. In addition, they suggested that intercurrent infections, for example with bacteria, could be necessary for the development of severe disease as seen naturally. It seemed likely that failure to produce clinical respiratory disease consistently merely reflected the lack of knowledge of the "appropriate conditions" under which to carry out experimental infections.

Recently, by optimising the potential variables, Bryson and others (1979b) were able to consistently produce significant clinical disease and pathological lesions in experimental calves using a strain of PI3 virus isolated in Northern Ireland. They attributed their success to using repeated doses of low-passage virus in susceptible, colostrum-deprived calves. It was felt that these conditions reflected natural exposure more accurately than those in some of the previous experiments by other workers. Using similar procedures they successfully infected calves with a strain

of bovine RS virus and again were able to produce significant, sometimes severe, clinical disease and extensive pulmonary lesions (Bryson and others, 1982).

At the time when the RS viral infections described in this thesis were carried out the only strain of virus which was available (the Compton strain) had been repeatedly passaged in tissue culture. Its growth characteristics were poor with slow development of cytopathic changes and relatively low yields of infectious virus ($10^4$ to $10^5$ $TCID_{50}$/ml). Nevertheless, it was important to try and infect calves experimentally with this virus in order to test the methods of recovery of virus from nasopharyngeal swabs and tissues. As experimental infections with RS virus by other authors had produced only mild clinical and pathological responses, it was decided to try and increase the susceptibility of the lung to viral damage by infection with the cattle lungworm Dictyocaulus viviparus. It was felt that combined infection with both RS virus and D.viviparus could result in more severe damage than infection with either agent alone. In one experiment (A) the viral inoculum preceded the parasite inoculum and in the other (B) this order was reversed. Whether or not synergism between these two agents occurs under natural conditons is not clear. No cases of dual infection were detected in the field investigations, however, RS viral infection was seen in suckler calves which had been at grass. Although it is unlikely that these animals had ingested sufficient larvae to cause clinical disease they could have had some tissue damage due to the parasite.

Experimental animals were obtained from farms in the West of Scotland which had no history of severe respiratory disease. All animals were screened for serum neutralising antibody to RS virus using the test described in Section Two. Unfortunately it was not possible to find animals which were free of antibody to RS virus, even those which were three of four months of age. At this time one would have expected antibody derived from colostrum to have

declined to negligible levels. Finding antibody could mean either that there initial titre after colostrum intake had been high or that they had been exposed to RS virus. This could have reduced the clinical and pathological effects of the experimental infections, although some authors have reported that experimental infections in calves with serum antibody have produced more severe disease than in those without (Smith and others, 1975).

In December 1980 a strain of PI3 virus was isolated from the lungs of a calf with severe pneumonia (see section Three, Outbreak H). An inoculation procedure similar to that described by Bryson and others (1979b) was used to infect calves with this virus. In addition viral excretion and the relationship between viral antigens and the histopathological features in infected lungs was studied. Groups of two weeks old (experiment C) and four months old (experiment D) calves were used.

RS VIRAL INFECTIONS

EXPERIMENT A

EXPERIMENT B

EXPERIMENT A

## Introduction

The object of this experiment was to infect a defined segment of the lung with RS virus and then to initiate tissue damage in this area by infection with Dictyocaulus viviparus. Respiratory syncytial virus can be present in the lungs of calves for up to 21 days after an experimental infection (Mohanty and others, 1975). Damage to the lung caused by D.viviparus starts at approximately seven to ten days after infection so in this experiment it was hoped that RS virus would still be present in the lung when damage was being produced by the parasite. This could result in a potentiation of the pathogenic effect of the virus, making the viral lesions more extensive.

The area of the lung chosen for infection with the virus was the right caudal lobe. An endobronchial catheter could readily be introduced into this region using a fibreoptic endoscope as described in Section Two.

## EXPERIMENTAL DESIGN

### Experimental Animals

Five three months old calves (EA1 to EA5) were used in this experiment. All had been housed since birth and none had been vaccinated against parasitic bronchitis. Their reciprocal neutralising antibody titres to RS virus were less than 500 prior to viral infection.

### Experimental Protocol

On d y 0 of the experiment three calves (EA1, EA2 and EA3) were each inoculated endobronchially with 20ml of a suspension of

RS virus. Two calves (EA4 and EA5) were each inoculated with 20ml of tissue culture medium. For inoculation the calves were sedated with xylazine (Rompun, Bayer) administered by intravenous injection. They were then restrained in lateral recumbency on their right side with head and neck extended. A fibreoptic endoscope (Olympus Instruments) was introduced into the right ventral nasal meatus and passed via the larynx and trachea into the right caudal bronchus. A sterile flexible catheter was then passed down the endoscope and advanced as far as possible into the bronchus. When in position the inoculum was slowly injected through the catheter into the right caudal lung lobe over a period of two minutes. The endoscope were then removed and the calf was allowed to recover.

On day 4 of the experiment four calves (EA2, EA3, EA4 and EA5) were infected, per os, with D. viviparus third stage larvae. All calves were slaughtered on day 20.

Viral inoculum

The viral inoculum was prepared from a stock suspension of the Compton Strain of bovine RS virus. Freshly monolayered FBK cells in 2 litre rolling bottles were inoculated with this virus at a multiplicity of infection of 0.1 $TCID_{50}$/cell. After adsorbing for one hour at 37°C the inoculum was removed and the cell sheet was washed twice with EFC0. Fresh EFC5 (150ml) was added and incubation was continued at 37°C for 24 hours. The medium was then changed and incubation was continued until maximum cpe had developed. The cells were then scraped into the medium and were stored in aliquots at -70°C. A portion was kept for titration and was checked for viral, bacterial and mycoplasmal contamination.

When required aliquots were thawed and were centrifuged at 3000g for 10 minutes at 4°C to remove cell debris. The supernatant was used as inoculum. The titre of the inoculum was $10^5 TCID_{50}$/ml and 20ml of this was administered. The total amount of virus given was $10^{6.3}$ $TCID_{50}$. The control calves were inoculated with

uninfected tissue culture medium prepared from FBK cells in the same manner as the viral inoculum.

Parasite inoculum

Faeces from calves with patent D.viviparus infection was collected. First stage larve of the parasite were harvested using the Baermann technique (La Page, 1962). The larvae were then cultured by incubation in a shallow layer of water for five to seven days at 26°C. During this time development into the infective third stage (L3) took place. Viable larvae were counted and an inoculum containing $3.10^3$ D.viviparus L3 was prepared for inoculation of the experimental calves.

Sampling of Calves

Nasopharyngeal swabs for viral isolation and blood samples for serology were taken from all of the calves on day 0. Thereafter nasopharyngeal swabs were taken from calves EA2, EA3, EA4 and EA5 on days 2, 4, 6, 8, 10, 12, 14 and 16. Blood samples were taken from all calves at slaughter on day 20.

RESULTS

Clinical Findings

Calf EA1 died one hour after inoculation with virus on day 0. Death occurred during recovery from sedation whilst not under observation by a clinician. In calves EA2, EA3, EA4 and EA5 there were no significant clinical signs until day 14. After this time they became increasingly tachypnoeic and hyperpnoeic and coughed frequently. From day 18 adventitious sounds, crackles and occasional squeaks, were heard in the caudal areas of both lung fields.

Pathological Findings

Calf EA1

Gross: There was congestion and oedema of the lungs with frothy oedema fluid in the trachea and major bronchi. There was a segmental area of overinflation and interstitial emphysema extending from the lobar bronchus to the caudal border of the right caudal lobe (Fig. 24). On cross section this area was severely congested and oedematous with gas present in the interlobular septae.

Microscopic: There was alveolar overinflation with patchy congestion and oedema. Oedema fluid was present in the lumen and in the lamina propria of the major airways. The lymphatics contained oedema fluid and mononuclear cells and a few neutrophils were present in some blood vessels. There were occasional aggregates of lymphoid cells in peribronchial and peribronchiolar tissues. No inclusion bodies or epithelial syncytia were seen in any of the sections examined.

Immunofluorescence: Sections of lung were negative for RS viral and PI3 viral antigens.

Calves EA2, EA3, EA4 and EA5

The lesions in these calves were similar.

Gross: There were areas of overinflation and consolidation in both caudal lobes and in the accessory lobe. Consolidated areas were slightly raised above the surface of normal lung and had a mottled surface. On cross section the lesions were congested and slightly oedematous with frothy mucopus exuding from cut bronchi. A few small lungworms were present in the lobar bronchi of the caudal lobes.

Microscopic: There was congestion and oedema, alveolar collapse and alveolar overinflation. A patchy infiltrate of macrophages, neutrophils and eosinophils was present in alveoli. A few syncytia with peripherally placed nuclei and abundant cytoplasm were seen in alveolar airspaces. None contained eosinophilic intracytoplasmic inclusion bodies or were convincingly part of the alveolar epithelium. The alveolar epithelium was focally hyperplastic. The bronchiolar epithelium was dysplastic and hyperplastic but no inclusion bodies were seen in the epithelial cells. There were aggregates of lymphocytes, plasma cells and a few eosinophils in peribronchiolar tissues. There was a marked bronchitis with epithelial dysplasia and increased numbers of lymphocytic cells and eosinophils in the lamina propria. Neutrophils were seen between epithelial cells. Lungworms were present in the larger bronchi.

Immunofluorescence: Tissues from each lung lobe of each animal were examined for RS viral and PI3 viral antigens. All were negative.

Microbiological Findings

No viruses were recovered from any of the nasopharyngeal swabs examined or from any of the tissues examined at post-mortem.

Serological Findings

The reciprocal neutralisation titre to RS virus in paired sera from these calves is presented in Table 24. Calves EA2 and EA3 seroconverted to RS virus. All calves had serum antibody at the beginning of the experiment.

Table 24: Neutralisation titre to respiratory syncytial virus in paired sera from the calves in Experiment A.

| Calf Number | Day of Experiment 0 | 20 | CF |
|---|---|---|---|
| EA1 | 160 | NS | - |
| EA2 | 50 | 250 | 5.00 |
| EA3 | 200 | 420 | 2.10 |
| EA4 | 100 | 90 | 0.90 |
| EA5 | 120 | 85 | 0.71 |

CF = Conversion Factor

## Discussion

The object of this experiment was to infect a defined segment of the lung with RS virus and then to initiate tissue damage in this area by infection with D.viviparus. The area of lung to be infected with virus was identified by endoscopy so that it could be specifically sampled at post-mortem. Calves were not killed until day 20 in order to allow sufficient time for a serological response to develop.

The serum antibody titres to RS virus in calves EA2 and EA3 rose after endobronchial inoculation whereas those in the control calves (EA4 and EA5) fell. In calf EA2 there was a significant four-fold or greater rise in titre and in calf EA3 a two-fold rise. Despite the lack of a significant seroconversion in Calf EA3 the rising titres suggest that both calves became infected with RS virus. As no virus was recovered in nasopharyngeal swab infection must have been limited to the lower respiratory tract. However, at post-mortem examination no virus could be detected, by either isolation or immunofluorescence, in the lung and there were no pathological lesions which could be attributed specifically to RS viral infection. There are several possible reasons for this, some of which might be of significance in explaining the difficulties encountered in the diagnosis of natural respiratory disease outbreaks. Viral infection in the lower respiratory tract may not necessarily be reflected by infection in the upper respiratory tract. In this experiment RS virus was known to be present in the lung but this was not reflected by viral isolations from nasopharyngeal swabs. Against this the route of infection was not natural and whether RS viral infection of the lung without infection in the nasopharynx can occur naturally is not clear. In this instance there was no evidence of transfer of virus between the two sites.

In experimental infections RS virus may be present in the lung 21 days after infection (Mohanty and others, 1975), although

how frequently this is the case is not clear. In most cases virus has been recovered only up to day 10 or 14 after experimental infection (Jacobs and Edington, 1975; Bryson and others, 1982). Bearing this in mind it may not be surprising that virus was not present in the lungs of the experimental calves EA2 and EA3 20 days after infection. Also, by this time both calves had increasing amounts of serum antibody and this may have precluded isolation of virus as suggested by Wellemans (1977). However, no viral antigens were found using immunofluorescent staining, a test which should not be affected by the presence of antibody as only intracellular antigens are detected. It is more likely, therefore, that the virus had been eliminated by this time.

The lesions present at day 20 could not be specifically attributed to RS viral infection. There were a number of syncytia present in the alveoli but these did not contain RS viral antigens. Mohanty and others (1975) considered multinucleated giant cells to be "characteristic of RS viral multiplication". However, these authors did not apparently consider that syncytia can be derived from different cell types, e.g. epithelia and macrophages, and may be present in different pneumonias e.g. parasitic bronchitis (Jarrett and others, 1957b), PI3 viral pneumonia (Omar and others, 1966).

The clinical response and pathological lesions were no worse in calves EA2 and EA3 than in calves EA4 and EA5. It did not seem, therefore, that damage to the lung caused by D.viviparus significantly potentiated the pathogenic effects of RS virus.

Calf EA1 died very quickly within one hour after being inoculated with RS virus. At post-mortem examination there were two types of lesion present in the lungs. Death was due to severe generalised pulmonary oedema. In addition there was a localised reaction at the site of viral inoculation consisting of severe congestion and oedema, focal intra-alveolar haemorrhage and interstitial emphysema. Emphysema in the bovine probably develops following increased airways resistance (Pirie, 1978). As the

emphysema was localised then it is likely that the obstructive lesion was also localised, in this case to the segment of lung inoculated with virus. In this area there was oedema of the lamina propria of the larger bronchi. There could also have been bronchospasm prior to death but this would not be detected post-mortem due to relaxation of the bronchial musculature. The combination of thickening of the lamina propria with oedema fluid and bronchospasm could have caused sufficient obstruction to air flow in the bronchi of this segment to lead to the development of overinflation and emphysema. The generalised pulmonary oedema is more likely to have been caused by a systemic anaphyllactic reaction (Aitken and Sandford, 1969). Both the localised and the generalised reactions could possibly have been caused by an absorbed antigen or antigens from the infected segment. Whether these were viral antigens or tissue culture medium constituents could not be stated with certainty but the two calves (EA4 and EA5) which received tissue culture medium alone did not react in this way. Prior to inoculation calf EA1 had serum antibody to RS virus. This could have been residual maternal antibody or conceivably could have resulted from a previous RS viral infection which might have sensitised the animal to further exposure to RS virus. It is interesting to note that some calves dying of natural RS viral infection (Holzhauer, 1978; Pirie and others,1981a) have some of the features present in this experimental calf, namely emphysema, oedema and a bronchial lesion.

EXPERIMENT B

## Introduction

The object of this experiment was to see whether calves in which there was pre-existing lung damage were more susceptible to the pathogenic effects of RS viral infection. Pulmonary damage was induced by infecting the calves with D.viviparus. When the lesions had reached maximum severity RS virus was administered by repeated intra-nasal and intra-tracheal inoculations. This was thought to reflect natural exposure more accurately than a single large inoculum.

## EXPERIMENTAL DESIGN

### Experimental Animals

Six, four months old calves (EB1 to EB6) were used in this experiment. All had been housed since birth and none had been vaccinated against parasitic bronchitis. Their reciprocal antibody titres to RS virus were less than 500 prior to viral inoculation.

### Experimental Protocol

The calves were divided into three groups (Groups 1, 2 and 3). Groups 1 and 2 were housed together. The day on which viral inoculations commenced was considered day 0 and all timings are given relative to this date. All calves were inoculated twice daily on three successive days.

Group 1 (calves EB1 and EB2): these were inoculated with $3.10^3$ D.viviparus 36 days prior to day 0. They also received RS virus.

Group 2 (calves EB3 and EB4): these received RS virus only.

Group 3 (calves EB5 and EB6): these received tissue culture medium from FBK cells prepared and administered in the same way as the viral inoculum.

## Viral inoculum

The Compton strain of bovine RS virus was used as prepared for experiment A. Twice daily on three successive days Groups 1 and 2 received 10ml viral inoculum intra-nasally and 10ml intra-tracheally. The total amount of virus given was $10^{7.25}$ $TCID_{50}$.

## Parasite inoculum

$3.10^3$ *D.viviparus* L3 larvae, prepared as for experiment A.

## Sampling of Calves

Nasopharyngeal swabs for viral isolation and blood samples for serum were taken from each calf prior to viral inoculation on day 0. Thereafter nasopharyngeal swabs were taken on days 4, 6, 8, 12, 15 and 19. The calves were slaughtered on day 21. Blood samples for serum and tissues for pathological and microbiological examination were taken at this time.

# RESULTS

## Clinical Findings

Group 1: Prior to being inoculated with *D.viviparus* their respiratory rates and rectal temperatures were within the normal range. Seven days after inoculation with *D.viviparus* their respiratory rates began to rise until, at the time of inoculation with RS virus, the calves were markedly tachypnoeic (EB1 78/min and EB2 84/min) hyperpnoeic and were coughing frequently. They were only slightly pyrexic (103.5°F). After being given RS virus their respiratory rates stayed fairly constant. Nine days after

infection with RS virus calf EB1 became dyspnoeic and died.

Group 2: The respiratory rates and rectal temperatures of these calves stayed within the normal range throughout the experiment. Both calves developed a slight serous nasal discharge 3 days after infection with RS virus.

Group 3: These calves remained clinically normal throughout the experiment.

Pathological Findings

Group 1

The gross and microscopic findings in these two calves were similar although more extensive in calf EB1, which died as a result of respiratory disease.

Gross: There was consolidation in the caudal lung lobes and also in the cranial and the middle lobes. Consolidated areas were slightly collapsed and firm to cut. Adjacent normal lobules were slightly overinflated and there was gas in the interlobular septae. On cross section mucopus exuded from cut bronchi and large numbers of mature D.viviparus were present in the lobar bronchi.

Microscopic: There was alveolar collapse and congestion with foci of neutrophils, eosinophils and macrophages present in alveolar spaces. Macrophage-type syncytia were present in some alveolar areas. The alveolar epithelium was markedly hyperplastic, especially in calf EB1. No epithelial syncytia or inclusion bodies were seen in either animal. There was a marked bronchiolitis with inflammatory cells plugging the lumen and neutrophils were present between epithelial cells. The epithelium was dysplastic and focally necrotic. There was a mixture of eosinophils, lymphocytes and plasma cells in the peribronchiolar tissues. In some areas there were aggregates of lymphocytes in peribronchiolar and in peribronchial tissues. There was a severe bronchitis with

epithelial dysplasia. Neutrophils and eosinophils were present between epithelial cells. The lamina propria was oedematous and contained excess numbers of lymphocytes, plasma cells and eosinophils. Occasional lungworms were present in the bronchi. In the cranioventral parts of the caudal lobes in calf EB1 there were lobular areas of parenchymal necrosis.

The tracheal and nasal epithelia were dysplastic with neutrophils and eosinophils between the cells and excessive numbers of lymphocytes and plasma cells in the lamina propria.

Immunofluorescence: No RS or PI3 antigens were detected in the lungs and trachea of these calves.

Group 2

The changes in the two calves in this group were similar.

Gross: The lungs were slightly over-inflated, expecially in the caudal lobes. In view of the gross appearance a sample of bronchial mucus was taken and was found to contain immature D.viviparus larvae.

Microscopic: There was bronchitis and bronchiolitis with plasma cells and eosinophils around the airways. There were areas of alveolar collapse with congestion and oedema. A few macrophages and eosinophils were present in the alveolar air spaces.

Immunofluorescence: No RS or PI3 viral antigens were detected in the lungs and trachea of these calves.

Group 3

There were no macroscopic or microscopic lesions in the lungs of these animals and no RS or PI3 viral antigens were detected by immunofluorescence.

## Microbiological Findings

Respiratory syncytial virus was isolated from nasopharyngeal swabs as indicated in Table 25. The virus was also recovered from the trachea, but not from other tissues, of calf EB1.

## Serological Findings

The neutralisation titre to RS virus in paired sera from these calves is presented in Table 26. Calves EB2 and EB4 seroconverted to RS virus. In calf EB3 the titre of antibody rose but not significantly.

Table 25: The isolation of respiratory syncytial virus from masopharyngeal swabs from the calves in Experiment B

| Calf Number | Day of Experiment 0 | 4 | 6 | 8 | 12 | 15 | 19 |
|---|---|---|---|---|---|---|---|
| EB1 | - | + | + | + | - | - | - |
| EB2 | - | + | + | + | - | - | - |
| EB3 | - | + | + | + | - | - | - |
| EB4 | - | + | + | + | - | - | - |
| EB5 | - | - | - | - | - | - | - |
| EB6 | - | - | - | - | - | - | - |

+ RS virus isolated

- RS virus not islolated

Table 26: Neutralisation titre to respiratory syncytial virus in paired sera from the calves in Experiment B

| Calf Number | Day of Experiment 0 | 21 | CF |
|---|---|---|---|
| EB1 | 320 | NS | - |
| EB2 | 140 | 680 | 4.85 |
| EB3 | 250 | 950 | 3.80 |
| EB4 | 180 | 800 | 4.44 |
| EB5 | 150 | 100 | 0.66 |
| EB6 | 400 | 120 | 0.30 |

CF = Conversion Factor

NS = No sample

## Discussion

All calves challenged with RS virus were successfully infected. Virus was present in nasal secretions for at least eight days after infection and the calves responded serologically. These findings are similar to those in experimental infections by other authors in which a single inoculum was given (Jacobs and Edington, 1975). Thus repreated inoculations did not seem to prolong the period of viral shedding significantly. On day 21 no virus could be detected by either isolation or immunofluorescence in the lungs of three successfully infected calves. This is in contrast to the findings of Mohanty and others (1975).

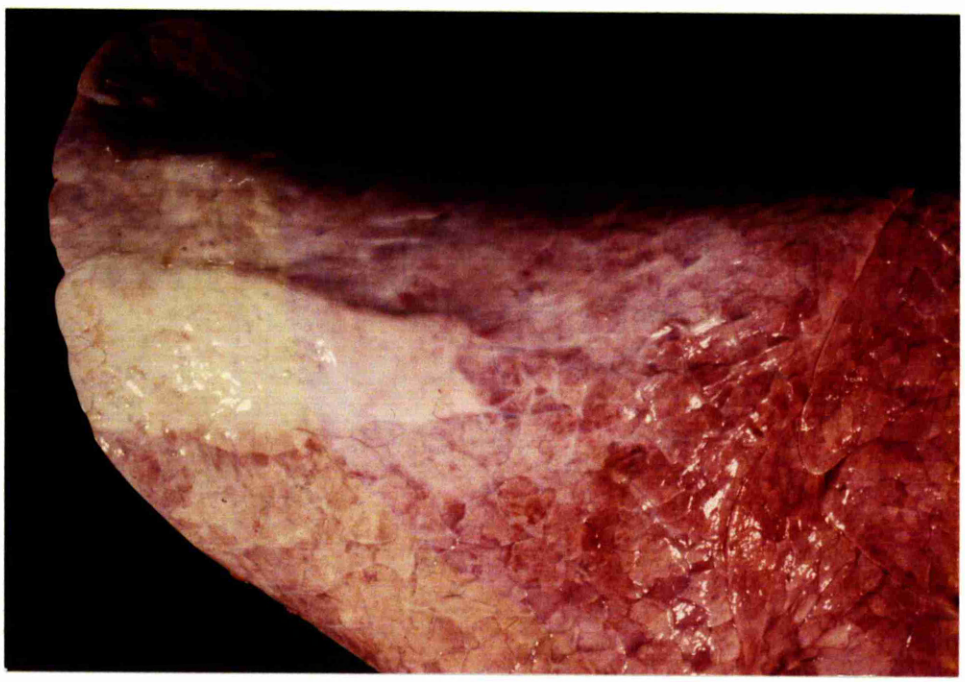
Severe clinical disease was produced only in those calves given D.viviparus and RS virus. One calf (EB1) died nine days after initial viral infection and virus was isolated from the trachea at post-mortem examination. There was no specific evidence of viral infection of the lung and the pathological lesions present were those usually associated with D.viviparus infection. Certain features, however, could also have been due to viral infection e.g. alveolar epithelial hyperplasia, bronchiolar epithelial damage, and interstitial emphysema (Omar, 1964; Holzhauer and van Nieuwstadt, 1976). This highlights the difficulties in inferring aetiology from the morphological appearance of a lesion. Calf EB1 had a severe lungworm infection. In the absence of specific evidence of RS viral infection in the lung it was not possible to say whether the cause of death was D.viviparus infection alone or was a result of synergism between the two organisms.

The calves in Group 2 did not become clinically ill despite being infected with RS virus and there were no specific viral lesions at slaughter on day 21. There were, however, lesions suggestive of D.viviparus infection and this was confirmed by examination of bronchial mucus. This was unexpected but presumably was due to the ingestion of bedding or hay which had been contaminated by the Group 1 calves.

RS VIRAL INFECTIONS

FIGURE 24

FIG. 24: The caudal lobe of the right lung of calf EA1 one hour after being inoculated endobronchially with RS virus. Note the segmental area of interstitial emphysema and the pulmonary oedema.



PI3 VIRAL INFECTIONS

EXPERIMENT C

EXPERIMENT D

## EXPERIMENT C

### Introduction

The object of this experiment was to establish an experimental model of PI3 viral infection in the bovine. This was considered essential in order to study the pathogenesis of the disease produced by this virus and to develop and evaluate diagnostic tests which could be applied to natural outbreaks of respiratory disease. A similar method to that described by Bryson and others (1979b) was used to infect experimental calves involving repeated inoculations of PI3 virus, although in this experiment both intra-nasal and intra-tracheal routes were used.

## EXPERIMENTAL DESIGN

### Experimental Animals

Four Friesian cross Holstein male calves (EC1 to EC4) were used in this experiment. By arrangement with the farmer the calves were removed from their dams as soon as possible after birth and a blood sample was taken. They were reared on milk substitute and were kept in isolation from other calves on the farm until approximately two weeks of age. They were then transferred to the Veterinary School where nasopharyngeal swabs for viral isolation and blood samples for serum were taken. On arrival one calf (EC1) was diarrhoeic and it died shortly after admission. Samples were taken from its lungs for pathological and microbiological examinations.

Three calves (EC2, EC3 and EC4) were inoculated with PI3 virus intranasally (10ml) and intra-tracheally (10ml) on five occasions over three days. The day on which the first viral inoculum was given was day 0. Two calves (EC2 and EC3) were slaughtered on day 4 and one (EC4) on day 11.

Viral Inoculum

A strain of PI3 virus, isolated from the lungs of a three months old calf with acute respitratory disease (see Section Three, outbreak H, calf H2) was propagated from a terminal dilution of the original viral isolate. Freshly monolayered FBL cells in 2 litre rolling bottles were inoculated with this virus at a multiplicity of infection of 0.1 $TCID_{50}$/cell. After adsorbing for one hour at 37°C the inoculum was removed and the cell sheet was washed twice with EFC0. Fresh EFC5 (150ml) was added and incubation was continued at 37°C for 24 hours. The medium was then changed and incubation was continued until maximum cpe had developed. The cells were then scraped into the medium and were stored in aliquots at -70°C. A portion was kept for titration and was checked for viral, bacterial and mycoplasmal contamination.

When required aliquots were thawed and were centrifuged at 3000g for 10 minutes at 4°C to remove cell debris. The supernatant was used as inoculum.

The titre of the inoculum was $10^{6.5}TCID_{50}$/ml. The total amount of virus administered was $10^{8.5}TCID_{50}$.

Sampling of Calves

Nasopharyngeal swabs for viral isolation and blood samples for serum were taken from calves EC2, EC3 and EC4 on day 0. Thereafter nasopharyngeal swabs were taken from calf EC4 on days 4,5,7,9 and 11. Blood samples were taken from all calves at

slaughter.

RESULTS

Clinical Findings

Calves EC2 and EC3

In these calves the rectal temperature rose from 101.5°F to 103.5°F by day 3. Both calves coughed occasionally. Respiratory rates increased from approximately 50/min to 72/min. Calf EC3 was diarrhoeic on day 4 and on this day its rectal temperature dropped to 100°F.

Calf EC4

The rectal temperature of this calf rose gradually after viral inoculation and was 104°F on day 9. Over this period its respiratory rate rose from 54 to 78/min and it became hyperpnoeic. On day 4 it developed a serous nasal discharge and on day 6 this became mucopurulent. On days 9 and 10 the calf was inappetant.

Pathological Findings

Calf EC1

This calf died shortly after arrival at the Veterinary School. It was dehydrated and diarrhoeic, this being the cause of death.

Gross: There were areas of consolidation in the cranial lung lobes. These were deep red and firm with a sharply demarcated border.

Microscopic: There was a severe acute exudative pneumonia. The alveolar and bronchiolar epithelia were normal and no intracytoplasmic or intranuclear inclusion bodies were detected.

Immunofluorescence: No PI3 or RS viral antigens were detected in the lungs of this calf.

Calves EC2 and EC3

These calves were slaughtered on day 4. At post mortem examination the pneumonic lesions in these two calves were similar in appearance and were confined to the cranial lobes, the middle lobe, the accessory lobe and the cranial parts of the caudal lobes (Fig. 25).

Gross: There were multifocal, and coalescing sub-lobular and lobular consolidated areas which were deep red in colour and were slightly collapsed with a smooth surface. On cross section they were slightly oedematous. There was little exudate in the airways. The bronchial lymph nodes were slightly enlarged and there was slight excess grey/white mucus in the nasal passages.

Microscopic: The outstanding features were congestion and oedema with epithelial changes. There was a mild rhinitis with focal necrosis of epithelial cells. A few eosinophilic intracytoplasmic inclusion bodies were present in the epithelial cells and neutrophils were present between the cells. The lamina propria was oedematous and contained a few lymphocytes, plasma cells and neutrophils. In the nasopharynx there was ballooning of the epithelial cells and some contained eosinophilic intracytoplasmic inclusion bodies. There was an infiltrate of neutrophils in the epithelium and the lamina propria and a few lymphocytes and plasma cells were present in lamina propria. There was a mild tracheitis with neutrophils present in the lamina propria and between epithelial cells. Many epithelial cells had ballooned, clear cytoplasm and some contained intracytoplasmic inclusion bodies. A few lymphocytes and plasma cells were present in the lamina propria and around the submucosal glands.

In the lung the major lesions were collapse, either partial or total, of some lobules and congestion and oedema in others.

There were marked changes in the epithelum of the bronchi, the bronchioles and the alveoli with hydropic degeneration of the cytoplasm of many cells. Many epithelial cells contained eosinophilic intracytoplasmic inclusion bodies, usually one per cell but occasionally more. These were especially noticeable in the bronchiolar epithelium (Fig. 27). They were generally round or slightly oval and present in the luminal cytoplasm. Occasional irregularly shaped inclusions were noted. In some bronchioles nearly every cell contained an inclusion body. No intranuclear inclusions were seen. A few syncytia, usually with three or four nuclei but occasionally as many as eight, were formed in the alveolar and the bronchiolar epithelium. These were not, however, a marked feature of the lesions. A few necrotic syncytia were seen in the alveolar and the bronchiolar lumena.

In addition to these epithelial features a few lymphocytes and plasma cells were present in the interstitium of some lobules. In others there was thickening of the intralveolar septae.

Immunofluorescence: Parainfluenza type 3 viral antigens were detected in epithelial cells in the nasal mucosa, the nasopharynx, the trachea, the bronchi, the bronchioles and the alveoli. No viral antigens were detected in the bronchial lymph node.

In the nasal mucosa antigen was present in the lumenal cytoplasm of single epithelial cells and occasionally in groups of adjacent cells. In the nasopharynx and trachea the distribution of antigens was similar to that in the nasal mucosa. In the bronchi relatively few epithelial cells were infected. In the bronchioles and the alveoli there was a patchy distribution of viral antigens. In some areas virtually all epithelial cells at both sites were infected whereas in other areas only a few cells were infected (Figs. 28, 29 and 30). Antigens were seldom seen in cells free in alveolar or bronchiolar lumina. They were never seen in cells in the interlobular septae or blood vessels.

Cells with positive staining had both diffuse and granular intracytoplasmic fluorescence. Antigen granules varied markedly in size. Some of the larger ones corresponded to eosinophilic intracytoplasmic inclusion bodies as seen by staining with H&E.

Calf EC4

This calf was slaughtered on day 11.

Gross: There were pneumonic lesions in the cranial, the middle, the accessory and the cranio-ventral parts of the caudal lobes of the lung. The lesions were dark purple and slightly collapsed.

Microscopic: There was dysplasia of the epithelium in the nasal mucosa, nasopharynx and trachea. No inclusion bodies were noted in epithelial cells. Neutrophils were present between epithelial cells and in the lamina propria. A few plasma cells and lymphocytes were present in the lamina propria.

The pulmonary lesions were similar in all lobes. There was collapse of the alveoli and foci of alveolar epithelial hyperplasia. In some areas there were focal infiltrates of neutrophils and macrophages. The walls of the bronchioles were thickened with proliferation of fibroblasts and infiltration with plasma cells. The epithelium was dysplastic and a few neutrophils were present between the cells. In some lobules there was early bronchiolitis obliterans. In some bronchi the epithelium was dysplastic and neutrophils were present between the cells; in others it was normal. Occasionally the lamina propria was slightly thickened in some intrapulmonary bronchi and contained plasma cells and lymphocytes. No inclusion bodies or syncytia were seen in the epithelia of the bronchi, the bronchioles or the alveoli.

Immunofluorescence: The tissues from this calf were all negative for PI3 viral antigens.

## Microbiological Findings

The results of microbiological investigations on tissues from calves EC1, EC2, EC3 and EC4 are presented in Table 27. Nasopharyngeal swabs were taken from calf EC4 up until day 11. Parainfluenza type 3 virus was isolated on days 4,5,7 and 9.

## Serological Findings

One day after birth calves EC3 and EC4 had serum HAI antibody titres to PI3 virus of 40. In calf EC2 the titre was 20. In all animals on day 0 of the experiment the titre was 20 and this did not alter in the two calves (EC2 and EC3) killed on day 4. In calf EC4 the titre rose to 80 by day 11.

TABLE 27: The pathogenic viruses, bacteria and mycoplasmas detected in the respiratory tract tissues from the calves in Experiment C.

| Calf Number | Tissue | Viruses | Bacteria | Mycoplasmas |
|---|---|---|---|---|
| EC1 | NM | Negative | P.multocida | Negative |
| died before | TM | Negative | P.multocida | M.bovis |
| experiment | L | Negative | P.multocida | Negative |
| began | | | | |
| EC2 | NM | PI3 virus | Negative | Negative |
| DAY 4 | TM | PI3 virus | Negative | Negative |
| | L | PI3 virus | Negative | Negative |
| EC3 | NM | PI3 virus | Negative | Negative |
| DAY 4 | TM | PI3 virus | Negative | Negative |
| | L | PI3 virus | Negative | Negative |
| EC4 | NM | Negative | Negative | Negative |
| DAY 11 | TM | Negative | Negative | M.dispar |
| | L | Negative | Negative | M.dispar |

NM = Nasal Mucosa
TM = Tracheal Mucosa
L = Lung

## Discussion

Three, two weeks old calves were successfully infected with PI3 virus and all developed clinical signs of respiratory disease and lesions in their lungs.

In calves EC2 and EC3, which were killed on day 4, PI3 virus was isolated from the lungs at post-mortem and by using immunofluorescencent staining was shown to be localised to the epithelia of the pneumonic areas. No other recognised respiratory pathogens were isolated from the lungs at this time. The evidence shows that the pneumonia was caused by PI3 virus and confirms the experimental results of others (Omar and others, 1966; Bryson and others, 1979b).

The histopathological features of the pneumonia in calves EC2 and EC3 were similar. Many epithelial cells contained eosinophilic intracytoplasmic inclusion bodies but no intranuclear inclusions were seen. Epithelial syncytia with between two and eight nuclei were present but were not numerous. Some syncytia had detached and were lying free in the lumen of alveoli or bronchioles. These findings were similar to those in a naturally infected three weeks old calf (case RC17, Section Three) showing that this experimental model is representative of at least some natural PI3 viral infections.

The detection of viral antigens by immunofluorescence was a more sensitive indication of viral infection than the presence of cellular changes seen by staining with H&E. Antigens were detected in epithelial cells and also in cells free in the lumen which were either detached epithelial cells or alveolar macrophages. No alveolar septal cells were seen to contain antigens. This is in contrast to the findings of van der Maaten (1969) who detected PI3 viral antigens in the cells in the alveolar septae. Some of the intracytoplasmic inclusion bodies seen by H&E staining also stained by immunofluorescence. The relatively few epithelial syncytia seen was surprising in view of the quantity of

viral antigen which was present.

The conditions under which paramyxoviruses induce epithelial cells to form syncytia *in vivo* are not known. In tissue culture syncytia form by fusion of cells, rather than by disordered proliferation,and this is a function of a specific viral glycoprotein (Sheid and Choppin, 1974). This property is thought to be important in the pathogenesis of disease caused by this and related viruses. In general where fusion is more marked, disease is more severe (Merz and others, 1980). Whether or not different strains of PI3 virus vary in their capacity to induce fusion *in vivo* is not known. The relative lack of syncytia at day 4 also could be due to the stage of infection. Omar and others (1966) found syncytia present at day 5 but not at day 7. However Bryson and others (1979b) killed calves serially over 21 days and still concluded that syncytium formation was not a marked feature of experimental PI3 viral infection.

In the calf killed on day 11 there was no specific evidence of viral infection. No virus was isolated from the lungs, no syncytia or inclusion bodies were present in the respiratory epithelia and no viral antigens were detected by immunofluorescence. The calf had, however, been infected as virus was isolated from nasopharyngeal swabs taken up to day 7 and there was a significant rise in the level of serum HAI antibody to the virus. The lesions were similar in distribution and extent to those in calves EC2 and EC3 although the microscopic appearance was different. The striking feature was a diffuse cellular thickening around the bronchioles with proliferation of fibroblasts, and infiltration with lymphocytic cells, principally plasma cells. A similar lesion was present in the calves in the outbreak of respiratory disease from which the strain of PI3 virus was recovered (Outbeak H, calves H2 to H4). In this outbreak no viral antigens were detected in the peribronchiolar tissues and the lesion occurred both in the presence and in the absence of the bronchiolar epithelial cells being infected with PI3 virus. Other micro-organisms were also present in the lungs with this lesion.

There were no consistent bacteria present but M.dispar was isolated from calf EC4 and Ureaplasma sp. from the calves in outbreak H. Whether this lesion reflects PI3 viral damage per se or is due to its interaction with another organism is not clear. The end results of this lesion could be peribronchiolar fibrosis and, if the muscle layer was significantly damaged, bronchiolectasis.

Omar and others (1966) found that virus was present in the lung at day 5 but not at day 7. In their experiments Bryson and others (1979b) found virus present as late as day 12, although two calves killed prior to this (on days 8 and 10) were negative. This highlights a potential difficulty in the investigation of outbreaks as calves with pneumonia due to PI3 virus may have no specific evidence of viral infection if examined later in the course of the disease. This would make the pathological changes difficult to interpret in other than general terms.

Plasma cells and lymphocytes were present in the lesions at day 4 and the former cell type was numerous at day 11. By day 11 calf EC4 had seroconverted to PI3 virus. The presence of antibody may have interfered with attempts to isolate virus from lung tissue as has been suggested for RS virus (Wellemans, 1977). However, no viral antigens were detected by immunofluorescence at this stage suggesting that virus had been completely eliminated.

EXPERIMENT D

## Introduction

After successfully infecting and producing respiratory disease in two-weeks old calves with PI3 virus it was decided to try a similar experiment in four months old calves. This age group represented more closely the calves involved in the outbreak of respiratory disease from which the strain of PI3 virus was originally recovered. In-contact control calves were included to determine whether infection could spread naturally from experimentally infected calves.

## EXPERIMENTAL DESIGN

### Experimental Animals

Eight, four months old calves were used in this experiment. They were selected on the basis of having reciprocal serum HAI antibody titres to PI3 virus of 20 or less. All came from farms with no history of severe respiratory disease and none had been vaccinated against respiratory pathogens. The calves were randomised into three groups. Group 1 consisted of four calves (ED1 to ED4), Group 2 of two calves (ED5 and ED6) and Group 3 of two calves (ED7 and ED8).

### Experimental Protocol

The calves in Group 1 were inoculated with PI3 virus intra-nasally (10ml) and intra-tracheally, (10ml) on five occasions over three days. The day on which the first viral inoculum was given was day 0. Two calves (ED1 and ED2) were slaughtered on day 4 and two (ED3 and ED4) on day 11.

The calves in Group 2 were given no inoculations and were put in with the Group 1 calves on day 3. Both calves were slaughtered on day 15.

The calves in Group 3 were inoculated with tissue culture medium intra-nasally (10ml) and intra-tracheally (10ml) on five occasions over three days. One calf (ED7) was slaughtered on day 4 and one (ED8) on day 11.

## Viral Inoculum

The viral inoculum was prepared from the same strain of PI3 virus and in the same manner as that used in Experiment C. The titre of the inoculum was $10^{6.5}$ $TCID_{50}$/ml. The total amount of virus administered was $10^{8.5}$ $TCID_{50}$.

The tissue culture medium given to Group 3 calves was prepared from uninfected FBL cells in the same way as the viral inoculum.

## Sampling of Calves

Nasopharyngeal swabs for viral isolation and blood samples for serum were taken from all calves prior to inoculation on day 0. Thereafter on days 4,7,9,11 and 14, nasopharyngeal swabs were taken from surviving calves. Blood samples for serum and tissues for microbiological and pathological investigation were taken at slaughter.

# RESULTS

## Clinical Findings

### Group 1

All calves developed a moderate pyrexia (104°F) by day 3. There was a serous nasal discharge, which became mucopurulent in calves ED3 and ED4. All calves became tachypnoeic, slightly hyperpnoeic and coughed increasingly more frequently as the experiment progressed.

Group 2

The rectal temperatures of both calves rose gradually from day 4 and were 103.8°F on day 9. Both calves became hyperpnoeic and tachypnoeic, developed a slight mucopurulent nasal discharge and began to cough.

Group 3

Calf ED7 remained clinically normal throughout the experiment. Calf ED8 had signs of respiratory disease before the experiment began. It coughed occasionally and had harsh respiratory sounds cranioventrally on the right side of the chest. Its temperature was normal at the beginning of the experiment but rose to 104°F on day 4.

Pathological Findings

Group 1

Gross: The gross lesions in this group were similar. In all animals there were lobular and sublobular patches of consolidation whch were either slightly raised (calves ED1 and ED2; Fig. 26) or slightly collapsed (calves ED3 and ED4) in relation to the surface of the normal surrounding lung. The surface of the lesion was slightly mottled in calf ED4. The extent of these lesions varied between the calves but was always confined to the ventral parts of the cranial lobes, the middle lobe and the cranial parts of the caudal lobes. On cross section the lesions were slightly oedematous and small quantities of mucopus could be expressed from the bronchi. The tracheal and the nasal mucosae were slightly congested and there was a slight excess of grey mucopus on their surfaces.

In addition to these features there were other, morphologically distinct, lesions in calf ED1. The cranial part of the right cranial lobe was consolidated and firm with a pale

knobbly surface. On cross section the large and small bronchi were excessively prominent and there were small spots on the surface of the lung lobules. Excess mucopus exuded from cut bronchi.

Microscopic: The lesions of calves ED1 and ED2 (killed on day 4 ) were distinct from those in calves ED3 and ED4 (killed on day 11). They were, in each case, similar to the lesions seen in the calves killed at equivalent times after viral inoculation in Experiment C. Briefly, in calves ED1 and ED2 there was bronchitis, bronchiolitis and alveolitis, with epithelial damage at each site. Inclusion bodies were present in single epithelial cells and in occasional syncytia. There was collapse, congestion and oedema of alveoli with focal infiltration by neutrophils and macrophages. A few plasma cells and lymphocytes were seen in alveolar septae and in the peribronchiolar tissues.

In calves ED3 and ED4 there was bronchiolar epithelial dysplasia and hyperplasia and focal alveolar epithelial hyperplasia. Plasma cell and lymphocyte infiltrates around the bronchioles were more marked and there was thickening of the lamina propria due to fibroblast proliferation and plasma cell infiltration. In some sections there was early bronchiolitis obliterans. No syncytia or inclusion bodies were seen.

The morphologically distinct lesion in the right cranial lobe of calf ED1 was characterised by alveolar collapse and infiltration with neutrophils. Lymphocytic infiltration around the bronchi and the bronchioles was marked, often forming "cuffs" with germinal centres. The epithelia of bronchi and bronchioles were substantially intact but slightly hyperplastic. In some lobules there was bronchiolitis obliterans.

Immunofluorescence: Parainfluenza type 3 viral antigens were detected in the bronchial, the bronchiolar and the alveolar epithelial cells in calves ED1 and ED2. In calf ED1 no viral antigens were detected in tissue from the lesions in the cranial part of the right cranial lobe. The lesions in this area were

morphologically distinct from the other lesions in the lungs. Viral antigens were not detected in calves ED3 and ED4.

Group 2

In the lungs of calf ED5 were a few lobular and sublobular areas of collapse. In these areas there was a mild bronchitis and bronchiolitis with increased numbers of plasma cells and lymphocytes. There was alveolar collapse with occasional foci of macrophages and neutrophils. No syncytia or inclusion bodies were seen.

The lungs of calf ED6 were macroscopically normal. Microscopically there were occasional aggregates of lymphoid cells adjacent to the bronchi and the bronchioles. No syncytia or inclusion bodies were seen.

Immunofluorescence: No viral antigens were detected.

Group 3

The lungs of calf ED7 were normal. Calf ED8 had patches of consolidation in the cranial and middle lobes and mucopus exuded from the cut bronchi. Histologically there was alveolar collapse and infiltration with neutrophils and macrophages. The lamina of the bronchioles and the bronchi were obstructed with degenerating inflammatory cells but the epithelium was intact. Aggregates of lymphocytes were present around the bronchioles and the bronchi and occasionally these contained germinal centres. No inclusion bodies or viral syncytia were seen.

Immunofluorescence: No viral antigens were detected.

Microbiological Findings

Prior to being divided into groups for the experiment the

calves were in direct contact with each other. During this time nasopharyngeal swabs, taken from all the calves yielded only one isolation of a pathogenic micro-organism, Mycoplasma dispar, which was isolated from calf ED1 10 days prior to day 0. The results of the isolation of PI3 virus from nasopharyngeal swabs taken after 0 are presented in Table 28.

No pathogenic bacteria or mycoplasmas were isolated from the nasopharyngeal swabs taken after day 0. The micro-organisms detected in the respiratory tract tissues of the calves were:-

Group 1

Parainfluenza type 3 virus was isolated from the upper and lower respiratory tract of calf ED1 and from the lower respiratory tract of calf ED2. Mycoplasma dispar and M.bovis were isolated from the upper and from the lower respiratory tract of calf ED2.

No pathogenic micro-organisms were recovered from calf ED3 but P.multocida and M.dispar were recovered from the lower respiratory tract of calf ED4.

Group 2

No viruses or pathogenic bacteria were recovered from the respiratory tracts of these two calves. Mycoplasma dispar was isolated from the lower respiratory tract of calf ED6.

Group 3

No viruses or pathogenic bacteria were recovered from the respiratory tracts of these two calves but, in both cases, M.dispar was isolated from the trachea.

Serological Findings

The HAI titre to PI3 virus in sera from the calves are presented in Table 29. Calves ED3 and ED4 seroconverted to PI3 virus.

TABLE 28: The isolation of parainfluenza type 3 virus from the nasopharyngeal swabs taken from the calves in Experiment D.

| Calf Number | Group Number | Day of Experiment 0 | 4 | 7 | 9 | 11 | 13 |
|---|---|---|---|---|---|---|---|
| ED1 | 1 | - | | | | | |
| ED2 | 1 | - | | | | | |
| ED3 | 1 | - | + | + | - | | |
| ED4 | 1 | - | + | + | + | | |
| ED5 | 2 | - | + | + | - | - | - |
| ED6 | 2 | - | + | + | - | - | - |
| ED7 | 3 | - | - | - | - | | |
| ED8 | 3 | - | | | | | |

TABLE 29: Haemagglutination-inhibition titre to parainfluenza type 3 virus in sera from the calves in Experiment D.

| Calf Number | Group Number | Day of experiment 0 | 4 | 11 | 15 |
|---|---|---|---|---|---|
| ED1 | 1 | 40 | 40 | | |
| ED2 | 1 | 20 | 40 | | |
| ED3 | 1 | 40 | 40 | 160 | |
| ED4 | 1 | 20 | 40 | 160 | |
| ED5 | 2 | 40 | 40 | | 80 |
| ED6 | 2 | 40 | 40 | | 40 |
| ED7 | 3 | 20 | 10 | | |
| ED8 | 3 | 20 | 20 | | |

Discussion

All the calves which were challenged with PI3 virus were successfully infected as judged by viral isolation from nasopharyngeal swabs and lung tissue or by serology. The calves developed clinical signs of respiratory disease and lesions were produced in the lungs. The lesions produced were similar in type to those in experiment C but were generally less extensive. The amount of virus administered in the two experiments was similar. In this experiment older calves were used. They were selected on the basis of having low levels of serum HAI antibody to PI3 virus. By using this criterion it was hoped that calves which had been infected with PI3 virus could be avoided and colostral antibody would have declined to insignificant levels. However, following natural PI3 viral infections Thomas (1973) found that antibody titres declined rapidly and therefore a low titre in an animal which is four months old is not necessarily indicative of it never having been exposed to the virus. This could have important implications in the selection of experimental animals, particularly those representative of the age range in which many respiratory disease outbreaks occur.

In calf ED2 virus was detected in the middle and the caudal lobes by immunofluorescence but was not isolated from these sites. Van der Maaten (1969) found viral isolation to be more sensitive than immunofluorescence for the detection of PI3 virus in the lungs of experimentally infected calves. Several factors could be important here. For viral isolation a larger amount of lung tissue is sampled than for immunofluorescence and this should increase the chances of detecting virus, especially when only small amounts are present. Against this, however, viral infectivity could be blocked if there is neutralising antibody present in the serum in the tissue. Immunofluorescent staining avoids this particular problem and the size of the sample can be increased by examining multiple sections of lung. In this experiment the principal aim of using the immunofluorescence staining technique was to relate the presence of virus directly to the histopathological changes which

it produced at a cellular level. This is not possible using viral isolation alone.

Viral infection was transmitted from the experimentally infected calves to the two calves (ED5 and ED6) in direct contact with them. Their nasopharyngeal secretions contained virus by as early as 27 hours after the initial exposure. Mild clinical signs of respiratory disease were associated with viral shedding and at post-mortem small lesions were present in the lungs of these calves. Virus was not isolated from the lungs at post-mortem nor were viral antigens demonstrated in the lesions, however, as the calves were killed 11 days after their initial exposure to virus this was not entirely surprising.

The pulmonary lesions were limited in extent and the clinical signs of respiratory disease were mild. The period of viral shedding was shorter than that in calves ED1 to ED4. This could be explained by the fact that two sources of viral infection were removed from contact with the calves after only 24 hours thus reducing the level of viral challenge. A heavier, more sustained challenge might have increased the severity of the pathological lesions and resulted in more marked clinical disease.

Other respiratory pathogens were isolated from the lungs of these calves e.g. P.multocida. M.dispar and M.bovis. It is therefore possible that synergism between these organisms and PI3 virus could have been responsible for some of the lesions present at post mortem. This highlights the problems of using "conventional" calves in experiments. However, these calves were thought to be more representative of those which are involved in many outbreaks of respiratory disease than either gnotobiotic calves or very young calves. Using the immunofluorescence technique it was possible to relate the presence of viral antigens directly to the pathological changes present in the respiratory tract. This demonstrates that PI3 virus is a pathogen in that it can damage the respiratory epithelium. It does not discount the possibility that other organisms may contribute to the lesions and,

therefore, to the clinical disease. However, comparing this experiment with experiment C where PI3 virus was present in the lesions and respiratory disease was produced in the absence of other pathogens being present, it can be seen that these other organisms are not necessary for the development of lesions.

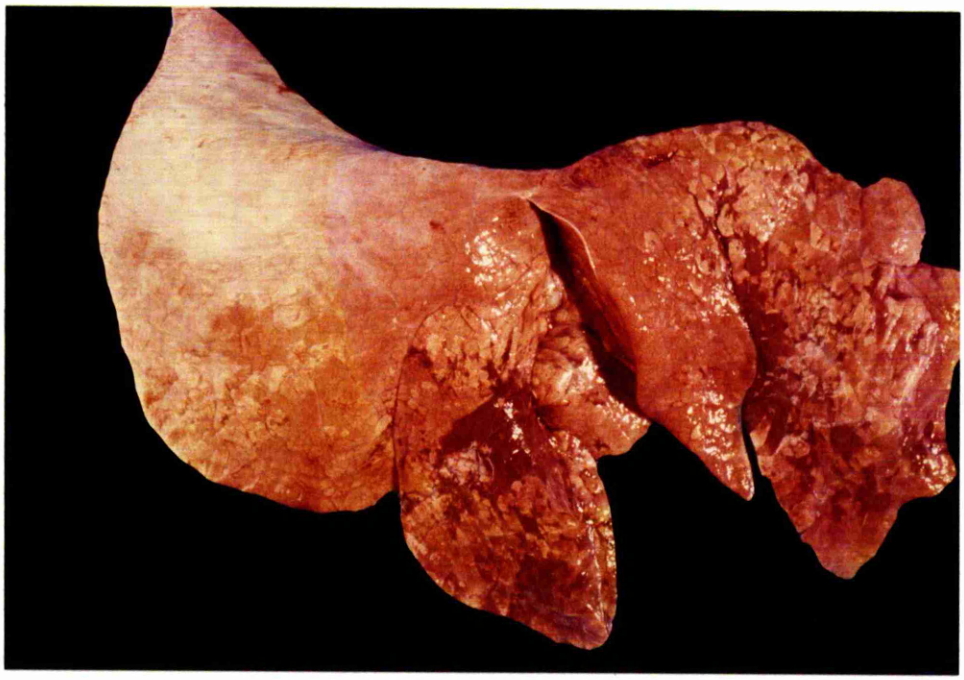
This experiment showed that clinical signs of respiratory disease and pathological lesions can be produced by infecting four months old calves with PI3 virus. Although the disease was not severe it probably represents accurately the majority of PI3 viral infections which occur naturally. Viral infection was transmitted to in-contact calves by natural means and mild clinical signs of respiratory disease resulted.

PI3 VIRAL INFECTIONS

FIGURES

FIG. 25: Experimental PI3 viral infection - the right lung from calf EC2. There are lobular and sub-lobular lesions in the cranial and middle lobes and in the cranial parts of the caudal lobe.

FIG. 26: Experimental PI3 viral infection - detail of pneumonic lesions in calf ED1. The surface of the lesion is smooth and is spotted. Normal or slightly overinflated lobules are present in the pneumonic areas.



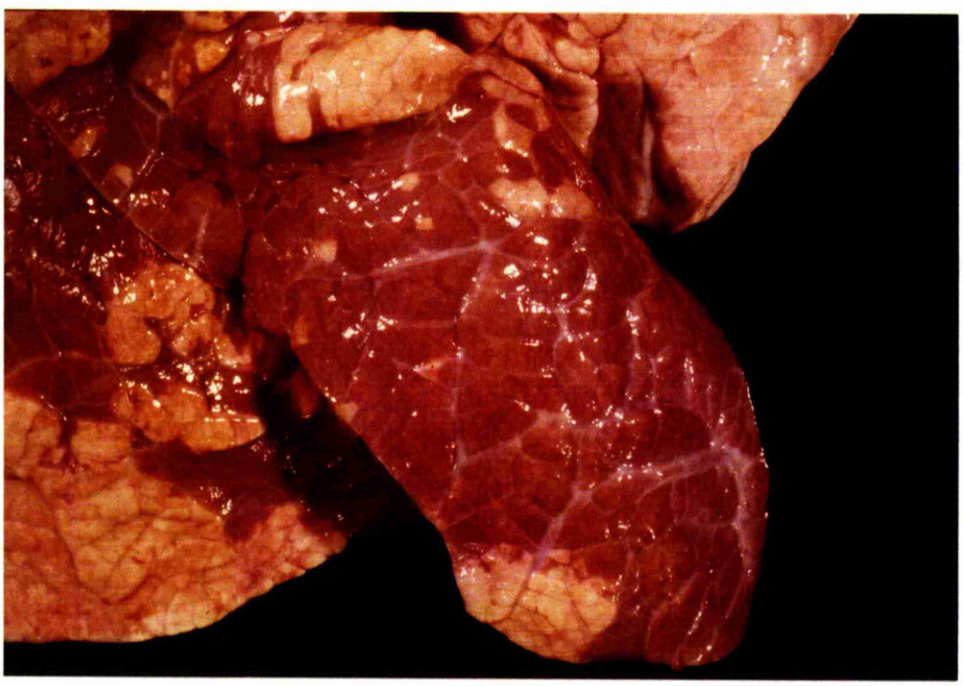
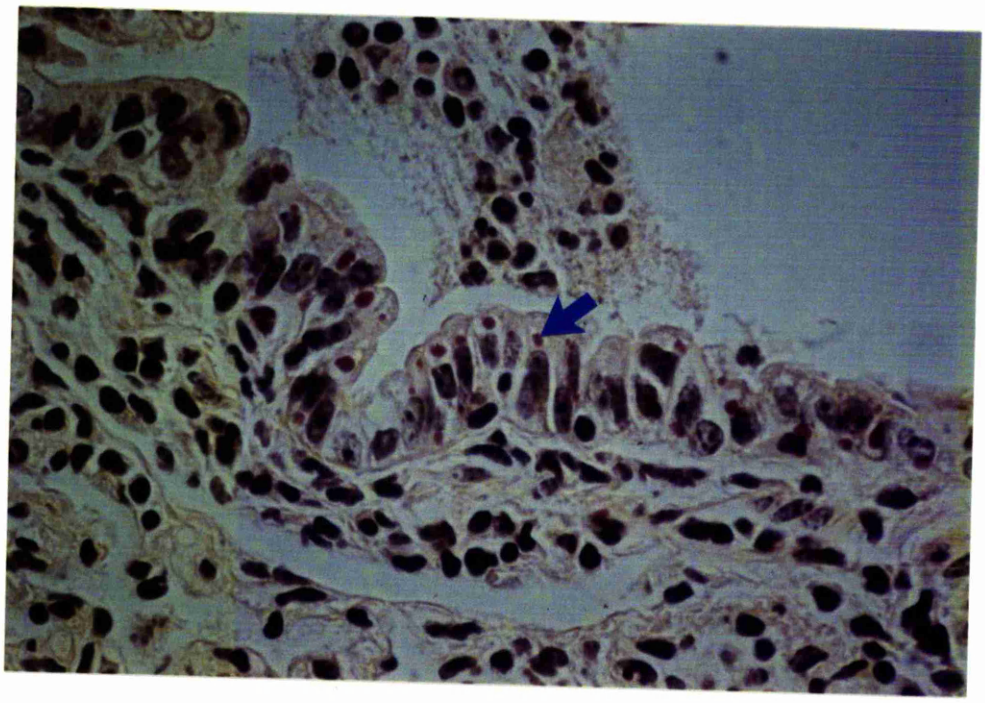


FIG. 27: Bronchiolar epithelium infected with PI3 virus in calf EC2. Most of the epithelial cells contain at least one intracytoplasmic inclusion body (blue arrow). There are no syncytia.

PTI x 400

FIG. 28: Bronchiolar epithelium infected with PI3 virus in calf EC2. Viral antigens are present in all the epithelial cells and the bronchiolar lumen is collapsed.

Acetone-fixed frozen section, FITC x 250



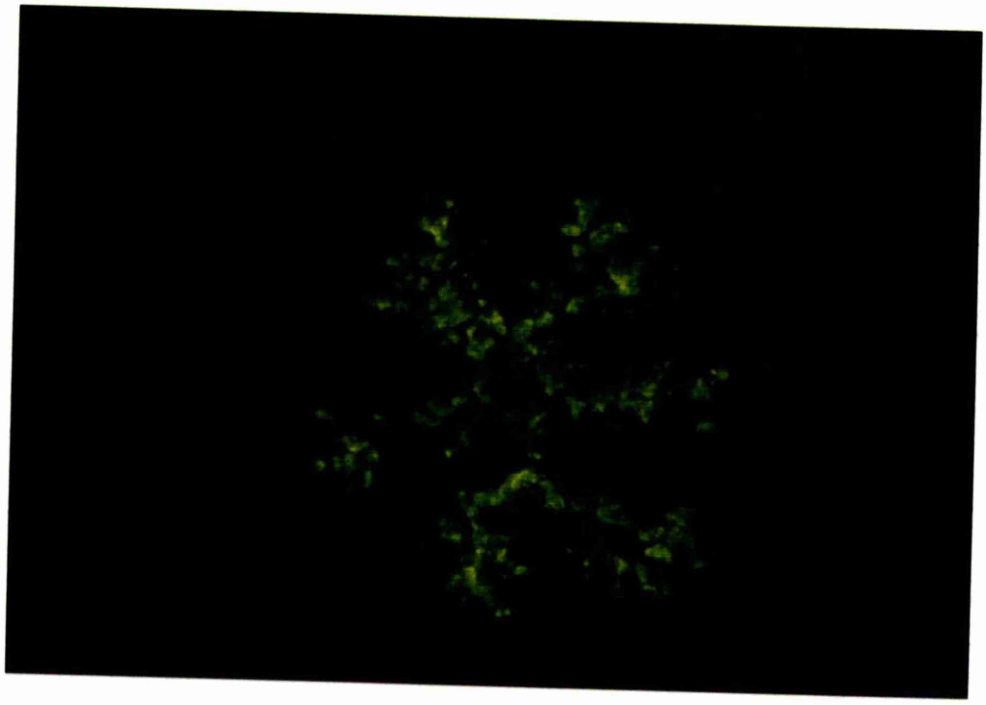
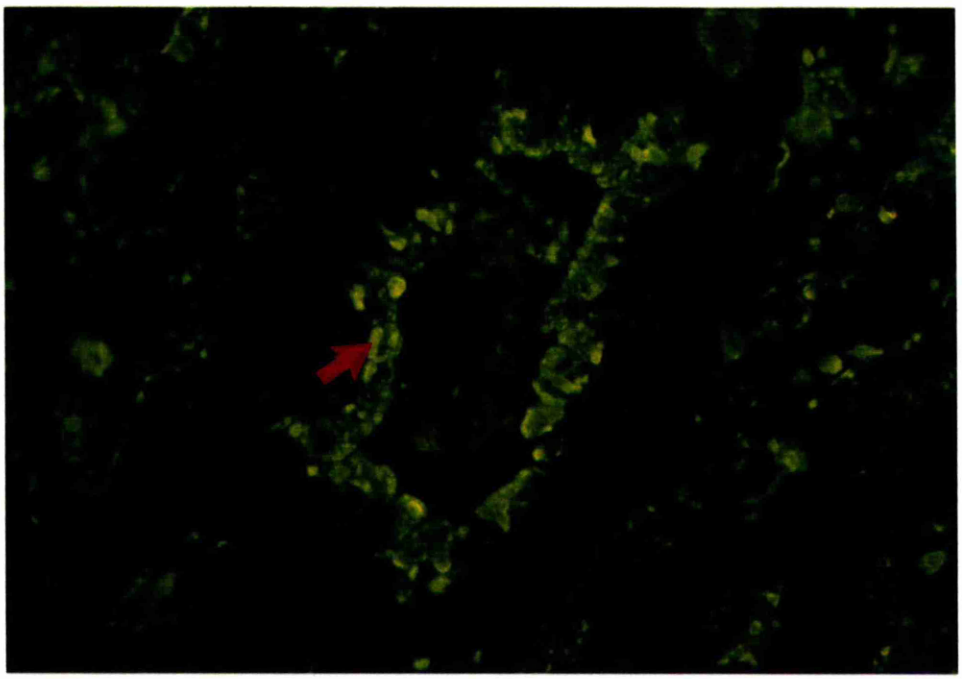


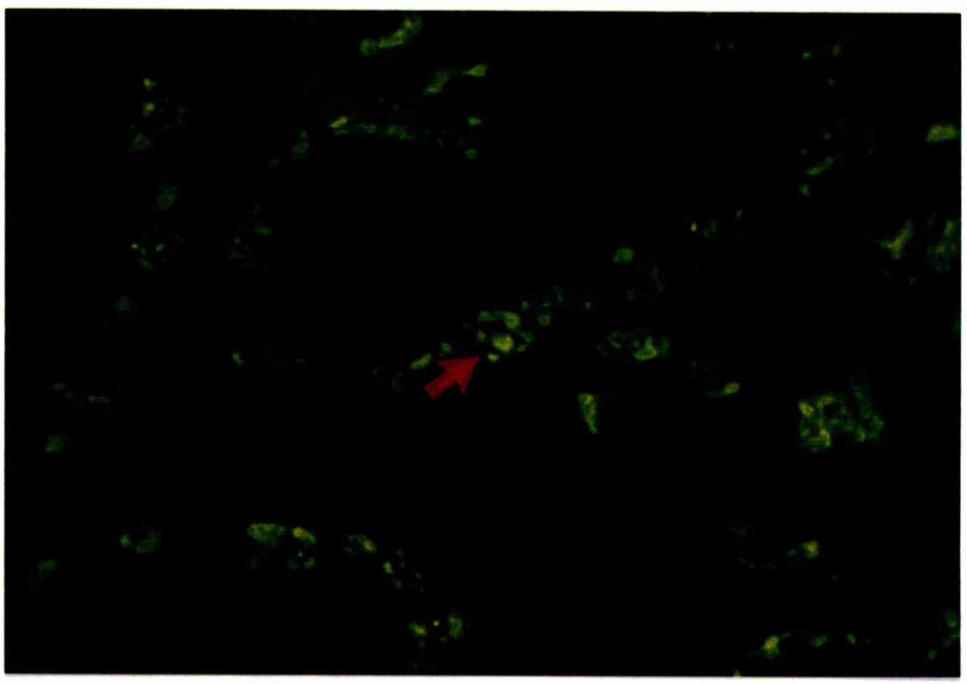
FIG. 29: Bronchiolar and alveolar epithelial cells infected with PI3 virus in calf EC2. Some of the cells contain large granules of antigen (red arrow) which correspond to eosinophilic intracytoplasmic inclusion bodies in sections stained with H&E.

Acetone-fixed frozen section, FITC x 400

FIG. 30: Alveolar epithelium infected with PI3 virus in calf EC2. A small syncytium is present in the centre of the photograph (red arrow)

Acetone-fixed frozen section, FITC x 400





SECTION FIVE

# GENERAL DISCUSSION

# GENERAL DISCUSSION

The aim of this thesis was to investigate the part played by RS virus and PI3 virus in respiratory diseases of cattle with particular reference to the pathogenesis, diagnosis and differential diagnosis of these two infections in naturally occurring outbreaks of disease. These primary aims were to be accompanied by attempts to produce viral infection in experimental calves using isolates of virus obtained from the field investigations. These aims were largely achieved for PI3 virus. It was realised from the outset that RS virus would pose the greater problem and although the field investigations were successful there was, unfortunately, no opportunity to use the isolates of RS virus which they yielded in experimental infections. Nevertheless, experimental calves were successfully infected with a strain of bovine RS virus which was obtained from another laboratory.

Infection with RS virus was detected in four outbreaks of acute respiratory disease and in a total of 21 individual animals. In both the outbreaks and the individual cases there was a temporal association between the presence of viral infection and the development of clinical signs of respiratory disease. More significantly, where animals were available for post-mortem examination, there was a direct association between virus-infected cells and the pathological lesions which were present in the lower respiratory tract of affected animals. These were present in areas of acute inflammation, particularly in those areas in which the inflammatory process was in its earliest stages. Specific cellular changes, due to viral infection, were present in the bronchiolar, the alveolar and the bronchial epithelium. They comprised syncytium formation and cells containing eosinophilic intracytoplasmic inclusion bodies. Infected cells became necrotic and detached from the epithelium.

Superimposed on these changes were the vascular and exudative reactions characteristic of inflammation with congested blood vessels and oedema fluid and increased numbers of neutrophils and macrophages in the alveolar and bronchiolar lumina. In other areas of the same lung, or even the same lobe of the lung, severe pathological lesions could be present in which no viral antigens could be detected. However, these areas were in the later stages of inflammation with significant numbers of plasma cells and lymphocytes in the tissue and early evidence of resolution or healing of the lesions. The association of RS virus with the earlier stages of inflammation indicates that it is a primary pathogen. In the majority of cases where material was available for full microbiological examination no other recognised respiratory pathogens were present. This is not to say that other factors could not have contributed to the development and severity of the disease and in some cases other respiratory pathogens e.g. Mycoplasma bovis were present which could have modified the disease process.

There is no evidence as yet that isolates of RS virus vary in their virulence for the bovine respiratory tract (Jacobs and Edington, 1975). However, the susceptibility of individuals or groups of animals and the conditions under which they are exposed to the virus must vary widely. In experimental infections animals challenged with equivalent amounts of virus have shown a range of severities of disease suggesting that, for whatever reasons, individuals vary in their susceptibility to infection. Within groups of animals on farms there may be individuals of dissimilar ages and with different histories of exposure to the various respiratory pathogens, particularly where animals have been brought together from several separate sources. The closer the contact between animals the more opportunities there are for the spread of viruses. The infectivity of the viruses may be better preserved in the environment under certain conditions of temperature and humidity. The combination of these factors i.e. susceptibiltity of the individuals and the group as a whole, and the survival and opportunities for spread of virus within the environment occupied

by the animals, will affect the level of viral challenge. This in turn will determine the way in which the disease develops.

Previous exposure to the virus does not prevent re-infection (Stott and others, 1980). By analogy with the human reinfections can be expected to result in less severe disease than primary infections (Beem and others, 1960). This may, to some extent, account for the variation in severity of disease, particularly when considering older animals which are more likely to have been exposed to the virus before. Reinfections are difficult to specifically identify although a high rate of rise of antibody may give an indication that this has occurred. Susceptibility to RS virus may also be affected by intercurrent infections. Combined experimental infections with BVD virus and RS virus produced more severe disease than with RS virus alone (Thomas and Stott, 1977) although the mechanism involved was not clear nor was its significance in natural disease. In the present investigations there was no significant evidence of BVD viral infection in outbreaks A to D and when combined infection was recorded in a group of animals which were being monitored serologically (Section Three, farm M) there was no evidence of severe respiratory disease.

Concurrent infection in the respiratory tract with other organisms could affect the severity of disease by producing lesions additional to those due to the viral infection. In this respect, although other recognised pathogens were absent from most individual cases of RS viral infection, in four Mycoplasma bovis was present. It is difficult to rule out the possibility of synergism between these two organisms although from histopathological observations the majority of the lesions present could be attributed directly or indirectly to RS viral infection and there was no unequivocal evidence that synergism did occur.

Considering the individual animals in which RS viral infection was detected the most striking histopathological feature was epithelial damage, principally in the bronchioles and the alveoli. This was present in all of the cases in which RS viral

antigens were detected in the lungs by immunofluorescence. In the early stages of infection the epithelial changes consisted of swollen epithelial cells, syncytium formation, individual cells containing intracytoplasmic inclusion bodies and focal necrosis and detachment of cells. At this stage viral antigens were present in the epithelium and therefore the lesions could be specifically recognised as being due to viral infection. Eventually the infected cells were shed and epithelial regeneration began with flattened basophilic cells spreading over the denuded basement membrane. There were often outgrowths of fibroblasts into the lumen and organisation of the exudate to form obliterative plugs which eventually became covered in new epithelium.

These lesions could affect both the bronchiolar and the alveolar epithelium but appeared, in some cases at least, to affect them sequentially with viral spreading peripherally from the bronchioles. Viral antigens could be present either solely in the bronchiolar epithelium or solely in the alveolar epithelium. Similar types of staining have been noted by others (Thomas and Stott, 1981; van den Ingh and others, 1982). By combining the observations in immunofluorescent staining with those of conventional H&E staining it was noted that when there were virus-infected cells only in the alveolar epithelium then the bronchiolar epithelium was abnormal, with a combination of necrosis and dysplasia, indicating prior viral infection.

A reaction in the bronchi also seemed important in the pathogenesis of the severe disease as most of the animals which died were dyspnoeic and had severe interstitial emphysema, both of which can imply increased resistance to airflow in the bronchi. Although viral antigens were rarely detected in the bronchiolar epithelium of those animals which had died, bronchitis was a feature of all cases with congestion and oedema of the lamina propria which contained increased numbers of lymphocytes and plasma cells. There was also epithelial dysplasia and this suggests that viral infection had at some time been present. In this respect it is interesting that van den Ingh and others (1982) noted that

coughing, which can be indicative of bronchitis, preceded the onset of severe clinical signs by approximately three days in natural RS viral infections. Thus it is possible that by the time animals die virus has disappeared from the bronchial epithelium. Whether or not uncomplicated bronchitis per se could account for the severe respiratory distress seen in some animals is not clear. Constriction of the bronchial musculature could have been present prior to death but this would not have been evident post-mortem. Serum taken from some animals after death had significant amounts of neutralising antibody to RS virus but more work is necessary to determine whether this could take part in an antibody-mediated hypersensitivity reaction leading to bronchoconstriction.

In some cases animals developed extensive lesions involving widespread diffuse epithelial damage in the caudal lobes. There was congestion and oedema of the alveoli with hyaline membrane formation. The epithelium in the alveoli was focally or diffusely hyperplastic but the bronchiolar epithelium was relatively undamaged. These lesions were not directly associated with viral replication as they did not contain virus-infected cells as judged by immunofluorescence. Their significance is not clear but they resemble those previously described as atypical interstitial pneumonia (Omar and Kinch, 1966). This lesion may also be present in other pneumonias and the significance of its relationship to these cases of RS viral infection remains to be determined.

Diagnosis of RS viral infection was relatively straight forward provided appropriate samples were available. Viral isolation, as expected, yielded few diagnoses, even in those animals which were subsequently proven to have been infected by other tests. Serological examinations yielded many diagnoses but interpreting these results in relation to the clinical disease was difficult. Infection could not be localised specifically to the lower respiratory tract using this method therefore the possibility of the virus being an incidental finding present only in the upper respiratory tract had to be considered. The proportion of the animals which seroconverted in the outbreaks of disease varied but

was always 25% or over. This figure, of course, could be affected by many factors the most critical of which is probably the timing of the first blood sample in relation to the start of the infection. Following from this acute respiratory diseases which are investigated promptly are the most likely to yield results which can be interpreted. Diagnosis is less easy in those with a slower onset or when the "acute" phase is missed. There were 14 outbreaks for which no specific diagnosis could be made and in some cases this could have been due to delayed investigation.

Histopathological and immunofluorescence techniques were only available if animals had died and/or could be obtained for post-mortem examination. However, when dealing with outbreaks of naturally occurring respiratory disease, dead animals are frequently available and may be the only material on which to establish a diagnosis. The latter technique was essential for establishing a specific diagnosis and, in conjunction with conventional histopathology, could establish the significance of the viral infection in the pathogenesis of the disease. The distribution of viral antigens within the lesions could be determined. The ease with which this could be done was greatly improved by the development of a technique to detect viral antigens in material which had been fixed for conventional histopathology. Retrospective studies of stored material could be made and tissues could be received from other laboratories. Most importantly a differential diagnosis between RS viral and PI3 viral infection could be established. Examination of H&E stained sections was the most useful technique when examining animals which had died later in an outbreak when the tissues were unlikely to contain virus. Although a specific diagnosis could not be reached a viral aetiology could be suggested by the nature, extent and distribution of the epithelial damage.

Using these techniques to confirm viral infection it was possible to establish tentative criteria for the diagnosis of RS viral infection on the basis of macroscopic features of the affected lungs. Common findings in animals which had died were

acute pneumonic lesions in the cranial parts of the lung lobes and severe interstitial emphysema with bulla formation in the caudal lobes. These lesions were present without extensive necrosis, fibrinous pleurisy or suppuration. In animals which have been at grass the possibility of D.viviparus infection must be eliminated although in this condition pneumonic lesions are more likely to be present in the caudal rather than the cranial parts of the caudal lung lobes. As the lesions due to RS viral infection were not considered pathognomonic the diagnosis was always corroborated by histopathological and immunofluorescence techniques. Clinically RS viral infection was suspected where there was an acute outbreak of respiratory disease, in housed calves and in which a prominent feature was sudden death or severe respiratory distress.

The epidemiology of the virus within populations of animals was difficult to elucidate. The source of infection in individual outbreaks was impossible to identify conclusively although circumstantial evidence incriminated several possible alternatives including older animals on the same farm and the farmer's vet. There was no specific evidence that persistent infection of individual animals could occur. However, this would be difficult to either prove or disprove with the techniques used in this study. Pre-natal infection with bovine RS virus has not been proven but a proportion of individual foetal sera do contain antibody (Gould and others, 1978). The role of other species in the epidemiology of RS viral infection in the bovine is not known. Ovine sera have been found to contain antibody to RS virus (Berthiaume and others, 1973) and this species is susceptible to experimental infection with bovine RS virus (Lehmkuhl and Cutlip, 1979). Whether this is of any significance naturally is not known.

In general, experimental infections have not produced severe clinical signs of respiratory disease (Jacobs and Edington, 1975; Mohanty and others, 1975). In view of the findings in the field investigations reported here this is not surprising as experimental infections have not reproduced within the lung the quantities and distribution of viral antigens which are seen in natural cases of

RS viral infection. Until this is achieved conclusions drawn from experimental infections regarding the ability, or inability, of the virus to produce severe disease as seen naturally may be misleading. Many natural infections in the present investigations resulted in mild clinical disease and this has frequently been reproduced by experimental infection. Another consideration is that to be successful the conditions under which experimental infections are carried out must reflect those occurring naturally in terms of virulence and dose of the infecting virus, route of challenged and susceptibility of the animal. Few of these conditions are fully understood in natural infections and therefore cannot be reproduced experimentally. Nevertheless in the experiments performed in this thesis calves were successfully infected with RS virus. No severe clinical signs of respiratory disease were attributable to this infection but virus was shed in nasal secretions and in both experiments the animals responded serologically. The strain of virus used had been repeatedly passaged in cell culture and may have lost pathogenicity. There was no specific evidence of exacerbation of the RS viral infection by combined infection with D.viviparus, whether the parasite was given before or after the virus. Allowing the animals to live long enough to develop a clinical or serological response meant that the chances of detecting viral antigens in lung tissue at post-mortem were greatly reduced. This meant that the relationship between viral infection and the pathological changes in the respiratory tract could not be assessed. In the one calf which died with combined RS viral and D.viviparus infection (calf EB1) the lesions present at post mortem were largely due to the parasite.

The experimental animals chosen all had pre-existing serum antibody to RS virus. In fact all animals which were screened for use in experimental infections had significant pre-existing levels of serum antibody. In addition, during the investigation of the respiratory disease oubtreaks only 14 of approximately 260 calves were found to be free of antibody. Selecting animals for experimental infection was difficult if "conventional" calves of a similar age to that in which the natural disease occurred were

required. The presence of antibody may have reduced the pathogenic effects of the virus. Although some workers have suggested that pre-existing antibody may be involved in immunologically-mediated respiratory tract damage in the calf (Smith and others, 1975) this has yet to be confirmed. Colostrum-deprived calves were susceptible to RS viral infection in experiments by Bryson and others (1982) and these may be a more successful experimental animal. There are, however, difficulties in obtaining and rearing this type of calf. Also, in terms of age, they are not representative of the majority of animals which, in the present investigations, succumbed to natural RS viral infection.

The general concepts influencing the development of disease due to PI3 viral infection are similar to those already described for RS virus. Infection with PI3 virus was detected in four outbreaks of respiratory disease and in a total of three individual animals. In both the outbreaks and the individual cases there was a temporal association between the presence of viral infection and the onset of the clinical signs of respiratory disease. More significantly, in one outbreak (H) and in the individual cases there was a direct association between the presence of PI3 viral antigens and the pathological lesions which were present in the lower respiratory tract of some of the affected animals. Virus-infected cells were present in areas of acute inflammation but not in areas with further advanced lesions. This strongly suggests that PI3 virus is a primary pathogen. Histopathological features which were characteristic, but not pathognomonic, of PI3 viral infection were eosinophilic intracytoplasmic inclusion bodies which were present in epithelial cells of the bronchi, the bronchioles and the alveoli. Epithelial syncytia were also occasionally seen in the bronchioles and the alveoli.

Epithelial damage in uncomplicated PI3 viral infection was not as severe as that due to RS virus and consisted of hyperplasia and dysplasia with necrosis of individual epithelial cells. Syncytium formation was not as marked as with RS viral infection in spite of the large amounts of viral antigen present in the

epithelium. This could relate to the apparently lower pathogenicity of PI3 virus when compared with RS virus. Cell fusing activity is thought to be very important in the pathogenesis of disease caused by members of the Paramyxoviridae (Merz and others, 1980) and in vivo at least certain strains of PI3 virus seem to produce fewer syncytia than do strains of RS virus. The pneumonias in which PI3 virus was involved were usually complicated by the presence of other pathogens, particularly Pasteurella haemolytica and Pasteurella multocida. It has been suggested that PI3 viral infection may interfere with pulmonary defence mechanisms and in vivo studies have shown that epithelia cilia are lost (Bryson and others, 1979b) and the clearance of bacteria e.g. Pasteurellae is impaired (Lopez, and others, 1976). Alveolar macrophages may be infected in vivo (Tsai and Thompson, 1975) and this could lead to dysfunction as has been found in the mouse where macrophages from lungs infected with parainfluenza type 1 virus had reduced bacteriocidal activity (Jakab and Green, 1972; 1976). If secondary bacterial infection is more important in the development of severe disease than is the viral infection itself then it is not surprising that the association of virus with disease is not as clear as with RS virus. Outbreaks will, in general, be investigated later in their course therefore limiting the efficiency of techniques for demonstrating viral infection. Also the histopathological features at this stage will principally be of exudation with the initiating viral infection being obscured.

Experimental infections with PI3 virus were more successful than those using RS virus. Animals with low levels of antibody to PI3 virus were readily available and the strain of PI3 virus used was a recent field isolate which grew to a relatively high titre in cell culture. Both two-weeks old and four-months old animals were susceptible to infection. Using the immunofluorescence staining technique on either acetone-fixed frozen sections of lung or on formalin-fixed paraffin-embedded sections virus-infected cells could be specifically identified. Epithelial cells in the bronchi, the bronchioles and the alveoli became infected but syncytium formation was not a marked histopathological feature despite there

being considerable numbers of infected cells present. Clinical respiratory disease was produced in both ages of animals and in animals in-contact with them. Virus was shed in nasopharyngeal secretions for up to nine days after infection.

The histopathological examination of sections of pneumonic lung is an important technique in the diagnosis and study of bovine respiratory diseases. In sections stained conventionally with haematoxylin and eosin both RS virus and PI3 virus have been associated with the formation of epithelial syncytia and eosinophilic intracytoplasmic inclusion bodies in the bronchioles and alveoli (Betts and others, 1964; Pirie and others, 1981a). It is therefore important to establish criteria by which these two important infections can be recognised and distinguished from each other. In the present series epithelial syncytia were present in the lungs of 20 animals with pneumonia and of these 19 were due to RS viral infection and only one was due to PI3 viral infection. The morphological characteristics of the syncytia produced by both viruses were similar. The nuclei overlapped and were generally centrally placed within the cytoplasm. The ratio of nuclei to cytoplasm was high. They were larger than the nuclei of normal cells and were slightly oval in shape with hyperchromatic nuclear membranes. The nucleoli were prominent and were surrounded by a clear or pale stained area. Syncytia due to RS viral infection generally contained between four and ten nuclei but could contain up to 20. Those due to infection with PI3 virus tended to contain fewer nuclei, usually between three and six with a maximum of 12.

Eosinophilic intracytoplasmic inclusion bodies were present in 21 cases. Nineteen were due to RS viral infection and two to infection with PI3 virus. No intranuclear inclusion bodies were detected in any animal although these have been reported in experimental PI3 viral infections (Dawson and others, 1965; Omar and others, 1966; Bryson and others, 1979b). Intracytoplasmic inclusion bodies were present in a proportion of syncytia and also in some individual cells. There was some variation in size of the inclusion bodies in both infections. Those in RS viral infections

were round or slightly oval. In one case of PI3 viral infection (RC17) there was a wide variation in both the size and the shape of the inclusions with large crescentic perinuclear inclusions being a prominent feature.

In cattle in the UK epithelial syncytia and eosinophilic intracytoplasmic or intranuclear inclusion bodies in the respiratory tract are histopathologial features which, as far as is known, are specific to either RS or PI3 viral infection. However, when using these two features alone it was not possible to distinguish between the two infections with absolute confidence. The conclusive diagnosis could only be made by specifically staining the viral antigens using the immunofluorescence technique. There were, however, features of the cases which could suggest infection with one or other of the viruses. Epithelial damage tended to be more flagrant in RS viral infections with dysplasia and necrosis of the bronchiolar and alveolar epithelium rather than the dysplasia and hyperplasia seen in PI3 viral infection.

A cellular infiltrate in the peribronchiolar tissues which was predominantly composed of lymphocytes was more typical of RS viral infection. In PI3 viral infections plasma cells were usually the predominant cell type. Neutrophilic infiltrates seemed to be more marked in PI3 viral infections. However, whether these were a response to viral damage or to secondary bacterial infection was not clear. Epithelial vacuoles containing aggregates of neutrophils and cellular debris were present in one case of PI3 viral infection but were not present in any of the RS viral cases. The significance of the observations with regard to PI3 viral infection must be interpreted with caution as only three individual cases in which viral antigens were present were detected.

There is no doubt that these two viruses are important causes of respiratory disease in the bovine. The pathogenesis of the infections is still not fully elucidated but the observations in these investigations have highlighted several areas worthy of

closer study, particularly in relation to the epidemiology of the virus in popuations of animals. Identification of potential sources of viral infection will be important in the development of effective disease control measures, whether these are based on housing, management or vaccination.

SECTION SIX

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APPENDIX 1

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## APPENDIX 1

### TISSUE CULTURE MEDIA

The tissue culture media which were used were based on Eagle's medium (Glasgow modification) supplemented with foetal calf serum (virus and mycoplasma screened; Gibco-Biocult, U.K.). Extra antibiotics were added for virus transport medium (VTM) and for the early stages of viral isolation (EFC2.5XAB). The concentrations of antibiotics used were:-

| | | |
|---|---|---|
| VTM | Penicillin | 100IU/ml |
| | Streptomycin | 100IU/ml |
| | Tylosine | 60µg/ml |
| | Amphotericin B | 2.5µg/ml |
| EFC2.5XAB | Tylosine | 60µg/ml |
| | Amphotericin B | 2.5µg/ml |

### HISTOPATHOLOGICAL FIXATIVES

The two fixatives used were 10% buffered neutral formalin (BNF) and corrosive formal (corrosive). The formulae for these were:-

| | | |
|---|---|---|
| BNF | Sodium dihydrogen phosphate (anhydrous) | 8.75g |
| | Di-sodium hydrogen phosphate (anhydrous) | 16.25g |
| | Formaldehyde (40% w/v) | 250ml |
| | Distilled water | 2250ml |
| Corrosive | Saturated mercuric chloride | 900ml |
| | Formaldehyde (40% w/v) | 100ml |