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A search for new microorganisms in calf pneumonia by the inoculation of gnotobiotic calves

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In a detailed microbiological and pathological study of eight outbreaks of calf pneumonia, which included the inoculation of respiratory material into 18 gnotobiotic calves, a total of five viruses, four species of mycoplasma and 19 species of bacteria were identified. The only microorganism not previously associated with bovine respiratory disease was a coronavirus. The experimental disease produced in the majority of the gnotobiotic calves by the intratracheal inoculation of unpassaged respiratory material, closely resembled the natural disease. However statistical analysis of the results could not ascribe the disease seen to a single micro-organism or to a particular combination of micro-organisms. Assuming therefore that no micro-organisms were missed in the study, it was concluded that the microbiological cause of the experimental disease lay amongst the organisms already found and that one or more of three other factors, discussed in the paper, accounted for the experimental disease produced.

A LARGE number of different microorganisms: bacteria (Omar 1966, Gourlay et al 1970), mycoplasmas (Gourlay and Howard 1979) and viruses (Omar 1966, Phillip and Darbyshire 1971, Stott et al 1980), have been isolated from calves with respiratory disease. None of these organisms on their own will reliably reproduce the natural disease in experimental animals. One explanation is that important pathogens may yet be undiscovered, another that a combination of different organisms may be required.

In order to examine these possibilities a detailed microbiological and pathological study was carried out on material from eight outbreaks of naturally occurring calf pneumonia and this included passage of the material collected into gnotobiotic calves and the use of electron microscopy and organ culture.

Materials and methods

Outbreaks of disease

Eight early acute outbreaks (A-H) with a recognisable time of onset were selected. All outbreaks, with the exception of outbreak G, were sampled within 10 days of the onset of clinical signs. All outbreaks were in housed or yarded cattle, aged less than six months (Table 1).

Sampling procedure at outbreaks

Eight animals showing a range of clinical signs were sampled from each outbreak. Nasopharyngeal samples (Thomas and Stott 1975) were taken into 4 ml transport medium without antibiotics. Paired blood samples for serum were taken three weeks apart from each animal. In some outbreaks less than eight pairs were taken due to the intervening death of an animal.

Post mortem examinations were carried out within three hours of death on animals that died within seven days of the first visit. Lung wash samples were collected, by filling the tracheobronchial tree with 1000 ml phosphate buffered saline (PBS) and then inverting the lung, 300 to 400 ml of lung washings was usually recovered. Before washing, two or three lobes or parts of lobes were clamped off, for subsequent fixation and histopathological examination or freezing and fluorescent antibody (FA) staining.

Preparation and storage of samples

Cells in the lung wash samples were concentrated by centrifugation at 1000 rpm (330 *g*) for 15 minutes at 4°C and resuspended in 20 ml Eagle's basal medium (EBM) with 5 per cent heated fetal calf serum (FCS). Supernatant fluids were stored at —70°C. Concentrated lung wash cells and nasopharyngeal samples were stored in liquid nitrogen.

Histopathology

Specimens for histopathological examination were fixed in 12 per cent neutral buffered formalin before processing and embedding in paraffin wax. Sections were cut and stained by haematoxylin and eosin.

TABLE 1: Summary of the eight outbreaks of calf pneumonia investigated

Farm outbreak	Year	Number of animals in group	Number died from pneumonia (%)	Age (months)	Husbandry notes
A	1975	80	1 (1·3)	0-3	Dairy calves, housed
B	1976	28	7 (25)	1	Single suckled, yarded with dams
C	1976	106	6 (5·7)	5	Beef calves, housed
D	1976	80	2 (2·5)	3	Beef calves, housed
E	1976	180	0	2-4	Single suckled, yarded with dams
F	1977	72	0	3-4	Beef calves, housed
G	1976	52	2 (4)	1	Single pens, calf nursery
		50	0	2	Beef calves, yarded
H	1977	40	0	3	Beef calves, yarded

Immunofluorescence

Frozen lung sections were cut and stained with hyperimmune bovine sera to respiratory syncytial virus (RSV) and parainfluenza type 3 (Pi3) virus. The sera were conjugated with fluorescein isothiocyanate (FITC) (Thomas and Stott 1981).

Virus isolation

Cells from lung washings and nasopharyngeal samples were inoculated into cultures of secondary calf kidney and serially passed calf testis cells and maintained and examined as previously described (Stott et al 1980). Adenoviruses were identified by neutralisation with specific antiserum. Coronaviruses and Pi3 virus were identified by haemagglutination inhibition or specific immunofluorescence with specific antisera. Periodically coverslips of the calf testis cells were fixed in cold acetone for 10 minutes and stained by the indirect immunofluorescence test (IFT) using gnotobiotic antisera raised against RSV and bovine virus diarrhoea virus (BVDV).

Cells from lung wash samples were also cultured in 4 oz medical flat bottles at 2×10^6 cells/ml in EBM containing 0·1 per cent sodium bicarbonate, 5 per cent heated FCS, 5 per cent tryptose phosphate broth (Difco), 100 μg/ml ampicillin, 100 μg/ml kanamycin, 50 units/ml fungizone, 2·5 per cent 1 M HEPES buffer adjusted to pH 7·2 with sodium hydroxide. Every 14 days coverslips were prepared from these cultures, fixed and stained by the IFT. Where possible cells were cultured for eight weeks before being considered negative.

Tracheal organ cultures were prepared and maintained as detailed by Thomas et al (1976). They were inoculated with 0·1 ml of one of the following: the gnotobiotic calf inoculum, a pool of the nasopharyngeal samples, the lung washing sample diluted 1/10 in PBS. The medium was changed at three, seven, 10, 14 and 17 days after inoculation. Organ culture fluids were harvested on days 7, 14 and 21, tested for haemagglutinin against rat erythrocytes and inoculated into secondary calf kidney and calf testis cells for virus isolation as detailed above.

Virus detection by electron microscopy

Lung wash supernatant fluids, lung wash cells and nasopharyngeal samples were prepared by differential centrifugation followed by centrifugation through a 30 per cent (w/w) sucrose solution (Bridger et al 1978). The daily nasopharyngeal samples, taken from the gnotobiotic calves on days 0 to 10 after inoculation, were pooled, mixed using a syringe and needle, then frozen and thawed before centrifugation. Up to 350 ml of lung wash supernatant fluids were used. Lung wash cells, as available, were suspended in approximately 10 ml of distilled water, frozen and thawed once and mixed using a syringe and needle before centrifugation. Material from the original outbreaks was examined from outbreaks D and G only.

In some instances immune electron microscopy was conducted with a pool of sera from convalescent calves from the original outbreak. Fifty μl of a sample prepared for electron microscopy, as described above, was incubated with 10 μl of a 1:10 dilution of the serum pool for two hours at 37°C followed by overnight at +4°C. Immune electron microscopy was also conducted with dilutions of a convalescent antiserum to a British bovine enteric coronavirus (Bridger et al 1978).

Mycoplasma isolation

Lung wash samples were examined for mycoplasmas essentially as described by Gourlay et al (1970) by titration in glucose-serum broth (Gourlay and Leach 1970) containing ampicillin (1 mg/ml) instead of penicillin, U4 broth (Howard et al 1978) and U4 broth containing arginine (0·5 per cent) instead of urea with the pH adjusted to 7·0. Mycoplasma isolates were identified by biochemical, cultural and serological methods (Gourlay and Howard 1979).

Bacteriology

Serial 10-fold dilutions of lung wash and nasopharyngeal samples were made in PBS up to 10^{-4} and

0·1 ml of each dilution was spread onto half an ox blood agar plate. Duplicate plates were used, one incubated aerobically and the other anaerobically under hydrogen contalning 10 per cent carbon dioxide. Predominant colonies were identified by the methods of Cowan (1974).

Virus serology

Antibodies against RSV, Pi3 virus, bovine rhinoviruses 1 and 2 (RV1, RV2), BVDV, infectious bovine rhinotracheitis (IBR) virus and reoviruses 1 and 2 were titrated as detailed by Stott et al (1980). Complement fixing (CF) antibodies to chlamydia were measured by the method of Bradstreet and Taylor (1962) using a psittacosis CF antigen kindly supplied by the Public Health Laboratory, Colindale. A fourfold or greater rise in titre between paired serum samples was taken as evidence of recent infection.

Mycoplasma serology

Sera were examined for antibody to *Mycoplasma dispar* and *M bovis* by the single-radial-haemolysis (SRH) technique. The test was slightly modified from that described previously (Howard et al 1977). Bovine, instead of rabbit, erythrocytes were used. Indubiose A37 (Uniscience) was used instead of agarose and chromic chloride was used to couple antigens to erythrocytes.

Experimental calves

Friesian or Aberdeen Angus gnotobiotic calves were procured by hysterotomy and maintained as described by Dennis et al (1976). Eighteen calves, between eight and 28 days of age, were inoculated with material collected either directly from outbreaks or from experimental animals. Where outbreak material was used the inoculum comprised the nasopharyngeal samples (2 ml of each) and lung wash cells (2 ml of each sample) where available (Table 2), pooled then diluted to a final volume of 30 ml in PBS. Where experimental material was passed only lung wash material was inoculated. The inoculum was given once by both intratracheal (20 ml) and

TABLE 2: Microorganisms isolated from eight naturally occurring outbreaks of calf pneumonia

Outbreak	Number of samples examined LW	NP	Viruses (number of isolates)	Mycoplasmas†	Titre (log_{10})	Bacteria†	Titre (log_{10})
A	0	8	RSV* (1)	*M bovirhinis*	5	None	<2
				M dispar	6		
B	3	6	Pi3 (3)	*M bovirhinis*	4	None	<2
			RSV (2)	*M dispar*	5		
C	2	8	Entero (1)	*M bovirhinis*	6	*Branhamella catarrhalis*	6
			RSV	*M dispar*	8		
				Ureaplasma sp	7		
D	1	8	BVDV (1)	*M bovirhinis*	7	*Streptobacillus actinoides*	6
				M dispar	7	*Neisseria* sp	5
				Ureaplasma sp	1	*Pasteurella haemolytica*	3
E	0	8	Pi3 (3)	*M bovirhinis*	5	*Branhamella catarrhalis*	4
				M dispar	4	*Pasteurella haemolytica*	4
				Ureaplasma sp	3	*Staphylococcus* sp	4
F	0	8	Pi3 (1)	*M dispar*	4	*Lactobacillus* sp	4
				M bovis	3	*Lactobacillus* sp	3
				Ureaplasma sp	2	*Pasteurella haemolytica*	3
G	1	8	Adeno (2)	*M bovirhinis*	3	*Gemella haemolysans*	7
			Coronavirus‡			*Streptococcus pneumoniae*	7
						Staphylococcus epidermis	6
						Escherichia coli	6
						Aerococcus viridans	6
						Streptococcus faecium	6
H	0	8	Entero (1)	*M bovirhinis*	1	*Bacteroides* sp	4
			RSV (2)				

LW Lung wash
NP Nasopharyngeal swab
RSV Respiratory syncytial virus
Pi3 Parainfluenza type 3 virus
BVDV Bovine virus diarrhoea virus

* RSV isolated from a lung wash sample not used in passage experiment

† Isolations made from pool of nasopharyngeal and lung wash samples after storage in liquid nitrogen (as used to inoculate calves). Titres expressed as colour change units/ml (mycoplasmas) and colony forming units/ml^2 (bacteria)

‡ Particles detected by electron microscopy

intranasal routes (5 ml) except for calves L74 and L65 which were inoculated only intranasally.

Daily rectal temperatures were taken from five days before inoculation up to slaughter. Blood samples for routine haematology were taken into EDTA at five to six day intervals from two weeks before inoculation up to slaughter.

Nasopharyngeal samples were taken for virus isolation on days 0, 2, 4, 8, 10, 14 after inoculation and for electron microscopy on days 0 to 10 inclusive following inoculation. Serum samples were taken immediately before inoculation and at slaughter.

Calves were slaughtered 21 days after inoculation or sooner if severe clinical signs, including lateral recumbency and pyrexia, were exhibited. The extent of pneumonia at slaughter was expressed as a percentage of the dorsal surface of the lung affected and was calculated from drawings prepared at autopsy on a standard lung outline. Lung washing samples and lung tissue were taken and prepared as described above for the field specimens.

Statistical analysis

In order to investigate the relationship between the extent of pneumonic consolidation and the micro-organisms isolated calves were classified as infected or uninfected with each of RSV, Pi3 virus, corona-virus, BVDV, *Mycoplasma bovirhinis*, *M dispar*, ureaplasmas, *Streptobacillus actinoides*, *Escherichia coli* or *Pasteurella haemolytica* and infected and uninfected groups compared for each organism using a *t* test.

Calves were also grouped according to the presence or absence of a virus (RSV, Pi3), a mycoplasma (*M dispar*, ureaplasmas) and a bacterium (*S actinoides*) resulting in five non-void groups: virus plus mycoplasma plus bacteria, virus plus mycoplasma, virus plus bacteria, mycoplasma, none of these. Mean pneumonic consolidations in various pairs of these groups were then compared using *t* tests.

Results

SURVEY OF EIGHT OUTBREAKS OF CALF PNEUMONIA

Clinical findings

The animals affected, their ages and the husbandry systems used are shown in Table 1. The clinical severity of the eight outbreaks may be inferred from the mortality rate. Clinical signs included widespread coughing, a variable degree of nasal discharge and inappetance in the majority of animals. Severer signs, including dyspnoea, tachypnoea, abnormal lung sounds and fever, were observed in a smaller proportion of animals. In outbreak G, visited some three weeks after the onset of clinical disease, respiratory signs were less widespread than in the other seven outbreaks. It was reported that neonatal diarrhoea had been a problem earlier with these calves. Respiratory disease was also reported in older groups of calves on this farm, one group of which contributed to half of the paired serum samples taken.

Autopsy findings

Macroscopic. In 17 of 18 carcases examined (one carcase was not available from outbreak B), 80 to 90 per cent of the lung tissue was red, hepatised and consolidated. The cut surface of the lungs frequently had a grey, stippled appearance and purulent material exuded from the incised bronchioles especially of the anterior lobes. Abscess formation was occasionally present in these anterior lobes (outbreaks C and G). Interlobular emphysema was present in the caudal lobes of eight of the 17 lungs examined. Emphysematous bullae were not apparent. A fibrinous pleurisy was present in six of the 17 lungs examined and the bronchial and mediastinal lymph nodes were grossly enlarged in all carcases. A frothy exudate frequently occluded the tracheal lumen and was usually accompanied by gross congestion of the tracheal mucosa.

In addition, the lungs from outbreak B were excessively heavy and oedema fluid exuded on incision. The interlobular septa were very prominent and the lung tissue had grey hepatisation.

Other post mortem findings in all carcases included enlargement of the heart with petechial or ecchymotic haemorrhages in the myocardium, congestion of the liver, spleen, adrenal cortex and regional lymph nodes, accumulation of sero-sanguinous fluid in both body cavities and a fevered, deep red, occasionally cyanotic appearance to the carcase flesh.

Microscopic. In the lung tissue the microscopic changes varied depending on the location of the sample examined. In the anterior lobes a suppurative bronchopneumonia with microabscessation and occasionally fibrosis was often the dominant lesion whereas in the more caudal lobes, at the interface between the relatively normal and the pneumonic tissue an acute alveolitis and bronchiolitis was present (Fig 1).

In these more acute lesions, thickening of alveolar walls was due to infiltration by inflammatory cells and the bronchiolar and alveolar lumina were filled with a catarrhal or purulent exudate that contained alveolar macrophages, polymorphs and small round cells (Fig 2). Hyaline membranes, giant cells, oedema and intra-alveolar haemorrhage were seen in lung tissue from outbreak B. Giant cells were present in

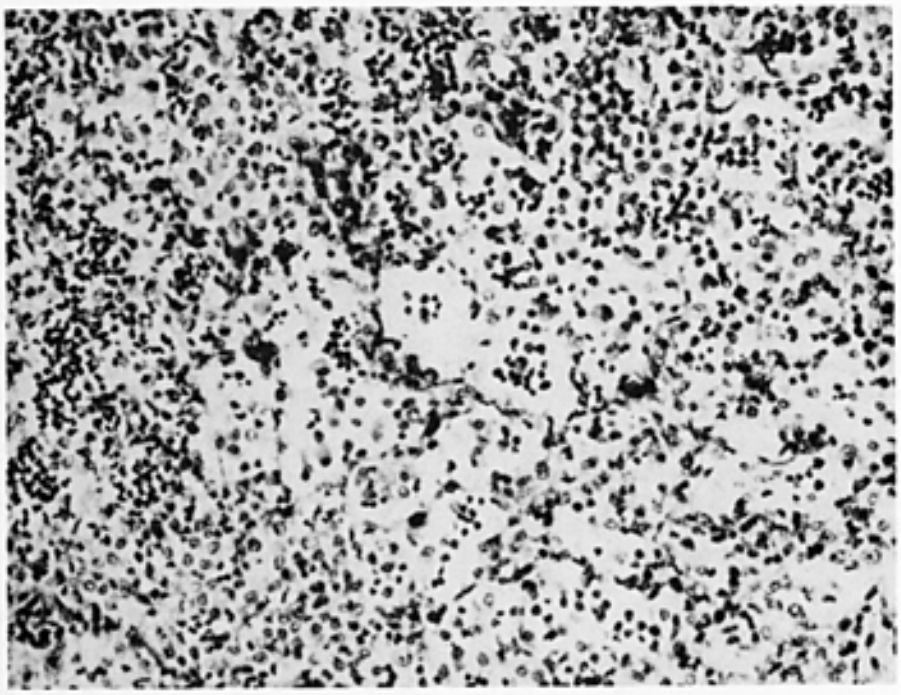


FIG 1: Typical exudative alveolitis in the lung of a calf from outbreak D. H&E × 57

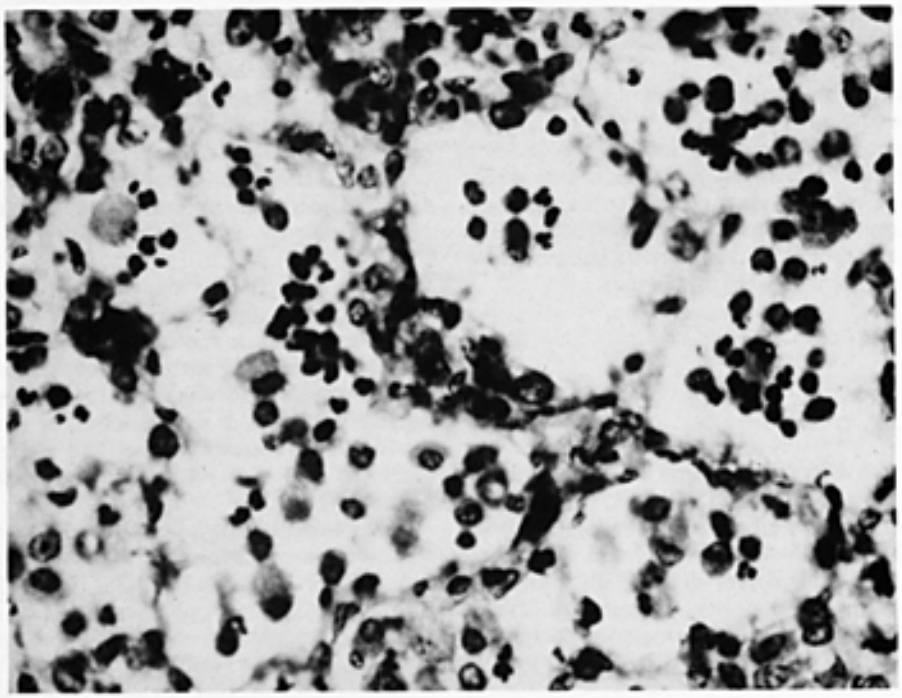


FIG 2: Detail of Fig 1 showing exudate comprising alveolar macrophages and neutrophils. H&E × 570

some but not all of the lungs in which RSV antigen was detected.

Microbiological findings

Viruses. In four of eight outbreaks (A, B, C and H) RSV was isolated from either the nasopharynx or lung material of affected animals (Table 2). In three outbreaks (B, D and G) fluorescent RSV antigen was located in the lung tissue. In outbreak D, RSV was identified in lung washings by electron microscopy and in four outbreaks (B, C, D and H) a significant serological response to RSV was found in at least half of the paired samples taken (Table 3). Thus RSV infection was identified in six (A, B, C, D, G and H) of eight outbreaks studied by one or more of the methods used.

Infection with Pi3 virus was identified in four outbreaks (B, E, F and H). The virus was isolated from three of these outbreaks (B, E and F) but significant serological responses were found only in four animals from three outbreaks. No Pi3 virus antigen was found in lung material from any of the outbreaks by immunofluorescent studies.

Infection with BVDV was demonstrated by virus isolation once only (outbreak D) and by serology in a further three animals from two outbreaks (C and E). No infection with IBR virus was found either by serology or virus isolation.

Infection with RV1 was found only by serology in seven animals from four outbreaks (A, E, F and H) and RV2 in six animals from four outbreaks (A, D, F and G). Coronavirus particles were identified in a lung wash sample from outbreak G by electron microscopy. Chlamydial infection was detected serologically in only one animal from outbreak D.

Mycoplasmas. M bovirhinis was isolated from seven, *M dispar* from six, *M bovis* from one and ureaplasmas from four of the eight outbreaks at titres as shown in Table 2. Serological responses to *M bovis* were detected in paired samples from outbreak F and to *M dispar* in samples from outbreaks A, B, C, D, E and G (Table 3).

Bacteria. Fourteen different species of bacteria were isolated from six outbreaks at titres greater than 10^2/ml of lung wash sample (Table 2). Bacteria were not isolated in significant numbers from outbreaks A and B. *P haemolytica* was the commonest isolate (three of eight outbreaks).

INOCULATION OF GNOTOBIOTIC CALVES

Clinical findings

Clinical signs observed, in the 13 calves killed between two and 21 days after inoculation (Table 4), included pyrexia (maximum 41·7°C), dyspnoea, tachypnoea, apathy, anorexia and collapse. The severity of the signs and the speed of onset are indicated by the day on which the animal was killed. The decision to kill an animal was made when collapse and lateral recumbency were exhibited. The five animals surviving to 21 days after inoculation showed only mild clinical signs within 10 days of inoculation.

White cell counts were depressed by 50 per cent or more of the preinoculation mean in calves K198, L74, K242, L65, L71 and L268. Three of these calves received material from outbreak D, the remainder from outbreaks B, C or F. No other significant changes were noted in the haematology.

TABLE 3: Serology of eight outbreaks of calf pneumonia (1975-77)

Outbreak	Number paired samples	Serology (number seroconversions) Viruses RSV	Pi3	RV1	RV2	BVDV	IBR	Reo	Mycoplasmas *M dispar*	*M bovis*
A	8	1	0	2	1	0	0	0	4	0
B	4	3	0	0	ND	0	0	0	3	0
C	8	4	0	0	0	2	ND	0	1	0
D	7	4	0	0	2	0	0	ND	1	0
E	7	0	1	3	0	1	0	ND	1	0
F	8	0	1	1	1	0	0	ND	0	4
G	8	0	0	0	2	0	0	ND	2	0
H	8	7	2	1	0	0	0	ND	0	0
Total positive		5	3	4	4	2	0	0	6	1

RSV Respiratory syncytial virus
Pi3 Parainfluenza type 3 virus
RV1 Rhinovirus 1
RV2 Rhinovirus 2
BVDV Bovine virus diarrhoea virus
IBR Infectious bovine rhinotracheitis virus
ND Not done

TABLE 4: Microbiological findings and experimental results following inoculation of gnotobiotic calves with material from eight outbreaks of calf pneumonia

Outbreak	Calf number	Inoc	Day killed	% pn con	Virus (Table 5)	Microorganisms: Mycoplasma	Titre§ $\log_{10}$	Bacteria	Titre§ $\log_{10}$
A	L224	O/B	6	18	None†	*M bovirhinis*	5	*Corynebacterium pyogenes*	4
						M dispar	5		
						Ureaplasma sp	6		
	L269	L224	13	34	None	*M bovirhinis*	5	*Streptococcus* sp	6
						M dispar	5	*Streptococcus pneumoniae*	6
						Ureaplasma sp	4	*Escherichia coli*	6
B	K169	O/B	8	30	PI3‖	*M bovirhinis*	5	*Escherichia coli*	5
					RSV	*M dispar*	6	*Staphylococcus* sp	5
	K198	K169	21	16	PI3‡	*M bovirhinis*	4		
						M dispar	5	*Streptobacillus actinoides*	5
C	K239	O/B	11	36	RSV	*M bovirhinis*	5	*Streptobacillus actinoides*	5
					CV	*M dispar*	5	*Cardiobacterium* sp	5
						Ureaplasma sp	4		
	L74	K239 +	21	9	None	*M bovirhinis*	5	*Streptobacillus actinoides*	7
						M dispar	4	*Cardiobacterium* sp	6
						Ureaplasma sp	5	*Proteus* sp‖	
	L82	K239	10	40	RSV	*M bovirhinis*	6	*Streptobacillus actinoides*	6
						M dispar	7	*Cardiobacterium* sp	4
						Ureaplasma sp	2		
D	K242	O/B	5	3	RSV	*M bovirhinis*	4	*Haemophilus haemolyticus*	5
					CV	*M dispar*	5	*Streptobacillus actinoides*	5
					BVDV	*Ureaplasma* sp	5	*Pasteurella haemolytica* (T)	3
	L65	K242 +	9	0	RSV	*M bovirhinis*	3	*Streptobacillus actinoides*	7
					CV	*M dispar*	4	*Haemophilus haemolyticus*‡	6
					BVDV	*Ureaplasma* sp	4	*Pasteurella haemolytica*	5
	L71	K242	8	37	RSV	*M bovirhinis*	5	*Haemophilus haemolyticus*	5
					CV	*M dispar*	5	*Streptobacillus actinoides*‡	
					BVDV	*Ureaplasma* sp	5	*Pasteurella haemolytica*	3

E	L179	O/B	4	32	Pi3	*M bovirhinis*	4	*Streptococcus pyogenes*	4
						M dispar	5	*Pasteurella haemolytica* (A)	4
						Ureaplasma sp	5	*Enterobacter/Hafnia* sp	2
	L230	L179	3	16	None†	*M bovirhinis*	3	*Bacteroides* sp	6
						M dispar	2	*Pasteurella haemolytica*	5
						Ureaplasma sp	2	*Streptococcus pyogenes*	4
								Streptobacillus actinoides	4
	M2	L179	3	14	Pi3	*M bovirhinis*	3	*Bacteroides* sp	7
						Ureaplasma sp	3	*Escherichia coli*‖	6
								Gram + ve coccus	6
								Streptococcus pyogenes	6
								Citrobacter freundii	5
F	L183	O/B	21	12	Adeno	*M bovis*	6	*Lactobacillus* sp	6
					BVDV	*Ureaplasma* sp	5	*Escherichia coli*	5
	L268	L183	21	4	Adeno	*M bovis*	6	*Streptococcus* sp	4
						Ureaplasma sp	4	*Escherichia coli*	3
G	L151	O/B	4	5	CV†	*M bovirhinis*‡	4	*Lactobacillus fermenti*	5
								Corynebacterium pyogenes	5
								Streptococcus faecium	4
	L154	O/B	2	1	None†	None†		*Staphylococcus epidermis*	6
								Escherichia coli	6
								Corynebacterium pyogenes	5
H	L225	O/B	21	0	RSV§	*M bovirhinis*	4	*Pasteurella multocida*	5
						M dispar	4	*Bacteroides* sp	5

pn con Pneumonic consolidation
O/B Outbreak material (Table 2)
* By intranasal route only
† Bacterial overgrowth in cultures
‡ Nasopharyngeal isolate
§ Titre expressed as colour change units per ml LW sample (mycoplasmas) or as colony forming units per ml LW sample (bacteria)
‖ Isolated from rectal swab prior to inoculation
Pi3 Parainfluenza type 3 virus
RSV Respiratory syncytial virus
CV Coronavirus
BVDV Bovine virus diarrhoea virus

Autopsy findings

Ten of the 13 calves killed between two and 21 days after inoculation had 14 to 40 per cent of lung tissue consolidated, one had also a severe fibrinous pleurisy (Table 4). The remaining three animals had less than 5 per cent of pneumonic lung and apart from some petechial haemorrhages in the heart and congestion of the adrenals, liver and intestinal blood vessels no other remarkable findings were made. Collapse in these animals was attributed to a toxaemia.

The five animals which survived to 21 days were killed, while apparently healthy, and showed between 0 and 16 per cent pneumonic consolidation.

Material from six of eight outbreaks was shown therefore to produce extensive pneumonic consolidation (at least 12 per cent) in at least one of the inoculated animals. Material from the other two outbreaks induced a severe toxaemia (outbreak G) or no lesions at all (outbreak H).

The microscopic findings in the consolidated lung tissue were of an acute exudative bronchopneumonia that closely resembled the natural disease (Figs 1 and 2).

Virological findings

By cell culture techniques, RSV was the most common isolate recovered from six of the 18 gnotobiotic calves representing four of eight outbreaks. Other viruses isolated were Pi3 (four isolates from two outbreaks), BVDV (four isolates from two outbreaks) and adenovirus (two isolates from one outbreak) (Tables 4 and 5). Bacterial overgrowth of cultures precluded virus isolation on three occasions.

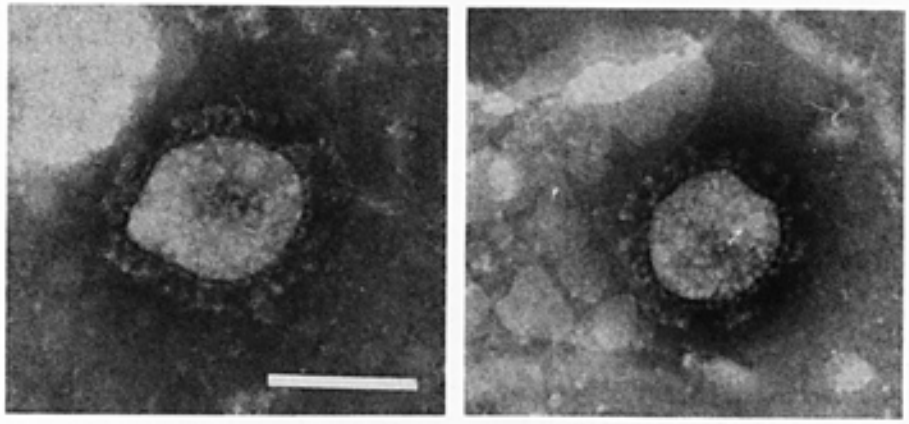
Fluorescent antibody staining of lung tissue revealed RSV or Pi3 antigen in some lungs and these correlated closely with virus isolations (Table 5). One additional infection with RSV was detected by this method (calf L82).

By electron microscopy, particles characteristic of a coronavirus, an adenovirus and a paramyxovirus

TABLE 5: Virology of gnotobiotic calves inoculated with material from eight outbreaks of calf pneumonia

Outbreak	Gnotobiotic calf	Isolated LW or NP	Sero-conversion	IF	EM LW	EM NP	Organ culture Passes	Organ culture cse	Organ culture Isolated
A	L224	–	–	–	–	–	2	–	–
	L269	–	–	–	–	–	1	–	–
B	K169	Pi3, RSV	–	RSV	–	–	2	+	Pi3
	K198	Pi3	Pi3	Pi3	–	–	1	–	–
C	K239	RSV	RSV	RSV	–	–	2	+	CV*
	L74	–	–	–	–	–	2	–	–
	L82	–	–	RSV	–	–	2	–	–
D	K242	RSV, BVDV	–	RSV	RSV CV*	CV	3	–	CV*
	L65	RSV, BVDV	–	–	CV	CV	2	–	CV
	L71	RSV, BVDV	–	RSV	CV	CV	2	–	CV
E	L179	Pi3	–	Pi3	–	–	2	+	Pi3
	L230	Cont	–	–	–	–	1	+	Cont
	M2	Pi3	–	–	–	–	1	–	–
F	L183	BVDV Adeno	–	–	–	–	1	+	–
	L268	Adeno	–	–	–	Adeno	1	–	–
G	L151	Cont	–	–	CV	CV	2	+	Cont
	L154	Cont	–	–	–	–	2	+	Cont
H	L225	RSV	RSV	RSV	–	–	2	–	–

* Antigen detected by ELISA
Cont Contaminated/bacterial overgrowth
cse Ciliastatic effect
IF Immunofluorescence detected in lung
LW Lung wash
NP Nasopharyngeal swab
EM Electron microscopy
Pi3 Parainfluenza virus type 3
RSV Respiratory syncytial virus
BVDV Bovine virus diarrhoea virus
CV Coronavirus



FIGS 3a and b: Coronavirus particles from lung wash sample of calf K242. Bar indicates 100 nm

were detected in lung wash and nasopharyngeal samples (Table 5). The latter two findings correlated with isolation of an adenovirus and RSV from calves L268 (outbreak F) and K242 (outbreak D) respectively. Coronavirus particles (Fig 3) were detected in a total of four gnotobiotic calves representing two outbreaks (D and G), their presence in outbreak D being confirmed by isolation in organ culture (see below); electron microscopy failed to detect the coronavirus found by organ culture inoculation in calf K239. Coronavirus particles, which were mainly spherical and approximately 120 nm in diameter, appeared to possess a double fringe, approximately 20 nm wide. The paramyxovirus-like particles were pleomorphic, and resembled RSV with a densely packed fringe 12 nm in length (Fig 4). Immune electron microscopy with pooled sera from the original outbreaks revealed no additional viruses but immune electron microscopy with antiserum to bovine enteric coronavirus showed that the corona-

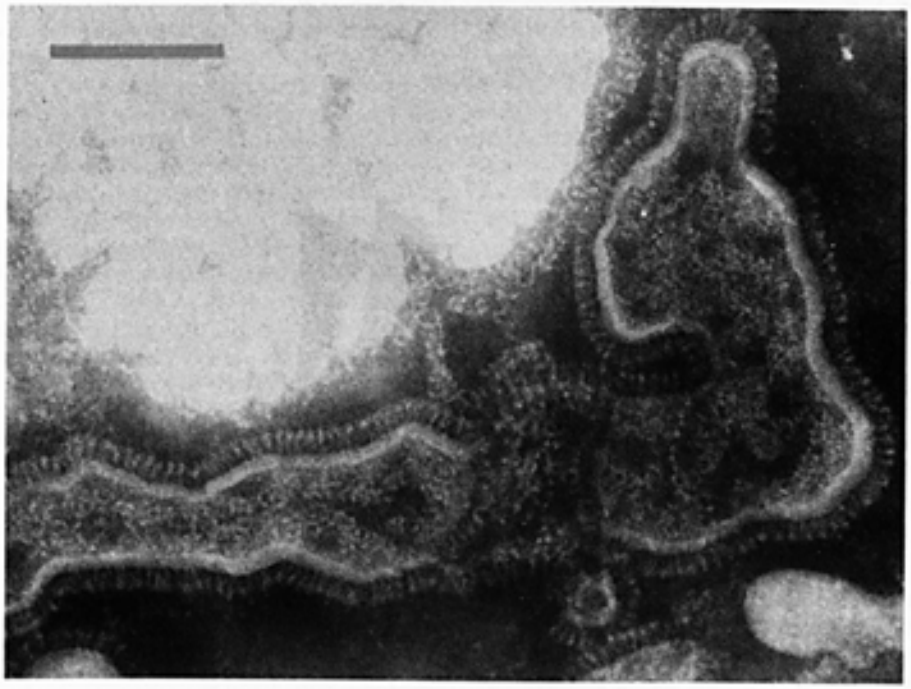


FIG 4: Respiratory syncytial virus-like particle from lung wash sample of calf K242. Bar indicates 100 nm

virus identified in outbreak D was related to the enteric virus — the respiratory virus was clumped by this antiserum and the morphology of the projections was obscured.

Passage of respiratory material (inoculum, nasopharyngeal and lung wash samples) from all 18 calves, separately, at least once in organ culture produced a ciliastatic effect on seven occasions (Table 5). Pi3 virus was subsequently isolated in tissue culture from two of these organ cultures. In three organ cultures the ciliastatic effect was attributed to contamination by fungi or bacteria, in one, a coronavirus was identified and in the seventh, no cytopathic viruses were identified. However BVDV and an adenovirus had been isolated previously from the lung wash of this calf (L183) and it was assumed that one of these, probably the adenovirus, was responsible for the ciliastatic effect. From three other organ cultures (outbreak D) a coronavirus was identified although no ciliastatic effect was observed.

Thus, with the exception of a coronavirus from K239, no additional cytopathic agents were revealed by organ culture that had not previously been detected by other methods. The presence of a coronavirus in organ cultures inoculated with K239 and K242 material and the lung wash of K242, prepared for electron microscopy, was confirmed by an ELISA for the detection of the bovine enteric coronavirus (Table 5). The finding of coronavirus-like particles in lung wash and nasopharyngeal material from L151 could not be confirmed by ELISA.

No ciliastatic effect was observed in K198 organ culture although inoculated with material known to contain a ciliastatic virus, namely Pi3, because the inoculum had been treated with a Pi3 hyperimmune serum so that other viruses might be revealed if present.

Mycoplasmological findings

M bovirhinis was the most common isolate from the experiments (15 of 18). *M dispar* (14), ureaplasmas (13) and *M bovis* (two) comprised the other mycoplasma isolates, being derived from seven, seven, five and one outbreak respectively (Table 4).

Bacteriological findings

Nineteen different species of bacteria were isolated from the 18 gnotobiotic calves; the most commonly isolated were *Streptobacillus actinoides* from nine calves (four outbreaks), *E coli* (five and four) and *P haemolytica* (five and two). The 16 other isolates were made from less than four calves from two or one different outbreak.

Microorganisms isolated and the degree of pneumonic consolidation

The mean percentage of pneumonic consolidation in calves infected with each of the more commonly isolated microorganisms (Table 4) was compared with the mean for uninfected calves. For this comparison, calf L230 was assumed to have been infected with Pi3 and L154 with *M bovirhinis* (bacterial overgrowth of cultures having precluded their probable isolation) (Table 4).

The only single microorganism shown to be significantly associated with pneumonic consolidation was *M dispar*, the means of the infected and uninfected groups being 23·6 per cent and 7·2 per cent respectively, the standard error of the difference 6·95 per cent and the significance level of the *t* test 0·03.

Calves from which all of virus, mycoplasma and bacteria were isolated had a higher percentage of pneumonic consolidation (30·4 per cent) than those from which none of these was isolated (14·7 per cent) but the significance level of the *t* test was $P = 0{\cdot}06$, so that the test was not conclusive. The standard error of the difference between the means was 6·65.

Discussion

The microbiological investigations reported here were broader and more intensive than in any previously published work. However, apart from the coronaviruses, no new microorganisms have been revealed in association with calf pneumonia. Microbiological findings from both the farm survey calves and the 18 experimentally inoculated, gnotobiotic calves revealed five different viruses, four species of mycoplasma and 19 species of bacteria. Included among which were those currently regarded by us as most significant in the aetiology of calf pneumonia, that is, RSV, Pi3 virus, *M dispar*, *Ureaplasma* sp and *P haemolytica* (Gourlay et al 1979, Thomas 1979, Stott et al 1980).

The clinical response and pathology of the experimental disease produced by material from six of the eight outbreaks closely resembled the field disease except for the absence of emphysema and pleurisy. And yet, as will now be discussed, pure cultures of the organisms do not reproduce the field disease.

M dispar, *M bovis* and *Ureaplasma* sp have been shown to produce pneumonia in gnotobiotic calves following intratracheal or endobronchial inoculation (Howard et al 1976, Gourlay et al 1976b, 1979). The pneumonia produced by these species was not the severe clinical disease observed in this experiment and so they cannot be regarded singly as the causal agents. *M bovirhinis*, although isolated from five of the six outbreaks, is recognised as being only mildly

pathogenic and is probably of little significance (Gourlay and Howard 1979).

Among the viruses isolated, a significant association between respiratory disease and infections has been demonstrated for RSV, Pi3 and BVDV (Stott et al 1980). Although claims have been made that these viruses are experimentally pathogenic (Pritchard 1963, Betts et al 1964, Dawson et al 1965, Inaba et al 1972, Smith et al 1975) few if any experiments have produced the severe clinical disease seen in the field. Furthermore the problem of excluding extraneous viruses, especially BVDV, from challenge cultures and the lack of sufficient experimental animals and controls (Thomas et al 1977) means that many of these experiments are incomplete or difficult to interpret.

Despite the use of organ cultures and electron microscopy, including immune electron microscopy, the only virus identified in this study not previously associated with bovine respiratory disease was the coronavirus. Bovine coronaviruses cause enteric disease in calves but respiratory disease has not been recorded in experimentally infected calves (Mebus et al 1973, Bridger et al 1978). The coronavirus identified here in the respiratory tract was similar to the enteric virus in that it appeared to have a double fringe, could be isolated in tracheal organ cultures and reacted with antiserum to the enteric virus. Another enteric coronavirus, transmissible gastroenteritis of pigs, is also known to be capable of replicating in the respiratory tract (Underdahl et al 1974) but further work will be necessary to establish if the respiratory and enteric bovine coronaviruses are identical and what role respiratory isolates of bovine coronavirus may play in the aetiology of calf pneumonia.

The roles of *S actinoides* and *P haemolytica*, two of the bacterial species most frequently isolated, are not clear. *S actinoides* was first isolated by Theobald Smith in 1918 and has been isolated only rarely since. Pathogenicity studies with this organism have given equivocal results (Smith 1921, Blakemore 1945, Levi and Cotchin 1950). A full description of the present isolates has been published (Gourlay et al 1982). *P haemolytica* has been isolated from pneumonic bovine lungs (Gourlay et al 1970, Bitsch et al 1976, Allan 1978) and from the blood of clinically affected animals (Thomas et al 1980) but it may also be found in relatively similar numbers in the nasal cavity of both pneumonic and non-pneumonic animals (Magwood et al 1969). Furthermore experimental work has been unable to confirm *P haemolytica* as a primary pathogen (Gale and Smith 1958, Hamdy et al 1963, Omar 1966). *E coli* is generally associated with septicaemia and enteric disease in calves (MAFF 1964, Sojka 1971) although it has been implicated in pneumonia complicating white scours (Jarrett 1956). Thus, as with the viruses and mycoplasmas discussed above, it is not possible to ascribe the severe disease in this experiment to a single species of bacterium.

Failure of pure cultures of an organism to produce a severe pneumonia may be for one of three reasons: (1) combinations of organisms are required for disease; (2) passage of the organisms, necessary for purification, causes their attenuation; and (3) material in the respiratory secretions other than the organisms identified, is required for disease (and this could include organisms undetected by the present detailed study).

Taking these points in turn: there is no evidence from our small number of outbreaks that any particular combination of microorganisms correlated statistically with pneumonia. However other workers (Heddleston et al 1962, Hamdy et al 1964) claim to have produced severe experimental disease using a combination of Pi3 virus or IBR virus and *Pasteurella* sp with superimposed environmental stressors.

Purification of individual microorganisms from crude respiratory secretions without passage in vitro is technically difficult and perhaps accounts for the little attention that has been given to the problem in the past. But it is the only way to resolve the question of whether microorganisms become attenuated when grown in vitro. In experiments using antibiotics to remove bacteria from unpassaged inocula, disease was considerably reduced indicating that the remaining unpassaged mycoplasmas were not alone capable of producing severe clinical disease (Gourlay et al 1976a). Furthermore sterile lung material does not appear to be the essential ingredient for disease production in combination with an inoculum of uncloned mycoplasmas (Howard et al 1976). Both of which observations argue in favour of the 'combinations' hypothesis at least for mycoplasmas and bacteria.

Use of untreated respiratory material, and all that it may contain, is a return to the method of Lamont and Kerr (1939) and Jennings and Glover (1952), and appears both then and now reliably to reproduce the natural disease. It indicates that the type of experimental animal and the route of inoculation are appropriate and that particular environmental stresses are not necessarily required for experimental production of the field disease.

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