THE ROLE OF BACTERIA IN CANINE RESPIRATORY DISEASE

by

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SUMMARY

The morbidity and mortality associated with contagious canine respiratory disease have provided a strong stimulus for investigations into the aetiology and pathogenesis of this disease complex. Investigations by previous workers have established that a number of viral agents are of importance in such disease, but the role of bacteria has remained obscure.

Part I of this thesis consists of combined pathological, bacteriological and virological studies of naturally occurring cases of the 2 major respiratory disease syndromes of the dog i.e. canine distemper and kennel cough. These studies establish that bacterial infection of the lower respiratory tract, especially with the bacterium Bordetella bronchiseptica, is of importance in both syndromes and indicates that in kennel cough, Bordetella bronchiseptica may play a primary aetiological role.

Part II of the thesis comprises a detailed evaluation of the pathogenicity of Bordetella bronchiseptica for the respiratory tract of the dog. It shows that, under experimental conditions, infection of young dogs with this bacterium results in a severe tracheobronchitis with the development, in some dogs, of an exudative pneumonia. The clinical, pathological, bacteriological, immunofluorescence, and serological features of the experimental infection are described.

The control of contagious respiratory disease is of great practical importance. In Part III is contained an account of attempts to immunise young dogs against experimental infection with Bordetella bronchiseptica. The results of these investigations indicate that it may be possible to provide some measure of protection from this bacterial respiratory disease by parenteral vaccination with an aluminium hydroxide adjuvanted preparation.

The administration of antimicrobial agents is one of the main forms of treatment used in cases of contagious respiratory disease. Part IV of this thesis details the results of treatment of experimental Bordetella bronchiseptica infection in young dogs with two different antibiotics, oxytetracycline and amoxycillin. The treatment regimes employed failed to alter significantly the course of disease in experimental animals and it may be that effective chemotherapy of Bordetella bronchiseptica infection will necessitate the administration of high doses of antibiotics at frequent intervals.

GENERAL INTRODUCTION

In the dog, as in man and many other of his domesticated animals, contagious respiratory disease is recognised as a major problem. In research laboratories, military training establishments, boarding and breeding kennels, indeed wherever dogs are gathered together in any numbers, outbreaks of respiratory disease are a significant cause of morbidity and mortality, with consequent delay and disruption of research programmes, concern to individual dog owners and general economic loss.

Intensive investigations of respiratory disease outbreaks have led to the isolation of a number of viral agents. Canine distemper virus has long been recognised as a major cause of respiratory disease in the dog and, since 1962, canine adenovirus, canine herpesvirus, canine reovirus and canine parainfluenza (SV5) virus have also been incriminated.

In recent years little attention has been paid to the role of bacterial and mycoplasma organisms in canine respiratory disease. Many different bacteria have been regularly recovered from the nasopharynx of dogs with respiratory disease but attempts to reproduce such disease using these agents have not been consistently successful and they have been generally regarded as secondary invaders complicating primary viral damage to the respiratory epithelium.

The purpose of these investigations was to ascertain the importance of the role of bacteria in canine respiratory disease. The first part of this thesis consists of a pathological, virological and bacteriological survey of dogs with such disease; during this survey, it became apparent that the bacterium Bordetella bronchiseptica was of importance in this disease complex. The remainder of the work comprises of an investigation of the pathogenicity of Bordetella bronchiseptica for the dog and an evaluation of the effects of prophylactic and therapeutic measures upon this bacterial component of canine respiratory disease.

PART 1 : THE ROLE OF BACTERIA IN CONTAGIOUS CANINE RESPIRATORY DISEASE

SECTION I : INTRODUCTION AND REVIEW OF THE LITERATURE

Contagious respiratory disease is a major cause of morbidity and mortality in the canine population : Binn et al., (1967) found that 53 of 75 mixed-breed dogs, newly acquired from a commercial vendor, developed a spontaneous respiratory disease which was fatal in 10 cases. Such respiratory disease is generally considered to exist in two distinct clinical forms (Appel et al., 1970; Thompson et al., 1975). Firstly, respiratory disease is a major component of canine distemper, a systemic paramyxovirus infection in which involvement of lymphoid, alimentary and nervous systems may contribute to the overall clinical picture. Secondly, contagious respiratory disease exists as a less well-defined clinical syndrome, which, because of its tendency to occur in groups of kennelled animals, has become popularly known as "kennel cough" : this syndrome is restricted to the respiratory tract and is characterised by a harsh, often paroxysmal rasping cough, which persists for a number of weeks (Prier, 1956; O'Brien and Todd, 1971).

Recent research into the aetiology of canine respiratory disease has concentrated on the role played by viral agents and the literature on these agents has been extensively reviewed (Appel et al., 1970; Thompson et al., 1975). Canine distemper virus (CDV) has long been recognised as a major cause of respiratory disease in the dog (Dunkin and Laidlaw, 1926) but recently a number of other viruses have been isolated from dogs with respiratory disease and, under experimental conditions, these have been shown capable of inducing pathological changes in the canine respiratory tract. Thus, canine adenovirus type 1 (CAV-1) (Wright et al., 1971; Studdert and Studdert, 1972; Wright et al., 1972), canine adenovirus type 2 (CAV-2) (Ditchfield et al., 1962; Binn et al., 1967), canine reovirus (CRV) (Lou and Wenner, 1963; Massie and Shaw, 1966), canine herpesvirus (CHV) (Motohashi and Jajima, 1966; Karpas et al., 1967; Karpas et al., 1968), canine parainfluenza (SV5) virus (Binn et al., 1967, Crandell et al., 1968; Binn et al., 1968) and human influenza virus type A2/Hong Kong (Bibrack, 1974; Bibrack, 1975) have all been incriminated to varying degree in the aetiology of kennel cough. The role played by bacteria and mycoplasmas in canine respiratory disease is, however, much less certain.

In the latter half of the nineteenth century, during the search for the actiological agent of canine distemper, many organisms were isolated from the respiratory tracts of diseased dogs; the findings of these early investigators were reviewed in some detail by Torrey and Rahe (1913). From the descriptions given, the bacteria recovered appear to have been staphylococci, streptococci, Pasteurella-like organisms and various Gram-negative bacilli, but there was little agreement among the different workers as to the relative importance of these organisms in the disease syndrome : each investigator championed a different organism.

Later, in the early years of the twentieth century, the bacterium originally called Bacillus bronchicanis (Ferry, 1910), and subsequently renamed Bacillus bronchisepticus (Ferry, 1912), gained widespread recognition as being of importance in canine distemper. This organism could be regularly recovered, in pure culture, from the lower respiratory tract of affected dogs, and inoculation of apparently healthy dogs with the organism appeared to result in a distemper-like syndrome (Ferry, 1910; Ferry, 1911; McGowan, 1911a; Torrey and Rahe, 1913). As early as 1905, however, Carré had considered a virus to be the cause of canine distemper and the conclusive confirmation of the viral aetiology of the disease (Laidlaw and Dunkin, 1926) resulted in the relegation of Bacillus bronchisepticus to a position of secondary significance in the pathogenesis of canine respiratory disease.

Subsequent investigations of canine distemper (Schlingman, 1932; Shoetensack, 1934; Hsiung and Stafseth, 1952; Lauder et al.,1954a and b) have confirmed that a variety of bacterial and mycoplasmal species may be recovered from the respiratory tracts of dogs with this disease. An equally wide range of bacteria has been recovered from the nose and throat of diseased dogs during investigations of the kennel cough syndrome (Mann and Bjotvedt, 1964; Binn et al.,1968; Snow et al.,1969; Wilkins and Helland,1972).

In evaluating the significance of bacteria in the aetiology and pathogenesis of canine respiratory disease, several points must be considered : firstly, the constitution of the normal microbial flora of the

canine respiratory tract; secondly, whether the microflora of the respiratory tract changes in diseased animals; thirdly, whether the presence of the micro-organism being evaluated modifies the development of respiratory disease due to agents of established primary pathogenicity; and, finally, whether the microorganism, on its own, is capable of inducing pathological changes in the respiratory tract of normal dogs.

As a first step to understanding the role of bacteria in respiratory disease it is important to establish the normal bacterial flora in the canine respiratory tract. A remarkably varied bacterial flora is known to exist in the upper respiratory tract and oropharynx of normal animals (Austrian, 1968) and a number of studies have been made of the nasal and pharyngeal flora of the normal dog. It is important that in such studies the normal animal should have a known history of freedom from clinical respiratory disease and from exposure to a diseased population which might result in subclinical infection or mechanical carriage of potential pathogens. Smith (1961) surveyed the aerobic bacterial flora of the nose and tonsillar area of dogs submitted for euthanasia; these were healthy household dogs allowed minimal contact with other animals and may be considered as a normal population. Clapper and Meade (1963) made a study of the normal flora of nose, throat and intestine of 25 healthy colony beagles and Brennan and Simkins (1970) investigated the throat flora of beagles in a closed breeding colony. The findings of these authors are summarised in Table 1 ; briefly, the organisms most frequently recovered were Staphylococcus spp., Streptococcus spp., Neisseria spp., Pasteurella spp., Corynebacterium spp., Coliforms and Mycoplasma spp.; the other organisms were less consistently isolated.

Singh and Parnaik (1965) investigated the nose and throat flora of dogs suffering from a variety of non-respiratory conditions admitted to a Veterinary Hospital but since no investigation of the possible exposure to respiratory disease prior to hospitalisation of the dogs was made, and since spontaneous outbreaks of respiratory disease frequently develop in Veterinary Hospital conditions, it is doubtful whether this population could be regarded as "normal". Nonetheless, this investigation, like those above, found the flora to be composed predominantly of Staphylococcus spp., Streptococcus sp.,

Staphylococcus spp.	:	Smith, 1951; Clapper and Meade, 1963; Brennan and Simkins, 1970.
Streptococcus spp.	:	" " "
Neisseria spp.	:	" " "
Pasteurella spp.	:	" " "
Corynebacterium spp.	:	" " "
Coliforms	:	" " "
Bacillus spp.	:	" " "
Haemophilus spp.	:	Smith, 1951; Clapper and Meade, 1963.
Alcaligenes spp.	:	" "
Lactobacillus spp.	:	" "
Proteus spp.	:	Smith, 1951; Brennan and Simkins, 1970.
Achromobacter spp.	:	" "
Falvobacterium spp.	:	Smith, 1951.
Mima spp.	:	Clapper and Meade, 1963.
Pseudomonas spp.	:	"
Klebsiella (Aerobacter) spp.	:	"
Paracolobactorum spp.	:	"
Micrococcus spp.	:	Clapper and Meade, 1963; Brennan and Simkins, 1970.
Mycoplasma spp.	:	Brennan and Simkins, 1970.

Table 1 : Aerobic bacteria recovered from the nose and throat of normal dogs

diptheroids and coliforms.

In studies of the incidence of individual microorganisms, Smith (1955) found Pasteurella septica in the nose of 10% and the tonsil of 55% of normal dogs while Blouse et al.(1964) recovered Staph. aureus from 64 of 150 healthy dogs. Edward and Fitzgerald (1951) reported the recovery of four strains of mycoplasma from the throat and vagina of normal dogs. These four strains, now designated Mycoplasma canis, Mycoplasma maculosum, Mycoplasma spumans (Edward and Freundt, 1956) and Mycoplasma edwardii (Tully et al.,1970) were also isolated from the larynx of a total of 71% of 93 apparently normal dogs by Barile et al., (1970) who considered them normal commensal inhabitants of the dog. These mycoplasmas have also been found in the nose and throat of normal dogs by Brennan and Simkins (1970) and Koshimuzu and Ogata (1974).

In contrast to the mixed bacterial flora of the normal oro - and nasopharynx, the lower respiratory tract of both man and animals is generally regarded as sterile (Brumfitt et al.,1957; Lees and McNaught, 1959; Laurenzi et al.,1961). The sterility of the normal lower respiratory tract in the dog was commented on by Ferry (1911) who had examined the trachea and bronchi of living healthy animals by means of a bronchoscope. In a similar fashion, Heuer and Dunn (1920) found the respiratory mucosa at the level of the tracheal bifurcation in healthy experimental dogs to be almost invariably sterile.

No comprehensive, controlled survey has yet been carried out into the incidence of bacteria in both the upper and lower respiratory tracts of dogs with respiratory disease. However, extensive investigations have been made of the nasopharyngeal flora of dogs with a kennel cough-type syndrome (Mann and Bjotvedt, 1964; Binn et al. 1968; Snow et al.,1969; Baker and Huebner, 1970; Wilkins and Helland, 1972). A wide range of bacteria were isolated by these authors but, of the species they recovered, only Bordetella (Bacillus) bronchiseptica is not included in Table 1, i.e. is not also regularly found in normal dogs. Pasteurella spp. (Stevenson and Hilbert, 1937) and Beta-haemolytic streptococci (Hare, 1946; Townson, 1947) were recovered from tonsillar swabs from dogs with respiratory disease and were considered by the authors to be of significance in the

pathogenesis of the disease. Snow et al.,(1969) compared the incidence of bacterial species in the nasal flora of 97 dogs newly acquired from various commercial sources and showing no clinical evidence of respiratory disease with that of those 25 dogs which subsequently developed respiratory disease. Whilst the incidence of most bacteria was unchanged, that of Staph. aureus and Bord. bronchiseptica rose from 69% and 12.5% respectively in the asymptomatic period to 84% and 36% respectively in the diseased period.

As there is some difficulty in interpreting results from nasopharyngeal swabs in diseased dogs since the normal flora is subject to considerable variation, more significance might be attached to organisms isolated from the normally sterile lower respiratory tract. Investigations of lower respiratory tract bacteriology in dogs with kennel cough are, however, few. Armstrong et al.(1972) found Pasteurella spp., Pseudomonas spp., Beta-haemolytic streptococci, coliforms and other bacteria in dogs with spontaneous respiratory disease, while Swango et al.,(1970) recovered Bord. bronchiseptica, Past. multocida, Pseudomonas aeruginosa and streptococci from the lungs of dogs experimentally infected with canine adenovirus.

More extensive studies of lower respiratory tract bacteriology have been made during investigations of canine distemper. Thus, Ferry (1911) recovered Bord. bronchiseptica from the lower respiratory tract of 97 dogs with distemper; the organism was also found by McGowan (1911) in 93% of 29 dogs and by Torrey and Rahe (1913) in 81% of 80 dogs with distemper. Later Schoichi (1923) and Hardenberg (1925) found Bord. bronchiseptica in the lungs of 85% and 87% of distemper cases respectively. Schlingman (1932) found streptococci, staphylococci and organisms of the colon-typhoid group to form the predominant flora in dogs examined, while Bord. bronchiseptica was also present. In a more recent survey (Lauder et al.,1954) of the lungs of 70 dogs with distemper, 63% of lungs were sterile whilst streptococci, Bacterium coli, Proteus vulgaris and Staph. albus were most common in the remaining 37%. Bord. (Haemophilus) bronchiseptica was not found. These authors considered that Bord. bronchiseptica had become uncommon in the dog, but since the tracheobronchial tree, the optimum site for isolation of this organism (Winsser 1960), was not examined, the validity of this conclusion must be open

to doubt. The current incidence of bacteria in the lower respiratory tract of dogs with distemper is unknown.

Mycoplasmas have been recovered from the upper (Shoetensack, 1934; Greig, 1954; Binn et al.,1968) and lower respiratory tract (Armstrong et al.,1970; Armstrong et al.,1972; Rosendal, 1972) of dogs with respiratory disease and it may be of significance that one of the mycoplasmas found in pulmonary lesions differed from the four normally-occurring strains described previously. This new strain was subsequently designated Mycoplasma cynos (Rosendal, 1973).

Klebsiella pneumoniae has been isolated from the lungs of individual dogs with pneumonia (Ludford and Stevens, 1958; Cole, 1964).

Table 2 summarises the above isolations of bacteria recovered from the upper and lower respiratory tracts of dogs with respiratory disease. Of these bacteria, Bord. bronchiseptica, Streptococcus spp., Staphylococcus spp., Klebsiella spp., and Pseudomonas spp. are considered to be most significant in canine respiratory disease (Jubb and Kennedy, 1963; Pennock and Archibald, 1968; Aronson and Kirk, 1971). However, their role, if any, in the pathogenesis of such disease has yet to be conclusively established.

Canine distemper was first reliably reproduced in the dog by Dunkin and Laidlaw (1926) by inoculation of bacteria-free material into healthy dogs. Under these experimental conditions, clinical signs referable to the respiratory tract were, in contrast to those found in field cases of the disease, almost invariably mild, and although at post-mortem examination there was in some animals evidence of mild bronchitis with small scattered patches of bronchopneumonia, the severe and extensive pneumonias recognised in the field disease were not present. The authors concluded that naturally occurring CDV infection was often complicated by secondary bacterial infections which were responsible for much of the clinical disease. More recently, a wide range in the severity of clinical signs associated with experimental CDV infection in conventionally reared dogs have been recorded by Cornwell et al (1965a) and Appel (1969) while

Bordetella bronchiseptica	:	Ferry, 1911; Torrey and Rahe, 1913; Hardenberg, 1925; Snow et al., 1969; Appel et al. ,(1970); Wilkins Helland, 1972.
Klebsiella spp.	:	Mudford and Stevens, 1958; Cole, 1964; Mann and Bjotvedt, 1964.
Streptococcus spp.	:	Hare, 1946; Townson, 1947; Snow et al., 1969; Baker and Huebner, 1970; Wilkins and Helland, 1972.
Staphylococcus spp.	:	Mann and Bjotvedt, 1964; Snow et al., 1969; Baker and Huebner, 1970; Wilkins and Helland, 1972.
Pasteurella spp.	:	Stevenson and Hilbert, 1937; Brennan et al., 1965; Wilkins and Helland, 1972.
Proteus spp.	:	Mann and Bjotvedt, 1964; Snow et al., 1969; Appel et al., 1970a; Wilkins and Helland, 1972.
Pseudomonas spp.	:	Mann and Bjotvedt, 1964; Snow et al., 1969; Appel et al., 1970a; Wilkins and Helland, 1972.
Neisseria spp.	:	Snow et al., 1969; Wilkins and Helland, 1972.
Haemophilus spp.	:	Wilkins and Helland, 1972.
Corynebacterium spp.	:	Wilkins and Helland, 1972.
Coliforms	:	Mann and Bjotvedt, 1964; Binn et al., 1968; Snow et al., 1969; Wilkins and Helland, 1972.
Alcaligenes spp.	:	Wilkins and Helland, 1972.

Table 2 : Bacteria recovered from the respiratory tract ot dogs with respiratory disease

Bacillus spp.	:	Snow et al., 1969.
Mima spp.	:	Wilkins and Helland, 1972.
Achromobacter spp.	:	Wilkins and Helland, 1972.
Flavobacterium spp.	:	Wilkins and Helland, 1972.
Paracolobactrum spp.	:	Mann and Bjotvedt, 1964; Snow et al., 1969.
Micrococcus spp.	:	Baker and Huebner 1970; Snow et al., 1969.
Mycoplasma spp.	:	Shoetensack, 1934; Armstrong et al., 1970; Rosendal et al., 1972; Wilkins and Helland, 1972.

Table 2 (continued) : Bacteria recovered from the respiratory tract of dogs with respiratory disease.

Gibson et al.,(1965) investigating CDV infection in gnotobiotic animals found that disease signs were limited to pyrexia and decreased appetite; the latter authors hypothesised that the microbial flora of the non-gnotobiotic animal was necessary for the development of many commonly recognised clinical signs. A similar view was held by Appel (1970) although Bussel (1970) considered that mild symptomatology in experimental animals may have been due either to non-optimal routes of infection or to rapidly developing immunity.

Secondary bacterial infection might well be expected in CDV infection since the virus induces marked lymphoid depletion and thymic atrophy (McCullough et al.,1973) and it may be that much of the clinical features of respiratory disease associated with CDV is due to bacterial invasion of the respiratory tract in these immunosuppressed animals.

In the kennel cough syndrome, there is uncertainty as to the role played by bacteria, the disease being generally recognised as of viral aetiology (Wilkins and Helland, 1972). Over the years, a number of viruses have been incriminated (Appel et al., 1970; Thompson et al., 1975) but it is interesting to note that the clinical signs recorded in experimental studies of respiratory disease induced by these agents have often been mild, transient or even inapparent; this is in marked contrast to the severe cough, often persisting for several weeks, which is characteristic of many naturally occurring cases of kennel cough (Mosier, 1955; Prier, 1956; Pennock and Archibald, 1968).

Experimental reovirus infection has resulted in either mild rhinitis (Lou and Wenner, 1963; Massie and Shaw, 1966) or inapparent clinical disease (Holzingier and Grisemer, 1966; Thompson et al., 1970); in only one animal has coughing been recorded (Lou and Wenner, 1963). Similarly, experimental herpesvirus infection has resulted in only a serous or mucoid rhinitis (Appel et al., 1969; Thompson et al.,1972) although spontaneous coughing was recorded in 30% of 13 dogs inoculated intranasally (Karpas et al., 1968). In experimental SV5 infection, some authors have recorded inapparent infection (Black and Lee, 1970) and mild rhinitis (Crandell et al., 1968; Lazar et al., 1970); rhinitis was also noted by Appel

and Percy (1970) in 25% of 20 dogs, 35% of which also developed a mild cough for up to 6 days. Rosenberg et al.(1971) also described coughing of 2 to 12 days' duration in 60% of experimental dogs infected with SV5, although Binn and Lazar (1970) considered inapparent infection with SV5 to be more common than overt disease. Swango et al.(1970) reported that experimental CAV-2 infection resulated in paroxysmal coughing but, in this experiment, many potentially pathogenic bacteria were also recovered from the lungs of the infected animals : in contrast, in dogs infected with CAV-1, when the lungs were bacteriologically sterile, spontaneous coughing was recorded for only 4 days in 75% of 16 infected dogs although, in 19% coughing could be induced by tracheal palpation for up to 12 days (Wright et al., 1971).

In the few studies where dogs infected with a virus have also been given bacteria, clinical signs have been much more severe than in dogs given virus alone, with coughing of several weeks' duration developing in some animals (Appel and Percy, 1970; Appel et al., 1970). It may, therefore, be possible that, like distemper, supervening bacterial infections are responsible for much of the clinical progression of the kennel cough syndrome (Karpas et al., 1968; Appel et al., 1970; Wilkins and Helland, 1973; Wright et al., 1974).

Limited attempts have been made to establish the primary pathogenicity of bacteria for the canine respiratory tract. Ferry (1911), McGowan (1911) and Torrey and Rahe (1913) claimed to have produced respiratory disease in dogs by inoculation of Bord. bronchiseptica via the respiratory tract, but as no control animals were employed and there was no method of eliminating CDV infection, these results cannot be regarded as conclusive. Hardenberg (1925) also inoculated dogs with Bord. bronchiseptica via the respiratory tract, but was unconvinced that the subsequent disease in these dogs was in fact associated with this bacillus as his uninoculated control animals developed an identical disease. Ray (1948) considered kennel cough to be due to infection with Bord. bronchiseptica while Townson (1947) believed that Beta-haemolytic streptococci, recovered from the lungs of affected dogs,were primary pathogens. Unsuccessful attempts to produce respiratory disease in dogs by inoculation of these agents either

alone or in combination have, however, been recorded (Greig, 1954; Mosier, 1955; Chappel et al., 1956).

More recently, Appel et al.(1970) failed to induce disease in dogs by intranasal inoculation of Bord. bronchiseptica alone or with an untyped mycoplasma, although these organisms had been recovered from a naturally occurring outbreak of respiratory disease from which no known canine virus could be isolated. However Appel et al.(1970) cited Harris as finding that intratracheal rather than intranasal inoculation with Bord. bronchiseptica had resulted in respiratory disease in a dog.

In summary, a wide variety of bacterial species can be recovered from the upper respiratory tract in dogs with respiratory disease; however, these same bacteria, with the possible exception of Bord. bronchiseptica can also be found in the same situation in normal, healthy dogs. There is evidence that bacterial infection may be at least partly responsible for the clinical progression of distemper and kennel cough, the two main forms of contagious canine respiratory disease. The incidence of bacteria in the lower respiratory tract, normally sterile, of dogs with these conditions has not, however, been established nor is it definitely known whether the presence of bacteria at this site is correlated with more severe pathological lesions than are found in uncomplicated viral infections. The primary pathogenicity of bacteria for the canine respiratory tract has yet to be proved.

In order to elucidate the role of bacteria in canine respiratory disease, a combined pathological, virological and bacteriological study of dogs suffering from the two main clinical forms of contagious respiratory disease i.e. distemper and kennel cough, was undertaken.

SECTION 2 : MATERIALS AND METHODS

Survey Populations

Two separate groups of animals were examined.

The first group consisted of animals diagnosed clinically as having canine distemper. Cases were obtained for necropsy from a small animal clinic staffed by members of the University of Glasgow Veterinary School; all these cases were drawn from the central, urban area of Glasgow. A clinical diagnosis of distemper was made on the basis of the presence, in the affected animal, of a minimum of 4 of the 6 following clinical signs i.e. 1) hyperkeratosis of pads or nose, 2) nervous signs, 3) discharge from eyes and/or nose, 4) respiratory signs, 5) diarrhoea and 6) minimum duration of illnesses of three weeks; if hyperkeratosis was present, a total of three of these signs was considered sufficient to make a firm diagnosis. Dogs were examined at various points in the disease process as, in all cases, the owners had requested that their dog be humanely destroyed. Some animals had received antibiotic therapy prior to destruction.

The second group was composed of dogs submitted for necropsy from a large kennel housing stray animals drawn from suburban and rural districts to the west of Glasgow. Only apparently healthy dogs were admitted to this kennel and, on arrival, all dogs were inoculated with measles vaccine (Kavac M : Duphar Veterinary Ltd., Southampton). Dogs were held in the kennel for 7 to 10 days and, unless claimed or bought within this period, were then humanely destroyed. Nonetheless, this kennel, with its everchanging dog population, had a persistent problem of contagious respiratory disease. Disease outbreaks were frequent and, at such times, many of the dogs presented for destruction were coughing, whilst dogs returned to their owners or sold required subsequent veterinary attention for persistent coughing. Between such "outbreaks", there were periods of less overt clinical disease. This kennel thus presented an opportunity to study a "kennel cough" - type population. During the period of this survey an outbreak of respiratory disease had occurred in the kennels and approximately 75% of dogs submitted for further examination in the present study had clinical respiratory disease at the time of destruction. No animal had received antibiotic therapy.

Necropsy Procedures

All animals were killed by rapid intravenous injection of pentobarbitone sodium (Euthatal : May and Baker Ltd., Dagenham). A full post mortem examination was carried out as soon after death as possible; in all cases this was within 24 hours of death. All systems were examined and samples of tissue were taken for further examination as required.

Representative samples of lung were selected for histological examination whether there was gross evidence of disease or not; blocks of trachea, tracheobronchial and retropharyngeal lymph nodes, tonsil, liver, kidney, spleen and bladder were also routinely examined. In cases of distemper with clinical evidence of encephalitis, the brain was removed and fixed whole.

One entire lung lobe was taken for bacteriological examination; this was normally the right cardiac lobe but in some cases the intermediate lobe was used; the lobe was removed at the level of the lobar bronchus, care being taken not to cut the lung substance. Trachea, tonsil and tracheobronchial and retropharyngeal lymph nodes were also examined by bacteriological techniques.

Small (1 cm x 1 cm x 1 cm) blocks of lung, including a bronchus, tonsil and retropharyngeal lymph node were taken for examination by fluorescent antibody techniques. In cases with clinical evidence of encephalitis, small blocks of hind-brain were also taken.

An entire lung lobe, normally the intermediate lobe, was taken and stored at -20^{o}C until virological isolation procedures could be carried out.

Histological Procedures

Lung tissue was fixed in 10% neutral buffered formol saline (NBFS). The fixative was instilled into entire lung lobes via the bronchus until the pleural surface of the lobe was wrinkle free; the bronchus was then ligated and the lobe immersed in 10% NBFS. After 48 hours, all the tissues were trimmed and transferred to fresh NBFS for a further 24 hours.

Tissues were dehydrated in a phenol/methanol-absolute alcohol-chloroform/xylene series and were then embedded in paraffin wax; lung blocks were embedded overnight in a vacuum oven.

Sections were cut at 4-6μm and stained routinely with Mayer's haemalum and eosin (HE); to demonstrate collagen and fibrin, selected sections were stained with Martius-Scarlet Blue (MSB) and to demonstrate distemper virus inclusion bodies the haemalum-phloxine-tartrazine method (PTI) of Lendrum (1947) was employed.

Bacteriological Procedures

The tissues taken for bacteriological examination were sampled as described below.

Lung : a) Bronchus : Bronchial exudate was aspirated into a Pasteur pipette or the mucosa was washed with sterile normal saline (SNS). One drop of this material was used for inoculation.

b) Lung substance : The pleural surface of the lung lobe was heat-seared and the lung substance obtained for inoculation by stabbing through the seared area with a Pasteur pipette.

Trachea : The tracheal mucosa was massaged vigorously with a sterile wire loop and the material so obtained was subsequently used.

Lymph nodes and tonsil : These tissues were seared on their external surfaces and then transected with a sterile scalpel blade. The exposed surface was then punctured and tissue aspirated and then used for inoculation as described above.

Primary isolation of bacteria was carried out by inoculation of the above samples onto paired Nutrient Blood Agar (NBA) and MacConkey Agar plates, these plates then being aerobically incubated at $37^{o}C$ for 24-48 hours. Direct smears of the material inoculated on to the agar plates were made on glass slides; these smears were heat-fixed, stained by Gram's method and examined for the presence of bacteria. Some smears were also stained by Loeffler'sMethylene Blue.

Representative bacterial colonies appearing at primary isolation were examined and identified according to the methods of Cowan and Steele (1965).

Media : Nutrient Blood Agar was made up using Nutrient Agar Base (Oxoid Ltd., London); 7% sterile horse blood (Burroughs Wellcome Ltd.; Kent) was added to the molten Agar base at 55°C.

MacConkey Agar was made from MacConkey Agar Base (Oxoid Ltd.: London) according to manufacturer's instructions.

Sterile Normal Saline was a 0.85% saline solution sterilised by autoclaving at 15 lb/sq. inch. for 15 minutes.

Immunofluorescence Procedures

The small blocks of tissue taken at post mortem examinations were placed in individual glass containers, snap frozen in a mixture of solid carbon dioxide and 2-methylbutane, and stored at -20°C until examined. For examination 3-4μm thick sections were cut on a Slee cryostat at -20°C, dried in air, fixed in acetone for 10 minutes and stained with fluorescent antisera specific for canine distemper virus (CDV) and canine adenovirus (CAV). The antiserum was layered onto the fixed sections and these were left in a moist chamber for 30 minutes at room temperature. The sections were then washed in phosphate buffered saline (PBS) pH 7.4 for 30 minutes, mounted in PBS and examined on a Leitz "Orthoplan" fluorescence microscope equipped for incident light fluorescence.

The specific fluorescent antisera were prepared as described below.

a) Canine distemper virus antiserum. Young, healthy rabbits were inoculated intramuscularly with 1 ml of an equal volume of an emulsion of virus suspension and Freund's complete adjuvant. Six weeks later, the rabbits were given suspension intravenously. They were bled after a further 10 days and the sera pooled. The virus suspension used was derived by repeated purification in sucrose density gradients from tissue culture fluids obtained from a continuous dog kidney cell line which had been infected with the Glasgow 841 strain of distemper virus (Cornwell et al., 1965b).

b) Canine adenovirus antiserum. A fourteen week old dog was inoculated subcutaneously with 1 ml of virus suspension emulsified with 1 ml of Freund's complete adjuvant. A further 1 ml dose of virus was given intravenously 6 weeks later and after a further 10 days the dog was bled and the serum separated. The strain of virus used (CAV-1; infectious canine hepatitis virus) was obtained by repeated freezing and thawing of a dog kidney cell line infected with a strain of adenovirus isolated from a dog with interstitial nephritis (Wright et al., 1973a).

Serum fractionation : Equal volumes of serum and saturated ammonium sulphate were thoroughly mixed and left for 10 minutes at $4^{o}C$. The precipitate, containing the globulin fraction, was separated by centrifugation at $4^{o}C$ and resuspended in PBS to one third of the original serum volume. The protein solution was dialysed for 48 hours at $4^{o}C$ against PBS to remove ammonium ions.

Serum conjugation : The globulin fraction was conjugated at $4^{o}C$ with 10% fluorescein isothiocyanate (FITC) on celite powder (Calbiochem : California, U.S.A.) by the method of Rinderknecht (1962). 10 mg of dry FITC powder was added to 2 ml of globulin and 2 ml of carbonate/ bi carbonate buffer pH9, and stirred in an ice bath for 5 minutes. Free dye was removed by passing the conjugate through a column of Sephadex G25 fine grade (Pharmacia; Uppsala, Sweden). The conjugated serum was absorbed at $4^{o}C$ with 100 mg dog tissue powder/ml conjugate.

Tissue powder for absorption was prepared from dog liver and kidney tissue of young healthy dogs. Tissues were homogenised in a Waring blender and washed repeatedly in isotonic saline before several washes in large volumes of acetone. The supernatant was removed and the remaining material dried overnight at $37^{o}C$, ground in a mortar, coarse material removed by sieving and the remaining fine powder stored, in airtight containers, at $4^{o}C$.

Virological Procedures

The lung lobe taken at necropsy was finely chopped with scissors and a 20% suspension of this tissue was made up in maintenance medium. The suspension was thoroughly shaken, allowed to settle, and the supernatant was used to inoculate primary cultures of dog kidney cells in 8 oz bottles; 4 ml of supernatant was inoculated per bottle, allowed to absorb at 37°C for 2 hours and was then decanted. The cell sheet was washed with PBS and maintenance medium added. The cell cultures were changed daily for the first 7 days and then twice weekly. The bottles were examined daily for evidence of cytopathic effect. Haemadsorption tests were also carried out on inoculated bottles after 12 days' incubation.

Maintenance medium : Earles Balanced Salt Solution (Burroughs Wellcome Ltd., ; Kent) was made up to manufacturer's instructions : 0.5% lactalbumin hydrolysate was added. Immediately before use 5% calf serum (Burroughs Wellcome Ltd. : Kent) and the following antibiotics were added :

100 units/ml	Penicillin
100 µg/ml	Streptomycin
50 µg/ml	Polymixin B
2.5 µg/ml	Amphotericin B
10 µg/ml	Chloretracycline

Haemadsorption test : An 8 oz bottle inoculated 12 days previously was reseeded onto coverslips in roller tubes; 6 coverslips were obtained from 1 bottle. The next day, i.e. 13 days post inoculation, maintenance medium was decanted from the coverslips and the cell sheets washed in PBS at 4°C; 0.2ml of a 0.4% suspension of freshly obtained guinea-pig red blood cells in Alsevers solution was added to each coverslip preparation, allowed to incubate at 4°C for 30 mins and was then decanted. The cell sheet was washed twice in cold PBS and examined under a microscope for adsorption of the red cells to the kidney cells.

SECTION 3 :

A SURVEY OF DOGS WITH CANINE DISTEMPER VIRUS INFECTION

Survey Design

The object of this survey was to investigate the incidence and nature of bacterial involvement in the respiratory component of CDV infection in the dog and to investigate the effect of such bacterial involvement on the macroscopic and microscopic pathological changes associated with the primary viral infection.

A total of 50 dogs (D1 - D50) were selected for examination in this survey on the basis of the clinical criteria described in Section 2 above. Relevant background data on these 50 dogs are presented in Table 3. Mongrel or crossbred dogs and dogs up to 1 year of age comprised a high proportion, 64 per cent and 98 per cent respectively, of the dogs examined in this survey; the ratio of male to female animals in the survey population was approximately 2 : 1.

Each dog was submitted to a full post mortem examination at which samples were taken, as described in Section 2, for immunofluorescence examination for CDV antigen and for bacteriological and histopathological investigation.

Pathological Findings

Macroscopic : The major pathological changes found at post mortem examination were present in the respiratory tract and associated structures.

In the lungs, 3 different types of change were recognisable and the incidence and severity of these changes are summarised in Table 4. The most frequent change was the presence of pulmonary oedema; affected areas of the lungs were darker than normal in colour (Figs. 1 and 2), did not collapse as completely as uninvolved areas when the thoracic cavity was opened and were of a pliable, rubbery consistency. When areas of oedematous lung tissue were transected, clear, often frothy fluid could easily be expressed from the cut surface which, itself, had a glassy appearance (Fig. 3). Oedema varied in severity from the

Dog Number	Breed	Age (in months)	Sex	Dog Number	Breed	Age (in months)	Sex
D1	Mongrel	12 mths	F	D26	Collie X	$3\frac{1}{2}$ mths	M
D2	Mongrel	7 mths	M	D27	Alsatian X	3 mths	M
D3	Foxhound	4 mths	M	D28	Mongrel	6 mths	F
D4	Mongrel	4 mths	M	D29	Mongrel	4 mths	M
D5	Labrador X	12 mths	F	D30	Collie X	6 mths	M
D6	Terrier X	8 mths	M	D31	Alsatian	8 mths	F
D7	Alsatian	4 mths	M	D32	Alsatian	11 mths	F
D8	Collie X	6 mths	M	D33	Mongrel	6 mths	F
D9	Collie X	6 mths	M	D34	Alsatian	14 mths	F
D10	Labrador	4 mths	M	D35	Mongrel	4 mths	M
D11	Labrador	12 mths	M	D36	Foxhound	7 mths	M
D12	Collie X	6 mths	F	D37	Alsatian	3 mths	F
D13	Labrador X	4 mths	M	D38	Alsatian	3 mths	M
D14	Terrier X	5 mths	M	D39	Mongrel	8 mths	F
D15	Mongrel	6 mths	M	D40	Labrador	7 mths	F
D16	Mongrel	4 mths	F	D41	Shetland Sheep dog	8 mths	F
D17	Mongrel	9 mths	M	D42	Mongrel	6 mths	M
D18	Mongrel	7 mths	M	D43	Poodle	$4\frac{1}{2}$ mths	M
D19	Mongrel	6 mths	M	D44	Alsatian	3 mths	M
D20	Mongrel	6 mths	M	D45	Shetland Sheep dog	2 mths	F
D21	Mongrel	10 mths	M	D46	Labrador	3 mths	M
D22	Labrador X	4 mths	M	D47	Beagle	6 mths	F
D23	Mongrel	4 mths	M	D48	Mongrel	8 mths	M
D24	Mongrel	3 mths	F	D49	Mongrel	6 mths	M
D25	Mongrel	4 mths	M	D50	Beagle	12 mths	M

Table 3 : Canine distemper survey - breed, age and sex of each dog examined.

Dog Number	Oedema	Exudative Pneumonia	Non-exudative Pneumonia	Dog Number	Oedema	Exudative Pneumonia	Non-exudative Pneumonia
D1	+	++	-	D26	++	-	-
D2	++	-	-	D27	+	-	++
D3	+	+++	-	D28	Lungs contained multiple greyish-white nodules 2mm in diameter		
D4	++	-	-	D29	++	-	-
D5	+++	-	-	D30	+	++	-
D6	++	-	-	D31	+++	-	-
D7	++	-	++	D32	+	-	-
D8	+	+++	-	D33	+	-	-
D9	+++	-	-	D34	+	+++	-
D10	+++	-	-	D35	+	-	-
D11	+	-	+	D36	+	++	-
D12	++	-	-	D37	++	-	-
D13	+	++	-	D38	+	++	-
D14	++	+	+	D39	-	-	-
D15	+	++	-	D40	-	-	-
D16	+	-	+	D41	++	-	-
D17	+	++	+	D42	+	+++	-
D18	++	++	-	D43	+	+++	-
D19	+	+++	-	D44	++	-	-
D20	+	++	-	D45	+	-	-
D21	+	-	-	D46	+	-	+
D22	++	-	++	D47	+	-	++
D23	++	-	-	D48	+	++	-
D24	++	-	-	D49	+	++	-
D25	++	-	-	D50	+	-	+++

- = Not present Lesions graded + to +++ pm severity (see text)

Table 4 : Canine distemper survey - macroscopic findings in the lungs at post mortem examination

presence of patchy areas scattered throughout the lung lobes (designated "+" in Table 4) to diffuse involvement of the majority of the lung tissue (designated "+++" in Table 4). Excessive amounts of clear or frothy fluid were frequently found in the tracheobronchial tree of dogs with moderate or severe pulmonary oedema.

The second most frequent finding in the lungs was the presence of areas of exudative pneumonia which were found almost exclusively in the anterior, dependent portions of the lungs i.e. the apical, cardiac and anteroventral portion of the diaphragmatic lobes (Figs. 1 and 4). These areas were mottled red-brown or red-yellow in colour and were often surrounded by a peripheral zone of hyperaemia (Fig. 2); they were firm in consistency and were usually well-demarcated from the surrounding lung tissue (Fig. 5), although in some dogs they appeared as patchy, coalescing foci (Fig. 6); on section, there was exudation of fluid from the cut surface and a purulent exudate could easily be expressed from the bronchial tree. The severity of exudative pneumonia varied from involvement of only a single, or part of a single, lobe (designated "+" in Table 4), to almost complete involvement of the whole anterior portions of the lungs (designated "+++" in Table 4).

The third type of change which was recognised in the lungs also consisted of areas of pneumonia but these were different in nature from the hyperaemic, exudative areas previously described. These areas of non-exudative pneumonia could be found in all lobes of the lungs but were more common in the anterior lobes where they were often present at the edges, rather than in the centre, of the lung lobes (Fig. 7). These areas were firm in consistency and a homogenous grey-pink in colour; they were usually well-defined from the surrounding tissue and were sometimes slightly raised above the level of the adjacent, deflated lung; on section, there was no exudation of fluid from the cut surface and pus could not be expressed from the bronchial tree. Areas of non-exudative pneumonia varied from the presence of individual, or a few, small foci (designated "+" in Table 4) to involvement of up to approximately 40% of the lung (designated "+++" in Table 4).

From Table 4, it can be seen that pulmonary oedema was present in 47 of the dogs examined, exudative pneumonia in 18, non-exudative pneumonia in 10 and that all 3 changes could be found in any individual animal e.g. D14 (Fig. 1). In only 1 dog (D28) was there a different macroscopic appearance : in this animal multiple, 2 mm. diameter, grey-white, nodules were found on the pleural surface and throughout the substance of all the lung lobes; these nodules contained a rather crumbly, greyish material. In 2 dogs (D39 and D40) no macroscopic pathological changes could be detected; further investigation of the clinical history of these dogs revealed that they had, apparently, recovered from the disease only to relapse with severe neurological signs.

In the majority of the dogs examined, the tracheobronchial and retropharyngeal lymph nodes were enlarged up to twice normal size and were often congested; the palatine tonsils were also enlarged in approximately half the dogs.

Other macroscopic pathological changes which were regularly found were thymic atrophy and hyperkeratosis of either or both the rhinarium and foot pad. Complete or almost complete thymic atrophy (Figs. 8 and 9) was found in 17 animals and in many other dogs the thymus seemed smaller than would have been expected in that age of animal; no attempt was made to quantify this change. Hyperkeratosis of the rhinarium was often associated with a mucopurulent nasal discharge.

Microscopic : Microscopic pathological changes were invariably present in the lungs of all dogs examined; 4 distinct forms of change could be recognised, although not all of these were necessarily present in any individual dog. The incidence and severity of these changes are summarised in Table 5.

The most consistent finding was the presence of some form of bronchitis and, more especially, bronchiolitis. In its mildest form, this consisted of focal bronchial and bronchiolar epithelial disorganisation and necrosis; eosinophilic intracytoplasmic inclusion bodies could, occasionally, be found in epithelial cells in such areas. There was often

Dog Number	Macroscopic Findings	Microscopic Findings			
		Bronchitis/Bronchiolitis	Oedema	Exudative pneumonia	Proliferative interstitial pneumonia
D39	NAD	+	-	-	-
D40	NAD	+	-	-	-
D2	O	++	++	-	-
D4	O	++	++	-	-
D5	O	++	++	-	-
D6	O	+	++	-	-
D9	O	+++	+++	-	-
D10	O	++	++	-	-
D12	O	++	++	-	+
D21	O	+	+	-	-
D23	O	+++	+++	-	-
D24	O	+++	+++	-	-
D25	O	++	+++	-	-
D26	O	++	+++	-	-
D29	O	+	++	-	-
D31	O	+	++	-	-
D32	O	+	++	-	-
D33	O	+	++	-	-
D35	O	+	++	-	+
D37	O	+	++	-	-
D41	O	++	++	-	-
D44	O	++	++	-	-
D45	O	++	+++	-	+

O = Oedema P = Non-exudative pneumonia NAD = No abnormalities detected Ex = Exudative pneumonia = Not present

Table 5 : Canine distemper survey - Microscopic findings at post mortem examination and their relationship to the macroscopic appearance of the lungs.

Dog Number	Macroscopic Findings	Microscopic Findings			
		Bronchitis/Bronchiolitis	Oedema	Exudative pneumonia	Proliferative interstitial pneumonia
D1	O, Ex	++	++	+++	-
D3	O, Ex	++	+	+++	-
D8	O, Ex	++	+	+++	-
D13	O, Ex	++	++	+++	-
D15	O, Ex	+++	+	++	++
D18	O, Ex	+	+	+	+++
D19	O, Ex	+++	++	+++	-
D20	O, Ex	++	++	++	-
D30	O, Ex	+++	+	+++	+++
D34	O, Ex	++	+	+++	-
D36	O, Ex	++	+	+++	-
D38	O, Ex	+++	++	+++	-
D42	O, Ex	+++	+	+++	-
D43	O, Ex	+++	+	++	+++
D48	O, Ex	++	++	+++	-
D49	O, Ex	+++	++	+++	++
D7	O, P	++	++	-	+++
D11	O, P	+	+	-	++
D16	O, P	++	++	-	+
D22	O, P	++	+	-	+++
D27	O, P	++	+	-	++
D46	O, P	++	++	-	++

Table 5 (continued) : Canine distemper survey - Microscopic findings at post mortem examination and their relationship to the macroscopic appearance of the lungs.

Dog Number	Macroscopic Findings	Microscopic Findings			
		Bronchitis/Bronchiolitis	Oedema	Exudative pneumonia	Proliferative interstitial pneumonia
D47	O, P	+++	++	-	+++
D50	O, P	+++	+	-	+++
D14	O, P, Ex	++	++	++	++
D17	O, P, Ex	++	++	+	+++
D28	Multiple nodules	Focal necrosis with interstitial pneumonia			

Table 5 (continued) : Canine distemper survey - Microscopic findings at post mortem examination and their relationship to the macroscopic appearance of the lungs

some congestion of vessels in the underlying lamina propria of such areas and slight infiltration of macrophages into the epithelium and lumen. This type of change was designated as "+" in Table 5. In more severe forms ("++"), there was more extensive epithelial denudation and increased cellular infiltration. In cases designated as "+++" there was extensive disorganisation and sloughing of the bronchial epithelium with infiltration by macrophages and a few polymorphonuclear leucocytes and almost complete loss of the bronchiolar epithelium. In the bronchi and larger bronchioles, vessels in the underlying lamina propria were usually congested and a cellular infiltrate, composed mainly of lymphocytes with some macrophages and a few plasma cells, was present in the lamina propria and submucosa. In the smaller bronchioles, there was also infiltration by lymphocytes and macrophages and many macrophages were present in the surrounding alveolar airspaces.

In dogs in which, histologically, exudative pneumonia was also present, changes in the bronchial tree were the more complex and will be described with those exudative changes (see below). In 2 dogs (D39 and D40) changes in the bronchial tree took the form of focal peribronchial and peribronchiolar accumulations of lymphocytes with some plasma cells and macrophages; similar cellular aggregations were also found around small venules in the lungs of these dogs; this change was also designated as an "+" bronchitis/bronchiolitis.

Oedema was recognised in almost all the dogs examined. It varied in severity from patchy intra-alveolar oedema ("+") to diffuse intra-alveolar oedema associated with severe congestion of the alveolar mural capillaries and oedema of the peribronchial and perivascular connective tissue ("+++"); in dogs with severe oedema, increased numbers of alveolar macrophages were usually present in the alveoli. Hyaline membranes were not recognised in the dogs examined.

Changes associated with an exudative pneumonia were found in a total of 18 dogs. In these animals there was a moderate or severe bronchitis and bronchiolitis in which, in addition to the changes described above, there was a polymorphonuclear leucocyte response; the

lamina propria was congested and oedematous and polymorphonuclear leucocytes as well as mononuclear cells were present in the lamina propria, epithelium and lumen (Fig. 10). In many of these dogs, bacterial colonies could be seen in the luminal exudate and, in several animals (D3, D8, D36, D42 and D49) bacteria were found amongst the cilia of remaining bronchial epithelial cells (Fig. 11). Associated with these changes in the bronchial tree were surrounding areas of mixed polymorphonuclear leucocyte and macrophage infiltration into the alveolar airspaces (Fig. 10). The alveolar mural capillaries were congested and the alveolar walls were slightly thickened as a result of the presence of polymorphonuclear leucocytes and mononuclear cells within them. Intra-cytoplasmic and, occasionally, intranuclear bodies could also be found in bronchial and bronchiolar epithelial cells (Figs. 11 and 12) and in alveolar macrophages.

Exudative pneumonia varied in severity from a localised distribution around severely affected bronchioles ("+") to involvement of a majority of the lung tissue in the sections examined ("+++").

The final change which was recognised on histopathological examination of the lungs was the presence of some degree of a proliferative interstitial pneumonia. In mild cases ("+") there was thickening of the alveolar septae by oedema and increased numbers of mainly mononuclear cells while the alveolar epithelial cells themselves were hyperplastic (Fig. 13). In more severe cases ("++") there was more marked thickening of the alveolar walls and more extensive alveolar epithelial hyperplasia, the classical "epithelialisation" lesion of distemper; large mononuclear cells, possibly alveolar macrophages or desquamated epithelial cells were often present in the alveolar lumen (Fig. 14).

In the most severe cases of interstitial pneumonia ("+++") there was marked thickening of the alveolar walls, often as a result of fibrosis; the alveoli were lined by thick squamous or low cuboidal cells which gave the appearance of epithelial proliferation (Fig. 15). The alveolar airspaces contained a mixture of large cells, again possibly desquamated

epithelial cells or alveolar macrophages; these cells and the hyperplastic alveolar lining cells were, not uncommonly, binucleate and, occasionally, giant cells were found within the alveoli. Lymphocytes and a few polymorphonuclear leucocytes, were also found in the alveolar lumina and a few polymorphonuclear leucocytes were also found in the alveolar lumina and walls. Eosinophilic intracytoplasmic and intranuclear inclusion bodies were frequently present in cells in the alveoli. The bronchiolar epithelium in areas with severe proliferative interstitial pneumonia was often dedifferentiated, having an almost stratified squamous appearance. (Fig. 16).

Examination of Table 5 shows that the 4 different histopathological changes which were recognised occurred in a total of 5 different combinations.

The most frequently occurring combination (18 dogs) was that of bronchitis/bronchiolitis and pulmonary oedema; the severity of these changes were, in general, fairly closely related i.e. severe diffuse pulmonary oedema was normally associated with severe bronchitis/ bronchiolitis. Dogs with this combination of microscopic changes had macroscopic evidence of pulmonary oedema.

Bronchitis/bronchiolitis, oedema and exudative pneumonia were found together in a total of 11 dogs; the exudative pneumonic component was usually severe (in 10 dogs). Macroscopically, this combination of histopathological findings was recognised as exudative pneumonia with oedema.

Bronchitis/bronchiolitis, oedema and proliferative interstitial pneumonia were also found in combination in a total of 11 dogs. In those cases in which the proliferative interstitial pneumonia was graded as moderate or severe (7 animals), the corresponding macroscopic findings were oedema and non-exudative pneumonia. Of the 4 dogs in which there was only mild, proliferative, interstitial pneumonia, however, only 1 had macroscopically appreciable pneumonia; in the remaining 3 animals, only pulmonary oedema was recognisable at post mortem examination.

All 4 histopathological changes were found in combination in a total of 7 animals. In all of these dogs the proliferative interstitial pneumonia was classified as either moderate or severe, but in only 2 dogs (D14 and D17) was the proliferative interstitial pneumonia recognised macroscopically as distinct foci of non-exudative pneumonia. In the remaining 5 dogs and also in some areas of the lungs of D14 and D17 there was a mixed pneumonic reaction with components of the exudative and proliferative interstitial lesions being present in the same areas (Fig. 17); macroscopically such mixed areas were indistinguishable from the simple exudative pneumonia.

The final histological grouping was that which was seen in D39 and D40 in which only a slight bronchitis and bronchiolitis characterised by focal mononuclear cell accumulations were found. Macroscopically, the lungs of these animals, which had been killed late in the course of disease, had appeared normal; the mononuclear cell accumulations may represent an end-stage lesion.

In only 1 dog (D28) did the histopathological changes in the lung differ from the lesions described above. In D28, multiple foci of necrosis were found throughout the lung substance; in these necrotic areas, small, often intracellular, toxoplasma-like organisms could be seen; between the necrotic areas, the lung tissue was collapsed and oedematous with thickening of the alveolar walls by a mixed cellular infiltrate of polymorphonuclear leucocytes and mononuclear cells.

Histopathological changes associated with CDV infection were also present in other organs examined. Tracheitis was frequently present and the changes associated with this were usually similar to those found in the bronchial tree of the same animal. Tracheitis thus varied in severity from focal areas of epithelial disorganisation and necrosis with congestion and mononuclear cell infiltration (Fig. 18) to extensive areas of epithelial necrosis, severe congestion and oedema of the underlying tissues and mixed polymorphonuclear leucocyte and mononuclear cell infiltration.

Eosinophilic intracytoplasmic and, occasionally, intranuclear inclusion bodies were found in degenerating tracheal epithelial cells.

In dogs in which bacteria were found in the cilia of the bronchial epithelium (D3, D8, D38, D42, D49), bacteria could also be located in the cilia of tracheal epithelial cells; a heavy polymorphonuclear leucocyte infiltrate of the lamina propria and epithelium was usually associated with this finding. In D39, small groups of bacteria were found scattered in tracheal epithelial cilia; these were associated, in this dog, with only slight polymorphonuclear infiltration - the main finding was the presence of scattered cell aggregations in the lamina propria and submucosa.

Histopathological changes were consistently found in the lymph nodes and tonsils of all dogs examined. These changes most commonly took the form of varying degrees of lymphocytolysis with consequent loss of normal lymph node and tonsillar structure (Figs. 19 and 20). Many macrophages were found amongst the disrupted and pyknotic lymphoid cells and the lymphatic sinusoidal lining cells were large and active, often containing phagocytosed debris. Congestion and sinusoidal oedema were frequently present and, in some animals, polymorphonuclear leucocytes were found, in some numbers, in the sinuses and medullary cords.

The macrophages present in the lymph nodes and tonsil were often binucleate and, in 6 dogs, multinucleated giant cells were also present (Fig. 21). Intracytoplasmic inclusion bodies were, occasionally, found in these macrophages and in the hyperplastic sinusoidal lining cells. The tonsillar epithelium was frequently vacuolated and infiltrated by mononuclear cells and polymorphonuclear leucocytes; intracytoplasmic inclusions could be found in tonsillar epithelial cells (Fig. 22). In a few animals, notably D39 and D40, lymphocytolysis was not a feature; in these dogs, lymphoid follicular hyperplasia was evident in the cortex and large numbers of plasma cells were found in the medullary cords.

In those dogs with clinical neurological disturbances (a total of 17 animals including D39 and D40) there were varying degrees of encephalitis mainly involving the hind brain and the areas around the ventricular system. These areas were characterised by demyelination, gliosis and perivascular cuffing by mononuclear cells; eosinophilic intranuclear and intracytoplasmic inclusion bodies could be found in cells in the affected areas.

Immunofluorescence Findings

The results of immunofluorescence examination of the lung, tonsil and lymph node for CDV antigen are presented in Table 6. In 43 dogs, CDV antigen was demonstrated at all 3 sites whilst, in an additional 4 dogs, antigen was demonstrated at 2 sites. Even at relatively low powers of magnification, positive immunofluorescence was distinctive, the CDV antigen appearing very bright apple green against the dark, unstained, background tissue (Fig.23); at higher powers of magnification, fluorescence was seen to be intracytoplasmic and to have a characteristic granular pattern (Fig. 24).

In sections from tonsil, individual affected cells could be located in the tonsillar crypts and epithelium (Fig. 23) but, in the underlying lymphoid tissue, fluorescence tended to occur in groups of cells and was often extensive (Fig.23) with a majority of the cells present being stained. In sections from lymph node, extensive areas of fluorescence were often present but again, individual fluorescent cells could be located, usually in the subcapsular sinus. In the lung, CDV antigen was consistently present in bronchial and bronchiolar epithelial cells (Fig.24) and was also demonstrated in cells present in the alveolar walls and alveolar airspaces.

In only 3 dogs (D21, D39 and D40) was there failure to demonstrate CDV antigen in lung, tonsil or lymph node. These 3 dogs were amongst those which had been destroyed following the development late in the course of the disease of severe neurologic disturbances; further immunofluorescence investigations demonstrated the presence of CDV antigen in tissues taken from the hind brain of these animals.

Dog Number	Lung	Tonsil	Lymph Node	Dog Number	Lung	Tonsil	Lymph Node
D1	++	+++	+++	D26	++	++	++
D2	++	+	+	D27	++++	++++	++
D3	++++	++	++	D28	++	++	+++
D4	++	+++	+++	D29	++	+	+
D5	+	++	+	D30	+++	+++	+
D6	-	+	+	D31	+	++	++
D7	++	+++	++++	D32	++	-	+
D8	+	++	++	D33	++	++	+
D9	++	++++	++	D34	+	+	++
D10	+++	++++	++++	D35	+	+	+
D11	++	+	+	D36	+	++	+
D12	++	++	+++	D37	++	+++	++
D13	+	++	++	D38	+	++	++
D14	+	+	++	D39	-	-	-
D15	+	+	++	D40	-	-	-
D16	-	+	+	D41	+	++	++
D17	+	++	++	D42	++	+++	+++
D18	+	+	++	D43	++	++	++
D19	+	+	+	D44	+	+++	++
D20	+	+++	+++	D45	++	+	+
D21	-	-	-	D46	++	++	+
D22	-	+++	+++	D47	+	++	++
D23	+	+++	++++	D48	++	+++	+++
D24	++	+	++++	D49	++	++	+++
D25	+++	+	++	D50	++	++	++

Results graded + to ++++ on amounts of antigen present.

Table 6 : Canine distemper survey - results of immunofluorescence examination for CDV antigen

Bacteriological Findings

The results of bacteriological examination of the trachea, bronchus and lung substance of each dog are shown in Table 7. The bacterial species recovered from these sites were Bord. bronchiseptica (21 dogs), E. coli (10 dogs), Staph. spp. (6 dogs), beta-haemolytic streptococci (3 dogs), non-haemolytic streptococci (2 dogs), Proteus spp. (2 dogs) and a Mycoplasma spp. (1 dog); in 14 dogs no bacteria could be isolated from any of the respiratory tract sites examined.

Bord. bronchiseptica, the organism most frequently isolated, was found, often in heavy culture, in the bronchus of 21 dogs; in 18 of these animals, Bord. bronchiseptica was the only bacterium recovered from this site. Bord. bronchiseptica was recovered almost as frequently, in 20 dogs, from the trachea; at this site, it was again often present in heavy culture but in only 13 dogs was it the only microorganism present. Bord. bronchiseptica was found in the lung substance of 16 animals; in 15 of these dogs it was present in pure culture but often at this site there was only very sparse growth of the organism.

Of the other microorganisms recovered, beta-haemolytic Strep. spp. and Proteus spp. were, when isolated, usually present in large numbers and at more than 1 level of the lower respiratory tract. In contrast, E. coli and Staph. spp. were usually present in only small numbers and were often found only in the trachea; similarly the non-haemolytic Strep. spp. and the Mycoplasma sp. were isolated only from the trachea and again were present in only small numbers. As there was, in some of the animals examined in this survey, a lapse of up to 24 hrs. between death and post mortem examination, it was possible that some of the sparse bacterial cultures isolated from the trachea were the result of post mortem spread from the contiguous oropharynx.

Dog Number	Bacteria recovered from		
	Trachea	Bronchus	Lung substance
D1	Bord. bronchiseptica, E. coli	Bord. bronchiseptica	Bord. bronchiseptica
D2	-	-	-
D3	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
D4	E. coli	-	-
D5	Bord. bronchiseptica	Bord. bronchiseptica	-
D6	Proteus sp.	Proteus sp.	Proteus sp.
D7	-	-	-
D8	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
D9	Bord. bronchiseptica, Staph. sp.	Bord. bronchiseptica	Bord. bronchiseptica
D10	-	-	-
D11	-	-	-
D12	Staph. sp. Proteus sp.	Staph. sp. Proteus sp.	Staph. sp.
D13	Bord. bronchiseptica, Mycoplasma sp.	Bord. bronchiseptica	Bord. bronchiseptica
D14	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
D15	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
D16	E. coli	-	-
D17	Bord. bronchiseptica, Staph. sp.	Bord. bronchiseptica	-
D18	Bord. bronchiseptica, β -haem Strep. sp.	Bord. bronchiseptica, β -haem Strep. sp.	Bord. bronchiseptica
D19	Bord. bronchiseptica, E. coli	Bord. bronchiseptica, E. coli	Bord. bronchiseptica
D20	Bord. bronchiseptica	Bord. bronchiseptica	-
D21	Staph. sp.	-	-
D22	E. coli	-	-
D23	Bord. bronchiseptica	Bord. bronchiseptica	-
D24	-	-	-
D25	-	-	-

Table 7 : Canine distemper survey - recovery of bacteria from the respiratory tract at post mortem examination. - = No growth

Dog Number	Bacteria recovered from		
	Trachea	Bronchus	Lung substance
D26	-	-	-
D27	Staph. sp.	-	-
D28	-	-	-
D29	E. coli	E. coli	E. coli
D30	-	Bord. bronchiseptica	Bord. bronchiseptica
D31	Strep. sp. (non-haemolytic)	-	-
D32	Bord. bronchiseptica	Bord. bronchiseptica	-
D33	-	-	-
D34	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
D35	-	-	-
D36	Bord. bronchiseptica E. coli	Bord. bronchiseptica, E. coli	Bord. bronchiseptica, E. coli
D37	-	-	-
D38	E. coli	E. coli	E. coli
D39	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
D40	-	-	-
D41	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
D42	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
D43	β -haem Strep. sp	β -haem Strep. sp	β -haem Strep. sp
D44	E. coli	E. coli	E. coli
D45	E. coli	E. coli	E. coli
D46	Strep. sp (non-haemolytic)	-	-
D47	-	-	-
D48	Staph. sp	Staph. sp. β -haem. Strep. sp	Staph. sp
D49	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
D50	-	-	-

Table 7 : (contd.) Canine distemper survey - recovery of bacteria from the respiratory tract at post mortem examination.
- = No growth

It was considered that the situation prevailing in the live animal was more likely to be accurately represented, in this survey, by the results of examination of the bronchial tree and lung substance and in Table 8, the pathological findings in the lungs of these 50 dogs examined are related to the recovery of bacteria from these sites. It is apparent, from Table 8, that the presence of an exudative pneumonia in the dogs with distemper was invariably associated with the presence of bacteria in the lower respiratory tract. Bord. bronchiseptica was the bacterium most frequently recovered from the 18 dogs in which exudative pneumonia was present; this bacterium was isolated from 15 of these 18 dogs, being present in pure culture in 12, in combination with E. coli in 2 and with a beta-haemolytic streptococci in 1. E. coli, a staphylococcus and a beta-haemolytic streptococcus were each isolated in pure culture from the lower respiratory tract of 1 dog with exudative pneumonia.

Of the 32 animals in which exudative pneumonia was not present, bacteria were recovered from the lower respiratory tract of only 11. Bord. bronchiseptica was, once again, the bacterium most frequently isolated, being present in pure culture in 6 of these 11 dogs. It may be significant that the degree of bronchitis/bronchiolitis present in the 6 dogs from which Bord. bronchiseptica was isolated, was graded as moderate or severe with the exception of D39 in which the pathological changes appeared to be late-stage resolving lesions. The other bacterial species recovered were E. coli from 3 dogs, Proteus sp. from 2 dogs and a staphylococcus from 1 dog; the degree of bronchitis/bronchiolitis associated with these bacteria was either mild or moderate.

Bacteriological examination was also carried out on samples of tracheobronchial and retropharyngeal lymph nodes and palatine tonsils taken from each dog. The bacteria recovered from these sites were non-haemolytic, alpha- and beta-haemolytic streptococci, Pasteurella spp. E. coli and staphylococci; growth of bacteria from these sites was never heavy, approximately two-thirds of the samples examined were

Dog Number	Microscopic findings	Bacteria recovered from bronchus & lung
D39	Br	Bord. bronchiseptica
D40	Br	-
D2	Br, O	-
D4	Br, O	-
D5	Br, O	Bord. bronchiseptica
D6	Br, O	Proteus sp.
D9	Br, O	Bord. bronchiseptica
D10	Br, O	-
D21	Br, O	-
D23	Br, O	Bord. bronchiseptica
D24	Br, O	-
D25	Br, O	-
D26	Br, O	-
D29	Br, O	E. coli
D31	Br, O	-
D32	Br, O	Bord. bronchiseptica
D33	Br, O	-
D37	Br, O	-
D41	Br, O	Bord. bronchiseptica
D44	Br, O	E. coli
D7	Br, O, Pr	-
D11	Br, O, Pr	-
D12	Br, O, Pr	Staph. sp. Proteus sp.
D16	Br, O, Pr	-
D22	Br, O, Pr	-
D27	Br, O, Pr	-
D35	Br, O, Pr	-
D45	Br, O, Pr	E. coli
D46	Br, O, Pr	-
D47	Br, O, Pr	-
D50	Br, O, Pr	-
D1	Br, O, Ex	Bord. bronchiseptica
D3	Br, O, Ex	Bord. bronchiseptica
D8	Br, O, Ex	Bord. bronchiseptica
D13	Br, O, Ex	Bord. bronchiseptica
D19	Br, O, Ex	Bord. bronchiseptica
D20	Br, O, Ex	Bord. bronchiseptica
D34	Br, O, Ex	Bord. bronchiseptica
D36	Br, O, Ex	Bord. bronchiseptica E. coli
D38	Br, O, Ex	E. coli
D42	Br, O, Ex	Bord. bronchiseptica
D48	Br, O, Ex	Staph. sp.

Br = Bronchitis/Bronchiolitis O = Oedema Ex = Exudative pneumonia Pr = Proliferative interstitial pneumonia - = No growth

Table 8 : Canine distemper survey - Relationship of microscopic findings to lower respiratory tract bacteriology.

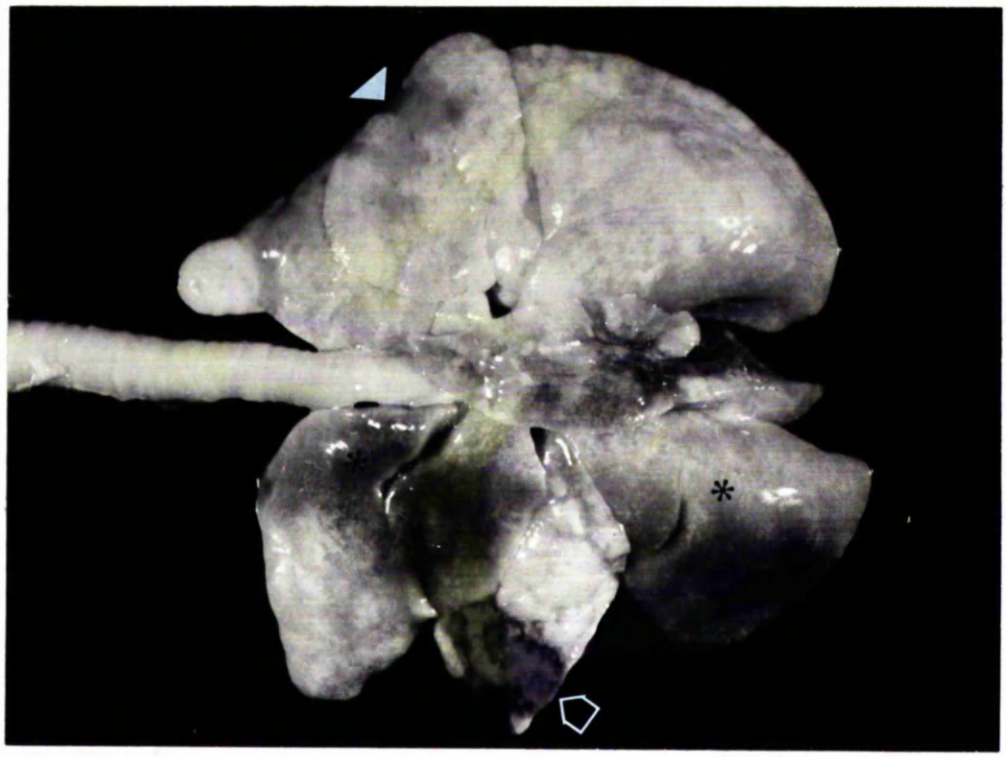
Dog Number	Microscopic findings	Bacteria recovered from bronchus & lung	Dog Number	Microscopic findings	Bacteria recovered from bronchus & lung
D14	Br, O, Ex, Pr	Bord. bronchiseptica	D30	Br, O, Ex, Pr	Bord. bronchis ptica
D15	Br, O, Ex, Pr	Bord. bronchiseptica	D43	Br, O, Ex, Pr	β - haem. Strep. sp.
D17	Br, O, Ex, Pr	Bord. bronchiseptica	D49	Br, O, Ex, Pr	Bord. bronchiseptica
D18	Br, O, Ex, Pr	Bord. bronchiseptica -haem. Strep. sp	D28	Multiple necrotic foci	-

Table 8 (continued) : Canine distemper survey - Microscopic findings at post mortem examination and their relationship to the macroscopic appearance of the lungs.

bacteriologically sterile and only occasionally was more than 1 bacterial species recovered from any individual sample. The recovery of bacteria from these sites appeared to be independent of the respiratory disease status of the animals.

Fig. 1 : Canine distemper - lungs of D14. Dark areas of pulmonary oedema (starred) are visible. Areas of exudative pneumonia (open arrow) and non-exudative pneumonia (closed arrow) are also present.

Fig. 2 : Canine distemper - lungs of D14. There is marked oedema (starred) of the apical and diaphragmatic lung lobes. An area of exudative pneumonia in the cardiac lobe is surrounded by a dark zone of hyperaemia (open arrow).



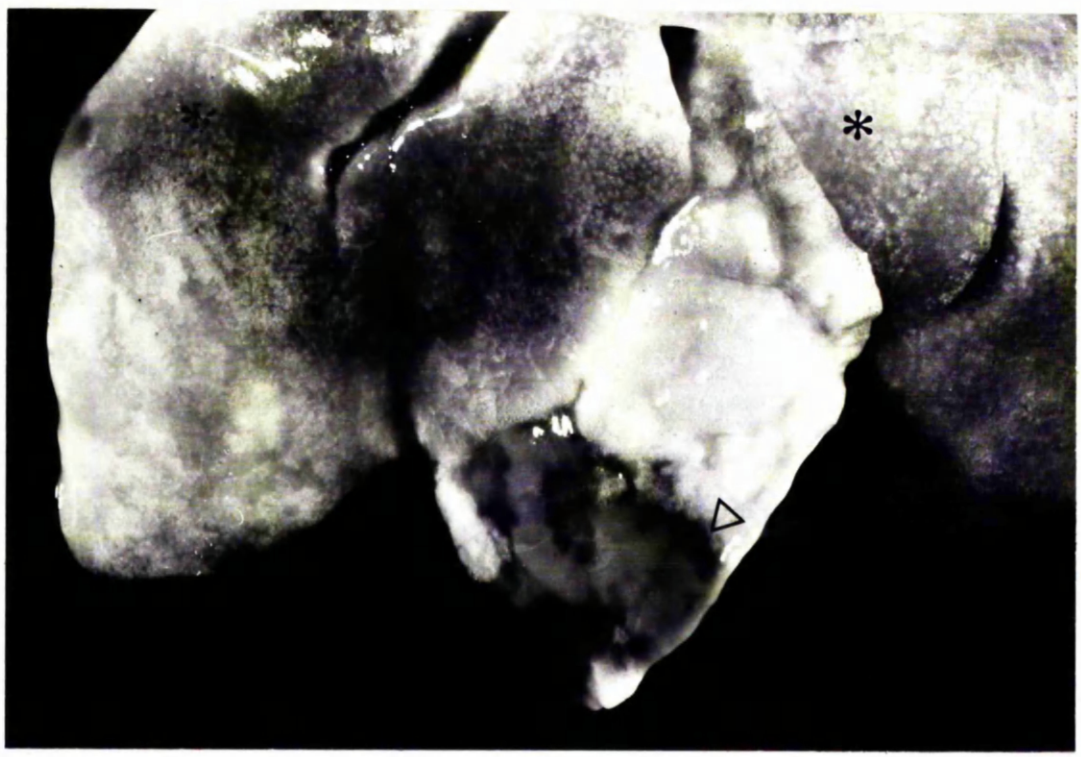


Fig. 3 : Canine distemper - transverse section diaphragmatic lung lobe, D14. The cut surface of the lobe has a translucent, glassy appearance; frothy oedema fluid is visible on the surface.

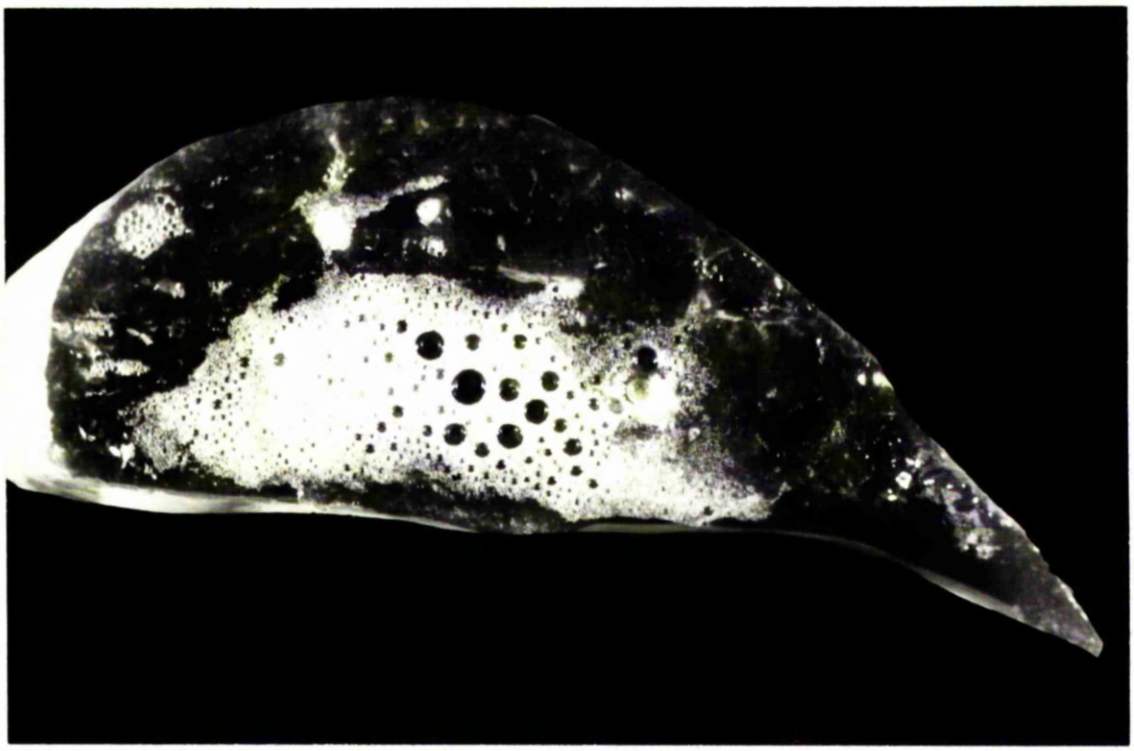


Fig. 4 : Canine distemper - lungs of D13. Well-demarcated areas of exudative pneumonia are present in both left and right anterior lung lobes (arrows). Pulmonary oedema is also evident, particularly in the diaphragmatic lobes.

Fig. 5 : Canine distemper - apical and cardiac lung lobes, D13. Dark areas of exudative pneumonia have a well-demarcated junction (arrows) with remaining lung tissue.

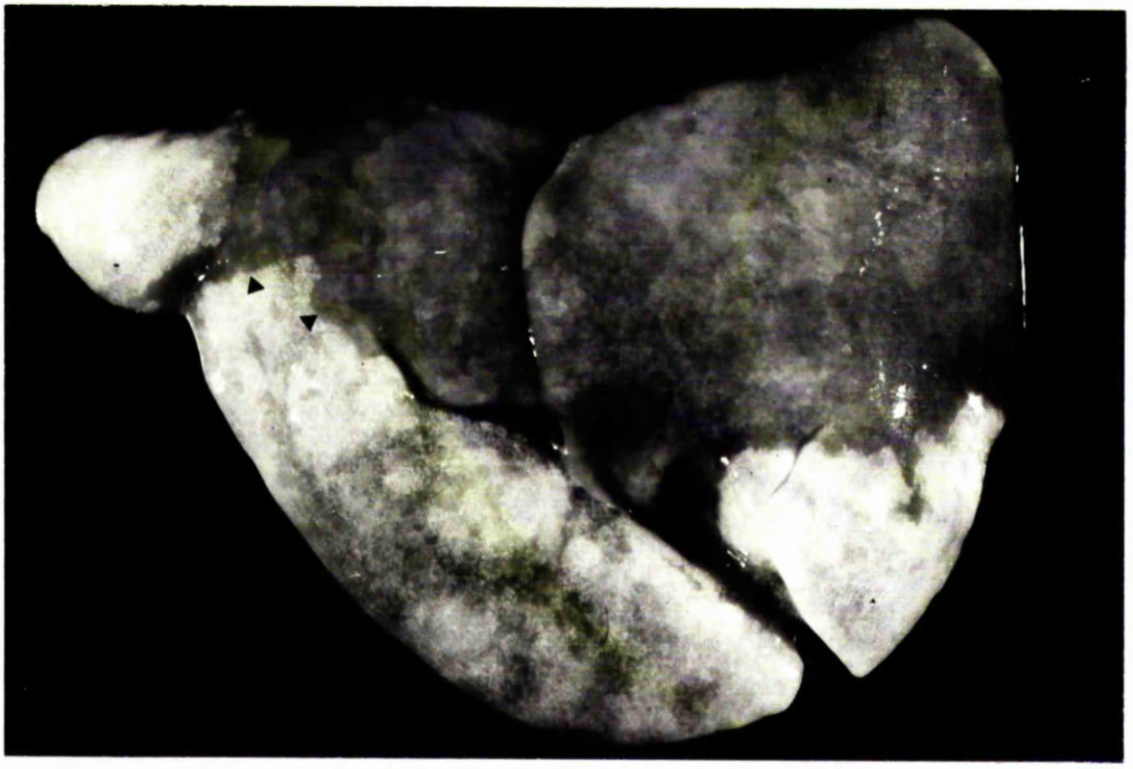


Fig. 6 : Canine distemper - lungs of D20. Patchy, coalescing, dark areas of exudative pneumonia are present in the cardiac and anterioventral diaphragmatic lung lobes.

Fig. 7 : Canine distemper - lungs of D14. Areas of non-exudative pneumonia (arrows) are situated at the edges of both apical and cardiac lung lobes.

Fig. 8 : Normal dog - thymus. The thymus of this 6 month old, healthy dog can be seen (arrowed) in the precardial mediastinum.

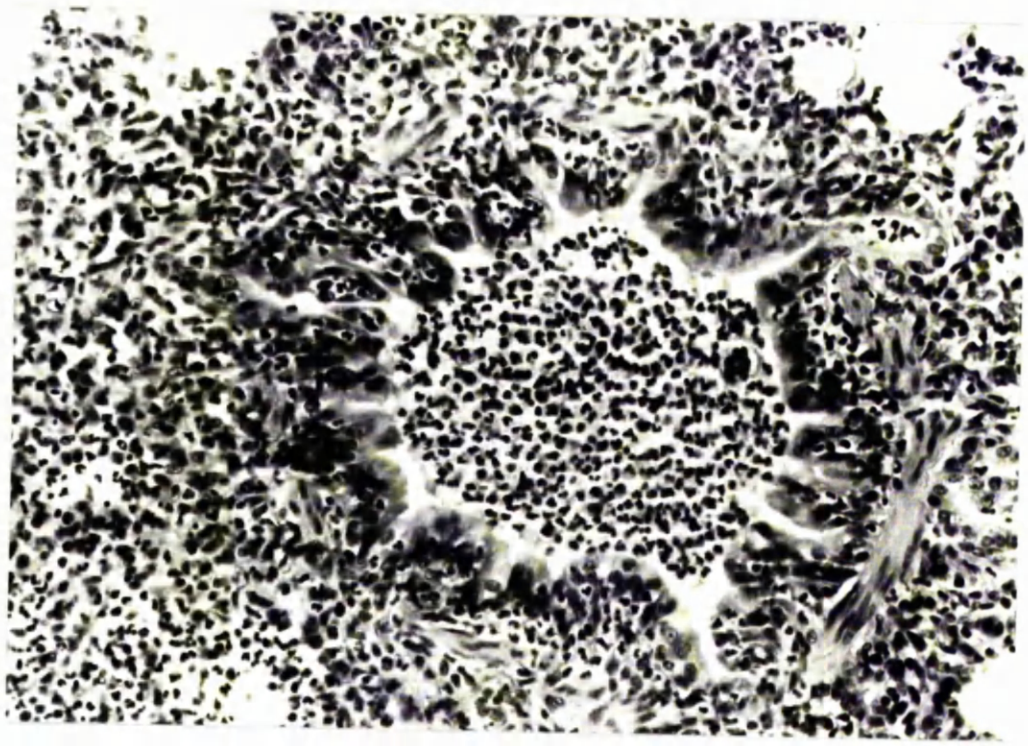
Fig. 9 : Canine distemper - thymic area D14. In this 5 month old dog with canine distemper virus infection there is thymic atrophy. The precardial mediastinum is thin and transparent with blood vessels easily visible within it (arrows); no thymic structure can be distinguished.

Fig. 10 : Canine distemper - bronchiolitis D49. The bronchiolar lumen and the surrounding alveolar air spaces are filled by polymorphonuclear leucocytes and macrophages. The bronchiolar epithelium is heavily infiltrated by polymorphonuclear leucocytes.

(HE, x 250).

Fig. 11 : Canine distemper - bronchitis D49. The bronchial epithelial cells are vacuolated and contain many intracytoplasmic inclusion bodies (small arrows). Small clumps of bacteria are visible amongst remaining epithelial cilia (large arrows). Polymorphonuclear leucocytes and macrophages are present within the epithelium and in the lumen.

(HE, x 400).



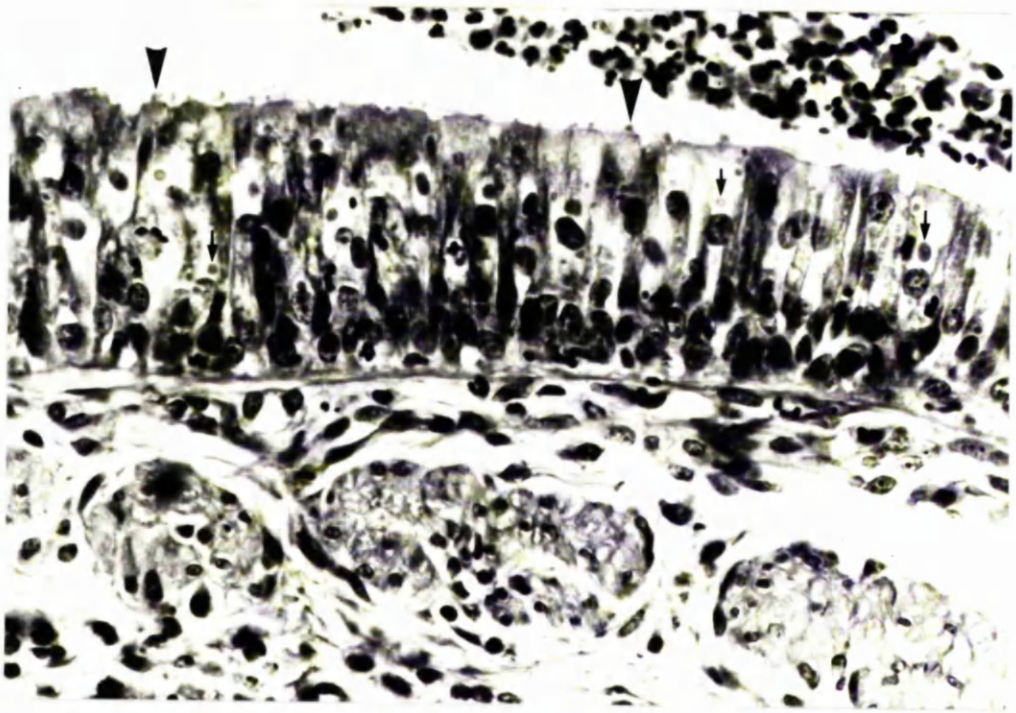


Fig. 12 : Canine distemper - bronchitis D49. Large numbers of bright red intracytoplasmic inclusion bodies (arrow) can be seen within the bronchial epithelial cells. Polymorphonuclear leucocytes are present in the epithelium and in the lumen.

(P. T. I., x 300)

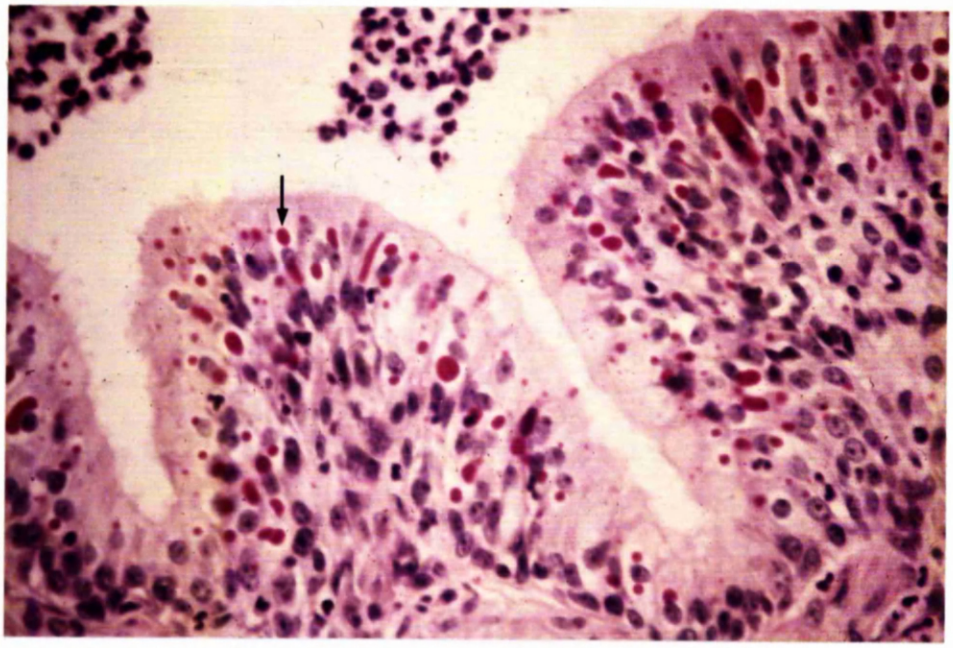
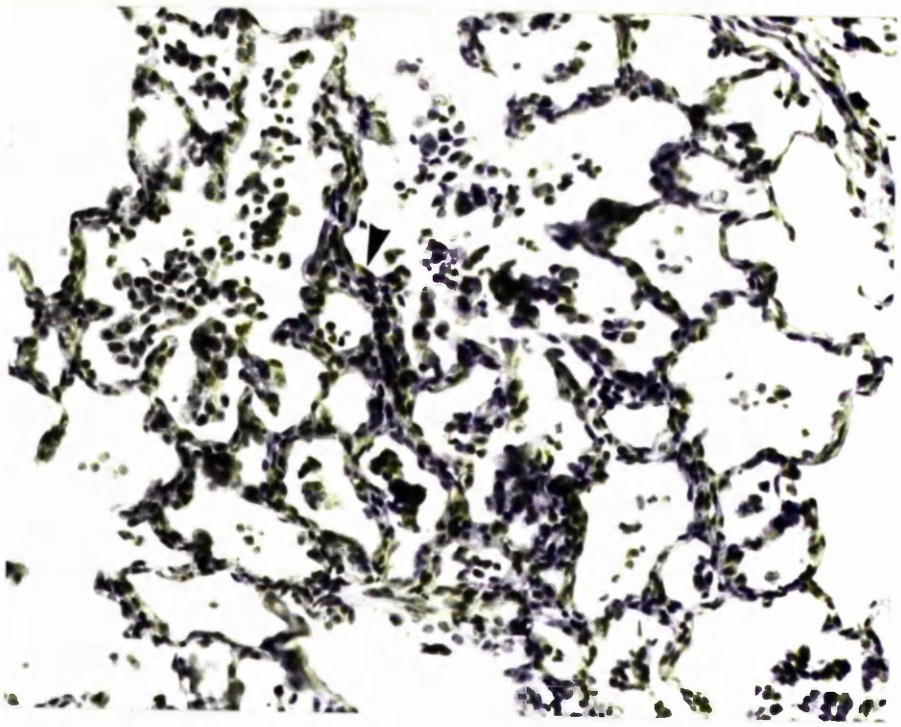


Fig. 13 : Canine distemper - proliferative interstitial pneumonia D12. There is alveolar epithelial hyperplasia. The alveolar septae contain increased numbers of mononuclear cells (arrow) and the alveolar air spaces contain a cellular infiltrate of large mononuclear cells.

(HE, x 250).

Fig. 14 : Canine distemper - proliferative interstitial pneumonia D27. Alveolar epithelial hyperplasia is present and the alveolar septae are markedly thickened by oedema and cellular infiltration. Large mononuclear cells are present in the alveolar air spaces. Note the apparently desquamating alveolar cell (arrow).

(HE, x 300).



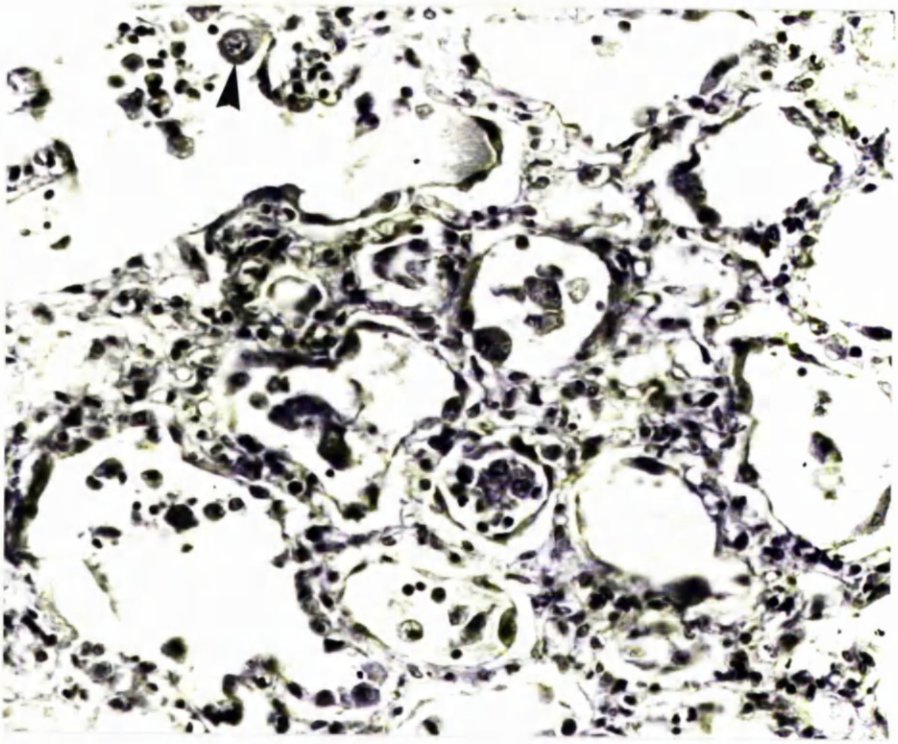
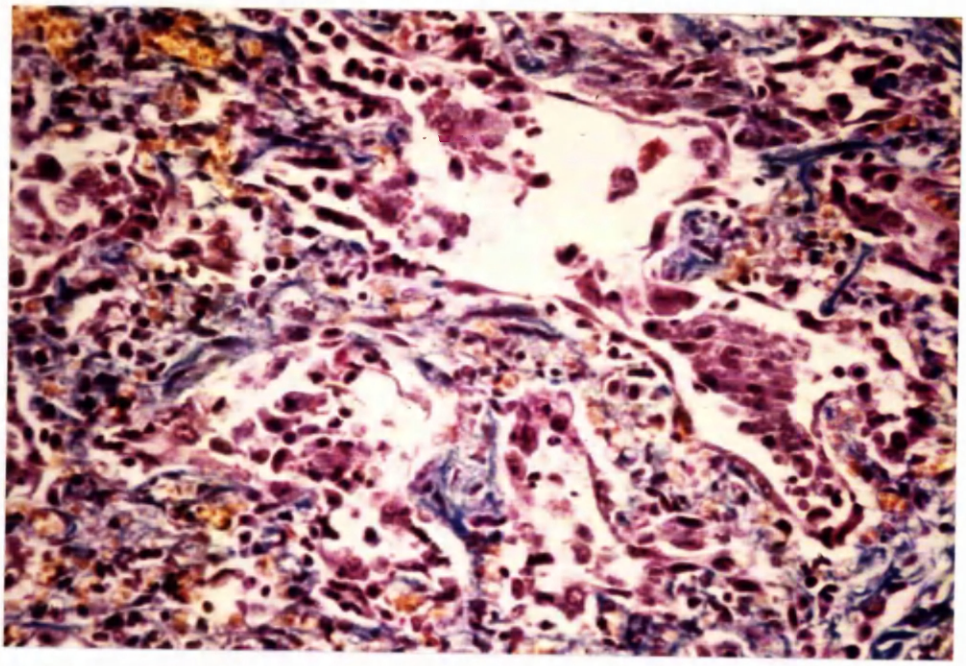


Fig. 15 : Canine distemper - proliferative interstitial pneumonia, D50. There is thickening of the alveolar walls, partially as a result of fibrosis. The alveoli are lined by thick squamous and low cuboidal cells and large mononuclear cells are present in the air spaces.

(MSB, x 300).

Fig. 16 : Canine distemper - bronchiolitis, D50. The bronchiolar epithelium in this area of proliferative interstitial pneumonia is hyperplastic and de-differentiated with, in places, an almost stratified squamous appearance. Note the binucleate cell (small arrow). The adjacent alveolar epithelium is also hyperplastic (large arrow).

(HE, x 250).



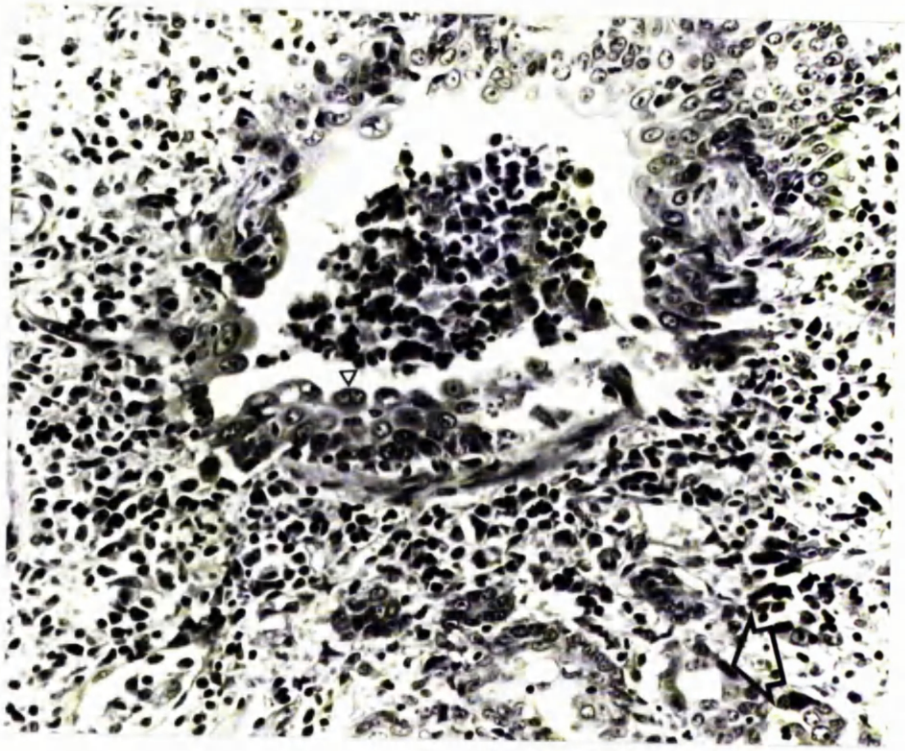
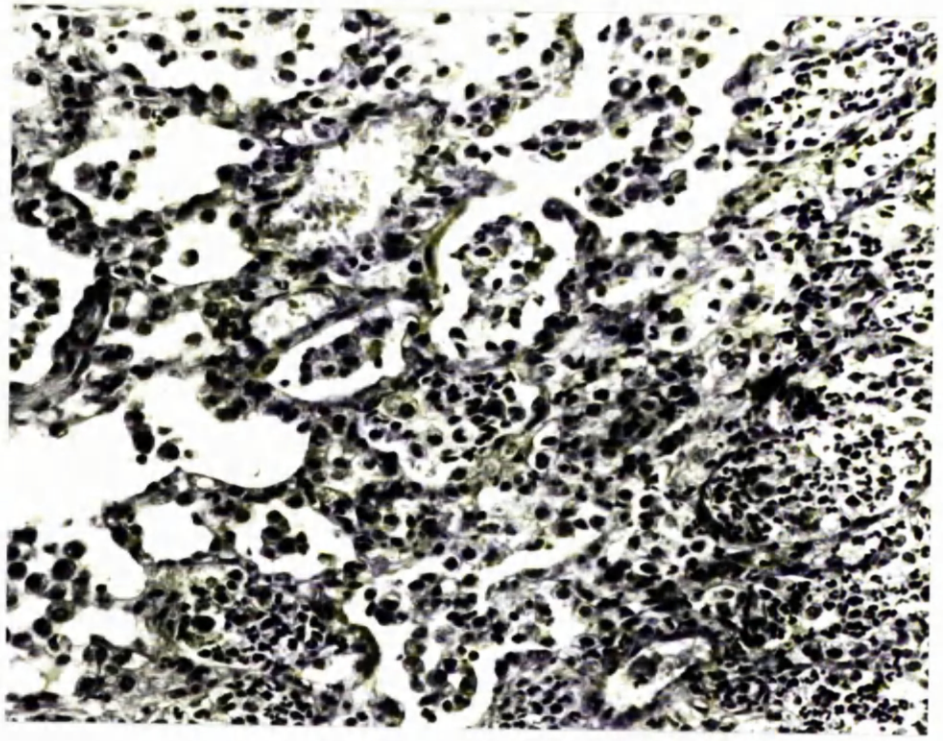


Fig. 17 : Canine distemper - mixed pneumonia, D30. At the top left of this photomicrograph is a proliferative interstitial pneumonia with alveolar epithelial hyperplasia; at the bottom right, the pneumonic reaction is characterised by exudation of polymorphonuclear leucocytes into the alveolar air spaces. A mixed reaction is evident centrally.

(HE, x 250).

Fig. 18 : Canine distemper - tracheitis, D12. There is vacuolation, disorganisation and necrosis of the tracheal epithelium. An intracytoplasmic inclusion body is present in a degenerate epithelial cell (arrow).

(HE, x 250).



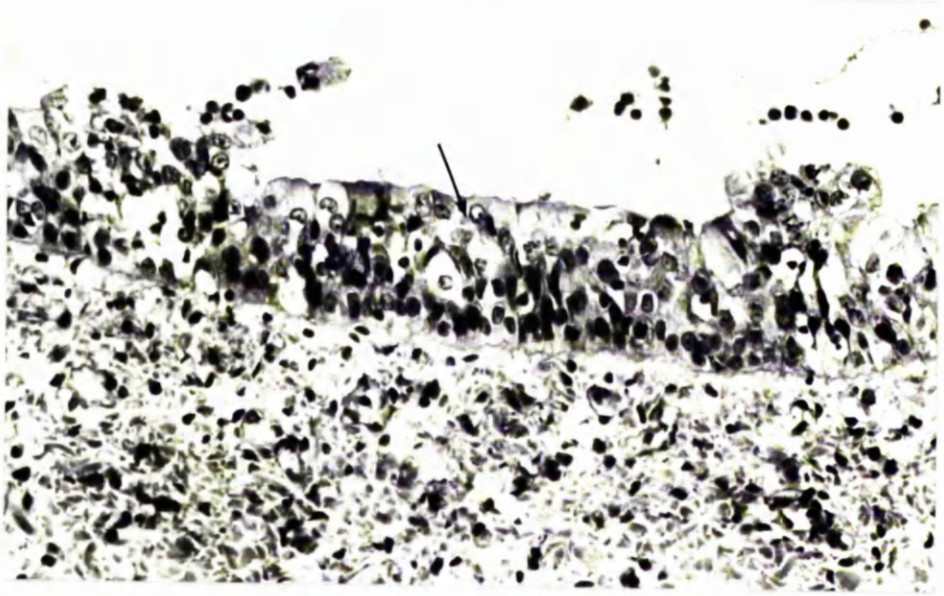
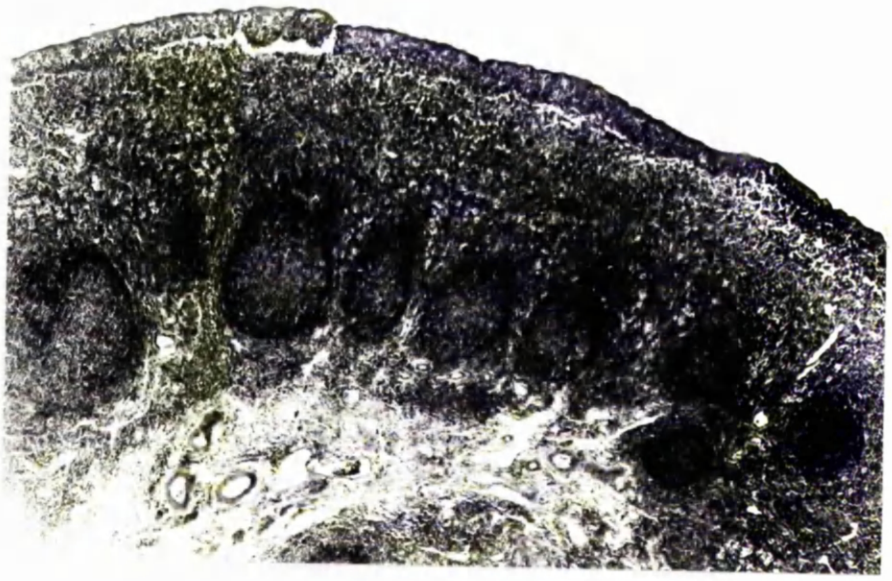


Fig. 19 : Normal dog - tonsil. A dense population of cells is present beneath the tonsillar epithelium and a number of germinal follicles can be seen.

(HE, x 35).

Fig. 20 : Canine distemper - tonsil. There has been extensive lymphocytolysis and the tonsil consequently appears depleted of cells. No germinal follicles are present.

(HE, x 35).



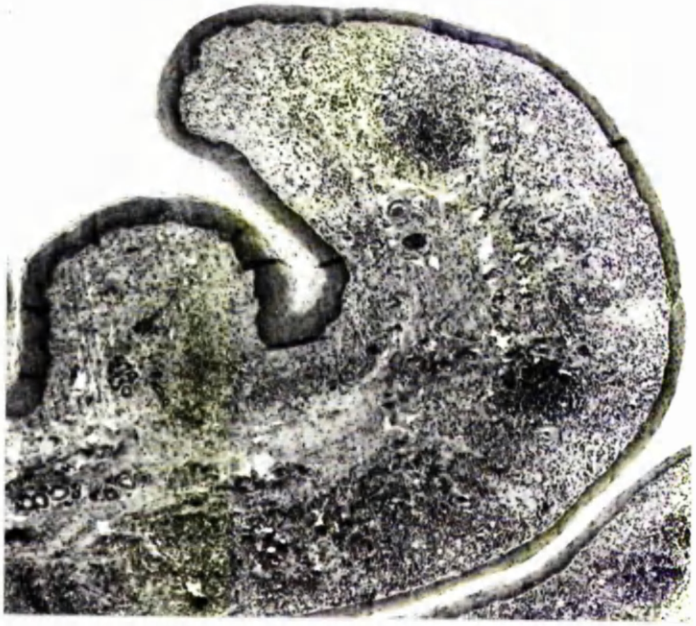
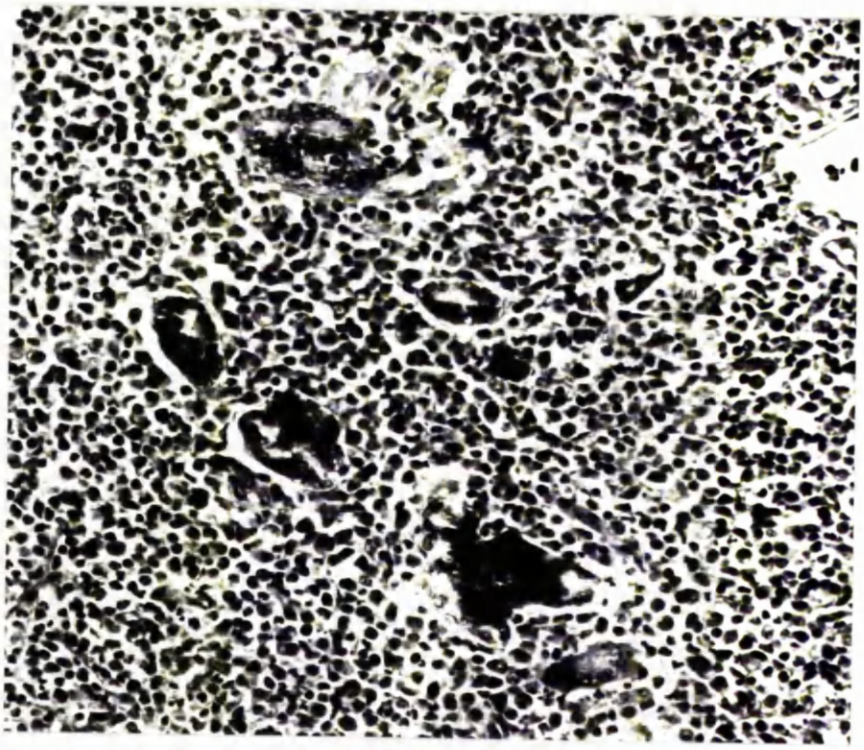


Fig. 21 : Canine distemper - tonsil. The cell population is composed predominantly of macrophages and polymorphonuclear leucocytes; a number of multinucleate giant cells are present.
(HE, x 250).

Fig. 22 : Canine distemper - tonsil. The tonsillar epithelial cells are vacuolated and a number of intracytoplasmic inclusion bodies are visible (arrow).
(HE, x 400).



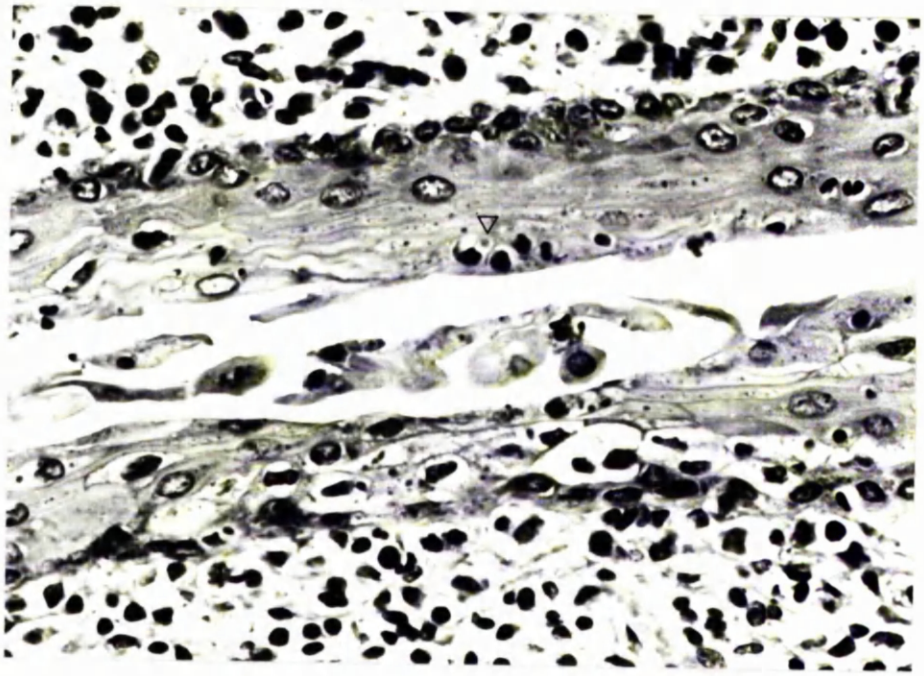


Fig. 23 : Canine distemper - Immunofluorescence in tonsil.

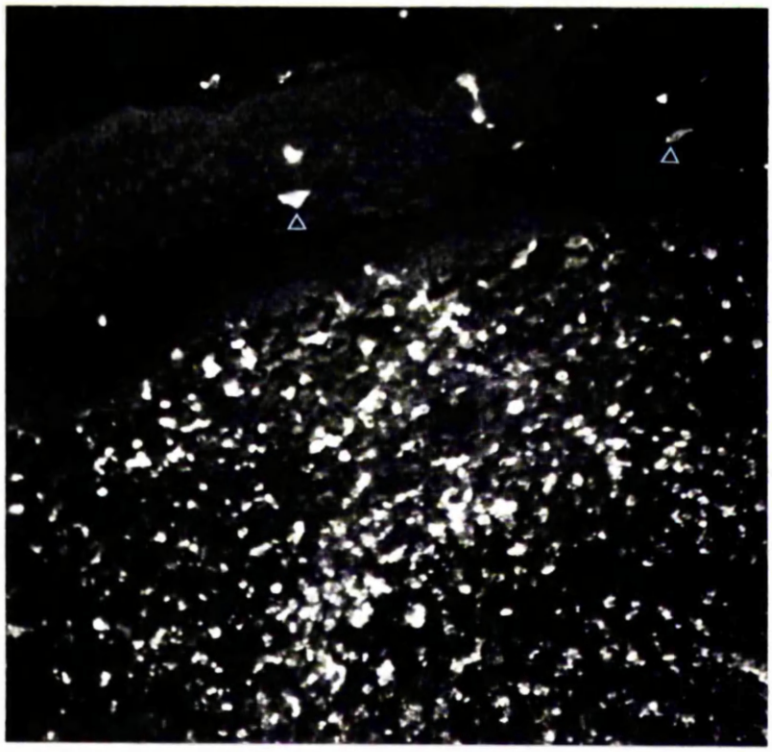
Individual, positively-stained cells are present in the tonsillar crypt and epithelium (arrows). Large numbers of positively stained cells are present in the subepithelial lymphoid tissue.

(Fluorescent antibody, x 200).

Fig. 24 : Canine distemper - Immunofluorescence in bronchus.

Canine distemper virus antigen is present in most of the epithelial cells of this small bronchus. The positive fluorescence has a distinctive granular appearance.

(Fluorescent antibody, x 400).



SECTION 4 : A SURVEY OF DOGS WITH CONTAGIOUS RESPIRATORY DISEASE

Survey Design

The object of this survey was to investigate the incidence and nature of bacterial involvement in the aetiology and pathogenesis of contagious respiratory disease in a group of dogs.

A total of 35 dogs (M1-M35) were obtained, as described in Section 2, from a large kennel housing stray animals. General background data on these animals are presented Table 9: cross-bred dogs formed a high proportion (21 out of 35) of the dogs examined whilst a majority were young animals - either young adults (17 dogs) or puppies (8 dogs). The ratio of male to female animals was approximately 2 : 1.

Each dog was submitted to a full post mortem examination at which samples were taken for histopathological examination, immunofluorescence examination for CDV and CAV antigens and for bacteriological and virological investigations.

Pathological Findings

Macroscopic : At post mortem examination significant pathological changes were found in the respiratory tract and associated structures of many of the dogs examined.

Within the respiratory tract 2 changes were frequently recognised. The first was tracheobronchitis characterised by congestion of the tracheal and bronchial mucosae and the presence, in the lumen of the tracheobronchial tree, of a mucous or mucopurulent exudate. The second was the presence of patchy, often coalescing, foci of exudative pneumonia in the apical, cardiac and/or anterior regions of the diaphragmatic lobes. These foci were reddish-brown in colour, firm in consistency and, on section, fluid, often purulent in nature, could be expressed from the bronchial tree and the cut surface of the lung substance. Tracheobronchitis was recognised in a total of 15 dogs in 6 of which exudative pneumonia was also present (Table 10).

Dog Number	Breed	Age	Sex	Dog Number	Breed	Age	Sex
M1	Collie X	Young adult	F	M19	Rough Collie	Adult	M
M2	Terrier X	Young adult	F	M20	Alsatian	Puppy	F
M3	Terrier X	Aged	M	M21	Collie X	Young adult	M
M4	Labrador	Young adult	M	M22	Border Collie	Adult	F
M5	Irish Setter	Young adult	M	M23	Labrador X	Adult	F
M6	Alsatian X	Young adult	M	M24	Labrador X	Adult	F
M7	Collie X	Young adult	M	M25	Labrador X	Aged	M
M8	Alsatian X	Young adult	M	M26	Alsatian X	Puppy	M
M9	Labrador	Young adult	M	M27	Labrador X	Young adult	M
M10	Poodle	Puppy	M	M28	Border Collie	Young adult	F
M11	Labrador X	Young adult	M	M29	Collie X	Adult	M
M12	Collie X	Young adult	M	M30	Labrador	Adult	M
M13	Terrier X	Puppy	F	M31	Labrador	Adult	M
M14	Terrier X	Puppy	F	M32	Alsatian	Puppy	M
M15	Terrier X	Puppy	M	M33	Collie X	Young adult	M
M16	Labrador X	Adult	M	M34	Collie X	Young adult	F
M17	Alsatian	Young adult	M	M35	Alsatian	Young adult	M
M18	Labrador	Puppy	F				

Puppy = < 9 mths.
Young adult = 9 mths - 2 yrs
Adult = 2 yrs - 7 yrs
Aged = > 7 yrs

M = Male
F = Female

Table 9 : Contagious respiratory disease survey - breed, estimated age and sex of each dog examined.

Dog Number	Tracheobronchitis	Exudative Pneumonia	Dog Number	Tracheobronchitis	Exudative Pneumonia
M1	+	+	M19	+	+
M2	+	+	M20	+	+
M3	+	-	M21	-	-
M4	+	-	M22	-	-
M5	-	-	M23	-	-
M6	-	-	M24	-	-
M7	+	-	M25	+	-
M8	+	+	M26	-	-
M9	-	-	M27	+	-
M10	-	Petechiation	M28	-	-
M11	+	Oedema	M29	+	-
M12	+	+	M30	-	-
M13	-	Petechiation	M31	-	-
M14	-	Oedema	M32	+	-
M15	-	-	M33	+	-
M16	-	-	M34	-	-
M17	-	-	M35	-	-
M18	-	-			

+ = Present

- = Not present

Table 10 : Contagious respiratory disease survey - macroscopic findings in respiratory tract of dogs at post-mortem examination

In 2 dogs (M11and M14) there was diffuse pulmonary oedema which was associated in one animal (M11) with tracheobronchitis; in a further 2 animals (M10 and M13) petechiae were found scattered over the pleural surface of all the lung lobes. In 17 dogs no abnormalities could be detected in the tracheobronchial tree of lung substance.

In the majority of those dogs in which tracheobronchitis was recognised there was enlargement, up to twice normal size, and congestion of the palatine tonsils and bronchial and retropharyngeal lymph nodes; similar changes were found, although less consistently, in dogs without tracheobronchitis.

Microscopic: On histological examination, the main findings were varying degrees of tracheobronchitis and exudative pneumonia; these lesions were present either individually or in combination in all dogs with macroscopic changes and in 9 of the 17 dogs in which no macroscopic change had been detected (Table 11).

Tracheobronchitis was present in 27 of the 35 dogs examined; this varied in severity and the trachea and bronchial tree in the same animal were not necessarily affected to the same degree (Table 11). Two distinct types of change were recognisable.

The first change, seen in 12 dogs (M5, M7, M10, M13, M14, M15, M17, M18, M32, M23, M25 and M34). consisted of focal epithelial degeneration and necrosis (Fig. 25). There was vacuolation and pyknosis of the epithelial cells with loss of their normal pseudostratified organisation and sometimes almost complete breakdown of the epithelium. Reaction in the underlying lamina propria was limited to congestion and slight cellular infiltration by lymphocytes and macrophages. These lesions occurred as small, scattered foci ("+") and as more frequent and extensive, coalescing areas ("+ +") although even in these latter cases there was still apparently normal epithelium present in other areas.

Dog Number	Macroscopic findings	Tracheitis	Bronchitis/ Bronchiolitis	Exudative pneumonia	Dog Number	Macroscopic findings	Tracheitis	Bronchitis/ Bronchiolitis	Exudative pneumonia
M5	NAD	-	+	-	M4	Tb	+++	++	-
M6	NAD	-	-	-	M7	Tb	++	+	-
M9	NAD	-	+	+	M25	Tb	++	+	-
M15	NAD	-	+	-	M27	Tb	++	++	+
M16	NAD	-	-	-	M29	Tb	+++	+	-
M17	NAD	+	+	-	M32	Tb	+++	++	-
M18	NAD	++	+	-	M33	Tb	+++	+	-
M21	NAD	+	-	-	M1	Tb, Ex	+++	+++	++
M22	NAD	+	+	+	M2	Tb, Ex	++	++	+++
M23	NAD	-	+	-	M8	Tb, Ex	+++	+++	++
M24	NAD	-	-	-	M12	Tb, Ex	++	+++	++
M26	NAD	-	-	-	M19	Tb, Ex	+	++	+
M28	NAD	-	-	-	M20	Tb, Ex	++	+	+
M30	NAD	-	-	-	M11	Tb, O	+	++	Oedema
M31	NAD	-	-	-	M14	O	+	++	Oedema
M34	NAD	+	+	-	M10	P	-	+	Haemorrhage
M35	NAD	-	-	-	M13	P	+	+	Haemorrhage
M3	Tb	+	++	-					

NAD = No abnormalities detected
Tb = Tracheobronchitis
Ex = Exudative pneumonia
O = Oedema
P = Petechiation

Table 11 : Contagious respiratory disease survey - microscopic findings in respiratory tract of dogs at post-mortem examination and their relationship to the macroscopic appearance of the lungs.

In the remaining 15 dogs with tracheobronchitis, there was congestion and oedema of the lamina propria which contained a cellular infiltrate composed of polymorphonuclear leucocytes, lymphocytes and some macrophages. The epithelium was also infiltrated by polymorphonuclear leucocytes and was disorganised in severely affected areas. A mucopurulent exudate was usually present in the lumen. In cases designated as "+++" tracheitis or bronchitis, there were areas of epithelial denudation. In 4 dogs (M4, M22, M27 and M33) clumps of bacteria were visible amid the cilia of the tracheobronchial epithelium (Fig. 26). In the 2 dogs with oedema (M11 and M14) eosinophilic intracytoplasmic inclusion bodies, characteristic of CDV infection, were found in the bronchial epithelial cells. In 1 animal (M3) an aged Terrier cross-bred dog, certain of the morphological characteristics of chronic bronchitis were also found in the bronchial tree i.e. apparent increase in the volume of the bronchial mucous glands and extension of mucous glands to the bronchiolar level.

In 9 dogs there was an exudative pneumonia. This was characterised by alveolar mural capillary congestion and exudation of fluid, polymorphonuclear leucocytes and macrophages into the alveolar airspaces. In some dogs, exudative pneumonia was restricted in distribution to areas around affected bronchioles, designated "+"; in others it was more severe and in 1 dog (M2), designated "+++", most of the lung tissue in sections taken from the anterior lung lobes was affected.

In dogs M11 and M14 there was patchy intra-alveolar oedema, associated with alveolar capillary congestion, whilst in M10 and M13 scattered areas of intra alveolar haemorrhage was found. In 8 dogs (M6, M16, M24, M26, M28, M30, M31 and M35) no abnormalities were detected in any part of the respiratory tract on histopathological examination.

The bronchial and retropharyngeal lymph nodes of many of the dogs examined appeared reactive : lymphoid follicular hyperplasia was present in many animals and large numbers of plasma cells were found in the medullary cords. In addition, in some animals, these lymph nodes were

congested and polymorphonuclear leucocytes were identified in the afferent lymphatic vessels and the medullary sinuses. In the palatine tonsils, the epithelium was often vacuolated and heavily infiltrated by polymorphonuclear leucocytes and macrophages; lymphoid follicular hyperplasia was also evident in some dogs and severe congestion was commonly present.

In 2 dogs (M11 and M14) the main finding in the lymph nodes and tonsils was lymphocytolysis with loss of normal follicular structure.

Immunofluorescence Findings

CDV antigen was demonstrated by immunofluorescence examination in 12 of 35 dogs examined (Table 12). In only 2 dogs (M11, M14) was antigen detected at all 3 sites examined i.e. the lung, palatine tonsil and retropharyngeal lymph node. In the remaining 10 dogs, antigen was found only in the tonsil and/or retropharyngeal lymph node : in the tonsil, positive fluorescence was restricted to scattered individual cells, often located in the tonsillar epithelium except in 1 dog (M16) in which a small focus of fluorescent cells was also found in the tonsillar substance; in the retropharyngeal lymph node, positive fluorescence was also found in scattered individual cells.

In Table 13, the instance and nature of pathological changes in the respiratory tract are related to the detection of CDV antigen. Antigen was demonstrated in the tonsil and lymph node of dogs whether or not the respiratory tract pathology was also present. It may, however, be significant that in both those dogs in which diffuse pulmonary oedema was recorded, CDV antigen was detected in the lungs.

Immunofluorescence examinations of similar samples for CAV antigen were consistently negative except in 1 dog (M9), in which positive intranuclear fluorescence was detected in a few cells in the tonsillar epithelium. CAV antigen was not detected at any other site examined in this dog.

Dog Number	Lung	Tonsil	Lymph Node	Dog Number	Lung	Tonsil	Lymph Node
M1	-	+	+	M19	-	+	+
M2	-	+	-	M20	-	-	-
M3	-	-	-	M21	-	-	-
M4	-	-	+	M22	-	-	-
M5	-	+	+	M23	-	-	-
M6	-	+	+	M24	-	-	-
M7	-	-	-	M25	-	-	-
M8	-	-	-	M26	-	-	-
M9	-	-	-	M27	-	-	-
M10	-	-	+	M28	-	-	-
M11	+	+	+	M29	-	-	-
M12	-	-	-	M30	-	-	-
M13	-	+	+	M31	-	-	-
M14	++	++++	++	M32	-	-	-
M15	-	-	-	M33	-	+	-
M16	-	++	-	M34	-	-	-
M17	-	-	-	M35	-	-	-
M18	-	-	-				

Results graded + to ++++ on amounts of antigen present.

Table 12 : Contagious respiratory disease survey - results of immunofluorescence examination for CDV antigen

Dog Number	Microscopic findings	Detection of CDV antigen	Dog Number	Microscopic findings	Detection of CDV antigen
M6	NAD	+	M14	T,Br(+oedema)	+(lung)
M16	NAD	+	M17	T,Br	-
M24	NAD	-	M18	T,Br	-
M26	NAD	-	M25	T,Br	-
M28	NAD	-	M29	T,Br	-
M30	NAD	-	M32	T,Br	-
M31	NAD	-	M33	T,Br	+
M35	NAD	-	M34	T,Br	-
M21	T	-	M9	Br, Ex	+
M5	Br	+	M1	T,Br, Ex	+
M10	Br(+haemorr-hage	+	M2	T,Br, Ex	+
M15	Br	-	M8	T,Br, Ex	-
M23	Br	-	M12	T,Br, Ex	-
M3	T,Br	-	M19	T,Br, Ex	+
M4	T,Br	+	M20	T,Br, Ex	-
M7	T,Br	-	M22	T,Br, Ex	-
M11	T,Br(+oedema)	+(lung)	M27	T,Br, Ex	-
M13	T,Br(+haemorr-hage)	+			

NAD = No abnormality detected
T = Tracheitis
Br = Bronchitis/bronchiolitis
Ex = Exudative pneumonia

+ = CDV antigen detected in tonsil and/or lymph node
+(lung) = CDV antigen detected in lung and tonsil and/or lymph node
- = No CDV antigen detected

Table 13 : Contagious respiratory disease survey - relationship of microscopic findings to detection of CDV antigen

Bacteriological Findings

The results of bacteriological examination of the trachea, bronchial tree and lung substance of each of the dogs in this survey are presented in Table 14.

Bacteria were recovered from 1 or more of the 3 sites examined in 15 dogs. The most frequently isolated bacterium was Bord. bronchiseptica which was found, in pure culture in the trachea and bronchial tree of 11 dogs; in 3 of these animals, this organism was also isolated from the lung substance.

Pasteurella multocida, Proteus sp., E. coli and an alpha-haemolytic Strep. sp. were each isolated from dogs M2, M18, M3 and M8 respectively. Only Pasteurella multocida was present at all 3 levels examined; the Proteus sp. was recovered from both trachea and bronchus but the other two microorganisms were present only in the trachea. In 20 dogs all 3 sites sampled were bacteriologically sterile.

The samples of bronchial and retropharyngeal lymph nodes examined by bacteriological techniques were, in general, sterile. In a few animals, however, small numbers of bacteria were isolated, usually from the retropharyngeal lymph nodes; non-haemolytic and alpha-haemolytic streptococci, E. coli, a Pasteurella-like organism and, in 2 cases Bord. bronchiseptica were all recovered from individual samples. Bacteria were also isolated, although more frequently, from the palatine tonsils; the species recovered were similar to those found in the lymph node samples except that Bord. bronchiseptica was not isolated from this site and beta-haemolytic streptococci were also present.

In Table 15, the recovery of bacteria from the lower respiratory tract is related to the presence of histopathological changes within it. It is notable that bacteria were not recovered from any of those dogs in which no microscopic abnormalities were detected although bacteria were present in over half of those dogs with respiratory tract pathology. In particular, bacteria were recovered from 7 of the 9 dogs in which exudative pneumonia was recorded.

Dog Number	Bacteria recovered from		
	Trachea	Bronchus	Lung substance
M1	Bord. bronchiseptica	Bord. bronchiseptica	-
M2	Pasteurella multocida	Pasteurella multocida	Pasteurella multocida
M3	E. coli	-	-
M4	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
M5	-	-	-
M6	-	-	-
M7	-	-	-
M8	*a* -haemolytic Strep. sp	-	-
M9	-	-	-
M10	-	-	-
M11	Bord. bronchiseptica	Bord. bronchiseptica	-
M12	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
M13	-	-	-
M14	-	-	-
M15	-	-	-
M16	-	-	-
M17	-	-	-
M18	Proteus sp.	Proteus sp.	-
M19	-	-	-
M20	Bord. bronchiseptica	Bord. bronchiseptica	-

- = No growth

Table 14 : Contagious respiratory disease survey - recovery of bacteria from the respiratory tract at post-mortem examination

Dog Number	Bacteria recovered from		
	Trachea	Bronchus	Lung substance
M21	-	-	-
M22	Bord. bronchiseptica	Bord. bronchiseptica	-
M23	-	-	-
M24	-	-	-
M25	-	-	-
M26	-	-	-
M27	Bord. bronchiseptica	Bord. bronchiseptica	Bord. bronchiseptica
M28	-	-	-
M29	Bord. bronchiseptica	Bord. bronchiseptica	-
M30	-	-	-
M31	-	-	-
M32	Bord. bronchiseptica	Bord. bronchiseptica	-
M33	Bord. bronchiseptica	Bord. bronchiseptica	-
M34	Bord. bronchiseptica	Bord. bronchiseptica	-
M35	-	-	-

Table 14 (continued) : Contagious respiratory disease survey - recovery of bacteria from the respiratory tract at post-mortem examination

Dog Number	Microscopic findings	Bacteria recovered from lower respiratory tract	Dog Number	Microscopic findings	Bacteria recovered from lower respiratory tract
M6	NAD	-	M14	T,Br(+oedema)	-
M16	NAD	-	M17	T,Br	-
M24	NAD	-	M18	T,Br	Proteus sp.
M26	NAD	-	M25	T,Br	-
M28	NAD	-	M29	T,Br	Bord. bronchiseptica
M30	NAD	-	M32	T,Br	Bord. bronchiseptica
M31	NAD	-	M33	T,Br	Bord. bronchiseptica
M35	NAD	-	M34	T,Br	Bord. bronchiseptica
M21	T	-	M9	Br, Ex	-
M5	Br	-	M1	T,Br, Ex	Bord. bronchiseptica
M10	Br(+haemorrhage)	-	M2	T,Br, Ex	Pasteurella multocida
M15	Br	-	M8	T,Br, Ex	α-haemolytic Strep. sp.
M23	Br	-	M12	T,Br, Ex	Bord. bronchiseptica
M3	T,Br	E. coli	M19	T,Br, Ex	-
M4	T,Br	Bord. bronchiseptica	M20	T,Br, Ex	Bord. bronchiseptica
M7	T,Br	-	M22	T,Br, Ex	Bord. bronchiseptica
M11	T,Br(+oedema)	Bord. bronchiseptica	M27	T,Br, Ex	Bord. bronchiseptica
M13	T,Br(+haemorrhage)	-			

NAD = No abnormalities detected
T = Tracheitis
Ex = Exudative pneumonia
- = No growth
Br = Bronchitis/bronchiolitis

Table 15 : Contagious respiratory disease survey - relationship of microscopic findings to respiratory tract bacteriology

Virological Findings

Virological investigations of the lung lobes taken from each animal at necropsy resulted in the isolation of an haemadsorbing agent from a number of dogs (Table 16). This agent was identified as a canine parainfluenza SV-5 virus (Cornwell et al., 1976). The virus was recovered from 10 of the 35 dogs examined. In Table 16, the recovery of SV-5 is related to microscopical changes in the lungs. SV-5 was present in only 1 dog in which no histological evidence of respiratory disease was found but it was recovered from 9 of the 27 in which microscopic changes were present in the respiratory tract. In both dogs (M10 and M13) in which intra-alveolar haemorrhage was recorded, SV-5 virus was isolated from the lungs.

Combined Findings

In Table 17 are presented the combined results of the microscopical, immunofluorescence, bacteriological and virological examinations carried out on samples taken from the 35 dogs examined in this survey.

Of the 8 dogs in which no histological evidence of respiratory disease was found SV-5 virus was isolated from 1 and CDV antigen was detected in 2 dogs.

Histopathological evidence of respiratory disease was found in the lower respiratory tract of 27 dogs and a number of microorganisms were detected either alone or in combination, in samples taken from these dogs. The bacterium Bord. bronchiseptica was the microorganism most frequently recovered on its own from dogs with respiratory disease (from 7 dogs); SV-5 virus (3 dogs), CDV (2 dogs), CAV (1 dog) E. coli (1 dog) and a Proteus sp. (1 dog) were also demonstrated, in the absence of other microbiological agents, from dogs with respiratory disease. Combinations of different microorganisms were also detected in dogs with respiratory disease. The most frequent combinations were those of Bord. bronchiseptica with CDV (3 dogs) and SV-5 virus with CDV (3 dogs). In 3 animals with respiratory disease no known microbiological agent was detected in any of the samples examined.

Dog Number	Microscopic findings	Isolation of SV5	Dog Number	Microscopic findings	Isolation of SV5
M6	NAD	-	M14	T,Br (+ oedema)	+
M16	NAD	-	M17	T,Br	-
M24	NAD	-	M18	T,Br	-
M26	NAD	+	M25	T,Br	+
M28	NAD	-	M29	T,Br	-
M30	NAD	-	M32	T,Br	-
M31	NAD	-	M33	T,Br	-
M35	NAD	-	M34	T,Br	-
M21	T	+	M9	Br, Ex	-
M5	Br	-	M1	T,Br, Ex	-
M10	Br(+haemorrhage)	+	M2	T,Br, Ex	+
M15	Br	-	M8	T,Br, Ex	+
M23	Br	-	M12	T,Br, Ex	-
M3	T,Br	-	M19	T,Br, Ex	-
M4	T,Br	-	M20	T,Br, Ex	-
M7	T,Br	+	M22	T,Br, Ex	-
M11	T,Br(+oedema)	+	M27	T,Br, Ex	-
M13	T,Br(+haemorr-hage)	+			

NAD = No abnormalities detected
T = Tracheitis
Br = Bronchitis/bronchiolitis
Ex = Exudative pneumonia

+ = SV-5 virus isolated
- = SV-5 virus not isolated

Table 16 : Contagious respiratory disease survey - relationship of microscopic findings to the isolation of parainfluenza virus SV-5 from the lung.

Dog Number	Microscopic findings	Canine distemper virus	Canine adenovirus	SV-5 virus	Bacteria in lower respiratory tract
M6	NAD	+	-	-	-
M16	NAD	+	-	-	-
M24	NAD	-	-	-	-
M26	NAD	-	-	+	-
M28	NAD	-	-	-	-
M30	NAD	-	-	-	-
M31	NAD	-	-	-	-
M35	NAD	-	-	-	-
M21	T	-	-	+	-
M5	Br	+	-	-	-
M10	Br+haemorr-hage	+	-	+	-
M15	Br	-	-	-	-
M23	Br	-	-	-	-
M3	T,Br	-	-	-	E. coli
M4	T,Br	+	-	-	Bord. bronchiseptica
M7	T,Br	-	-	+	-
M11	T,Br+oedema	+	-	+	Bord. bronchiseptica
M13	T,Br+haemorr-hage	+	-	+	-

NAD = No abnormalities
T = Tracheitis
Br = Bronchitis/bronchiolitis
Ex = Exudative pneumonia

+ = present
- = not present

Table 17 : Contagious respiratory disease survey - combined results of microscopic, immunofluorescence, bacteriological and virological examinations.

Dog Number	Microscopic findings	Canine distemper virus	Canine adenovirus	SV-5 virus	Bacteria in lower respiratory tract
M14	T,Br(+oedema)	+	-	+	-
M17	T,Br	-	-	-	-
M18	T,Br	-	-	-	Proteus sp
M25	T,Br	-	-	+	-
M29	T,Br	-	-	-	Bord. bronchiseptica
M32	T,Br	-	-	-	Bord. bronchiseptica
M33	T,Br	+	-	-	Bord. bronchiseptica
M34	T,Br	-	-	-	Bord. bronchiseptica
M9	Br, Ex	-	+	-	-
M1	T,Br, Ex	+	-	-	Bord. bronchiseptica
M2	T,Br, Ex	+	-	+	Pasteurella multocida
M8	T,Br, Ex	-	-	+	∝ haem.Strep.sp.
M12	T,Br, Ex	-	-	-	Bord. bronchiseptica
M19	T,Br, Ex	+	-	-	-
M20	T,Br, Ex	-	-	-	Bord. bronchiseptica
M22	T,Br, Ex	-	-	-	Bord. bronchiseptica
M27	T,Br, Ex	-	-	-	Bord. bronchiseptica

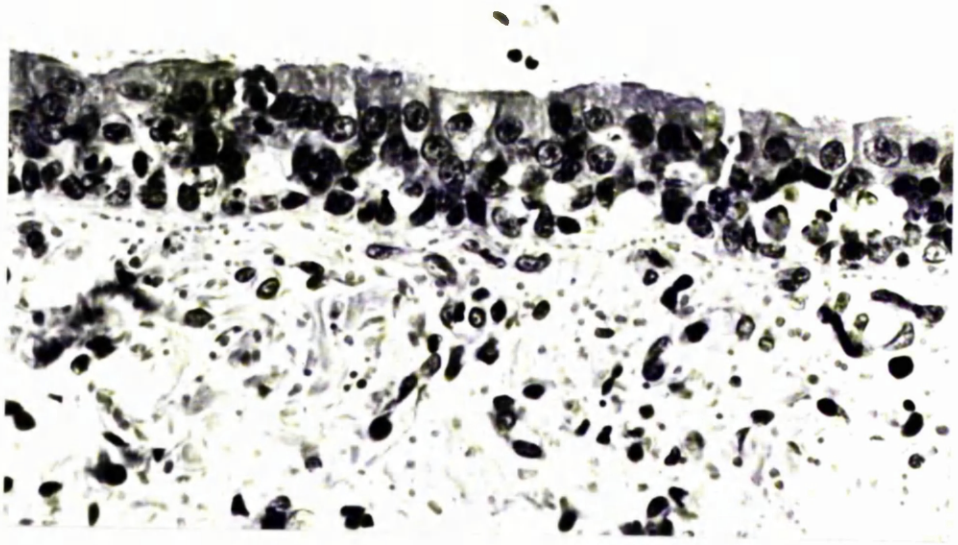
Table 17 (continued) : Contagious respiratory disease survey - combined results of microscopic, immunofluorescence, bacteriological and virological examinations.

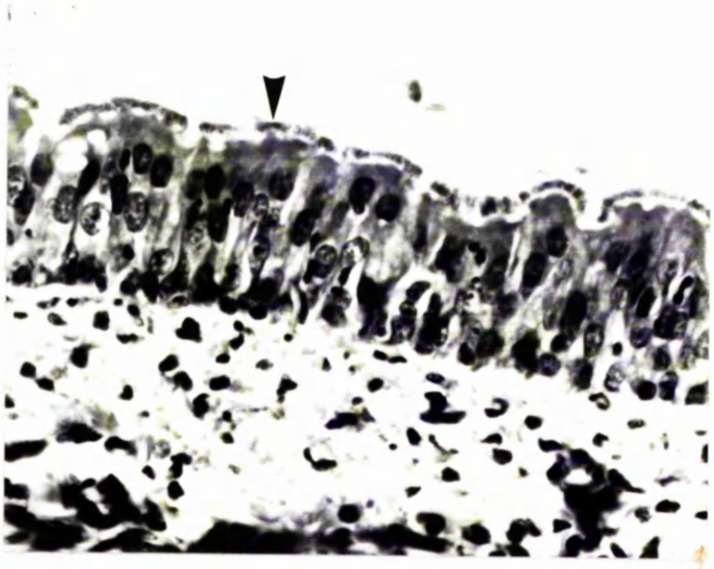
Fig. 25 : Kennel cough - tracheitis, M7. There is vacuolation and disorganisation of the tracheal epithelium.

(HE, x 400).

Fig. 26 : Kennel cough - tracheitis, M27. Masses of bacteria (arrow) are present in the cilia of the tracheal epithelium which is infiltrated by a number of polymorphonuclear leucocytes.

(HE, x 400).





SECTION 5 : DISCUSSION

The surveys described in Sections 3 and 4 were undertaken in an attempt to establish the incidence, nature and significance of bacterial involvement in the 2 major clinical respiratory disease syndromes of the dog i.e. canine distemper and kennel cough.

Because of the difficulties which may arise in evaluating the significance of bacterial isolates from the upper respiratory tract, with its normal, widely variable commensal flora, it was decided, in both the above surveys, to limit bacteriological examination of the respiratory tract to sampling of those lower levels i.e. trachea, bronchial tree and lung parenchyma, where bacteria are not normally present; this also reduced the possibility, always present in samples taken from the upper respiratory tract, of overgrowth of significant microorganisms by normal commensal inhabitants. Bacteriological examination of the lower respiratory tract sites also allowed direct comparison to be made between the isolation of bacteria from these sites and the presence in the lower respiratory tract of pathological changes likely to result in significant clinical respiratory disease.

In the survey of dogs with distemper, all 50 of which had pathological evidence of respiratory disease, bacteria were isolated from the lower respiratory tract of 36 animals; in the survey of dogs taken from a population in which contagious respiratory disease of the kennel cough type was known to exist, bacteria were recovered from the lower respiratory tract of 15 of the 27 animals with pathological evidence of respiratory disease. In both surveys, therefore, the incidence of bacterial infection in dogs with pathological evidence of respiratory disease exceeded 50%. These results in diseased dogs form a marked contrast both to the sterility which, it is generally accepted, exists in the lower respiratory tract of the normal dog and to the absence of bacteria from the lower respiratory tract of all those 8 dogs, examined in Section 4, in which neither macroscopic nor microscopic evidence of respiratory disease could be found.

In both surveys, only a limited number of bacterial species were recovered from the lower respiratory tract of diseased dogs : Bord. bronchiseptica, E. coli, Staph. spp., Strep. spp., Proteus spp. and a mycoplasma-like organism from dogs with distemper; Bord. bronchiseptica Pasteurella multocida, E. coli, a Strep. sp. and a Proteus sp. from dogs with kennel cough type respiratory disease. In addition, only 1 or 2 bacterial species were recovered from the lower respiratory tract of any individual animal, a situation distinct from that in the upper respiratory tract, where a multiplicity of bacterial species is known to exist. All except 1of the bacterial species present in the lower respiratory tract of diseased dogs are amongst those which have been regularly recovered from the upper respiratory tract of healthy animals; the exception, Bord. bronchiseptica, was, however, the single most frequently isolated bacterium in both the distemper survey, where it was found in 21/50 dogs, and in the kennel cough type disease survey where it was present in 11/35 dogs.

It appears, therefore, that the microflora of the lower respiratory tract in dogs with canine distemper and kennel cough differs significantly from that which previous investigators have found to occur in the respiratory tract of dogs without respiratory disease. The significance of the microflora present in the lower respiratory tract of dogs with respiratory disease can, however, be fully evaluated only when the results of the other investigations carried out in the distemper and kennel cough surveys are also considered.

In the survey of dogs with canine distemper, dogs were killed and examined at various stages of the disease process. In all cases, the clinical diagnosis could be confirmed by immunofluorescence examination although, in a few late stage cases, CDV antigen could be located only in the brain. The macroscopic and microscopic pathological findings in organs other than those of the respiratory tract were similar, in all 50 dogs examined, to those which have been described by previous investigators.

The macroscopic and microscopic pathological findings in the lungs of those dogs in which bacteria were not recovered from the lower respiratory tract were also similar to those which have been described by other authors (Lauder et al. 1954b; Watrach, 1958; Jubb and Kennedy, 1970). In almost all dogs there was bronchitis and, more especially, bronchiolitis with intra-alveolar oedema; in some dogs there was, in addition, an interstitial pneumonia characterised by hyperplasia of the alveolar epithelial cells; in the few late stage cases, there was persistence of foci of lymphocytes and macrophages in peribronchial and perivascular tissues.

In those dogs with distemper in which bacteria were recovered from the lower respiratory tract, the same pathological changes described in dogs without bacteria could be found in some areas of the lungs, but, in many cases, there were also areas of exudative pneumonia with moderate or severe, acute, purulent bronchitis and bronchiolitis with massive infiltration of polymorphonuclear leukocytes into the surrounding alveolar airspaces. These exudative pneumonic changes were seen both in dogs in which only bronchiolitis and oedema were otherwise present and in dogs in which proliferative interstitial pneumonia was found; these exudative changes were invariably associated with the presence of bacteria, usually Bord. bronchiseptica, in the bronchial tree and lung parenchyma. In those animals without exudative pneumonia in which bacteria were isolated from the lower respiratory tract there was usually a severe bronchitis and bronchiolitis with some degree of polymorphonuclear leucocyte infiltration into the bronchial tree. The bacterium Bord. bronchiseptica was also isolated from the bronchial tree of 1 dog with late stage, resolving bronchitis and bronchiolitis.

It would, therefore, appear that bacterial infection of the lower respiratory tract may occur at any stage in the course of canine distemper and is associated with exacerbation of the bronchitis and bronchiolitis present in the primary viral infection with progression, in some cases, to an exudative bronchopneumonia. Although bacteria recognised as normal commensal inhabitants of the upper respiratory tract were occasionally isolated, the organism most often associated with bacterial infection of the

lower respiratory tract was Bord. bronchiseptica, a finding which is of course, consistent with the result obtained by the early investigators of canine distemper (Ferry, 1911; McGowan, 1911; Torrey and Rahe, 1913). Bord. bronchiseptica did not, however, appear to be present in the respiratory tract of dogs with distemper in Glasgow 20 years ago (Lauder et al., 1954a and b); it may be that the failure of these workers to isolate the organism was a result of their method of sampling. In the present survey, Bord. bronchiseptica could be isolated, when present, in large numbers from the bronchial tree and also, though in smaller numbers, from pneumonic lung parenchyma; failure to examine both these specific sites may easily result in failure to detect the bacterium.

One of the earliest reports of the histopathological features of canine distemper (Rhea, 1913) describes the presence of large numbers of bacteria in the cilia of the tracheobronchial epithelium in dogs from which the microorganism, Bacterium bronchisepticium had been isolated. It is, therefore, of particular note that bacteria were seen in the tracheobronchial cilia of 5 dogs in the present survey and that in all 5 Bord. bronchiseptica (alias Bacterium bronchisepticium) was isolated in pure culture from the tracheobronchial tree.

In the survey of dogs from a population with contagious respiratory disease of the kennel cough type, there was a wide range in the severity of pathological changes in the respiratory tracts of the dogs examined. In dogs in which there was evidence of respiratory disease, this took the form of some degree of tracheobronchitis with or without an exudative pneumonia; in these dogs a number of different microbiological agents were identified, both alone and in combination.

Three different viral agents were identified in dogs with respiratory disease. Small amounts of CAV antigen were identified by immunofluorescence in the tonsil of 1 dog (M9) in which there was mild bronchitis and early exudative pneumonia. Both CAV-1 and CAV-2 have been shown capable of causing respiratory disease in the dog; however, antigen was not demonstrable by immunofluorescence examination in the lung of this dog,

nor did histopathological examination reveal any of the characteristic basophilic intranuclear inclusion bodies associated with canine adenovirus infection at any level of the respiratory tract. The role of CAV in the pathogenesis of the respiratory disease found in this individual animal is, therefore, unclear. It appears certain, however, that CAV did not play a major role in respiratory disease syndrome seen in the more general population under examination in this survey.

The virus most frequently identified in this survey was CDV which was present in 10 of the 27 dogs with respiratory disease. In 2 of these animals, CDV appeared to be the primary cause of the respiratory disease : antigen was present in the lungs and in these dogs (M11 and M14) histopathological features characteristic of CDV associated disease were present both in the respiratory tract and in the lymph nodes and tonsil. In the remaining 8 animals and, indeed, in 2 animals in which no evidence of respiratory disease was found, CDV antigen was present in only small amounts in the tonsils and/or retropharyngeal lymph node and there were no histopathological changes specifically associated with CDV in either the respiratory tract or the other organs examined. The immunofluorescence appearance in these dogs was similar to that which has been described in the few days immediately following experimental aerosol infection with CDV (Appel, 1969) and it may be that these small amounts of antigen are associated with naturally occurring virus challenge from dogs (such as M11 and M14) with flagrant CDV infections; whether these dogs would, in turn, have developed flagrant CDV infection would have depended on their individual immune status, hopefully high as a result of the vaccination policy adopted at this particular kennel. It seems unlikely that the small amounts of distemper antigen present in these animals was the only, or even the primary, cause of the sometimes severe respiratory disease from which they were suffering at the time of death.

The third virus detected in the dogs in this survey was canine parainfluenza virus SV5 which was recovered from the lungs of 9 dogs with respiratory disease and from 1 apparently normal animal. Canine parainfluenza virus SV5 has been considered to be one of the main

aetiological agents of the kennel cough syndrome in the United States since its initial isolation in that country (Binn et al., 1967), but until the present investigations, the virus had not been isolated from dogs with respiratory disease in Great Britain. In dogs from which SV5 virus was isolated in this survey, there was, in general, mild to moderate tracheobronchitis; in 2 dogs (M2 and M8), there was severe tracheobronchitis with exudative pneumonia but bacteria were also present in the lower respiratory tract of these dogs; in 2 dogs (M10 and M13 in which CDV was also present, although not in the lung), there was petechiation of the lungs at post mortem examination, a finding which has been recorded in experimental SV5 infection in the dog (Appel and Percy, 1970).

Since SV5 virus has been shown capable, experimentally, of producing respiratory disease in the dog and since the virus was isolated in the absence of other microbial agents from the lungs of at least 3 dogs (M7, M21 and M25) with respiratory disease, it is likely that this virus was involved, to some extent, in the pathogenesis of the respiratory disease syndrome in the population of dogs under examination. In experimental SV5 virus infection (Appel and Percy, 1970) it was found that virus could not be recovered from the lungs for longer than 9 days post-infection although both clinical and pathological evidence of respiratory disease persisted after this time. It is possible, therefore, that the incidence of SV5 involvement in this survey was, in fact, greater than was revealed by the virus isolation techniques employed, since dogs infected early in their stay in the kennels could, theoretically, have shed infection by the time of death.

Bacteria were isolated from the lower respiratory tract of 15 dogs with respiratory disease whether or not viruses were also present in these animals. In general, the presence of bacteria in the lower respiratory tract was associated with a more severe respiratory disease than was found when the lower respiratory tract was bacteriologically sterile; in particular, the presence of exudative pneumonia was almost invariably associated with the presence of bacteria in the lower respiratory tract.

Bord. bronchiseptica appears to be the most significant bacterial isolate in this survey. It was recovered in large numbers, and in pure culture, from more than 1 level of the lower respiratory tract in 11 animals, many of which had severe tracheobronchitis and 5 of which also had an exudative pneumonia. Moreover, in 7 of these dogs, Bord. bronchiseptica was the only microbial agent which could be detected in these animals.

Of the remaining bacterial isolates, Pasteurella multocida, a microorganism widely recognised as a potential pathogen, was present in heavy, pure culture throughout the lower respiratory tract of 1 dog with severe respiratory disease and is likely to have been involved in that disease pathogenesis; E. coli, the alpha-haemolytic Strep. sp. and the Proteus sp., however, are recognised commensals in the canine upper respiratory tract and seem unlikely to be of major significance in the pathogenesis of the respiratory disease with which they were associated since they were present in only small numbers in the trachea and, in 1 dog, the bronchus.

It is apparent that the aetiology of the contagious respiratory disease syndrome present in the population examined in this survey is complex. Indeed, it is possible that the aetiology in these dogs is even more complex than would appear from the results obtained : in 3 dogs with respiratory disease, no known microbial agent was isolated ; it may be that, an as yet unknown, fastidious respiratory tract pathogen remains undetected in these animals.

Nonetheless, the results obtained in this survey did provide some insight into the actiology and pathogenesis of the respiratory disease syndrome in this population. Known respiratory viral pathogens were detected in some cases, but it is of note that the presence of severe tracheobronchitis and exudative pneumonia was usually associated with bacterial infection of the normally sterile lower respiratory tract. The organism most frequently associated with such infection was Bord. bronchiseptica and, in some cases of severe respiratory disease, this bacterium was the only detectable microorganism in the respiratory tract.

In summary, in both canine distemper and contagious canine respiratory disease of the kennel cough type, the microflora of the lower respiratory tract differs from that found in dogs without respiratory disease. Moreover, bacterial infection of the lower respiratory tract results, in canine distemper, in exacerbation of the disease picture present in the uncomplicated viral infection whilst, in kennel cough, it is associated with a more severe pathological disease than is generally found in dogs in which the lower respiratory tract is bacteriologically sterile. In both surveys, bacterial infection of the lower respiratory tract was occasionally caused by organisms recognised as normal commensal inhabitants of the upper respiratory tract but in the majority of cases it was due to Bord. bronchiseptica a bacterium which has, in the past, been isolated in other outbreaks of respiratory disease.

It appears, therefore, that bacteria, in particular Bord. bronchiseptica, are of significance in the pathogenesis of the 2 major respiratory disease syndromes of the dog. In canine distemper, the role of bacteria appears to be that of secondary invaders complicating primary viral damage to the respiratory tract in animals which are probably immunosuppressed as a result of viral insult of the lymphoid system. In kennel cough, bacteria did occur in dogs in which known viral respiratory pathogens were present; however, Bord. bronchiseptica was associated with respiratory disease in dogs in which no known canine virus was detected and it seems possible that this bacterium might play a primary role in aetiology of such disease.

PART I I : PATHOGENESIS OF CANINE BORDETELLOSIS

SECTION 1. INTRODUCTION AND REVIEW OF THE LITERATURE

The bacterium now recognised as Bordetella bronchiseptica was first isolated and described by Ferry (1910, 1911) in the United States during his investigations of canine distemper; from its common site of occurrence he named it Bacillus bronchicanis. In 1912, after further work, involving other animal species, Ferry renamed his bacterium Bacillus bronchisepticus. Organisms similar to Bacillus bronchisepticus had previously been recovered from dogs with distemper (Lignieres, 1901) and from the respiratory tract of guineapigs (Tartakowsky, 1897-8; Martini, 1900; Selter, 1906).

McGowan (1911), working independently in Edinburgh, also found Bacillus bronchisepticus in the bronchial tree of dogs with canine distemper and, like Ferry, considered it the causal agent of the disease. In the succeeding twenty years, the organism was repeatedly isolated from dogs with distemper (Torrey and Rahe, 1913; Schoichi, 1923; Hardenberg, 1925; Schilingman, 1932) but the demonstration by Laidlaw and Dunkin (1926) of the primary viral aetiology of canine distemper resulted in the relegation of Bord. bronchiseptica to the role of a secondary invader.

In more recent years, Bord. bronchiseptica has been frequently recovered from dogs with kennel cough (Snow et al., 1969; Wilkins and Helland, 1972; Appel et al., 1970) and as early as 1948 was considered to have a primary role in this complex (Ray, 1948). Investigations of the flora of the nose and throat of healthy dogs with a known history of freedom from disease have shown that Bord. bronchiseptica is not present in the normal commensal flora of such animals (Smith, 1961; Clapper and Meade, 1963; Brennan and Simkins, 1970) although it may be found in the flora of dogs in contact with, but not necessarily showing signs of, respiratory disease. (Parnaik and Singh, 1965; Snow et al., 1969). However, attempts to demonstrate the primary pathogenicity of Bord. bronchiseptica for the dog have not been consistently successful (Greig, 1954; Chappel et al., 1956) and although the early workers claimed to have reproduced respiratory disease in dogs by inoculation of Bord. bronchiseptica

(Ferry, 1910, 1911; McGowan, 1911; Torrey and Rahe, 1913), this bacterium, in the recent past, has been generally regarded as an opportunistic pathogen complicating primary viral infections.

Since its identification in 1910, Bord. bronchiseptica has undergone several changes of name and classification. After finding that the organism occurred in the respiratory tracts of several animal species with respiratory disease, Ferry changed the original name of Bacillus bronchicanis (Ferry, 1910) to Bacillus bronchisepticus (Ferry, 1912). In 1918, Evans found the organism to have physiological and serological relationships to bacteria of the contagious abortion - Malta fever group and assigned the canine species to this group as Bacterium bronchisepticum. In the reclassification of the genus Bacterium, the organism was placed in the genus Alcaligenes as a result of its inability to ferment carbohydrates (Bergey, 1923) but Alcaligenes bronchisepticus was subsequently reclassified as Brucella bronchiseptica (Bergey, 1939) on the basis of its antigenic relationship to the contagious abortion organisms previously noted by Evans (1918). However, because of the very close antigenic relationship of the organism to the whooping-cough (Pertussis) bacillus (Ferry and Noble, 1918; Ferry and Klix, 1918; Evans and Maitland, 1939; Eldering, 1941) it was later assigned to the Haemophilus group (Wilson and Miles, 1955), which, at that time, included the pertussis bacillus. In 1957, Haemophilus bronchisepticus was placed with the antigenically related pertussis and parapertussis organisms in the new genus Bordetella (Bergey, 1957) and it is as Bord. bronchiseptica that the organism is best known today, its position in this genus being recently confirmed by Johnson and Sneath (1973). These name changes are summarised in Table 18.

Ferry's initial isolation of Bord. bronchiseptica from the tracheobronchial tree of dogs with respiratory disease stimulated bacteriological investigations of the respiratory tracts of other animal species showing similar clinical signs of respiratory disease (Table 19). McGowan (1911) recorded the recovery of Bord. bronchiseptica not only from dogs but also from a variety of other species including cats, ferrets, rabbits, guinea-pigs and monkeys. McGowan's observations on the occurrence of the organism in laboratory animals were confirmed by other workers (Ferry, 1912; Ferry, 1913;

Bacillus bronchicanis	Ferry, 1910
↓	
Bacillus bronchisepticus	Ferry, 1912; Torrey and Rahe, 1913.
↓	
Bacterium bronchisepticum	Evans, 1918
↓	
Alcaligenes bronchisepticus	Bergey, 1923
↓	
Brucella bronchiseptica	Bergey, 1939
↓	
Haemophilus bronchisepticus	Wilson and Miles, 1955
↓	
Bordetella bronchiseptica	Bergey, 1957; Johnson and Sneath, 1973

Table 18 : Bord. bronchiseptica - résumé of nomenclature

Pig	:	Phillips, 1943; Ray, 1950; Dunne et al., 1961; L'Ecuyer et al., 1961; Ross et al., 1963a.
Guinea-pig	:	McGowan, 1911a; Ferry, 1912; Ferry, 1913; Smith, 1913; Griffin, 1955; Winsser, 1960; Ganaway et al., 1965; Woode and McLeod, 1967.
Rabbit	:	McGowan, 1911a; Ferry, 1912; Ferry, 1913; Ferry and Hoskins, 1920; Bull and McKee, 1928; Winsser, 1960.
Rat	:	Hoskins and Stout, 1919-20; Griffin, 1955; Winsser, 1960; Switzer et al., 1966; Burek et al., 1972.
Ferret	:	McGowan, 1911a; Ferry, 1913; Spooner, 1938.
Cat	:	McGowan, 1911a and b; Fisk and Soave, 1973; Snyder et al., 1973.
Monkey	:	McGowan, 1911a; Ferry, 1912; Ferry, 1913; Winsser, 1960; Graves, 1968; Siebold et al., 1970.
Horse	:	Gallaghar, 1965; Saxegaard et al., 1971.
Man	:	Brown, 1926; McGowan, 1911a; Ferry, 1913.
Gerbil	:	Winsser, 1960.
Hedgehog	:	Edwards, 1957.
Vole	:	Bacon et al., 1956.
Fox	:	Switzer et al., 1960.
Oppossum	:	Switzer et al., 1960.
Skunk	:	Switzer et al., 1960.
Racoon	:	Switzer et al., 1960.

Table 19 : Species other than the dog from which Bord. bronchiseptica has been isolated.

Smith, 1913; Bull and McKee, 1928) and the organism is now well-recognised as a cause of respiratory disease in the laboratory species of guinea-pig, rabbit and rat in which it tends to show a high degree of infectivity but a relatively low degree of virulence (Griffin, 1955; Winsser, 1960).

In the guinea-pig, an acute fatal bronchopneumonia is commonly associated with Bord. bronchiseptica infection; one or more lung lobes may be completely consolidated and there may be overlying areas of pleurisy (Woode and McLeod, 1967; Nikkels and Mullink, 1971) but histological examination indicates that the initial lesion is a purulent bronchitis (Nikkels and Mullink, 1971). In rabbits Bord. bronchiseptica has been associated with both pneumonia and "snuffles" (infectious nasal catarrh) (Winsser, 1960), whilst experimental infection in the rat resulted in a multifocal bronchopneumonia which took up to eight weeks to resolve (Burek et al., 1972). Winsser (1960) reported that an asymptomatic carrier state could occur in laboratory animal species with consequent difficulties in identification and elimination of infected animals. Ganaway et al., (1965) also recorded a carrier state in the guinea-pig and noted that it could be accurately detected only by bacteriological examination of the trachea at post mortem examination; nose and throat cultures from the living animal were unreliable indicators of infection.

Bord. bronchiseptica has also been isolated from ferrets (Spooner, 1938) and European hedgehogs (Edwards, 1957) kept as laboratory animals and respiratory disease has been reproduced by intranasal inoculation of the organism in the ferret and Mongolian gerbil (Winsser, 1960). Mice (Griffin, 1955; Winsser, 1960) and hamsters (Winsser, 1960) appear, however, to be resistant to infection with at least some strains of Bord. bronchiseptica, neither overt disease nor a carrier state developing after intranasal inoculation. Poultry would seem to be completely resistant to the organism.

More recently, respiratory disease associated with Bord. bronchiseptica infection has been reported in cats kept in laboratory facilities. In a study of cats admitted to a Californian laboratory (Fisk and Soave, 1973), the carrier rate of the organism, as established by swabbing the nasopharynx, rose from 10% on admission to 48% after 3 weeks of close communal confinement. At the same laboratory 10 cats died, 7 having shown clinical signs of respiratory disease, with firm red areas of consolidation in one or more lung lobes (Snyder et al., 1973); the histological picture was one of bronchopneumonia with an inflammatory exudate present in the bronchial tree and alveoli; Bord. bronchiseptica was recovered from the trachea and lungs of all 10 cats.

Bord. bronchiseptica has also been recovered from a fatal case of bronchopneumonia in an African green monkey (Graves, 1968). Subsequently this author undertook a survey of agglutinating antibodies to the bacterium in the sera of monkeys obtained before, during and after the epizootic in which the above-mentioned fatality occurred (Graves, 1970); antibody titres were still positive in 46% of animals 19 months after the epizootic although the organism was never recovered from the nose or throat of any animal. Siebold et al. (1970) have reported the recovery of Bord. bronchiseptica from 36 fatal cases of bronchopneumonia in New World monkeys.

Of the larger domestic animals, Bord. bronchiseptica has been isolated from only the horse and the pig. Gallagher (1965) reported the recovery of Bord. bronchiseptica from 7 of 15 thoroughbred horses with recurrent respiratory disease characterised by coughing and a muco-purulent nasal discharge; serological studies showed a rising agglutinin

titre to the bacterium but no change in titre to three strains of equine influenza virus. Saxegaard et al. (1971) isolated the organism from the lungs of a 3 month-old foal with bronchopneumonia; neither mycoplasma nor viruses could be recovered from the lung tissue of this animal. In contrast to the sporadic recovery of Bord. bronchiseptica from the horse, the bacterium has been regularly recovered from outbreaks of respiratory disease in the pig, in which species it is regarded as a pathogen of economic significance.

Bord. bronchiseptica-like organisms were first isolated from the pig by Spray (1922) who found them in both pneumonic and apparently normal lungs. Daugherty (1941) associated the organism with cases of rhinitis in American swine, while Phillips (1943, 1946) recovered the bacterium in pure culture from the lungs of pigs with pneumonia in 8 Ontario piggeries and also, with other organisms, from the nasal cavity of pigs with rhinitis. The recovery of Bord. bronchiseptica from pneumonic pig lungs has since been repeated by Ray (1950), Dunne et al. (1961), L'Ecuyer (1961) and Goodwin and Whittlestone (1962), while Switzer (1956), Cross and Cafflin (1962) and Ross et al. (1963a) have isolated the organism from additional field cases of atrophic rhinitis (AR). The unthriftiness and stunting associated with both AR and pneumonia (Switzer, 1970) has provided the economic stimulus for research into the aetiology of those conditions and, in particular, the role played by Bord. bronchiseptica in porcine respiratory disease.

Pneumonia was produced in conventionally-reared young pigs by inoculation with Bord. bronchiseptica by L'Ecuyer (1961), Goodwin and Whittlestone (1964) and Duncan et al. (1966b). Goodwin and Whittlestone were reluctant to specify the bacterium as the primary cause of the disease, but the other groups of workers considered they had produced a primary bacterial pneumonia by intratracheal inoculation. The primary role of the bacterium was confirmed by Meyer and Beamer (1973) who consistently produced a pneumonia, identical to that described by the above authors, by intranasal inoculation of Bord. bronchiseptica into germ-free piglets; no other microbiological agent could be isolated from either infected or control animals. The pneumonia was characterised by bronchitis

and bronchiolitis, vasculitis and alveolar oedema and haemorrhage with infiltration of inflammatory cells into the alveolar air spaces; peribronchial, perivascular and interstitial fibrosis with alveolar epithelialisation were also marked especially in the later stages of the disease, and small peribronchial and peribronchiolar mononuclear cell infiltrates were found. These changes were identical to those described in field cases of pneumonia associated with Bord. bronchiseptica (Dunne et al., 1961) and isolates of the organism from various animal species have been shown capable of reproduing the condition in pigs (Ross et al., 1967).

Atrophic rhinitis has been induced by intranasal inoculation of Bord. bronchiseptica by Switzer (1956), Cross and Caflin (1962), Ross et al. (1963b), Duncan et al. (1966a) and Shimizu et al. (1971). Noticeable turbinate atrophy was reported to occur within two weeks of infection in animals inoculated at 3 days of age. (Ross et al., 1963b; Duncan et al., 1966a). The histological lesions consisted of epithelial hyperplasia and metaplasia, fibroplasia and collagen deposition in the lamina propria and bony resorption with replacement fibrosis of the osseous core (Duncan et al., 1966a). Bacteria were identified by a fluorescent antibody technique among the cilia of the epithelium but not in the underlying tissues; (Maeda and Shimzu, 1974). Duncan et al. (1966a) postulated that the changes in the underlying tissues were due to some bacterial product, possibly an endotoxin.

The atrophy produced by experimental infections was more severe in animals inoculated at 3 days rather than at 4 weeks of age (Ross et al., 1963b); Duncan et al. (1966a) considered the increased severity in animals infected at a very early age to be due to suppression of the very rapid growth of the turbinate bones which normally occurs in the first few weeks of life. Several other agents including a virus and Pasteurella multocida (Switzer, 1956) have also been implicated in AR outbreaks and it may well be that any agent interfering with normal turbinate development in the young pig will produce a similar gross appearance which is dysplastic rather than atrophic in origin.

Bord. bronchiseptica persisted for up to 6 weeks after infection in the turbinates and trachea of pigs (Ross et al., 1963b) but could be recovered from only the turbinates after 8 weeks (Ross et al., 1963b; Shimizu et al., 1971). It should be emphasized that infection with Bord. bronchiseptica will result in the colonisation of both turbinate and lower respiratory tract mucosa and that AR and pneumonia are merely two aspects of the same disease process (Ross et al., 1967); which aspect becomes predominant may depend upon other factors such as the age of the pig at the time of infection.

Reinfection of swine herds from which Bord. bronchiseptica had been eliminated, even in the absence of contact with other pigs, has been reported (Switzer et al., 1966). Investigation of wild life led to the sporadic isolation of the organism from skunk, opposum, fox, racoon and cat and from rats (Switzer et al., 1966); in addition, the bacterium has been recovered from a wild vole (Bacon et al., 1956) : it was concluded that, in certain circumstances, rats might be responsible for infection of Bord. bronchiseptica-free pig herds.

Bord. bronchiseptica has occasionally been recovered from humans with a respiratory disease diagnosed clinically as whooping-cough (Brown, 1926; Chang, 1950); Brown (1926) ascribed the infection in a 5 year-old child to close contact with a pet rabbit suffering from "snuffles" due to Bord. bronchiseptica. McGowan (1911), Ferry (1917) and Winsser (1960) have recorded respiratory disease due to Bord. bronchiseptica in animal attendants working with infected stock. Lautrop and Lacey (1960) estimated the organism to be responsible for 0.1% of whooping cough cases in London and considered that infection with Bord. pertussis, Bord. parapertussis and Bord. bronchiseptica in man were indistinguishable without bacteriological or serological examinations. Since these infections in man are clinically indistinguishable and the organisms responsible are very closely related, the features of classical whooping cough i.e., Bord. pertussis infection in man will be briefly reviewed.

Whooping-cough is a communicable, infectious disease of childhood, characterised by violent paroxymsms of coughing ending in the characteristic inspiratory "whoop" which gave the disease its name. The "whoop", however, is a feature of only the later stages of the disease; in the earlier stages, clinical signs consist of coughing, sneezing and non-specific signs of upper respiratory infection (Robbins, 1974). Clinical signs normally persist for 6 to 8 weeks. In mild forms or in cases in older children and adults, the symptomatology is often limited to mild coughing and the "whoop" may never occur (Morse, 1968).

The condition is highly communicable within a susceptible population with transfer of infection occurring in 80-90% of susceptible family contacts and 25-50% of susceptible contacts outside the home (Gordon and Hood, 1951). Traditionally, epidemics of whooping-cough originated in young school children who then transmitted the disease to their pre-school siblings (Gordon and Hood, 1951), but intensive vaccination has considerably changed this epidemiological pattern. Pertussis outbreaks are becoming increasingly recognised in older age groups and amongst patients fully vaccinated in childhood (Lambert, 1965; Morse, 1968; Kurt et al., 1972); Lambert (1965) indicated that 95% of vaccinated individuals were fully susceptible 12 years after their last dose of vaccine. Clinical recognition of the disease in these groups may be difficult since, as mentioned above, the course is frequently atypical and consequently liable to be diagnosed as bronchitis (Kurt et al., 1972; Linneman et al., 1974); the definitive diagnosis of pertussis in such cases requires specialised bacteriological examination dictated by the fastidious nutritional requirements of the organism; many cases probably go unrecognised and the importance of pertussis in adult humans awaits clarification.

The histological features of naturally occurring Bord. pertussis infection have been difficult to ascertain as most cases recover and many fatalities are due to complicating factors such as supervening infections by other microorganisms. Although Bord. pertussis was described and associated with whooping-cough by Bordet and Gengou as early as 1906, it was not until 1912 that there was an initial description of the histological

lesions in the respiratory tract. Mallory and Hornor (1912) described the presence of large numbers of minute coccobacilli amid the cilia of epithelial cells in the tracheal and bronchial mucosa; in addition there was damage to or complete loss of cilia and migration of polymorphonuclear leukocytes into the epithelium and lumen of the trachea and bronchi; the submucosa was infiltrated by lymphocytes and plasma cells. These features were also found by Smith (1927) who, in addition, noted marked enlargement of tracheobronchial lymph nodes and considered that bronchopneumonia could be due to the direct action of the pertussis bacillus, without secondary bacterial invaders, since he isolated the organism in pure culture in the alveolar exudate of such cases.

In attempts to elucidate the histological features of the disease, Mallory et al. (1913) inoculated dogs, monkeys and rabbits intratracheally with pure cultures of Bord. pertussis. Signs of respiratory disease occurred in some, but not all cases; bacteria were seen among the cilia of the respiratory tract and an organism with many of the characteristics of Bord. pertussis was recovered from the lungs. The validity of this experimental work was questioned by Rhea (1913) who commented on the similarity of the lesions produced by Mallory et al. (1913) to those seen in naturally occurring cases of animals suffering from respiratory disease associated with Bord. bronchiseptica; Smith (1913) claimed to have seen Bord. bronchiseptica between the epithelial cilia in guinea-pigs as early as 1899 and Rhea (1915) described identical lesions to those seen in natural whooping-cough in dogs dying of "distemper" associated with Bord. bronchiseptica infection. Mallory (1913) acknowledged and confirmed Rhea's findings in the dog and suggested that bacteriological examination should be used to eliminate Bord. bronchiseptica infection in experimental animals.

Since then, clinical respiratory disease has been induced in man (MacDonald and MacDonald, 1933) and in experimental animals (Sauer and Hambrecht, 1929; Shibley, 1934; Rich et al., 1936) by inoculation of Bord. pertussis in the absence of Bord. bronchiseptica; in addition, histological lesions have been produced in mice (Burnett and Timmins, 1937; Bradford, 1938) and embryonic chick lung (Gallavan and Goodpasture, 1937) by infection

with Bord. pertussis. These studies have resulted in a more exact knowledge of the pathological features of whooping-cough. The pulmonary lesions are now generally agreed to be characterised by growth of Bord. pertussis in the ciliated epithelial border with midzonal and basal epithelial necrosis and, in severe cases, epithelial desquamation; there is infiltration of polymorphonuclear leucocytes into the epithelium with accumulation of a mucopurulent exudate in the lumen and peribronchial mononuclear cell accumulations are found. The alveolar walls may also be thickened by a mixed cellular infiltrate of polymorphonuclear leucocytes and mononuclear cells and cellular infiltration into the alveolar air spaces may occur (Robbins, 1974).

These lesions are similar to those seen in natural and experimental infections with Bord. bronchiseptica in the pig and laboratory animals. The similarity in the clinical and pathological features of Bord. pertussis and Bord. bronchiseptica infections is probably due to the biological similarities of the organisms. A close antigenic relationship between them was demonstrated as early as 1918 (Ferry and Noble, 1918; Ferry and Klix, 1918). They have since been shown to have antigenically related agglutinogens (Eldering and Kendrick, 1938; Anderson, 1953), to produce haemagglutinins (Keogh, et al., 1947) and identical heat labile toxins (Evans, 1940); both organisms, in common with other Gram-negative bacteria, have a heat stable endotoxin (Munoz, 1963; Harris et al., 1968). The heat labile toxin (HLT) is lethal for rabbits, mice and guinea-pigs by intravenous or intraperitoneal injection and produces dermal necrosis in rabbits when injected by the sub- or intra-cutaneous routes. Many authors (Lapin, 1943; Munoz 1963; Bradford, 1965) have considered HLT to be involved in the pathogenesis of whooping-cough; Asada (1953a; 1953b) claimed to have produced identical lesions by intranasal instillation of Bord. pertussis and HLT in mice and dogs. More recently, Harris et al. (1968) demonstrated that Bord. bronchiseptica endotoxin interfered with mitochondrial metabolism and suggested that this effect might be responsible for some of the changes seen in atrophic rhinitis.

In summary, the bacterium Bord. bronchiseptica has been recovered from a wide range of animal species in which it is associated with a respiratory disease characterised by inflammation of the ciliated respiratory epithelium. A clinically and pathologically similar disease, whooping-cough, in man is recognised as being due to infection with the related bacterium Bord. pertussis. While Bord. bronchiseptica is recognised as a primary respiratory pathogen in the pig and some laboratory animal species, in the dog it has until recently been regarded as a microorganism incapable of mounting a primary infection. In view of its primary pathogenicity in other species and the frequency, as described in Part I of this thesis, with which it was recovered from dogs with naturally-occurring respiratory disease, sometimes in the absence of any other recognised canine respiratory pathogens, it was decided to undertake a critical re-evaluation of the pathogenicity of Bord. bronchiseptica for the dog.

SECTION 2 : MATERIALS AND METHODS

Experimental Animals

In the investigation of contagious respiratory disease, it is important that the experimental animals employed should not only be free from clinical respiratory disease but also that there should not have been any possibility of exposure to infected animals: 6 to 12 week old, unvaccinated puppies, bred on isolated farms and obtained through a commercial source were, therefore, used throughout these experiments.

On arrival, all animals were maintained in isolation for a minimum period of one week. During this isolation period, serum samples were obtained in order to measure antibody titres to Bord. bronchiseptica. On at least two occasions during isolation nose and throat swabs were obtained from each animal and were examined by bacteriological techniques for the presence of Bord. bronchiseptica.

All dogs were housed indoors at an ambient temperature of 20^{o}C. They were fed a commercial dog food (Lassie : Pedigree Pet Foods Ltd., Leicester),proprietary dog meal and reconstituted dried milk.

Neuroleptanalgesia was induced with "Immobilon" (Reckitt and Coleman, Pharmaceutical Division, Hull) to facilitate restraint during aerosolisation and jugular venipuncture.

Aerosolisation Procedure

Dogs were infected with Bord. bronchiseptica by exposure to an aerosol of a 24 hour nutrient broth culture of the organism.

Dogs were sedated and placed in a clear Perspex chamber to which was attached a polythene tube. This tube was connected to a Wright nebuliser unit powered by a CFI electric compresser unit (Aerosol Products Ltd., Colchester). The nebuliser was, in turn, attached to a universal container in which was a broth culture of Bord. bronchiseptica. Dogs were exposed to the aerosol produced in this manner for 15 minutes :

2.5ml of the culture was aerosolised in this time. The aerosol particle size was < 8μm.

Necropsy Procedures

All animals were anaesthetised by intravenous injection of pentobarbitone sodium (Euthatal; May and Baker Ltd, m Dagenham) and exsanguinated by severing the jugular veins. A full post mortem examination was immediately performed. The trachea, oesophagus, heart and lungs were removed; the oesophagus and heart were separated from the respiratory tract.

The entire left lung and the right diaphragmatic lung lobe were routinely taken for histopathological examination. Blocks were also taken from the remaining lobes if lesions occurring in these lobes were not represented in the left lung. Samples for histopathological examination were also taken from the trachea, palatine tonsil, tracheobronchial and retropharyngeal lymph nodes, soft palate, adenoid, nasal turbinate, rhinarium, liver, kidney, spleen and brain.

The following were taken for bacteriological examination : right cardiac lung lobe, trachea, nasal turbinates, tracheobronchial and retropharyngeal lymph nodes, palatine tonsil.

Small blocks of lung (including a bronchus), tonsil and retro-pharyngeal lymph node were taken for immunofluorescence. Blocks of trachea were occasionally taken.

The intermediate lung lobe was taken and stored at -20^{o}C until virus isolation could be attempted.

Where ultrastructural examination was to take place, small blocks of bronchial and tracheal mucosa were removed as soon after euthanasia as possible and processed immediately.

Histological Procedures

The left lung was fixed in 10% NBFS. The fixative was instilled into the main bronchus until the lung was inflated to its normal size (Medical Research Council, 1975); the bronchus was then ligated and the whole lung submerged in fixative. In selected cases the fixative was also infused via branches of the pulmonary artery. The right diaphragmatic lobe was similarly fixed with mercuric chloride-formol. Portions of other organs were fixed in NBFS.

Tissues were trimmed after 48 hours and transferred to fresh fixative for a further 24 hours. A minimum of 11 blocks were taken from the left lung: 3 blocks from the apical and cardiac lobes and 5 from the diaphragmatic lobe. All tissues were then processed as described in Part I Section 2.

Sections were cut at 4μm and routinely stained by Mayer's haemalum and eosin (HE). Selected sections were also stained by MSB to demonstrate collagen and fibrin and by the Gram-Twort method (Ollett, 1947) to demonstrate bacteria.

Ultrastructural Procedures

Fixation : Small pieces of bronchial and tracheal mucosae were removed as soon after death as possible and placed in drops of chilled para-formaldehyde/glutardehyde fixative on blocks of dental wax. These tissues were then chopped into blocks approximately 0.5mm thick using a grease free razor blade and were then transferred to glass vials containing chilled fixative. The tissues were fixed in the paraformaldehyde/ glutaraldehyde mixture at 4^{o}C (Karnovsky, 1965) for $4\frac{1}{2}$ hours. The tissue was then rinsed overnight in a cacodylate rinsing solution and postfixed in 2% osmium tetroxide for 1 hour.

Paraformaldehyde/glutcraldehyde mixture : 1.3% paraformaldehyde and 1.6% glutaraldehyde in cacodylate buffer pH 7.2 - 7.4.

Paraformaldehyde	2g
Distilled water	25 ml.
IN Sodium hydroxide	2 - 3 drops
25% gluteraldehyde	10 ml.
Cacodylate buffer	115 ml.
Anhydrous calcium chloride	25 mg.

Cacodylate buffer : This was prepared as a 0.1M solution of sodium cacodylate (21.4 g/litre) and adjusted to pH 7.4 - 7.6 by a few drops of concentrated hydrochloric acid.

Cacodylate rinsing solution : Sucrose was added to cacodylate buffer (34.2 g/l) resulting in a 0.1M solution of sucrose; pH was adjusted to 7.2 - 7.4.

Osmium tetroxide : 1% osmic acid (BDH Chemical Ltd., Poole, England) in Millonig's buffer, pH 7.2 - 7.4.

Millonig's phosphate buffer : This was prepared as follows:

Sodium dihydrogen phosphate (2.26%)	83 ml.
Sodium hydroxide (2.52%)	17 ml.
Distilled water	10 ml.
Sucrose	0.54 g.

Embedding : Fixed tissue was dehydrated in an ascending series of 70 percent, 90 percent and absolute alcohol, and was then rinsed in propylene oxide. The tissues were soaked for 1 hour in a mixture of equal parts propylene oxide and Araldite and left overnight in an 80% Araldite mixture. Individual tissue blocks were then embedded in Araldite in gelatine capsules, the resin being polymerised at 57^{o}C for 48 hours.

Araldite mixture : Equal parts Araldite resin (CY212) and Araldite hardener (HY 964) were mixed by stirring overnight and then stored at 4^{o}C. Before use in embedding 0.6 ml of accelerator (DH 064) and 2.4 ml of di-n-butyl-phthalate were added to 57 ml of the above mixture and the whole stirred for 30 minutes.

Staining : Sections 1μm thick were cut on an LKB Mark III ultra-microtome using glass knives, mounted on glass slides, and stained with toluidine blue according to the method of Trump et al.(1961). Fields for ultramicroscopy were selected and the original blocks trimmed accordingly.

Ultrathin sections were then cut on the ultra-microtome and mounted on uncoated Athene 483 copper specimen grids (Smethurst High Light Ltd., Bolton); they were stained with uranyl acetate, (Wilson, 1958), rinsed in methanol, 50% methyl alcohol and distilled water and dried on filter paper. They were then stained for 10 minutes with lead citrate, (Reynolds, 1963), rinsed with 0.02N sodium hydroxide and distilled water and again dried on filter paper. They were then examined using an AE1 6B electron microscope.

Uranyl acetate : a 20% solution (May and Baker Ltd., Dagenham) was made up in 100% methanol.

Lead citrate : lead nitrate (1.33g) and sodium citrate (1.75g) were dissolved in separate 15 ml volumes of distilled water. The solutions were then mixed, the lead citrate precipitate shaken for 1 minute and allowed to stand, with periodic stirring, for 30 minutes. The precipitate was solubilised by adding 8 ml of IN sodium hydroxide and was diluted to 50 ml with distilled water. The final pH was 12 ± 0.1.

Bacterial cells were processed in a similar way except that the colonies of Bord. bronchiseptica to be examined were washed off agar plates with chilled fixative and then centrifuged at 1500 rpm for 10 minutes. The pellet of cells was then dislodged from the glass and treated as a tissue block.

Bacteriological Procedures

The strain of Bord. bronchiseptica used throughout this study was isolated from the lungs of a dog suffering from bronchopneumonia; it was designated 52498/3 (Wright et al., 1973b). The organism was Gram-negative with a tendency to bipolar-staining. It was motile at 37°C, grew well on nutrient agar and MacConkey agar (MA) and produced beta-haemolysis on nutrient blood agar (NBA). After 24 hours aerobic incubation at 37°C, the

colonies produced were tiny, clear and glistening, and, where growth was confluent, beta-haemolysis was apparent on NBA (Fig. 27); MA plates showed a characteristic yellowing of the medium underlying these colonies. After 48 hours incubation, colonies were larger, 1-2 mm in diameter, and beta-haemolysis was apparent under individual colonies (Fig. 28). The biochemical characteristics of Bord. bronchiseptica used to confirm the identity of isolate 524983 are shown in Table 20.

A third passage culture of this isolate of Bord. bronchiseptica was freeze-dried in sterile horse serum (Burroughs Wellcome Ltd., Kent) using a Speedivac Model 5PS freezer drier (Edwards High Vacuum Ltd.). When a culture of bacteria was required an ampoule was revived in sterile serum broth and inoculated onto NBA; colonies were then picked from NBA plates and inoculated into containers of sterile nutrient broth. After 24 hours incubation, these cultures were used for aerosolisation procedures. Total viable counts by a plate dilution method showed that, under the conditions employed, the infecting cultures contained approximately 5×10^8 organisms/ml. The nasal and pharyngeal flora of experimental animals was sampled by swabbing these areas with sterile cotton swabs (Exogen Ltd., Glasgow) moistened with sterile nutrient broth. In swabbing the nasal area, care was taken to introduce the swab completely into the common nasal meatus; for the pharyngeal sample, a separate swab was introduced as far back into the tonsillar area as possible. The swabs were then inoculated onto paired NBA and MA plates, incubated and examined as described in Part I.

At necropsy of experimental animals, primary isolation of bacteria was attempted from bronchus, lung substance, trachea, palatine tonsil, bronchial and retropharyngeal lymph nodes as described in Part I Section 2. In addition, the middle-turbinate was sampled; a sterile wire loop was rubbed vigorously over the turbinate mucosa and the material obtained used for inoculation and preparation of direct smears as in Part I. Nasopharyngeal exudates were also examined.

Test	Result
Oxidase test	+
Catalase test	+
Urease activity	+
Indole production	-
Gelatin hydrolysis	-
Sugar fermentation	-
Citrate utilisation	+
Litmus milk reaction	Alkaline

Table 20 : Biochemical characteristics of Bord. bronchiseptica

NBA, MA and Nutrient broth were supplied by Oxoid Ltd., London, and were constituted according to the manufacturer's instructions. Serum broth was made by adding 1 ml sterile horse serum (Burroughs Wellcome Ltd., Kent) to 10 ml of nutrient broth.

Immunofluorescence Procedures

Tissues taken for immunofluorescence were prepared and stained for CDV and CAV as described in Part I. In addition, tissues were stained with a specific antiserum against Bord. bronchiseptica. This antiserum was prepared as described below.

Two ml of a 48 hour broth culture of Bord. bronchiseptica 52498/3 were emulsified in complete Freund's adjuvant and inoculated subcutaneously into rabbits free from Bord. bronchiseptica infection as judged by negative culture of the nasopharynx. Four weeks later, a further 7 ml of a 24 hour culture was administered; 4 ml was given subcutaneously and 3 ml intraperitoneally. After a further 14 days, the rabbits were bled, and the pooled sera tested for antibody against Bord. bronchiseptica by a serum agglutination test (see below) and by an indirect immunofluorescence technique; the latter used acetone fixed smears of Bord. bronchiseptica as antigen (see below). The serum was also tested against a range of Gram-positive and Gram-negative bacteria isolated from the nose and throat of dogs; specific fluorescence was found only with Bord. bronchiseptica. The serum was then fractionated and conjugated as in Part I, Section 2.

Smears of nasopharyngeal exudate were examined by the method of Donaldson and Whitaker (1960).

Staining procedures using Bord. bronchiseptica specific antiserum were identical to those using CDV and CAV antiserum (see Part I Section 2).

Serological Procedures

Antibodies to Bord. bronchiseptica were detected by two methods : a serum agglutination test and an indirect fluorescent antibody test.

Serum agglutination test : The tube agglutination test employed was based on the method recommended by Lautrop and Lacey (1960). Doubling serum dilutions from 1: 8 upwards were made in 0.2 ml volumes in small tubes; to each dilution was added 0.3 ml of a standard antigen suspension. The tubes were then incubated overnight in a water bath at 37^{o}C. Agglutination was of a finely granular type and the endpoint was usually clear cut.

The antigen suspension was prepared from the Bord. bronchiseptica strain 52498/3. A 48 hour growth of Bord. bronchiseptica on NBA was washed off with sterile normal saline (SNS). This suspension was centrifuged for $1\frac{1}{2}$ hours at 67 x g on an MSE refridgerated centrifuge and the precipitate resuspended in SNS. This washing was repeated three times. The final precipitate was resuspended in SNS and diluted to a concentration of 10×10^{9} organisms/ml using standard opacity tubes (Burroughs Wellcome Ltd., Kent), previously graduated for use with this organism by means of total bacterial counts. Formaldehyde was added to a final concentration of 0.15% and the suspension allowed to stand at room temperature for a few days before checking for inactivation. Each batch was tested for sterility before use.

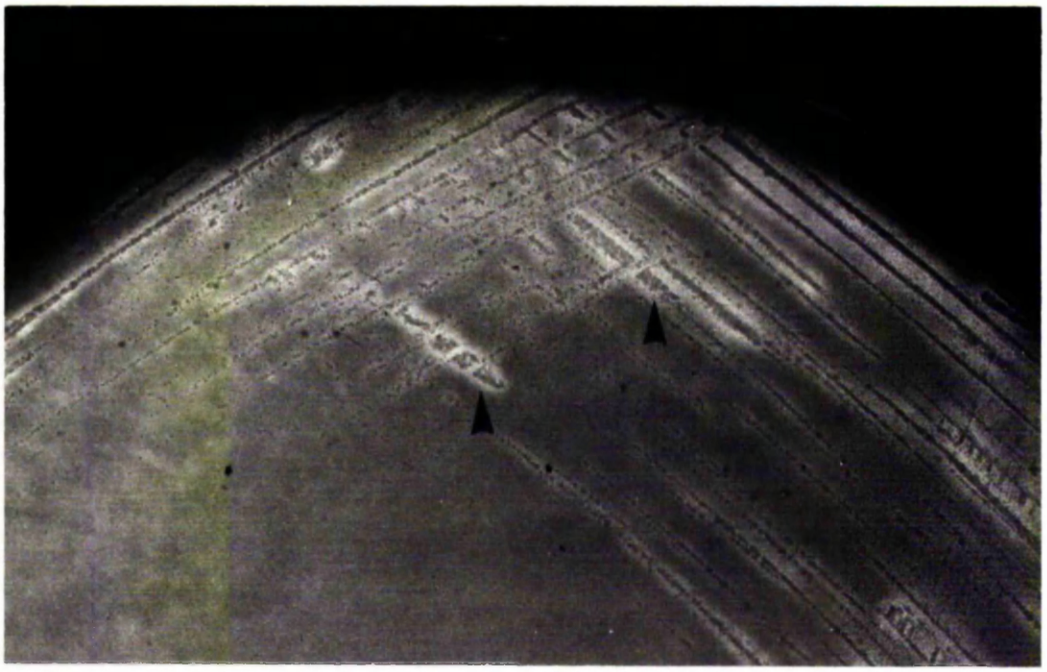
Indirect fluorescent antibody test : This test was performed according to the method of Edwards et al.(1970) with the exception that the smears of Bord. bronchiseptica on glass slides used as antigen were fixed in acetone for 10 minutes rather than by heat. Smears were stained for 30 minutes with doubling dilutions of test sera, washed in PBS for 10 minutes and then counterstained with commercially prepared FITC rabbit antidog globulin (Sera Services Ltd., Berks) for 30 minutes. After a final wash in PBS the smears were mounted in this buffer and examined under a fluorescence microscope.

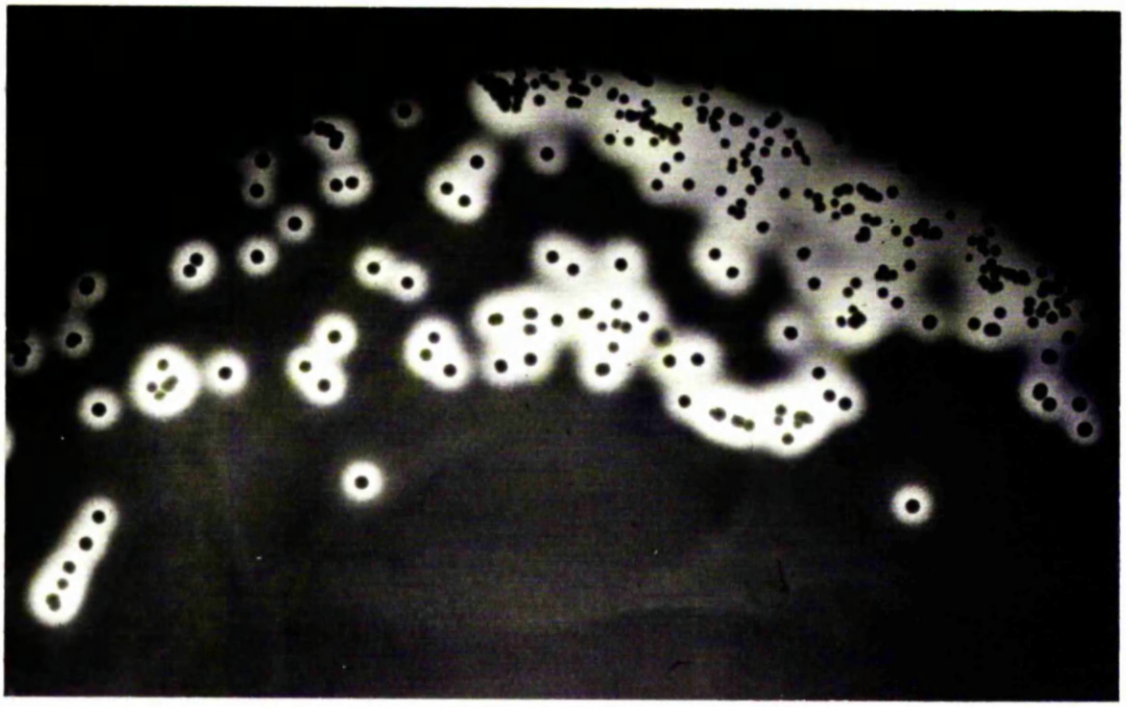
Virological Procedures

Lung lobes were examined for the presence of viruses as described in Part I, Section 2.

Fig. 27 : Bord. bronchiseptica - growth on NBA. After 24 hrs aerobic incubation at 37°C small, clear zones of beta-haemolysis (arrows) are evident around areas of confluent growth.

Fig. 28 : Bord. bronchiseptica - growth on NBA. After 48 hrs aerobic incubation at 37°C, distinct zones of beta-haemolysis are visible around individual colonies.





SECTION 3 : EXPERIMENT ONE - 12 WEEK OLD DOGS

Experimental Design

This experiment was designed to assess the pathogenicity of Bord. bronchiseptica for the respiratory tract of the dog and to investigate the pathology and pathogenesis of any disease produced by infection with this microorganism.

A total of 11 healthy, 12 week old puppies, randomly divided into 3 groups, were used in this experiment. The first group, of 5 dogs, was exposed to an aerosol containing Bord. bronchiseptica as described in Section 2; this was the infected group. The second group (the contact control) consisted of 3 pups which, although not exposed to the infective aerosol, were housed in the same airspace as the infected group, in an adjoining pen. The third group, also of 3 pups, was the unexposed control group; these animals were exposed to an aerosol of sterile nutrient broth and, after aerosolisation, were maintained in an airspace separated from the other groups.

All dogs were examined daily for clinical evidence of disease. Investigation of the disease pathogenesis was facilitated by sequential killing of dogs at intervals from 3 days to 12 days after infection. At necropsy, pathological, bacteriological and virological examinations were carried out as described in Section 2.

Clinical Findings

In the infected group, 4 dogs had developed clinical signs of respiratory disease by 3 days after exposure to the infective aerosol (Fig. 29). The predominant clinical sign was coughing which varied from an occasional soft cough to severe paroxysms; coughing was easily precipitated by excitement or exercise. The remaining animal (No. 3) had started to cough by 5 days after infection and, in all animals in this group, coughing persisted throughout the remainder of the experiment. At 8 days post infection, the 2

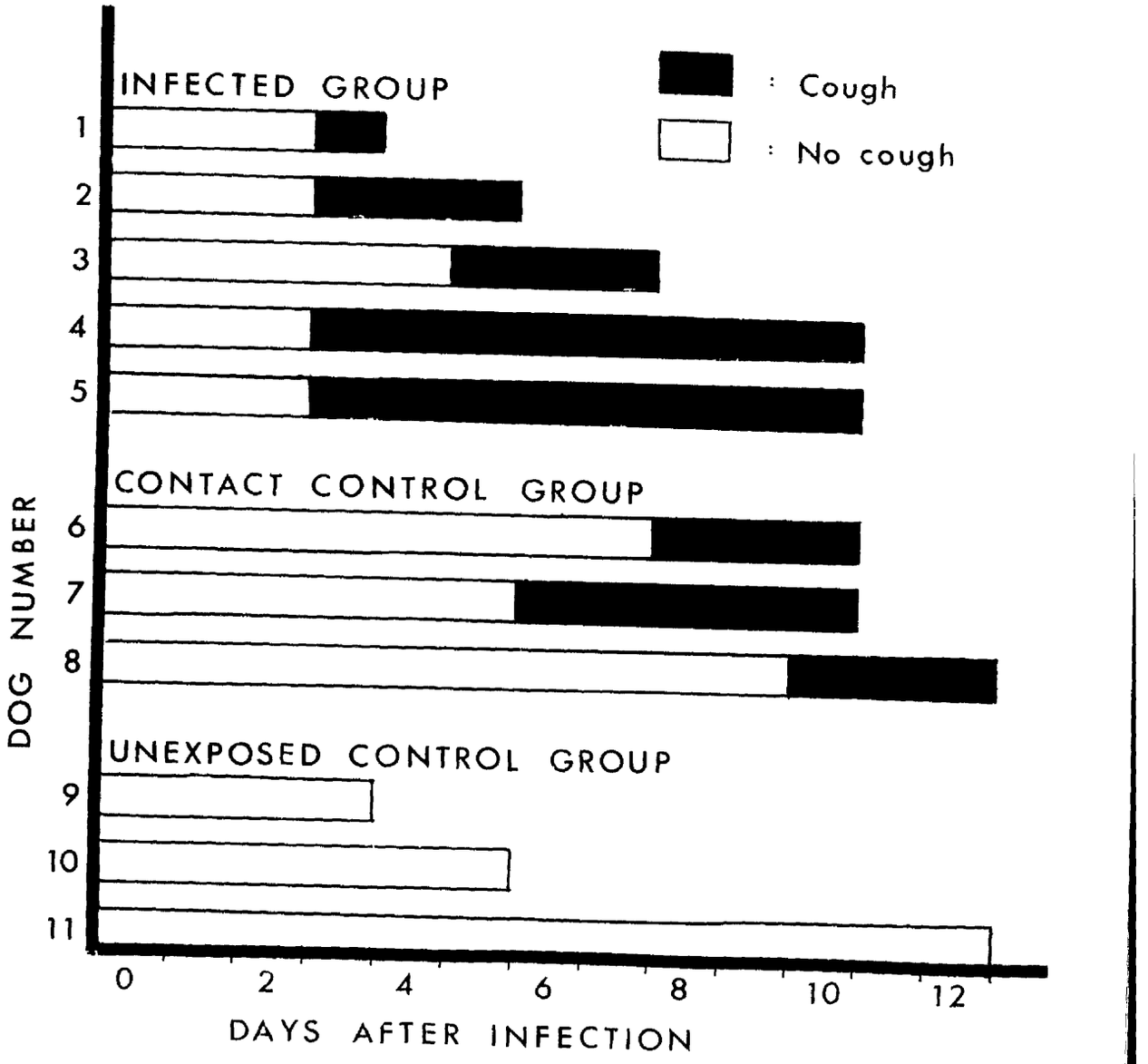


Fig. 29: Experiment one - incidence of coughing.

remaining infected dogs (Nos. 4 and 5) developed a mucopurulent nasal discharge which was still present when, at day 10, they were killed. All 5 dogs remained bright with a good appetite throughout the experiment.

All 3 dogs of the contact control group developed a similar cough at intervals from 6 to 10 days after exposure to the infected group (Fig. 29); coughing varied in severity but was occasionally paroxysmal. One dog (No. 8) also developed a mucopurulent nasal discharge on day 10 which persisted until it was killed on day 12.

No evidence of disease was found in the unexposed control group.

Pathological Findings

Macroscopic findings: significant findings at post mortem examination were confined to the respiratory tract and associated structures.

In all 5 dogs of the infected group, many pinpoint, red foci were found scattered throughout the lungs, while the bronchial and retropharyngeal lymph nodes were enlarged up to twice their normal size and were oedematous. A catarrhal exudate was found in the pharynx of both dogs killed on day 10 and mucopus was found overlying the turbinate bones and crusted on the rhinarium of these animals.

In the contact control group, no macroscopic lesions were found in the lungs but the bronchial and retropharyngeal lymph nodes in all 3 dogs were enlarged and oedematous. In dog No. 8, killed on day 12, the turbinate mucosae were congested and overlain by a purulent exudate; mucopus was present in the pharynx of this dog.

There was no gross evidence of disease in the unexposed controls at post mortem examination.

Microscopic findings: these were confined to the respiratory

tract and associated lymph nodes and are summarised in Table 21.

In the infected group, there was inflammation at all levels of the respiratory tract. In particular, there was infiltration of polymorphonuclear leucocytes into the mucosae and lumina of the conducting airways and masses of small bacilli were visible in the cilia of the respiratory epithelium; these bacteria were shown, by the Gram Twort staining technique, to be Gram-negative (Fig.30). Similar Gram-negative colonies were also seen in mucopurulent exudates overlying both ciliated and non-ciliated respiratory epithelia.

In dogs killed at 3 and 5 days after infection, there was tracheitis, bronchitis and bronchiolitis characterised by congestion and oedema of the lamina propria with infiltration by polymorphonuclear leucocytes and a few mononuclear cells but the epithelium, although also heavily infiltrated by polymorphonuclear leucocytes, remained intact. Bacteria were easily seen in the epithelial cilia. In dogs killed on days 7 and 10, focal areas of epithelial necrosis had developed. These were most marked in the bronchi and bronchioles where, in addition, the lumen frequently contained a cellular exudate of polymorphs and macrophages. Where the epithelium remained intact, it was heavily infiltrated by inflammatory cells and there was loss of cilia. Bacteria were less obvious in areas of ciliary loss but could still be seen adherent to the remaining short stubby cilia. The bronchial mucous glands were distended but contained little exudate and were lined by only a low cuboidal epithelium.

Involvement of the lung parenchyma was mainly confined to alveolar capillary congestion but in some areas, especially around affected bronchioles, there were foci of exudative pneumonia with alveolar macrophages and polymorphonuclear leucocytes in the alveolar air spaces; in dog No. 4 killed on day 10 there were also patchy areas of intra-alveolar oedema. There was slight oedema of pulmonary vessel walls.

Rhinitis, although consistently present, was most severe in the 2 infected dogs killed on day 10. Bacteria were present in the cilia of the

Dog Number	Day Examined	Rhinitis	Tracheitis	Bronchitis	Exudative pneumonia	Lymphadenitis
1	3	+	+	++	+	+
2	5	+	+++	++	+	++
3	7	+	+++	++	+	++
4	10	+++	+++	+++	+	++
5	10	+++	+++	++	++	++
6*	10	+	+	+	+	+
7*	10	+	++	++	+	++
8*	12	+++	+++	+++	+	++
9**	3	-	-	-	-	-
10**	5	-	-	-	-	-
11**	12	-	-	-	-	-

* Contact control ** Unexposed control Lesions graded + to +++ on severity

Table 21 : Experiment one - histopathological findings

turbinate epithelium and there was extensive infiltration of polymorphonuclear leucocytes and lymphocytes into the mucosa; a mucopurulent exudate was present in the lumen.

In the bronchial and retropharyngeal lymph nodes of dogs killed on days 3 and 5, there was marked sinusoidal oedema; many macrophages and a few polymorphonuclear leucocytes were also present in the lymph nodes. At later stages, oedema was less marked and there was evidence of lymphoid hyperplasia.

Histological changes in the contact control group were identical in form and degree to those described in the infected group. No histological lesions were found in the respiratory tracts of the unexposed controls.

Bacteriological Findings

Bacteria recovered from tissues taken at post-mortem examination are shown in Table 22. Bord. bronchiseptica was recovered, often in pure culture, from the turbinates, bronchi and lung parenchyma of both infected and contact control groups of dogs. Isolation of Bord. bronchiseptica from lymphoid tissues was less consistent and other bacterial species were also regularly isolated from these sites. In contrast the lower respiratory tract of unexposed controls was uniformly sterile although staphylococci and streptococci were recovered from the turbinates and retropharyngeal lymph nodes.

Virological Findings

No known canine virus could be isolated from the lungs of any dog used in this experiment.

Dog Number	Day Examined	Turbinate	Bronchi	Lung Parenchyma	Bronchial Lymph Node	Retropharyngeal Lymph Node
1	3	-	+	-	NT	-
2	5	+	+	+	+St	+St
3	7	+	+	+	-	NT
4	10	+St	+	+	-St	+St
5	10	+St, C	+St	+	-	+St, Sa
6*	10	+	+	+C	-	-St, P
7*	10	+	+	+	-St	-St
8*	12	+	+	-St	+	-
9**	3	-	-	-	-	-
10**	5	-St	-	-	-	-St
11**	12	-St	-	-	-	-St, Sa

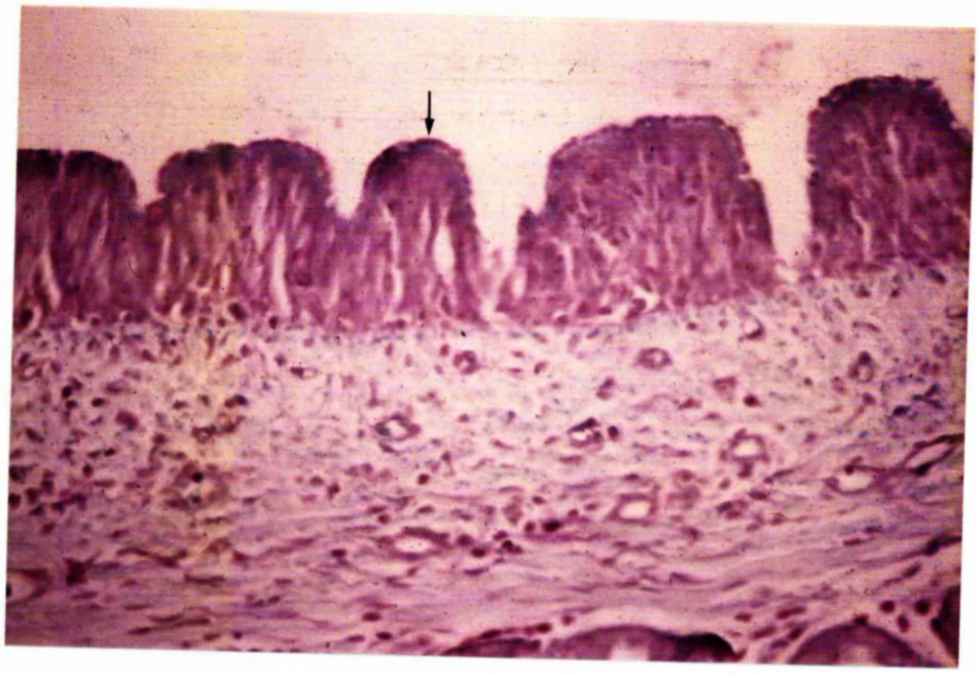
* Contact control
** Unexposed control
NT Not tested

\+ = Bord. bronchiseptica recovered
\- = " " not recovered
Sa = Staphylococcus sp.
St = Streptococcus sp.
C = Coliforms
P = Pasteurella sp.

Table 22 : Experiment one - recovery of bacteria at post mortem examinations

Fig 30 : Experiment one - tracheitis, dog 2. Masses of red-stained, Gram-negative bacteria (arrow) are present in the epithelial cilia.

(Gram-Twort, x 300).



SECTION 4 : EXPERIMENT TWO - 6 WEEK OLD DOGS

Experimental Design

This experiment was designed to confirm and extend the results described in Section 3. The dogs used were of a different age, the experiment was continued for a longer period of time and additional investigations were carried out both during the course of the experiment and at post mortem examination.

A total of 12 healthy, 6 week old puppies were used in this experiment; these animals were randomly divided into an infected group of 6 dogs, a contact control group of 4 dogs and an unexposed control group of 2 dogs. The groups were aerosolised and maintained as in the previous experiment and members of the 3 groups were killed at intervals from 4 days to 21 days after infection.

Clinical examination of the dogs was carried out daily as before and, in addition, the morning rectal temperature of each animal was noted. The aerobic nasal and pharyngeal bacterial flora of each dog was monitored daily from 3 days before infection until death. At necropsy, pathological, bacteriological, immunofluorescent, ultrastructural and virological studies were undertaken. Serological investigations were performed on paired samples from each dog obtained before infection and at necropsy.

Clinical Findings

As in the previous experiment, the main clinical finding was coughing (Fig.31). All of the infected group were coughing by 5 days after infection; coughing was most severe, as judged by the number and duration of paroxysmal episodes, between days 6 and 12, but persisted in each animal until death, as late as 21 days after infection. Excessive amounts of a serous nasal discharge were recorded in 5 of the 6 infected dogs on day 5 and

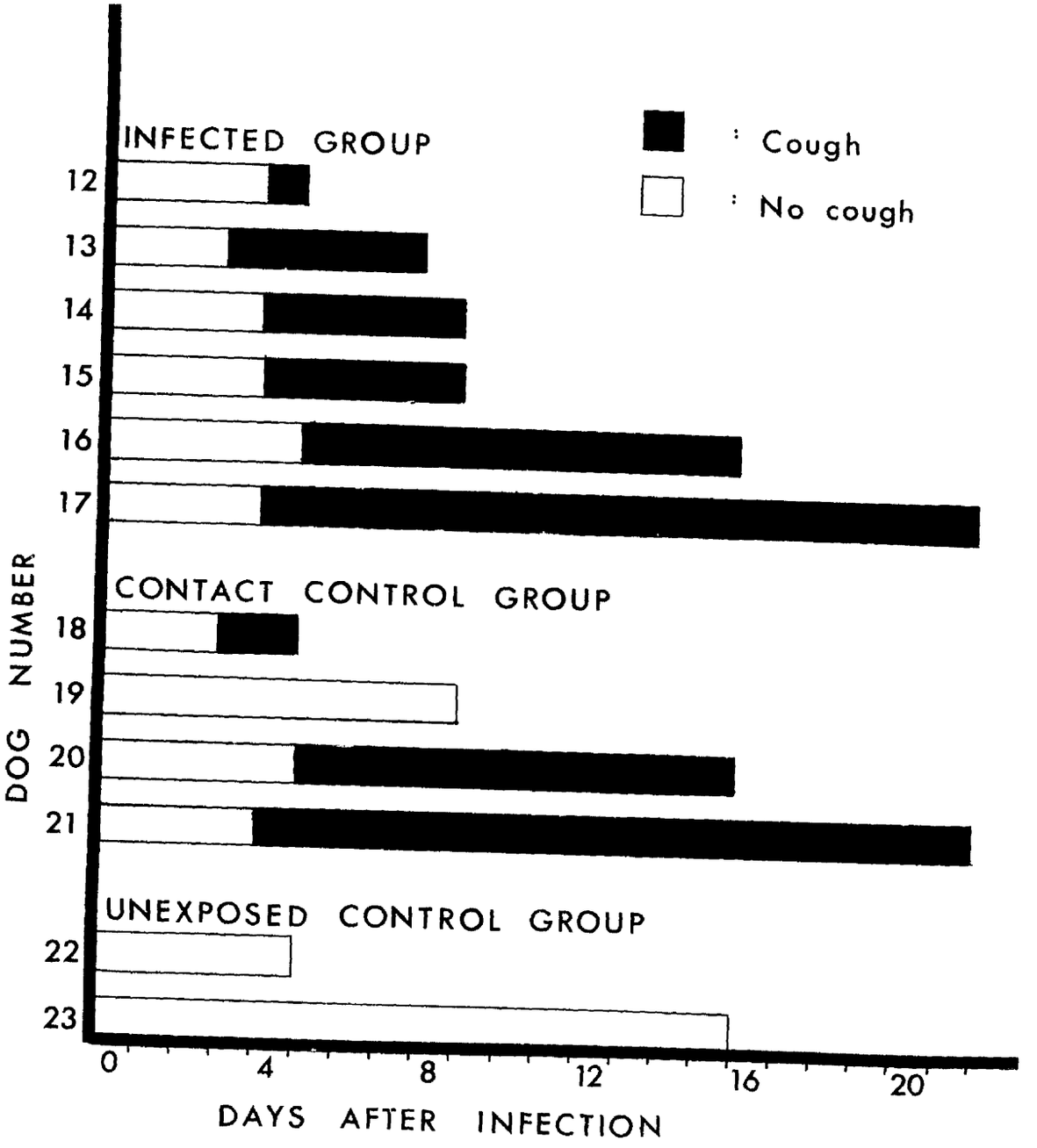


Fig. 31 : Experiment two - incidence of coughing.

sporadically from all dogs thereafter; occasionally this clear discharge was rather mucoid in consistency. On day 10, the two remaining infected dogs (Nos. 16 and 17) developed a mucopurulent nasal discharge (Fig. 32) which persisted in both animals until day 14.

Similar signs of respiratory disease were seen in the contact control group. Spontaneous paroxysmal coughing developed in 3 of these 4 dogs (Fig. 31) and a serous or mucoid discharge was inconsistently found from day 6 onwards. In dog No. 20 a mucopurulent nasal discharge was present on days 11 and 12.

No clinical signs of disease were observed in the unexposed control group.

The morning rectal temperature of all dogs varied slightly from day to day but was consistently within the range 100.5°F to 102.5°F. All dogs remained bright and active throughout the experiment.

Pathological Findings

Macroscopic findings : As in the previous experiment macroscopic changes in experimental animals were confined to the respiratory tract; changes were similar to those described in experiment one.

In the infected group, small greyish-red foci were found throughout the lungs of all animals and, in dog No. 16, killed at 15 days, there were raised areas of consolidation, dark red in colour, in the anterior lung lobes (Figs. 33 and 34). A mucopurulent exudate was found in the tracheobronchial tree of dogs killed from day 7 onwards (Fig. 35 and 36). The bronchial and retropharyngeal lymph nodes were consistently enlarged up to twice normal size throughout infection and were sometimes oedematous (Figs. 33 and 35); tonsillar enlargement was noted in 5 dogs (Nos. 13 to 17) with distinct haemorrhagic foci present in 3 of these (Nos. 13, 15 and 16). The turbinate mucosae were congested and overlain by a sticky, sometimes purulent exudate in 5 dogs (Nos. 12 to 16) and in 3 of these (Nos. 12, 13 and 15) a purulent exudate was also present in the nasopharynx, the adenoid region

of which was prominent and congested (Fig. 37).

Similar changes were seen in 3 of the 4 contact control dogs (Nos. 18, 19 and 20), but no macroscopic changes were found in either of the unexposed controls.

Microscopic findings: Histopathological changes in the respiratory tract and lymph nodes of dogs examined in this experiment are summarised in Table 23. As in experiment one, there was, in the infected group of dogs, inflammation at all levels of the respiratory tract.

The most prominent finding was tracheobronchitis which was present as early as 4 days after infection. At this time, there was acute inflammation of the tracheobronchial tree (Figs. 38 and 39); there was congestion and oedema of the lamina propria with heavy infiltration of polymorphonuclear leucocytes into the lamina propria and epithelium; the epithelium was, however, intact. Bacteria were evident among the epithelial cilia (Figs. 39 and 40) and a purulent exudate was present in the tracheobronchial lumen. The bronchial mucous glands were distended but were devoid of secretion and, in some, polymorphonuclear leucocytes were present in the lumen. At 7 and 8 days after infection, polymorphonuclear leucocytes were still evident in the tracheobronchial epithelium but, in addition, there were areas of disorganisation, ciliary loss and necrosis of the epithelium (Fig. 41) and, in such areas, bacteria, although still present, were less prominent. The lamina propria was less congested and oedematous than at 4 days but still contained a heavy cellular infiltrate which was composed mainly of polymorphonuclear leucocytes with some lymphocytes. The bronchial mucous glands were still distended.

At 15 and 21 days, tracheobronchitis was still present. The tracheobronchial mucosa was thickened and, in the bronchial tree, was thrown into folds (Fig. 42): the epithelium was hyperplastic and was also infiltrated by polymorphonuclear leucocytes (Fig. 43); the lamina propria and submucosa contained a cellular infiltrate composed mainly of lymphocytes

Dog Number	Day Examined	Rhinitis	Tracheitis	Bronchitis	Exudative pneumonia	Lymphadenitis
12	4	++	++	++	+	+
13	7	++	++	++	+	+
14	8	+	+++	+++	+	++
15	8	++	+++	++	+	++
16	15	+	++	+++	+++	++
17	21	+	+++	++	++	++
18*	4	+	+	-	-	-
19*	8	+	++	+	+	+
20*	15	++	++	++	+	++
21*	21	+	+	+	-	+
22**	4	-	-	-	-	-
23**	15	-	-	-	-	-

* Contact control. ** Unexposed control. Lesions graded + to +++ on severity.

Table 23 : Experiment two - histopathological findings

with some macrophages and plasma cells and a few polymorphonuclear leucocytes (Figs. 43 and 44). Bacteria were still present, in small, scattered clumps, in the epithelial cilia (Fig. 44). The bronchial glands still appeared distended.

In infected dogs killed from 4 to 8 days after infection there was alveolar capillary congestion and, especially around severely affected bronchioles, small foci of exudative pneumonia with infiltration of macrophages and polymorphonuclear leucocytes into the alveolar air spaces (Fig. 45). In dog No. 16, killed on day 15, there were more extensive areas of exudative pneumonia (Fig. 46) with surrounding areas of congestion and alveolar oedema. In dog 7, killed on day 21, there were small foci of oedema with accumulation of polymorphonuclear leucocytes in the alveoli.

In dogs killed from days 4 to 8, there was rhinitis, characterised by congestion, oedema and infiltration by polymorphonuclear leucocytes of the lamina propria and epithelium (Figs. 47, 48 and 49): a mucopurulent exudate was usually present in the lumen. In the dogs killed on days 15 and 21, rhinitis was less marked but increased numbers of lymphocytes and some plasma cells were present in the lamina propria.

In the bronchial and retropharyngeal lymph nodes at 4 days after infection, there was congestion and sinusoidal oedema and polymorphonuclear leucocytes were present in the afferent lymphatics and medulla (Fig. 50). Similar changes were present at 7 and 8 days after infection, but at 15 and 21 days after infection, there was lymphoid follicular hyperplasia (Fig. 51) and the medullary cords contained large numbers of plasma cells. In the palatine and adenoid tonsils, there was usually congestion with heavy infiltration by polymorphonuclear leucocytes and macrophages of the overlying epithelium; in dogs killed on days 15 and 21 there was also lymphoid follicular hyperplasia.

No abnormalities were detected in the other organs examined.

Histopathological changes in the dogs of the contact control group were similar in nature to those described in the infected group.

Histopathological changes were not found in the respiratory tract of the unexposed control dogs.

Bacteriological Findings

The recovery of Bord. bronchiseptica from daily nasal and pharyngeal swabs is shown in Table 24. The number of colonies of Bord. bronchiseptica recovered varied from day to day and from dog to dog, but, in general, more bacteria were found in the earlier phases of infection, from days 3 to 10, than in the later stages. Nonetheless, bacteria could still be isolated from infected and contact control dogs at 21 days after infection.

The isolation of Bord. bronchiseptica from samples taken at necropsy is detailed in Table 25. It is noticeable that profuse, pure cultures of Bord. bronchiseptica could be recovered from the tracheobronchial tree of both infected and contact control dogs as late as 15 and 21 days after infection when recovery from the upper respiratory tract, as seen in daily swabs, had become less consistent. While profuse growth of Bord. bronchiseptica was often obtained from cultures of the turbinate mucosae, the bacterium was seldom present in pure culture; Staphyloccocus spp, Streptoccocus spp. Klebsiella spp. and Pasteurella spp. were also recovered from this site. These other bacterial species and coliform-like organisms were also recovered in small numbers from the lymph nodes and tonsils where Bord. bronchiseptica was only inconsistently present. The lower respiratory tract of unexposed controls was consistently sterile.

Gram staining of direct tissue smears taken at post-mortem examination demonstrated small, Gram-negative bacilli or cocco-bacilli, singly or in small groups, in samples taken from sites which, on cultural examination, yielded Bord. bronchiseptica (Fig.52); in some microscopic fields bacteria could be identified within or attached to polymorphs.

Immunofluorescence Findings

Fluorescent antibody staining of lung and tracheal samples

Dog Number	Days after infection. -3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
12	-	-	-	-	N	NP	NP	NP																	
13	-	-	-	-	-	P	P	NP	N	P	P														
14	-	-	-	-	N	N	P	NP	N	N	NP	N													
15	-	-	-	-	NP	NP	NP	NP	NP	NP	NP	NP													
16	-	-	-	-	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	N	P						
17	-	-	-	-	NP	P	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	N	NP	NP	-	NP	P	P	NP	P
18 *	-	-	-	-	-	-	-	P																	
19 *	-	-	-	-	NP	N	-	NP	N	NP	NP	NP													
20 *	-	-	-	-	NP	P	NP	NP	NP	NP	P	NP	NP	NP	NP	NP	NP	NP	NP						
21 *	-	-	-	-	N	N	NP	NP	NP	P	NP	P	P	NP	P	P	P	N	P	-	P	P	N	P	P
22**	-	-	-	-	-	-	-	-																	
23**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						

* Contact control

** Unexposed control.

N = Bord. bronchiseptica recovered from nasal swab.

P = Bord. bronchiseptica recovered from pharyngeal swab.

\- = No Bord. bronchiseptica recovered.

Table 24 : Experiment two - recovery of Bord. bronchiseptica from daily nasal and pharyngeal swabs.

Dog Number	Day Examined	Turbinates	Trachea	Bronchus	Lung Parenchyma	Bronchial Lymphnode	Retro-Pharyngeal Lymphnode.	Tonsil
12	4	++++	++++	++++	++	+	-	+ St.
13	7	++++ Sa, St	++++	++++	++	- St	-	-
14	8	++++ Sa, St	+++	++++	+++	- St, P	-	- St, P, C
15	8	+++	+++ Sa, C	++++	++	- St	- St	+ St
16	15	++++ Sa, K	++++	++++	++ St	- Sa, K	+	+ Sa
17	21	++++	++++	++++	++	-	-	- K, St
18 *	4	+++ Sa, K, St	++ C	-	-	- C, St	- C, St	+ Sa
19 *	8	+ P, Sa, St	+++	+++	++	+	-	- C, P, St
20 *	15	++++	++++	++++	++	-	-	+
21 *	21	-	+	++++	++	-	-	-
22 **	4	- Sa, St	-	-	-	- St	-	- Sa, St
23 **	15	-	-	-	-	- Sa	-	- C, St

* Contact control
** Unexposed control.

\+ = Bord. bronchiseptica recovered from sparse + to profuse +++ culture
\- = No Bord. bronchiseptica recovered.

St = Streptococcus spp.
Sa = Staphylococcus spp.
C = Coliforms
P = Pasterurella spp.
K = Klebsiella spp.

Table 25 : Experiment two - bacteriological findings at post mortem examination.

allowed the specific identification of Bord. bronchiseptica at these sites. In the early stages of infection i.e. 4 to 8 days after infection bacteria were found on the surface of tracheal (Fig. 53) and bronchial (Fig. 54) epithelium, where they were so massed as to produce an almost solid fluorescent outline of these structures. In areas of epithelial necrosis bacteria were found extending down to basal epithelial layers (Fig. 55) but, for the most part, they were confined to the luminal epithelial surface. Bacteria were also found in the luminal exudate and occasional small clumps were found in the alveoli. Bord. bronchiseptica could be identified on tracheal, bronchial and bronchiolar epithelium in both infected and contact control dogs until 21 days after infection although, in the later stages, they appeared in small clumps (Fig. 56), rather than the solid masses seen earlier. Very occasionally a few individual bacteria could be found in the subcapsular sinuses of the bronchial lymph nodes.

Smears of nasopharyngeal exudates revealed masses of bacteria apparently free in mucus and within or attached to polymorphonuclear leucocytes (Fig. 57).

Bord. bronchiseptica was not found in tissue samples from un-exposed control dogs.

Lung, lymph node and tonsillar samples stained with CDV and CAV specific antisera were consistently negative for these viruses in infected, contact control and unexposed control animals.

Ultrastructural Findings

The ultrastructure of bronchial epithelium found in samples from unexposed control dogs was in general agreement with that previously reported (Frasca et al., 1968; Wheeldon, 1974). Five cell types were regularly seen: ciliated cells, which had both cilia and microvilli along the luminal border (Fig. 58); goblet cells; basal cells, immediately above the basement membrane; intermediate cells; and, infrequently, non-ciliated cells with only microvilli on the luminal edge. Ciliary structure was, in the main, similar to that reported for cilia in many different sites

(Fawcett, 1967) with the typical axial filament complex of nine peripheral double tubules and two central tubules contained within the plasmalemma (Fig. 59). Compound cilia were occasionally seen; these were composed of multiple sets (sometimes as many as 12, but usually only 3 or 4) of axial filaments contained within a common external membrane (Fig. 59). These compound cilia have previously been reported in normal dogs (Wheeldon, 1974). Occasionally, bizarre compound cilia with incomplete or irregularly orientated axial filaments were found.

Infection with Bord. bronchiseptica, as seen in infected and contact control animals resulted in a number of changes in the bronchial epithelium. Increased vacuolation of apical cytoplasm and swelling of mitochondria were consistently found in epithelial cells early in infection whether bacteria were present in the immediately overlying cilia or not (Fig. 60). With increasing duration of infection, this mitochondrial swelling and cytoplasmic vacuolation became more severe and there were apical protrusions of cytoplasm, sometimes containing ciliary tubules, into the lumen where debris from necrotic epithelial cells could be found (Fig. 61); these cytoplasmic protrusions were not seen in control material. Polymorphonuclear leucocytes could be found in the epithelium (Fig. 62) and in the lumen where they could be seen engulfing bacteria (Fig. 63 and 64). Goblet cells were often seen to discharge prematurely with the release of cytoplasmic organelles as well as secretory granules (Fig. 63).

From 8 days onwards, cilia appeared to be decreased in number (Figs. 61 and 62). In addition, atypical ciliary formations were found more frequently than in control material; incomplete sets of axial filaments were often seen in the cytoplasmic projections mentioned above (Fig. 61), and bizarre compound cilia were also often present (Fig. 62). Microvilli did not appear to increase in number but were more obvious due to loss of cilia. Atypical ciliated cells with irregular orientated microtubules present throughout the cytoplasm (Fig. 65) were found in dog 16 killed at 15 days.

Epithelial intercellular spaces were more distended in infected animals, especially in the basal epithelial area but tight junctions and desmosomes

seemed to remain intact. Polymorphonuclear leucocytes were more numerous in the lamina propria of infected dogs than of unexposed controls.

The most striking feature seen in infected dogs, however, was the presence of bacteria among the cilia and microvilli of surface epithelial cells (Figs. 66, 67 and 68). Bacteria were found very closely apposed to both cilia and microvilli and were possibly adherent to both these structures by means of the fine filamentous processes (Figs. 67 and 68) mentioned below. The bacterial structure seen in both bronchial tissue samples and bacterial colonies removed from agar plates, was similar to that described for other Gram-negative bacteria (Fuhs, 1965; van Iterson, 1965) and for a strain of Bord. bronchiseptica (Richter and Kress, 1967). Bacteria (Figs. 67, 68 and 69) varied considerably in length, from 0.5μ - 1.5μ, but less in width, about 0.5μ. The cell wall was composed of several layers, the external surface was rather irregular or wavy in appearance and fine filamentous processes, almost certainly pili, could be seen radiating from the cell surface. The peripheral cytoplasm contained a well defined zone of electron-dense ribosomes, whilst the more electron-lucent central nucleoid was composed of a fine irregular fibrillar mesh in which coarsely granular electron-dense material could be seen.

Serological Findings

The results of both serum agglutination tests and indirect fluorescent antibody tests are summarised in Table 26. Antibodies were not present in the sera of any dog before exposure to infection but were found in the sera in both infected and contact control dogs killed from day 7 onwards.

In the agglutination test, the end points were easily read in spite of a cloudiness of sera associated with the lipaemia frequently seen in young dogs. The indirect immunofluorescence test correlated well with the agglutination test but was more laborious to perform due to difficulty in obtaining adequately fixed smears of Bord. bronchiseptica; it was decided to limit future serological studies to an agglutination test.

Virological Findings

No known canine virus was found in the lungs of any dog used in the experiment.

Dog Number	Day Killed	Serum Agglutination Pre-infection	Necropsy	Indirect Fluorescent Antibody Pre-infection	Necropsy
12	4	< 8	NT	-	NT
13	7	< 8	32	-	32
14	8	< 8	32	-	32
15	8	< 8	8	-	8
16	15	< 8	16	-	32
17	21	< 8	16	-	16
18*	4	< 8	< 8	-	-
19*	8	< 8	8	-	8
20*	15	< 8	8	-	16
21*	21	< 8	32	-	32
22**	4	< 8	< 8	-	-
23**	15	< 8	< 8	-	-

* Contact control
** Unexposed control
NT Not tested

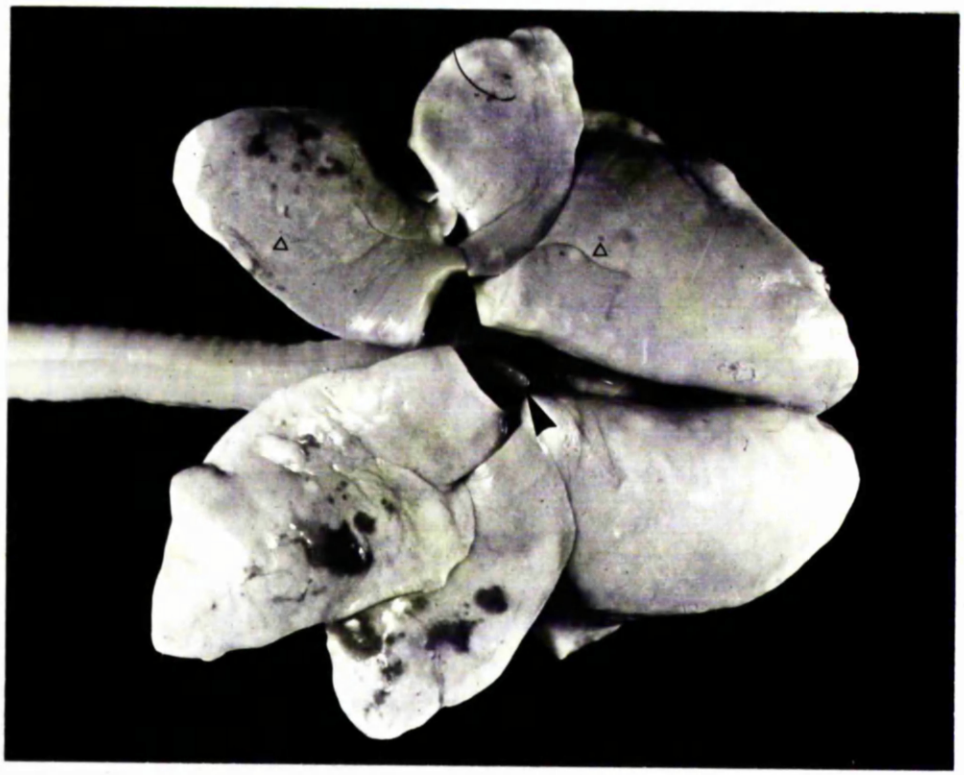
Titres expressed as reciprocal of serum dilution

Table 26 : Experiment two - serological findings

Fig. 32 : Experiment two - mucopurulent nasal discharge, dog 17.

Fig. 33 : Experiment two - lungs of dog 16. Small foci of congestion (open arrows) are visible on the pleural surface of all the lung lobes and larger areas of pneumonia are present in both left and right anterior lobes. The bronchial lymph nodes (closed arrow) are enlarged.

Fig. 34 : Experiment two - lungs of dog 16. Dark, irregular areas of pneumonia are present in the apical and cardiac lung lobes (arrow). Smaller, 1-2 mm foci of congestion are visible scattered over the pleural surface.



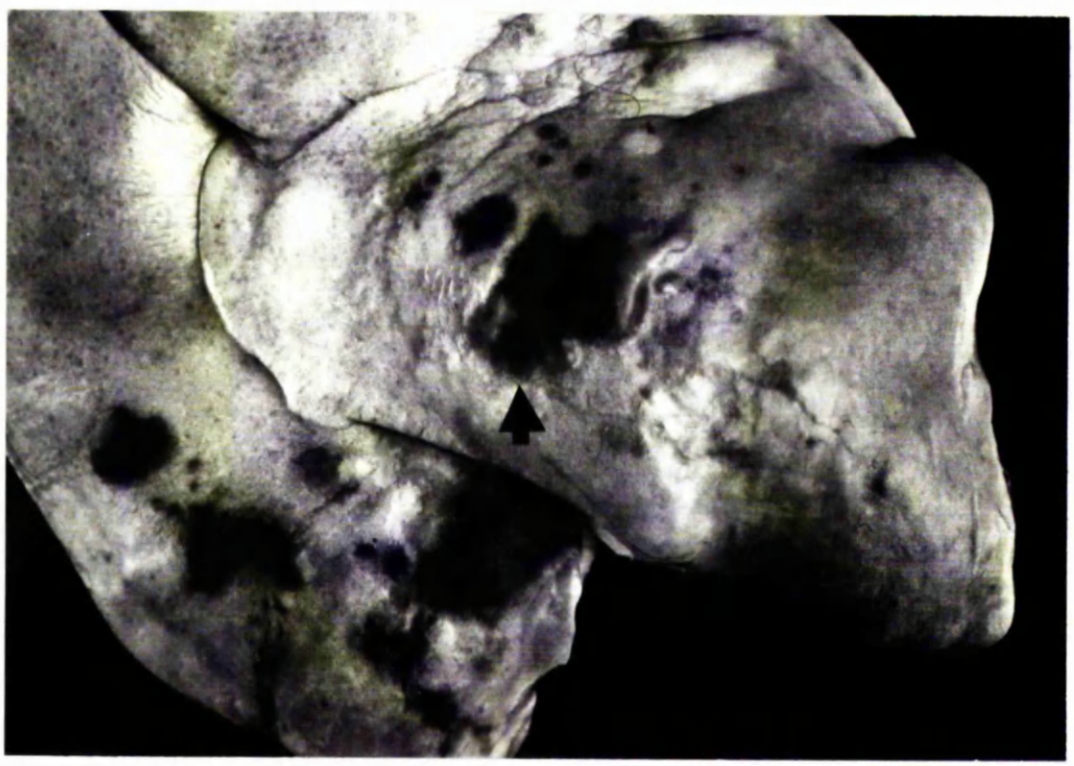


Fig. 35 : Experiment two - trachea of dog 14. A thick, mucopurulent exudate (arrow) is present in the trachea. The bronchial lymph nodes are enlarged.

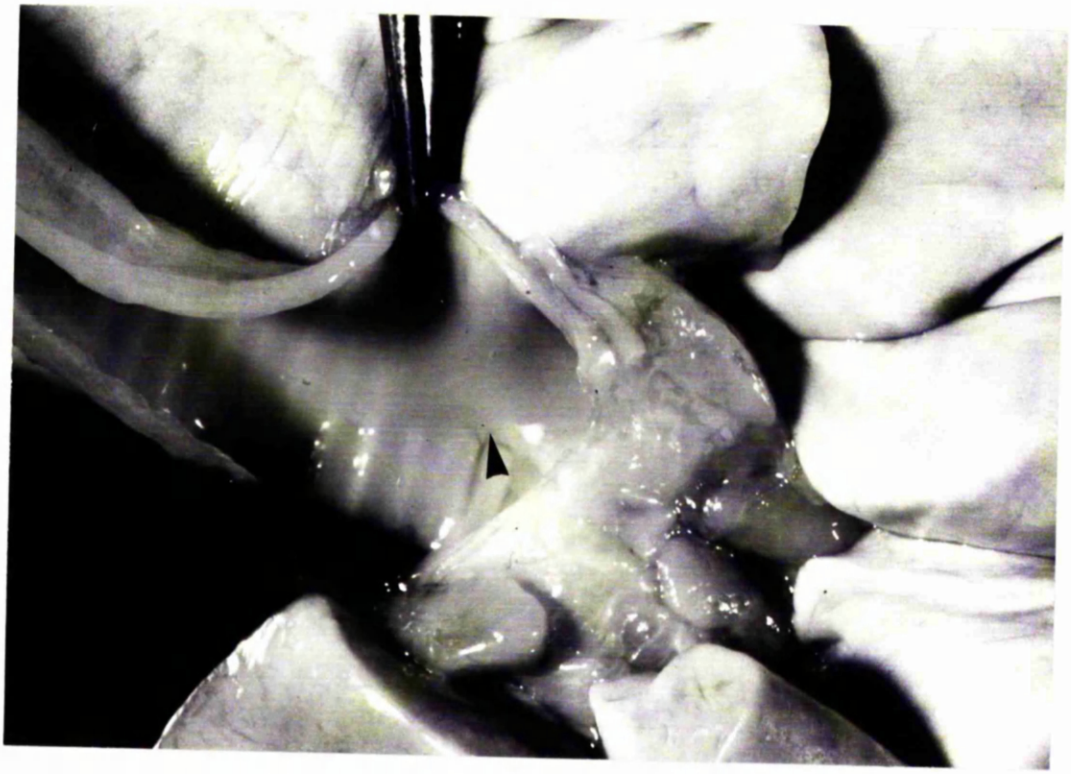
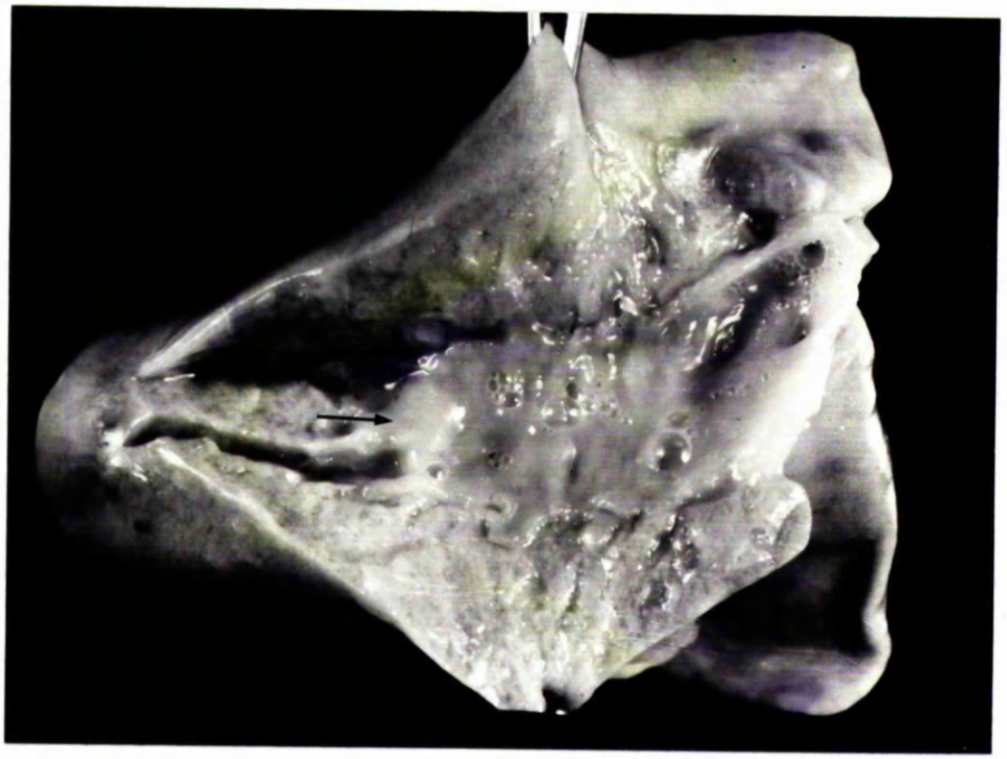


Fig. 36 : Experiment two - bronchus of dog 14. The main bronchus of the right cardiac lung lobe has been partially opened along its length. A slightly frothy, mucopurulent exudate is present on the bronchial epithelial surface. A plug of exudate (arrow) blocks the lumen of the unopened portion of the bronchus.

Fig. 37 : Experiment two - nasopharynx of dog 15. A pool of mucopus (arrow) is present in the nasopharynx overlying a hyperplastic adenoid area (star).



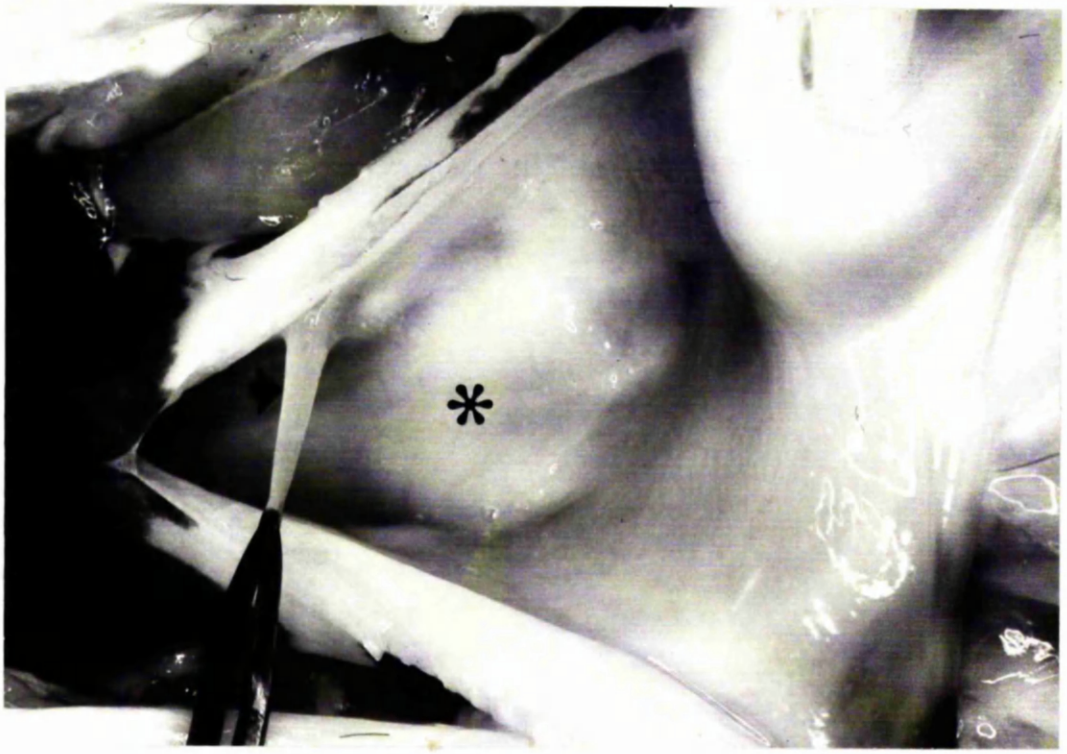
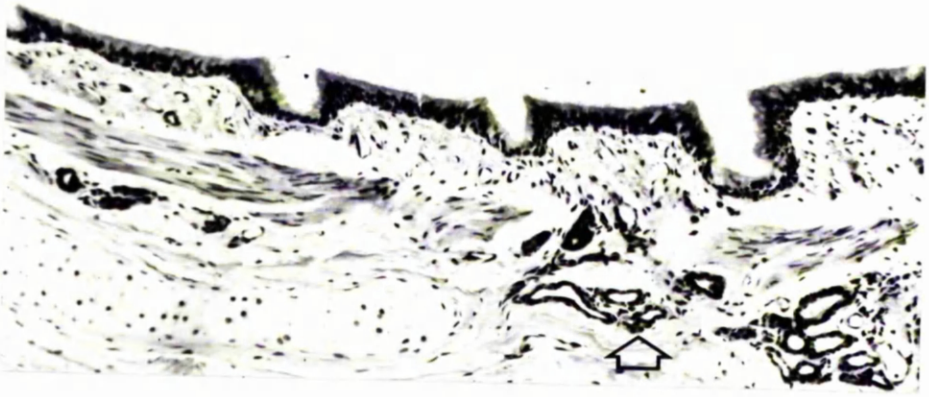


Fig. 38 : Normal dog - bronchial wall. The epithelium is thin, approximately 3 cell layers thick and the lamina propria contains very few cells. The bronchial glands (arrow) are small.

(HE, x 110).

Fig. 39 : Experiment two - bronchitis, dog 12. Four days after aerosolisation, bacteria are present in the epithelial cilia. The lamina propria is oedematous and polymorphonuclear leucocytes are infiltrating the epithelium. A purulent exudate is present in the lumen.

(HE, x 400).



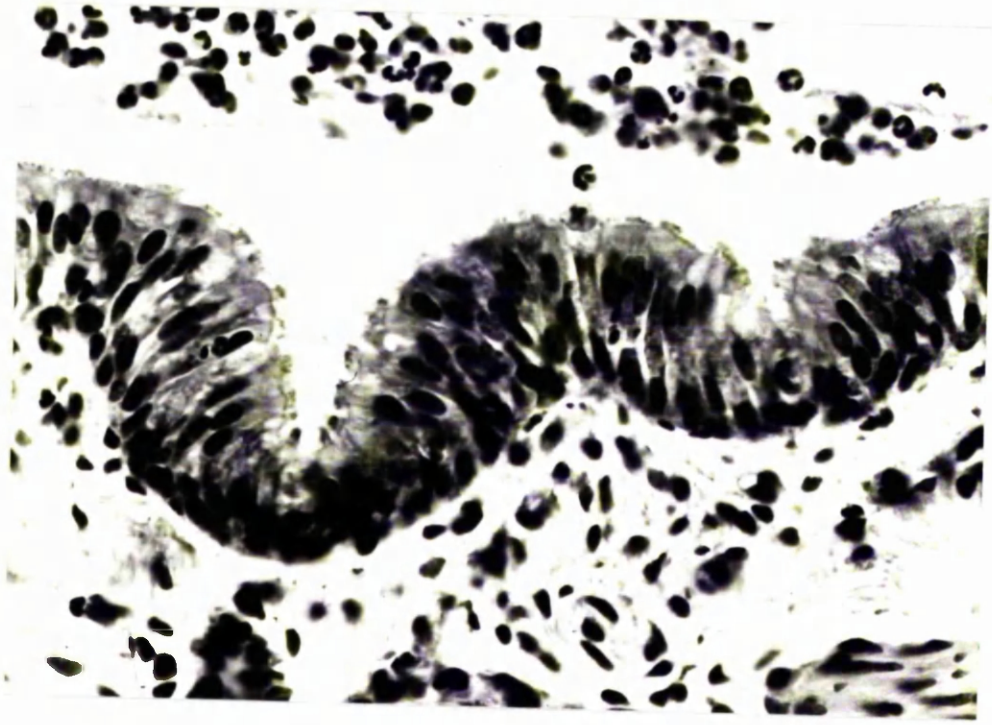
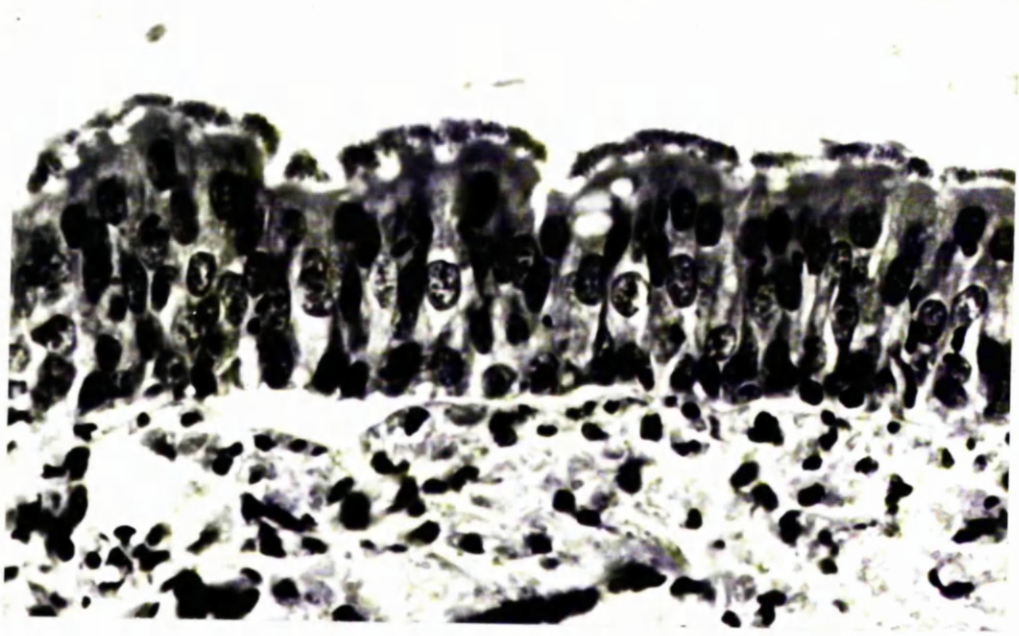


Fig. 40 : Experiment two - tracheitis, dog 12. Four days after infection, masses of bacteria are visible among the epithelial cilia. The epithelium is infiltrated by polymorphonuclear leucocytes.

(HE, x 450).

Fig. 41 : Experiment two - tracheitis, dog 14. The tracheal epithelium is generally disorganised and many of the epithelial cells are necrotic; there has been extensive ciliary loss. The lamina propria is congested and polymorphonuclear leucocytes are present in both the lamina propria and the epithelium.

(HE, x 400).



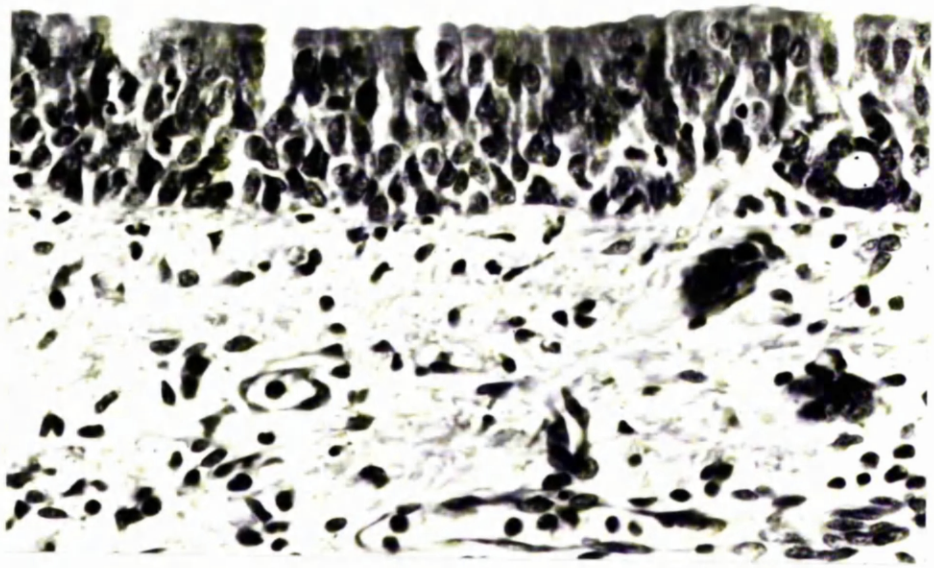


Fig. 42 : Experiment two - bronchitis, dog 17. Twenty-one days after infection the bronchial wall is thickened (cf Fig. 38). The epithelium is thrown into folds and a dense cellular infiltrate is present in the lamina propria and submucosa. The bronchial glands are distended.

(HE, x 35).

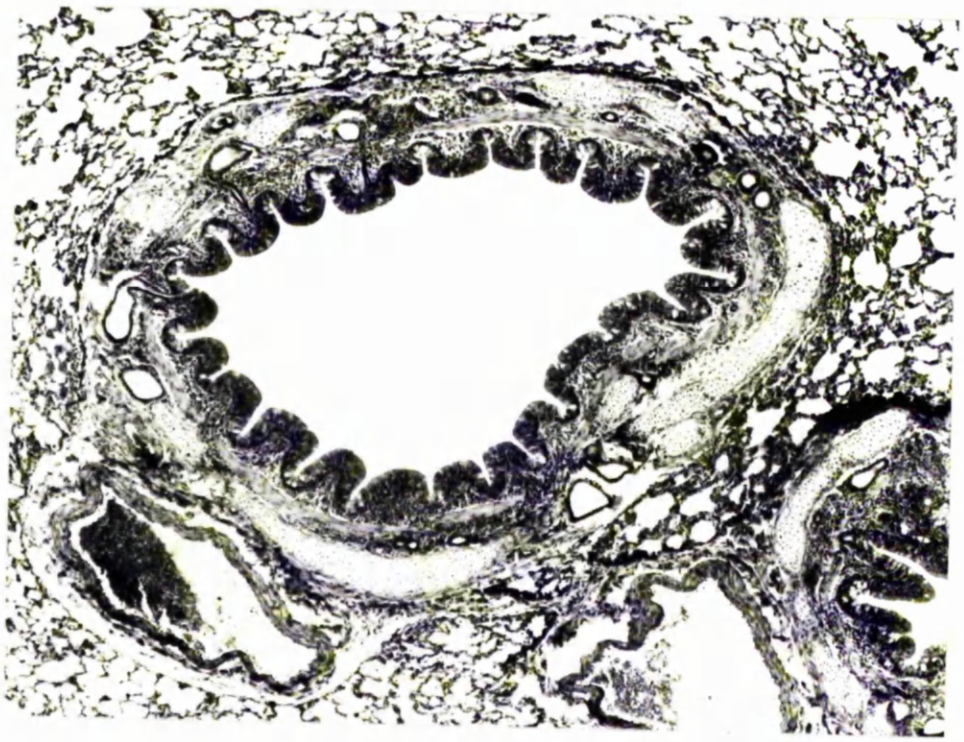


Fig. 43 : Experiment two - bronchitis, dog 17. Foci of degeneration and necrosis are visible within the hyperplastic bronchial epithelium which is also infiltrated by polymorphonuclear leucocytes. The lamina propria is congested and contains a heavy cellular infiltrate.

(HE, x 250).

Fig. 44 : Experiment two - bronchitis, dog 17. Bacteria are still evident in the epithelial cilia. The cellular infiltrate in the lamina propria is composed predominantly of lymphocytes. Note marginating polymorphonuclear leucocytes in vessel in lamina propria (arrow).

(HE, x 400).

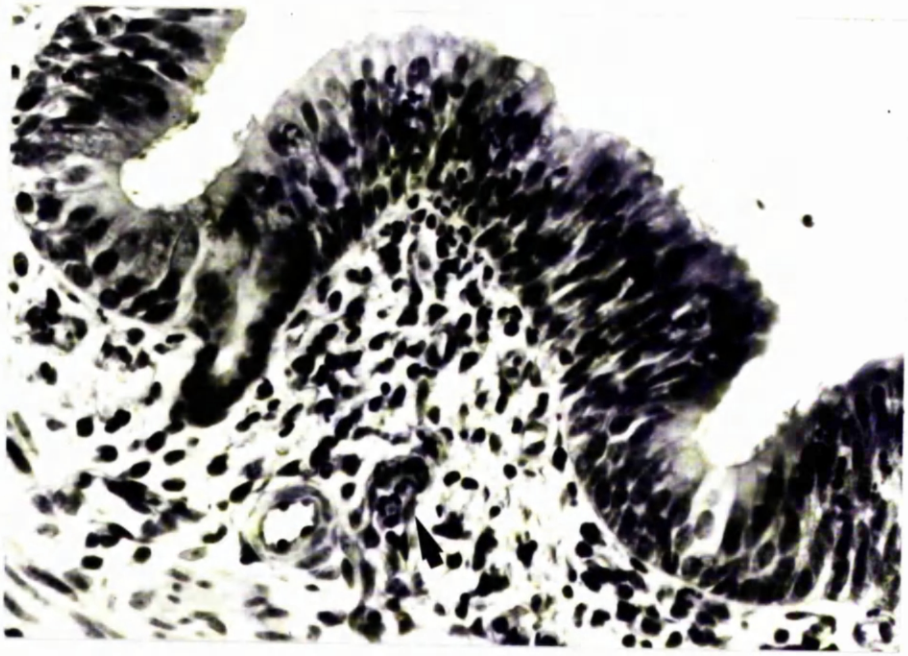


Fig. 45 : Experiment two - exudative pneumonia, dog 14.

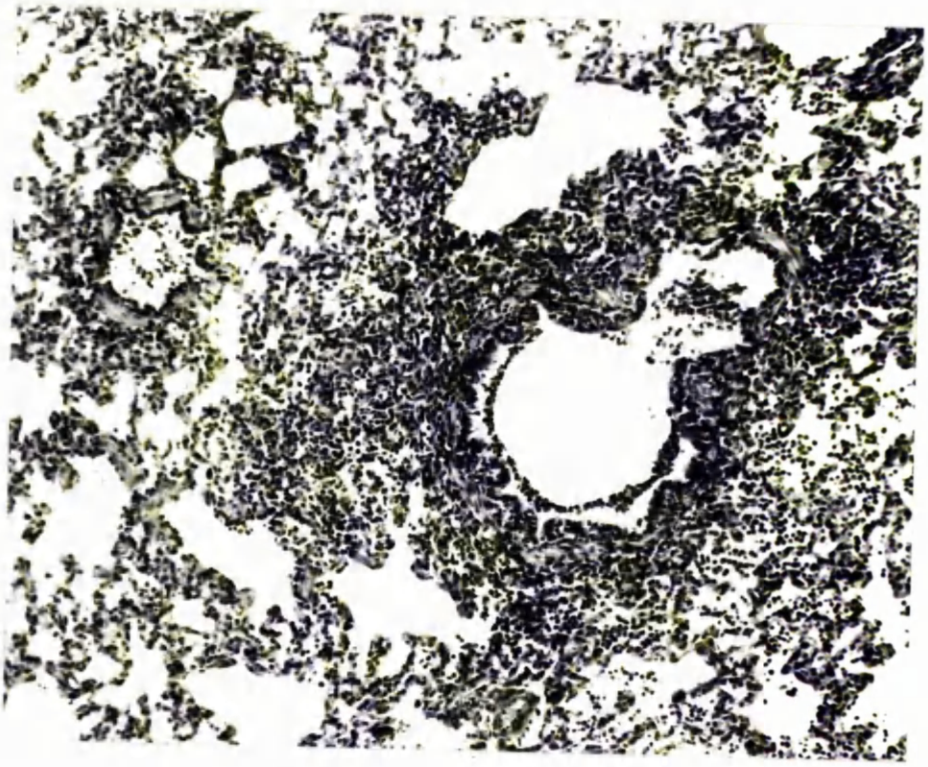
Eight days after infection, foci of exudative pneumonia are present around severely affected bronchioles; the alveolar air spaces contain large numbers of polymorphonuclear leucocytes and macrophages.

(HE, x 110).

Fig. 46 : Experiment two - exudative pneumonia, dog 16.

Fifteen days after infection, the structure of this area of lung tissue is almost completely obscured by the infiltration of large numbers of polymorphonuclear leucocytes and macrophages.

(HE, x 110).



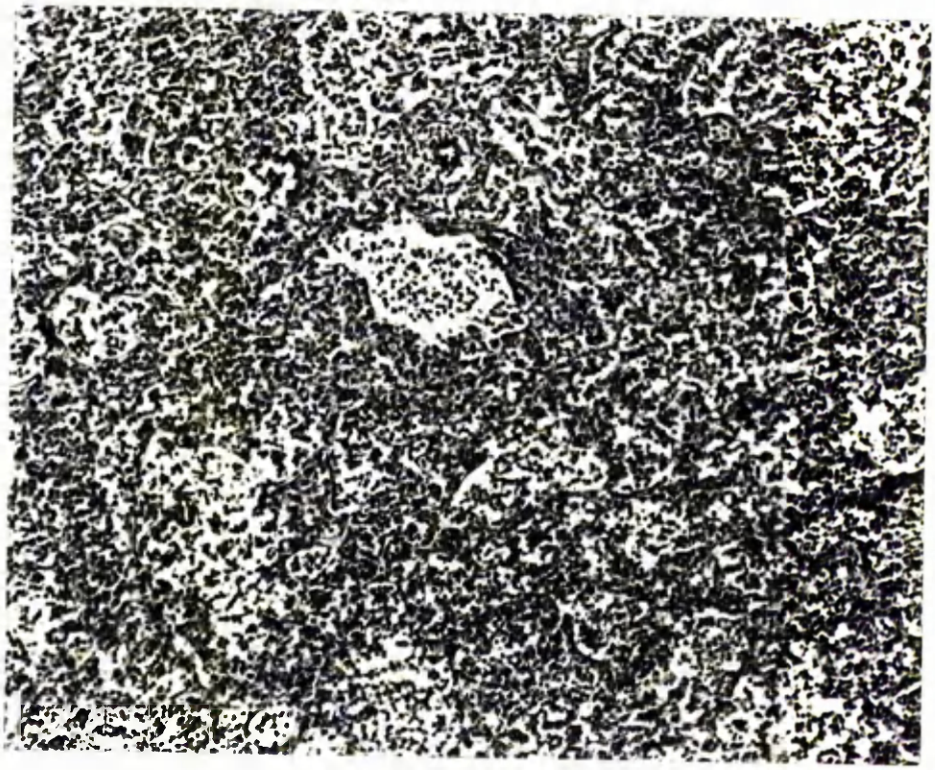


Fig. 47 : Normal dog - nasal cavity. The nasal vestibule is lined by a stratified squamous epithelium.

(HE, x 110).

Fig. 48 : Experiment two - rhinitis, dog 15. A purulent exudate is present in the lumen of the nasal vestibule 8 days after infection and the stratified squamous epithelium is heavily infiltrated by polymorphonculear leucocytes. Polymorphonuclear leucocytes are also present in the lamina propria which is congested and oedematous.

(HE, x 110).

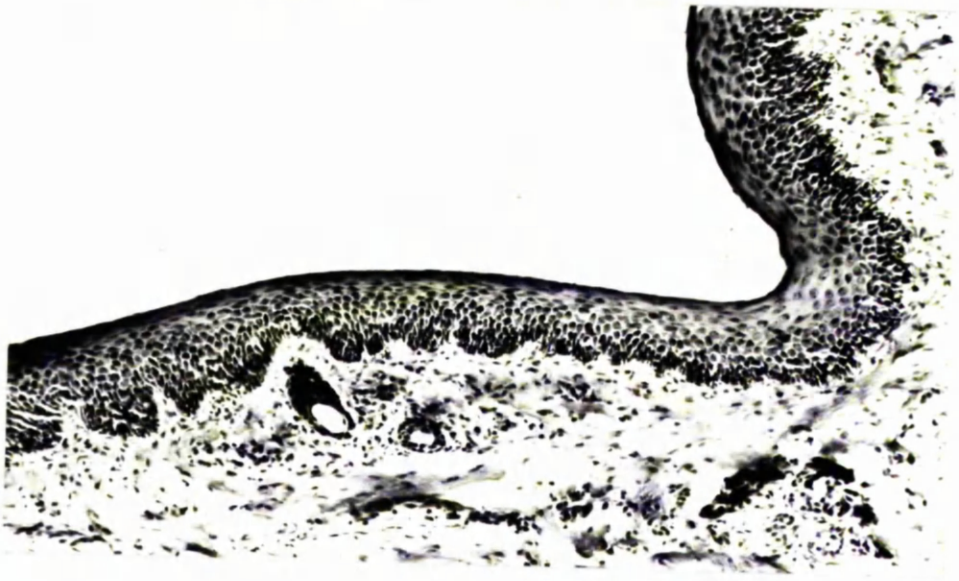


Fig. 49 : Experiment two - rhinitis, dog 15. The turbinate epithelium is infiltrated by polymorphonuclear leucocytes and the lamina propria is oedematous. A mucopurulent exudate is present in the lumen between the scrolls of the turbinate bones.

(HE x 110).

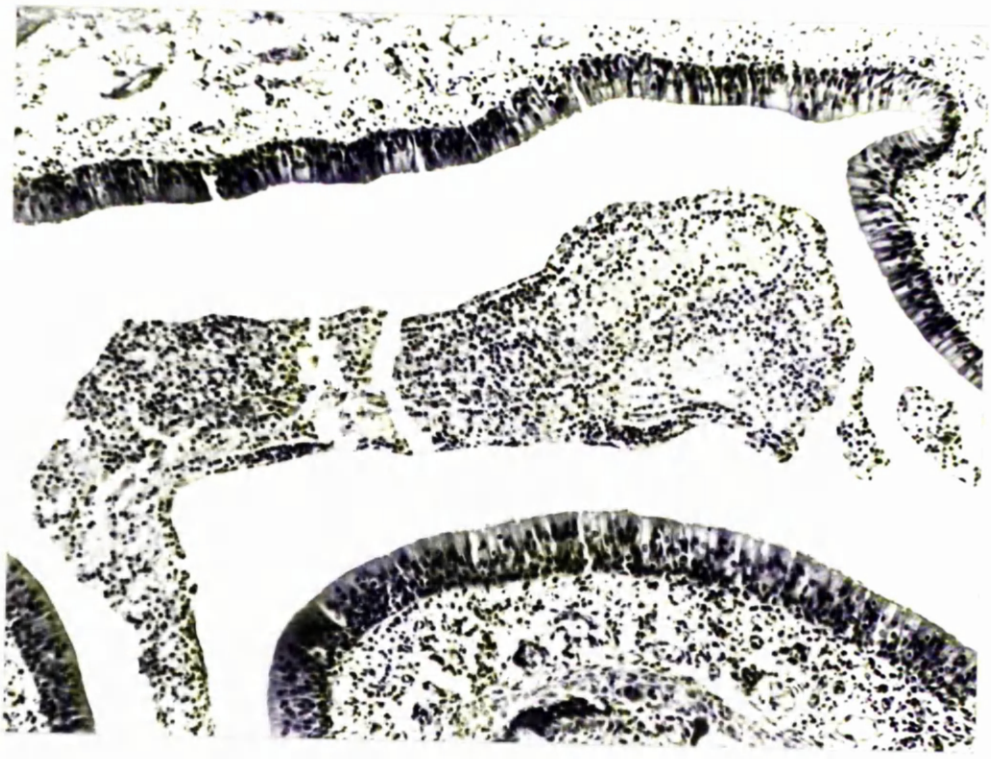


Fig. 50 : Experiment two - lymphadenitis, dog 12. Four days after infection, afferent lymphatic vessels and the subcapsular sinuses are dilated by oedema fluid; polymorphonuclear leucocytes are also present in the sinuses.

(HE, x 110).

Fig. 51 : Experiment two - lymphoid follicular hyperplasia, dog 17. Twenty-one days after infection, large numbers of lymphoid follicles are present in the cortex of the bronchial lymph node.

(HE, x 35).

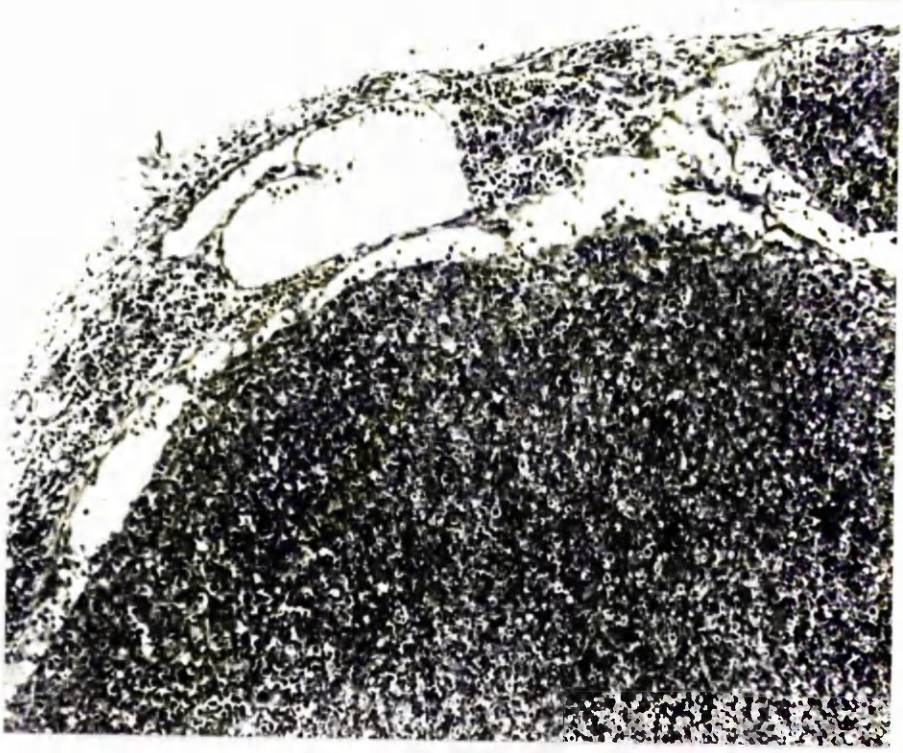


Fig. 52 : Experiment two - Gram stained smear of bronchial exudate. Small Gram-negative bacilli are present singly and in small groups throughout the field. Bacilli can be seen within or overlying polymorphonuclear leucocytes (arrow).

(Gram stain, x 1200).

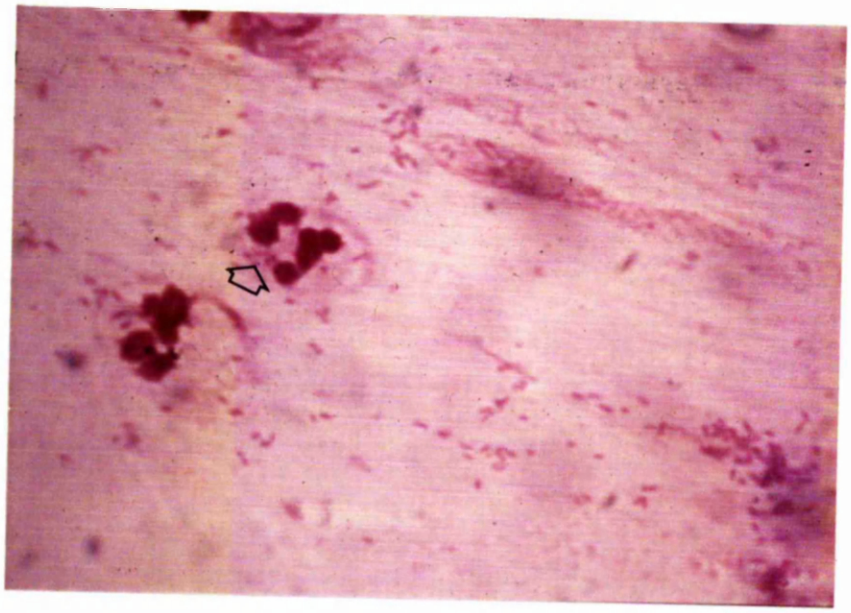


Fig. 53 : Experiment two - immunofluorescence, trachea.

Four days after infection, masses of positively-stained bacteria produce an almost solid line of fluorescence on the surface of the tracheal epithelium.

(Fluorescent antibody, x 200).

Fig. 54 : Experiment two - immunofluorescence, bronchus.

Seven days after infection, masses of fluorescent bacteria outline the surface of the folded bronchial mucosae.

(Fluorescent antibody, x 250).

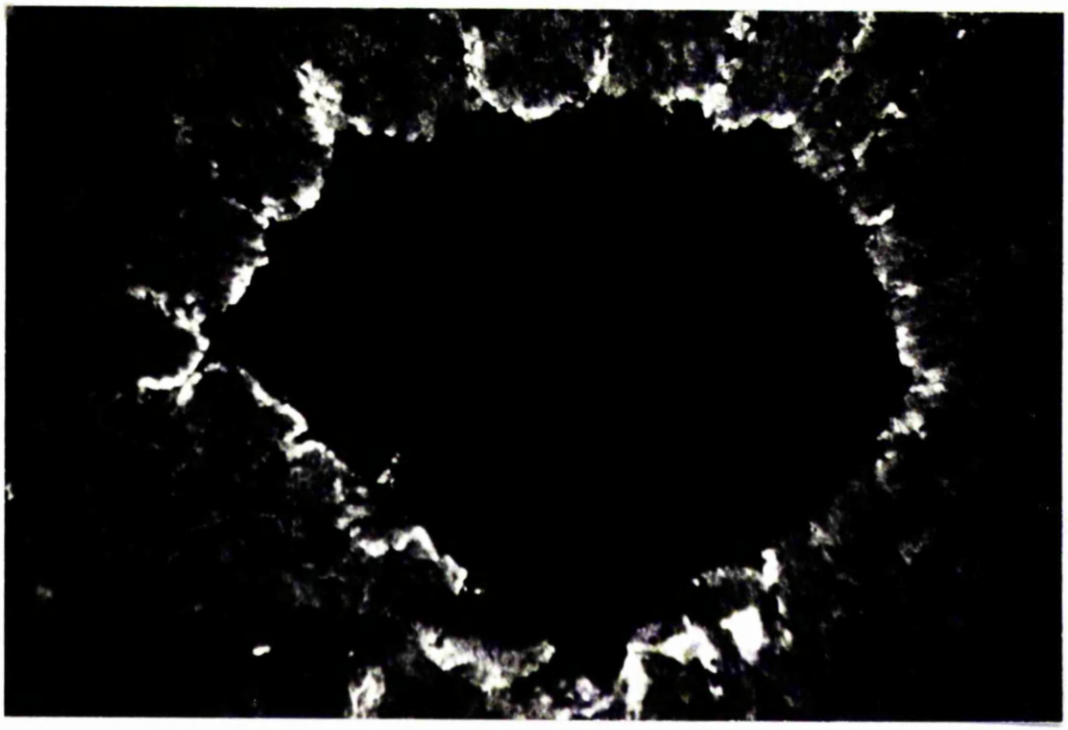


Fig. 55 : Experiment two - immunofluorescence, trachea. A focus of epithelial necrosis contains fluorescent bacteria almost to the level of the basement membrane (arrowed).

(Fluorescent antibody, x 300).

Fig. 56 : Experiment two - immunofluorescence, bronchial epithelium, dog 16. Clumps of fluorescent bacteria are present on the surface of the bronchial epithelium.

(Fluorescent antibody, x 400).

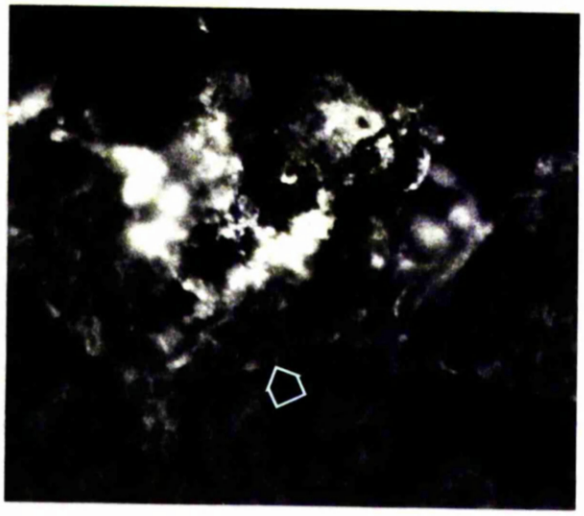


Fig. 57 : Experiment two - immunofluorescence, nasopharyngeal exudate. In this smear of nasopharyngeal exudate from dog 15, large numbers of fluorescent, rod-shaped bacilli can be seen. Some bacteria (arrow) appear to be attached to a polymorphonuclear leucocyte.

(Fluorescent antibody, x 1200).

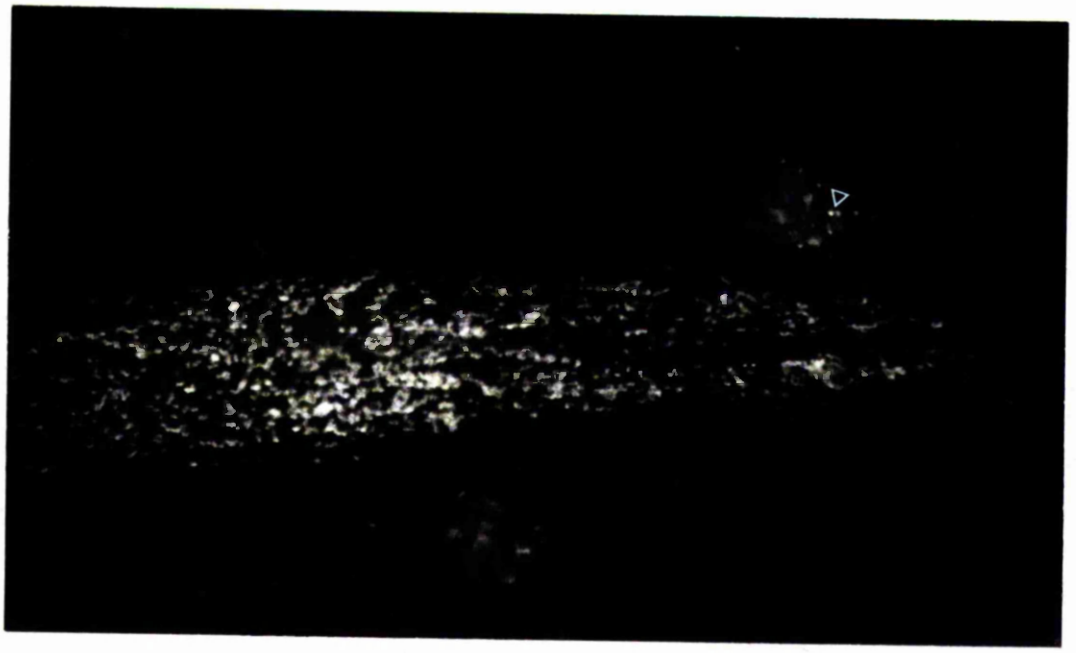


Fig. 58 : Normal dog - ciliated cell. The apical cytoplasm of this ciliated cell contains numerous mitochondria and many small vacuoles. The cilia (C) are regularly arranged along the luminal border and many slender, branching cytoplasmic processes, or microvilli (arrows), are present between the cilia.

(x 10,000).

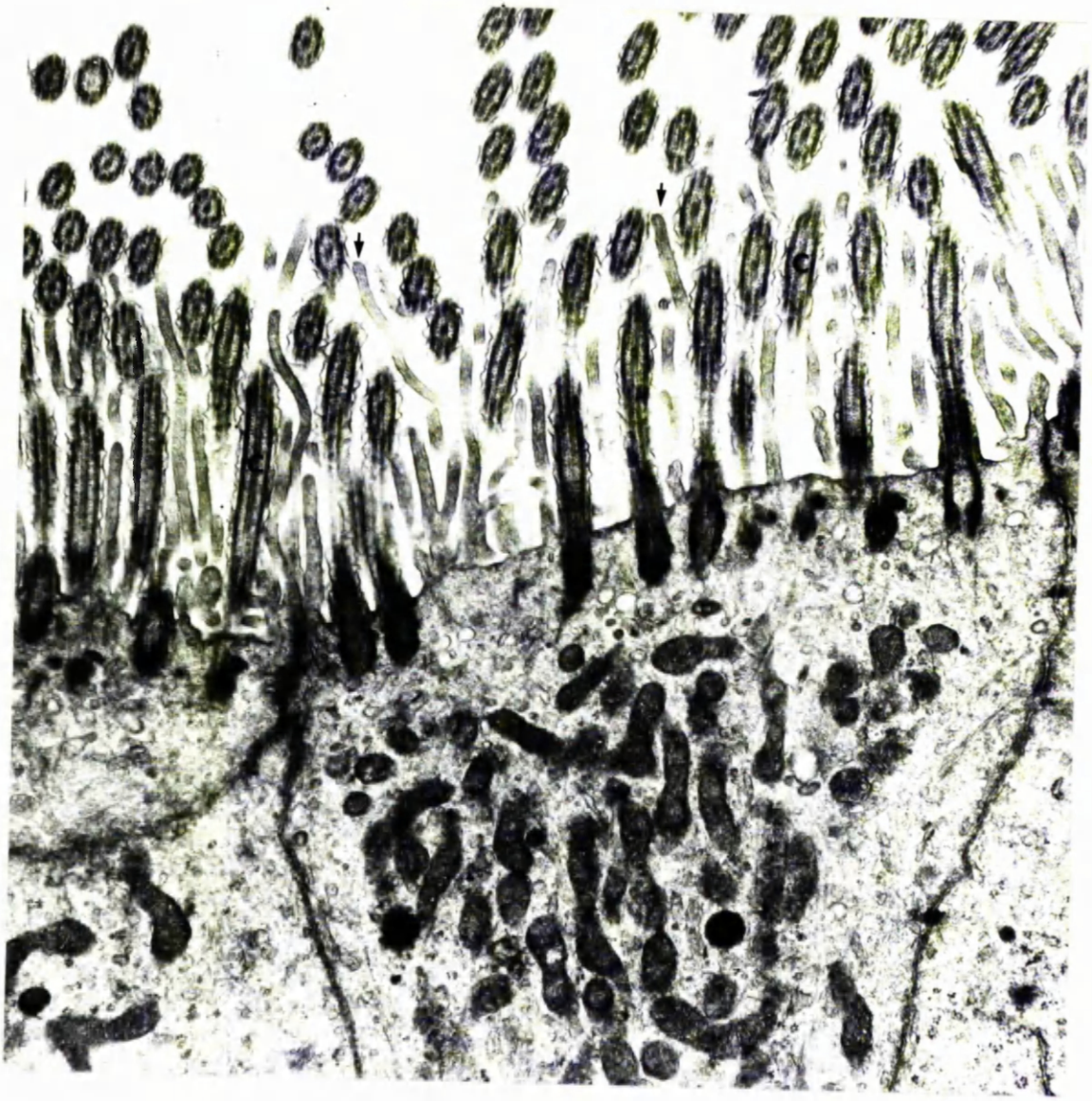


Fig. 59 : Normal dog - transverse section of cilia. Most cilia display the usual axial filament complex (arrow) of nine peripheral double tubules and two central tubules. Two compound cilia are present : they contain multiple, complete or almost complete sets of filaments regularly arranged within a common plasmalemma.

(x 20,000).

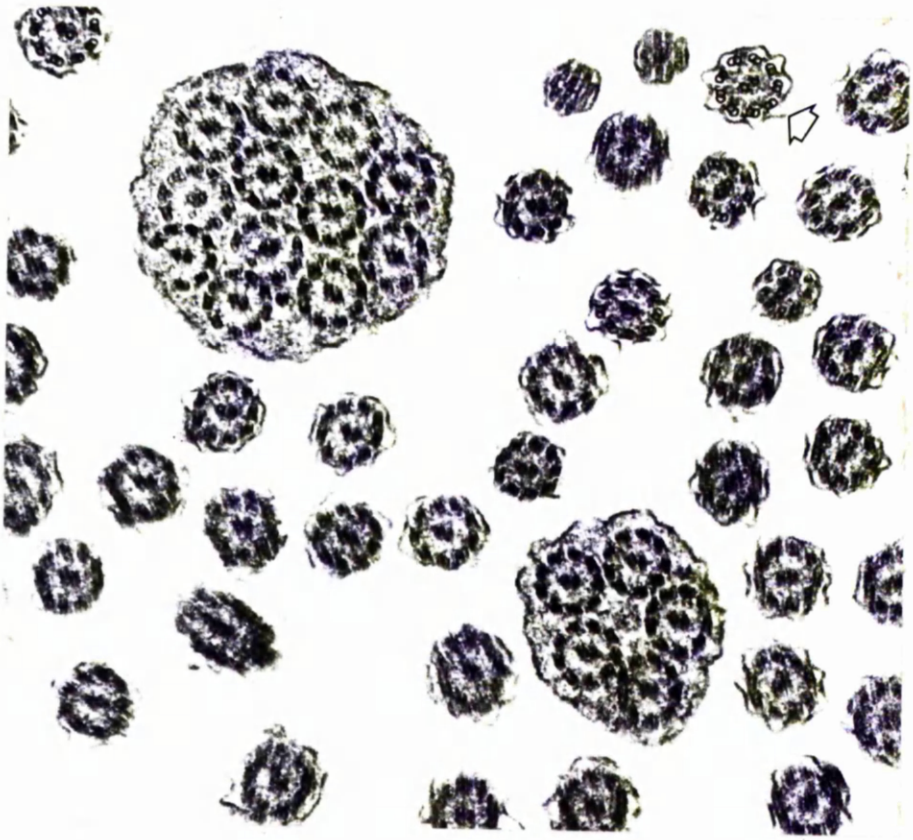


Fig. 60 : Experiment two - bronchial epithelium 4 days after infection. The apical mitochondria in these ciliated cells are swollen (arrow) and there is increased cytoplasmic vacuolation. Cilia are still regularly arranged along the luminal border. (x 10,000).

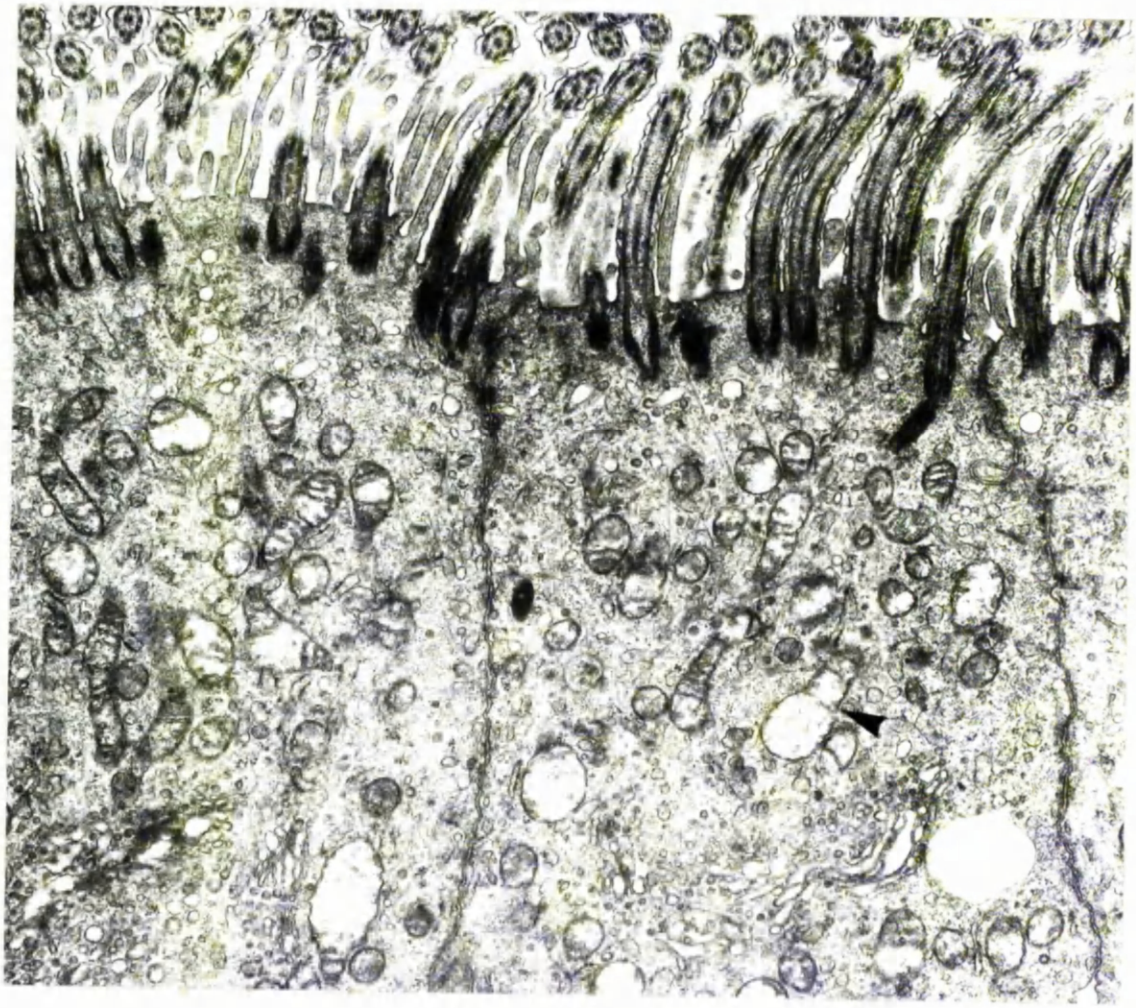


Fig. 61 : Experiment two - bronchial epithelium 8 days after infection. Mitochondrial swelling and cytoplasmic vacuolation are more severe than at 4 days. The cilia appear to be reduced in number (cf Fig. 58). The apical border of the cell is irregular in outline and a number of cytoplasmic projections into the lumen are visible; the largest of these (arrow) contains irregularly orientated ciliary tubules.

(x 5,000).

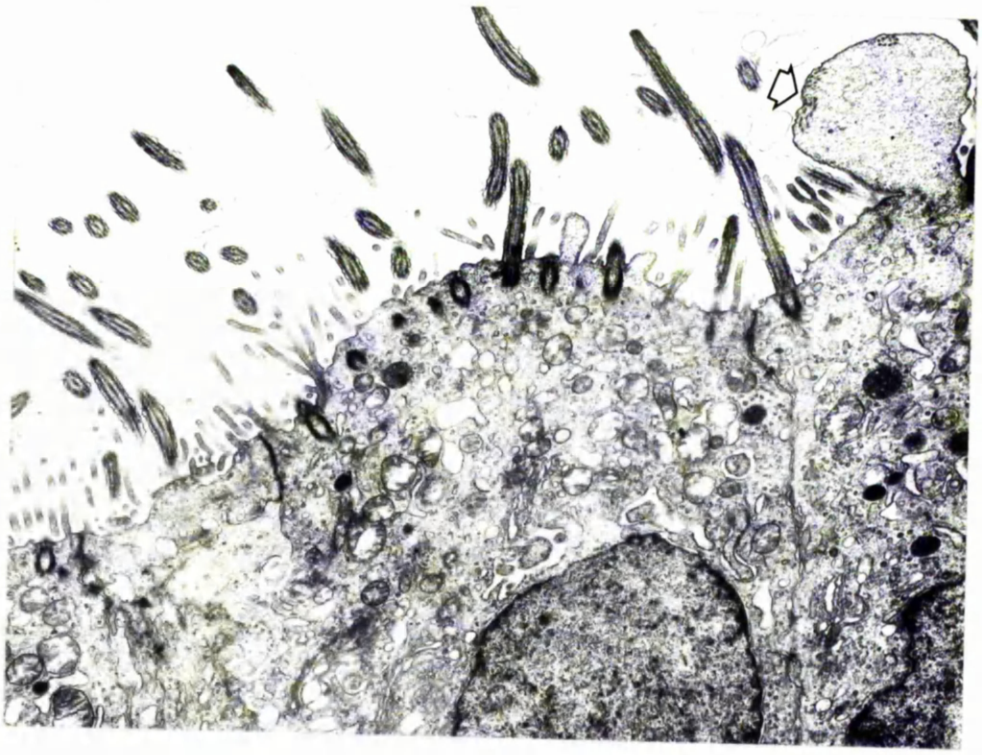


Fig. 62 : Experiment two - bronchial epithelium 8 days after infection. Two polymorphonuclear leucocytes (P) are infiltrating between vacuolated and degenerating ciliated cells (C). The luminal border of the ciliated cells is extremely irregular. A bizarre compound cilium containing sets of axial filaments in both longitudinal (open arrow) and transverse (closed arrow) section can be seen.

(x 7,500).

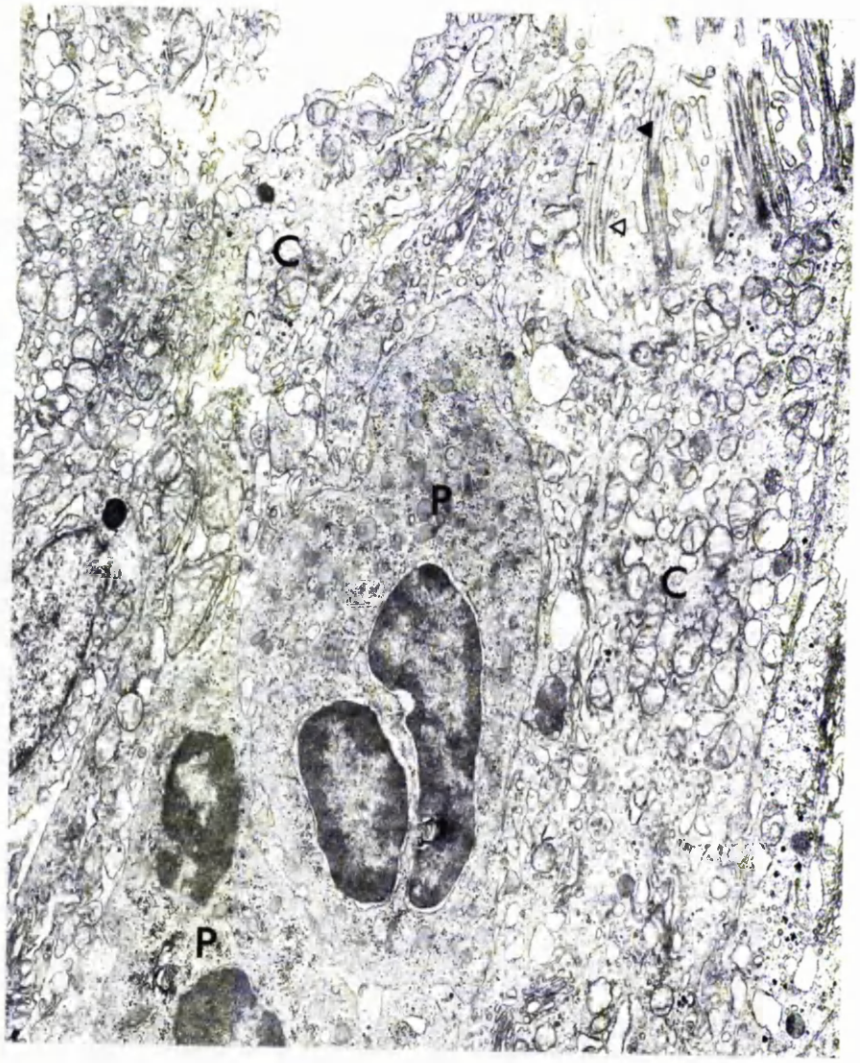

C
P
C
P

Fig. 63 : Experiment two - bronchial epithelium 8 days after infection. The ciliated cells (C) are severely vacuolated and few cilia are evident. Goblet cells (G) are protruding into the lumen and appear to be discharging large amounts of cytoplasmic material as well as some secretory granules (star). A polymorphonuclear leucocyte (P) is present in the lumen which also contains scattered cell debris.
(x 5,000).

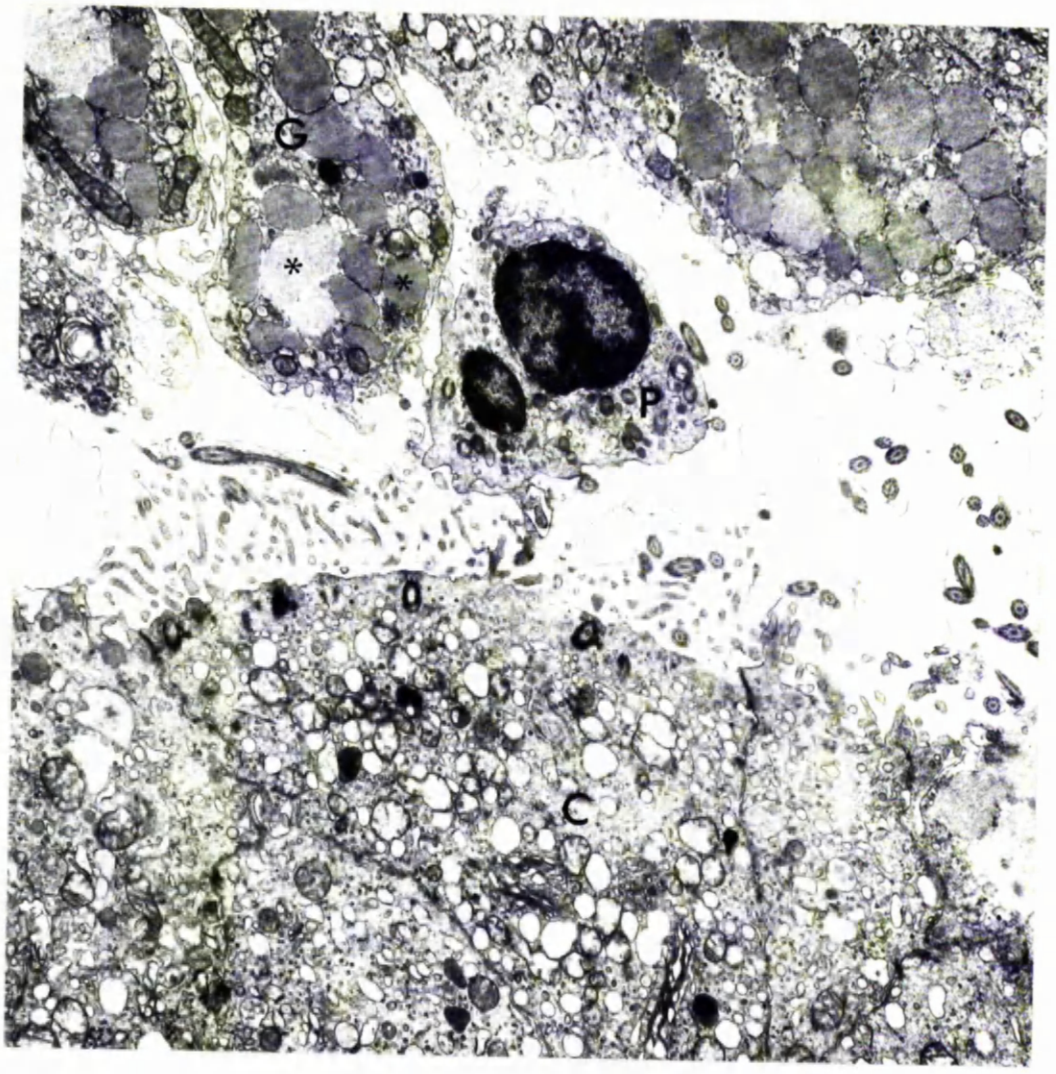

G
*
*
P
C

Fig. 64 : Experiment two - phagocytosis of bacteria.

Pseudopodia from this polymorphonuclear leucocyte in the bronchial lumen of an infected dog can be seen extending towards adjacent bacteria (B) and cell debris. Two phagosomes (arrows) containing engulfed and degenerating bacteria are visible within the cytoplasm.

(x 10,000).


B

Fig. 65 : Experiment two - bronchial epithelium 15 days after infection. In this atypical ciliated cell, irregularly orientated groups of filaments (arrows) are present throughout the cytoplasm.

(x 10,000).

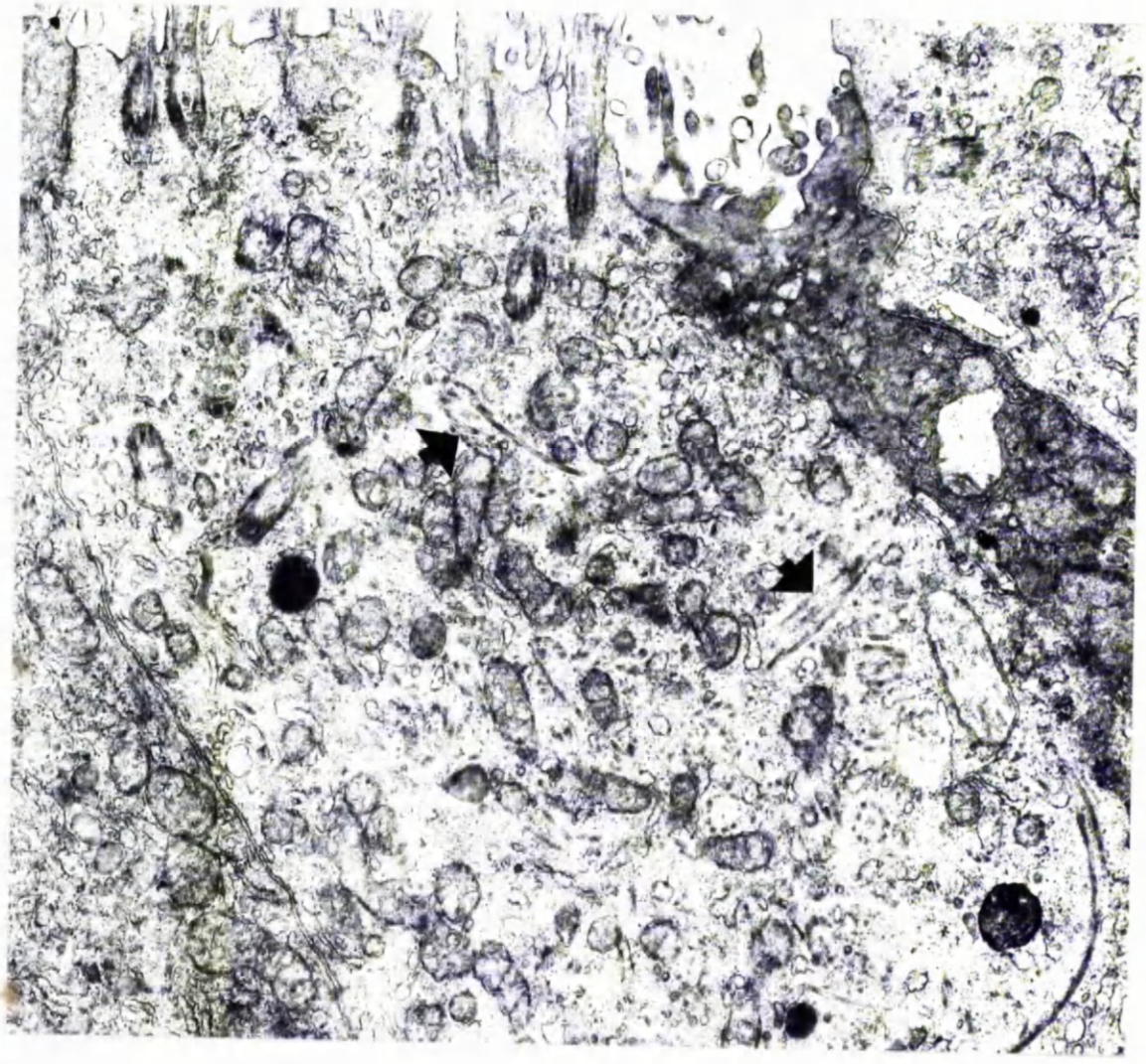


Fig. 66 : Experiment two - bronchial epithelium 4 days after infection. Many bacteria (B) are present among the cilia and microvilli of the ciliated epithelial cells. There is slight swelling of the apical mitochondria.

(x 7,500).

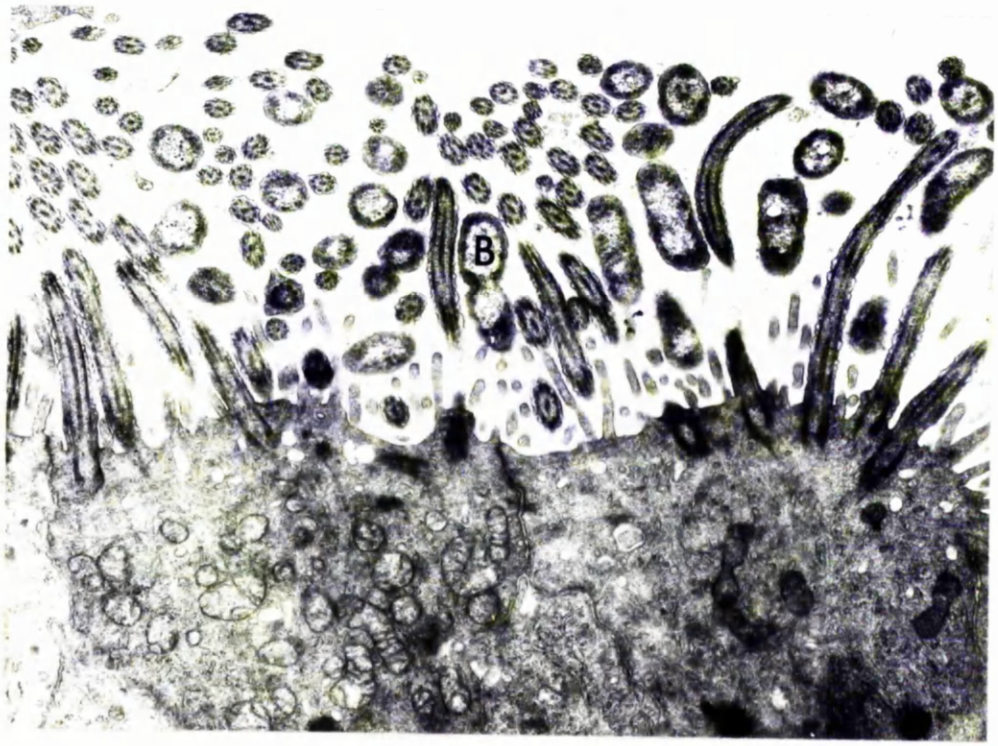

B

Fig. 67 : Experiment two - bronchial epithelium 8 days after infection. Bacteria (B) are present among the cilia and microvilli of an epithelial cell. The bacteria have a finely filamentous outer layer which may be adherent to the cilia and microvilli (arrows).
(x 20,000).

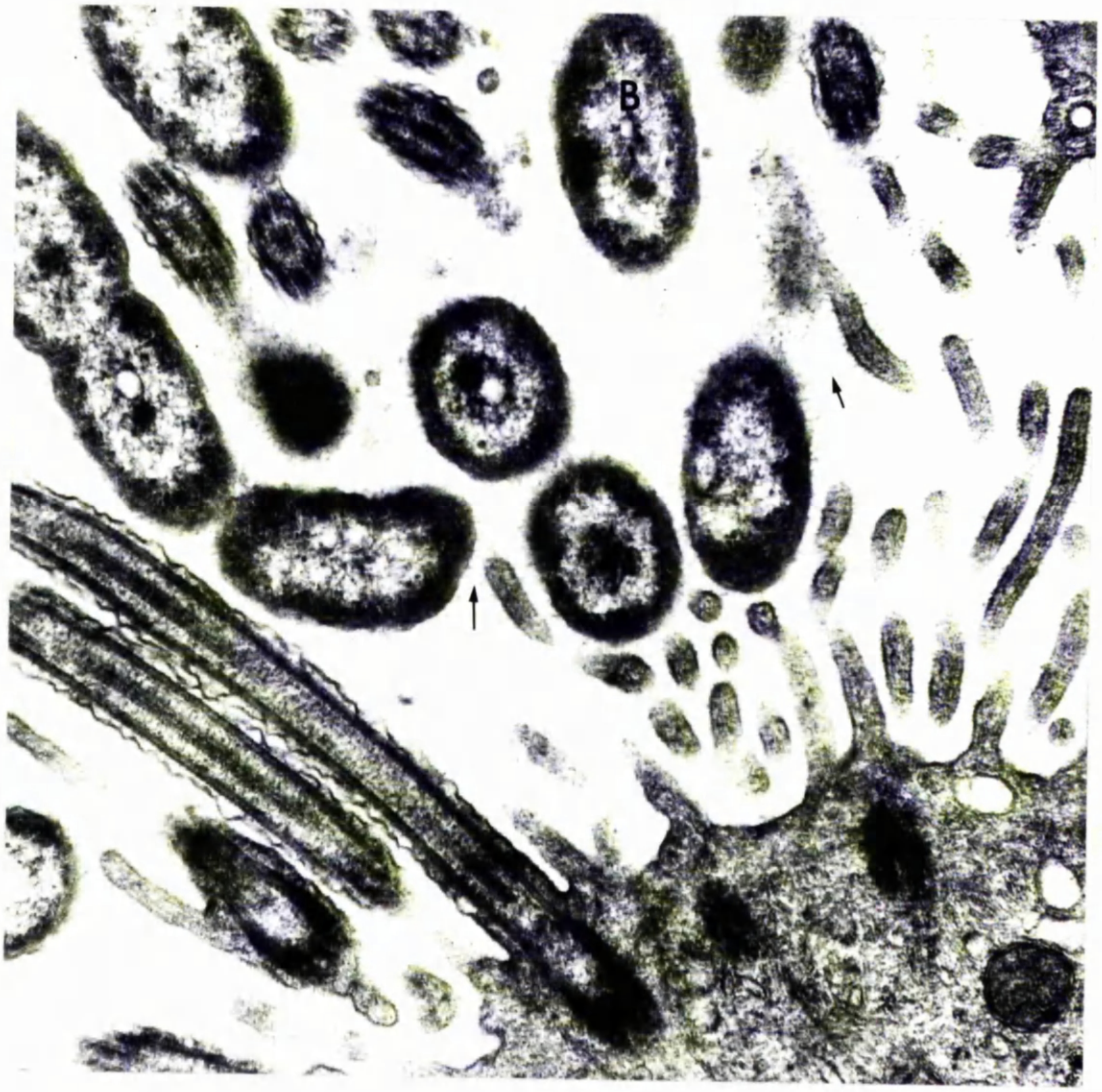

B

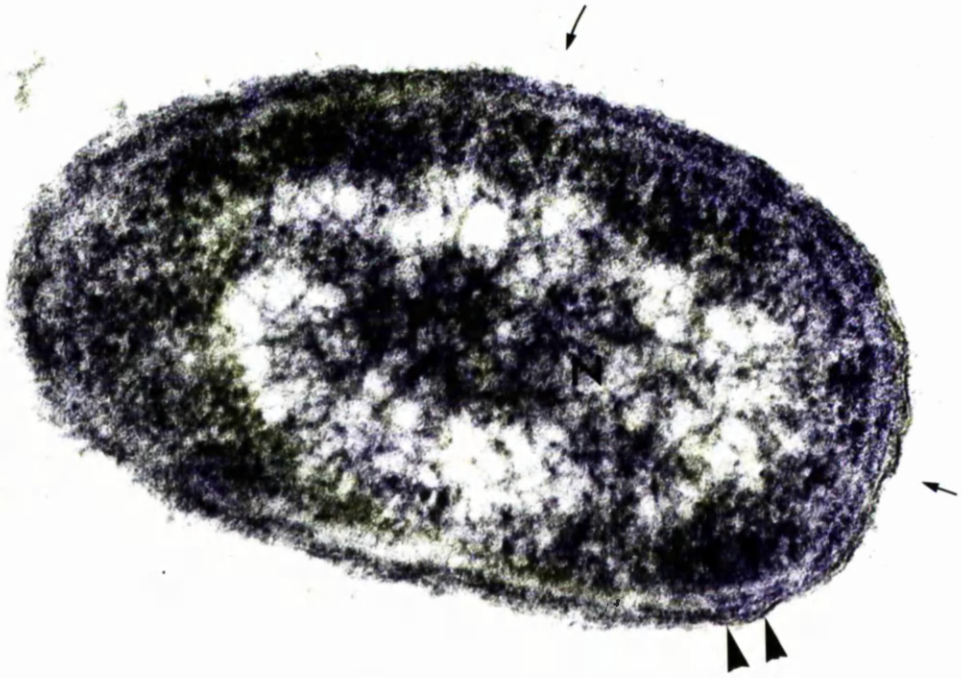
Fig. 68 : Experiment two - relationship of bacteria to cilia and microvilli. A bacterium (B) is present in close relationship to both cilia (C) and microvilli (M). Fine filamentous processes on the outer surface of the bacterium appear in some areas to be touching the plasmalemma of both cilia and microvilli (arrows).

(x 80,000).


C
B
M

Fig. 69 : Ultrastructure of Bord. bronchiseptica. The bacterial cell wall (double arrows) consists of a number of layers which can be recognised by their differing electron density. The outer surface of the cell wall is irregular in outline and fine filamentous processes (single arrows) extend from the cell surface. Beneath the cell wall is an electron dense zone of ribosomes (R). The central nucleoid (N) is primarily fibrillar in appearance.

(x 70,000).



SECTION 5 : DISCUSSION

The results obtained in both the above experiments show that Bord. bronchiseptica alone, unaided by viruses, can produce pathological changes in the respiratory tract of young dogs with the development of a clinically evident respiratory disease which spreads to in-contact animals. At least under these experimental conditions, Bord. bronchiseptica must, therefore, be regarded as a primary pathogen for the canine respiratory tract.

A number of factors may be considered as having had some part in the successful production of the bacterial respiratory disease described above. Firstly, the infecting bacterial strain had been freshly isolated from a clinical case of canine respiratory disease and had not been subjected to prolonged laboratory culture; loss of pathogenicity of Bordetella spp. following cultivation on artificial media is well recognised (Standfast, 1951; Nakase, 1957). Secondly, the experimental animals used were fully susceptible to infection; they had not previously been exposed to any naturally occurring form of respiratory disease and, as far as could be determined by serological examination and bacteriological examination of the nasopharynx, had no previous experience of the infecting organism. Thirdly the route of infection employed closely resembles natural spread of infection and ensures that the organism can reach all levels of the respiratory tract thereby allowing the bacterium to exert its maximal possible effect.

The clinical respiratory disease produced was characterised by coughing, often paroxysmal, in otherwise bright and active dogs. Clinical signs of respiratory disease in contact control animals developed only a few days later and were of equal severity to those found in animals infected by aerosol. There was no difference in the severity of clinical signs observed in 6 week and 12 week old animals. Coughing persisted, in both infected and contact control dogs, throughout the experiments until they were terminated at up to 21 days after infection; no dog made a clinical recovery during the

experimental period. In general, the clinical disease was similar to that described in naturally-occurring cases of "kennel-cough" (Pennock and Archibald, 1968).

The lower respiratory tract of normal unexposed control animals was almost invariably sterile in contrast to the upper respiratory tract from which a variety of organisms was recovered. Bordetella bronchiseptica persisted in both upper and lower respiratory tracts in both infected and contact control dogs throughout the experimental period; no dog cleared the infection. While the organism could be recovered from both nasal and pharyngeal swabs as late as 21 days after infection, in some samples only very few colonies were found, and, since large numbers of other bacterial species were also present in cultures from those sites, some difficulty was encountered in establishing the presence of Bord. bronchiseptica in these samples. In contrast, profuse, pure cultures of Bord. bronchiseptica were obtained from the tracheobronchial tree up to 21 days after infection. These findings are in agreement with those of Ganaway et al. (1965) who found nose and throat cultures from the living animal to be unreliable indicators of infection.

The pathological lesions induced in the lower respiratory tract of dogs by infection with Bord. bronchiseptica i.e., a tracheobronchitis with, in some cases, the development of focal areas of exudative pneumonia, are similar to those described in experimental infections with the microorganism in the pig (Duncan et al., 1966b; Meyer and Beamer, 1973) except that the pronounced vasculitis and pulmonary fibrosis present in some pigs were not found in experimental dogs. Similar lesions have also been described in Bord. bronchiseptica infections in the cat (Snyder et al., 1973), guinea-pig (Nikkels and Mullink, 1971), horse (Saxegaard et al.,1971), monkey (Graves, 1970) and rat (Burek et al.,1972) while Bord. pertussis infection (whooping cough) in man also has a strikingly similar histological picture (Robbins, 1974).

In the upper respiratory tract of the dog, Bord. bronchiseptica infection resulted in a purulent rhinitis but no macroscopic or microscopic turbinate atrophy was found. Nonetheless it is possible that this severe

rhinitis, if produced at an even earlier age, than that of the dogs used in these experiments could result in abnormal turbinate growth; in the pig (Ross et al.,1963b), turbinate atrophy was most noticeable in pigs infected with Bord. bronchiseptica in the first days of life, 4 week old pigs being far less affected.

Immunofluorescence examination showed that although infection with Bord. bronchiseptica provoked an extensive inflammatory reaction in the lamina propria and submucosa of respiratory airways, the bacteria themselves were limited to the ciliated epithelial border of the respiratory mucosae and did not extend into deeper structures except where there was frank epithelial necrosis; even then, they did not penetrate beyond the basement membrane. Only occasionally were bacteria found in alveolar airspaces or in lymph nodes. Similar results have been obtained using immunofluorescence techniques in the study of experimental Bord. bronchiseptica infection in the pig (Maeda and Shimizu, 1974).

Electron microscopy confirmed that bacteria were confined to the luminal surface of the epithelium but, in addition, showed that ultrastructural changes in the epithelial cells themselves were widespread, even where there were no immediately overlying bacteria. These features of the infection suggest that the widespread epithelial degeneration and necrosis may be due not to actual invasion by bacterial cells but to damage caused by a bacterial product, possibly the toxin described by Harris et al., (1968) which interferes with mitochondrial metabolism. The reduction in cilia seen on ultrastructural examination is also a feature of Bord. bronchiseptica induced atrophic rhinitis in the pig (Duncan and Ramsay, 1965); these authors also recorded the presence of atypical ciliary formations and abnormal ciliated cells not unlike those found in the present experiments. It seems likely that the increase in abnormal ciliated cells and cilia found in infected dogs is a result of either degenerative changes induced by the bacteria or their products or unsuccessful attempts at regeneration.

Among the most interesting aspects of the disease was the persistence of Bord. bronchiseptica in the tracheobronchial tree until 21 days

after aerosolisation. Bord. bronchiseptica has also been shown to persist in the respiratory tract of infected pigs for a minimum of 6 to 8 weeks (Ross et al., 1963b). This persistence of infection is reflected in both the clinical and histological features of the experimental disease: clinical respiratory disease was still noticeable at 21 days post infection and histological changes in the respiratory tract were still prominent at this time.

The late stage histological changes were, however, different from the acute inflammatory lesions of the early infection. There was evidence of tracheobronchial epithelial hyperplasia, possibly a response to the continued presence of bacteria although epithelial hyperplasia can also be seen in CAV infection at a time when the causal virus is no longer present (Wright et al., 1971). There was less congestion and oedema and, although polymorphonuclear leucocytes were still present in the lamina propria and epithelium, there was an extensive mononuclear cell infiltrate, mainly of lymphocytes with a few macrophages and plasma cells, in the lamina propria and submucosa; this lymphoid cell infiltrate may well reflect a continuing antigenic stimulus.

It is interesting to note that these late stage features of Bord. bronchiseptica infection i. e. thickening of the bronchial walls with epithelial hyperplasia and mononuclear cell infiltration of the lamina propria, are also features of chronic bronchitis in the dog (Wheeldon, 1974) especially since Bord. bronchiseptica is often isolated from dogs with this syndrome (Wheeldon, 1974). One of the outstanding features of chronic bronchitis is bronchial mucous gland hypertrophy and hyperplasia and although this was not present in experimental dogs with Bord. bronchiseptica infection, bronchial mucous gland distention was noted. The role of infection with Bord. bronchiseptica in the pathogenesis of canine chronic bronchitis is obviously worthy of further study.

The reasons for the persistence of Bord. bronchiseptica in the respiratory tract remain obscure. Resistance to infection in the lung is

associated with a wide range of factors both specific and non-specific (Green, 1970); among the most important of these are clearance of inhaled particles by the mucociliary apparatus, phagocytosis by alveolar macrophages (Laurenzi and Guarneri, 1963) and specific immunological mechanisms (Bienenstock and Perey, 1972; Cantey and Hand, 1974).

Mechanical clearance of particles deposited on the tracheobronchial mucosae by the mucociliary apparatus is normally rapid; Green (1970) estimated that 90% of material deposited in human subjects was physically transported to the larynx in under 1 hour. The localisation of Bord. bronchiseptica between epithelial cilia and microvilli, possibly with attachment to these structures, may protect these bacteria from this physical means of removal, and /or foci of "fixed" bacteria may continually renew the bacterial population despite the action of the mucus escalator. It is also possible that the heat labile toxin has a paralysing effect on the epithelial cilia and thus mechanically interferes with clearance.

Phagocytosis by alveolar macrophages has been shown to be one of the main mechanisms involved in the removal of bacteria from the distal lung (Green and Kass, 1964; Green, 1968). Large numbers of macrophages can be recovered from animal lungs, but these are more frequently seen in distal airways and alveoli than in the larger bronchioles, bronchi and trachea. The relative infrequency with which Bord. bronchiseptica were found in the alveolar air spaces even when they were numerous in adjoining airways may be related to a more efficient phagocytic system at the alveolar level and, conversely, a paucity of efficient phagocytic cells in the tracheobronchial tree may permit persistence and multiplication of organisms unable to be mechanically removed from that site.

Circulating agglutinating antibodies to Bord. bronchiseptica were detected in experimental animals as early as 7 days after infection; they indicate the ability of the dog to mount a systemic humoral immune response to this bacterium. These measurable antibodies in the systemic circulation need not however be direct proof of developing immunity to infection. Recent work has shown that local antibody production, mainly of secretory

immunoglobulin A (Tomasi and DeCoteau, 1969; Tomasi and Bienenstock, 1969; Bienenstock and Perey, 1972), and local cell mediated immune responses within the lung (Waldman and Henney, 1971; Cantey and Hand 1974) are better correlated with protection from infection against both viral (Waldman, 1969) and bacterial (Bellanti et al., 1967) respiratory pathogens than is the systemic immune response. Local immune responses in the lungs of experimental dogs were not measured directly in these experiments but any which were present appeared to have little effect in reducing or clearing the burden of infection in the tracheobronchial tree within the experimental period.

PART III : PROPHYLAXIS OF CANINE BORDETELLOSIS

Vaccination against Bord. spp. has been attempted, with varying success, in a number of species.

As early as 1923, vaccination was being used in attempts to control epidemics of whooping cough in man (Lapin 1943). An American study (Kendrick and Eldering, 1939) showed that the use of Bord. pertussis vaccine resulted in protection rates against whooping cough of 90% and 60% respectively of vaccinated children exposed to disease at school and in the family home when compared with non vaccinates. Field trials in Britain (Medical Research Council, 1956 and 1959) have also demonstrated that Bord. pertussis vaccines can be highly effective in controlling the clinical disease; widespread use of such vaccines has resulted in a very marked decrease in the reported incidence of pertussis in Britain (Edsall, 1975). Pertussis vaccines in routine use consist of killed, detoxified bacterial suspensions; vaccines prepared with aluminium-containing adsorbents as adjuvants have been especially widely used (Joo, 1969) since experimental evidence suggests that these adsorbents may increase the potency of simple bacterial suspensions.

Vaccination against Bord. bronchiseptica infection has been attempted in various animal species but has not been as consistently successful as has vaccination against Bord. pertussis in man. Wickert et al., (1958)used a simple, heat-killed, formalin-preserved bacterial suspension of Bord. bronchiseptica to vaccinate rats by a subcutaneous route. Agglutinating antibodies were detected in the sera of vaccinates, and challenge intranasal instillation or natural exposure to disease resulted in fewer deaths in vaccinates than in unvaccinated controls; total protection was not, however, achieved by this prophylactic regime.

As early as 1920, Ferry and Hoskins, although recognising that "snuffles" in rabbits was not due to the action of any one microorganism, nonetheless claimed that control of this disease could be achieved by inoculation with a triple vaccine prepared from Bord. bronchiseptica, Bact.

leptisepticum and Staph. albus. However, Bull and McKee (1928) reported that while subcutaneous injection of heat killed Bord. bronchiseptica resulted in detectable circulating antibody levels, animals immunised in this manner were as susceptible to infection as non-immunised controls.

In mice, protection from intranasal challenge and complete elimination of the challenge organism from the respiratory tract followed intraperitoneal inoculation with a vaccine prepared from the challenge strain of Bord. bronchiseptica (Winsser, 1960); the method of vaccine preparation was not specified.

In the guinea-pig, Ganaway et al. (1965) reported that a single intramuscular injection of a formalinised bacterial suspension of Bord. bronchiseptica emulsified in Freunds incomplete adjuvant resulted in the production of high serum levels of agglutinating antibody, protection from respiratory disease due to the bacterium and complete elimination of the organism from the respiratory tract. Nikkels and Mullink (1971), also recorded protection from disease and elimination of the organism after inoculation with a similarly adjuvanted vaccine.

Studies in the pig have shown that nasal and tracheal resistance to reinfection with virulent Bord. bronchiseptica develops after clearance of virulent Bord. bronchiseptica from the respiratory tract by sulphonamide therapy and after natural clearance from the respiratory tract of a low-virulence strain of Bord. bronchiseptica (Harris and Switzer, 1969). Circulating antibodies were not detected in the sera of animals which had cleared the low-virulence strain, even although they were fully resistant to reinfection. In the same series of experiments, resistance to reinfection was not produced by repeated intramuscular inoculation of a formalinised bacterial suspension, although high serum titres of agglutinating antibody were detected in immunised animals.

Subcutaneous inoculation of sonicated bacterial suspensions of Bord. bronchiseptica and Bord. pertussis emulsified with Freunds incomplete adjuvant similarly failed to produce resistance to intranasal infection with

virulent Bord. bronchiseptica (Harris and Switzer, 1972); vaccinated animals did, however, show accelerated clearance of the bacteria from the respiratory tract compared to unvaccinated controls. Accelerated clearance was not seen when a suspension of Bord. bronchiseptica in Freunds incomplete adjuvant was given to piglets already naturally infected with Bord. bronchiseptica (Koshimizu et al., 1973) but resistance to infection was found in the colostrum-fed piglets of sows previously vaccinated with this same adjuvanted suspension (Koshimizu et al., 1973).

From the above evidence, it would appear that parenteral vaccination with Bord. bronchiseptica can result in a degree of protection from disease due to this microorganism in at least some animal species. In view of this, and of the successful use of vaccination in the control of the related disease of whooping-cough in man, it was considered worthwhile to investigate the effects of vaccination on experimental infection with Bord. bronchiseptica in the dog. A series of experiments were therefore undertaken in which the incidence and course of infection with Bord. bronchiseptica were monitored in dogs vaccinated with preparations of the challenge strain of organism.

SECTION 2 : MATERIALS AND METHODS

Experimental Animals

Eight week old, healthy puppies were obtained, housed and maintained in isolation as described in Part II, Section 2.

Vaccine Preparation

Two types of vaccine were used in these experiments; the first was a simple, heat-killed suspension of Bord. bronchiseptica, the second consisted of a similar suspension adsorbed with aluminium hydroxide.

Heat-killed vaccine: aseptic techniques were used throughout the preparation of this vaccine. Typical, low-passage colonies of Bord. bronchiseptica 52498/3 were inoculated onto nutrient blood agar plates. After 48 hours aerobic incubation at 37°C the growth was washed off the agar plates with sterile normal saline (SNS). The suspension obtained was centrifuged for $1\frac{1}{2}$ hours at 670 x g on an MSE refrigerated centrifuge and the precipitate resuspended in fresh SNS. Centrifugation and resuspension was performed three times in all. The final bacterial suspension was adjusted to a concentration of 20×10^9 organisms/ml using previously calibrated opacity tubes and the bacteria were then heat-killed by incubating for 2 hours in a water bath at a temperature of 56°C. The vaccine was stored at 4°C until use which was within 3 weeks of preparation. Immediately before use the suspension was tested for sterility by bacteriological techniques.

Aluminium hydroxide adsorbed vaccine: a commercial formulation of aluminium hydroxide (Alhydrogel : Superfos Export Co., Denmark) was used in the preparation of the vaccine. A suspension of Bord. bronchiseptica 52498/3 was prepared by repeated centrifugation as described above. The final concentration was adjusted to 44×10^9 organisms/ml and the bacterial suspension was then heat-killed; 12 ml of Alhydrogel diluted 1 : 9 in sterile water was then added to each 10 ml of bacterial suspension; the proportions and concentrations of bacterial and Alhydrogel suspensions had been previously determined by repeated trial (Weir, 1973) to give maximum adsorption of

bacteria and the required final concentration of 20 x 10^9 organisms/ml. The adsorbed material appeared as a floccular precipitate which, when required for inoculation, could be resuspended by gentle shaking. The vaccine was stored at 4°C until use, again within 3 weeks of preparation. As with the simple heat-killed vaccine, the preparation was tested for sterility before use.

Aerosolisation Procedure

Necropsy Procedures

Histological Procedures

Bacteriological Procedures

These were identical to those previously described in Part II, Section 2.

Immunofluorescence Procedures

These were identical to those previously described in Part II, Section 2, but, in addition, samples of lung tissue were stained with a commercial preparation of fluorescein isothiocyanate conjugated rabbit anti-canine globulin globulin (Sylvana Co., Ltd., USA) in order to demonstrate the presence of globulin containing cells in the lung tissue. This commercial preparation was diluted 1 : 20 with phosphate buffered saline to overcome non-specific background fluorescence.

Serological Procedures

The serum agglutination test was used to detect circulating antibodies to Bord. bronchiseptica. The method used was identical to that described in Part II, Section 2.

Virological Procedures

Samples of lung taken at necropsy were examined for the presence of canine viruses as described in Part I, Section 2.

SECTION 3 : EXPERIMENT THREE - HEAT KILLED VACCINE

Experimental Design

A total of 13 puppies were used in this experiment. Routine bacteriological examination during the preliminary isolation period had shown that Bord. bronchiseptica was not present in the nose or pharynx of these dogs and serological examination failed to reveal any antibody to the bacterium in their sera. The puppies were randomly allocated to two groups. The first, vaccinated group consisted of 7 dogs which were to be inoculated with the heat-killed vaccine; the second group of 6 dogs acted as an unvaccinated control.

The heat-killed vaccine was given on 2 occasions at an interval of a fortnight. The dogs were 8 weeks old at the time of the first inoculation. On each occasion 1 ml of the vaccine was administered by injection deep into the biceps femoris muscle mass.

Two weeks after the second inoculation of vaccine, i.e. when the dogs were 12 weeks old, both vaccinated and unvaccinated control groups were challenged by exposure to an aerosol of Bord. bronchiseptica 52498/3. Both before and after challenge all dogs were maintained in a common airspace. Pairs of dogs, one from each group, were subsequently killed at the following intervals after challenge infection : 5 days; 9 days; 11 days; 16 days; 30 days. The remaining 3 dogs i.e. 2 vaccinated and 1 control, were killed 33 days after challenge infection.

All 13 dogs were monitored daily throughout the vaccination and challenge period for clinical evidence of disease. Nasal and pharyngeal swabs were taken from each dog at weekly intervals throughout the vaccination period and then daily from the day of challenge until death; these swabs were examined for the presence of Bord. bronchiseptica. Serum samples were obtained from each dog at intervals throughout the experimental period and also at post mortem examination; these were examined for agglutinating antibodies to Bord. bronchiseptica.

At necropsy, samples were taken from each dog for histopathological, immunofluorescence, bacteriological and virological examination.

Clinical Findings

All dogs, from both groups, remained healthy throughout the vaccination period. Inoculation with the heat-killed vaccine had no clinically discernible effect on the dogs. Pyrexia was not detected following vaccination, there was no tissue reaction at the site of injection and no dog became lame.

After challenge aerosolisation, both vaccinated and control groups developed clinical signs of respiratory disease. Signs of disease were confined to the respiratory tract and all dogs remained active and continued to eat normally following infection. At no time did any dog develop pyrexia.

As in previous experiments, the predominant clinical sign was coughing, the incidence of which is shown in Fig. 70. All members of the unvaccinated control group were coughing by 4 days after infection, the onset of coughing being almost synchronous in this group. Coughing was most severe between 8 and 12 days after infection but persisted spasmodically throughout the experimental period. By comparison, the onset of respiratory disease in the vaccinated group was delayed and was spread over a number of days coughing commencing as late as 8 days after infection in some dogs. Once coughing was evident in the vaccinated dogs it was, however, as severe and as persistent as in the unvaccinated animals. Dogs from both groups irregularly exhibited a serous or mucoid nasal discharge between 7 and 16 days after infection.

Pathological Findings

Macroscopic findings: At postmortem examination significant findings in the respiratory tract were similar in both vaccinated and unvaccinated dogs. There were no gross changes in the lungs of either dog killed five days after infection but in all dogs killed from 9 days onwards, red foci, up to 3 mm in diameter, could be seen on the pleural surface and in the

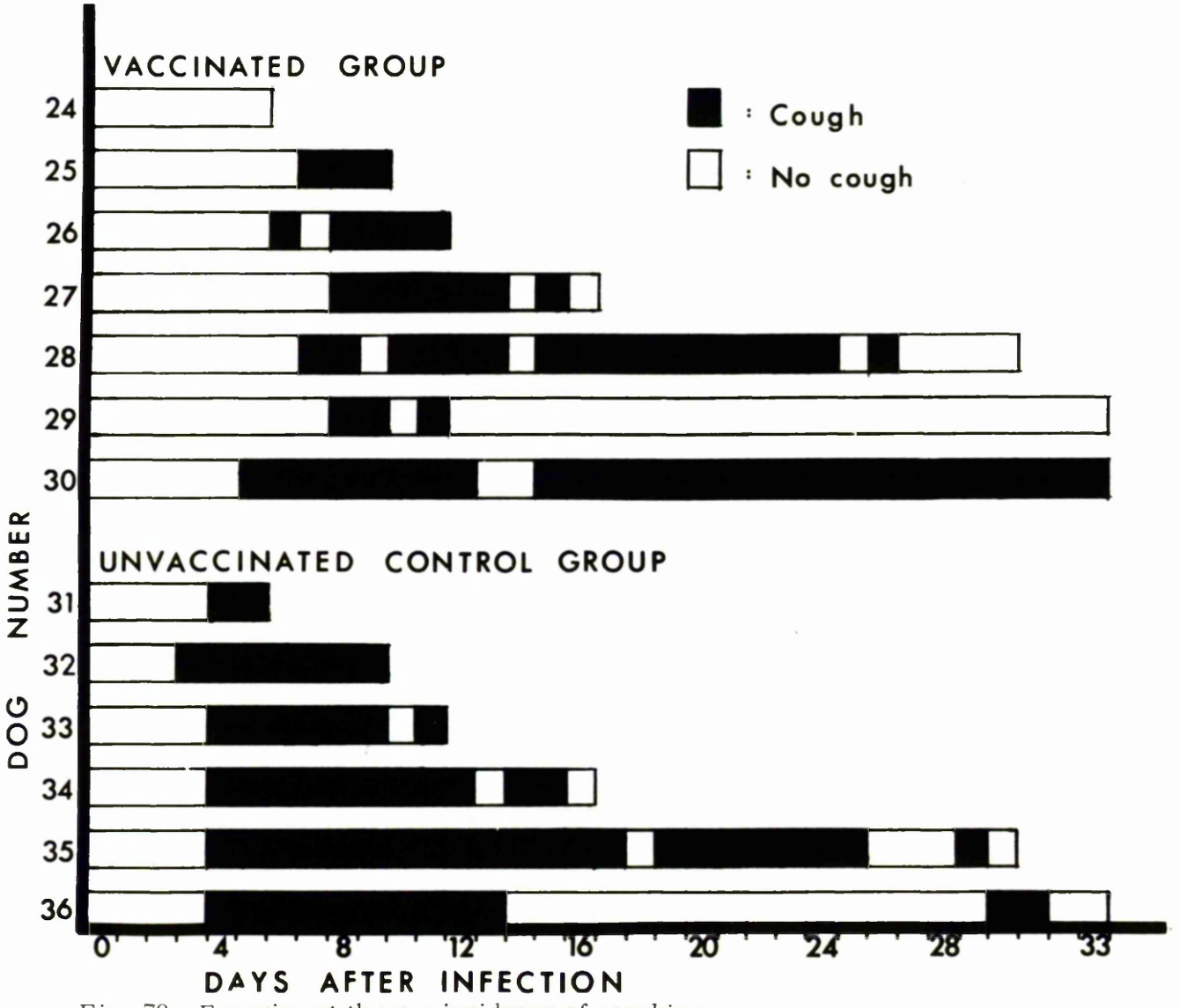


Fig. 70 : Experiment three - incidence of coughing.

substance of all lobes of the lungs. Quantities of muco-pus could be found in the trachea (Fig. 71) and large bronchi of both dogs killed 11 days after infection. Mucopus was also found in the posterior nasopharynx of vaccinated dogs killed on days 9 and 11 and of unvaccinated controls killed on days 5, 9, 11 and 16. The nasal-mucosa was congested except in dogs killed at 30 and 33 days after infection and mucopus could occasionally be seen in the scrolls of the ethmoid turbinate bones; there was no evidence of turbinate atrophy or distortion in any of the dogs.

The bronchial and retropharyngeal lymph nodes were enlarged to about twice normal size in dogs from both groups but whilst those of the vaccinated group were cellular, firm and white, those of the unvaccinated dogs killed from 5 - 11 days were congested and oedematous with haemorrhagic foci. The palatine tonsils were enlarged in three dogs from both groups whilst the adenoid region of all dogs appeared prominent (Fig. 72).

The only other significant findings were small areas of fibrous tissue in the biceps femoris around the sites of inoculation in vaccinated dogs.

Microscopic findings. Histopathological findings, summarised in Table 27, were qualitatively similar, in both groups of dogs to those previously described in experiments one and two. The lesions present in the respiratory tract of vaccinated dogs killed 5, 9, and 11 days after infection were slightly less severe than those seen in the unvaccinated control dogs. This sparing effect was evident only in the earlier phase of infection : in dogs killed from 16 days onwards, histopathological changes were of equal severity in both groups of dogs.

In the respiratory tract up until 16 days post infection these findings were similar to those which have been previously described in experiments one and two. There was rhinitis and tracheobronchitis with congestion, oedema and polymorphonuclear leucocyte infiltration of the lamina propria and epithelium; masses of Gram-negative bacteria could be seen among the epithelial cilia. In the bronchial tree, the mucous glands

Dog Number	Day Examined	Rhinitis	Tracheitis	Bronchitis	Exudative Pneumonia	Lymphadenitis
24*	5	+	++	++	+	+
25*	9	+	++	++	++	+
26*	11	++	+++	++	++	+
27*	16	++	++	++	+	++
28*	30	+	+	+	+	+
29*	33	+	+	+	+	+
30*	33	-	++	++	+	+
31**	5	+	+++	+++	+	++
32**	9	+	++	+++	++	++
33**	11	++	+++	+++	++	++
34**	16	++	++	++	+	++
35**	30	+	+	+	+	+
36**	33	-	++	+	+	+

* Vaccinated

** Unvaccinated control

Lesions graded + to +++ on severity

Table 27 : Experiment three - histopathological findings

were dilated and, with increasing duration of infection, the bronchial epithelium became thicker and thrown into folds, whilst mononuclear cells became more prominent in the cellular infiltrate in the lamina propria. In the dogs killed 30 and 33 days after infection, lymphocytes were still present in the lamina propria of the bronchial tree but larger numbers of lymphocyte were found in the submucosa where, occasionally, follicles were found(Fig. 73). Lymphocytes and plasma cells were present around the bronchial glands which, although no longer so obviously dilated, were still prominent(Fig.74). The epithelium was hyperplastic and was longitudinally folded with many plump goblet cells located at the base of these folds. Small foci of bacteria were still present among cilia and, underlying such areas, there was congestion of vessels in the lamina propria and infiltration of polymorphonuclear leucocytes into both lamina propria and epithelium. Exudate was still present in the lumen and was mainly mucoid in character. In the trachea at this stage, the main change was epithelial hyperplasia and dedifferentiation; in one vaccinated dog, No. 29, killed at 33 days, there were distinct areas of squamous metaplasia in the tracheal epithelium. In the lung parenchyma of dogs killed at 30 and 33 days there were increased numbers of macrophages in the airspaces and small accumulations of mononuclear cells, mainly small lymphocytes, around small venules and lymphatics. At this stage, changes in the turbinates were confined to small foci of congestion with occasional polymorphonuclear leucocyte infiltration of the epithelium: small foci of lymphocytes were also found in the lamina propria.

In the bronchial and retropharyngeal lymph nodes of unvaccinated dogs killed up to 11 days after aerosolisation there was congestion and oedema fluid was present in the sinuses where many macrophages and polymorphonuclear leucocytes were also present. The lymph nodes of vaccinated dogs killed at these times were less congested and oedematous but inflammatory cells were still evident in peripheral sinuses. In all vaccinated dogs and in unvaccinated dogs killed from 16 days onwards, many well developed follicles were present in the cortex and large numbers of plasma cells were found in the medullary cords.

In the palatine and adenoid tonsils there was marked lymphoid follicular hyperplasia in dogs killed on and after day 16.

Bacteriological Findings

The recovery of Bord. bronchiseptica from pre-mortem nasal and pharyngeal swabs is shown in Tables 28 and 29. In both groups of dogs, Bord. bronchiseptica was recovered up to the termination of the experiment. The bacterium was more readily isolated in the first fortnight of infection than in the later stages and, in the first 8 days, recovery was less consistent from the vaccinated dogs than from the unvaccinated controls. Profuse cultures of Bord. bronchiseptica were, however, recovered from individual swabs from both groups of dogs even during the last few days of the experiment.

The recovery of Bord. bronchiseptica from tissues sampled at postmortem examination is shown Table 30. Regardless of vaccination status or length of time after challenge, Bord. bronchiseptica was found throughout the length of the respiratory tract. In the lower respiratory tract Bord. bronchiseptica was present in pure culture, except in two dogs in which a few colonies of an Neisseria-like organism were also recovered from the tracheobronchial tree. In the upper respiratory tract, Bord. bronchiseptica was found in association with a number of other bacteria (see Table 30). Bord. bronchiseptica was recovered only sporadically and then only in sparse culture from the bronchial and retro-pharyngeal lymph nodes and tonsil; Staphylococcus spp. and Streptococcus spp. were the organisms most frequently recovered from these tissues, but even these were present only in sparse culture.

There was no evidence of increased clearance of Bord. bronchiseptica from the respiratory tract of dogs during the later phases of infection: heavy (+++) and profuse (++++) cultures were still obtained in dogs killed 30 and 33 days after challenge.

Immunofluorescence Findings

The results of studies carried out using Bord. bronchiseptica

Dog Number	Days from infection																								
	- 28	- 21	- 14	- 7	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
24	-	-	-	-	-	-	-	-	NP	N															
25	-	-	-	-	-	P	P	N	P	-	N	N	NP	N											
26	-	-	-	-	-	N	-	-	P	-	N	N	N	P	N	-									
27	-	-	-	-	-	-	P	-	P	N	NP	P	P	NP	NP	P	P	-	P	P	P				
28	-	-	-	-	-	P	P	P	NP	NP	P	P	N	NP	N	NP	-	-	P	P	NP	P	-	P	NP
29	-	-	-	-	-	P	P	N	P	P	P	P	NP	NP	NP	-	-	-	-	-	P	P	-	NP	N
30	-	-	-	-	-	N	N	-	P	N	P	NP	P	NP	P	P	-	P	NP	NP	P	NP	P	N	NP

	Days from infection												
	21	22	23	24	25	26	27	28	29	30	31	32	33
24													
25													
26													
27													
28	NP	P	N	P	NP	-	P	-	-	NP			
29	N	-	NP	-	N	-	N	P	P	NP	N	P	N
30	NP	N	P	NP	-	-	N	-	P	NP	NP	NP	NP

N = Bord. bronchiseptica recovered from nasal swab
P = Bord. bronchiseptica recovered from pharyngeal swab
- = No Bord. bronchiseptica recovered

Table 28 : Experiment three - recovery of Bord. bronchiseptica from nasal and pharyngeal swabs of vaccinated dogs.

Dog Number	Days from infection																								
	- 28	- 21	- 14	- 7	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
31	-	-	-	-	-	NP	NP	NP	NP	P															
32	-	-	-	-	-	NP	P	NP	NP	NP	NP	NP	NP	NP											
33	-	-	-	-	-	-	NP	-	NP	NP	NP	NP	N	NP	P	N									
34	-	-	-	-	-	NP	P	N	NP	NP	P	P	NP	NP	NP	N	P	N	NP	N	NP				
35	-	-	-	-	-	-	NP	NP	NP	P	NP	NP	NP	NP	NP	NP	NP	NP	NP	N	P	NP	NP	P	NP
36	-	-	-	-	-	N	N	N	N	NP	NP	P	NP	NP	P	N	P	N	P	N	-	N	-	-	N

	Days from infection												
	21	22	23	24	25	26	27	28	29	30	31	32	33
31													
32													
33													
34													
35	NP	N	NP	P	-	P	-	-	N	N			
36	NP	P	N	N	N	-	-	N	NP	N	NP	NP	P

N = Bord. bronchiseptica recovered from nasal swab

P = Bord. bronchiseptica recovered from pharyngeal swab

- = No Bord. bronchiseptica recovered

Table 29 : Experiment three - recovery of Bord. bronchiseptica from nasal and pharyngeal swabs of unvaccinated dogs.

Dog Number	Day Examined	Turbinates	Trachea	Bronchus	Lung Parenchyma	Bronchial Lymph Node	Retro Pharyngeal Lymph Node	Tonsil
24*	5	+++Sa	+++	+++	++	-Sa	+Sa	+Sa, St
25*	9	+++Sa, St	+++	+++	-	-Sa	-	+Sa, St
26*	11	++Sa, St	++	++	+	-St, C	-Sa, St	-St, C
27*	16	+++	+++	+++	++	-C	-	-St
28*	30	+++N, Sa	++N	++N	++	-	-	-St
29*	33	++Sa, P	++	++	+	+St	-	-St, C
30*	33	+++Sa, C	+++	+++	++	-St	-	-St, C, Sa
31*	5	+++Sa	+++	+++	++	+Sa	-Sa	+St, Sa
32**	9	+++Sa, C	+++	+++	++	-	-Sa	-
33**	11	+++Sa	+++	+++	++	+	-C	-St, C
34**	16	+++Sa, St	+++	+++	++	-	-Sa, St	-Sa, St, N
35**	30	+++N, Sa	++N	++N	++	-	-	-St
36**	33	+++Sa, C	+++	+++	+	-	-St	-St

* = Vaccinated
** = Unvaccinated control
\+ = Bord. bronchiseptica recovered from + sparse to +++ profuse culture
\- = No Bord. bronchiseptica recovered

Sa = Staphylococcus spp.
St = Streptococcus spp.
C = Coliform
N = Neisseria spp.

Table 30 : Experiment three - bacteriological findings at post-mortem examination.

specific fluorescent antisera were qualitatively and quantitatively similar in this experiment to those described in experiment two. Bord. bronchiseptica was demonstrated on the external surface of the tracheobronchial epithelium as late as 33 days after challenge, although, at this time, only small clumps of the organism were found in contrast to the almost continuous bacterial coating present earlier e.g. at 9 days after infection. There was no difference between the vaccinated and unvaccinated dogs. Small groups of fluorescent bacteria were occasionally localised in the peripheral sinuses of retropharyngeal lymph nodes or just beneath the tonsilar epithelium.

In sections of lung stained with specific, fluorescent anti-canine globulin globulin, positively stained plasma cells could be located in bronchial subepithelial tissues. These were present in greatest numbers in dogs killed at 30 and 33 days, and they were found, with equal frequency, in both vaccinated and unvaccinated dogs.

Staining of tissues with specific CAV and CDV anti-sera failed to reveal any viral antigen in any of the dogs used in this experiment.

Serological Findings

The results of the serum agglutination tests are shown in Table 31. Circulating antibodies to Bord. bronchiseptica were not detectable in any dog at the start of this experiment. Vaccination resulted in a rapid rise in circulating antibody, titres being detectable 7 days after initial vaccination. Titres continued to rise with the second inoculation of vaccine, reaching their highest levels one week before challenge; on the day of challenge, titres had fallen slightly from this peak but were still high at levels of 256 or 512. Aerosolisation of vaccinated dogs resulted in slightly increased antibody titres by day 9 but this increase was only to those levels previously achieved by vaccination alone, From day 9 until the experiment was terminated, the agglutinin titre tended to decline, although this was a gradual process. In the unvaccinated controls antibody was not detectable until after challenge infection which resulted in a rapid increase in antibody titre to levels comparable with those found in vaccinated dogs. Levels in

Dog Number	Days from infection											
	-28	-21	-14	-7	0	5	9	11	16	23	30	33
24*	<8	64	128	512	256	512						
25*	<8	64	128	512	256	ND	512					
26*	<8	32	64	512	512	ND	512	256				
27*	<8	64	128	512	256	ND	512	ND	512			
28*	<8	32	128	1024	512	ND	1024	ND	512	512	512	
29*	<8	32	128	512	256	ND	512	ND	256	256	128	128
30*	<8	32	64	512	256	ND	512	ND	512	256	128	256
31**	<8	<8	<8	<8	<8	<8						
32**	<8	<8	<8	<8	<8	ND	32					
33**	<8	<8	<8	<8	<8	ND	16	64				
34**	<8	<8	<8	<8	<8	ND	64	ND	64			
35**	<8	<8	<8	<8	<8	ND	64	ND	512	512	512	
36**	<8	<8	<8	<8	<8	ND	32	ND	256	128	128	128

* = Vaccinated N.B. 1st vaccination on day - 28
2nd " " " - 14
Challenge aerosolisation on day 0.

** = Unvaccinated control

ND = Not tested

Titres expressed as reciprocal of serum dilution

Table 31 : Experiment three - results of serum agglutination tests

vaccinated and unvaccinated dogs were very similar by 30 and 33 days after infection.

Virological Findings

No virus isolations were achieved from tissues from any dog used in this experiment.

Fig. 71 : Experiment three - trachea, dog 26. The tracheal mucosa is congested and a large amount of viscid, mucopurulent exudate is present in the lumen.

Fig. 72 : Experiment three - pharynx, dog 26. The palatine tonsils (arrows) are enlarged. The adenoid tonsil in the dorsal nasopharynx (starred) is prominent and congested.

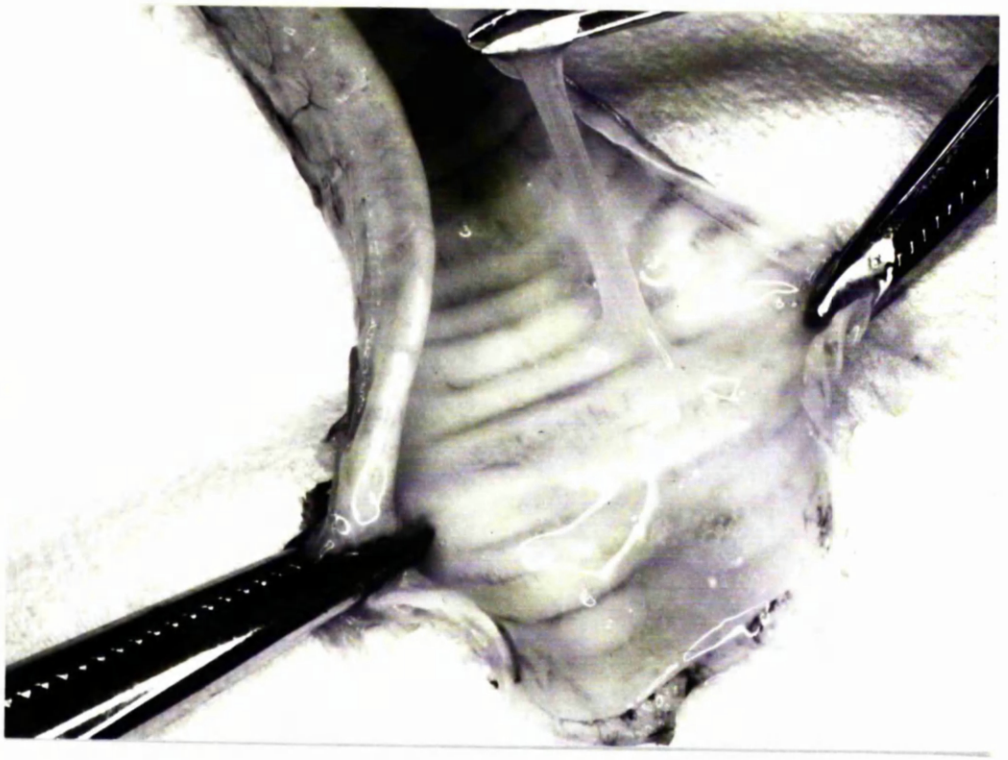
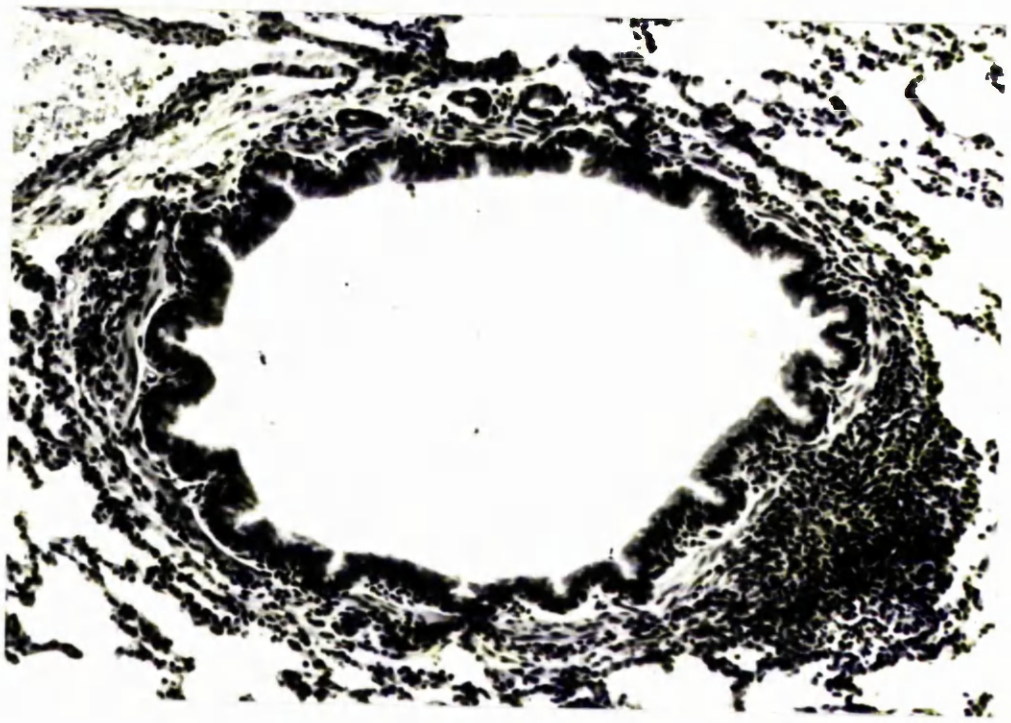


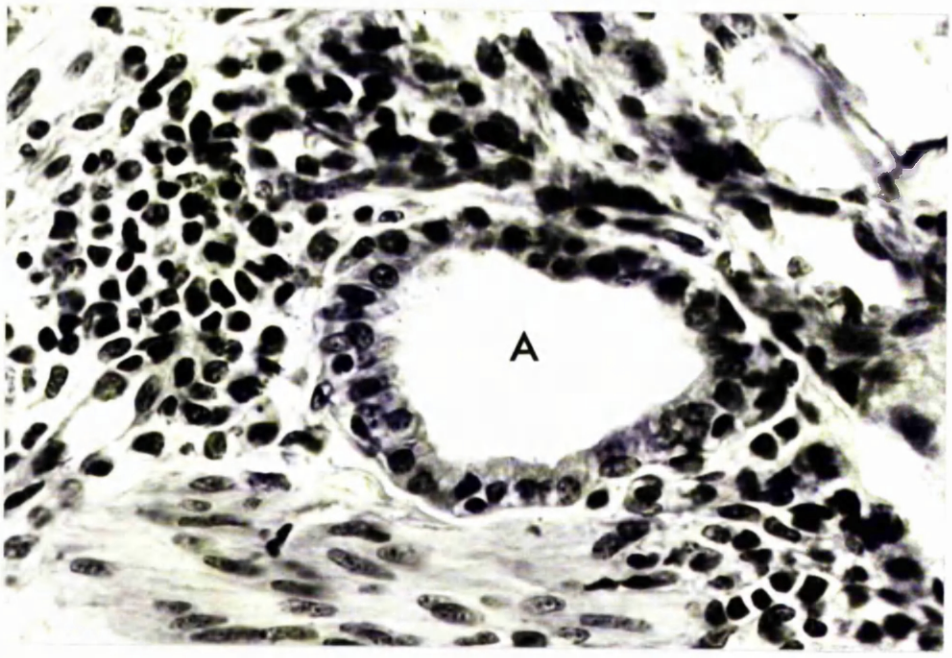
Fig. 73 : Experiment three - bronchiolitis, dog 29. The lamina propria of this bronchiole is hypercellular and a follicle-like aggregation of lymphocytes is present in the submucosa.

(HE, x 110).

Fig. 74 : Experiment three - bronchial gland, dog 29. A cellular infiltrate composed mainly of lymphocytes and plasma cells is present around a prominent bronchial gland acinus (A).

(HE, x 400).




A

SECTION 4 : EXPERIMENT FOUR - ALUMINIUM HYDROXIDE ADJUVANTED VACCINE

Experimental Design

12 puppies, randomly divided into 2 groups of 6 were used in this experiment. As in previous experiments the dogs did not harbour Bord. bronchiseptica in the nasopharynx and had no circulating antibodies to the organism.

The first, vaccinated group was inoculated with 1 ml of the aluminium hydroxide-adjuvanted vaccine by deep intramuscular injection on 2 occasions, a fortnight apart; the puppies were 8 weeks old at the time of the first inoculation. The vaccinated group and the remaining unvaccinated control group were subsequently challenged by exposure to an aerosol of virulent Bord. bronchiseptica 2 weeks after the second dose. After challenge, the dogs were maintained in a common airspace until 17 days after infection when the remaining vaccinated animals were moved to a clean, separate airspace.

One dog from each group was killed on days 5 and 7 after challenge while 2 dogs from each group were killed on days 13 and 20. Pre- and post-mortem investigations in these dogs were identical to those described for experiment three except that in immunofluorescence studies tissues were not stained with labelled rabbit anti-canine globulin globulin.

Clinical Findings

All 12 dogs remained healthy until after challenge. No clinical reaction to inoculation with the adjuvanted vaccine was noted in any of the vaccinated group.

After challenge, clinical signs of respiratory disease became apparent in all 6 unvaccinated control animals but in only 2 of the vaccinated dogs (Fig. 75). In the unvaccinated group 5 dogs were coughing by 4 days after infection and the remaining member of this group was also affected by 8 days after challenge. A harsh, paroxysmal cough persisted in each dog until it was killed i. e. up until 20 days after infection in two animals.

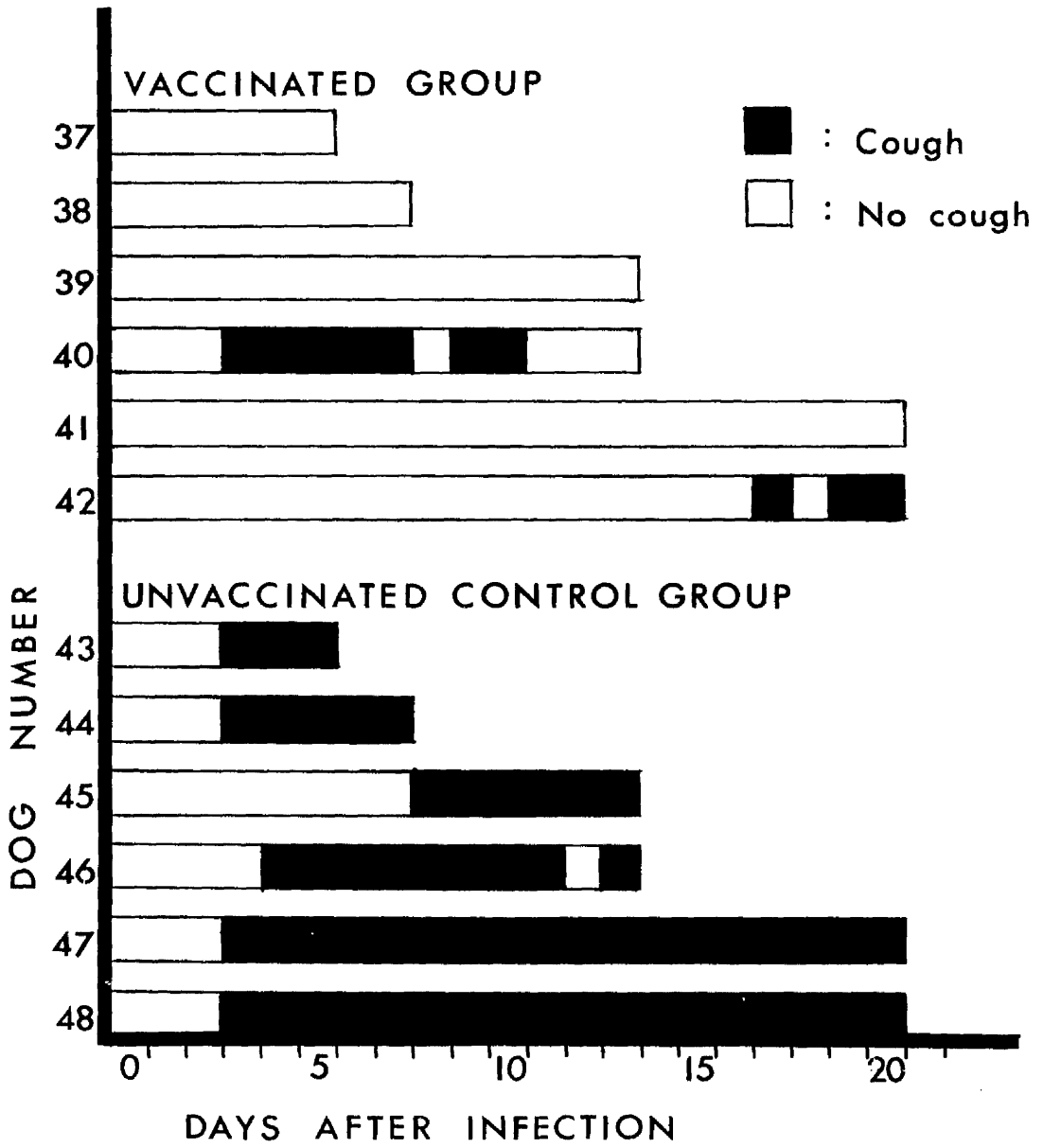


Fig. 75 : Experiment four - incidence of coughing.

Coughing was most severe in dogs 47 and 48 from 10 days after challenge onwards; while these animals remained playful and were never pyrexic their appetites were depressed from day 15 onwards. All control dogs developed an excessive serous or mucoid nasal discharge which was irregularly present between days 5 and 13.

In the vaccinated group, 4 dogs remained free of clinical evidence of respiratory disease throughout the experiment. Dog 40 was first found to cough 3 days after challenge but, in comparison with the controls coughing in this dog was of short duration, lasting only until day 10. The remaining member of the vaccinated group, dog 42, remained healthy until 17 days after challenge when a short, harsh cough developed. Paroxysmal coughing was not recorded in either of these dogs. An excessive serous discharge was occasionally found in 4 of the 6 dogs in this group. No other evidence of disease was found.

Pathological Findings

Macroscopic findings: There were marked differences in the severity of post-mortem findings between the vaccinated and unvaccinated control groups.

In the vaccinated group no gross abnormalities were detected in the lungs of dog 37, killed at 5 days, while the only finding in the lungs of the remaining 5 dogs was the presence of occasional small red foci, up to 1 mm in diameter, both on the pleural surface and in the lung substance (Figs. 76 and 78). The bronchial and retropharyngeal lymph nodes of all dogs in this group were firm and fleshy in consistency and enlarged to about $1\frac{1}{2}$ times normal size. The adenoids of dogs killed at 13 and 20 days were also enlarged and in one, dog 39 killed at 13 days, distinct haemorrhagic foci were present at this site.

In the unvaccinated control group, the post-mortem findings were much more severe. Mucopus was found both in the trachea and along the length of the bronchial tree in all 6 dogs. In addition, haemorrhagic foci up to 3 mm in diameter were found throughout the lungs of dogs killed on days 7 and 13 whilst larger consolidated areas of exudative pneumonia were evident in

the anterior lung lobes of both dogs killed on day 20 (Figs. 77 and 79). The bronchial and retropharyngeal lymph nodes of all dogs in this group were congested and enlarged to about twice normal size with occasional localised haemorrhagic areas. In all dogs except No. 48 killed at 20 days, quantities of mucopus were present in the nasopharynx overlying the adenoids; in both animals killed on day 13 a mucopurulent exudate was also present over the middle and ethmoid turbinates.

Microscopic findings: the histopathological findings in these groups of dogs are summarised in Table 32.

Histological changes in the vaccinated group of dogs were mild. At 5 days after infection the only abnormal finding in the lower respiratory tract was an occasional small group of bacteria in the cilia of the larger bronchi: reaction to these bacteria was confined to slight congestion and oedema of the underlying lamina propria with the presence of a few polymorphonuclear leucocytes and mononuclear cells in the epithelium and lumen in the immediate area of the bacteria. A few Gram-negative bacteria were also found on the turbinate epithelium of this dog with localised polymorphonuclear leucocyte infiltration of the adjacent epithelium from underlying areas of capillary congestion. In the bronchial and retropharyngeal lymph nodes, there was some peripheral sinusoidal oedema with some polymorphonuclear leucocytes present in the sinuses and medullary cords but there was also lymphoid follicular hyperplasia.

No abnormal histopathological findings were noted in the lower respiratory tract of the vaccinated dog killed at day 7 (Fig. 80). There was a mild rhinitis similar to that seen in the vaccinate killed on day 5; lymph node changes were also similar to those seen at 5 days.

At 13 days post infection there was more generalised oedema of the lamina propria along the length of the tracheobronchial tree. Gram-negative bacteria were occasionally identified in epithelial cilia, often surrounded by macrophages and a few polymorphonuclear leucocytes. The bronchial glands were prominent, some, though not all, being dilated and plasma

Dog Number	Day Examined	Rhinitis	Tracheitis	Bronchitis	Exudative Pneumonia	Lymphadeniti
37*	5	+	-	+	-	++
38*	7	+	-	-	-	+
39*	13	+	-	+	+	+
40*	13	+	-	+	+	+
41*	20	++	-	-	+	+
42*	20	+	-	+	+	+
43**	5	++	++	++	+	+
44**	7	++	+++	+++	+	++
45**	13	+++	+++	+++	++	++
46**	13	++	++	++	+	++
47**	20	+	+++	+++	+++	+
48**	20	+	+++	+++	+++	++

* = Vaccinated dog

** = Unvaccinated control dog

Lesions graded + to +++ on severity

Table 32 : Experiment four - histopathological findings

cells were found individually and in small groups around the glands. In the lung parenchyma, small foci of mononuclear cells, mainly lymphocytes, were found around small blood vessels. A mild focal rhinitis was present in these two dogs. In the bronchial and retropharyngeal lymph nodes and palatine and adenoid tonsils there was marked lymphoid follicular hyperplasia and many plasma cells were present in the medullary areas of the lymph nodes.

Changes at 20 days post infection were very similar to those at 13 days except that more mononuclear cells were present in the lamina propria of the tracheobronchial tree (Fig. 82) and the small, cellular accumulations in the lung parenchyma were more prominent (Fig. 84). These foci were often found around small blood vessels or next to terminal and respiratory bronchioles; they were composed mainly of lymphocytes but some macrophages and polymorphs were present, usually at the periphery of the foci.

The only other significant histopathological finding in tissues taken from vaccinated dogs was at the sites of vaccine inoculation. Many large active macrophages were found in the connective tissue of these areas; some plasma cells lymphocytes and a few polymorphs were also present.

In the unvaccinated group, infection with Bord. bronchiseptica resulted in a more severe histopathological picutre which was similar to that which has been described in previous experiments. There was severe tracheobronchitis (Figs. 81 and 83): initial acute inflammatory changes of congestion, oedema and polymorphonuclear leucocyte infiltration were followed by epithelial disorganisation, necrosis and hyperplasia with increasing mononuclear cell infiltration of the lamina propria and submucosa; Gram-negative bacteria were found in the cilia of the tracheobronchial epithelium in all 6 dogs examined. In the lung parenchyma there was initially alveolar mural capillary congestion with increased numbers of alveolar macrophages and some polymorphonuclear leucocytes present in the alveolar airspaces. However in dogs killed at 20 days after infection areas of exudative pneumonia had developed around severely affected bronchioles: the alveoli in these

areas were packed with polymorphonuclear leucocytes and macrophages and at the periphery of such areas there was alveolar congestion and oedema.

Rhinitis was present in all unvaccinated dogs but was most severe in those killed on day 13 when an extensive mucopurulent exudate was found overlying areas of turbinate epithelial necrosis with extensive congestion and inflammatory cell infiltration of the subepithelial tissues.

Lymphadenitis was present in the bronchial and retropharyngeal nodes: congestion, sinusoidal oedema and polymorphonuclear leucocyte infiltration was found up to 13 days after infection; at 20 days after infection, polymorphonuclear leucocytes were still noticeable in the medullary cords but there was also cortical follicular hyperplasia and large numbers of plasma cells were present in the medullary cords.

Bacteriological Findings

The recovery of Bord. bronchiseptica from nasal and pharyngeal swabs pre-mortem is shown in Table 33. In the unvaccinated group, the bacterium was recovered from almost every sample taken from one day after infection until the last dogs were killed on day 20. In previous experiments, bacterial recovery had tended to be less consistent and less profuse after the first 2 weeks of infection, but in this experiment Bord. bronchiseptica was isolated as consistently and in a profuse culture in the third week of infection as in the preceding 2 weeks.

In the vaccinated group, in the first 17 days of infection, Bord. bronchiseptica, although recovered from samples from all 6 animals was isolated much less consistently than from the control group although those vaccinated animals were in the same airspace as, and were, therefore, under constant challenge from the controls. After the remaining two vaccinates were moved to a clean airspace of one dog; Bord. bronchiseptica could not be identified in samples taken from the other dog.

Bacteriological examination of the samples taken at postmortem examination also revealed differences in the isolation of Bord. bronchiseptica

Dog Number	Days From Infection 28	21	14	7	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
37*	-	-	-	-	-	P	-	NP	-	NP														
38*	-	-	-	-	-	P	-	N	-	NP	NP	NP												
39*	-	-	-	-	-	-	N	N	P	N	NP	-	N	NP	P	-	-	P						
40*	-	-	-	-	-	P	N	P	-	NP	NP	P	NP	P	P	NP	NP	NP						
41*	-	-	-	-	-	-	NP	P	-	-	N	N	N	N	-	P	-	P	N	NP	N	P	-	P
42*	-	-	-	-	-	N	P	-	P	P	NP	-	-	P	NP	NP	P	P	-	-	P	-	-	-
43**	-	-	-	-	-	P	NP	NP	NP	NP														
44**	-	-	-	-	-	-	P	NP	NP	N	NP	NP												
45**	-	-	-	-	-	N	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP						
46**	-	-	-	-	-	-	NP	NP	NP	NP	NP	NP	N	N	NP	NP	NP	P						
47**	-	-	-	-	-	N	P	NP	N	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
48**	-	-	-	-	-	N	NP	N	N	P	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP

* = Vaccinated dog

** = Unvaccinated control dog

N = Bord. bronchiseptica recovered from nasal swab

P = Bord. bronchiseptica recovered from pharyngeal swab

\- = No Bord. bronchiseptica recovered

Table 33 : Experiment four - recovery of Bord. bronchiseptica from nasal and pharyngeal swabs of vaccinated and unvaccinated dogs.

between the vaccinated and unvaccinated groups (Table 34). In all unvaccinated dogs, Bord. bronchiseptica was recovered in profuse, almost invariably pure culture from the tracheobronchial tree. The bacterium could also be isolated from the lung parenchyma in pure, though less profuse culture and, in association with other bacteria from the turbinate mucosae.

In vaccinated dogs killed from 5 to 13 days after infection there was little difference in the recovery of Bord. bronchiseptica from the turbinates when compared with the unvaccinated group. In the lower respiratory tract, however, the bacterium was recovered only from the bronchi, where it was found in consistently sparser culture than in the control animals. This difference between the two groups was even more marked in the dogs killed at 20 days which had been in a clean airspace for 3 days before killing and were, therefore, no longer under constant challenge: Bord. bronchiseptica could be isolated in only very sparse culture from only one site in each dog.

Recovery of bacterial species from tonsil and lymph nodes was similar in both groups of dogs.

Immunofluorescence Findings

Bord. bronchiseptica was localised, by staining with specific fluorescent antisera, on the bronchial and bronchiolar epithelial surface in all 6 unvaccinated dogs. In the two unvaccinated dogs killed at 20 days after infection, which had areas of exudative pneumonia, small clumps of bacteria were also found in the alveolar airspaces.

In the vaccinated dogs, Bord. bronchiseptica could be found in only small numbers on only the bronchial epithelium of dogs killed from day 5 to 13. The organism could not be identified in either dog killed on day 20.

Staining with CDV and CAV specific antisera did not reveal any viral antigen in any tissue examined.

Dog Number	Day Examined	Turbinates	Trachea	Bronchi	Lung Parenchyma	Bronchial Lymph Node	Retropharyngeal Lymph Node	Tonsil
37*	5	++++Past, St, Sa	-	++	-	-	-	-C, St
38*	7	+++Sa, St	-	++	+	-	-Sa	-
39*	13	++++N	-	++	-	-Past	-	+C, Past, St
40*	13	++St, Past, N	-	++	-	-	-	-
41*	20	+Sa, C	-	-	-	-	-	-St, C
42*	20	-Past	-	+	-	-	-	-St, Past
43**	5	++++Past, St, Sa	+++	+++	++	-	-	-St
44**	7	++++Past, St	+++	+++	+	-	-Past	-
45**	13	++++Sa, St, C	+++	+++	+	-St	-	-St
46**	13	++++St, Sa	+++	+++	++	-	-	+St, N
47**	20	++++Past, Sa	+++	+++	+	-	-	-
48**	20	++++Past	++++St	+++	++	-	-	-

* = Vaccinated dog
** = Unvaccinated control dog

\+ = Bord. bronchiseptica recovered from + sparse to ++++ profuse culture
\- = No Bord. bronchiseptica recovered

Sa = Staphylococcus spp
St = Streptococcus spp
Past = Pasteurella spp
C = Coliform
N = Neisseria spp

Table 34 : Experiment four - bacteriological findings at post mortem examination.

Serological Findings

The results of the serum agglutination tests performed in the course of this experiment are shown in Table 35.

Circulating antibodies to Bord. bronchiseptica were not found in any dog at the start of this experiment. In the vaccinated group, the first inoculation of vaccine resulted in a slow rise in circulating agglutinin titre to Bord. bronchiseptica. Titres rose more sharply after the second inoculation and at the time of aerosol challenge, vaccinated animals had titres of 1 : 256 or 1 : 512 to the infecting bacterium. This level of circulating antibody was maintained or slightly increased in the vaccinated dogs in the 20 day period of infection.

In the unvaccinated controls serological examination for antibodies to Bord. bronchiseptica was consistently negative until after challenge infection; this resulted in a rise in titre to 1 : 64 at 20 days post infection.

Virological Findings

No known canine virus could be isolated from the lung tissue of any dog used in this experiment.

Dog Number	-28	-21	-14	-7	-1	5	6	7	13	20
37*	<8	<8	8	128	256	256				
38*	<8	8	16	256	512	ND	512	512		
39*	<8	8	16	128	256	ND	512	ND	512	
40*	<8	8	8	128	512	ND	512	ND	1025	
41*	<8	8	32	256	512	ND	512	ND	512	512
42*	<8	<8	8	128	256	ND	512	ND	512	512
43**	<8	<8	<8	<8	<8	<8				
44**	<8	<8	<8	<8	<8	ND	<8	<8		
45**	<8	<8	<8	<8	<8	ND	<8	ND	32	
46**	<8	<8	<8	<8	<8	ND	<8	ND	32	
47**	<8	<8	<8	<8	<8	ND	<8	ND	16	64
48**	<8	<8	<8	<8	<8	ND	<8	ND	10	64

* = Vaccinated N.B. 1st vaccination on day - 28
2nd vaccination on day - 14
Challenge aerosolisation on day 0.

** = Unvaccinated control

ND = Not tested

Titres expressed as reciprocal of serum dilution

Table 35 - Experiment four - results of serum agglutination tests.

Fig. 76 : Experiment four - lungs of dog 41. The lungs of this vaccinated dog killed 20 days after infection appear almost completely normal. Only a few scattered foci of congestion (arrows) are visible on the pleural surface.

Fig. 77 : Experiment four - lungs of dog 47. The lungs of this unvaccinated control dog killed 20 days after infection have extensive dark areas of exudative pneumonia in the apical, cardiac and anterior diaphragmatic lung lobes.

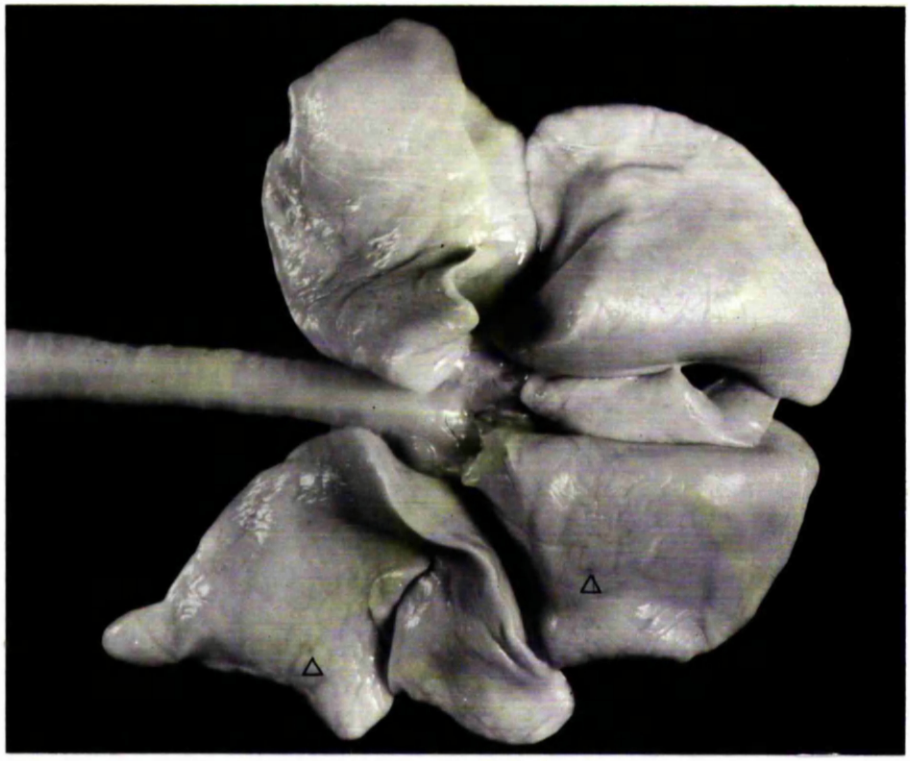
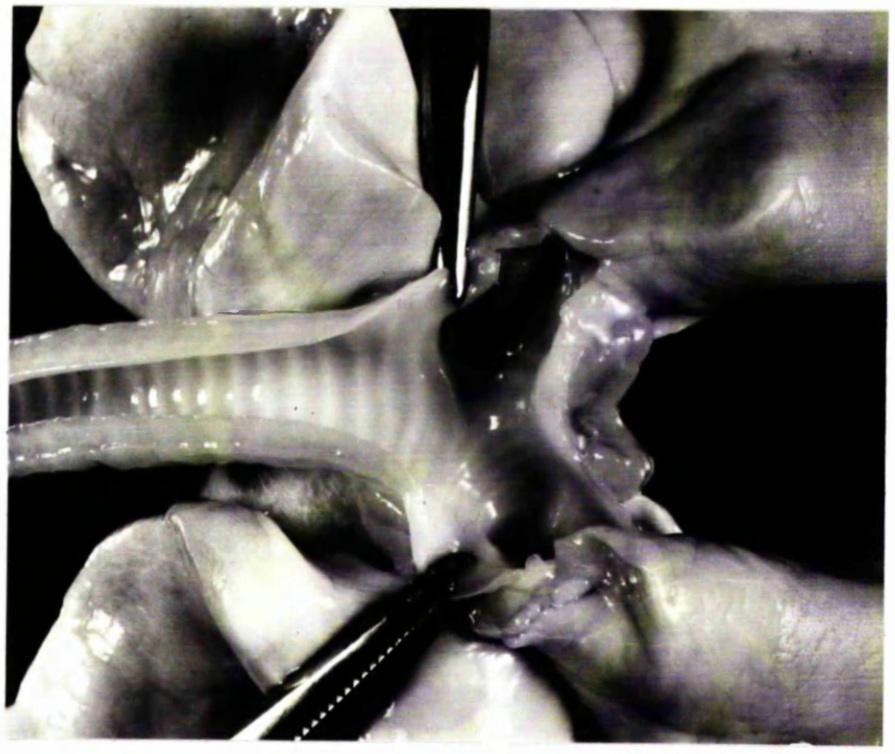


Fig. 78 : Experiment four - trachea of dog 41. The tracheobronchial tree of this vaccinated dog is free of exudate.

Fig. 79 : Experiment four - trachea of dog 47. In this unvaccinated control dog a frothy mucopurulent exudate is present along the length of the tracheobronchial tree.



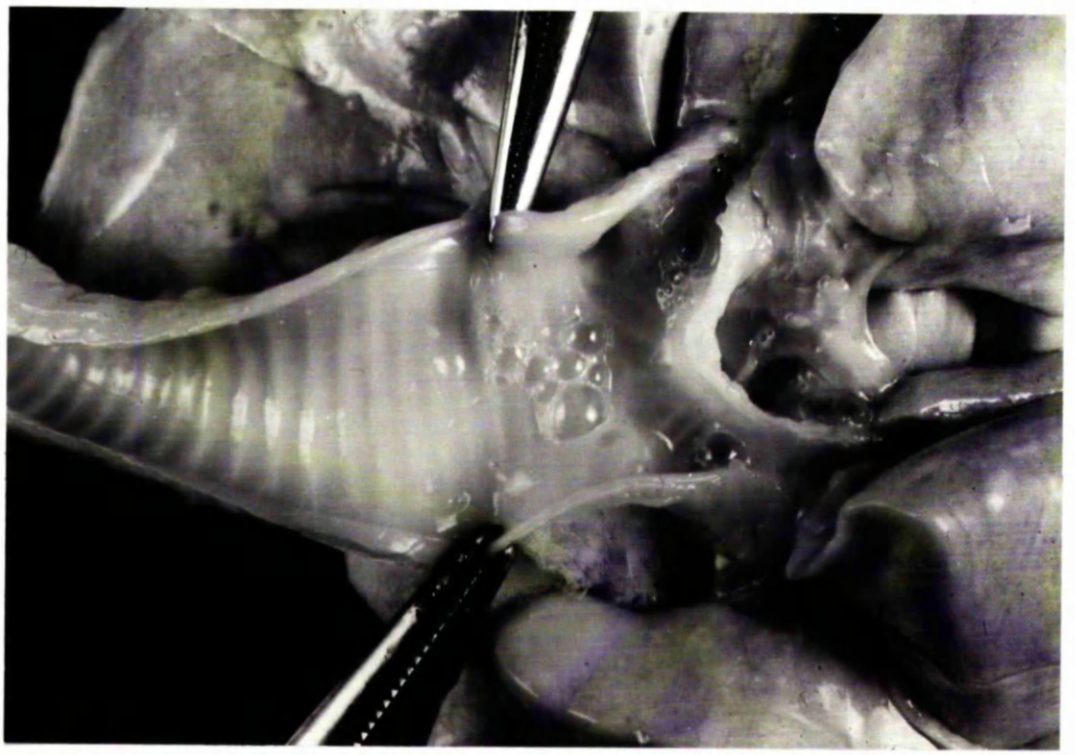
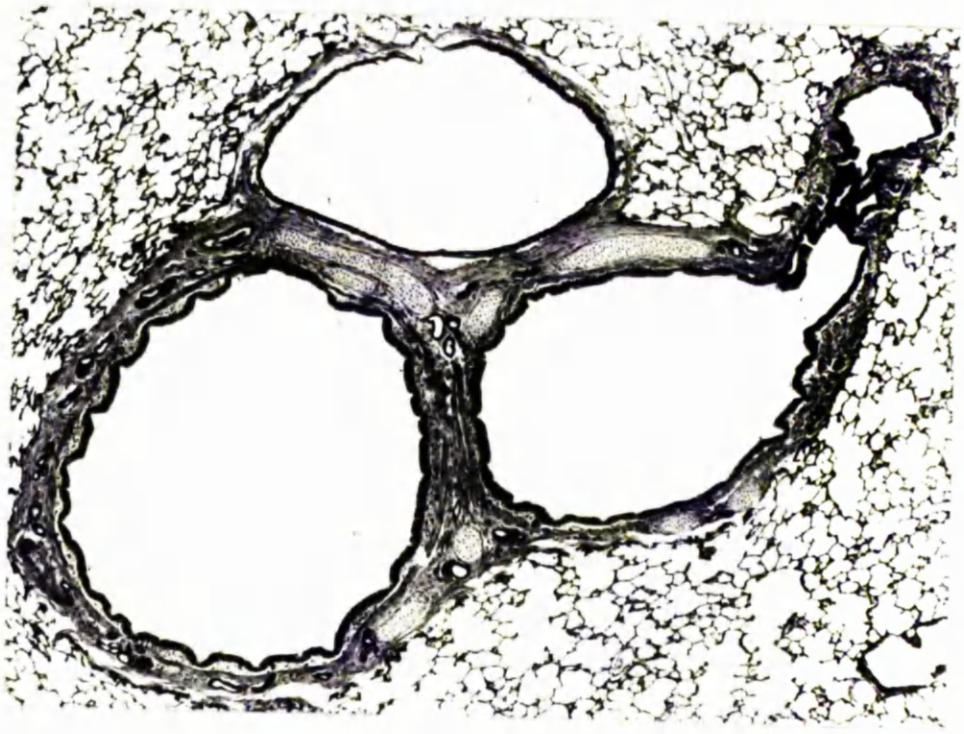


Fig. 80 : Experiment four - bronchi, dog 38. The bronchial tree of this vaccinated dog killed 7 days after infection appears normal.

(HE, x 35).

Fig. 81 : Experiment four - bronchi, dog 44. A severe bronchitis is present in this unvaccinated control dog killed 7 days after infection. A purulent exudate is present in the lumen and there is infiltration of cells into the lamina propria and epithelium. The bronchial glands are distended.

(HE, x 35).



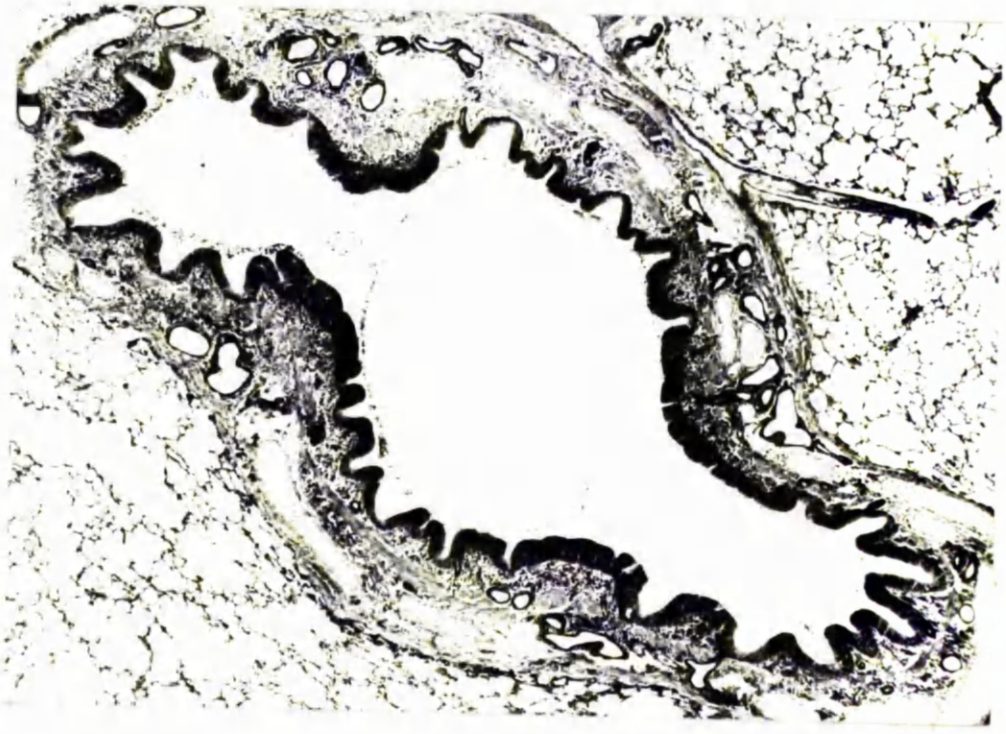
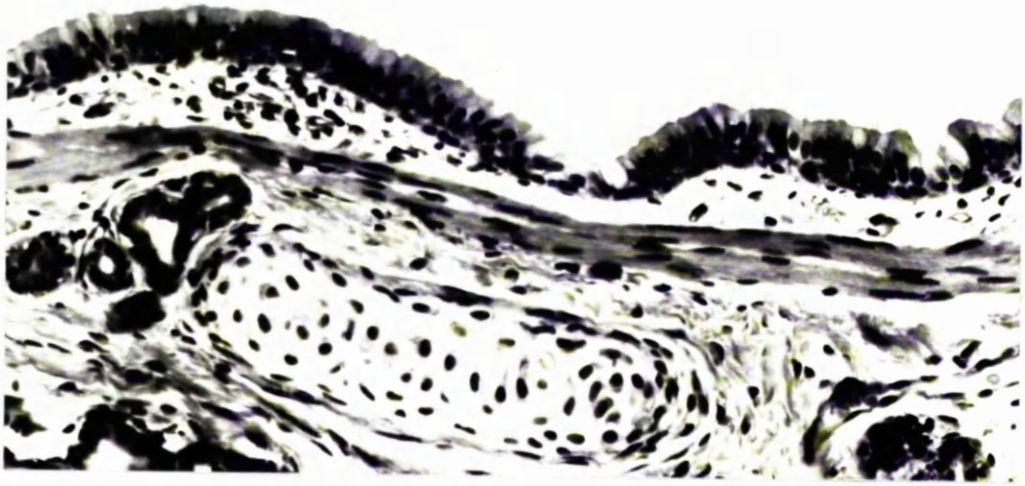


Fig. 82 : Experiment four - bronchial wall, dog 41. A small focus of lymphocytes in the lamina propria is the only notable feature in the bronchial wall of this vaccinated dog killed 20 days after infection.

(HE, x 250).

Fig. 83 : Experiment four - bronchial wall, dog 47. In this unvaccinated control dog killed 20 days after infection there is a severe bronchitis. The hyperplastic bronchial epithelium is infiltrated by polymorphonuclear leucocytes. Bacteria are visible in the epithelial cilia (arrow) and a mucopurulent exudate is present in the lumen. The lamina propria and submucosa contain a heavy cellular infiltrate.

(HE, x 250).



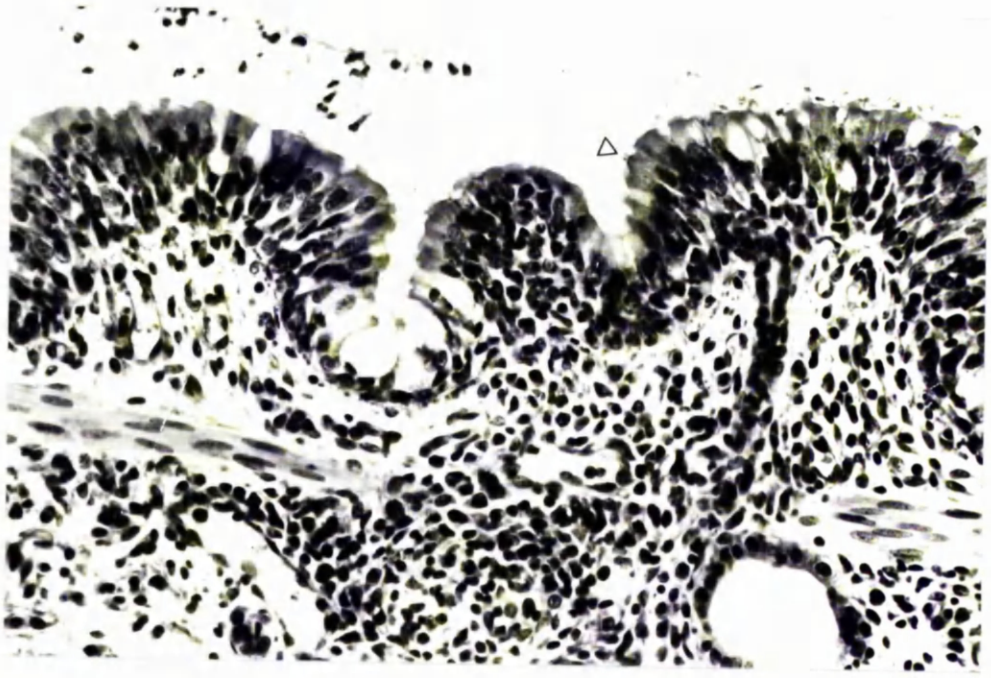
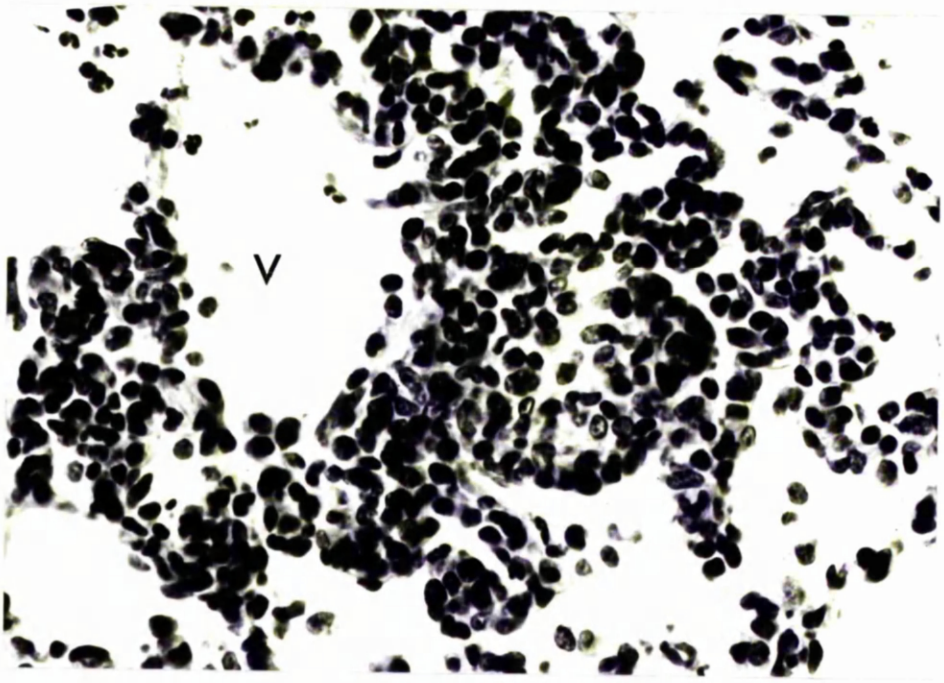


Fig. 84 : Experiment four - lung parenchyma, dog 41.

Twenty days after infection in a vaccinated dog, a small venule (V) in the lung parenchyma is surrounded by lymphocytes. A few macrophages and polymorphonuclear leucocytes are also present.

(HE, x 400).


V

SECTION 5 : DISCUSSION

In the experiments described above, attempts were made to immunise susceptible dogs against Bord. bronchiseptica infection. The vaccination regimes employed in these experiments were identical except for the mode of presentation of the antigen : in experiment three, the antigen was a simple bacterial cell suspension; in experiment four, the same quantity of the same bacterial cells was given by the same route, but the bacteria were adsorbed onto an aluminium hydroxide preparation. Despite this similarity in vaccination regimes, some differences in the clinical, bacteriological and pathological findings were observed.

The simple vaccine had little effect on the development of clinical respiratory disease; protection, if any, was limited to a delay in onset of respiratory disease of between 1 and 5 days when compared with the controls. In contrast, the adjuvanted vaccine resulted in a measure of protection from respiratory disease; clinical disease was evident in only 2 of the 6 vaccinated dogs whilst all 6 unvaccinated animals were affected. In addition, clinical signs recorded in the 2 vaccinates were of shorter duration and of lesser severity than those in the controls.

The superiority of the adsorbed vaccine was also demonstrated by both pre- and postmortem bacteriological findings. In experiment three, the only difference between vaccinated and control groups pre-mortem was that Bord. bronchiseptica was isolated less consistently from the nose and throat of the vaccinated dogs in the first 8 days of infection. There was no such difference in the recovery rate of Bord. bronchiseptica in the later stages of the experiment and the postmortem bacteriological findings in the respiratory tract were identical in both vaccinated and control groups. In experiment four, recovery of Bord. bronchiseptica from nose and throat swabs of vaccinated dogs was also less consistent than from controls, but this difference in recovery rate was present not merely in the initial stages but throughout the infection period. At necropsy there was also a considerable decrease in recovery of Bord. bronchiseptica from the lower respiratory tract of vaccinated dogs when compared with controls.

The macroscopic and microscopic findings showed marked differences in the 2 experiments. In experiment three, the pathological changes in both vaccinated and control groups were similar to those described in experiments one and two; the only effect of vaccination was a reduction in the severity of these changes in the initial stages of infection. In contrast, the pathological changes in the respiratory tract of vaccinated dogs in experiment four were much less severe when compared to those found in control animals. In particular, the severe tracheobronchitis so characteristic of the disease seen in the controls was not evident in the vaccinates nor were large numbers of Gram-negative bacteria found in the cilia of the respiratory epithelium. Pathological changes in the respiratory tract of vaccinated animals were confined to slight oedema of the lamina propria in the tracheobronchial tree, small foci of polymorphonuclear leucocyte and mononuclear cell infiltration of the epithelium, often beneath small groups of bacteria in the epithelial cilia, moderate mononuclear cell accumulation in the lung parenchyma and tracheobronchial lamina propria and submucosa and slight distention of the submucosal bronchial mucous glands.

From these results, it can be seen that the protection afforded against Bord. bronchiseptica infection by immunisation with a killed bacterial cell suspension is limited to a short delay in onset of clinical respiratory disease, a possible reflection of the initial sparing effect on the pathological and bacteriological features of the disease. Whether vaccination with the simple suspension used in experiment three would later have resulted in more rapid recovery from infection cannot be evaluated since, at the termination of the experiment, dogs from both groups were still harbouring bacteria; even at 33 days post-infection there was no evidence of clearance of the bacterium from the respiratory tract of either vaccinated or un-vaccinated dogs.

In contrast, immunisation with the same killed bacterial cell suspension adjuvanted with aluminium hydroxide resulted in substantial protection from respiratory disease due to Bord. bronchiseptica; protection was associated with decreased recovery of the infecting organism from the

respiratory tract and absence of pathological changes normally associated with Bord. bronchiseptica infection.

It may be that the degree of protection afforded by the vaccine in experiment three was challenge dose dependent i.e. a smaller challenge dose would have resulted in greater protection. Certainly, the challenge to which vaccinated dogs were subjected was very high, consisting of both exposure during aerosolisation and persistent, post-aerosolisation exposure in the form of continued contact with infected control dogs. The adjuvanted vaccine, however, provided a greater degree of protection despite similar high initial bacterial challenge and post-aerosolisation exposure. In the experiment with the adjuvanted vaccine the vaccinated dogs maintained increased clearance of Bord. bronchiseptica from the respiratory tract, compared with unvaccinated animals, throughout the experimental period; also, when the remaining vaccinated dogs were removed to a clean air space at 17 days after aerosolisation, and were therefore no longer subjected to constant challenge from infected controls, Bord. bronchiseptica was almost entirely eliminated from the respiratory tract within 3 days. This suggests that the recovery of bacteria from the respiratory tract of vaccinated dogs in the initial stages of the experiment was, to some extent, due to physical carriage of bacteria derived from continued exposure to unvaccinated, infected controls.

The superiority of the aluminium hydroxide adjuvanted vaccine in providing clinical, bacteriological and pathological protection from respiratory disease due to Bord. bronchiseptica was not paralleled by a similar superiority of the vaccine in stimulating the circulating antibody response, measured in these experiments by the serum agglutination test. The results of these tests show that in experiment three, at the time of challenge, serumagglutinin titreswere declining slightly from an earlier peak but were still high at 256 and 512; in experiment four, agglutinin titres were still rising at the time of challenge but the absolute levels present, 256 and 512, were identical to those in experiment three. It is difficult therefore, to ascribe the superiority of the adjuvanted vaccine solely to its effect on the systemic immune response; other immune

mechanisms must also be involved.

Successful vaccination is dependent upon efficient stimulation of those arms of the immune response concerned with protection against naturally occurring disease. Parenteral vaccination has long been recognised as providing substantial protection against disease caused by other major canine pathogens such as canine distemper virus and canine infectious hepatitis virus. In these diseases protection is well-correlated with the systemic immune response; parenteral vaccination with antigens derived from these viruses is effective in stimulating this response; hence, the vaccinated animal becomes resistant to the disease. The role of the systemic immune response in protection against those infectious diseases which exert their major effect at solely mucous surfaces is, however, much less certain and in recent years increasing attention has been paid to the role of local immune responses.

The concept of local immune responses, independent of the systemic response, was first postulated by Besredka in 1919. Later studies, demonstrated that in diseases such as cholera (Burrows et al., 1947) and influenza (Fazekas De St. Groth et al., 1951) resistance to infection was better correlated with the presence of antibodies in gastro-intestinal and respiratory tract secretions rather than with the presence of serum antibodies. More recently, it has been shown that the major class of immunoglubulin in external secretions is IgA (Tomasi et al., 1965) which, in serum, forms only a minor component of total immunoglobulin; moreover, it would appear that the IgA present in external excretions is produced locally, within the mucosae of the secretory surfaces (Tournville et al., 1969) and has distinct chemical and biological properties when compared with serum IgA (Tomasi and Bienenstock, 1968). As a result of these investigations there has been widespread acceptance of Besredka's postulate of a distinct local immune system which is involved in the protection of secretory surfaces from potential pathogens (Tomasi and De Coteau, 1969; Waldman, 1970; Bienenstock and Perey, 1972); as in the systemic immune response, the local response consists of both humoral (Tomasi, 1970) and cell - mediated (Henney and Waldman, 1970; Waldman and Henney, 1971) effector arms.

In the respiratory tract in particular, it has been shown that the local immune response is primarily involved in resistance to a number of viral and bacterial respiratory pathogens (Smith et al., 1966; Bellanti et al., 1967; Perkins et al., 1969 ; Waldman et al., 1969; Brunnel et al., 1974; Smith, 1975), these pathogens having in common their localization, at least initially, to the respiratory tract.

Investigations into the efficacy of various routes of vaccination in stimulating local immune responses within the respiratory tract have shown that, although the most efficient method is by direct application of antigen to the respiratory tract e.g. by using an aerosol to deposit antigen along the respiratory mucosa, nonetheless, parenteral vaccination can, in certain circumstances, stimulate the development of both local respiratory, humoral (Wigley et al., 1970; Waldman and Henney, 1971) and cell-mediated (Waldman and Henney, 1971; Waldman et al., 1972; Nash and Holle, 1972) immunity. The efficacy of parenteral vaccination in stimulating these local responses seems to be dependent on the nature of the antigen involved, the amount presented and the mode of presentation (Bienenstock and Perey, 1972; Kaltreider, 1976). The use of live i.e. replicating antigen, large doses of antigen or the incorporation of adjuvants appears to result in at least partial break-down of the relative independence of systemic and local immune systems.

Since, in infection with Bord. bronchiseptica, the bacterium is largely confined to the external surface of the respiratory mucosa, it seems likely that local immune responses within the respiratory tract will be important in recovery from infection and prevention of further disease. Evidence for this hypothesis is available both from the work of previous investigators and from the present series of experiments. In the pig (Harris and Switzer, 1969), it was found that animals with high circulating antibody titres to Bord. bronchiseptica as a result of parenteral vaccination with a killed bacterial cell suspension were not resistant to challenge infection by the respiratory route. However pigs inoculated intra-nasally with a low virulence strain were subsequently resistant to challenge even although no circulating antibodies to Bord. bronchiseptica could be detected in their sera. The development of local immune responses within the lung could well account for the resistance of these intra-nasally inoculated animals. This failure of high circulating

antibody levels to correlate with protection from aerosol challenge with Bord. bronchiseptica in the pig has been observed by other workers (Koshimizu et al., 1973). These findings in the pig would appear to be equivalent to the situation prevailing in experiment three above where parenteral vaccination resulted in a good circulating antibody response but little resistance to challenge infection.

Indirect evidence for the involvement of local immunity in Bord. bronchiseptica infection comes from the extensive mononuclear cell infiltrate found in the lamina propria and submucosa of the tracheobronchial tree of infected dogs. This infiltrate, seen in infected unvaccinated animals in experiments one, two three and four may be a reflection of developing local immune reactions within the respiratory tract as a response to established infection. The results of immunofluorescence examination of lung tissue in experiment three also indicate the presence of increasing immune responses within the respiratory tract : staining for canine globulin revealed the presence of positively stained cells (plasma cells) in sub-epithelial tissues, the number of positive cells increasing with the duration of infection.

In evaluating the results of experiments three and four, the probable importance of local immunity in Bord. bronchiseptica infection must be considered. In experiment three, there was, undoubtedly, stimulation of the systemic immune response evinced both by the high titres of circulating antibody and the histological response of lymph nodes and tonsils to vaccination; even at 5 days post challenge these lymphoid tissues were reactive, with lymphoid follicular hyperplasia and cellular proliferation in thymic dependent areas. The course of infection in the lung was, nonetheless, identical to that seen in control animals, the vaccinates, like the controls, developing a mononuclear cell infiltrate in the tracheobronchial tree. There was no clinical or histopathological evidence of a previously sensitized local immune system mounting an effective defence against infection. There was, however, even at 5 days post challenge marked inflammation with extensive congestion and oedema in the tracheobronchial tree of the vaccinated dogs. This early inflammatory reaction may have allowed leakage of serum immunoflobulins into the respiratory tract resulting in some anti-Bordetella activity. Such leakage

would occur extensively only after inflammation of the respiratory tract had developed; even then, the total amount of specific immunoglobulin reaching the respiratory secretions would not be large and these serum immunoglobulins, lacking the "protective" secretory piece of locally synthesized immunoglobulin, would be liable to rapid proteolysis and loss of activity : development of the already established disease process would thus tend to be retarded rather than suspended. Transudation of serum immunoglobulins could, however, account for the degree of protection achieved i.e. the decreased consistency of recovery of Bord. bronchiseptica from nose and throat swabs and the slightly lesser degree of severity of pathological changes observed in the initial phases of infection in the vaccinated group.

In experiment four, as in experiment three, there was serological and histological evidence of stimulation of the systemic immune response. The other results obtained, however, differed not merely quantatively but qualitatively from those in experiment three. Protection was associated with clearance of bacteria from the respiratory tract and almost complete absence of the inflammatory changes seen in the controls.

In the vaccinated dogs at 5 and 7 days post challenge, the lymphoid cells present in the lamina propria and submucosa of the tracheobronchial tree did not appear to be increased in number; those present could, however, have been sensitized to Bord. bronchiseptica by the vaccination procedure and therefore capable of mounting an effective defence against infection. It may be significant that although there was only slight cellular infiltration of the epithelium in these dogs, the infiltrate which was observed contained a high proportion of mononuclear cells in contrast to the controls in which the epithelial infiltrate was composed predominantly of polymorphonuclear leucocytes. At 13 and 20 days post challenge, mononuclear cells were more obvious in the lungs of the vaccinated dogs, although never reaching the numbers found in unvaccinated animals; they were found, in particular, in small foci around small vessels and bronchioles. This increased number of lymphoid series cells could be a reflection of response to the continued antigenic stimulation provided by repeated challenge from the infected controls.

These results, i.e. the lack of protection from disease in experiment three despite high circulating antibody levels, the degree of protection achieved in experiment four with apparently similar stimulation of the systemic immune response and the different cellular responses in the lungs following challenge in the two experiments, provide indirect evidence that, in experiment four, use of the aluminium hydroxide adjuvanted vaccine resulted in stimulation of additional immunological mechanisms to those operating following inoculation of the simple heat killed vaccine of experiment three. It is probable that these additional mechanisms comprise either or both of the local humoral and cell mediated immune responses which are involved in protection against many localized respiratory diseases. No direct evidence in support of this hypothesis was obtained in these experiments and confirmation will necessitate measurement of such parameters as immunoglobulin-mediated anti-Bordetella activity in bronchial secretions and Bordetella-specific cellular responses within the lung.

Experimental Bord. bronchiseptica infection in the dog would provide a good comparative model system for -further study of the immune response of the respiratory tract to bacterial infection; the role of various components of the immune response in resistance to infection; and the most effective methods of providing immunoprophylaxis against respiratory disease.

The increased efficacy of adjuvanted vaccine must depend on the effect of the aluminium hydroxide. Adjuvants are widely employed in the medical and biological sciences "..... in making bad antigens into good ones and persuading good ones to do even better." (White, 1966). Their effect on the local immune system is, like their precise mode of action, by no means clear; it is, however, of note that in those investigations in which parenteral vaccination has resulted in measurable local immune responses within the respiratory tract, adjuvants have usually been incorporated with the immunizing antigen (Waldman and Henney, 1971; Waldman et al., 1972).

The mode of action of aluminium hydroxide in particular is equally uncertain but there is some experimental evidence that Alhydrogel, the preparation used in this experiment, can, divorced from adsorbed antigen, have a direct stimulatory effect on the reticulo-endothelial system (Terry, et al., 1966).

PART IV : CHEMOTHERAPY OF CANINE BORDETELLOSIS

SECTION 1 : INTRODUCTION AND REVIEW OF THE LITERATURE

A wide range of chemotherapeutic agents is now available for use in the management of microbial disease. The rationale for the use of such drugs in contagious bacterial disease is twofold: firstly, to produce in the individual amelioration of symptoms and more rapid recovery from disease; secondly, to prevent further spread of disease in the population as a whole. In addition, in diseases of known viral aetiology the use of antibacterial chemotherapeutics may be indicated if there is evidence of superimposed bacterial involvement complicating the primary disease picture.

The choice of an appropriate antibiotic or other chemotherapeutic agent depends on a range of factors including the nature of the causal microorganism, the host animal and the type of disease involved. Thus, different species of bacteria vary in their sensitivity to different groups of antibiotics; an individual animal or species may be particularly susceptible to side effects produced by a given antibiotic; and treatment even with an antibiotic which is effective against the causal agent in vitro, may be ineffective if the disease involves an organ or system which, for anatomical reasons, that particular antibiotic is unable to reach in sufficient concentration. For these reasons, the only absolute criterion of efficacy of an antibacterial agent is the response of affected individuals to therapy.

In view of the frequency with which Bord. bronchiseptica is involved either on its own or with other agents in the causation of respiratory disease in the dog, it is important that the cheomtherapeutics used in such disease should be active against this bacterium.

There have been no recorded investigations of the efficacy of individual antibiotics against Bord. bronchiseptica infections in the dog. However, antibiotic sensitivity tests of strains of Bord. bronchiseptica recovered from dogs with clinical tracheobronchitis have shown that the organism is sensitive, at least in vitro, to a number of antibiotics : novobiocin, tetracycline, ampicillin, chloramphenicol, erythromycin and kanomycin (Wilkins and Helland, 1972). In addition, a number of studies have been

made of the effect of chemotherapy on non-specific respiratory disease in the dog in which various agents - nitrofurantoin, colistin, cephalothin, tylosin and ampicillin - have been shown to have some degree of success in controlling the disease process (Mosier, 1955; Mann and Bjotvedt, 1964; Snow et al., 1969; Baker and Huebner, 1970).

A number of reports have appeared in the literature commenting on the effect of chemotherapy on Bord. bronchiseptica infection in species other than the dog. Rosen et al., (1954) treated rats with a number of chemotherapeutics; streptomycin and sulphonamides were relatively ineffective but oxytetracycline given orally as a feed additive controlled both mortality and clinical disease. In guinea pigs (Ganaway et al., 1965) sulphamethazine given in drinking water was effective in controlling mortality due to Bord. bronchiseptica; this treatment did not however eliminate the disease since morbidity and mortality rose sharply when treatment was discontinued. In the pig it has been shown that sulphamethazine and sulphethoxypyridazine can eliminate the organism from the respiratory tract of both naturally and experimentally infected pigs (Switzer, 1963). Clearance of Bord. bronchiseptica from the respiratory tract of pigs has also been reported after administration, as a feed additive, of a mixture of chlortetracycline, sulphamethazine and procaine penicillin (Woods et al., 1972). Despite the initial promise of the sulpha drugs in clearance of Bord. bronchiseptica their widespread use as a feed additive in America failed to eliminate the bacterium from the national pig herd; this failure was attributed mainly to the appearance of sulphonamide resistant strains (Harris and Switzer, 1972).

The role of chemotherapy in the management of the related disease of whooping cough in man has been the subject of debate (Linnemann et al., 1974). Studies have shown that, in pertussis, chemotherapy has little effect on the clinical course of disease if treatment is instituted after clinical signs have become evident. However, a number of antibiotics, notably chloramphenicol, tetracycline and erythromycin, appear capable of clearing the bacillus from the respiratory tract thus preventing further spread of disease (Bass et al., 1969; Nelson, 1969; Linnemann et al., 1974; Islur et al., 1975). There is also some evidence that use of these drugs early in

infection, before the onset of symptoms, will abort the clinical disease (Bass et al., 1969).

From these reports it can be seen that chemotherapy of infections due to Bord. sp. has met with varying degrees of success. Nonetheless, antibiotic therapy is recommended in the management of those respiratory diseases in the dog in which Bord. bronchiseptica may be involved (Pennock and Archibald, 1968. O'Brien and Todd, 1971). In particular, the use of broad spectrum antibiotics e.g. chloramphenicol and the tetracyclines, has been advocated despite a lack of convincing evidence of their in vivo activity against Bord. bronchiseptica. It was considered worthwhile to investigate the effects of such antibiotic therapy on the experimental respiratory disease produced in dogs by aerosolisation with Bord. bronchiseptica.

SECTION 2 : MATERIALS AND METHODS

Experimental Animals

Complete litters of 8 week old, Collie-cross pups were obtained, housed and maintained as described in Part II, Section 2.

Bacteriological Procedures

Production of cultures for aerosolisation and examination of nasal and pharyngeal swabs and tissues taken at necropsy were processed as described in Part II, Section 2.

In addition, antibiotic sensitivity tests were performed against Bord. bronchiseptica 52498/3 according to the methods recommended by the World Health Organisation Expert Committee On Antibiotics (1961). Diagnostic Sensitivity Test Agar (DSTA) (Oxoid Ltd., London) was prepared with the addition of 7% defibrinated horse blood (Burroughs Wellcome Ltd., Kent) according to the manufacturer's instruction. DSTA plates were inoculated with a suspension of Bord. bronchiseptica which previous tests had shown would produce dense, though not confluent, growth. A number of different Antibiotic Sensitivity Test Discs (Oxoid Ltd., London) were then applied to the inoculated plates, each disc being applied to a separate plate. The DSTA plates were then held at room temperature for three hours and subsequently incubated overnight at 37°C. The plates were then examined for evidence of zones of inhibition of bacterial growth.

These tests showed that the test strain of Bord. bronchiseptica was sensitive, in vitro, to amoxycillin, ampicillin, chloramphenicol, colistin, kanamycin, oxytetracycline and a combination of sulphamethoxazole and trimethoprim; it was resistant to nitrofurantoin, penicillin G, streptomycin and sulphafurazole. Oxytetracycline and amoxycillin were selected for study of their activity in vivo against Bord. bronchiseptica and the results of these studies are recorded separately in Sections 3 and 4 respectively (vide infra). Oxytetracycline is frequently advocated for use in canine respiratory disease (Aronson and Kirk, 1971) whilst amoxycillin, a relatively new semi-synthetic penicillin, has been reported to be particularly effective in the treatment of sensitive bacterial respiratory infections in man,

possibly as the result of good penetration of the bronchial mucous membrane (May and Ingold, 1972).

In the course of these in vivo investigations further sensitivity tests against the antibiotic administered in each experiment were carried out on isolates of Bord. bronchiseptica recovered, at intervals throughout the experiment, from infected dogs. The procedure of these tests was as described above but care was taken to ensure that the inoculating suspensions of the different isolates were of equivalent concentration; this allowed direct comparison of the sensitivity of the different isolates to that antibiotic, since for a given antibiotic, the size of the zone of inhibition is inversely related to the minimal inhibitory concentration (McAllister, 1975).

Aerosolisation Procedures

Necropsy Procedures

Histological Procedures

Immunofluorescence Procedures

Virological Procedures

As in Part II, Section 2.

Serological Procedures

Serum samples obtained during these experiments were submitted to the serum agglutination test for antibodies to Bord. bronchiseptica described in Part II, Section 2.

SECTION 3 : EXPERIMENT FIVE - OXYTETRACYCLINE THERAPY

Experimental Design

A total of 10 healthy puppies, randomly divided into 2 groups of 5 dogs each, were used in this experiment. The first, treated group was to receive oxytetracycline; the second was to act as an untreated control group.

At 12 weeks of age, after routine pre-challenge tests, all 10 dogs were exposed to an aerosol of Bord. bronchiseptica 52498/3 . After this challenge, the dogs were maintained in a common airspace and examined daily for any clinical evidence of disease; the morning rectal temperature and presence or absence of cough and nasal discharge were routinely recorded. Nasal and pharyngeal swabs were taken daily from 2 days before challenge and submitted to bacteriological examination.

Evidence of respiratory disease (i.e. coughing and nasal discharge) became clinically apparent on day 3 post-challenge. On day 4, one member of the control group was killed to confirm the establishment of infection, the treated group was removed to a clean, separate airspace and treatment was instituted. Treatment consisted of a single, daily injection of oxytetracycline (Terramycin Q-Pfizer Ltd.) at a dosage rate of 5 mg/lb. for 5 days i.e. days 4-8 inclusively. The antibiotic was administered by deep injection into the biceps femoris muscle.

Clinical examination and sampling of nasal and pharyngeal flora was continued daily in both groups throughout the treatment period and until death. Pairs of dogs, one from each group, were killed on days 9, 11 and 13 post-challenge, the remaining 2 treated and 1 control dogs being killed on day 16. At necropsy, pathological, bacteriological, immunofluorescence and virological examinations were undertaken.

Serum agglutination tests were performed on paired samples from each dog taken at challenge and at death.

Sensitivity tests against oxytetracycline were performed on the challenge culture of Bord. bronchiseptica and on isolates of Bord. bronchiseptica recovered from the nose and pharynx of each dog during the treatment

period and from the bronchus of each dog at necropsy.

Clinical Findings

No clinical evidence of disease was apparent in any dog until after aerosolisation when signs of respiratory disease developed in all dogs from both groups. As in previous experiments, the predominant clinical sign of disease was coughing (Fig. 85).

In the untreated control group all 5 dogs were found to be coughing on the third day after infection. Coughing was most severe, with frequent, paroxysmal bouts, between days 5-10 after infection; thereafter, coughing persisted as a prominant clinical feature although in dog number 57, killed at 13 days, it became sporadic after day 10; coughing was still present at 16 days when the remaining member of this group was killed.

All members of the treated group were also coughing 3 days after infection. In one dog (No. 49), killed 9 days after infection, there was remission of clinical signs within the treatment period, coughing being absent in this dog on day 8 i.e. the last day of treatment, and day 9. In the remaining 4 dogs, however, coughing continued despite treatment until the time of death. In 2 of these dogs (Nos. 50 and 52), coughing was severe and paroxysmal on days 5-7 but became sporadic, though still significant, in later stages i.e. after the end of treatment; in the remaining 2 dogs (Nos. 51 and 53), coughing was most severe between days 5-7 and from day 12 until death with an intervening period, days 9-11 when paroxysms of coughing were less frequent and of shorter duration.

Dogs from both groups were occasionally found to have a slight serous or mucoid nasal discharge from days 8-10 after infection. In the treated group, intramuscular injection of Terramycin Q resulted in transient discomfort : the injected leg was carried for 5-10 minutes after injection and the dogs occasionally licked or gnawed at the injection site.

There was no evidence of systemic involvement : morning rectal temperatures remained within normal limits and all dogs were bright, with good appetites throughout the experiment.

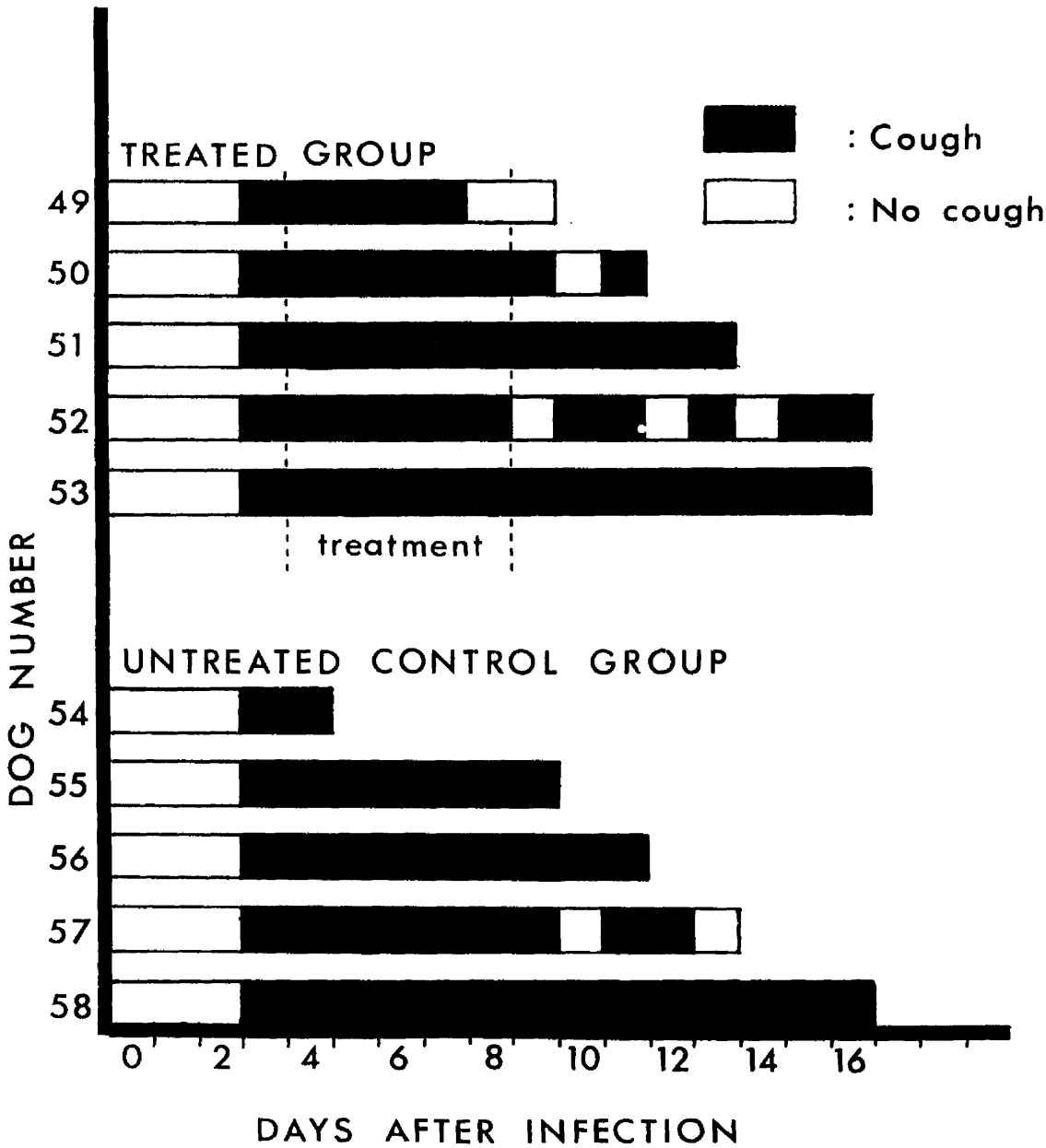


Fig. 85 : Experiment five - incidence of coughing.

Pathological Findings

Macroscopic: In the untreated dog (No. 54), killed on day 4, red foci up to 2 mm in diameter were found on the pleural surface and in the substance of the lungs. Small amounts of a mucopurulent exudate were present in the trachea and the posterior nasopharynx. The bronchial and retropharyngeal lymph nodes were enlarged to twice normal size and were very oedematous.

In the untreated dogs (Nos. 55 and 56), killed on days 9 and 11 respectively, small red foci were again found throughout the lungs and, in addition, there were firm, dark red areas of exudative pneumonia in the dependent portions of the apical and cardiac lobes and along the antero-ventral edges of the diaphragmatic lobes; these areas were more extensive in dog No. 56. Mucopus was present along the length of the tracheobronchial tree and nasopharynx : bronchial and retropharyngeal lymph nodes were enlarged and congested.

However, in the treated dogs (Nos. 49 and 50), killed at the same stage, only 1 mm red foci were found in the lung substance and although slight quantities of exudate were present in the tracheobronchial tree, this was of a clear, mucoid nature; the associated lymph nodes were enlarged and congested.

Exudative pneumonia was also a feature of untreated control dogs (Nos. 57 and 58), killed on days 13 and 16; in dog No. 57 this was confined to the antero ventral portion of the left diaphragmatic lobe and only scant quantities of a purulent exudate were found in the bronchi; in dog No. 58, however, all lobes were affected and mucopus was found along the length of the tracheobronchial tree and in the nasopharynx. Of the treated dogs killed at this time, dog Nos. 51 and 53 had patchy areas of exudative pneumonia present in all lobes of the lung and a mucopurulent exudate in the tracheo-bronchial tree and nasopharynx; in dog No. 52, however, multiple 1 mm red foci and a scant mucoid exudate were present. The bronchial and retropharyngeal lymph nodes were enlarged and firm in all dogs killed at this stage.

Microscopic: The histopathological findings in this experiment are summarised in Table 36. In both treated and control groups the histological features present in the respiratory tract of the aerosolised dogs were those which have been previously described in Bord. bronchiseptica infection (Part II, Sections 3 and 4): there was colonisation of the respiratory epithelium by Gram-negative bacteria with development of a severe tracheobronchitis and, in some dogs, an associated exudative pneumonia; rhinitis and inflammation of the lymph nodes draining the respiratory tract were also present.

In dog 54, the untreated control killed on day 4, these pathological changes in the respiratory tract were already well established: there was a moderate tracheobronchitis with congestion, oedema and polymorphonuclear leucocyte infiltration of the lamina propria; the epithelium was also infiltrated by these cells and many bacteria were present among the surface cilia.

In the control dogs (Nos. 55 and 56), killed on days 9 and 11, there was a more severe tracheobronchitis with epithelial necrosis; areas of exudative pneumonia were also present around severely affected bronchi and bronchioles. In contrast, only a mild to moderately severe tracheobronchitis was evident in treated dogs Nos. 49 and 50, also killed on days 9 and 11, bacteria were present along the epithelial surface but there was no epithelial necrosis and inflammatory changes in the mucosa were in general less severe; changes in lung parenchyma were confined to occasional foci of alveolar macrophages and a few polymorphonuclear leucocytes in alveolar air spaces.

By days 13 and 16, however, there was little difference in the histopathological findings in treated and untreated dogs (see Table 36); tracheobronchitis and exudative pneumonia were present in both groups. As in previous experiments, at this stage of infection with Bord. bronchiseptica the mucosa of the tracheobronchial tree was thickened with a mixed cellular infiltrate of polymorphonuclear leucocytes, lymphocytes and a few plasma cells present in the lamina propria; bacteria were still present on the surface of the overlying epithelium which was hyperplastic and infiltrated by polymorphonuclear leucocytes.

Dog Number	Day Examined	Rhinitis	Tracheitis	Bronchitis	Pneumonia	Lymphadenitis
49*	9	+	+	++	-	+
50*	11	+	+	+++	+	+
51*	13	++	+++	+++	+++	++
52*	16	+	++	++	+	+
53*	16	+	++	+++	+++	++
54**	4	+	++	++	+	++
55**	9	++	+++	+++	++	+
56**	11	++	+++	+++	+++	++
57**	13	+	++	++	++	++
58**	16	+	+++	+++	+++	+

* = Treated dog
** = Untreated control dog

Lesions graded + to +++ on severity

Table 36 : Experiment five - histopathological findings

Bacteriological Findings

The effects of treatment with oxytetracycline on the bacteriological features of Bord. bronchiseptica infection were limited to a reduction in the excretion of the bacteria during the treatment period.

The recovery of Bord. bronchiseptica from pre-mortem nasal and pharyngeal swabs is shown in Table 37. Institution of treatment with oxytetracycline resulted in a marked decrease in the recovery of Bord. bronchiseptica from these swabs on days 5 - 7 inclusive. On days 8 and 9 i.e. the last day of treatment and the following day, recovery of Bord. bronchiseptica from nasal and pharyngeal swabs from treated dogs was still less consistent than from controls, but from day 10 onwards i.e. after the end of treatment, there was no difference between the groups.

Treatment with oxytetracycline had little effect on the isolation of Bord. bronchiseptica at post-mortem examination (Table 38). Bord. bronchiseptica was recovered from the respiratory tract of treated dogs as frequently and in as profuse culture as from the untreated controls; Even in treated dog No. 49, killed only 1 day after the last dose of antibiotic, there was no evidence of clearance of Bord. bronchiseptica from the respiratory tract.

The results of the sensitivity test to oxytetracycline performed on the various isolates of Bord. bronchiseptica are shown in Table 39. The diameters of the zones of inhibition of bacterial growth were identical for the infecting culture of Bord. bronchiseptica and isolates of Bord. bronchiseptica recovered from nasal and pharyngeal swabs during the treatment period and from the tracheobronchial tree at post-mortem examination. The in vitro sensitivity of the infecting bacterium to oxytetracycline remained the same throughout the experiment: resistance to the antibiotic did not develop.

Immunofluorescence Findings

The results of immunofluorescence examination of sections of lung stained with specific, fluorescent, anti-Bordetella antiserum were identical to those of previous experiments. Fluorescent bacteria were found predominantly on the surface of the epithelium in the bronchial tree and in

Dog Number	Days from Infection: -2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Treatment																		
49*	-	-	-	P	NP	NP	NP	-	-	P	NP	NP							
50*	-	-	-	N	NP	NP	NP	-	P	-	N	N	N	P					
51*	-	-	-	N	NP	NP	NP	NP	P	NP	NP	NP	NP	NP	NP	NP			
52*	-	-	-	N	NP	NP	NP	-	P	-	NP	N	NP	NP	N	NP	NP	NP	NP
53*	-	-	-	N	NP	NP	NP	N	P	NP	P	N	NP	NP	NP	NP	NP	NP	NP
54**	-	-	-	-	NP	NP	NP												
55**	-	-	-	-	N	NP	NP	NP	NP	NP	NP								
56**	-	-	-	N	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP					
57**	-	-	-	-	N	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP			
58**	-	-	-	N	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP

* = Treated dog
** = Untreated control dog

N = Bord. bronchiseptica recovered from nasal swab
P = Bord. bronchiseptica recovered from pharyngeal swab
- = No Bord. bronchiseptica recovered

Table 37 : Experiment five - recovery of Bord. bronchiseptica from nasal and pharyngeal swabs of treated and untreated dogs.

Dog Number	Day Examined	Turbinates	Trachea	Bronchi	Lung Parenchyma	Bronchial Lymph Node	Retropharyngeal Lymph Node	Tonsil
49*	9	++++	++++	++++	+++	-	-	-
50*	11	++++	++++	+++	++	-	-	-C, St
51*	13	++++	++++	++++	++	+	-	-C
52*	16	++++	++++	++++	++	- St	-	-St, Sa
53*	16	++++	++++	++++	++	-	-	-Sa
54**	4	++++St	++++	++++	++	- Sa	+ St	-C, Sa
55**	9	++++Sa	++++	++++	+++	+	-	-
56**	11	++++	++++	++++	++	-	-	-
57**	13	++++	++++	++++	+++	-	- St	-
58**	16	++++	++++	++++	++	+ St	-	-C, St

* = Treated dog
** = Untreated control dog
\+ = Bord. bronchiseptica recovered from + sparse to ++++ profuse culture
\- = No Bord. bronchiseptica recovered
Sa = Staphylococcus spp.
St = Streptococcus spp.

Table 38: Experiment five - bacteriological findings at post mortem examination

Dog Number	Inhibition zone diameter	
	Isolate recovered during treatment period	Isolate recovered from bronchus at necropsy
49*	29 mm	28 mm
50*	28 mm	29 mm
51*	29 mm	29 mm
52*	29 mm	30 mm
53*	30 mm	28 mm
54**	30 mm	29 mm
55**	29 mm	29 mm
56**	29 mm	28 mm
57**	29 mm	29 mm
58**	29 mm	29 mm
52498/3***	29 mm	

* = Treated dog
** = Untreated control dog
*** = Culture of Bord. bronchiseptica used for infection

Diameter of inhibition zone to nearest millimetre

Table 39 : Experiment five - in vitro sensitivity to oxytetracycline of Bord. bronchiseptica isolates

exudate present in the bronchial lumen. Bacteria were also found in debris in areas of epithelial necrosis. Specifically stained bacteria were not found in the lymph nodes or tonsils of any animal in this experiment.

Staining of tissues was specific anti-CDV and anti-CAV antisera failed to reveal any viral antigen.

Serological Findings

The results of the serum agglutination tests performed in this experiment are shown in Table 40. Circulating antibody to Bord. bronchiseptica was not found in any dog before infection but developed in dogs from both groups after infection. Titres present ranged from 8 at 9 days after infection up to 32 at 16 days after infection.

Virological Findings

No known canine virus could be isolated from the respiratory tract of any dog used in this experiment.

Dog Number	Days from infection						
	-7	0	4	9	11	13	16
49*	<8	<8	NT	8			
50*	<8	<8	NT	NT	8		
51*	<8	<8	NT	NT	NT	16	
52*	<8	<8	NT	NT	NT	NT	32
53*	<8	<8	NT	NT	NT	NT	16
54**	<8	<8	<8				
55**	<8	<8	NT	<8			
56**	<8	<8	NT	NT	8		
57**	<8	<8	NT	NT	NT	8	
58**	<8	<8	NT	NT	NT	NT	16

* = Treated dog
** = Untreated control dog

Titres expressed as reciprocal of serum dilution

Table 40 : Experiment five - results of serum agglutination tests

SECTION 4 : EXPERIMENT SIX - AMOXYCILLIN THERAPY

Experimental Design

This experiment was similar in design to experiment five. 12 puppies, randomly allocated to 2 groups of 6, were used : the first group was to be treated with amoxycillin; the second was an untreated control.

The dogs were exposed to an aerosol of Bord. bronchiseptica 52498/3 at 12 weeks of age, after routine pre-challenge tests. After challenge, until the time of death, each dog was examined daily for clinical evidence of disease, the morning rectal temperature and presence or absence of cough and nasal discharge were noted and nasal and pharyngeal swabs were taken daily for bacteriological examination.

Amoxycillin therapy was instituted on day 5 after aerosolisation when clinical respiratory disease was established in both groups of dogs. Treatment consisted of oral administration of amoxycillin at a minimum rate of 10 mg per lb. twice daily. Each dog was weighed, the amount of amoxycillin required to attain this dosage rate computed and each dog given the nearest multiple of 25 mg of amoxycillin above this computed amount twice daily. Treatment was continued at intervals of 12 hours for 5 days i.e. days 5 to 9 inclusive. Both groups of dogs were maintained in a common airspace until treatment was begun, when the treated group was moved to a separate airspace, where they were kept until death.

One member of the untreated group was killed on day 4 to confirm the establishment of infection and pairs of dogs, one from each group, were killed days 7, 14, 15 and 16 after infection; 2 treated and 1 untreated dog were killed on day 10.

At necropsy, pathological, bacteriological, immunofluorescence and virological examinations were undertaken.

Serum agglutination tests were performed on paired serum samples taken from each dog at challenge and at death.

Sensitivity tests against amoxycillin were performed on the challenge culture of Bord. bronchiseptica and on isolated stains of Bord. bronchiseptica recovered from the nose or pharynx of each dog during the treatment period and from the bronchus of each dog at necropsy.

Clinical Findings

Both groups of dogs remained healthy until after aerosolisation when respiratory disease developed in all 12 animals. This respiratory disease was characterised, as in previous experiments, by a harsh spontaneous cough, the incidence of which is shown in Fig. 86.

Coughing was first noted in both treated and untreated groups on day 3 and by day 4, all dogs were affected (Fig. 86). Coughing continued in both groups of dogs until the time of death and in both groups, became severe, with frequent paroxysmal attacks, from day 7 onwards.

A nasal discharge was noted to occur irregularly in members of both groups from day 5 onwards; this was usually serous in nature but from days 5 to 12 it tended to be of a thick, mucoid consistency.

Morning rectal temperatures remained within the normal range and the dogs appeared bright and alert throughout the experiment.

Oral administration of amoxycillin was well accepted by the treated dogs and had no clinically discernable adverse effects upon them.

Pathological Findings.

Macroscopic : At post mortem examination there was no difference in the type, distribution or severity of the lesions found in treated and untreated groups.

In dogs killed up until day 10, small reddish foci up to 1 mm in diameter were found throughout the lung substance and on the pleural surface; from day 14 onwards, similar, pinpoint foci were found but in addition, solid, greyish-red foci of exudative pneumonia were present, mainly in the apical and cardiac lobes. Excessive amounts of exudate were found in the tracheobronchial tree of all 12 dogs; this exudate varied in quantity but was invariably mucopurulent in nature.

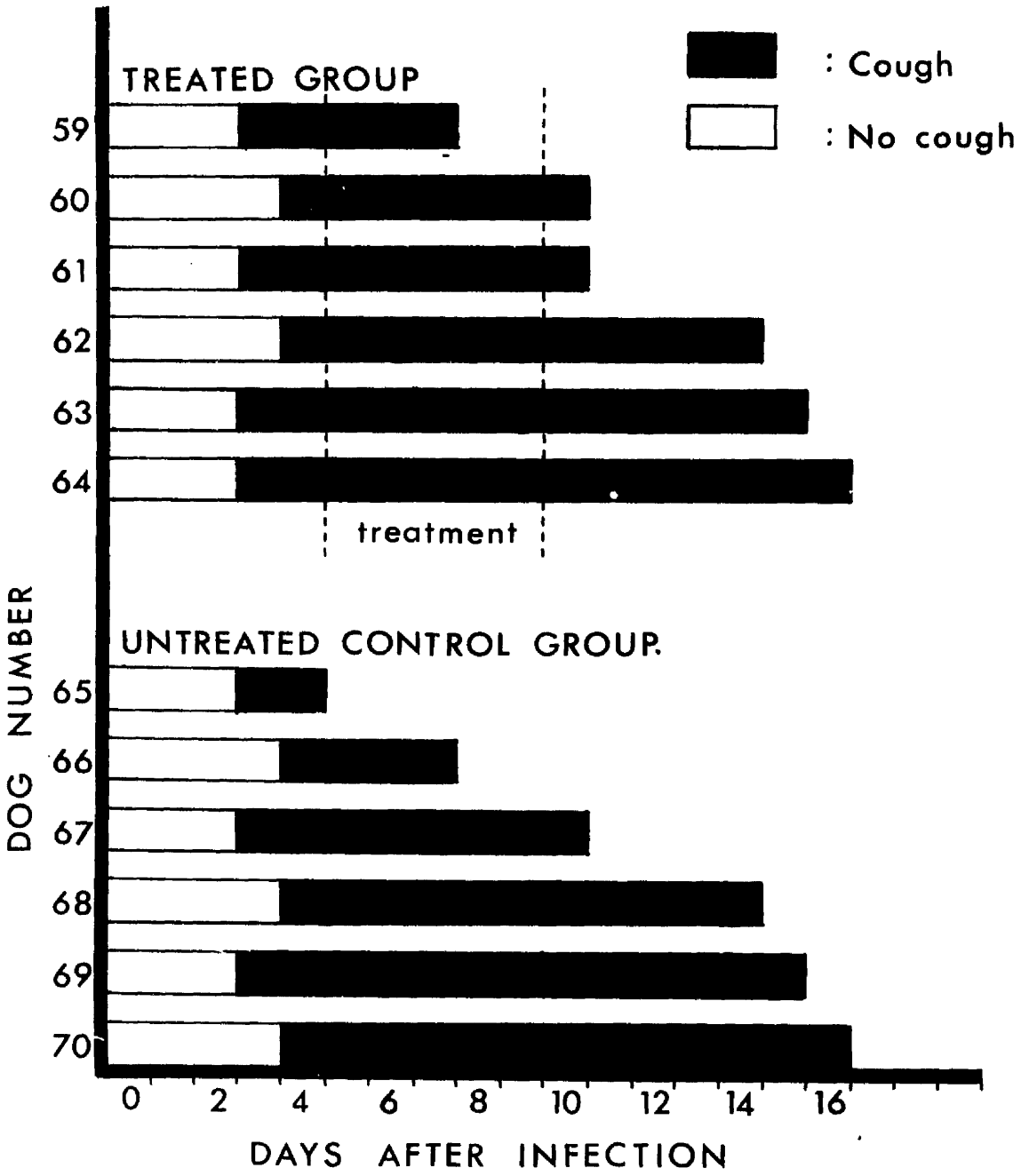


Fig. 86 : Experiment 6 - incidence of coughing.

Mucopus was also found in the nasal cavity of most dogs and was particularly prominent in the ethmoturbinate region; occasionally, the mucopus extended back along the roof of the nasopharynx to overlie the adenoids. The adenoids themselves were, in dogs killed from day 7 onwards, prominent and often congested. The palatine tonsils were only occasionally enlarged but, in dogs from both groups, the bronchial and retropharyngeal lymph nodes were consistently enlarged to about twice normal size; up until day 10, these lymph nodes were congested and oedematous but, thereafter, although congested areas were still found, they were firm in consistency.

Microscopic : The histological findings, shown in Table 41, were similar, in both groups of dogs, to those which have been associated with Bord. bronchiseptica infection in previous experiments. In both treated and untreated dogs, there was tracheobronchitis, characterised by the presence of Gram-negative bacteria among epithelial cilia and the infiltration of polymorphonuclear leucocytes into the lamina propria, epithelium and lumen of the tracheobronchial tree. In dogs killed from day 4 onwards, the epithelium seemed hyperplastic and increasing numbers of lymphocytes were found in the lamina propria.

In the lung parenchyma changes ranged from the presence of increased numbers of macrophages in the alveoli to foci of pneumonia, usually around affected bronchioles, where the alveoli were packed by polymorphonuclear leukocytes and macrophages. In the nasal cavity, there were inflammatory changes in the mucosae of the turbinate bones with Gram-negative bacteria visible in the cilia of an epithelium infiltrated by polymorphonuclear leucocytes.

Congestion, sinusoidal oedema and infiltration of polymorphonuclear leucocytes were the most striking findings in the lymph nodes of dogs killed up until 10 days; thereafter, lymphoid follicular hyperplasia became marked.

Dog Number	Day Examined	Rhinitis	Tracheitis	Bronchitis	Pneumonia	Lymphadenitis
59*	7	++	+++	+++	+	++
60*	10	++	+++	+++	+	++
61*	10	+	+++	+++	++	++
62*	14	+	+++	+++	++	++
63*	15	++	++	+++	+	+
64*	16	++	++	++	+	+
65**	4	+	++	++	-	+
66**	7	++	+++	+++	+	++
67**	10	++	+++	+++	++	++
68**	14	++	+++	+++	++	++
69**	15	++	++	+++	+	+
70**	16	+	++	+++	++	+

* = Treated dog

** = Untreated control dog

Lesions graded + to +++ on severity

Table 41 : Experiment six - histopathological findings

Bacteriological Findings

Treatment with amoxycillin had no effect on either pre-mortem or post mortem bacteriological findings.

The recovery of Bord. bronchiseptica from nasal and pharyngeal swabs is shown in Table 42 : in both treated and untreated control groups alike the bacterium was recovered from nasal and pharyngeal swabs from 1 day after infection until the time of death.

The recovery of Bord. bronchiseptica from samples taken at post-mortem examination is shown in Table 43. Bord. bronchiseptica was recovered in profuse, usually pure, culture from the tracheobronchial tree of all dogs irrespective of duration of infection or treatment status.

The results of sensitivity tests to amoxycillin performed on the isolates of Bord. bronchiseptica are shown in Table 44. All isolates examined had zones of inhibition of bacterial growth of almost identical diameter. Increased resistance of Bord. bronchiseptica to amoxycillin did not, therefore, develop during the period of infection.

Immunofluorescence Findings

Staining of sections of lung with specific fluorescent, anti-Bordetella antiserum resulted in the localisation of bacteria to the surface of bronchial and bronchiolar epithelium; fluorescent bacteria were also present in luminal exudate; there was no difference between the treated and untreated groups. Sections of lymph node and tonsil did not contain fluorescent bacteria. Sections of lung,lymph node and tonsil stained with specific anti-CDV antiserum and anti-CAV antiserum showed no evidence of virus infection.

Serological Findings

The result of agglutination tests on paired serum samples from each dog are present in Table 45. Titres rose from undetectable levels at the time of challenge to a maximum of 16 at 16 days after challenge.

Dog Number	Days from infection: -2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Treatment																		
59*	-	-	-	P	P	NP	NP	NP	NP	NP									
60*	-	-	-	P	P	NP	NP	NP	NP	NP	NP	NP	N						
61*	-	-	-	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP						
62*	-	-	-	N	P	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP		
63*	-	-	-	NP	P	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	
64*	-	-	-	NP	N	NP	NP	NP	N	NP	NP	N	N	NP	N	NP	NP	NP	NP
65**	-	-	-	P	NP	NP	NP												
66**	-	-	-	P	NP	NP	NP	NP	NP	NP									
67**	-	-	-	N	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP					
68**	-	-	-	P	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP		
69**	-	-	-	-	P	NP	NP	NP	NP	NP	NP	N	NP	NP	NP	N	N	NP	NP
70**	-	-	-	N	NP	NP	NP	NP	NP	NP	NP	N	NP	NP	NP	NP	NP	NP	NP

* = Treated dog

** = Untreated control dog

N = Bord. bronchiseptica recovered from nasal swab

P = Bord. bronchiseptica recovered from throat swab

- = No Bord. bronchiseptica recovered

Table 42 : Experiment six - recovery of Bord. bronchiseptica from nasal and pharyngeal swabs of treated and untreated dogs

Dog Number	Day Examined	Turbinates	Trachea	Bronchi	Lung Parenchyma	Bronchial Lymph Node	Retropharyngeal lymph node	Tonsil
59*	7	++++	++++	++++	++	-	-	-
60*	10	+++ +St, Sa	++++ C	++++	++	-	-	-C
61*	10	++++	++++	++++	++	-	-St	-
62*	14	++++	+++ +St, C	++++	+++	-	-	-
63*	15	++++	++++	++++	+++	-	-	-
64*	16	++++	++++	++++	+++	-	-St	-C
65**	4	+++ +St	++++	++++	+++	-	-	-St
66**	7	+++ +Sa, St	++++	++++	++	-	-	-St, Sa
67**	10	+++ +Sa, C	+++ +C, St	++++	+	-St	-	-C
68**	14	+++ +St	+++ +Sa	++++	++	-	-	-
69**	15	+++ +C, St	++++	++++	+++	-	-	-C, St
70**	16	+++ +St	++++	++++	++	-	-	-

* = Treated dog

** = Untreated control dog

\+ = Bord. bronchiseptica recovered from + to sparse to ++++ profuse culture

\- = No. Bord. bronchiseptica recovered

Sa = Staphylococcus spp.

St = Streptococcus spp.

Table 43 : Experiment six - bacteriological findings at post mortem examination

Dog Number	Inhibition zone diameter	
	Isolate recovered during treatment period	Isolate recovered from bronchus at necropsy
59*	32	31
60*	32	32
61*	32	32
62*	31	31
63*	32	32
64*	32	31
65**	31	31
66**	32	32
67**	32	32
68**	32	31
69**	31	32
70**	32	32
52498/3***	32 mm	

* = Treated dog
** = Untreated control dog
*** = Infecting culture of Bord. bronchiseptica

Diameter of inhibition zone to nearest millimetre

Table 44 : Experiment six - in vitro sensitivity to amoxycillin of Bord. bronchiseptica isolates.

Dog Number	Days from infection							
	-7	0	4	7	10	14	15	16
59*	<8	<8	NT	<8				
60*	<8	<8	NT	NT	<8			
61*	<8	<8	NT	NT	8			
62*	<8	<8	NT	NT	NT	16		
63*	<8	<8	NT	NT	NT	NT	8	
64*	<8	<8	NT	NT	NT	NT	NT	16
65**	<8	<8	<8					
66**	<8	<8	NT	<8				
67**	<8	<8	NT	NT	<8			
68**	<8	<8	NT	NT	NT	16		
69**	<8	<8	NT	NT	NT	NT	8	
70**	<8	<8	NT	NT	NT	NT	NT	16

* = Treated dog
** = Untreated control dog

Titres expressed as reciprocal of serum dilution

Table 45 : Experiment six - results of serum agglutination tests

Virological Findings

No known canine virus could be isolated from the respiratory tract of any dog used in this experiment.

SECTION 5 : DISCUSSION

The object of the experiments described in Sections 3 and 4 was to investigate the *in vivo* efficacy of 2 different antibiotic agents, oxytetracycline and amoxycillin, against *Bord. bronchiseptica* infection in the dog.

In Section 3 treatment with oxytetracycline resulted in only a slight decrease in the severity of clinical signs of respiratory disease and pathological changes in the respiratory tract. This decreased severity was apparent only by comparison with untreated controls; neither clinical respiratory disease, nor respiratory tract pathology was abolished by chemotherapy. In addition, the duration of this decreased severity was limited to the end of treatment and the immediate post-treatment phase; once treatment ceased, both clinical signs and pathological changes became as severe in treated as in control animals. Treatment with oxytetracycline also resulted in decreased recovery of *Bord. bronchiseptica* from nasal and pharyngeal swabs. This moderation in bacterial excretion was of very short duration and did not even extend to the end of the treatment period; by then, recovery of *Bord. bronchiseptica* from the nose and pharynx of treated animals was again as frequent as from untreated controls. Treatment had no effect on the recovery of *Bord. bronchiseptica* from the respiratory tract at post mortem examination.

In Section 4, treatment with amoxycillin failed to modify any of the clinical, pathological or bacteriological features of *Bord. bronchiseptica* infection which were recorded in this experiment: in particular, there was no amelioration of the disease syndrome and no reduction in the excretion of *Bord. bronchiseptica*.

From these results it is apparent that, despite the *in vitro* sensitivity of *Bord. bronchiseptica* to oxytetracycline and amoxycillin, under the conditions prevailing in these experiments neither antibiotic was able either to eliminate *Bord. bronchiseptica* from the respiratory tract or to alter significantly the course of disease in individual animals. There appears, therefore, to be little indication either on the grounds of improvement in signs of disease in individual animals or of protection of

the more general population for the use of either of these antibiotics, in the régimes used in these experiments, in the treatment of Bord. bronchiseptica infection in the dog.

Failure of antibiotic therapy may be due to any one, or, indeed, any combination, of a number of factors e.g. misdiagnosis, inappropriate antibiotic selection, development of bacterial resistance or inadequate dosage rate. The experimental nature of these investigations precluded the possibility of misdiagnosis; the causal agent of the respiratory disease was known to be bacterial and, therefore, susceptible, at least in theory, to antibiotic action. The selection of the antibiotics tested in these experiments was based on the results of the in vitro investigations of the susceptibility of Bord. bronchiseptica to antibiotic action; both oxytetracycline and amoxycillin were amongst those agents which would, in a clinical situation, have been recommended as likely to be of use against Bord. bronchiseptica. Bord. bronchiseptica did not, as far as could be determined, develop resistance to the action of either oxytetracycline or amoxycillin during the course of the experiments; strains of the bacterium recovered at post mortem examination had the same in vitro sensitivity as had the original infecting strains.

Since these factors i.e. misdiagnosis, inappropriate antibiotic selection and resistance, seem unlikely to be involved inthe failure of oxytetracycline and amoxycillin to produce improvement in respiratory disease caused by Bord. bronchiseptica, it is most probable that the lack of response to chemotherapy was associated with inadequate dosage régimes. In order for an antibiotic to exert its maximum effect against a susceptible bacterium in vivo it is first necessary that the antibiotic be present at the site of bacterial multiplication in at least the minimum inhibitory concentration of that antibiotic for that bacterium for a sufficient period of time. The concentration of antibiotic reaching the site of bacterial multiplication is dependent on the rate of absorption of antibiotic from the site of administration, the amount of antibiotic given and the distribution of antibiotic within the body tissues; the duration of a minimum concentration is related to the rate of breakdown of antibiotic and its rate of excretion from the body. The dosage régimes employed in the therapy of bacterial diseases must be

such as to ensure that, taking account of, for example, absorption and excretion rates, the required minimum inhibitory concentration is maintained for an adequate period of time at the appropriate site.

The dosage régime for oxytetracycline used in experiment five was based on the recommendations contained in the manufacturers' data sheets on the grounds that this would result in a therapeutic régime equivalent to that which would be used under field conditions. The highest daily dosage rate quoted, i.e. 5 mg/lb, was used and the treatment was continued for that period of time i.e. 5 days, in which some indication of resolution of disease might reasonably be expected (Smith, 1972). Whilst the minimum inhibitory concentration of oxytetracycline for the infecting strain of Bord. bronchiseptica is unknown, as is the exact concentration of oxytetracycline which would be achieved at the bronchial epithelial surface as a result of the dosage régime used in this experiment, nonetheless, the slight improvement seen in the clinical, pathological and bacteriological features of Bord. bronchiseptica in this experiment indicates that, for at least some period of time following treatment, at least the minimum inhibitory concentration was achieved at the respiratory epithelium. The failure of the treatment régime to produce more than this slight, transient improvement in the disease picture might well be the result of failure to maintain the minimal concentration for an adequate period of time. If this is so, then an increased dosage rate, possibly combined with increased frequency of treatment, might well result in successful oxytetracycline therapy of Bord. bronchiseptica infection in dogs.

The dosage régime used for amoxycillin in experiment six i.e. 10 mg/lb twice daily by mouth, was decided upon after consultation with the manufacturers (Beecham Animal Health, Middlesex) who also estimated the minimum inhibitory concentration of amoxycillin for the infecting strain of Bord. bronchiseptica to be 5 μg/ml. It has been shown that this concentration of amoxycillin i.e. 5 μg/ml is attained in the blood of dogs following oral administration at a rate of 5 mg/lb (Palmer et al., 1976); the antibiotic is, however, rapidly excreted from the body resulting in a rapid fall in blood concentration. The dosage rate used in experiment six i.e. 10 mg/lb was therefore sufficient to attain the minimum inhibitory concentration of

amoxycillin in at least the blood of the treated animals. Failure of amoxycillin in vivo might, therefore, be due either to the presence of a lower concentration of antibiotic at the respiratory epithelium than in the blood or to rapid excretion of the antibiotic resulting in a period of activity restricted to the few hours immediately following administration of the drug.

As with oxytetracycline, increased dosage rates and reduction of intervals between dosage might result in an increased efficacy of chemotherapy with amoxycillin, but additional animal experiments would be necessary to establish a valid treatment regime using either of these antibiotics.

The routes of administration of antibiotic used in the above experiments differed : the oral route of experiment six was better tolerated by the treated animals than the intramuscular route of experiment five which resulted in transient discomfort with carriage of the injected leg and gnawing of the injection site. Since effective chemotherapy of Bord. bronchiseptica infection may depend on frequent, high doses of an appropriate antibiotic, it is likely that oral administration of such antibiotics will be the method of choice.

Elucidation of an appropriate chemotherapeutic regime for cases of Bord. bronchiseptica infection in the dog will entail further experimental work. It may be that the severity of pathological changes which are likely to be present when an animal is presented for treatment will mean that elimination of Bord. bronchiseptica from the affected animal will not result in immediate recovery from disease : time will be needed for the resolution of those lesions already present. Nonetheless, elimination of Bord. bronchiseptica from an affected individual should promote more rapid recovery from disease and would, of course, eliminate the infection risk to other uninfected, in-contact animals. However, in the kennel situation in which the majority of contagious canine respiratory disease occurs, chemotherapy of a severely affected individual is unlikely to have its optimum effect unless treatment is combined with removal to a clean airspace where the treated dog will no longer be exposed to continual reinfection both from other inmates and from a contaminated environment.

CONCLUSIONS

In the introduction to this thesis, reference was made to the uncertainty which has, in the past, surrounded the role played by bacteria in contagious canine respiratory disease; the investigations described in this thesis have resolved some of this uncertainty and have provided data which, it is hoped, may lead to more effective management of naturally occurring outbreaks of respiratory disease in dogs.

The surveys which comprised Part I of this thesis established that bacterial infection of the lower respiratory tract was commonly present in naturally occurring cases of both the major clinical respiratory disease syndromes of the dog i.e. canine distemper and kennel cough. In both syndromes, bacterial infection was most often associated with the bacterium Bord. bronchiseptica; other bacterial species, E. coli, Staph. spp., Strep. spp., Pasteurella spp. and Proteus spp. were less frequently involved.

In both canine distemper and kennel cough, bacterial infection of the lower respiratory tract was generally associated with a more severe respiratory disease than was present in dogs in which the lower respiratory tract was bacteriologically sterile. It was evident that in both these disease syndromes bacteria played an important role in the progression of the naturally occurring disease. Moreover, it seemed possible that in kennel cough the bacterium Bord. bronchiseptica might play a primary aetiological role since this microorganism could be recovered in profuse, pure culture from the respiratory tract of dogs with respiratory disease in the absence, as far as could be determined, of any other known respiratory pathogen.

Further investigations, described in Part II of this thesis, confirmed that Bord. bronchiseptica could act as a primary respiratory pathogen in the dog. Experimental infection of young dogs by aerosol resulted in a clinical respiratory disease, characterised by coughing and similar to naturally occurring kennel cough, which was rapidly transmitted to in-contact animals. Pathologically, the experimental disease was characterised by rhinitis, tracheobronchitis and, in some dogs, a focal exudative pneumonia; bacteria could be localised between the cilia of respiratory epithelia by histological, immunofluorescence and ultrastructural techniques. The

disease persisted until at least 3 weeks after infection at which time Bord. bronchiseptica could still be recovered in profuse, pure culture from the tracheobronchial tree.

The attempts at immunoprophylaxis, described in Part III, established that protection from experimental canine bordetellosis could be obtained by parenteral vaccination with an aluminium hydroxide adjuvanted preparation of heat-killed bacteria; it remains to be seen whether a similar vaccine would be of value in the control of naturally occurring respiratory disease in the dog in which Bord. bronchiseptica is involved. Although the adjuvanted vaccine did produce high circulating agglutinin titres against Bord. bronchiseptica, protection from disease could not be ascribed solely to the systemic humoral immune response since dogs vaccinated with an unadjuvanted preparation developed equally high agglutinin titres but were not protected from disease. Further investigations of the mode of action of the adjuvanted vaccine should provide valuable information about those immunological mechanisms responsible for protection against Bord. bronchiseptica infection in particular and against respiratory diseases in general.

The experiments which comprised Part IV of this thesis demonstrated the difficulties which may arise in providing effective chemotherapy of bacterial infections of the respiratory tract. Despite the use of preliminary tests which confirmed the efficacy of both agents in vitro, neither oxytetracycline nor amoxycillin altered significantly the course of an experimental infection with Bord. bronchiseptica. The dosage rates and the time intervals between antibiotic treatments appear to be of critical importance in determining the success or failure of chemotherapy and further trials would be necessary to determine an optimal therapeutic regime. Nonetheless, it seems likely that successful chemotherapy of experimental or natural Bord. bronchiseptica infection in the dog will involve the use of high doses of antibiotics at frequent intervals.

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