STUDIES OF *CLOSTRIDIUM PERFRINGENS* TYPE A ENTERITIS IN THE PIG

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by

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PREFACE

The work described in this thesis was carried out in the Department of Veterinary Pathology, University of Glasgow from October 1982 to October 1986 under the able supervision of Dr. David John Taylor, MA. Vet.MB. PhD. MRCVS.

These studies represent original work carried out by the author, and have not been submitted in any form to any other university. Where use has been made of material provided by others, due acknowledgement has been made in the text.

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SUMMARY

Clostridium perfringens type A was isolated from 27 of 60 natural cases of diarrhoea and enteritis in pigs. In 14 cases C.perfringens type A was recovered in profuse culture and no other potential pathogens were isolated. Its presence was associated with enteritis in young piglets but accurate description of associated lesions was prevented by post-mortem change.

An isolate of C.perfringens type A was used to infect 8 day old hysterectomy-derived, colostrum-deprived (HDCD) piglets and found to produce diarrhoea and enteritis similar to that found in the field. The same isolate caused fatal infections in 5 out of 6 newborn HDCD piglets in a second experiment.

Deaths and diarrhoea could be prevented by the oral and parenteral administration of serum and the oral administration of milk and colostrum from sows immunised with a crude toxoid, a bacterin and a combination of these prepared from the challenge isolate. It was not clear from this study whether antitoxin or antibody to somatic antigens were responsible.

38 out of 42 isolates of C.perfringens type A isolated from cases of porcine enteritis were found to produce alpha toxin at levels varying from 4 haemolytic units to 256 haemolytic units. Alpha toxin was purified from the pathogenic isolate and found to produce no fluid accumulation but some inflammation in piglet gut loops. This change could be neutralised by antibody raised to the toxin. Antibody to alpha toxin was demonstrated by counterimmuno-electrophoresis in the sera of 45 out of 106 sows in a survey indicating that exposure to alpha toxin was widespread.

Spores of C.perfringens type A were detected in pig faeces and intestinal contents in 20 out of 23 cases of enteritis at levels of up to $5x10^6$/gram. Lower numbers of spores up to $2x10^4$/gram were present in 5 out of 10 samples from normal pigs. Enterotoxin was demonstrated by Vero cell assay in 5 of the 23 samples from diarrhoeic pigs but in none of the 10 from normal pigs. An

enterotoxin-producing isolate was used to prepare pure enterotoxin and antibody to it. Antibody to enterotoxin was demonstrated by counterimmunoelectrophoresis in 98 out of 106 sow sera from 4 herds.

The enterotoxin was found to produce fluid accumulation in piglet gut loops and a transient watery diarrhoea when given intraluminally to HDCD piglets. The enterotoxin-producing strain was used to infect neonatal HDCD piglets and both vegetative and sporulated cells caused a transient, non fatal diarrhoea. Sporulated cells and enterotoxin were demonstrated in their faeces. No protection was found when pig serum containing antibody to enterotoxin was given orally or parenterally.

These studies showed that both alpha toxin and enterotoxin-producing strains of C.perfringens type A of porcine origin could produce diarrhoea in neonatal HDCD piglets and that both the organisms and antibody to their toxin occurred widely in the pig population.

CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

1. INTRODUCTION

Infectious enteric disease is a major problem in the pig and outbreaks of enteric disease are a significant cause of morbidity, mortality and economic loss on pig farms. A number of microbial agents, Escherichia coli, Salmonella spp., Clostridium perfringens type C, Treponema hyodysenteriae, Transmissible Gastroenteritis virus and, Isospora suis have been considered major causes of enteric disease for many years. Recently agents such as Campylobacter spp., Rotavirus, cryptosporidia and the coronavirus of Porcine Epidemic Diarrhoea have also been incriminated.

C.perfringens type A has been largely ignored as a cause of enteritis in the pig. It has been recovered regularly from the intestinal tract of both diseased and normal animals, but few attempts have been made to correlate the presence of the bacterium with disease and it has been regarded generally as part of the normal intestinal flora. Recently C.perfringens type A has been associated with enteritis in calves, chickens, pigs and in food poisoning and some other enteric syndromes in man.

The literature on Clostridium perfringens in general and C.perfringens type A in particular is voluminous. Much of it relates to C.perfringens in animals other than the pig or to infections outside the gastrointestinal tract. The material selected for presentation in this review comprises work on the general properties of the organism, its biochemical and antigenic features and its relationship to enteric disease in the pig and was selected in 1985. More recent papers have been included, where relevant in the general discussion (Chapter 7).

The literature on the relationship between C.perfringens and the intestine has been reviewed separately with particular emphasis on the immune response of the gut to antigens and to C.perfringens in particular. This section concludes with a review of immune protection against enteric pathogens in the pig and other animals and C.perfringens in particular.

A final section gives an outline of the experimental approach adopted.

2. CLOSTRIDIUM PERFRINGENS

(a) General characteristics of the organism

Clostridium perfringens was discovered at the end of the last century. It was shown to be an important cause of gas gangrene in the First World War (Ispolatovskaya, 1971).

C.perfringens is a Gram-positive rod, about 4 x 1.5 µm, straight with parallel sides and rounded ends. Spores of C.perfringens are seldom seen in cultures grown on the usual laboratory media and their absence is one of the characteristic features of C.perfringens (Willis, 1977). When they do occur, they are oval in shape and usually subterminal in position, although terminal spores are sometimes seen (Smith, 1975a). Occasionally strains of C.perfringens are encountered which sporulate readily on ordinary media, and sporulating mutant strains have been induced by treatment with acridine orange or nitrosoguanidine (Duncan et al., 1972).

C.perfringens is not a strict anaerobe, growing readily in the presence of small amounts of oxygen. It is one of the most rapidly growing anaerobes, surface growth often being detectable after only 4-6 hours of incubation; growth in deep broth may be evident after only 2 hours (Willis, 1977). Colonial morphology is of value in identification. Colonies on the surface of blood agar after overnight incubation are 1 to 3 mm in diameter, low convex,

semi-opaque, glossy, with entire margins. On further incubation, the colonies are 3 to 8 mm in diameter, sometimes with a raised centre and a flattened periphery, or of comparatively even thickness except for the thinner periphery. Often, this periphery does not extend uniformly around the colony, but is much wider on one side than another, as if swarming were about to start, however, swarming is not observed with C.perfringens (Smith, 1975a).

Aberrant colony forms are sometimes encountered. Mucoid colonies are hemispherical, opaque, glistening, with sharp, regular margins. The organisms in mucoid colonies are encapsulated, particularly in young cultures and at the edge of the colonies. Rough cultures are 3 to 5 mm in diameter, slightly raised with a flat, rough surface, semi-translucent, with lobate margins. The cells are not encapsulated and tend to adhere to one another in the fashion of cells in rough colonies of other species. Pure cultures of C.perfringens frequently give rise to two or more colony forms (Smith, 1975a).

On horse blood agar many strains of C.perfringens produce zones of complete haemolysis due to the production of theta toxin (Willis, 1977). Partial haemolysis is frequently seen among type A strains, and absence of haemolysis is characteristic of some heat-resistant food poisoning type A strains, and of some type C strains, since they do not produce any theta toxin (Hobbs, 1965). Partial haemolysis on horse blood agar is due to the alpha toxin, which is relatively inactive against the red cells of the horse (Willis, 1977). On egg yolk agar C.perfringens produces diffuse opalescence, which is inhibited by C.perfringens anti-alpha toxin, no pearly layer is produced. In cooked meat medium growth is evident in a few hours, a fair amount of gas is produced and the meat particles are turned pink, there is no meat digestion. In ordinary milk medium rapid fermentation of the lactose occurs, with the subsequent development of a characteristic stormy clot reaction (Willis, 1977).

About three-fourths of the strains of C.perfringens have capsules demonstrable by negative staining with India ink. Some strains, produce capsules of appreciable size especially those forming mucoid colonies on nutrient agar containing 5 per cent glucose. Some strains do not seem to form capsules under any circumstances. Most strains, however, form demonstrable capsules if grown slightly above neutrality in the presence of fermentable carbohydrate and serum or milk (Smith, 1975a). Both species specific and strain-specific capsular antigens appear to exist. Cells of C.perfringens can sometimes be induced to lose the capsular antigen by cultivation in the presence of homologous antibody. When this happens the organisms become agglutinable by antisera to all C.perfringens strains, indicating that a somatic antigen underlying the capsule is common to all strains (Smith, 1975a).

C.perfringens ferments fructose, galactose, glucose, inositol, lactose, maltose, mannose, starch, and sucrose. A minority of the strains will ferment cellobiose, glycerol, insulin, raffinose, or salicin. It does not ferment adonitol, amygdalin, arabinose, cellulose, dulcitol, erythritol, esculin, mannitol, melezitose, meliziose, rhammose, ribose, sorbitol, sorbose, trehalose, or xylose. The fermentation products include acetic and butyric acids, with or without butanol (Willis, 1977).

Gelatin is hydrolysed, sometimes slowly. A few strains will hydrolyse casein. Urease is produced by some strains, particularly those of type B and E (Smith, 1975a). Type C strains isolated from animals do not produce this enzyme but two-thirds of the strains isolated from enteritis necroticans in man do so (Prevot et al., 1967). Nitrate is reduced by almost all strains, although this characteristic is dependent upon the medium that is used. Hydrogen sulphide and indol are not produced. Acetyl-methyl-carbinol is rarely produced (Smith, 1975a).

The optimum temperature for rapid growth of strains of types A, D and E is about 45°C, this is not, however, the optimum

temperature for the production of the various enzymes of this species, in every case the optimum temperature for the production of the enzymes is lower (Smith, 1972). The pH range over which growth most readily takes place is from 5.5 to 8.0 and the optimum pH value for maximum growth is about the same for all strains, being about 6.5 (Smith, 1972).

The highest redox potential at which growth of C.perfringens will take place is about +200 millivolts in a medium at pH 7.0. After the organism starts growing, the redox potential will drop sharply to a value near that of the hydrogen electrode, about -400 millivolts at pH 7.0 (Tabatabai and Walker, 1970).

C.perfringens produces at least 12 different toxins that may be involved in pathogenesis (McDonel, 1980; Table 1). Whether or not all these toxins are directly involved in the production of lesions or contribute to the pathogenesis of infections in animals or man is unclear (McDonel, 1980). At least eight of the toxins are believed to be lethal. The role in pathogenesis of eta, gamma, kappa, lambda, mu and nu toxins is uncertain (McDonel, 1980). Four of the lethal toxins, alpha, beta, epsilon and iota, are considered to be the major toxins and are used to group the species into five toxigenic types of A, B, C, D and E. Type A strains produce predominantly alpha toxin; type B, beta and epsilon toxins; type C, beta and delta toxins; type D, epsilon toxin; and type E, iota toxin (Stern and Warrack, 1964). A strain once classed as type F (Zeissler and Rossfeld-Sternberg, 1949) has been abandoned because of its essential similarity to type C (Stern and Warrack, 1964). Typing of the organism is accomplished with type-specific antisera. Type A antiserum neutralises only type A strains. Type B antiserum neutralises types A, B, C and D. Type C antiserum neutralises types A and C. Type D antiserum neutralises types A and D and type E antiserum neutralises types A and E (Carter, 1979). Treatment with trypsin usually inactivates, or reduces the potency of alpha and beta toxins, consequently, culture fluids of type C strains are often without toxic activity after trypsin treatment. Type B strains often yield culture filtrates that

TABLE 1

Toxins produced by the five different types of C.perfringens (McDonel, 1980)

Type	Major toxins				Minor toxins								Other toxins	
	alpha	beta	epsilon	iota	delta	theta	kappa	lambda	mu	nu	eta	gamma	entero-toxin	neura-minidase
A	+	-	-	-	-	+	+	-	+	+	?	-	+	+
B	+	+	(+)	-	+	+	+	+	+	+	-	?	-	+
C	+	+	-	-	+	+	+	-	+	+	-	?	+	+
D	+	-	(+)	-	-	+	+	+	+	+	-	-	+	+
E	+	-	-	(+)	-	+	+	+	-	+	-	-	-	+

\+ = produced by some strains of the type given. Quantities of toxin produced by different strains can vary.

\- = not known to be produced by any strains of the type given.

(+) = prototoxin, activation requires enzymes.

? = existence doubtful.

before trypsin treatment are neutralisable only by type B antiserum because they contain both beta and epsilon toxin; however, after trypsin treatment only epsilon toxin is left in active form and the culture fluids can then be neutralised by both types B and D antisera. Strains of B and D that are poor producers of epsilon toxin may require trypsin treatment before epsilon toxin can be demonstrated (Smith, 1975a).

(b) Distribution of the organism

C.perfringens is more widely spread than any other pathogenic bacterium (Prevot et al., 1967). Its principal habitats are the soil and the intestinal contents of man and animals. Only type A strains are found as part of the microflora of both soil and intestinal tracts; B, C, D and E strains seem to be obligate parasites, mostly of animals, although they may occasionally be found in man (Smith, 1975a).

Almost every sample of soil that has ever been examined, has been found to contain C.perfringens (Prevot et al., 1967). The number of cells of this organism vary considerably from one specimen to another, but any fertile soil can be expected to contain several thousand per gram, and may contain as many as fifty thousand per gram (Smith, 1975a). The existence of these strains of C.perfringens in the soil primarily as vegetative cells indicates that they are actively multiplying there and that the soil is a true habitat (Prevot et al., 1967). If cultures of C.perfringens types B, C, D and E are added to soil, they die within a few months, apparently being unable to compete with the type A strains that are native to soil (Smith, 1975a). It is reported (Beerens and Delcourte, 1958) that soil strains of type A differ from intestinal strains of type A in being more resistant to the toxic effect of sodium chloride.

C.perfringens has been found, in varying numbers, in the intestinal contents of almost every animal that has ever been investigated (Prevot et al., 1967). C.perfringens forms a part of

the normal intestinal flora of man and animals (Niilo, 1980) but is also associated with a number of disease syndromes in both man and animals. These may be extra intestinal e.g. Gas Gangrene in man and other species associated with C.perfringens type A or intestinal in origin (the enterotoxaemias) with clinical signs and lesions in a number of different organs systems as a result of C.perfringens infection in the gut of non-immune animals. These diseases are associated with the individual types of C.perfringens shown in Table 2.

(c) Clostridium perfringens in pigs

(i) Clostridium perfringens type A

C.perfringens type A is part of the normal intestinal flora of the pig, reaching levels of 10^6 cells per gram of faeces within 72 hours of birth and subsequently declining to levels of 10^3 cells per gram by 2 weeks of age (Mansson and Smith, 1962).

Both extra-intestinal infections and intestinal disease have been associated with C.perfringens type A. C.perfringens type A has been involved in cases of gas gangrene in pigs. Jaartsveld et al. (1962) reported the occurrence of C.perfringens infection in two herds following intramuscular injection of an iron preparation. Twelve of 25 piglets in one herd died hours after the injection. It was suggested that alpha toxin was responsible for the production of the lesions (Jaartsveld et al., 1962). When Mansson and Smith (1962) placed 8 week-old pigs on a diet high in protein, there was an increase in the number of C.perfringens type A from about 250 per gram of faeces on a normal diet to about 1,000,000 per gram in pigs on the high-protein diet. Another study (Mansson and Olhagen, 1967) demonstrated that when pigs were fed on a high-protein diet, the number of C.perfringens type A was dramatically increased in the faeces. In association with this increase, the animals showed skin lesions typical of parakeratosis and developed an arthritis involving mainly the phalangeal joints. They related the development of these joint and skin lesions to a

TABLE 2

The association of C.perfringens types with enteric disease

	Disease
Type A	Enterotoxin food poisoning and neonatal necrotic enteritis. Enteritis in cattle, chickens, dogs, goats, horses, rabbits, monkeys, pigs and necrotic enteritis in sheep.
Type B	Lamb dysentery, enterotoxaemia in goats and guinea pigs.
Type C	'Pig Bel' in man, 'Struck' in sheep, enterotoxaemia in cattle, pigs and chickens.
Type D	Pulpy kidney in sheep, enterotoxaemia in goats, cattle and guinea pigs.
Type E	Enterotoxaemia in sheep, calves, guinea pigs and rabbits.

rise in serum antibody to alpha toxin. On histological examination the lesions were found to consist of a highly vascularised granulation tissue. In the synovial membrane of the phalangeal joints, there was a slight accumulation of mononuclear cells. There was also distinct villous hypertrophy with a proliferation of the vascular tissue in particular. Bacteriological examinations of the synovia were negative. Mansson et al. (1971) later confirmed these results and in addition demonstrated a delayed hypersensitivity against intestinal C.perfringens type A in the experimental animals. Control pigs were negative but the hypersensitivity could be transferred to them by lymphocytes from an experimental pig. It was suggested that circulating soluble alpha toxin-anti alpha toxin complexes formed in antigen excess were responsible for the lesions produced (Mansson et al., 1971).

C.perfringens type A has been incriminated in enteric lesions in pigs from which no other pathogen could be demonstrated (Amtsberg et al., 1976). Another study (Ramisse et al., 1979) reported the isolation of C.perfringens type A from newborn piglets which died of diarrhoea. C.perfringens type A enteric disease was experimentally reproduced by Olubunmi (1982) in hysterectomy-derived, colostrum-deprived piglets. Profuse diarrhoea containing flecks of blood was noted in the inoculated piglets from the second day following infection. Two out of three inoculated animals died within 3 days of inoculation. Conventional weaned pigs were infected with the organism (Olubunmi, 1982). Loose faeces with varying amounts of mucus and blood were noticed. Poor bodily condition developed in the inoculated animals. The disease produced in this study was not fatal. Gross pathological changes attributable to C.perfringens type A infection were demonstrated in both experiments, the mucosa of the small intestine, particularly that of the jejunum was found to contain congestion and necrotic areas and villous atrophy was seen in all cases. Histological changes were found to be more severe in the hysterectomy-derived colostrum-deprived piglets than in the conventional weaned pigs. In the former animals the lesions included congestion, destruction of the villi and necrosis at small intestinal level. Epithelial

cell shedding and presence of inflammatory and red cells in the lumen was recorded. Necrosis of the epithelium and large areas of congestion were present in the large intestine. In conventional weaned pigs, histological changes were restricted to the small intestine with a mild congestion, oedema of the mucosa and villous atrophy. C.perfringens type A was recovered from the mucosa of the duodenum, jejunum and ileum, less frequently from stomach, caecum and colon. It was concluded that C.perfringens type A was capable of producing an enteric disease in inoculated pigs (Olubunmi, 1982). Another study (Nabuurs et al., 1983) on the aetiology of diarrhoea in piglets of one to three weeks of age revealed high numbers of C.perfringens type A in intestinal contents of severely affected animals. Experimental infections with hysterectomy-derived, colostrum-deprived piglets resulted in a clinical picture indistinguishable from the clinical signs observed under field conditions. The clinical signs were creamy diarrhoea, emaciation, abundant gas in the gut. Mortality was low. The predominant post-mortem findings were the presence of gas in the intestinal lumen, and superficial necrosis and villus atrophy in the mucosa.

A study in neonatal diarrhoea of piglets (Secasiu, 1984) associated an increase of the number of C.perfringens type A in intestinal contents of diseased piglets with the onset of diarrhoea. Most of the isolates were found to be strong alpha toxin producers and when tested in rabbit intestinal loops, 39 out of 178 were found to be toxigenic.

More recently, studies of enterotoxin have been carried out in pigs. Purified enterotoxin from a C.perfringens type A reference strain was found to induce fluid accumulation in the ileal loops of hysterectomy-derived piglets (Popoff and Jestin, 1985) suggesting that enterotoxin could be involved in diarrhoeas occurring naturally in young pigs. Jestin et al. (1985) reported a diarrhoeic syndrome in fattening pigs associated with C.perfringens type A enterotoxin in their faeces. A correlation was observed between the intensity of the diarrhoea and the level of C.perfringens type A enterotoxin in faeces. It was concluded that

C.perfringens type A enterotoxin was an important contributing factor of diarrhoea in fattening pigs.

(ii) Clostridium perfringens type C

C.perfringens type C is recognised as the cause of a highly fatal necrotic enteritis of young pigs. Young piglets less than one week of age are usually affected although sucking pigs up to a month old are occasionally affected (Bergeland, 1981). C.perfringens type C has been shown to attach to and invade the epithelium of the small intestinal villi, and to proliferate along the basement membrane. Attachment first occurs at the apices of jejunal villi (Arbuckle, 1972). This is accompanied by desquamation of the epithelium and complete necrosis of the lamina propria of the villi. In peracute cases, massive haemorrhage accompanies the necrosis. The majority of the bacteria remain attached to the necrotic villi and the villi, together with the adherent bacteria, are sloughed into the intestinal lumen (Bergeland, 1981). Beta toxin produced by the organism in the intestine is probably responsible for the inflammation of the intestine and the whole loss of mucosa (Meszaros et al., 1967). The disease may be associated with the inhibition of the proteolytic action of trypsin in the small intestine of neonates by a factor in the colostrum. Both trypsin and chymotrypsin have a notable inactivating effect on beta toxin of C.perfringens type C. The disease seems to be associated with low tryptic activity in the small intestine (Meszaros et al., 1967). It has been reported (Bergeland, 1972) that characteristic intestinal necrosis appears to result from active invasion of the mucosa by type C organisms rather than simply from the presence of toxin in the intestinal lumen. Intestinal necrosis was not observed in ligated intestinal loops injected with bacteria-free toxin, whereas focal areas of bacterial invasion and necrosis were seen in loops injected with whole broth culture of C.perfringens type C (Bergeland, 1972). Toxaemia may be a contributing factor in peracute cases in which the pigs collapse and die suddenly. Beta toxin is present in high concentration in the haemorrhagic intestinal content as well as the

peritoneal fluid of some of these pigs, and it is probable that some toxin is absorbed. Experimental intravenous infusion of type C toxin in high doses causes sudden death in young pigs (Bergeland, 1981). The mortality rate of pigs with clinical signs is usually very high, and complete recovery is rare. In the peracute form, sudden death occurs in piglets with little or no evidence of diarrhoea; others may have haemorrhagic diarrhoea and there may be perineal staining with bloody fluid, in these cases death usually occurs on the first or second day after birth. In the acute syndrome, affected animals may survive for two days after the onset of clinical signs, and commonly die within three days; throughout the course of the disease they have reddish brown liquid faeces that contain shreds of grey necrotic debris. In the subacute form, the affected animals have a persistent, non-haemorrhagic diarrhoea and usually die when 5-7 days old, their faeces tend to be soft and yellow at first and then change to a clear liquid containing flecks of grey necrotic debris. Chronic cases may have an intermittent or persistent diarrhoea for one or more weeks, faeces are yellowish-grey and mucoid, and the perineum and the tail may be coated with dried faeces. These pigs may eventually die after several weeks and the survivors fail to gain weight (Bergeland, 1981). The most consistent pathological feature in infected pigs is varying degrees of necrosis and desquamation of the epithelial layer of the mucous membrane of the jejunum, and sometimes of the ileum. In peracute cases, the jejunum is dark red and the lumen is filled with blood-stained fluid; the rest of the small and large intestine also contains bloody fluid. Microscopically, the villi in the jejunum are necrotic and are covered by large bacilli; the epithelium of the crypts may be necrotic. Profuse haemorrhages are present in the mucosal and submucosal layers. The jejunum may be emphysematous and there may be fibrinous peritonitis. Most of the villi are sloughed, leaving a necrotic membrane overlying the submucosa. Submucosal vessels are necrotic and may contain thrombi. The affected portion of the jejunum and ileum in piglets with subacute disease is thickened with a tightly adherent necrotic membrane in place of the mucosa. The intestine of pigs with chronic disease may appear normal from the serosal surface. Microscopically, there

is replacement of the mucosa by a necrotic membrane, with numerous bacteria on its underside. The submucosa and tunica muscularis are infiltrated by chronic inflammatory cells (Bergeland, 1981).

(d) Pathogenic mechanisms in Clostridium perfringens

The above review of the literature about clostridial infections in pigs has associated the adhesion of C.perfringens type C to the villi and the production of toxins to the damage caused. The mechanisms by which adhesion to epithelial cells occur have not been studied, it has merely been reported to occur in both human (Walker et al., 1980) and porcine (Arbuckle, 1972) infections with C.perfringens type C. In view of this scarcity of information this section of the review deals almost entirely with the exotoxins of C.perfringens.

(i) Alpha toxin

Alpha toxin (Lecithinase, Phospholipase C) is produced by all five types of C.perfringens, however type A strains usually produce the largest quantities (Ispolatovskaya, 1971). The majority of type A strains of C.perfringens produce amounts of alpha toxin detectable on horse blood agar or egg yolk agar but some isolates do not appear to do so. These isolates have the biochemical characters of C.perfringens but do not produce other toxins and were considered by Ispolatovskaya (1971) to be C.perfringens type A. The production of alpha toxin also varies considerably from one medium to another; alpha toxin production and the amount of growth are not necessarily correlated (Smith, 1975a). A study (Mollby et al., 1976) showed that the yields of alpha toxin produced by type A strains isolated from cases of gas gangrene and abdominal wounds were indistinguishable from those isolated from faecal samples from healthy persons. It was found in another study (Forget et al., 1969) that toxicity for mice and yield of alpha toxin production in culture supernatant fluids correlated with virulence of C.perfringens type A strains.

The genetic control of alpha toxin production is virtually unknown; one reported attempt to link its production to a plasmid was unsuccessful (Kramer and Schallehn, 1978).

The composition of the nutrient medium strongly influences growth and alpha toxin production of C.perfringens A. It has been shown that the cultivation of the organism on medium containing pieces of meat or meat extract promoted production of alpha toxin but not theta toxin (Takahashi et al., 1981).

Yields of alpha toxin production are influenced by the presence of aminoacids, carbohydrates, salts, vitamins and metal ions in the culture medium (Ispolatovskaya, 1971).

Production of alpha toxin is possible in most synthetic media only if peptides are present. However, a synthetic medium was devised (Murata et al., 1968) that supported some alpha toxin production, even though this was considerably less than that produced in a medium containing peptone. It has been shown (Ispolatovskaya, 1971) that cystine, tyrosine, glycine and arginine accelerate the growth of C.perfringens, but that alpha toxin production was stimulated by arginine exclusively and was further increased by histidine and tryptophan.

Nakamura et al. (1968) did not find alpha toxin produced in detectable amounts using a synthetic medium containing amino acids as a nitrogen source. The addition of some peptides, such as glycyl-DL-valine, glycyl-DL-norvaline, or D-leucyl glycyl glycine resulted in the production of alpha toxin. The addition of corresponding amounts of the individual acids composing these peptides did not result in alpha toxin production, nor did the addition of a number of other peptides. The importance of peptides for the formation of alpha toxin was also indicated in a study (Nekvasilova et al., 1970) which demonstrated that the substances which stimulated most active alpha toxin production in casein digest were tyrosine-containing peptides of low molecular weight. Starch, dextrin or fructose are the carbohydrates which produce the greatest increases in the yield of the toxin (Smith, 1975a).

The addition of a combination of vitamins, zinc and magnesium to the medium stimulates alpha toxin production (Ispolatovskaya, 1971). Murata et al. (1965) reported the production of alpha toxin by C.perfringens on a synthetic medium consisting of 19 amino acids, vitamins, and trace elements. Fructose was used as a carbohydrate source in this medium. It was observed that toxin production increased by the addition to the nutrient medium of arginine and was decreased in the presence of cystine. Zinc and magnesium had no influence on the growth of the bacteria but increased toxin production. An excess of manganese or of iron had a suppressive effect on toxin production. It was reported (Sato et al., 1978) that C.perfringens type A produces several distinct proteases that destroy alpha toxin produced in a zinc deficient medium. Alpha toxin without zinc is highly sensitive to proteases but resistant when combined with zinc.

The production of toxin components of C.perfringens depends upon the conditions of cultivation of the bacteria and the constituents of the nutrient medium (Ispolatovskaya, 1971). Quantitatively, alpha toxin production by C.perfringens is dependent upon the strain, the medium, the culture conditions, pH, temperature of incubation, and the length of incubation (Nakamura et al., 1969).

Roberts (1957) showed that there was less alpha toxin produced when the cultures were incubated at 43°C compared to 37°C.

It was reported (Weiss and Strong, 1967) that the alpha toxin activity of some strains decreased rapidly after reaching a maximum at 4-6 hours of incubation. However, Nakamura et al. (1969) reported that alpha toxin activity in older cultures (30-60 hours) was greater than the lecithinase activity in young actively growing cultures. It is possible that the composition of the medium used for the growth and production of alpha toxin plays an important role in the stability of the lecithinase after it is produced; in culture media deficient in nutrients alpha toxin may be broken down and metabolized (Ispolatovskaya, 1971).

Alpha toxin is readily inactivated by acid pH, being more resistant to alkaline pH values (Nakamura et al., 1969). The optimum pH of the culture medium for alpha toxin production has been reported to be between 7.6- 7.8 (Takahashi et al., 1981).

The alpha toxin activity of culture fluids may be assayed in several ways. The ability of the culture fluid to cause an increase in the turbidity of an egg yolk suspension may be used; the products of the reaction between the enzyme and lecithin can be titrated (Hanahan and Vercamer, 1954). Agar diffusion procedures can be used with egg yolk or blood agar as indicator, the toxin being placed either in holes cut in the agar with a cork-borer or on paper discs (Holdeman and Moore, 1973). The haemolytic activity can be titrated, or intraperitoneal inoculation of mice or intracutaneous injection of guinea pigs may be used to determine the action of the toxin in vivo (Smith, 1975a). The amount of alpha toxin antigen may be determined, rather than the enzyme or toxic activity, by combining tests with antiserum whose antitoxin value in international units is known, using egg yolk suspension or red blood cell suspensions as indicator of unneutralised alpha toxin (Smith, 1975a).

Alpha toxin has been demonstrated to have phospholipase C, lecithinase, lethal, necrotizing, haemolytic and cytolytic activities (Willis, 1977). It was the first bacterial toxin to be identified as an enzyme (a phospholipase C that catalyzes the hydrolysis of the phosphodiester bond in position 3 of the lecithin molecule to produce phosphorylcholine and water-insoluble 1, 2-diacylglyceride) (MacFarlane and Knight, 1941).

The molecular weight of alpha toxin has been variously determined as 106,000 (Meduski and Volkova, 1958); 31,000 (Bernheimer and Grushoff, 1967); 51,200 (Shemanova et al., 1968); 90,000 (Casu et al., 1971); 43,000 (Yamakawa and Ohsaka, 1977). The most reproducible value appears to be about 30,000 obtained by gel filtration chromatography (Mollby, 1978). The isoelectric point of alpha toxin has been reported to be at 5.0 (Bangham and

Dawson, 1961); 5.2 (Ispolatovskaya, 1971; 4.75 (Bird et al., 1974); 5.6 (Smyth and Arbuthnott, 1974). Using the technique of isoelectric focusing (Sugahara and Ohsaka, 1970) showed the existence of two forms of alpha toxin differing in charge with isoelectric points of 5.2 and 5.5 and 5.3 and 5.6 respectively.

Variations in published data on alpha toxin may result from the fact that many authors have used preparations of the enzyme from commercial or other sources with insufficient or no regard for the fact that these preparations may contain as many as 10 contaminating active substances. These may include theta haemolysin, mu and nu toxins, protease, alpha-glucosidase, N-acetyl-glucosamine, sulphatase, and β-glucuronidase (Mollby et al., 1973). Several of these contaminating substances have marked activity with respect to biological membranes (Smyth et al., 1975).

Alpha toxin is resistant to inactivation by heat. It can retain 45 per cent of its activity when exposed to 100°C (MacFarlane and Knight, 1941). It has been found (Smith and Gardner, 1950) that alpha toxin is greatly inactivated at 65°C, but can be partially reactivated by further heating to 100°C. It was suggested (Smith and Gardner, 1950) that enzymatically inactive complexes linked by calcium ions were formed at 65°C, however, at 100°C these complexes dissociated with the liberation of active toxin.

The activity of alpha toxin is calcium dependent (Bangham and Dawson, 1961), although the role of calcium appears to be limited to its effect on the substrate, not the enzyme (Dawson et al., 1976). Divalent cations and positively charged detergents are believed to activate the phospholipase action by giving the substrate a positive charge which optimizes attachment of the negatively charged enzyme (Dawson et al., 1976).

Alpha toxin is markedly haemolytic, although there is considerable variation from one species to another in erythrocyte susceptibility. Erythrocytes of cattle and mice seem most

susceptible, those of rabbit, sheep and men moderately susceptible, while those of horses and goats are comparatively resistant (Smith, 1975a). Apparently, either the type of phospholipid, lecithin, sphingomyelin, or other, or its position in the cell membrane determines the susceptibility or resistance of erythrocytes to lysis by alpha toxin (Smith, 1975a). The damage to the cell membrane may result directly in haemoglobin release but other factors may be involved. After exposure to low concentrations of alpha toxin at 37°C erythrocytes of many species of animals do not undergo lysis until they have been cooled to 4°C, a phenomenon called hot-cold haemolysis (Smith, 1975a). It was reported (Mollby, 1978) that only a slight hot-cold haemolytic phenomenon is detectable using highly purified alpha toxin from C.perfringens.

Aside from its effects upon red cells, alpha toxin also lyses platelets and leucocytes (Mihancea et al., 1970), stimulates histamine release from cells in vivo and in vitro (Strandberg et al., 1974) and damages membranes in fibroblasts (Mollby et al., 1974) and intact muscle cells (Boethius et al., 1973). Furthermore, it causes aggregation of platelets in vitro and in vivo (Ohsaka et al., 1978); this last study demonstrates that within one minute of topical application of alpha toxin to rat mesentery, pavementation of leucocytes occurred in venules but not arterioles. Thrombi then formed in venules, capillaries and eventually, in arterioles. This study concluded that thrombosis must be involved as an early important step in the pathogenesis of necrosis caused by alpha toxin and that these alpha toxin induced thrombi may be one of the factors involved in the causation of toxaemia often manifested in the late stage of gas gangrene (Ohsaka et al., 1978). It has also been shown (Ohsaka et al., 1978) that alpha toxin causes an increase in vascular permeability in guinea-pig skin due possibly to release of some mediators from aggregating platelets. Wilkinson (1975) demonstrated that the locomotor response of human monocytes is inhibited by the toxin.

The systemic effects of alpha toxin are affected by the route of administration (Smith, 1975a). When injected intravenously a 5

to 20 per cent drop in the red cell count occurs within a few hours followed shortly thereafter by death. Other effects are destruction of platelets, a drop in clotting time, and widespread capillary damage (Smith, 1975a). Under experimental conditions 90 per cent of the toxin injected disappears from circulating blood within 5 minutes of injection (Ellner, 1961). The toxin that disappears from the blood appears in the following organs: liver, 72 per cent; lungs, 15 per cent; kidneys, 8 per cent; and spleen, 5 per cent. Toxin does not appear to bind to skeletal muscle. There is little or no intravascular haemolysis after intramuscular administration of alpha toxin, and there are no systemic toxic effects unless massive doses are administered (Smith, 1979).

A disease in which alpha toxin alone appears to be the lethal factor is the post-abortion septicaemia caused by C.perfringens that occasionaly follows induced abortion (Willis, 1969). The organisms are introduced by unsterile instruments, and fragments of blood clot and necrotic tissue of foetal, placental, or maternal origin provide conditions suitable for the initiation of the infection. This type of septicaemia is usually marked by massive haemolysis with haemoglobinemia, haemoglobinuria, leukopenia, and renal involvement, all the result of the action of alpha toxin on the lipids of the cell membrane (Willis, 1969).

Rheumatoid arthritis and other collagen diseases have been associated with alpha toxin of C.perfringens (Olhagen and Mansson, 1968). Patients suffering from these diseases may show a higher C.perfringens faecal count than normal, as well as a higher than normal amount of antibody to C.perfringens alpha toxin in the serum (Olhagen and Mansson, 1968). Not all investigators have been able to demonstrate this association between increased levels of C.perfringens in the faeces of patients with rheumatoid arthritis (Sapico et al., 1973). Necrotic enteritis of chickens was reproduced by feeding C.perfringens type A (Al-Sheikhly and Truscott, 1977) and they considered that it was caused in large part by alpha toxin from C.perfringens type A. This was partly confirmed by Niilo (1978) who associated necrotic enteritis with

the isolation of strains of C.perfringens type A which were strong alpha toxin-producers. A different view was held by Smith (1975b) who reported the isolation of C.perfringens type C from necrotic enteritis but did not reproduce the disease.

Alpha toxin, purified and in concentrated culture supernatant fraction, failed to produce a response when inoculated in rabbit ileal loops (Duncan et al., 1968).

Inoculation of large amounts of alpha toxin in intestinal loops in lambs caused accumulation of fluid (Hauschild et al., 1968), however, the authors concluded that despite the demonstrated effect of alpha toxin on the sheep intestine, the toxin had no major role in C.perfringens type A enteritis in lambs.

Intravenous injections of crude alpha toxin in calves were fatal if large amounts were given in a short time; when slowly administered, the toxin was even less effective, probably due to its rapid catabolism and excretion (Niilo et al., 1963). The association between C.perfringens type A alpha toxin, high levels of dietary protein, arthritis and parakeratosis described by Mansson and Olhagen (1967) and Mansson et al. (1971) has already been reported above. In addition they reported an association between alpha toxin and proliferative glomerulonephritis and considered that all the changes were due to delayed hypersensitivity to alpha toxin, a somewhat surprising conclusion.

(ii) Beta toxin

Beta toxin is produced exclusively by C.perfringens types B and C (Sakurai and Duncan, 1978), which are the organisms most likely responsible for necrotic enteritis in man and animals.

Beta toxin can be demonstrated in the laboratory by growing a strain of type B or type C in cooked meat medium containing 2 per cent peptone, 0.5 per cent sodium phosphate, and 1 per cent glucose, adjusted to pH 7.2. The culture should be harvested at

the end of the log phase of growth, usually 4 to 5 hours after inoculation. If cultures are allowed to incubate overnight, the yield of beta toxin may be much reduced. Toxin synthesis is not related to growth yield of the organism (Sakurai and Duncan, 1979).

Sakurai and Duncan (1978) reported the purification of beta toxin from type C organisms by ammonium sulphate fractionation, gel filtration through sephadex G-100, isoelectric focusing on a pH 3 to 6 gradient, and immunoaffinity chromatography. The resultant purified toxin gave a single band with polyacrylamide gel electrophoresis. This study reported that the toxin appears to be a single polypeptide chain protein with a molecular weight of approximately 30,000 and a major component with an isoelectric point of 5.53 and a minor peak with an isoelectric point of 5.06. The toxin was found to be heat labile with 75 per cent loss of biological activity after 5 minutes at 50°C and destroyed by trypsin after 30 minutes at 37°C.

Necrotic enteritis caused by type C occurs in calves, lambs, pigs, and possibly feeder cattle (Kennedy et al., 1977); it is generally believed that beta toxin is the one primarily responsible for the symptoms noted in necrotic enteritis (Willis, 1977).

Beta toxin has also been incriminated as the cause of necrotising jejunitis in man (Lawrence and Walker, 1976). Parnas (1976) reported that beta toxin administered into the rabbit jejunum and ileum produces paralysis of the motor activity of the intestine which is believed to be similar to the paralyzing activity in the intestine noted in piglet dysentery (Parnas, 1976).

(iii) Epsilon toxin

C.perfringens types B and D do not produce epsilon toxin as such, instead they produce an only slightly toxic prototoxin that can be made highly toxic by treatment with certain proteolytic enzymes, such as trypsin (Smith, 1975a).

Worthington and Mulders (1977) reported a molecular weight of 32,700 for the prototoxin and 31,200 for the activated epsilon toxin. The isoelectric point for the major peak of prototoxin was 8.02. The activated toxin had two major peaks with isoelectric points of 5.36 and 5.74. The significant change in isoelectric point and the small change in molecular weight that resulted upon activation of the prototoxin suggested that a small highly basic peptide was being removed (Worthington and Mulders, 1977).

While activation is usually affected by the enzymatic activity of trypsin, some activation can occur spontaneously in growing cultures, presumably due to the activities of proteolytic enzymes such as kappa and lambda toxins produced by the bacteria (Smith, 1975a).

Epsilon toxin is responsible for acute enterotoxaemia in animals, most commonly in sheep but found also in goats and cattle (Hauschild, 1971). A contributing factor in enterotoxaemia is a sudden supply of food which results in the passage of undigested food and C.perfringens type D organisms from the rumen to the intestines, this is followed by multiplication and epsilon prototoxin production by the organism in the intestine where proteolytic enzymes activate the epsilon toxin. The highly toxic epsilon toxin, increases intestinal permeability, enters the blood in substantial quantities and causes typically swollen hyperaemic kidneys, lung oedema, and excess of pericardial fluid. The kidneys become pulpy in sheep, but not in cattle, within a few hours of death (Hauschild, 1971).

Nervousness is a principal clinical sign in sheep with acute cases of enterotoxaemia. It is believed (Buxton and Morgan, 1976) that epsilon toxin acts by binding the receptors in the walls of blood vessels in the brain causing vascular endothelial cells to degenerate, leading to leakage of serum proteins, and eventually red cells, to cause oedema. The oedema then produces the clinical signs of nervousness seen in cases of acute enterotoxaemia of sheep.

Buxton (1978) used a mouse model and found that epsilon toxin bound to the luminal surface of the endothelial lining of blood vessels in the brain, liver, lungs, heart, the distal convoluted tubules in kidney, and to the hepatic sinusoids. No binding was detected in the large or small intestines or smooth and skeletal muscles in the body. He suggested that the intestine does not have receptor sites for the toxin.

(iv) Iota toxin

Iota toxin, formed only by strains of type E, is produced as a relatively non-toxic prototoxin that can be activated by proteolytic enzymes. The prototoxin is formed during the period of active growth of the organisms and is usually activated by the proteolytic enzymes produced by them (Smith, 1975a). The prototoxin may be demonstrated in filtrates of young cultures, 3 to 8 hours old, especially if these have been grown in medium containing meat particles to lessen the activity of the native proteolytic enzymes (Smith, 1975a).

It has been reported (Orcutt et al., 1978) that iota toxin has an estimated molecular weight of about 70,000. Iota toxin is markedly heat-labile, being inactivated by temperatures as low as 55°C and it is rapidly inactivated by pH values between 5.2 and 4.2 (Craig and Miles, 1961).

Craig and Miles (1961) demonstrated that iota toxin causes an increase in capillary permeability when injected intradermally into a guinea-pig; larger doses injected intradermally cause necrosis. When injected intravenously, the toxin is lethal. It is believed (Stern and Batty, 1975) that types E strains do not appear to play a role in pathogenicity. However, Orcutt et al. (1978) showed that type E strains producing iota toxin may be responsible for enterotoxaemia, or haemorrhagic typhlitis in rabbits that have experienced diet-related stress. Another report (LaMont et al., 1979) described a toxin from caecal contents of rabbits with colitis that was neutralised by C.perfringens type E antiserum. It

was suggested that the toxic material might be iota toxin but C.perfringens type E was not isolated. Since then Borriello and Carman (1983) have shown Clostridium spiroforme could be isolated from caecal contents of affected rabbits and showed that it produced toxin that was neutralised by C.perfringens type E antitoxin. Wilkins et al. (1985) have suggested that C.spiroforme produces the iota toxin responsible for enterotoxaemia and colitis in rabbits and that C.perfringens type E is not responsible.

(v) Theta toxin

All types of C.perfringens produce theta toxin which is responsible for the zone of clear haemolysis that surrounds colonies on blood agar plates (Smith, 1975a). Theta toxin is cytolytic, haemolytic and lethal, belonging to a group of oxygen-labile haemolysins (Yamakawa et al., 1977). The same group contains streptolysin O, cereolysin, tetanolysin and listeriolysin produced by Streptococcus pyogenes, Bacillus cereus, Clostridium tetani and Listeria monocytogenes, respectively. These haemolysins share common properties, being activated by thiol compounds and inhibited by a small amount of cholesterol (Yamakawa et al., 1977). Close serological relationships have been demonstrated among these haemolysins (Bernheimer, 1976).

The molecular weight of theta toxin has been variously determined as 35,000 (Hauschild et al., 1973); 46,000 (Smyth, 1974); 51,000 (Yamakawa et al., 1977). The most reproducible value appears to be about 55,000 obtained by polyacrylamide gel electrophoresis (Smyth, 1975). The isoelectric point has been determined at pH 6.5 (Smyth and Arbuthnott, 1972).

Theta toxin is thermolabile and markedly temperature-dependent in that its activity rises sharply as temperature increases to 37°C but drops off sharply as the temperature is raised above 37°C (Duncan, 1975). The haemolytic activity is also markedly dependent upon pH with the optimum activity at pH 6.7-6.8. Hydrogen ion concentrations above or below these values cause rapid loss of activity (Smith, 1975a).

The haemolytic activity of theta toxin is abolished if the toxin is pre-incubated with erythrocyte membranes (Smith, 1975a). It has been shown (Hose et al., 1975) that theta toxin appears to bind to erythrocyte membranes and that the inactivating substances in membranes is lipid and must be a fraction containing cholesterol. Binding of theta toxin to cells appears, unlike haemolysis, to be independent of temperature. Binding occurs at 0°C while lysis does not. If cells bound with theta toxin are raised in temperature to 37°C they lyse (Hose et al., 1975).

Many studies on mechanisms of action reported for other substances such as C.perfringens alpha toxin and neuraminidase have been shown to be contaminated with theta toxin (Smyth, 1975). It has been shown (Smyth et al., 1975) that commercial preparations of alpha toxin caused arc-shaped structures to form in the membranes of horse erythrocytes. These same structures were found when the erythrocytes were treated with highly purified theta toxin, while they were absent when treated with highly purified alpha toxin (Smyth et al., 1975).

Theta toxin has been shown (Soda et al., 1976) to be lethal upon intravenous injection into mice. They postulated that theta toxin, when co-injected with alpha toxin into mice, enhances the speed of death even though the dose of toxin necessary to kill the animal is not lowered. Muscle tissue is not thought to adsorb theta toxin and may even suppress its production, thus theta toxin may not play a role in clostridial myonecrosis (Duncan, 1975).

(vi) Delta toxin

C.perfringens delta toxin is one of the three haemolytic toxins released by a number of type C strains and some type B strains (Alouf and Tixier, 1976). There appears not to be any correlation between delta toxin production and isolation of organisms from diseased animals (Smith, 1975a). Strains of type B associated with lamb dysentery have been shown to produce delta toxin while strains from enterotoxaemia of sheep and goats in Iran

reportedly have not (Smith, 1975a). Type C strains which have been isolated from sheep with struck in Britain, and cattle in Japan are producers of delta toxin (Yamogishi et al., 1971) while type C strains isolated from calves and lambs in the United States, and man in Germany and New Guinea do not appear to be active producers (Smith, 1975a).

Delta toxin has been shown to be haemolytic only for erythrocytes of sheep, goats, pigs and cattle but not for those of other animals (Alouf and Tixier, 1976). It is lethal but not necrotizing upon intradermal injection (Smith, 1975a).

The molecular weight of delta toxin has been estimated to be about 42,000 and the isoelectric point is at pH 9.1 (Alouf and Tixier, 1976).

Optimum production of delta toxin has been shown to occur during the logarithmic phase of growth of the organism within a narrow pH range between 7.5 and 8.0 (Alouf and Tixier, 1976).

Smith (1975a) considered that delta toxin is of no importance in the pathogenesis of necrosis of the intestine brought about by the strains of type C that produce it.

(vii) Kappa toxin

Kappa toxin, a collagenase and gelatinase, is produced by strains of types A, D and E and by some strains of types B and C (Smith, 1975a).

Kappa toxin was purified by Kameyama and Akama (1971) who concluded that the toxin is a protein, free of carbohydrate and phosphorus, heat labile being inactivated by 60°C for 10 minutes, and sensitive to low pH being inactivated at pH 4.5. The molecular weight was estimated to be about 80,000.

Kappa toxin has ben shown (Kameyama and Akama, 1971) to be lethal and necrotic when injected into mice. When injected subcutaneously into rabbits it produces haemorrhages which appear as early as 5 minutes after injection and necrosis at the site develops within a few days. Extensive haemorrhage in the lungs but not in other organs was noted after subcutaneous and intramuscular injection in guinea-pigs. Extensive destruction of connective tissue in the absence of visible changes in the muscle layer was noted in guinea-pigs injected subcutaneously with the toxin. The authors postulated from these observations that kappa toxin may play a role in the development of gas gangrene by softening muscle tissue and contributing to the spread of alpha toxin.

It has been reported (Kameyama et al., 1975) that guinea-pigs immunised with kappa toxin were resistant to challenge with type A organisms and kappa toxin but not the organisms and alpha toxin. On the other hand, guinea-pigs immunized against alpha toxin were resistant to challenge with the organisms and either alpha or kappa toxin. The authors concluded that alpha toxin is the main contributor to the development of local infection and gas gangrene.

(viii) Lambda, Mu, Nu and Neuraminidase toxins

Lambda toxin is a protease that is produced by strains of types B, E and some strains of D (Willis, 1977). Lambda toxin degrades numerous proteins such as gelatin, casein and haemoglobin. It can be distinguished from kappa toxin in that it does not degrade collagen (Willis, 1977). Lambda toxin is inhibited by normal non-immune serum, is destroyed by pH below 5 or above 9, is heat-labile, and is inhibited by egg albumin, cysteine, cyanide, and citrate (Smith, 1975a). It is believed (Smith, 1975a) that lambda toxin does not play a role in the pathogenesis of any infection although it may activate epsilon prototoxin in cases of enterotoxaemia.

Mu toxin, which is a hyaluronidase, is produced in largest amounts by strains of type B, but is also produced by strains of

types A and D, and possibly by type C (Smith, 1975a). It acts by releasing glucosamine from hyaluronic acid (Willis, 1977). Mu toxin is heat-labile being destroyed by temperatures above 50°C, is relatively resistant to high and low pH values (Smith, 1975a). Mu toxin has been demonstrated to have haemolytic, necrotic and lethal activities and has ben shown to increase skin permeability, therefore it may contribute to more rapid spread of infection in gas gangrene cases. However, no relationship has been found between mu toxin production and virulence of the organisms (Smith, 1975a).

Nu toxin, a deoxyribonuclease, is produced by strains from all five types of C.perfringens, though most frequently by types A and C (Smith, 1975a). Nu toxin is a leucocidin and its ribonuclease activity results in the destruction of nuclei of polymorphonuclear leucocytes and muscle cells when injected intramuscularly in rabbits. Nu toxin's involvement in gas gangrene appears to be reflected by the absence of a leucocyte response during the disease (McDonel, 1980).

Neuraminidase has been shown to be produced by all types of C.perfringens (Fraser, 1978). Three neuraminidase enzymes produced by C.perfringens with molecular weights of 310,000, 105,000 and 64,000 have been described (Rood and Wilkinson, 1976). Neuraminidase may contribute to the virulence and invasiveness of an organism but certain neuraminidase-negative organisms such as C.novyi type A are invasive and cause gas gangrene, and there are neuraminidase-positive organisms such as C.tertium that are not thought to be pathogenic (Fraser, 1978). The effects of neuraminidase in enteric infections are not known.

(ix) Enterotoxin

C.perfringens enterotoxin, responsible for one of the most common types of food poisoning in humans is mainly produced by C.perfringens type A strains (Duncan, 1970). Enterotoxin production by strains of type C (Skjelkvale and Duncan, 1975) and type D (Uemura and Skjelkvale, 1976) have been described but the

role in intestinal disease of enterotoxin produced by these strains is not clear.

The enterotoxin is not formed during the vegetative growth of C.perfringens but its synthesis is related to sporulation of the organism (Hauschild et al., 1970). Enterotoxin production appears to be directly related to the ability of the strain to sporulate, thus mutants with altered ability to sporulate also had altered ability to produce enterotoxin. A non-sporulating, non-enterotoxigenic strain that regained the ability to sporulate also regained the ability to produce enterotoxin (Duncan et al., 1972).

Enterotoxin from C.perfringens has only been demonstrated in sporulating cultures, and the toxin can be detected intracellularly about 3 hours after the inoculation of actively growing vegetative cells into a sporulation medium (Duncan, 1973). However, not all sporulating strains produce detectable levels of enterotoxin (Skjelkvale and Duncan, 1975). Difficulty in getting C.perfringens to sporulate in most laboratory media is well known (Labbe, 1981). Several media have been devised for sporulation by C.perfringens, the Ellner medium (Ellner, 1956) and the Duncan and Strong medium (Duncan and Strong, 1968) or minor modifications of it (Labbe and Rey, 1979; Levinson and Feeherry, 1978) have gained the widest acceptance. Inconsistency of sporulation by C.perfringens in different media has been reported (Skjelkvale and Duncan, 1975).

Enterotoxin production by C.perfringens varies according to the isolate. It has been reported (Yasukawa et al., 1975) enterotoxin production by 51 of 66 strains associated with five outbreaks of food poisoning; the percentage frequency of enterotoxin-producing strains from the faeces of healthy humans and soil was less than one per cent. In contrast Tsai et al. (1974) reported that 42 of 65 strains isolated from chicken faeces and the meat, intestine and mesenteric lymph nodes of cattle were enterotoxigenic. In another study Uemura (1978) demonstrated

that 11 of 35 healthy humans were carriers of enterotoxigenic C.perfringens. A study (Niilo, 1978) reported that 27 of 114 strains of C.perfringens isolated from the intestinal contents of cattle, sheep and chickens with enteritis were enterotoxigenic. A correlation between alpha toxin and enterotoxin production has been reported (Harmon and Kautter, 1976) suggesting that enterotoxin levels could be estimated by measuring the alpha toxin content of the food sample. In contrast, a study (Skjelkvale et al., 1979) reported that some strains of alpha toxin negative C.perfringens can produce large amounts of enterotoxin and Harmon and Kautter's work seems unlikely to be true.

Sporulation and enterotoxin production have been shown to be dependent upon pH, temperature and the availability of carbohydrate in culture (Labbe and Duncan, 1975).

C.perfringens mutants blocked in sporulation earlier than stage III do not produce enterotoxin while those blocked at stage III produce small amounts, and ones blocked at late stage IV and stage V are able to produce enterotoxin (Duncan et al., 1972). It is concluded that the enterotoxin is a sporulation-specific gene product (Duncan et al., 1972). It has been suggested (Labbe and Duncan, 1977) that enterotoxin formation, which may involve a stable messenger RNA, appears to begin approximately 2.5-3 hours after inoculation into sporulation medium. This corresponds to late stage II or early stage III of sporulation. Polysomes have been isolated from cultures one hour after inoculation that produce enterotoxin in vitro (Smith and McDonel, 1980). Duncan et al. (1973) described paracrystalline inclusion bodies found only in sporulating enterotoxin-producing cells which they considered might be an aggregate of enterotoxin or enterotoxin-like spore coat protein material. When they studied sporulation mutants the inclusions appeared only in cells blocked no earlier than stage IV of sporulation. The relationship of enterotoxin to spore coat proteins has been studied (Frieben and Duncan, 1975) finding that anti-enterotoxin serum precipitated protein material extracted from spores of enterotoxin-positive as well as negative strains, it was

concluded that the enterotoxin is a spore coat structural component. It has been shown (Smith and McDonel, 1980) that proteins synthesised *in vitro* by polysomes extracted from vegetative cells do not produce proteins precipitable by anti-enterotoxin serum

The molecular weight of enterotoxin has been determined to be 35,000 (Stark and Duncan, 1972), while the isoelectric point is at pH 4.3 (Stark and Duncan, 1972).

The enterotoxin molecule is heat labile, 60°C from 5 minutes destroys biological activity though serological activity can be retained even after 80 minutes at 60°C. Loss of serological activity is more rapid at 60°C if the pH is 6.0 or less (Naik and Duncan, 1978). If the enterotoxin is heat-inactivated in food samples, about 12 per cent of the serological activity destroyed by heating at 60°C can be restored by treatment with urea for one hour at room temperature (Naik and Duncan, 1978). The enterotoxin loses its activity during pronase and subtilisin treatment, but is insensitive to trypsin, chymotrypsin, lipase, steapsin, alpha-amylase, papain and neuraminidase (Duncan and Strong, 1969).

Several biological and serological assay systems have been developed for the detection and quantitation of enterotoxin (Table 3). Enterotoxin biological activity has been tested by the rabbit ligated ileal loop test (Duncan and Strong, 1969). The minimal dose detectable by this assay has been reported to be 140-200 erythemal units (EU) (Stark and Duncan, 1972). Hauschild (1970) reported that the enterotoxin, when injected intradermally, causes erythema in guinea-pig. The erythemal unit has been defined as that amount of enterotoxin that causes a zone of erythema 0.8 cm in diameter on the skin of a depilated guinea-pig which has received intradermal injections of the enterotoxin (Stark and Duncan, 1971). This assay can detect quantities of enterotoxin as low as 0.25-0.5 EU. Niilo (1975) reported that mouse lethality assay can detect about 5 EU when the enterotoxin is injected intravenously.

Enterotoxin has also been assayed by its effects on Vero cells grown in tissue culture (McClane and McDonel, 1979). They reported that this assay detects less than 0.25 EU of enterotoxin.

Ouchterlony double-immunodiffusion method allows identification of enterotoxin detecting 3 µg per ml (Genigeorgis et al., 1973). Duncan and Somers (1972) detected as little as 1 µg/ml of enterotoxin using electroimmunodiffusion. Counterimmunoelectrophoresis has been used to detect 0.2 µg per ml of enterotoxin (Naik and Duncan, 1977). Fluorescent antibody has been used to detect intracellular enterotoxin in small numbers of sporulating cells (Niilo, 1977). The limits of enterotoxin detection by some of these methods have been reviewed by Stringer (1985) and are given in Table 3.

It has been stated (Niilo, 1978) that there is no evidence that negative or weakly toxigenic strains of C.perfringens can become strong enterotoxin producers and, since enterotoxin is a spore-related toxin, the composition of the sporulation medium will have a considerable effect on the amount of toxin produced. Culture conditions and heat treatment prior to inoculation into the sporulation medium are important factors for enterotoxin production. It has been shown (Uemura, 1978) that enterotoxin production was greatly enhanced in two of five food poisoning strains of C.perfringens type A when the cultures were subjected to three successive heat treatments at 80°C for 10 minutes.

In a study (Uemura et al., 1975) where monkeys were orally infected with a C.perfringens type A enterotoxin-producer strain, enterotoxin was detected in most of the faecal specimens, but neither enterotoxin nor anti-enterotoxin could be detected in serum samples taken up to 21 days after challenge. It was concluded that a significant amount of enterotoxin was not absorbed from the intestine into the bloodstream. Skjelkvale and Uemura (1977) studied two incidents of food poisoning in which one person was involved in both. In the first outbreak, 13 to 16 µg of enterotoxin per gram of faeces could be detected and in the second episode 8 months later, 3 to 4 µg of toxin per gram of faeces was

TABLE 3

Sensitivity of some biological and serological methods for the detection and assay of C.perfringens enterotoxin

Method	Enterotoxin (µg)
Erythemal activity	0.125
Mouse lethality	1.8
Rabbit ileal loop	29.0
Single gel diffusion	0.3
Double gel diffusion	0.013
Electroimmunodiffusion	0.01
Counterimmunoelectrophoresis	0.002
Reversed passive haemagglutination	0.0005
Radioimmunoassay	0.001
Vero cells	0.001
ELISA	0.0001

(Stringer, 1985)

found. Enterotoxin was not detected in serum during the acute stage of the illness, but it was detected as a rise in antibody titre during the following 2 months.

It has been suggested (Dowell et al., 1975) that the detection of enterotoxin in faeces may serve as a diagnostic test for implicating C.perfringens type A as the cause of food-borne illness. A study (Itoh et al., 1979) of 130 faecal isolates associated with 13 outbreaks of food poisoning revealed that 109 contained enterotoxin 1-2 days after illness but not after 5 days and none of the faecal specimens from healthy subjects contained enterotoxin.

In a recent study in fattening pigs, Jestin et al. (1985) demonstrated the presence of enterotoxin in the faeces of 25 diarrhoeic pigs in which no other pathogens could be demonstrated. C.perfringens type A enterotoxin was not detected in the faeces of 9 healthy pigs.

Enterotoxin has been identified as the factor responsible for diarrhoea experimentally induced in lambs (Niilo, 1971), calves (Niilo, 1973a), chickens (Niilo, 1974), rats (McDonel, 1974), rabbits (McDonel and Demers, 1982), monkeys (Hauschild et al., 1971) and humans (Strong et al., 1971). The ability of the enterotoxin to cause fluid accumulation in intestinal loops was first demonstrated in rabbits (Duncan and Strong, 1969) and lambs (Hauschild et al., 1970). The erythemal reaction to the enterotoxin in guinea-pig skin (Hauschild, 1970) was used to develop an assay for its biological activity (Stark and Duncan, 1972), demonstrating also that the erythema is associated with an increase in capillary permeability. the increase in capillary permeability was reported (Niilo, 1971) to occur within 15-20 minutes of intradermal injection. In the same report he described the systemic effects of the enterotoxin when injected intravenously into rabbits, lambs and guinea-pigs. Lambs developed transient diarrhoea, lachrymation, salivation, nasal discharge, lassitude and dyspnoea within 1 to 5 hours after inoculation. Animals that died from the injections had hyperaemic small intestinal mucosa,

and congestion in the liver, lungs, spleen and kidneys. Rabbits and guinea-pigs responded in a similar manner. It was also reported that atropine and adrenaline alleviated the clinical signs in lambs and guinea-pigs. It was concluded, that the enterotoxin exhibits a parasympathomimetic mode of action characterised by increased capillary permeability and vasodilation in the intestine, and increased intestinal motility. A similar study was done in calves (Niilo, 1973a) that showed marked differences from the responses seen in lambs and rabbits (Niilo, 1971). Calves injected intravenously with enterotoxin did not develop lachrymation, salivation, nasal discharge, or diarrhoea, but, intestinal hyperaemia and some intestinal mucosal necrosis developed. These data were interpreted as indicative of a strong predilection of the enterotoxin for the intestinal capillary bed (Niilo, 1973a). Another report (Niilo, 1973b) established that fluid accumulation in bovine Thiry fistulas can occur within 30 minutes after exposure to the enterotoxin.

It was reported (McDonel, 1974) that in the rat ileum the enterotoxin induces a reversal of transport from absorption in controls, to secretion of fluid, sodium and chloride ions. Glucose absorption was inhibited while transport of potassium and bicarbonate was unaffected. Fluid secretion was found to increase with enterotoxin dose, up to a point beyond which excess of enterotoxin had no effect upon fluid secretion. This saturation phenomenon was confirmed in another report in which rabbit ileal loops were challenged with a range of 50 - minimum dose to cause fluid accumulation - to 1,000 EU of enterotoxin; the amount of fluid accumulation was indistinguishable in loops treated with each dose (McDonel and Duncan, 1977). Histological studies (McDonel, 1974) showed that desquamation of intestinal epithelial cells occurred at villus tips in enterotoxin-treated loops. It has been reported (Niilo, 1971) that epithelial desquamation occurs after the intravenous injection of enterotoxin.

It was reported (Yamamoto et al., 1979) that removal of enterotoxin from mouse ligated loops caused a reduction in fluid

accumulation, even if the loop was exposed to enterotoxin for 30 minutes prior to removal. It was concluded that the enterotoxin does not bind freely to the intestinal cells. In contrast a study (McDonel and Demers, 1982) demonstrated the binding of enterotoxin to the epithelial cells of the rabbit colon.

Studies with the rabbit have revealed that enterotoxin is most active in the ileum, mildly active in the jejunum, and nearly inactive in the duodenum (Hauschild et al., 1973). Another study (McDonel and Demers, 1982) showed that the colon of the rabbit did not present any apparent response to the enterotoxin in the transport of fluid and electrolytes as compared with untreated animals.

It is postulated (McDonel, 1979) that C.perfringens enterotoxin acts by direct cell membrane damage. A study showed (McDonel and Duncan, 1975) that cycloheximide, an inhibitor of protein synthesis, does not prevent tissue damage and fluid and glucose transport alterations induced by the enterotoxin. This evidence indicates that induction of new synthesis of protein does not play a role in the mode of action. It appears that, rather than activate new metabolic pathways, protein synthesis, or existing enzymes, the enterotoxin acts through interference with energy production and cellular maintenance of structure and function.

It has been reported (Gyobu and Kodama, 1978) that C.perfringens enterotoxin is cytotoxic for Vero cells. The cells have proven to be very sensitive in that enterotoxin alters their morphology, viability, and macromolecular synthesis. Gross morphological damage was observed within 30 minutes of exposure of monolayers to enterotoxin, and approximately 75 per cent of the cells had detached within 40 minutes. McDonel (1979) studied the action of the enterotoxin in Vero cells and found that, contrary to his in vivo findings (McDonel and Duncan, 1975), the enterotoxin inhibits protein, RNA and DNA synthesis of Vero cells within 15 to 30 minutes. Depending upon the dose used, the enterotoxin also

causes loss of viability and detachment of the cultured cells from the surface on which they are growing. The plating efficiency of the Vero cells can be significantly reduced by as little as 1 ng of enterotoxin per ml and doses of 1 µg cause reduction of the plating efficiency of 100 per cent of the cells (McDonel, 1979).

It is believed (McDonel, 1980) that the enterotoxin alters the configuration or structure of some components of the membrane which results in structural and functional changes in the membrane.

Enterotoxin binding studies have been performed (McDonel, 1980) with cells isolated from rabbit intestine, liver, kidney and brain. The enterotoxin was found to bind equally to cells from the intestine, liver and kidney but not the brain. Reversibility of the binding was not demonstrated, and it was postulated that the enterotoxin interacts with a compound in the membrane in such a way as to become trapped, or to be absorbed into the membrane of the cell.

3. PROTECTION AGAINST AND IMMUNITY TO ENTERIC MICROORGANISMS IN THE PIG

Protection against C.perfringens type A infections of the intestinal tract and immunity to them has not been studied in detail in the pig. There is, however, much information about the general mechanisms of protection and immunity in the gastro-intestinal tract of the pig and this is reviewed here with reference, where relevant, to C.perfringens. Protective immunity against infections with C.perfringens is well documented in other species and to some extent in C.perfringens type C infections in the pig. The subject has been reviewed here with particular reference to the pig.

(a) Non-specific defence mechanisms of the gastrointestinal tract

These mechanisms affect the entry of C.perfringens and its

products to the gastrointestinal tract, the establishment of the organism and the production of pathogenic determinants such as the toxins reviewed above.

(i) Gastric barrier

The stomach is a potent bactericidal trap limiting the number of viable organisms which gain access to the small intestine. At pH values less than 4.0 there is significant bactericidal activity which becomes total at pH 3.0. Bactericidal activity *in vivo* can apparently be ascribed entirely to the effect of pH since it is abolished by neutralisation. The susceptibility of microorganisms to the effect of pH is variable. *Staphylococcus epidermidis* and *Lactobacillus* spp. are relatively resistant while *E.coli* appears to be more susceptible (Newby and Stokes, 1984). The spores of *C.perfringens* are almost certainly unaffected by gastric pH but no information is available about its effect on vegetative cells. The raising of gastric pH has a significant effect upon infection of the intestinal tract. In the immediate neonatal period, gastric pH is nearly neutral. This may have importance in allowing the establishment of an early gut flora and it may prevent denaturation of the antibodies received through colostrum (Kruse, 1983). It is known from volunteer studies in man that *C.perfringens* enterotoxin is inactivated by gastric acid (Stringer, 1985).

(ii) Biliary secretions

The resident microflora of the small and large intestine are relatively resistant to bile salts. When secreted into the intestine most of the bile acids are in conjugated form, however, intestinal bacteria such as *Bacteroides*, *Enterococci*, *Clostridia* and *Bifidobacteria* are able to deconjugate bile salts, releasing free bile acids. Some intestinal species tolerant of conjugated bile salts may be inhibited by free bile acids and this inhibition is most prominent in mildly acid conditions. Thus, growth of bacteria in the small intestine may be limited by production of acid and by deconjugation of bile salts (Newby, 1984).

(iii) Epithelial barriers

The epithelial cells contribute to the integrity of the mucosal surface by their capacity to undergo rapid turnover and renewal (Eastwood, 1977). During infection of the small intestine the rate of turnover, including cell loss from the mucosa, increases, thus leading to removal of pathogen-infected cells (Eastwood, 1977). The rate of replacement of villous epithelium in the small intestine of pigs contributes to the innate age dependent receptivity to transmissible gastroenteritis (Moon et al., 1975). The apparent rate of regeneration of villi in the intestine of 3 week-old pigs is more rapid than in the intestine of newborn pigs. The accelerated replacement of villous epithelial cells in older pigs contributes to the resistance of the animal to the disease (Moon et al., 1975).

Adherence to the epithelium is important for intestinal colonization and for production of the diarrhoeal disease caused by toxigenic E.coli. In pigs the expression of receptors for the E.coli adhesin K88 is controlled by a single autosomal dominant gene and animals lacking this gene are not susceptible to the enteritis caused by K88 positive organisms (Sellwood, 1981). There is no information about the intestinal receptors for C.perfringens type C.

(iv) Mucus

The mucosal surface of the gastrointestinal tract is covered by a layer of mucus which is being continuously shed into the lumen and is continuously being replaced by Brunners' glands and the goblet cells of the intestinal epithelium. Mucus has a number of functions within the intestinal tract, it lubricates the epithelial surface, protects it from the low pH of the stomach, and acts as a medium in which digestion and absorption can occur. The mucus has direct antimicrobial effects by cleansing the epithelial surface. It can entrap bacteria and has been shown to possess regions mimicking the receptor sites for bacteria present on epithelial

cells and this may facilitate this trapping (Clamp, 1981). The relationship of C.perfringens to the mucus layer is not known.

(v) Lactoferrin and Lysozyme

These are found in intestinal secretions in association with mucus (Clamp, 1981). Lactoferrin, an iron-binding protein has bacteriostatic effects upon E.coli by successfully competing with the bacteria for iron (Reiter, 1978); it has been suggested that this protective effect may be subsidiary to its principle role as a regulator of iron absorption (Brock, 1980).

Lysozyme is synthesized within paneth cells at the base of the intestinal crypts. This enzyme has bactericidal activity by degrading the peptidoglycan of the cell wall of Gram-positive and the outer membrane of the Gram-negative organisms (Reiter, 1978). It may have some effect on clostridia in the gut.

(vi) Peristalsis

Peristalsis is probably the major factor reducing bacterial numbers within the small intestine. Bacteria introduced into the small intestine are cleared by peristalsis and drugs that inhibit peristalsis lead to increased bacterial numbers in the small intestine (Newby and Stokes, 1984). The effectiveness of bacterial clearance by peristalsis may be influenced by other factors. Villous movement in the small intestine appears to assist the mucous coating of bacteria on the surface of the mucosa. Antibody in intestinal secretions assists this mechanism both by agglutinating bacteria and inhibiting bacterial adherence to the mucosa (McClelland, 1979). Rises in clostridial counts have been reported in cases of reduced gut motility in man (George and Finegold, 1985).

(vii) Intestinal microflora

Bacteria colonise the intestine of the newborn pig within a

few hours of delivery and rapidly reach levels of 10^8-10^9 per gram of faeces. The bacteria concerned include facultatively anaerobic bacteria such as Streptococcus spp. and Escherichia, micro-aerophilic organisms such as lactobacilli, oxygen tolerant anaerobes such as C.perfringens and strictly anaerobic bacteria such as Fusobacterium spp, Peptostreptococcus spp. and later, Bacteroides (Ducluzeau, 1983). Initial levels of bacteria such as C.perfringens may be high (10^6/gram), but rapidly decline to reach levels of 2.5 x 10^3/gram (Mansson and Smith, 1962) in pigs receiving a conventional diet.

The normal flora can inhibit pathogenic organisms either through physical interference, occupation of the receptor sites, or by successfully competing for essential substrates. The indigenous flora can render the environment toxic to other species; Lactobacilli, by reducing the pH, inhibit the growth of E.coli, while many enteric bacteria produce substances such as colicins which are bactericidal for competing species (Newby, 1984).

Experiments with the colonisation of the neonatal gastro-intestinal tract of the pig have been carried out mainly with E.coli. Prior colonisation of the intestine with a K88 positive, non-enterotoxin-secreting E.coli can protect the animal from infection with the enterotoxin-producing variant, presumably by occupying all the K88 receptors (Ducluzeau, 1983) but such protection has not been described for C.perfringens. It has been shown (Tlaskalova et al., 1983) that under conventional conditions the presence of normal microbial flora effectively competes with colonization of the intestine with artificially introduced strains of bacteria, including known enteric pathogens. The colonization of conventionallly raised animals with various strains of enteric bacteria is extremely difficult and transitional success is achieved only with very high doses of bacteria (10^9 or more). This colonization resistance which represents a very important factor of anti-infectious resistance can be dramatically reduced, if normal flora is suppressed, for example, by suitable antibiotics, and completely eliminated under germ-free state conditions. Enteric

bacteria applied perorally to germ-free animals readily multiply, with generation time similar to *in vitro* conditions; bacterial counts in the intestine reach a plateau within one or two days with maximal values in colon and terminal ileum (Tlaskalova *et al.*, 1983). The ease with which germ-free and colostrum-deprived animals can be colonised indicates the importance of either active or passive specific immunity in protection against common enteric bacteria. This immune protection is reviewed below.

(viii) Diet

Diet may also affect the levels of enteric bacteria. Mansson and Smith (1962) described the effects of an increase in dietary protein from normal to a high level. *C.perfringens* counts rose from 2.5×10^2 per gram of faeces to 10^6 per gram. Similar changes have been recorded in sheep with *C.perfringens* Type D enterotoxaemia and 'Pig Bel' in man associated with *C.perfringens* type C infection (Walker, 1985) in which levels of clostridia remain constant on a low protein or restricted diet but rise dramatically when overeating or an increase in protein intake occurs.

It was reported (Stutz *et al.*, 1983) that an increase in the numbers of *C.perfringens* occurred when chickens were fed with a sucrose diet compared to chickens fed with a practical diet.

Another report (Stutz and Lawton, 1984) demonstrated that chickens fed with a standard corn and soya bean meal diet to which bacitracin was added, presented a significant weight gain and low numbers of *C.perfringens* in small intestine compared with chickens fed on the same diet without bacitracin. Growth depression was observed in these chickens and was correlated with an increase in the numbers of *C.perfringens* in the intestinal tract.

(b) Specific immune defence mechanisms of the gastro-intestinal tract

Antigens which enter the gut or are produced there by enteric bacteria stimulate immunity in the same way in most species. C.perfringens produces toxins which may penetrate the intestinal epithelium and give rise to systemic effects and systemic humoral response but the bulk of the antigens produced are restricted to the intestine and local immunity is most important in protection against them. For this reason the development of local immunity is reviewed in greatest detail along with passive protection of the neonate and the development of the immune response in the young piglet. The development of cellular immunity to enteric organisms is only touched upon here.

(i) IgA

Antigens from the lumen of the gut, stimulate precursor B-lymphocytes in the Peyer's patches to become antigen-stimulated IgA-precursor lymphoblasts (Genco et al., 1983). T-helper cells for IgA and T-suppressor cells for IgG, IgM and IgE are stimulated by antigens or mitogens that gain access to the Peyer's patches. The IgA-precursor cells then migrate through the intestinal lymphatics to the mesenteric lymph nodes which become enriched with IgA-plasma cells. From there, they enter the thoracic duct and circulation. The lymphoblasts in the circulation then home to the lamina propria of the gut and to the interstitial tissue of exocrine glands such as the lacrimal glands, salivary glands, mammary glands and to the respiratory, intestinal and genital urinary tracts (Genco et al., 1983). Antigens may function at several levels in mucosal immunity. An antigenic stimulation of gut-associated lymphoreticular tissue (GALT) such as Peyer's patches may occur from the intestinal lumen after oral administration of antigen. A parenteral inoculation may sensitise the GALT, including the Peyer's patches, with blood-borne antigen. This type of immune response is seen experimentally when animals are hyperimmunised parenterally and exhibit a secretory immune

response, but it is probably not a major mechanism for inducing secretory immunity. A local antigen application would provide an antigen-induced stimulation of the GALT leading to the maturation and persistence of IgA-producing lymphoblasts in the distal exocrine glands and in the lamina propria (Genco et al., 1983).

After release from plasma cells in the lamina propria, the IgA molecules bind to receptors composed of secretory components on the outside of the epithelial cell membrane. The secretory IgA thus formed then undergoes pinocytosis and is transported through the cytoplasm of the epithelial cells, whence it is released by reverse pinocytosis into the intestinal lumen (Brandtzaeg, 1981). The time lapse between the presentation of antigen to the intestinal epithelium and a measurable IgA response depends upon the techniques used. It may be hours in in vitro tests but in the gut, the primary response is usually considered to take 14-21 days. Specific IgA antibody production can be demonstrated for 120-360 days after immunisation. Memory of the response persists in the lamina propria in lymphoblasts and a secondary response to antigen takes 5 days to become detectable and lasts for 120-700 days (Porter et al., 1974).

Once released into the lumen, secretory IgA coats the epithelial cell surface by combining with mucin. Its capacity to bind to mucosal cell surfaces implicates its role in the inhibition of adherence of bacteria, and neutralization of viruses and toxins (McCaughan and Basten, 1983). IgA antibody raised against purified K88 pilus antigen and present in sow's milk protects newborn pigs from experimental infection with K88-bearing E.coli (Newby, 1984) and this protection is associated in vivo and in vitro with inhibition of adhesion between the bacteria and enterocyte i.e. the IgA antibody found in milk is specifically directed against antigens experienced by the sow.

Immunisation of rats with V.cholerae enterotoxin induces the appearance of secretory antibodies to the toxin which are protective against the effects of the toxin. Antibodies to the toxin present in the milk of immunised rats proved to be also

protective to their litters when challenged with the toxin (Rowley, 1983). This indicates the ability of secretory IgA to neutralise toxins in the gut. A suggestion that this type of protection may be relevant in C.perfringens disease has been made by Kliegman (1985) who associated diminished intestinal IgA levels with an increase in numbers of C.perfringens and other bacteria in neonatal necrotising enterocolitis. He considered that IgA might neutralise toxins produced by the organisms impeding their effects in the intestine.

Studies with V.cholerae killed whole cells demonstrated to some degree of protection to the disease after oral immunisation. A secretory IgA anti-toxin response was demonstrated in the immunised individuals (Rowley, 1983). A study showed that the formation of antigen-secretory IgA complexes in the intestinal lumen not only results in neutralisation of antigen but, in addition, causes stimulation of goblet cells with release of mucus; the mucus then interferes further with access of antigen to the epithelial cells by creating an impermeable barrier and by retaining the secretory IgA-antigen complexes in its glycocalyx for a sufficient period of time to allow degradation of antigen by intestinal enzymes (Ottaway et al., 1979).

Secretory IgA has been involved in bactericidal and parasiticidal activities by cooperating with lysozyme (Porter, 1979); with lactoperoxidase (Befus and Bienenstock, 1982); or with lactoferrin in IgA mediated bacteriostasis of E.coli (Newby, 1984). No such studies appear to have been carried out with Clostridium perfringens.

(ii) IgG

IgG plasma cells are present in significant numbers in the porcine intestinal lamina propria (Brown and Bourne, 1976), contributing significant immunoglobulin to the intercellular fluid of the lamina propria. Thus IgG may act locally in the lamina propria to neutralise antigen which penetrates the epithelial

barrier. It may also leak into the intestinal lumen through inflammatory lesions but its action here has not been studied in detail. Some of the locally-produced IgG may supplement plasma IgG levels produced elsewhere in the body (mesenteric lymph nodes, spleen and body lymph nodes) either from lymphoblasts primed in the lamina propria or from lymphoblasts primed in these other sites by antigen derived from gut infections.

A primary response of specific IgG secretion takes 7-14 days from presentation of the antigen to the gut to demonstration of antibody and is often preceded by IgM production. The primary response may last for 90-120 days in the absence of stimulation. A secondary response may develop within 5-7 days of re-stimulation and be detectable for 120-700 days (Porter et al., 1974).

The activities of IgG include those described by Newby (1984) i.e. that IgG antigen complex formation in the intestinal mucosa might produce a cellular inflammatory response resulting in collateral damage to tissues resulting in increased permeability of the mucosa.

It may also trigger the release of mucus from goblet cells by the formation of immune complexes on the mucosal surface, the increased flow of mucus may have pronounced flushing effects upon the epithelial surface and contribute to the removal of micro-organisms and parasites from the epithelial surface (Ottaway et al., 1979).

IgG also protects against systemic effects of toxins produced at intestinal level. Sheep immunised with C.perfringens epsilon toxin toxoid develop high serum IgG titres to the toxin which are correlated to the degree of protection to the effects of the disease (Cameron, 1980).

(iii) IgM

IgM is a major secretory immunoglobulin in the intestinal

tract of animals and during the first few weeks of life IgM-producing cells are numerically predominant to IgA cells at all levels of the intestine (Porter, 1979).

It is suggested (Allen and Porter, 1973) that IgM plays an initiating role in the onset of antibody response in mucosal immune system, in much the same manner as it does in the systemic response to parenterally administered antigens. Its appearance early in development, both of the individual and of an immune response, suggests that IgM may have the same role in early defence in secretions that is seen in serum (Porter, 1979). The low concentration reported for this immunoglobulin in secretions (Bourne, 1973) may be partly due to the rapid breakdown of this immunoglobulin in the intestinal lumen by proteolysis, and may not reflect its true concentration in intestinal secretions (Newby, 1984).

A primary response of IgM secretion takes 2-5 days from presentation of the antigen to the demonstration of antibody and lasts for 30-60 days when a switch to IgG production often occurs. Secondary responses take 1-2 days to develop and may last for 30-60 days (Porter et al., 1974). IgM antibody in porcine colostrum has been shown to have powerful opsonic activity against E.coli (Sellwood, 1981), suggesting the role it may play in antimicrobial defence.

(iv) IgE

IgE is present only in minute amounts in secretions being associated with mast cells. IgE responses are usually only seen following stimulation of the immune system with parasitic antigens (Jarrett and Miller, 1982).

It is suggested that the way in which IgE protects against parasitic infection in the intestinal tract is by degranulation of the mast cell as a result of cross-linking of surface IgE by antigen, producing the release of vasoactive amines that increase the vascularity of the mucosa and renders the epithelium more

permeable to serum proteins, which, entering the lumen of the gut, can attack the parasite (Jarrett and Miller, 1982).

IgE antibodies complexed to antigen within the intestinal lumen have also been shown to trigger the release of mucus from goblet cells, the increased mucus flow may prevent the parasite from coming into proper contact with the mucosa and also causes a significant proportion of parasites to become trapped in the mucus (Ottaway et al., 1979).

(v) Lymphokines

Lymphokines released by immune T-lymphocytes have been reported in the lamina propria of the pig following infection with transmissible gastroenteritis virus, porcine enterovirus and after oral immunisation, with protein antigen (Newby, 1984). It has been proposed (Ferguson and MacDonald, 1977) that lymphokines within the intestine may increase the rate of enterocyte turnover. In viral and some parasitic conditions where the enterocyte is the target cell of the pathogen, this increased shedding of cells might result in the expulsion of pathogens together with the epithelial cell.

Cepica and Derbyshire (1980) detected spontaneous cytotoxicity against transmissible gastroenteritis virus in intra-epithelial lymphocytes isolated from normal pigs. Peripheral blood mononuclear cells, transferred to neonatal pigs, partially protected the recipients from experimental challenge, and the recipients intraepithelial lymphocytes demonstrated spontaneous cytotoxicity.

(vi) Activated macrophages

Macrophages are common in the intestinal mucosa, thus some antigens are immediately available to macrophages upon completion of transport by the follicle-associated epithelium (Bockman et al., 1983). Intraepithelial macrophages are phagocytic when killed Salmonella typhi is administered orally to rabbits previously

sensitised intravenously (Kimura, 1977). Intraepithelial macrophages have also been shown to be capable of phagocytising intraluminally administered ferritin in non-immunised rats (Bockman et al., 1983).

It has been shown (Richman et al., 1981) that macrophages from Peyer's patches are capable of antigen presentation, thus they may participate in the induction of humoral immune responses at intestinal level.

(vii) Phagocytosis by neutrophils in the immune animal

Pigs immunised parenterally and subsequently challenged into gut loops with the same antigen showed a rapid influx of neutrophils into the lumen. These neutrophils were able to kill bacteria either by phagocytosis or by the release of lysozomal enzymes after their death and disruption in the lumen (Newby, 1984).

(c) Intestinal immunocompetence of the neonatal pig

The neonatal pig is practically devoid of defences to enteric organisms. Protection againt C.perfringens and its products has to be obtained by passive immunity transferred at birth from the dam to the new-born piglet.

Studies on the intestinal immunocompetence of the neonatal pig are reviewed below:

Lymphoid cells producing IgM antibodies are detected in Peyer's patches at day 55 of gestation, occurring prior to development of immunocytes in other organs. This lymphoid differentiation occurs in the absence of antigenic stimulus (Porter, 1981).

In the newborn pig there are very few lymphoid cells in the lamina propria and the lymphoid follicles are poorly defined.

After birth these structures develop and the lamina propria becomes infiltrated with lymphocytes and plasma cells probably due to responses to bacterial challenge, since there is no comparable development in germ-free piglets (Porter, 1981). Oral administration of E.coli antigens to germ-free piglets results in an early appearance of IgM-containing plasma cells, followed by those containing IgA. Thus IgM appears to play an initiating role in antibody response of the intestinal immune system (Porter, 1981). The predominant immunoglobulin-producing cells in the newborn pig intestine are of the IgM type for the first 3 to 5 weeks, then IgA-producing cells become established and are in greater numbers throughout adult life (Husband, 1980).

Humoral antibody to antigenic challenge exposure first appears within 10 days in the newborn pig. Without the continuous presence of the antigen, the antibody decreases to zero values by 4 weeks of life. On re-exposure, antibody appears again but as a primary immune response, indicating that memory cells do not develop in the intestinal-associated lymphoid tissue (Husband, 1980).

(d) Transmission of passive immunity to the newborn pig

The transmission of passive immunity from sow to piglet occurs postnatally by way of colostrum. It is established that there is normally no transmission of maternal immunoglobulins across the placenta of the pig. As a result the pig is born devoid of immunoglobulins and relies on colostrum as a source of circulating antibody during the early part of its life (Kruse, 1983).

If immunoglobulins are found in foetal or newborn pig serum they must be of foetal origin following antigen stimulation or have been passively transferred from the sow across a damaged placenta. It has been demonstrated that the foetal pig, like many other mammalian foetuses, is immunocompetent to a variety of antigens when exposed either naturally or by artificial means. However, under normal circumstances antigen exposure does not occur until soon after birth. In consequence, the newborn pig is equipped with

a rudimentary immune system, which has not hitherto been primed to function (Kruse, 1983).

In the sow, antigen-sensitised cells migrate from the intestinal tract to the mammary gland. The immunoblasts become antigen primed at the level of Peyer's patches. These immunoblasts destined to secrete IgA antibodies migrate via the mesenteric lymph nodes and thoracic ducts into the general circulation, with the mammary gland as one of their target sites (Opdebeeck, 1982). This intestinal-mammary immunological link has been observed in orally immunised sows with E.coli (Evans et al., 1980).

Newborn piglets have very low levels of serum immunoglobulins and hence obtaining immunoglobulins from colostrum reduces their vulnerability to disease. The immunoglobulin concentration of colostrum is highest at birth and then declines, the rate of decline depending on the amount of colostrum which is being removed. In sows which have been sucked, the immunoglobulin concentration drops to 50 per cent by 5 hours after the first piglet is born and the level is close to that of normal milk by 16 hours (Bourne, 1969).

The importance of colostral immunoglobulins in the resistance to infectious diseases in newborn pigs is universally recognised (McCallum et al., 1977; Bourne et al., 1978). It has been reported that 90 per cent of piglets deprived of colostrum died before weaning (McCallum et al., 1977).

There is approximately 15g of whey proteins in 100 ml of colostral whey from sows and 60-70 per cent of these proteins are immunoglobulins (Kruse, 1983). Colostral proteins fed to the newborn pig are not degraded and used as a food source but reach the small intestine intact. Because the proteolytic activity in the digestive tract is low, and is further minimised by the presence of trypsin inhibitor in colostrum (Jensen, 1978). IgG-rich colostral whey passes quickly through the stomach and into the small intestine. For the first 24-36 hours after birth the

small intestine is lined with highly vacuolated, immature mucosal epithelial cells which are capable of absorbing macromolecules. The colostral immunoglobulin is thus transported from the lumen of the intestine, via the absorptive epithelial cells and intestinal lymphatic system, to the blood. Colostral immunoglobulins in the lumen of the intestine are first internalised by enterocytes via pinocytosis in the uptake phase, and internalised macromolecules are thereafter transported to the blood (Kruse, 1983). The uptake and transport of macromolecules is qualitatively non-selective in the pig and includes a variety of heterologous and homologous proteins, dextrans and polyvinylpyrolidone. Proportions of the different classes of immunoglobulins in serum after ingestion of colostrum reflect therefore the proportions in colostrum when absorption is completed (Kruse, 1983).

The intestinal epithelium of the newborn pig retains the ability to pinocytose macromolecules for only a short time before the highly vacuolated epithelium is replaced by mature epithelial cells (Moog, 1979). The ability to absorb immunoglobulins drops to a low level after 24 hours although starved piglets may still be able to absorb immunoglobulins up to 4 days of age (Broom, 1983). In general, permeability is highest immediately after birth and declines rapidly thereafter.

The cessation of pinocytosis, a phenomenon called closure, apparently signals a shift in the membrane producing capacity of the epithelial cells, the endocytotic type of membrane is no longer being synthesised (Moog, 1979). The intestinal cells that absorb immunoglobulins are replaced by a more mature cell population with enzymes that characterise the mature microvillus surface and the cells are no longer engaged in pinocytosis. Closure appears to be controlled by endocrine influences and coordinated with the development of the enzymes that characterise the mature microvillus surface (Moog, 1979). Cessation of the uptake of colostral immunoglobulins occurs from the proximal to the distal part of the small intestine. There is evidence that pinocytosis in the epithelium disappears 2 hours after birth in the duodenum but

not before 72 hours in the ileum. The period of absorption, however, is not limited to a certain critical time period after birth but is dependent upon the type of food ingested (Kruse, 1983).

IgG accounts for about 80 per cent of the total immunoglobulin content in colostrum of pigs but rapidly declines so that a 30-fold decrease occurs during the first week of lactation, and remains a minor component of milk during the remainder of lactation (Curtis and Bourne, 1971). Although the level of IgA in colostrum is only 16 per cent of that of IgG, it declines only 3-fold and soon becomes the predominant immunoglobulin in milk (Curtis and Bourne, 1971). IgM is at low levels in both colostrum and milk. In colostrum, nearly all of the IgG and 40 per cent of IgA are derived from serum; but in milk more than 90 per cent of IgA and IGM, and 70 per cent of IgG are produced in the mammary gland (Bourne and Curtis, 1973). The transition from colostrum to milk occurs during the first 3 to 7 days of lactation, with the predominant change occurring during the first 24 hours (Bourne and Curtis, 1973).

Sows immunised parenterally with C.perfringens type C toxoid provide good levels of protective antibodies in colostrum and milk (Djurickovic et al., 1975). These antibodies are possibly induced by the presentation of processed antigen by circulating lymphocytes or macrophages to the immunoblasts present at intestinal level or by a direct stimulation of immunoblasts present in the mammary gland (Opdebeeck, 1982).

Milk immunoglobulins ingested by the suckling pig bathe the mucous membranes of the intestine and provide passive protection against enteric infection (Bourne and Curtis, 1973). Studies in fistulated pigs (Porter et al., 1970) have shown that after a single feed IgA continues to pass along the intestine for more than 90 minutes, thus milk IgA provides an almost continuous coating of antibody for the small intestine.

Although the major immunoglobulin in porcine milk is IgA and is largely responsible for protection, both IgG and IgM antibodies are present often in significant amounts and can provide significant protection (Newby, 1984).

Using neonatal E.coli diarrhoea of pigs as a model, it has been shown that immune milk from immunised sows protects the young from experimental challenge, inhibits the adhesion of bacteria to enterocytes in vitro and prevents their attachment to the intestinal mucosa in vivo (Newby, 1984).

(e) Immunisation of sows against enteric organisms to protect the newborn pig

Studies on immunisation of sows against certain enteric organisms such as E.coli are extensive, but those on immunisation to C.perfringens are concerned, in the case of the pig, only with immunisation against C.perfringens type C infection. General sow immunisation studies are reviewed below with reference to C.perfringens where possible.

To stimulate a mucosal immune response that is protective against enteric infection, a variety of rates of antigen presentation and combination of adjuvants have been used. Effective vaccination commonly requires previous infection with the live organism. Oral administration of dead bacterial antigens may lead to the production of IgA antibodies in the gut secretions of pigs (Porter et al., 1974). By making use of the link that exists between the gut and mammary gland, it has been possible to compare in lactating sows the effects of oral immunisation with live and heat-killed E.coli. The IgA response in both milk and gut secretions to heat-killed organisms was both reduced and delayed in appearance (Evans et al., 1980).

Oral immunisation of sows with E.coli lipopolysaccharide antigen followed by parenteral immunisation leads to an antibody response in serum primarily of the IgM class and can provide the

suckling piglet with colostral antibody which is protective (Chidlow and Porter, 1978).

In general, inactivated microorganisms given orally produce a relatively poor immune response. The response is strongly dependent upon the size of the administered dose, and in order to maximise the local response, very large amounts of antigen are required. In pigs, the optimal dose of bacteria is of the order of 1×10^{11} but even these extremely large doses of antigen give a response which is inferior to that produced by viable organisms (Evans et al., 1980). It appears that active colonisation of the intestine is essential since a response is only seen in animals in which the bacteria can be cultured from the faeces (Newby, 1984), and pigs lacking the binding receptor for K88 do not mount a significant response to the K88 bearing E.coli (Sellwood, 1981).

However, certain inactivated vaccines have been shown to produce a degree of immunity protecting against challenge. It has been reported that pigs orally immunised with antigens of E.coli were partially protected against challenge (Newby, 1984). A study (Porter and Linggood, 1983) reported that the daily administration of antigenic extracts of heat-inactivated E.coli to pregnant sows, from day 60 of gestation to parturition with an intramuscular injection on day 95, effectively protected the progeny of the vaccinated sows against challenge. The protection was associated with high titres of antibody to the O-antigen of E.coli predominantly of the IgM class.

It was reported (Furer et al., 1983) that pregnant sows immunised intramuscularly with heat-inactivated cholera toxin, 5 weeks and 2 weeks prior to parturition, conferred protection to their progeny against challenge with pathogenic K88 E.coli. High titres of antibodies of the IgG class against the cholera toxin were found in colostrum and serum of vaccinated animals which neutralised the LT toxin of E.coli.

Ciosek et al. (1983) failed to find effective protection of

piglets against E.coli infection when pregnant sows were parenterally immunised with commercial multi-adhesin E.coli vaccines. Nagy and Walker (1983) reported the immunisation of pregnant gilts with a multi-adhesin vaccine combining the components K88, K99 and 987P plus the beta toxoid of C.perfringens, the animals were immunised subcutaneously 6 and 2 weeks before parturition. The progeny of vaccinated gilts was effectively protected against challenge with K88, K99 and 987P E.coli cultures.

Injections of C.perfringens type C antitoxin into newborn pigs have been effective in preventing the disease, both in field cases, and in experimentally produced infections. The antitoxin is effective if injected either subcutaneously or intraperitoneally (Bergeland, 1972). Sows vaccinated 4 and 2 weeks before parturition with a commercial C.perfringens type C toxoid produced detectable antitoxin levels in milk and colostrum which were transferred to their progeny (Djurickovic et al., 1975). In a study (Hogh, 1976) serum from immunised sows with C.perfringens type C toxoid was administered subcutaneously to piglets after birth, resulting in protective serum titres of beta antitoxin. In the same study, immunised sows developed high titres of antitoxin detectable in colostrum and milk and when passively transferred to their progeny, resulted in an effective protection of the piglets against C.perfringens type C challenge.

(f) Immunisation of species other than pigs against C.perfringens components

Immunisation to C.perfringens cells and toxins in species other than pig is reviewed below.

Sheep can effectively be protected against the enterotoxaemia caused by C.perfringens type D by the immunisation with an aluminium hydroxide-absorbed epsilon toxoid (Jansen, 1967). This immunity is of relatively short duration, and in order to maintain a constant level of protection, repeated immunisations are required. A study (Cameron, 1980) reported the immunisation of

pregnant ewes with 2 doses of an oil adjuvant-absorbed toxoid followed by a single dose of aluminium hydroxide-absorbed toxoid, elicited a good immunity and high antitoxin titres persisted for 12-15 months. Immunisation of lambs against enterotoxaemia has been achieved successfully with single doses of formalin-killed, aluminium-hydroxide absorbed whole culture and with aluminium hydroxide-absorbed toxoid, both preparations being equally effective to protect against enterotoxaemia (Pierson, 1968).

In a study (Bousicaux et al., 1974), the immunisation of sheep with 2 doses of a multi-component bacterin containing formalin-killed whole cultures of C.perfringens types A, B, C and D, elicited serum titres of antibodies to alpha, beta and epsilon toxins which were considered to be protective in field trials.

It has been shown (Fleenor and Stott, 1983) that the parenteral immunisation of pregnant cows, 6 and 3 weeks prior parturition, with a bacterin containing C.perfringens types C and D induced a significant rise in serum and colostrum of antibodies to beta and epsilon toxins which were passively transferred to their calves and demonstrated in sera.

In a study (Lozano et al., 1970) calves were given subcutaneous inoculations with horse serum C.perfringens beta antitoxin; calves of a second group were given the same antitoxin orally, and a third group received colostrum from immunised cows. All calves given the subcutaneous antitoxin inoculations developed detectable amounts of antitoxin in serum only 2 of 9 calves given the antitoxin orally presented detectable antitoxin in serum and, all calves that ingested colostrum showed antitoxin in serum. When challenged with C.perfringens type C all the animals of the first 2 groups developed diarrhoea, whereas the calves of the third group were partially protected, developing a mild form of the disease.

Alpha toxin of C.perfringens has been considered to be a poor antigen (Oakley, 1970). The effectiveness of alpha antitoxin in prevention of experimental gas gangrene in guinea-pigs was reported

(Evans, 1943). There have been conflicting data, however, as to the efficacy of the active immunisation with alpha toxoid in prophylaxis of gas gangrene. It was reported (MacLennan, 1962) that it was impossible to convert C.perfringens alpha toxin to a toxoid. The inconsistent immunogenicities of alpha toxin toxoid were pointed out raising a serious problem in hyperimmunisation of horses (Yamamoto et al., 1970). It was reported (Ito, 1968) that alpha toxin was easily destroyed by formalin and that a careful control of various conditions during toxoiding was necessary to maintain the antigenicity. Ito (1970) showed that alpha toxin could be converted to a toxoid of high immunogenicity and could protect immunised guinea-pigs against gas gangrene.

Niilo et al. (1971) immunised lambs parenterally with formalised cell extract, or bacterin, prepared from sporulating cultures of an enteropathogenic strain of C.perfringens type A. This procedure induced an antibody in the serum which neutralised the enteropathogenic activities of the cell extract. However, protective immunity was not evident when the immunised lambs were challenged by the intraduodenal and the ligated intestinal loop methods with toxic cell extract or with whole cells of C.perfringens.

It has been shown (Uemura et al., 1975) that intraluminal enterotoxin challenge in monkeys confers no detectable serum anti-enterotoxin titres in the animals. Anti-enterotoxin sera have been prepared (Bartholomew and Stringer, 1983) by immunisation of rabbits with a mixture of purified enterotoxin and Freund's complete adjuvant at 7-day intervals for a period of 7 weeks. These antisera, however, have not been proved to be protective to entero-toxin challenge.

4. CONCLUSIONS FROM THE LITERATURE AND OUTLINE OF THE EXPERIMENTAL APPROACH

C.perfringens type A is a potential pathogen of the

gastrointestinal tract of a number of animal species, but little attention has been paid to the role of the bacterium and its toxins in the pig. The strongest association between disease in the pig and C.perfringens type A is in neonatal diarrhoea and diarrhoea in young pigs. The occurrence of C.perfringens type C diarrhoea and its association with vegetative cells and toxin suggests that vegetative cells and alpha toxin may be involved in C.perfringens type A infections in the enteric tract of the neonate. There is some evidence for the involvement of alpha toxin in enteric disease in poultry and cattle but some of the evidence is not convincing.

Recently French workers have described the association of enterotoxin with diarrhoea in fattening pigs and there is a well-marked association between enterotoxins and diarrhoea in several species, principally man.

In view of these conclusions, the role of C.perfringens type A in gastroenteritis was studied. At first (Chapters 3 and 4) studies were carried out on vegetative cells and alpha toxin by isolation from clinical and post-mortem samples. Experimental studies which included animal infection, and protection experiments were carried out. Detailed studies of the relationship of alpha toxin to the disease were carried out and are described in Chapter 5.

Finally, the role of sporulating C.perfringens type A and enterotoxin in porcine gastroenteritis was studied and the results are presented in Chapter 6.

CHAPTER 2

MATERIALS AND METHODS

1. PIG POPULATIONS SURVEYED FOR ORGANISMS AND ANTIBODY

The populations surveyed fell into 3 groups:

(a) 3 local pig farms. Farms 1, 2 and 3.

(b) Diagnostic material.

(c) 4 large pig farms in Eastern Scotland (Farms 4,5,6 and 7)

(a) Local pig farms

Farm 1 was initially a 120 sow herd but grew to 220 sows during the period of study. It fattened all progeny to bacon weight and had a high perinatal mortality problem with some diarrhoea in neonates, diarrhoea at 10 days of age and post-weaning diarrhoea. C.perfringens type A had been isolated by Olubunmi (1982) and this farm was the source of his experimental isolate. Parvovirus, coccidia, K88+ve E.coli, Campylobacter mucosalis, C. hyointestinalis, C.coli and some non-T.hyodysenteriae spirochaetes had all been isolated from enteric disease on this unit.

Farm 2 was a 30-35 herd stocked with National Pig Development High Health Pigs fattening progeny to pork weight but had a perinatal mortality problem and diarrhoea in young pigs 10 days of age. Incidents of C.perfringens type C enteritis had occurred and coccidia, cryptosporidia, rotavirus, porcine epidemic diarrhoea virus and non-T.hyodysenteriae spirochaetes had all been isolated previously. This farm was used as a source of sows for hysterectomy and for those used in vaccination studies.

Farm 3 is a 200 sow herd fattening all progeny to cutter or bacon weight. Perinatal mortality and post-weaning mortality is low but incidents of post-weaning diarrhoea caused by K88+ E.coli had been recorded in the past and were controlled by vaccination. Fewer details of other enteric pathogens were available.

(b) Diagnostic material

This consisted of carcases, organs, faecal samples or faecal swabs submitted to 3 laboratories for diagnosis. The three laboratories were: Glasgow University Veterinary School (whole period of this study), Leeds Veterinary Investigation Centre (4 weeks August - September 1983) and Norwich Veterinary Investigation Centre (2 weeks November 1985).The farm of origin was recorded for each sample and some details such as the problem, the nature of the sample and the results of other examinations performed were available. No history of treatment of the individual pigs providing the samples were available. Material was obtained from 6 farms in the Glasgow area including Farms 1, 2 and 3, from 2 farms at Leeds and from 15 farms at Norwich, a total of 84 samples.

(c) 4 large Eastern Scottish pig farms

All 4 farms belonged to the Pig Health Scheme and were essentially healthy with no obvious enteric disease problems. Only one of these was visited - Farm 4, samples from the remaining farms were taken by Mr. D. Wood the Ministry of Agriculture Veterinarian on routine Pig Health Scheme visits.

Farm 4 was of 500 sows fattening all progeny to bacon weight. No antimicrobial treatments were being used and the management was good.

Farms 5, 6 and 7 were all breeder-fatteners and no enteric disease problems were reported.

2. NECROPSY PROCEDURES

Throughout this study pigs for post-mortem examination were killed and examined according to the following procedure, whether the animals were submitted for diagnosis or were experimental in origin. All examinations could not be performed in every case.

(a) Euthanasia

All live animals were killed by the injection of pentobarbitone sodium (Euthatal May and Baker Ltd., Dagenham) into the anterior vena cava and then exsanguinated. Blood samples were taken from the anterior vena cava prior to euthanasia.

(b) Post-mortem examination

(i) Gross examination

A post-mortem examination was carried out as soon after death as possible; in all cases this was within 24 hours of death. Particular attention was paid to the gastrointestinal tract and the organs of the abdominal and thoracic cavity were examined for the presence of gross lesions. The gross changes noted were recorded.

The appearance of the mucosal surfaces and the consistency of the contents were noted. Sample lengths of the gastrointestinal tract were opened and the various segments were examined for the location and distribution of the gross lesions.

Samples of small intestine were opened in sterile physiological saline and viewed by low power microscopy to observe the villous architecture.

(ii) Histological procedures

Samples for light microscopy were fixed in 10 per cent neutral

buffered formol saline for at least 48 hours before trimming and embedding. The samples were embedded in paraffin wax and sections were cut at 4-6 μm and stained routinely with haematoxylin and eosin. All the stained sections were examined under the light microscope and the results recorded.

Frozen sections of tissue were prepared for immunofluorescence by excision in blocks from the intestine or spleen of freshly-killed pigs. They were placed in individual plastic containers and embedded in O.C.T. compound (Tissue Tek, Ames Laboratories) for all examinations other than for alpha toxin studies and frozen in liquid nitrogen immediately. Samples were then stored at -70°C until sectioning. 4-6 μm sections were prepared in a cooled cryostat at -20°C and placed on slides for staining by direct and indirect fluorescence using the materials and methods described below under immunofluorescent techniques. All sections were fixed in cold acetone.

(iii) Bacteriological procedures

Both rectal faeces samples or swabs and material taken at post-mortem examination were examined bacteriologically. A general procedure was adopted which is given here. Special examinations e.g. for spores and for the presence of toxins were carried out but are given in the appropriate section below.

Direct smears. Direct smears were made on glass slides and heat fixed. The material was taken from faeces directly but that from the stomach, duodenum, jejunum, ileum, caecum, colon and liver was taken with a stiff bacteriological loop after the surface of the organ had been seared. Fixed smears were stained by Gram's method and examined by light microscopy using oil immersion. Direct smears could not be made from swabs submitted in transport medium.

Cultural examination. A limited number of routine cultural procedures was carried out on all specimens. All samples were inoculated into 7 per cent sheep blood agar plates (Oxoid Blood

Agar Base No. 2, Oxoid CM271 with added formolised sheep blood, Gibco Biocult Ltd.) and MacConkey Agar Plates (Oxoid CM7 with added 1 per cent neutral red) for aerobic incubation. A 7 per cent horse blood agar plate (containing 7 per cent defibrinated horse blood Oxoid SR50) was inoculated for anaerobic incubation. A 5 per cent Egg Yolk Agar Plate (5 ml Egg Yolk Agar Suspension, Oxoid SR47, added to sterile molten Nutrient Agar Base No. 2, Oxoid at 55°C) was also inoculated for anaerobic incubation. A Campylobacter medium plate (7 per cent horse blood agar supplemented with antibiotic SR69, Oxoid Ltd.) was also inoculated and incubated microaerophilically.

A single sample from the gut of each animal and each faecal sample was examined for the presence of Salmonella spp. by the inoculation of tetrathionate broth (Oxoid, CM29) incubation for 24 hours and plating on Salmonella Shigella Agar (Oxoid CM99) or Desoxycholate Citrate Agar (Oxoid CM35). Non-lactose-fermenting colonies were examined further to confirm their identity as salmonellae.

Atmospheric conditions. Cultures were incubated in aerobic, microaerophilic and anaerobic conditions. Cultures for aerobic incubation were placed directly into the incubator.

Microaerophilic conditions were produced by evacuating loaded anaerobic jars with no catalyst (Whitley Scientific Ltd) to a pressure of 20 inches of mercury, using a vacuum pump. The jars were then flooded with a 5 per cent carbon dioxide and 95 per cent hydrogen mixture (British Oxygen Co. Ltd., Special Gases Division).

Anaerobic conditions were produced by evacuating anaerobic jars containing the cultures and fitted with cold catalysts to a pressure of 24 inches of mercury using a vacuum pump. The evacuated jars were then filled with the carbon dioxide-hydrogen mixture. Gassed jars were evacuated once more and gassed again in order to flush out any remaining oxygen.

Temperature and period of incubation. Cultures were incubated at 37°C for 24 hours except those in Selective medium for Campylobacters which were incubated for 48 hours.

Examination of inoculated plates and the identification of bacterial isolates. This was carried out on a presumptive basis in most cases. Examination of colonies was made by the naked eye and under dissecting microscope. The colonies seen were described in terms of their morphological characters such as size, elevation, outline, colour, consistency and their effect on the medium. Colonies were presumptively identified by these characters. Smears were made from colonies on slides, air dried and heat fixed. They were stained by Gram's method. The stained slides were examined under the oil immersion lens. This method made it possible to determine the cellular morphology of the bacteria and their reaction to Gram's stain. Further identification of bacteria was carried out in more detail using the criteria for identification of Cowan and Steel (1974) in the initial stages of the study and when considered necessary later. The only exception to this was the identification of C.perfringens which is described in detail below.

(iv) Methods for the identification of Clostridium perfringens

Colonial morphology. Colonies with the morphology and haemolysis characters described for C.perfringens in Chapter 1 were considered to belong to that species on presumptive grounds.

Cellular morphology and Gram staining reaction. Smears of colonies considered to be C.perfringens were prepared and stained by Gram's method. The presence of bacteria with the morphological characters of C.perfringens was considered to be additional evidence of identification.

Confirmation by biochemical tests. Colonies presumed to be those of C.perfringens were subjected to the biochemical tests outlined by Cowan and Steel (1974). These were carried out in anaerobic conditions at 37°C.

Utilisation of sugars was tested using sugar broth base No. 2 prepared using the following ingredients in the basal medium; Lab Lemco beef extract (Oxoid) 5g; Oxoid bacteriological peptone 10g; Sodium chloride (May and Baker) 3g and disodium hydrogen phosphate May and Baker) 2g were dissolved in 1 litre of deionised water and steamed for 30 minutes. When the medium was cool, the pH was adjusted to 7.2 to 7.3 and 12 ml of indicator (0.1g Bromothymol blue, May and Baker; 2.5 ml 0.1 M NaOH (Analar) and 47.5 ml of distilled water) were added per litre. This medium was dispensed in 100 ml volumes. One gram of the appropriate sugar (Glucose, Maltose, Lactose, Salicin and Sucrose) was added to 100 ml of this medium. When each sugar was dissolved, it was dispensed in 4 ml volumes into Bijou bottles containing Durham tubes. The bottles were then sterilised by tyndallisation. Bijou bottles were inoculated with cultures of bacteria under test. The results were recorded after 24 hours but inoculated cultures were not discarded until 7 days after inoculation.

Indole production was determined by growing the culture in 1 per cent peptone water (Oxoid) and testing with Kovacs reagent.

Nitrate reduction was determined on cultures grown in nutrient broth supplemented with 0.1 per cent potassium nitrate (Analar) and incubated for 5 days. They were then tested for the presence of nitrite.

Hydrogen sulphide production was assessed on Triple Sugar Iron agar (TSI) - Oxoid. Slopes of TSI were inoculated and incubated for 48 hours. Production of hydrogen sulphide was indicated by a black colour.

Litmus milk (Oxoid) was used in 10 ml quantities and incubated for 7 days at 37°C when the result was recorded.

Gelatin liquefaction was determined using Nutrient gelatin (Oxoid) after 15 days of incubation at 37°C. Cultures were placed in a refrigerator at 4°C for 2 hours before being recorded as positive.

Urease production was determined by the inoculation of Urea Broth Medium (Oxoid) with added Urea solution at 40 per cent (Oxoid). Inoculated broth was incubated and examined after 24 hours.

The appearance of cultures on egg yolk agar was determined by inoculation onto plates and incubated for 24 hours. The changes in the medium were recorded.

Digestion of cooked meat was determined following the inoculation of Cooked Meat Medium (Difco) and incubation at 37°C for 14 days. During the study both Cooked Meat medium (Oxoid) and Cooked Meat medium (Difco) were used. Both media were prepared with deionised water and dispensed in 10 ml volumes in Universal glass bottles and in 50 and 100 ml volumes in medical flat bottles.

Tests for toxin production. Haemolytic toxin effects were determined by streaking cultures on 7 per cent sheep blood and 7 per cent horse blood agar plates. These were incubated anaerobically at 37°C for 24 hours and haemolytic activity around colonies was recorded.

Nagler's reaction. On one-half of an egg yolk agar plate two or three drops of C.perfringens antitoxin A (Wellcome Reagents Ltd.) were spread and allowed to dry. The plate was then streak-inoculated with cultures of clostridia. Plates were examined after anaerobic incubation for 24 hours. Inhibition by antiserum of the production of an opalescent halo was a positive reaction.

Serum neutralisation tests in mice. Cultures were seeded in cooked meat medium previously enriched with 1 ml of a sterile glucose solution (10 per cent weight-volume) and 0.5 ml of a sterile solution of calcium chloride (25 per cent weight-volume) and incubated overnight. 23g white mice were challenged intraperitoneally with 0.5 ml of the supernatant previously

filtered through a cellulose acetate filter of approximate pore diameter (APD) 0.22 μm (Millipore Ltd.). The identity of the toxin was determined by neutralisation tests involving the incubation of 1.3 ml volumes of the culture supernatant with 0.3 ml volumes of specific antisera anti C.perfringens types A, B, C, D and E (Wellcome Reagents Ltd.), and with filtered supernatants treated with 0.1 per cent trypsin (Difco 1:250) similarly neutralised with the various antisera; these mixtures were allowed to stand at 37°C for 30 minutes and 0.5 ml was inoculated intraperitoneally into each of two mice. The animals were observed from time to time for the next few hours and kept for 48 hours.

Criteria for the identification of an isolate as C.perfringens type A. Isolates identified presumptively as C.perfringens were confirmed as such by the biochemical tests described above. Identification as type A was based on the absence of toxins of other types by the neutralisation test.

Maintenance of cultures of C.perfringens Type A. Pure cultures of Clostridium perfringens type A were maintained in Cooked Meat medium in a dark place at room temperature. All isolates were subcultured into a fresh Cooked Meat medium at least once every 6 months.

(v) Virological and parasitological procedures

Virological examinations. These were carried out from intestinal contents taken from a small number of piglets presenting diarrhoea. Each sample was suspended in sterile physiological saline (0.85 per cent of sodium chloride in deionised water) and centrifuged at 11,000g for 15 minutes as a clarification run. The supernatant was taken and centrifuged at 30,000g for 45 minutes. The pellets were resuspended in a few drops of physiological saline and negatively stained. These samples were examined using an AEI 6B electron microscope for the presence of viral particles. Presence or absence of viral particles was recorded.

Cases examined at Norwich V.I. Centre were examined by the laboratory staff for Transmissible Gastroenteritis and Porcine Epidemic Diarrhoea viruses by immunofluorescence on frozen sections.

Parasitological examinations. Scrapings of ileal mucosa and faecal samples were examined for Cryptosporidium using light microscopy. Giemsa stained smears of faeces or intestinal scrapings were used to detect cryptosporidia. The presence or absence of cryptosporidia was recorded. Histological sections of the small intestinal mucosa were examined for the presence of Isospora suis when such sections were available.

3. STUDIES WITH CLOSTRIDIUM PERFRINGENS TYPE A

Isolates of C.perfringens type A obtained in local pig farms and diagnostic materials stored as described above were used for these studies. Two types of study were carried out. One was on the vegetative cells to determine alpha toxin production and to produce crude and later pure alpha toxin. The other was on the ability of strains to sporulate and to produce enterotoxin and the production of pure enterotoxin.

(a) Studies on vegetative cells and alpha toxin

(i) Tests of C.perfringens type A isolates for alpha toxin production

Stored cultures were plated on horse blood agar and incubated anaerobically for 16 hours. A single colony was picked from each isolate for further investigation. This colony was then used to inoculate peptone medium containing meat particles and incubated for 6 hours at 37°C. The media were centrifuged at 20,000g for 30 minutes at 4°C. The supernatants were then filtered through a cellulose acetate filter of A.P.D. 0.45 µm (Millipore Ltd.) and tested for the presence of alpha toxin activity. Alpha toxin activity was tested by means of turbidity in saline extract of egg

yolk (Smyth and Arbuthnott, 1974); haemolysin acitivity in sheep erythrocytes suspension (Möllby et al., 1976) and the neutralisation test in mice.

Turbidity in saline extracts of egg yolk. The substrate was prepared from egg-yolk emulsion (Oxoid), diluted 1 in 10 with physiological saline solution. The saline extracts were adjusted to neutrality with Sodium bicarbonate. The test was performed by adding 0.5 ml of the egg-yolk solution to an equal volume of serial twofold dilutions of alpha toxin samples to be tested. The tubes were incubated for one hour at 37°C and examined for turbidity which indicated lecithinase activity. Suppression of the reaction by anti-alpha toxin antiserum or by C.perfringens type A diagnostic antiserum was performed as evidence of the presence of alpha toxin in samples.

Haemolysin activity in sheep erythrocytes. Sheep erythrocytes were washed three times by centrifugation at 1,000g in 0.02M Tris-HCl buffer pH 7.0 containing 0.15M of Sodium Chloride, the packed erythrocytes were resuspended as a 1 per cent suspension in the same buffer. Serial twofold dilutions of test samples were made in microtitre plates and an equal volume of erythrocyte suspension was added. Haemolysis was estimated visually after one hour at 37°C and finally determined after 2 hours at 4°C when the erythrocytes had settled. Anti-alpha toxin antiserum was used for neutralisation of haemolysis.

Mouse Lethality Test. This test was carried out by making serial twofold dilutions of the supernatant from a culture of each isolate in physiological saline solution. 1 ml amounts were injected intraperitoneally into 23g Swiss White mice. Each dilution was tested in 2 mice and the mice were observed for 48 hours for signs of illness or death. A mouse lethal dose was a reciprocal of the highest dilution of the toxin which was lethal to the 2 mice within 48 hours.

(ii) The preparation of crude alpha toxin

C.perfringens type A isolate used was isolate No. 7, also used for the experimental infection of piglets described below and in Chapter 3. It was grown in a peptone medium containing meat particles according to the method of Takahashi et al. (1981). The medium contained the following constituents per litre (5 litres were prepared): 45g of proteose peptone (Difco), 14.32g of Na_2HPO_4, .12 H_2O, 1.36g of KH_2PO_4, 0.2g of $MgSO_4$. $7H_2O$, 0.5g of yeast extract (Difco), 5.75 mg of $ZnSO_4$. $7H_2O$, 3.94 mg of $MnCl_2$. $4H_2O$, 10g of fructose, 7 mg of $FeSO_4$. $7H_2O$, 1g of $KHCO_3$, 0.01g of sodium thioglycollate, and 5g of meat particles (Difco). The medium was adjusted to pH 7.6 with 10 N NaOH and sterilised in the autoclave. When the medium cooled down to 37°C, it was inoculated with the C.perfringens type A isolate. After incubation for 6 hours at 37°C, the organisms were removed by centrifugation at 10,000g for 60 minutes at 4°C. The sediment was discarded and the supernatant was precipitated with solid ammonium sulphate at 50 per cent saturation in a cold water bath at 4°C, the ammonium sulphate was added slowly to the supernatant, starting with a saturation of 5 per cent and increasing by 5 per cent until 50 per cent saturation was reached. The precipitate was centrifuged at 10,000g for 60 minutes at 4°C. The supernatant was discarded and the sediment was dissolved in one litre of 0.05M Tris-HCl buffer pH 7.5 and dialysed exhaustively against the same buffer. The dialysis residue was centrifuged to remove the precipitate formed and the supernatant fluid was concentrated to a 100 ml volume by dialysis against polyethylene glycol (molecular weight 20,000, Analar) at 4°C. The dialysis tubing was rinsed in tap water to remove the excess of polyethylene glycol and the concentrated fluid was collected.

Toxin levels were determined by dilutions of the concentrated toxin in physiological saline injected intraperitoneally in 20-23g Swiss white mice in 1.0 ml quantities. The toxin titre was the highest dilution causing death in 24 hours.

(iii) The preparation of pure alpha toxin

The Clostridium perfringens type A isolate used in the preparation of alpha toxin described above was used for the production of pure alpha toxin. The steps described above for the production of crude concentrated alpha toxin were followed, the only variable was the production of alpha toxin from 10 litres of medium instead of 5.

100 ml of concentrated toxin were dialysed against 0.05M Tris-HCl buffer pH 7.5 containing 0.15M of Sodium Chloride. The toxin was applied to a column (2.6 x 90 cm) packed with Ultrogel AcA-34 (LKB Instruments Ltd.) equilibrated with the same buffer. Filtration proceeded at a flow rate of 7.2 ml/hour. Fraction and exclusion volumes were 5 and 110 ml respectively. A plot of absorbance at 280 nm and enzyme activity of the fractions was recorded. Fractions containing alpha toxin activity from ten column runs were pooled and concentrated in dialysis tubing against polyethylene glycol-20,000.

Isoelectric focusing of concentrated fractions. The concentrated fractions (20 ml in total) were subjected to preparative isoelectric focusing following the method described by Johnstone and Thorpe (1982). The concentrated material was dialysed against a solution of 1 per cent glycine for 18 hours. 4g of Ultradex gel (LKB Instruments Ltd.) were added to a solution containing the concentrated fractions to be focused, 20 ml, distilled water, 80 ml and 5 ml of carrier ampholytes (Ampholine pH 5-8 , LKB Instruments Ltd.) at a final concentration of 5 per cent. This solution was placed onto a glass plate (20 x 12 x 0.4 cm). The gel layer was air dried until 20 per cent of water was evaporated. The plate was mounted horizontally on a cooling block with circulating water at 4°C and contact with phosphoric acid 1M on the anodic side and potassium carbonate 1M in the cathode was made by paper strips. Focusing was carried out at a constant voltage of 200V for 4 hours followed by a constant voltage of 600V for 12 hours. The gel was removed from the chamber and covered

with a piece of filter paper to blot focused proteins. The paper was removed, dried and washed in several changes of 10 per cent trichloroacetic acid and then stained with Coomassie brilliant blue at 0.025 per cent. Protein bands in the gel were located by comparison with the stained paper. Proteins localised in the gel were scraped off, resuspended in phosphate buffered saline pH 7.2, mixed thoroughly and centrifuged at 400g for 5 minutes. The supernatants of different fractions were removed and precipitated with solid ammonium sulphate at 95 per saturation then centrifuged at 2,500g for 10 minutes. The pellets were resuspended in small volumes of phosphate buffered saline pH 7.2 and passed through a Sephadex G-25 column for rapid desalting. Protein content in fractions was estimated by their absorbance at 260 nm and 280 nm in a spectrophotometer.

Collected fractions were tested for their alpha toxin activity by haemolysis of sheep erythrocytes and by turbidity in egg yolk suspensions.

The focused fraction which presented alpha toxin activity was subjected to purity tests by double diffusion and by immunoelectrophoresis by the methods described in the section on immunological methods below and by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Crude ammonium sulphate precipitated alpha toxin, fraction eluted from Ultrogel AcA-34 colum and isoelectric focusing purified alpha toxin were electrophoresed on a polyacrylamide gel according to the method described by Johnstone and Thorpe (1982). Purified alpha toxin (Phospholipase C XII, Sigma London Chemical Company Ltd.) was used as a reference standard. Samples were dialysed against 0.125M Tris-HCl buffer pH 6.8 for 24 hours. Supporting medium for electrophoretic separation consisted of a homogeneous mixture of 7 per cent acrylamide and 0.16 per cent NN'-methylene bisacrylamide in distilled water. 50 µl of N,N,N,N'-tetramethylethylene diamine (TEMED) and 25 mg of ammonium persulphate were added for each 100

ml of the reaction medium. The gel solution was poured into a gel mould (LKB Instruments Ltd.) The gel plates used were 16 x 16 cm, 3 mm thick. A stacking gel containing 3 per cent of acrylamide and 0.08 per cent of bisacrylamide was polymerised on top of the separating gel. The samples were adjusted to a protein concentration of 300 µg per ml and loaded into the slots of the stacking gel. Electrophoresis was carried out in a Tris-glycine buffer pH 8.3 at 50V for 30 minutes and then the voltage was increased to a constant value of 200V for 6 hours. The mould was cooled with circulating tap water. Bromophenol blue was used to monitor the progress of the electrophoresis. The polyacrylamide gel was stained with Coomassie brilliant blue at 0.025 per cent.

Purified alpha toxin was stored frozen at -20°C until required.

(b) Studies with sporulating cells and enterotoxin

(i) Culture media

All C.perfringens type A isolates were tested for their ability to sporulate and to produce enterotoxin. All isolates were tested in 3 sporulation media: Duncan and Strong medium (Duncan and Strong, 1968) comprised of: 1.5 per cent proteose peptone, 0.4 per cent yeast extract, 0.1 per cent sodium thioglycollate (Oxoid L120), 0.4 per cent raffinose (BBL) and 1.0 per cent Na_2HPO_4. $7H_2O$; Ellner medium (Ellner, 1956) consisting of: 1.0 per cent proteose peptone, 0.3 per cent yeast extract, 0.3 per cent starch (BBL), 0.01 per cent $MgSO_4$, 0.15 per cent KH_2PO_4, and 5 per cent Na_2HPO_4. $7H_2O$; and Tsai medium (Tsai et al., 1974) comprised of: 1.5 per cent proteose peptone, 0.3 per cent yeast extract, 0.3 per cent starch, 0.01 per cent $MgSO_4$, 0.1 per cent sodium thioglycollate, and 1.1 per cent Na_2HPO_4. $7H_2O$.

(ii) Cultural methods for sporulation studies

Stock cultures in cooked meat medium were kept in the dark at

room temperature. 2 ml of stock culture were inoculated into 10 ml of fluid thioglycollate medium (BBL Code 11720), heat-activated at 80°C for 10 minutes and incubated at 37°C for 18 hours. 2 ml of this culture were then transferred to 10 ml of fluid thioglycollate, heat-activated at 80°C for 10 minutes and incubated for 6 hours at 37°C. Volumes of 0.5 ml of this culture were inoculated into each of 10 ml volumes of the 3 sporulation media. These media were previously heated in boiling water and then cooled rapidly in ice before being inoculated. The inoculated media were incubated at 37°C for 6-12 hours to obtain maximum numbers of sporulating cells, or for 24 hours to test enterotoxin released into the culture supernatant.

Smears of sporulation cultures were stained by Gram's stain. Vegetative cells and spores were observed and enumerated in the transmitted light microscope. Percentage of sporulation in the cultures was obtained from the ratio of the number of spores to the total number of vegetative cells and spores.

(iii) Methods for the enumeration of vegetative cells and spores in faecal samples from pigs

Porcine faecal specimens were examined for vegetative cells and spores as follows: 1g of the sample was added to 10 ml of sterile phosphate buffered saline and homogenised. Ten-fold serial dilutions were carried out in sterile phosphate buffered saline, then 30 µl amounts of the dilutions were inoculated onto the surface of a commercially prepared tryptone-sulphite agar medium (Oxoid, CM543) containing a concentration of D-Cycloserine (Sigma, C6880) of 400 µg/ml. For assessment of spores the same dilutions were heated in a water bath at 80°C for 10 minutes before inoculation of tryptone-sulphite agar plates. Inoculated plates were left to dry at room temperature and then inoculated anaerobically for 24 hours. Following incubation, the plates were examined for the presence of blackened colonies which were counted. The count was considered to give the number of colony forming units. (Miles and Misra, 1938).

(iv) The production and purification of enterotoxin

C.perfringens type A isolate No. 29 previously tested for its ability to sporulate and produce enterotoxin as described was used for the production and purification of enterotoxin. The isolate which was maintained in cooked meat medium was inoculated onto horse blood agar plates and incubated anaerobically at 37°C for 18 hours. Three to five colonies from the horse blood agar plate were then inoculated into freshly steamed cooked meat medium, and incubated at 37°C for 18 hours; the culture was heat-activated at 80°C for 10 minutes and 1 ml inoculated into 10 ml of fluid thioglycollate medium and incubated at 37°C for 18 hours, the culture was then heat-activated at 80°C for 10 minutes and inoculated into 100 ml of freshly prepared thioglycollate medium and incubated at 37°C for 6 hours. The entire culture was then inoculated into 10 litres of Duncan and Strong medium. No special precautions were taken to obtain anaerobic conditions other than the inclusion of sodium thioglycollate and stationary incubation of the culture. The culture was incubated at 37°C and after 4 hours samples were removed at various time intervals and examined by Gram's stain for growth and sporulation. When more than 50 per cent of the cells had produced spores, (usually 5 to 8 hours) the culture was cooled at 4°C, the cells were harvested by centrifugation at 10,000g for 60 minutes at 4°C and washed twice, by centrifugation at the same speed, using phosphate buffered saline.

Extraction of enterotoxin from sporulated cells. The washed cell pellet was resuspended in 400 ml of phosphate buffered saline, and the suspension was subjected to intervals of ultrasonic treatment at an amplitude of 14 μm by means of an ultrasonic disintegrator until all sporangia were disrupted and only free spores were detected. Sonication was monitored by the examination of Gram's stained smears. The suspension was centrifuged at 15,000g for 30 minutes at 4°C. The sediment was discarded and the supernatant precipitated with solid ammonium sulphate at 40 per cent saturation. The precipitate was centrifuged at 10,000g for 60

minutes at 4°C and the sediment was redissolved in 100 ml of phosphate buffered saline. The protein solution was adjusted to 15 per cent saturation with respect to ammonium sulphate, centrifuged at 10,000g for 60 minutes at 4°C and the resulting precipitate dissolved in 30 ml of phosphate buffered saline. This solution was dialysed overnight against the same buffer and any precipitate removed by centrifugation.

Gel filtration on Sephadex G-200. The protein solution from the preceding step was applied to a column (2.6 x 90 cm) packed with Sephadex G-200 (Pharmacia) equilibrated with phosphate buffered saline 0.02M pH 7.2. The filtration proceeded with the same buffer at a flow rate of 8.7 ml/hour. Fraction and exclusion volumes were 4.5 and 90 ml respectively. A plot of absorbance at 280 nm of the fractions was recorded. The fractions presenting enterotoxin activity from three column runs were pooled, freeze-dried and stored at -20°C.

Polyacrylamide gel electrophoresis. The ammonium sulphate precipitates and the active fraction eluted from Sephadex G-200 column were electrophoresed on a polyacrylamide gel, following the method previously described. All samples were adjusted to a protein concentration of 500 µg per ml. Purified enterotoxin provided by M. Stringer, Central Public Health Laboratory, London, was used as a reference standard.

(v) Methods to test enterotoxin activity

Toxicity test in mice. For the toxicity test in mice, serial double dilutions of crude or purified enterotoxin in 0.02M phosphate buffered saline pH 7.2, were inoculated intraperitoneally in 0.5 ml amounts into each of two mice per sample. Swiss White mice of either sex weighing 20 to 25g were used. The animals were observed at intervals for 48 hours to determine lethality of the samples. The mouse lethal dose (MLD)/ml was expressed as the reciprocal of the highest dilution which was lethal to two mice within 48 hours.

Toxicity test in Vero cells. Vero (African green monkey kidney) cells were purchased (Gibco Europe Ltd.) and subcultured in 75 cm^2 tissue culture flasks (Gibco) with Medium 199 supplemented with 5 per cent foetal calf serum (Gibco) and 0.75 per cent sodium bicarbonate. When monolayers reached confluency they were removed by trypsinization with 0.25 per cent trypsin in calcium and magnesium-free Hank's balanced salts solution (Gibco). Trypsinized cells were then subcultured in the same supplemented Medium 199 to a microplate-96 wells (Gibco). Each well was inoculated with 150 µl of a cell suspension containing approximately 5 x 10^3 cells/ml. Culture plates were incubated for 24 hours, at 37°C in a humidified atmosphere containing 5 per cent CO_2 in air, allowing growth to confluency. The cells were washed once with 150 µl of calcium and magnesium-free Hank's balanced salts solution, and dilutions of samples to be tested for enterotoxin in Medium 199 without serum added. Samples were either filtrates of cultures or filtrates of pig faeces.

Plates were read after 16 hours' incubation under an inverted microscope. Morphological damage to Vero cells was recorded. Toxic units (TU)/ml were expressed as the reciprocal of the highest dilution of enterotoxin which produced morphological changes of Vero cells.

4. IMMUNOLOGICAL METHODS

(a) Preparation of antigen

(i) Preparation of whole culture antigen

Clostridium perfringens type A (Isolate No. 7) was grown in the peptone medium with meat particles described above. Five litres of the medium were inoculated and incubated for 16 hours at 37°C. The culture was centrifuged at 2,000g for 30 minutes to remove meat particles. The supernatant was taken and the sediment discarded.

Viable counts of bacteria present in the suspension were determined.

One ml 0.6 per cent formaldehyde was added per 100 ml of whole culture for its inactivation and kept at 37°C for 14 days, after this time the whole culture bacterin was concentrated to a 600 ml suspension in a dialysis tubing covered by polyethylene glycol 20,000.

Sterility tests were performed by inoculating 1.0 ml amounts of the bacterin in 5 flasks containing 50 ml of thioglycollate broth and incubating them at 37°C for 8 days. The incubated broths were inspected visually for signs of bacterial growth and cultured on horse blood agar plates incubated aerobically and anaerobically. The presence of colonies was considered to indicate contamination or incomplete inactivation.

Detoxification was assessed by the intraperitoneal injection of 0.5 ml of the formalised suspension into 2 White Swiss Mice 23g in weight. These were observed for signs of illness for 48 hours. In the absence of clinical signs the material was considered inactivated.

The protein concentration of the suspension was estimated by the method of Lowry et al. (1951). The bacterin was absorbed to a suspension of aluminium hydroxide (Alhydrogel, Superfos Export Co. Denmark) to give a final concentration of 2.18 mg of aluminium hydroxide per ml of bacterin. The pH of the adsorbed material was adjusted to pH 7.0 with IN HCl. Agitation was constant during its addition to the bacterin. The bacterin was preserved with thiomersal at 0.005 per cent, dispensed in 10 ml Universal bottles and kept at 4°C. The finished product appeared as a floccular precipitate which could be resuspended by gentle shaking.

(ii) Preparation of freeze-dried whole cell oral antigen

Clostridium perfringens type A isolate No. 7 was grown in the

peptone medium with meat particles described above. 10 litres of the medium were inoculated and incubated for 16 hours at 37°C. The culture was centrifuged at 10,000g for 60 minutes. The supernatant was discarded and the sediment which contained the bacterial cells was resuspended in sterile phosphate saline buffer, the cells were washed twice with the same buffer by centrifugation and resuspended in 250 ml of the buffer.

Viable counts of bacteria present in the suspension were determined by the method described by Miles and Misra (1938). The bacterial cells were inactivated by the addition of 1 ml 0.6 per cent formaldehyde per 100 ml of suspension and kept at 37°C for 14 days.

Sterility and completeness of detoxification were tested as described above.

The protein concentration of the suspension was determined by the Lowry method and recorded.

The bacterial cell suspension was freeze-dried in 10 ml volumes in glass Universal bottles using a Modulyo Freeze Dryer (Edwards). The freeze-dried material was stored in sealed sterile containers at 4°C until used. 95g freeze-dried cells were mixed with 950g Maize starch (Cornflour, Fine Fare Ltd.).

(iii) Preparation of crude toxoid

Crude alpha toxin was prepared as described above. Detoxification was carried out using the method of Ito (1968) in which 0.2 ml of formaldehyde 40% w/v (Koch Light Labs) to 100 ml of the crude toxin to which l-Lysine (BDH) had been added to give a final concentration of 0.05M. The toxin was kept at a temperature of 28°C for 8 days. The inactivated crude toxin was then tested for the presence of residual toxicity every 4 days by mouse inoculation and haemolysis of sheep red cells until complete inactivation was attained.

Sterility was assessed using the method described above.

Protein concentration of the toxoid was estimated by the Lowry method .

The toxoid was adsorbed to a commercial formulation of aluminium hydroxide giving a final concentration of 2.18 mg of aluminium hydroxide per ml of toxoid. The pH of the adsorbed material was adjusted to 7.0 with IN HCl. The toxoid was preserved with thiomersal at 0.005 per cent, dispensed in 10 ml Universal flasks and kept at 4°C. The adsorbed toxoid appeared as a floccular precipitate which, when required for inoculation, could be resuspended by gentle shaking.

(iv) Preparation of pure alpha toxin and enterotoxin

These products were prepared as above but not inactivated before use.

(v) Preparation of pure porcine secretory IgA

Secretory IgA was purified, to produce an antiserum against it, according to the method described by Bourne (1969).

Treatment of sow's milk. Milks from 3 sows from Farm B were pooled (220 ml) and centrifuged at 20,000g for one hour then the whey between the floating fatty layer and the pellet was collected (140 ml) and mixed with 70 ml of physiological saline and centrifuged again at 20,000g for one hour. The clarified whey was collected. 100 ml of clarified whey were precipitated with solid ammonium sulphate at 50 per cent saturation under gentle agitation at 4°C for 6 hours then the precipitate was centrifuged at 7,000g for 30 minutes. The supernatant was discarded and the pellet resuspended in 10 ml of physiological saline and left in dialysis against the same solution for 16 hours. The dialysed solution was stirred and the pH was brought to 4 by the addition of hydrochloric acid, and the solution was centrifuged at 30,000g for 30 minutes at

4°C. The pellet was discarded and the supernatant neutralised with 2M Tris. The solution was centrifuged again at 30,000g for 30 minutes and the supernatant passed through a 0.45 µm filter to give a final volume of 5 ml.

Gel filtration on Sephadex G-200. This 5 ml of purified whey solution was applied to a column (3.2 x 100 cm) packed with Sephadex G-200 (Pharmacia Ltd.) equilibrated with 0.1M sodium phosphate buffer pH 6.8. Filtration was carried out with the same buffer at 5.6 ml/hour. The exclusion volume was 260 ml. A plot of absorbance at 280 nm was recorded (Fig. 1). Fractions eluted from the first major peak were pooled together and concentrated by dialysis against polyethylene glycol MW 20,000.

Ion exchange chromatography. The sample was dialysed against .02M Tris-HCl buffer pH 7.4 and applied to a column (1.5 x 20 cm) of diethylaminoethyl (DEAE) cellulose (Pharmacia Ltd.) equilibrated in the same buffer. The column was eluted with about 400 ml of the buffer followed by stepwise elution with the same buffer at different molarities (0.02, 0.05, 0.1, 0.125, 0.15, 0.2, 0.3 and 0.5M). Absorbance at 280 nm was monitored (Fig. 2). Peaks eluted with 0.1M and 0.125M buffers were collected and subjected to IgA purity tests by means of immunoelectrophoresis and double diffusion tests as described below. A single arc of precipitation was observed when the presumed IgA was tested by immunoelectrophoresis against commercial rabbit anti-swine whole serum (Miles) (Fig. 3) and by double immunodiffusion (Fig. 4). The 0.125M peak contained most IgA. The purified IgA was stored at -20°C in sealed vials until required.

(b) The production of antisera and other immune products

(i) Animal species used

Antisera were prepared in mice, rabbits and pigs. Both 4 month old weaned pigs and pregnant sows were used.

Mice. Mice used in the studies were Swiss White mice bred in

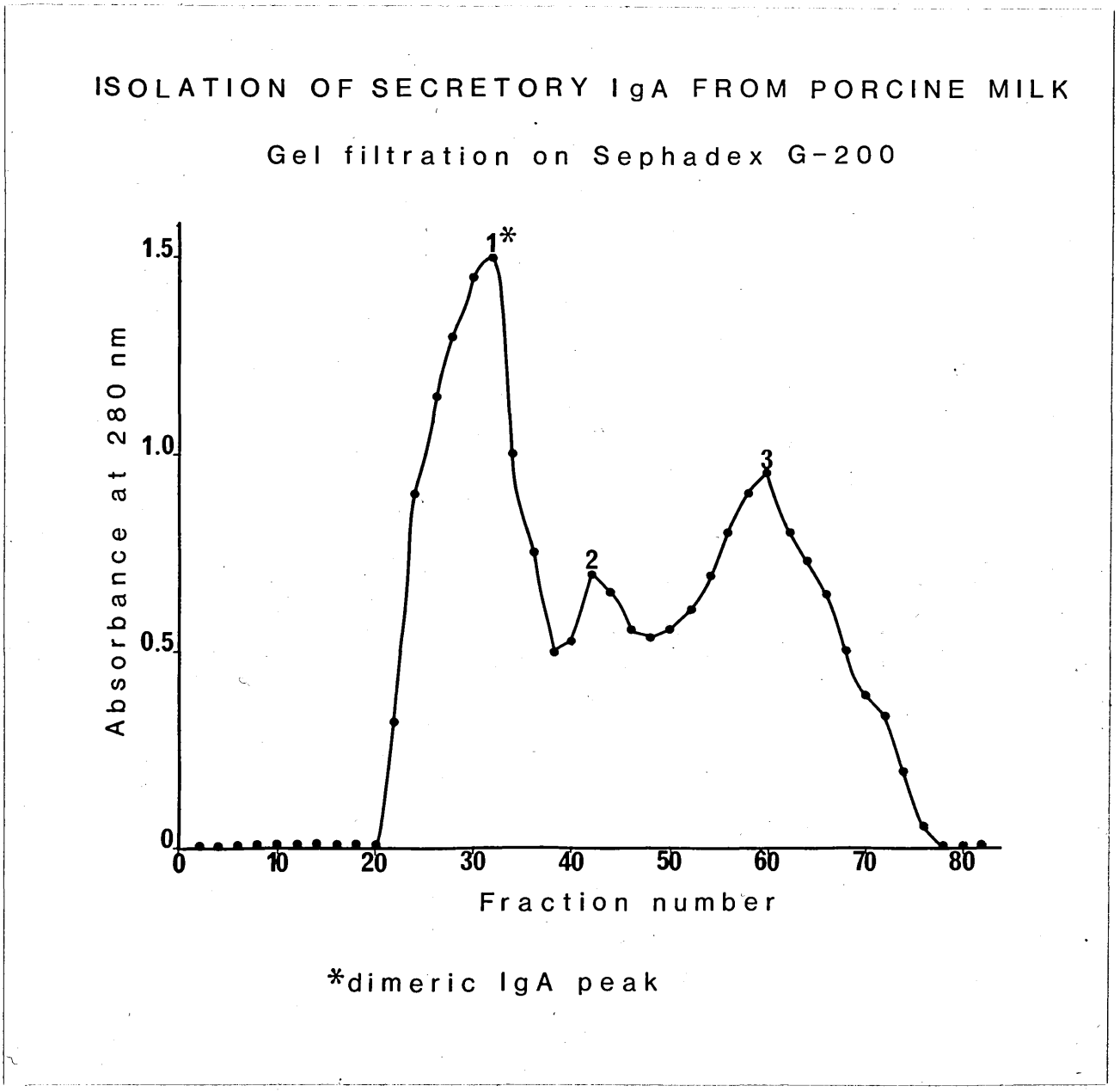

FIG. 1: Separation of secretory IgA from porcine milk on sephadex G-200.

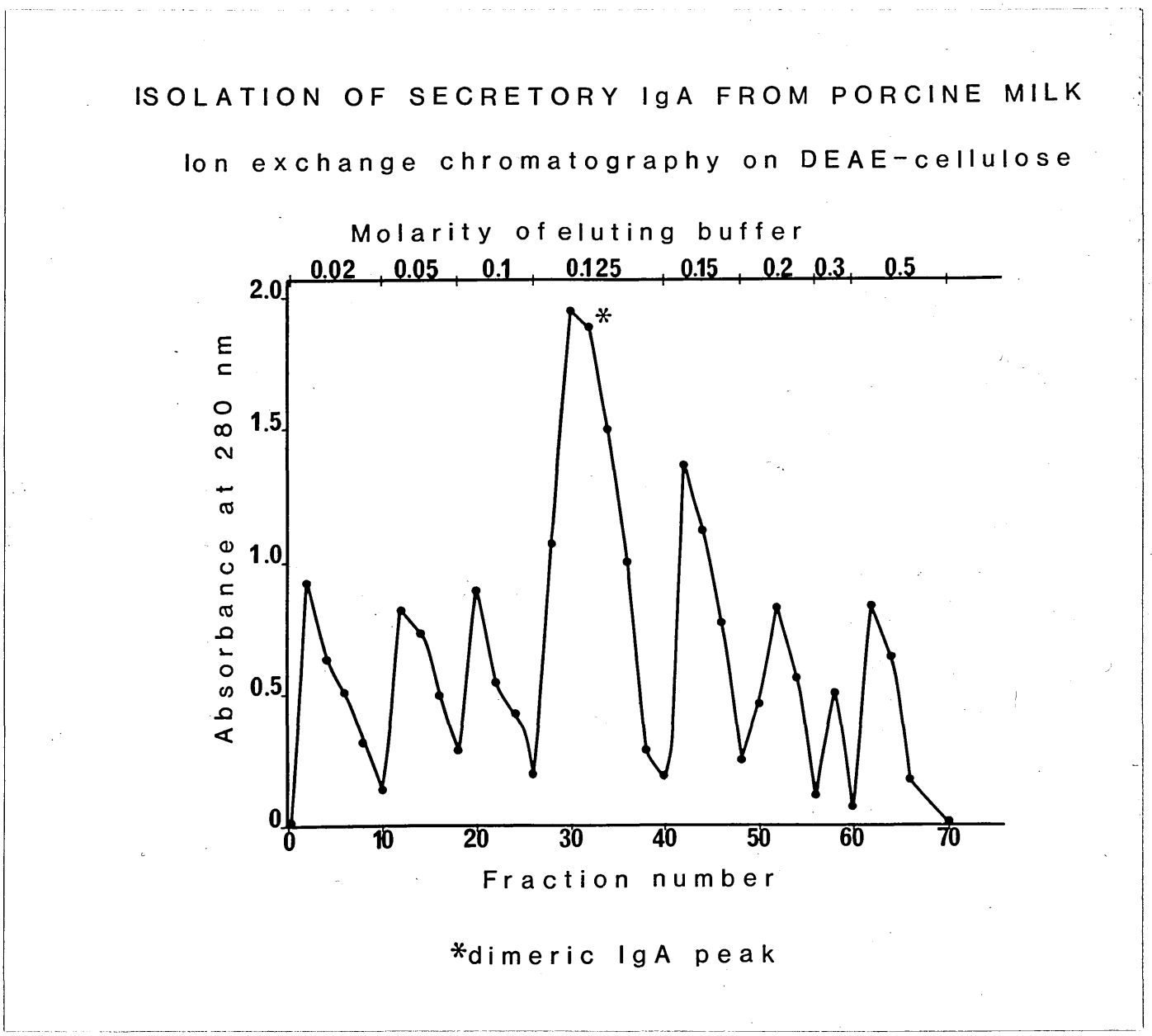

FIG. 2: Separation of secretory IgA from porcine milk on DEAE-cellulose.

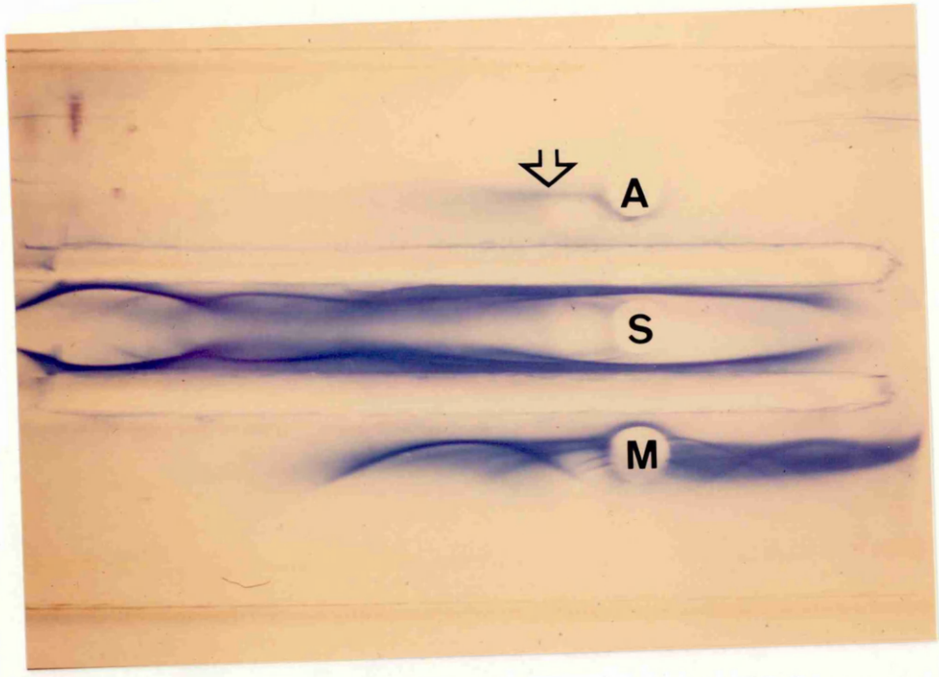

FIG. 3: Immunoelectrophoresis pattern of purified secretory IgA (A) compared to normal porcine serum (S) and porcine milk (M). Rabbit anti-porcine serum in the longitudinal troughs.
Note the single line of precipitation produced by the purified secretory IgA (arrow).

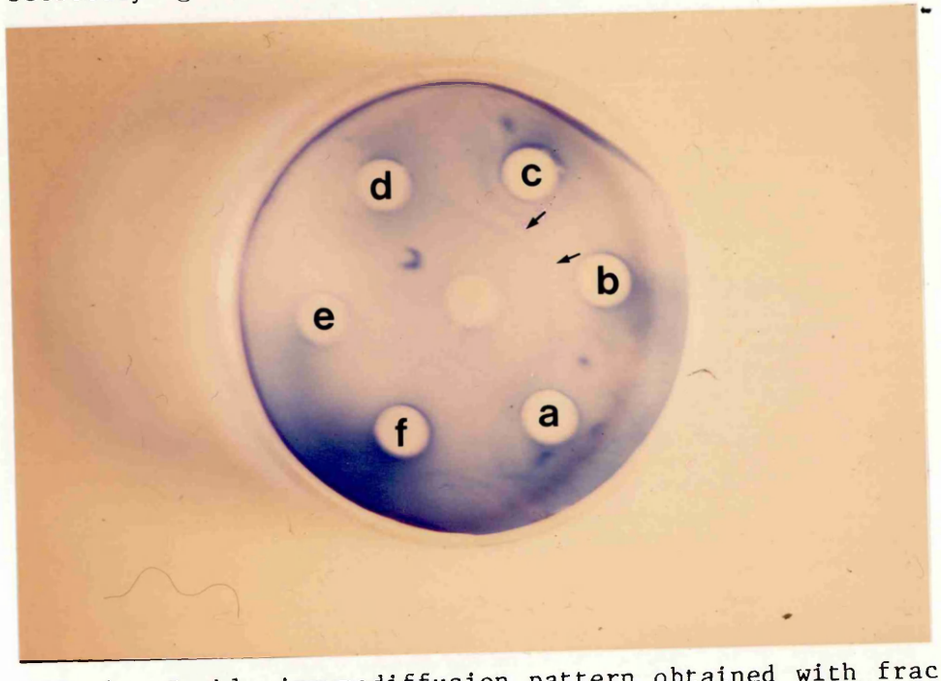

FIG. 4: Double immunodiffusion pattern obtained with fractions eluted from DEAE-cellulose column. Molarity of eluted fraction: (a) 0.05; (b) 0.1; (c) 0.125; (d) 0.15; (e) 0.2; (f) 0.3. Rabbit anti-porcine serum in the central well.
Note lines of identity produced by the 0.125M and 0.1M fractions (arrow). Most of the secretory IgA was contained in the 0.125M fraction.

the Animal House of the Department of Veterinary Pathology, University of Glasgow. Adult mice (20-25g) of both sexes were used. They were young, fed on a laboratory mouse diet (diet 41, J.Ogston Ltd.). The mice were housed in polythene cages containing wood shavings and bedding. Feed and water were available ad libitum.

Rabbits. The rabbits used for the experiments were New Zealand White purchased from Olac Ltd. Young adult animals of both sexes were used. They were fed on a laboratory rabbit diet (diet 18, J. Ogston Ltd.). Each rabbit was individually housed in a floor slatted cage containing no bedding. Feed and water were freely available.

Pigs. All pigs used in the study were of minimal disease origins (National Pig Development High Health Status) and were obtained from the University of Glasgow, Animal Husbandry Department. They were of 2 types, conventional weaned pigs aged 12 weeks, and conventional pregnant sows.

Conventional weaned pigs were produced by normal farm practice and when obtained had been weaned and maintained in flatdeck accommodation since weaning at 3 weeks of age. All were individually identified using a numbered ear tag. They were housed in unheated pens with concrete floors and straw bedding. They were fed on a barley-based ration containing no non-nutrient additives standard to the Department of Animal Husbandry. Water was freely available.

All the pregnant sows were kept in the dry sow house in individual stalls at an ambient temperature of 20°C. The animals were fed on a barley-based ration for pregnant sows standard to the Department of Animal Husbandry. Water was freely available. Three-four days before farrowing, each sow was moved to an individual farrowing cage where the temperature was 22-24°C until weaning.

(ii) Blood sampling procedures

Blood samples were taken from mice only after cervical dislocation. The blood was taken from the heart. Rabbits were bled from the ear vein and at the end of the study were anaesthetised with pentobarbitone sodium and large volumes were taken by cardiac puncture. Pigs were bled from the jugular vein and, after stunning, from the stick wound at slaughter.

Blood samples were allowed to clot, serum was removed and centrifuged at 1,600g for 10 minutes. The clear serum was removed and stored at -20°C in sealed Bijou or Universal bottles.

(iii) Milk and colostrum samples

Milk and colostrum samples were taken by milking sows after the intramuscular injection of oxytocin (Intervet Labs. Ltd.). Samples were spun at 10,000g for 1 hour and the middle fraction collected and stored at -20°C until required.

(iv) Immunisation procedures

Pre-inoculation blood samples were taken from rabbits and pigs, but not from mice, and the sera stored.

Immunisation of mice was performed by inoculating groups of 6 mice each with the adjuvanted toxoid and whole culture bacterin, each mouse receiving a dose of 0.5 ml of the preparations. A second immunisation was given two weeks later; fourteen days after the mice were bled out by cardiac puncture, the sera were collected, pooled and stored at -20°C. Sera from non-immunised mice were collected as a control.

Immunisation of rabbits was carried out after the pre-inoculation bleed by the subcutaneous or intramuscular injection of the antigens given in Table 4. The volumes and the adjuvants used and the injection schedule are given in the table.

TABLE 4 Antigens and immunisation procedures for the production of rabbit antisera

Antigen	Adjuvant	Inoculation	Inoculation schedule	
Crude alpha	Alhydrogel	s/c	2.0 ml	D0
toxin toxoid		s/c	2.0 ml	D14
22.9 mg Protein/ml			Bleed	D28
Whole culture	Alhydrogel	s/c	2.0 ml	D0
bacterin		s/c	2.0 ml	D14
73.6 mg Protein/ml			Bleed	D28
Pure alpha toxin	Freund's	s/c	2 ml	D0
.590 mg Protein/ml	Complete 1:1			
	Freund's			
	incomplete 1:1	s/c	2 ml	D14
			Repeated	D28
			Repeated	D42
			Repeated	D56
			Bleed	D70
Pure enterotoxin	Freund's	4 sites	0.5 ml/	D0
.200 mg Protein/ml	Complete 1:1	2 s/c, 2 i/m	site	
			Repeated	D28
			Repeated	D42
			Bleed	D56
Secretory IgA	Freund's 1:1	4 sites	0.5 ml/	D0
.180 mg Protein/ml	Complete	2 s/c, 2 i/m	site	
	Freund's	4 sites	0.5 ml/	D14
	Incomplete 1:1	2 s/c, 2 i/m	site	
			Repeated	D28
			Repeated	D42
			Repeated	D56
			Bleed	D70

s/c = subcutaneous i/m = intramuscular D = Day

Immunisation of pigs was carried out after the pre-inoculation bleed by the subcutaneous, intramuscular or oral administration of the antigens given in Table 5. The volumes and the adjuvants administered and the schedule of administration are given in Table 5, as are the immune products harvested.

(v) The preparation of fluorescent antibody to secretory IgA

Rabbit antibody to secretory IgA was prepared using the antigens and the immunisation schedule above. The serum was precipitated with 40 per cent solid ammonium sulphate. The precipitate was dissolved in 0.01M phosphate buffered saline pH 7.2 at approximately one-third the original volume of serum. The residual ammonium sulphate was removed by column chromatography on Sephadex G-25 (Pharmacia Ltd.) using the same phosphate buffer for elution.

The eluted fraction was dialysed in a 0.25M sodium carbonate buffer containing 0.1M sodium chloride. The protein concentration of the globulins was adjusted to 20 mg per ml with the same sodium carbonate buffer. Fluorescein isothiocyanate (Sigma London Chemical Company Ltd.) was used at the ratio of 0.05 mg per mg of protein. The dry fluorescein powder was dissolved in the sodium carbonate buffer pH 9.0 (5 per cent volume of the protein solution) and added by drops to the protein solution. The mixture was stored at 4°C for 20 hours. The pH was adjusted to 7.5 and the mixture passed through a Sephadex G-25 column to remove excess of fluorescein and carbonates, elution was done with 0.01M phosphate buffered saline pH 7.5. Globulin estimation and fluorescein/protein ratios were determined spectrophotometrically by the method of Johnstone and Thorpe (1982). The final conjugated globulins were filtrated through a 0.22 µm membrane filter and stored at -20°C. The conjugate was titred to find the dilution most suitable for use.

The dilution most suitable for use was determined using fixed

TABLE 5 Antigens and immunisation procedures for the production of pig immune sera and milks

Antigen	Adjuvant	Inoculation	Inoculation schedule	
			A. Weaned pigs	
Crude alpha toxin toxoid 22.9 mg Protein/ml	Alhydrogel	s/c	5.0 ml	D0
		s/c	5.0 ml	D14
			Bleed	D28
			B. Sows	
Crude alpha toxin toxoid 22.9 mg Protein/ml	Alhydrogel	s/c	5.0 ml	D0
		s/c	5.0 ml	D14
			Bleed at Farrowing Milk	
Whole culture bacterin 73.6 mg Protein/ml	Alhydrogel	s/c	As for crude Alpha toxin toxoid (sows and weaned pigs)	
Crude alpha toxin toxoid and whole culture bacterin 1:1	Alhydrogel	s/c	As above for weaned pigs and sows	
Crude toxoid and whole culture mixture as above	Alhydrogel	s/c	5.0 ml	D0
Washed freeze-dried *C.perfringens* cells 10% in corn-starch	None	oral in feed	3.75g/day farrowing	D0
			Bleed at Farrowing Milk	
Pure enterotoxin .200 mg Protein/ml	Freund's Complete 1:1	s/c	2.0 ml	D0
	Freund's Incomplete 1:1	s/c	2.0 ml	D14
			Repeated	D28
			Bled	D56

s/c = subcutaneous i/m = intramuscular D = Day

frozen sections of pig spleen. The anti-porcine secretary IgA conjugate was applied to the tissue and incubated in the dark for 30 minutes at room temperature in a moist chamber. The slides were washed twice for 15 minutes each in phosphate buffered saline. Slides were mounted with cover slips using 10 per cent glycerol in phosphate buffered saline pH 9.0 and observed with the fluorescence microscope. Intensity of specific fluorescence was recorded.

(vi) The absorption of sera

Aliquots of antisera used on intestinal tissue sections (anti-alpha toxin and anti-enterotoxin) were absorbed with pig liver powder to prevent non-specific fluorescence. Sera from some experimental animals were also absorbed with vegetative C.perfringens cells.

Absorption with pig liver powder. Antiserum was absorbed according to the method of Johnson (1961). Pig liver powder was prepared by homogenizing 500g of fresh pig liver in a 500 ml volume of physiological saline in a stomacher. The homogenized tissue was transferred to a measuring cylinder and precipitated with 5 volumes of acetone. The supernatant was removed by suction and washed three times with saline by filling the cylinder, allowing the tissue particles to sediment and removing the supernatant. The lipids were removed by further washing with acetone and then transferred to a Büchner funnel and washed through with acetone. The material was spread on filter paper and allowed to dry in an incubator at 37°C. The material was ground in a mortar and passed through a fine sieve to remove fibrous particles. Phosphate buffered saline pH 7.2 was added to the powder, centrifuged at 2,000g and the supernatant discarded. The powder was stored at -20°C until used.

Serum was absorbed by mixing two volumes of serum to one volume of liver pig powder. The mixture was gently rotated for 20 minutes at 4°C and then centrifuged at 2,000g, collecting the

supernatant. The absorbed sera were stored at -20°C until required.

Absorption of sera with vegetative C.perfringens type A.

Vegetative cells of C.perfringens type A isolate 29 (used for enterotoxin production and experimental infections) were grown in thioglycollate medium for 12 hours. The cells were harvested by centrifugation at 8,000g. The pellet was washed in PBS, pH 7.2, centrifuged and washed again. After a final centrifugation the cells were resuspended in the sera to be absorbed to a concentration of 2 x 10^7 cells/ml, incubated at 37°C for 30 minutes and then centrifuged again at 8,000g. The supernatant was filtered through a cellulose acetate filter of APD 0.22 µm and stored at -20°C until required.

(vii) Inactivation of sera

All sera used for immunofluorescence were inactivated by heating at 56°C for 30 minutes.

(c) Immunological techniques

The following techniques and methods were used throughout the study to evaluate the serological response elicited by C.perfringens and its products in field and experimental material.

(i) Counterimmunoelectrophoresis

Agarose (L.K.B. Instruments Ltd.) at one per cent concentration was dissolved by heat in 0.025 ionic-strength barbital-acetate buffer pH 8.6. Slides were layered with melted agarose and allowed to harden for at least 20 minutes and stored in a moist chamber until used.

Barbital-acetate buffer pH 8.6 was prepared in distilled water. The composition of stock buffer was 3.09 per cent sodium barbital, 0.552 per cent barbital and 1.23 per cent sodium

acetate. The buffer solution was heated to approximately 90°C for 15 minutes to dissolve the buffer salts and was then diluted to a 0.025M ionic-strength buffer with distilled water. Parallel rows of wells 0.3 cm in diameter were cut 0.5 cm apart in the agar. Ten microlitres of antigen were placed in wells near the cathode. The samples (sera, colostra and milks) to be tested were placed in the wells near the anode in similar volumes. the electrode vessel contained barbital-acetate buffer of the same ionic strength, (0.025M) as was used for the gel preparation. Electrophoresis was carried out in an electrophoresis chamber (Gelman Instrument Co.) at room temperature with a constant current of 6mA per slide for 60 minutes. Filter paper was used as an electrode wick. After electrophoresis, the slides were removed to a moist chamber and allowed to develop for 30 minutes. For a permanent record the slides were washed for 24 hours in phosphate buffered saline and for 6 hours in distilled water before staining with Coomassie brilliant blue at 0.025 per cent.

(ii) Immunoelectrophoresis

Slides covered with a layer of one per cent agarose 1.0-1.5 mm thick containing 0.01 per cent thiomersal in 0.025 ionic-strength barbital-acetate buffer pH 8.6. The slides were set on a levelling platform and allowed to harden for at least 20 minutes. Troughs 0.4 cm wide by 5 cm long and wells 0.3 cm in diameter were cut out of the agar with a cork borer and a scalpel. The distance between the troughs and the wells was 0.3 cm. A drop of bromothymol blue added to one well acted as an electrophoretic marker. Separation was achieved with a potential of 100-150V for 3-4 hours, the gel being cooled with tap water. The agar was then removed from the troughs, antiserum was placed in the troughs and diffusion of antiserum and antigens allowed to take place over the central compartment of the electrophoretic tank or in petri dishes lined with moist blotting paper at room temperature. After an incubation time of 18-24 hours the slides were washed for 24 hours in phosphate buffered saline and for 6 hours in distilled water. The slides were allowed to dry up at room temperature before staining with Coomassie brilliant blue at 0.025 per cent.

(iii) Double immunodiffusion

Plastic petri dishes 3.5 cm in diameter were covered with a layer of one per cent agarose 1.0 - 1.5 mm thick containing 0.01 per cent thiomersal in phosphate buffered saline pH 7.2. The agar was allowed to solidify and wells 0.3 cm in diameter and 0.4 cm apart were cut out of the agar. Ten microlitres of samples to be tested were placed in the wells and diffusion of antiserum and antigens allowed to take place for 24-48 hours at room temperature. The petri dishes were washed in phosphate buffered saline and distilled water, the gel allowed to dry at room temperature and stained with Coomassie brilliant blue at 0.025 per cent.

(iv) Immunofluorescence

Indirect immunofluorescence was used in a number of studies in this thesis.

Indirect immunofluorescence on bacterial smears. Colonies of Clostridum perfringens type A grown for 16 hours in horse blood agar were picked and resuspended in a small amount of physiological saline solution then the suspension was smeared on pre-cleaned microscope slides, air-dried and fixed in acetone pre-cooled at 4°C, for 10 minutes. Smears of other Clostridia (C.tertium, C.chauvoei, C.septicum and C.haemolyticum) were used as controls. Dilutions of sera, colostra or milks to be tested were spread over the smear and incubated in the dark room for 30 minutes at room temperature in a moist chamber. After incubation, the slides were washed twice for 15 minutes each in phosphate buffered saline pH 7.2. Fluorescein-conjugated rabbit anti-swine IgG, rabbit anti-swine IgM, rabbit anti-mouse IgG and sheep anti-rabbit IgG (Miles Laboratories Ltd.), and the fluorescein conjugated rabbit anti-porcine secretory IgA prepared by the methods described above were all used. The fluorescent antisera were placed on the smear and incubated again in the dark for 30 minutes at room temperature. The slides were washed twice for 15 minutes each in

phosphate buffered saline pH 7.2 and mounted with cover slips using 10 per cent glycerol in phosphate buffered saline (pH 9.0) and UG-1 filters. The intensity of the specific fluorescence produced was recorded.

Indirect immunofluorescence on frozen sections. Specific antiserum was layered on the sections and incubated in the dark for 30 minutes at room temperature in a moist chamber. After incubation, the slides were washed twice for 15 minutes each in phosphate buffered saline pH 7.2 and fluorescein conjugated anti-rabbit IgG applied to the sections which were incubated 30 minutes at room temperature then they were mounted and observed in the fluorescence microscope as described above. A negative rabbit serum was used as a control in each case.

Indirect immunofluorescence on Vero cells. Vero cells were cultured into Lab-Tek (Miles Laboratories) chamber slide wells (1 cm^2 surface area/well) and allowing growth to confluency (24 hours). The cells were washed once with 1 ml of calcium and magnesium-free Hank's balanced salts solution, and enterotoxin in Medium 199 without serum added. After 4-6 hours of incubation at 37°C in a humidified atmosphere containing 5 per cent CO_2 in air, the slides were fixed in cold acetone for 10 minutes. Decomplemented anti-enterotoxin serum (56°C - 30 minutes) was layered on the slides and incubated in the dark for 30 minutes at room temperature in a moist chamber. After incubation the slides were washed in phosphate buffered saline pH 7.2 and commercial fluorescein conjugates (anti-rabbit IgG or anti-pig IgG) was applied to the slides which were incubated 30 minutes at room temperature and washed in phosphate buffered saline pH 7.2. They were mounted and observed in the fluorescence microscope as described above. Fluorescence was recorded.

(v) Toxin neutralisation studies

These were carried out in mice, Vero cells and by the prevention of haemolysis.

Toxin neutralisation studies in mice. Toxin neutralisation tests were carried out in mice using antibody from immunised animals of other species (pigs) or in the mice themselves as a means of assessing the effectiveness of immunisation.

Toxin neutralisation tests. Antitoxin activity of sera, colostra or milks was tested by the neutralisation of specific amounts of alpha-toxin. The procedure consisted of making serial two-fold dilutions of the secretion to be tested in physiological saline solution. To each of the series of dilutions 1 ml of toxin containing 16 mouse lethal doses was added. A mouse lethal dose was the reciprocal of the highest dilution of the toxin which was lethal to two mice within 48 hours. The reactants were mixed and allowed to stand at room temperature for 30 minutes.

Groups of two mice were used, one for each dilution to be tested. Each mouse received 1 ml of the toxin:antitoxin mixture. A positive control was carried out using the toxin and dilutions of an antiserum of known antitoxic activity. Dilutions of normal pig serum and toxin provided a negative control. The mice were observed for 48 hours and the results were recorded.

Mouse protection tests. Mice were immunised in order to test the antigenicity of the adjuvanted crude alpha toxoid and the adjuvanted whole culture bacterin prepared as described above. Groups of five mice were inoculated subcutaneously with 0.5 ml of dilutions of the crude toxoid or the whole culture bacterin. A second immunisation using the same dilutions was given 14 days later. Fourteen days after the second immunisation the mice were challenged intraperitoneally with 0.5 ml of a C.perfringens type A supernatant culture containing 16 M.L.D/ml. The animals were kept under observation for 48 hours and the number of animals which died was recorded.

Neutralisation of haemolysis. Serum neutralisation of specific amounts of haemolysin activity of alpha toxin was used to determine the antitoxin content of sera. Serial doubling dilutions of sera to be tested were made in physiological saline solution in microtitre plates (Becton, Dickinson and Co.). To each of the wells in the series of dilutions, 60 µl of toxin containing 64 sheep erythrocyte haemolytic units were added. A haemolytic unit was the reciprocal of the highest dilution of the toxin which caused a complete haemolysis of a one per cent erythrocyte suspension. The mixture was allowed to stand at room temperature for 30 minutes then 60 µl of a one per cent suspension of washed sheep erythrocytes in physiological saline solution was added to each well. The reactants were mixed and placed at 37°C for one hour, and then the microtitre plates were placed at 4°C for 18 hours. Specific neutralisation of toxin was achieved with standardized antitoxin for each titration. Reciprocals of serum dilutions neutralising 64 haemolytic units were considered as the anti-haemolytic levels of assayed serum samples.

5. PIG EXPERIMENTAL STUDIES

Two types of study were carried out. In the first, hysterectomy-derived colostrum-deprived (HDCD) animals were infected and maintained for some days for observation. In the second both conventional and HDCD piglets were anaesthetised and gut surgery was performed.

(a) Source and maintenance of animals

(i) Hysterectomy-derived, colostrum-deprived piglets

The hysterectomy-derived, colostrum-deprived piglets were produced and reared by the general methods described by Betts et al. (1960). Sows were obtained from the Veterinary School Farm at Cochno and were of National Pig Development High Health stock.

Hysterectomy was carried out on the 112th day of pregnancy. After stunning the sow electrically, the whole uterus was removed aseptically into an antiseptic solution in a large bin. The piglets were removed from the uterus on a disinfected operating table. The umbilical cords were clamped (Piglet umbilical clamps, Veterinary Drug Co. York) after respiration was established.

Hysterectomy-derived piglets thus produced were retained, for gut loop inoculation studies in a clean but not sterile environment at 35°C, and those intended for infection studies were placed in a clean, non-sterile box lined with sterile paper and moved to a formalin fumigated loose box containing racks of 9 cages and heated by electricity and gas to a constant temperature of 35°C. The piglets were placed in groups of 2 animals per cage and arranged in the racks according to the experimental protocol (see Chapters 3, 4 and 6). All piglets were fed on evaporated tinned full cream milk (Fine Fare Ltd.), diluted as appropriate with tapwater. The feeding started about 2 hours after delivery with 20 ml diluted milk. The piglets were fed 3 times per day. Water was available ad libitum. The feeding was supplemented with vitamins (Abidec Multi-vitamins, Park Davis Ltd.) for the first three days of life. After each feed, the feeding trays were washed in a solution of Chloros (10 per cent alkaline sodium hypochlorite, I.C.I. Ltd.) and then rinsed in water. The milk fed was gradually increased in amount to 50 ml at each feed. Animals were individually identified using skin marks and cage numbers.

(ii) Conventional newborn piglets

Two newborn piglets were obtained from the Cochno herd (Farm 2) and were segregated from the sow before they were thought to have ingested colostrum. The animals were placed in clean boxes as were the hysterectomy-derived piglets, and maintained at 35°C until required.

(b) Infection studies with Clostridium perfringens type A and its products

A total of 3 experiments was carried out according to the protocols described in Chapters 3, 4 and 6. Control animals were housed in separate cage racks in the same room and negative controls were handled first during feeding and sampling procedures.

(i) Sampling procedures

Clinical signs including body condition, skin colour and faecal consistency were recorded in all 3 experiments. Rectal temperatures were recorded only in the experiment described in Chapter 4 (Experiment 2). Morbidity and mortality were recorded.

Rectal faecal samples were taken daily using cotton-tipped swabs with Amies charcoal transport medium and the samples examined by the procedures outlined in Section 2 of this chapter.

(ii) Inoculation procedures

Some pigs were protected using sterile immune products given by parenteral injection into the peritoneum and some by oral dosing with the product prior to the first feed to avoid the closure phenomenon. The amounts of immune products given and its identity are given in the appropriate chapters. Infection was always oral and measured volumes of the culture were given before feeding which followed 15-30 minutes later.

(iii) Post-mortem examinations

These were performed as described in Section 2 of this chapter.

(c) The intestinal inoculation of anaesthetised piglets

Piglets were anaesthetised for the intraluminal inoculation of toxins and bacterial cultures into both gut loops and directly, by laparotomy into the intestinal lumen.

Both hysterectomy-derived and conventional supposed colostrum-deprived piglets were used.

(i) Anaesthesia and analgesia

Anaesthesia was induced with halothane using a conical face mask and piglets were maintained on a halothane-oxygen mixture for the duration of the surgical procedures. After surgery piglets were allowed to recover and given Pentazocine (Fortral, Sterling Research) every two hours intramuscularly to maintain analgesia.

(ii) Surgical procedure

Anaesthetised piglets were tied in a lateral position, the skin was washed and disinfected with an iodine-based scrub (Pevidine Veterinary Scrub) and a lateral abdominal approach carried out. The small intestine was exteriorised and kept moist by the use of sterile saline on gauze packs. The region of the intestine exteriorised was identified and inoculated with toxin in the case of intraluminal inoculation studies or made into gut loops. Gut loops were prepared by ligating the small intestine with nylon at 10 cm intervals beginning at a point at least 20 cm proximal to the ileocaecocolic junction. The prepared loops were inoculated.

Inoculated intestines were replaced in the abdominal cavity and the cavity closed with nylon sutures.

(iii) Observation period

Recovered animals were observed for 12 hours and given intra-

peritoneal injections of saline at hourly intervals to counter dehydration. Clinical signs which developed during this period were observed. At the end of the period the animals were killed by the intracardiac injection of pentobarbitone sodium.

(iv) Post-mortem examination

Animals were opened immediately after death and the appearance of the intestine recorded. Each ligated loop was examined externally checking for fluid accumulation, then each loop was slit lengthwise for examination of the contents. Segments of the loops were preserved in 10 per cent neutral buffered formol saline and also kept frozen at -70°C for immunofluorescence studies. The intestines inoculated intralumenally were examined by similar procedures.

CHAPTER 3

INITIAL STUDIES OF THE DISTRIBUTION OF CLOSTRIDIUM PERFRINGENS TYPE A IN PORCINE GASTROINTESTINAL DISEASE AND THE EXPERIMENTAL REPRODUCTION OF DISEASE IN PIGLETS USING A PURE CULTURE

1. INTRODUCTION

The studies described in this chapter comprised an initial survey of pig diagnostic material to confirm the work of Olubunmi (1982) who had found C.perfringens type A to be common in the faeces and intestines of pigs with enteritis. This initial survey provided a number of isolates of C.perfringens type A for further study. One of these was used to infect hysterectomy-derived, colostrum-deprived pigs in order to test the pathogenicity of the isolate for pigs.

2. INITIAL SURVEY OF THE INCIDENCE AND DISTRIBUTION OF C.PERFRINGENS TYPE A IN DIAGNOSTIC MATERIAL FROM PIGS

(a) Material surveyed

The material surveyed in this study comprised that submitted for diagnosis to Glasgow University Veterinary School from Farms 1, 2 and 3 and from other sources and material submitted to Leeds Veterinary Investigation Centre (Chapter 2, Section 1). It consisted of faecal samples and dead piglets. More than 1 sample (whether faecal or carcase) was submitted in most cases and each submission was examined in detail. The details of the submissions are given in Table 6. All samples in this survey were from diarrhoeic animals.

(b) Methods used

The methods used in the survey have been described in detail in Chapter 2, Sections 1, 2 and 3.

TABLE 6

Samples examined in the initial survey; farm details and history

Submission number	Farm number	Number of samples	Age of animals affected	Nature of sample Carcase	Faeces
1	1	4	2 weeks		+
2	3	4	1 week		+
		5	3 weeks		+
3	1	6	4-7 days		+
4	3	3	3 days	+	
5	2	2	3 weeks		+
		1	6 days		+
		3	1 day		+
6	D.M.L.	2	3 months		+
7	2	3	3 days	+	
8	D.M.G.	1	1 week	+	
9	2	3	2-5 days	+	
10	D.M.G.	4	2 weeks		+
11	1	2	3-5 days	+	
12	D.M.G.	4	5-7 days	+	
13	D.M.G.	1	5 days	+	
14	D.M.G.	4	2 weeks		+
15	2	3	2-3 days		+
16	1	3	2 weeks		+
17	2	2	3 days	+	

D.M.L. = Diagnostic Material from Leeds Veterinary Investigation Centre

D.M.G. = Diagnostic Material from Glasgow University Veterinary School

(c) Results

C.perfringens was isolated from 30 of 60 samples. The results are given in Table 7. Positive samples were identified in 11 of the 17 submissions. The pigs concerned were aged from 2 days of age to 3 months of age.

Isolates of C.perfringens obtained in this survey and stored for further study were numbered in order of their isolation (Table 7). One colony was picked from each plate and stored as described in Chapter 2.

The other bacteria isolated are indicated in Table 7. Campylobacter coli was isolated from 8 samples from diarrhoeic pigs (Submisions 1, samples 1, 2, 3 and 4; Submissions 5, samples 26 and 27; Submission 11, samples 42 and 43). None of these samples yielded C.perfringens. The organism was not found in profuse culture in any case. However, as no other potentially pathogenic agent was found, the conditions observed were correlated with the presence of this bacterium. β-haemolytic E.coli were isolated in profuse numbers from 15 animals of 2 days to 3 months of age. It was isolated from 5 of the 17 submissions. It was present both in the presence of C.perfringens (Submission 9) and in its absence (Submission 10). None of the other bacteria isolated: non-haemolytic Escherichia coli; Streptococcus spp.; Clostridium spp.; Bacillus spp.; Proteus spp.; Peptostreptococcus spp.; Bacteroides spp.; Fusobacterium spp.; Lactobacillus spp.; Staphylococcus spp.; Pseudomonas spp.; Corynebacterium spp.; Sarcina spp., were considered significant and no protozoa or viruses were demonstrated in these samples.

(i) The C.perfringens isolates

Twenty seven of the 30 isolates of C.perfringens were identified as belonging to type A and 3 isolates were identified as belonging to type C. These were all isolated from the same submission, Submission 7 and were recovered from all 3 samples.

TABLE 7

Results of the survey

Submission number	Sample number and type	C.perfringens isolated	Other agents demonstrated
1	1 (F)	-	B, C, E, N
	2 (F)	-	B, C, E, N
	3 (F)	-	B, C, E, N
	4 (F)	-	B, C, E, N
2	5 (F)	+	B, C, K
	6 (F)	+	B, C, K
	7 (F)	++	B, C, E, K
	8 (F)	++	B, C, E
	9 (F)	-	B, C, K, L
	10 (F)	-	B, C, E, L
	11 (F)	-	B, C, I, L
	12 (F)	-	B, E, L
	13 (F)	-	B, C, E, I, K, L
3	14 (F)	+	B, C, E, F, H, I
	15 (F)	-	B, C, E, I, K
	16 (F)	-	B, C, E, F
	17 (F)	++	B, C, E, F, H, I, K
	18 (F)	-	B, C, E, F
	19 (F)	-	B, C, I, K
4	20 (C)	++	B, C, I
	21 (C)	++	B, C, I
	22 (C)	++	B, C, I
5	23 (F)	-	A, B, C, H, I, L
	24 (F)	-	A, B, C, H, L
	25 (F)	-	B, C, H, I
	26 (F)	-	B, C, E, K, N
	27 (F)	-	B, C, E, I, N
	28 (F)	-	B, C, E, K

TABLE 7 (Continued)

Submission number	Sample number and type	C.perfringens isolated	Other agents demonstrated
6	29 (F)	+	A, B, E
	30 (F)	++	A, B, E, M
7	31 (C)	++*	B, E, I, K, L
	32 (C)	++*	B, C, E, K, L
	33 (C)	++*	B, E, I, L
8	34 (C)	+	B, F, J, K, L
9	35 (C)	+	A, B, C, D, F, G, M
	36 (C)	++	A, B, C, G, L, M
	37 (C)	++	A, B, C, D, F, L
10	38 (F)	-	A, B, E, F, K, L
	39 (F)	-	A, B, F, I, I.
	40 (F)	-	A, B, E, F, I, L
	41 (F)	-	A, B, E, F, I, K
11	42 (C)	-	B, C, E, L, N
	43 (C)	-	B, C, E, L, M, N
12	44 (C)	+	B, C, E, I, L
	45 (C)	++	B, E, H, I, L
	46 (C)	++	B, C, E, H, I, L
	47 (C)	+	B, C, E, H, I, L
13	48 (C)	-	B, C, L, O
14	49 (F)	-	A, B, E, H, L
	50 (F)	-	A, B, C, E, H
	51 (F)	-	A, B, C, E, L
	52 (F)	-	A, B, E, H, L
15	53 (F)	+	B, C, E, I, L
	54 (F)	++	B, C, E
	55 (F)	+	B, C, L
16	56 (F)	++	B, C, E, M
	57 (F)	+	B, C, E, H, I, J
	58 (F)	++	B, C, E, H, J, L

TABLE 7 (Continued)

Submission number	Sample number and type	C.perfringens isolated	Other agents demonstrated
17	59 (C)	+	B, C, F, L
	60 (C)	+	B, C, I, L

(F) = faeces (C) = carcase

Number of C.perfringens colonies in horse blood agar plates

\+ = 1-8 colonies. ++ = more than 8 colonies.

++* = C.perfringens type C.

A = β-haemolytic E.coli
B = Non-haemolytic E.coli
C = Streptococcus spp.
D = Peptostreptococcus spp.
E = Bacillus spp.
F = Clostridium spp.
G = Corynebacterium spp.
H = Proteus spp.
I = Bacteroides spp.
J = Fusobacterium spp.
K = Lactobacillus spp.
L = Staphylococcus spp.
M = Pseudomonas spp.
N = Campylobacter coli
O = Sarcina spp.

Of the 27 C.perfringens type A isolates, 25 were haemolytic C.perfringens type A, and 3 were non-haemolytic C.perfringens type A.

C.perfringens type A was presumptively identified in primary cultures by its morphology and haemolysis pattern. Double zones of haemolysis were characteristically produced on horse blood agar plates (Fig. 5). Non-haemolytic C.perfringens type A was recognised by its colonial morphology. The organism was further tested for its biochemical reactions, and the identity of the organism as Clostridium perfringens type A confirmed by the methods described in Section 2.

(ii) Clinical signs associated with the isolation of C.perfringens in the absence of other obvious pathogens

Eight submissions and 25 samples yielded C.perfringens as the only potential pathogen to be identified (Submissions 2, 3, 4, 7, 8, 12, 15, 16, 17). In all cases in which large numbers of colonies of C.perfringens type A were isolated alone, the animals had a history of diarrhoea and the majority of animals were aged one week or less. The faeces of the piglets were yellowish to brownish and creamy in consistency (Fig. 6).

The bodily condition of the pigs ranged from poor to fair, at the time of examination. The flanks of most of the live and dead piglets were hollow and the hindquarters were stained with faeces (Fig. 7). In the case of Farm 1, a history of successful treatment of the diarrhoea by the use of parenteral penicillin injections was also available.

The three cases in Submission 7 from which C.perfringens type C was isolated were typical of C.perfringens type C disease in that the piglets had died suddenly or after a brief period of blood stained diarrhoea by 3 days of age.

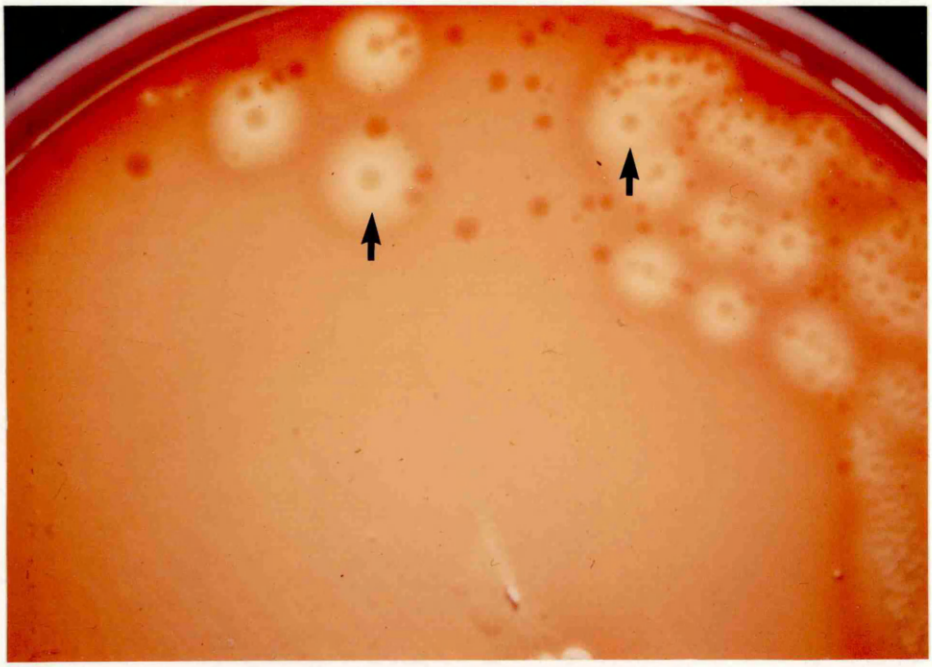

FIG. 5: C.perfringens type A colonies isolated from sample 7. 24 hour primary isolation culture on horse blood agar.
Note the double zone of haemolysis around the colonies (arrow).

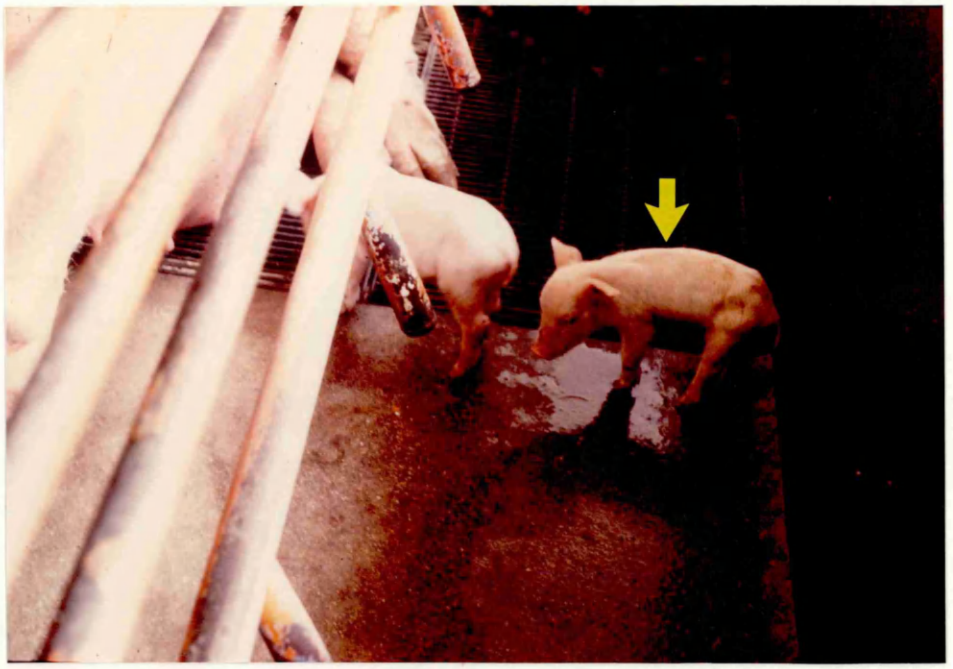

FIG. 6: Piglet with diarrhoea (sample 7) from which C.perfringens type A was isolated in profuse culture. Note the bodily condition of the animal (arrow).

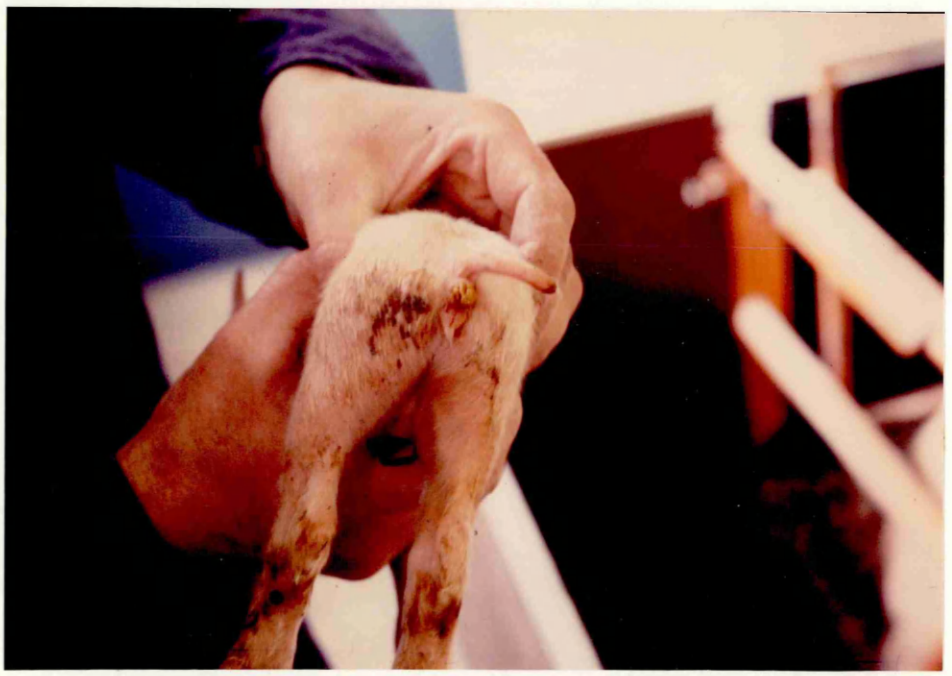

FIG. 7: Perineal region of a diarrhoeic piglet (sample 17) from which C.perfringens type A was isolated in profuse culture. Note the staining of the hindquarters with faeces.

Post-mortem findings associated with the isolation of C.perfringens in the absence of other pathogens

Five submissions consisted of carcases from which no other pathogenic organisms were isolated (Submissions 4, 7, 8, 12 and 17). The 3 piglets in Submission 7 were cases of C.perfringens type C enteritis and were typical of that disease. The carcases were pale, in fair bodily condition and slightly decomposed. Blood-stained faeces were present on the perineum and more could be expressed from the rectum. The gross post-mortem findings were severe in the small intestine, the serosal surface of which was dark red (Fig. 8). The large intestinal serosa was less severely affected. The intestinal contents were blood stained and the small intestinal mucosa haemorrhagic. Large numbers of Gram-positive bacteria resembling C.perfringens were seen in smears made from these contents. C.perfringens type C was isolated from the regions of the gut shown in Table 8. No histological examination was performed.

The remaining 4 submissions contained the carcases of 10 animals with the following pathological features.

Affected animals were aged 3 days to 1 week. The bodily condition ranged from poor to fair with hollowed flanks and hindquarters stained with creamy or brown faeces. Gross changes were confined to the gastrointestinal tract (Fig. 9).

The stomach was either empty or half-filled with feed. The changes seen in the gastric mucosa ranged from slight localised hyperaemia to moderate congestion in some.

The small intestine was often distended with fluid contents, and the serosa was usually congested to a variable extent. Changes were least obvious in the duodenum in which the contents were fluid and often normal in appearance; some cases presented a mild congestion of the mucosa. The contents of the jejunum were fluid and on occasions, contained small particles of necrotic material. The mucosal surface was congested in all cases and haemorrhagic in a few cases. Villi were usually reduced in height and occasionally

TABLE 8

Sites from which C.perfringens was isolated in animals examined at post-mortem and the abundance of colonies

Submission number	Sample number	Site of isolation					
		Stomach	Duodenum	Jejunum	Ileum	Caecum	Colon
4	20	+	++	++	++	++	+
	21	-	+	++	++	++	+
	22	-	+	++	++	++	+
7	31*	-	-	++	++	+	+
	32*	-	+	++	++	++	+
	33*	-	-	++	++	+	+
8	34	+	+	+	+	+	+
12	44	-	+	+	+	+	+
	45	+	++	++	++	+	+
	46	+	+	+	++	++	+
	47	-	+	+	+	+	+
17	59	-	-	+	+	+	-
	60	-	+	+	+	+	+

Number of C.perfringens colonies in horse blood agar plates.

- = no colonies detected
+ = 1-8 colonies
++ = more than 8 colonies
* = isolation of C.perfringens type C

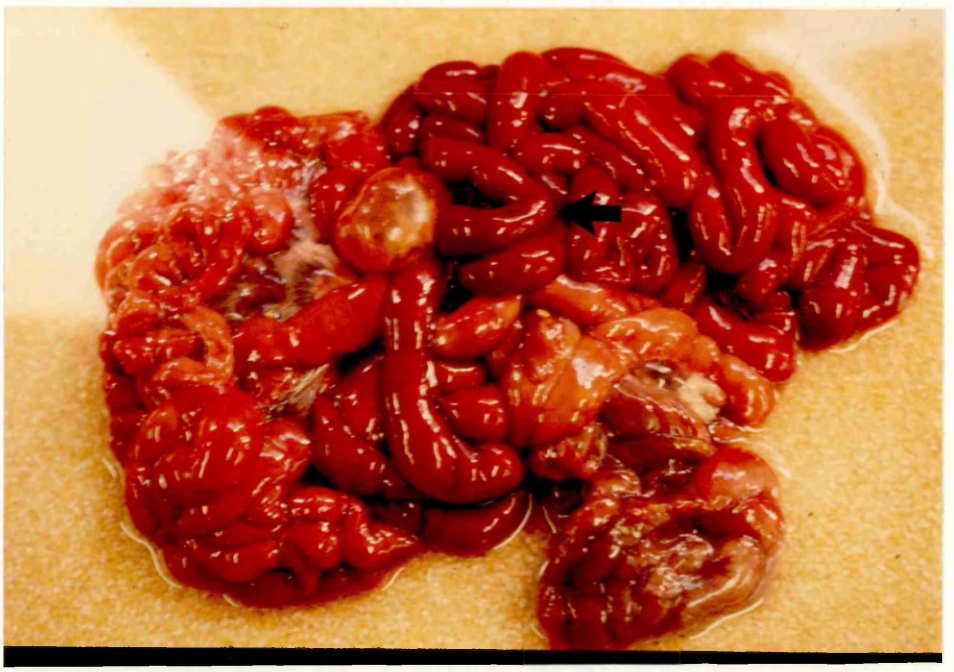

FIG. 8: Gross appearance of the abdominal viscera of a piglet from which C.perfringens type C was isolated (sample 32). Note the reddened small intestine (arrow).

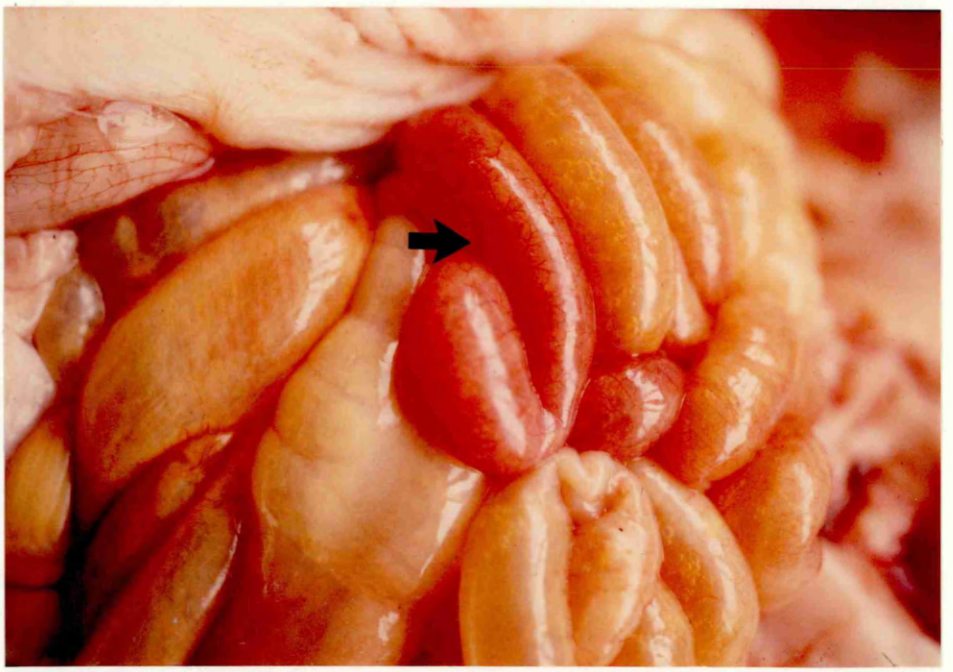

FIG. 9: Gross appearance of the intestine of a piglet (sample 21) from which C.perfringens type A was isolated in profuse culture. Note distension and congestion of the small intestine (arrow).

covered with flakes of fibrinous or necrotic material. The ileum was similar in appearance although the contents differed slightly in that they contained gas bubbles and were foamy in appearance. The mucosal appearance resembled that of the jejunum.

The large intestinal contents were fluid and bulky in some cases while in others they were pasty and adherent to the mucosal surface. The mucosal changes varied from slightly generalised congestion to localised areas of hyperaemia and pinpoint haemorrhages.

The mesenteric blood vessels were in most cases congested and a variable degree of enlargement and congestion was seen in the mesenteric lymph nodes.

In the few cases (Submissions 4, 8, and 17) that histopathology was carried out, the histological changes were most pronounced in the small intestine, particularly in the jejunum and ileum. In every case the mucosa was congested and villi slightly shortened. An excess of mononuclear cells and polymorphs was present in the lamina propria. Changes in the caecum and colon were less apparent. The mucosal surface was intact in most cases. Fibrin and cell debris could be seen on the lumenal surface of the epithelium. In some cases there was slight congestion of blood vessels of the lamina propria.

In Gram-stained smears made from the small intestinal contents, large numbers of Gram-positive stout rods were seen. Large numbers of colonies of C.perfringens type A were isolated from the mucosa of the duodenum, jejunum and ileum of some animals. The organism was isolated less commonly from the gastric mucosa but was recovered in small numbers from the large intestinal mucosa of most animals in which it was present (Table 8). It was never recovered from sites outside the gastrointestinal tract.

In all cases C.perfringens type A was accompanied by other bacteria such as non-haemolytic E.coli, faecal streptococci,

Bacillus spp., Bacteroides spp. and Lactobacillus spp. as the most common species isolated. In certain cases, however C.perfringens type A colonies clearly outnumbered those of other bacterial species.

3. EXPERIMENTAL INFECTION OF HYSTERECTOMY-DERIVED, COLOSTRUM-DEPRIVED PIGLETS WITH CLOSTRIDIUM PERFRINGENS TYPE A ISOLATE 7 (EXPERIMENT 1)

(a) Experimental design

Five HDCD piglets 8 days of age produced by the methods described in Chapter 2 were available for this study. They were housed individually in a block of 6 cages in the same rack and numbered U1-U5. Two animals were kept as uninfected controls U1 and U2, and the other 3 were infected.

Isolate 7, obtained from a case of C.perfringens type A enteritis, was used in this study. It had been cloned and stored in cooked meat medium for 8 weeks. It was passaged twice on horse blood agar prior to use (Fig. 10). It was then subcultured into a 200 ml bottle containing cooked meat medium enriched with glucose and calcium chloride as described in Chapter 2. The culture was incubated at 37°C for 16 hours. Viable cell counts present in the inoculum were determined according to the method of Miles and Misra (1938).

5 ml of inoculum containing approximately 3.0×10^8 organisms/ml were given to each piglet after it had been fasted for 18 hours.

After an observation period of 8 days the piglets were killed and post-mortem examinations were carried out. The methods used for clinical, bacteriological and pathological examination are described in Chapter 2.

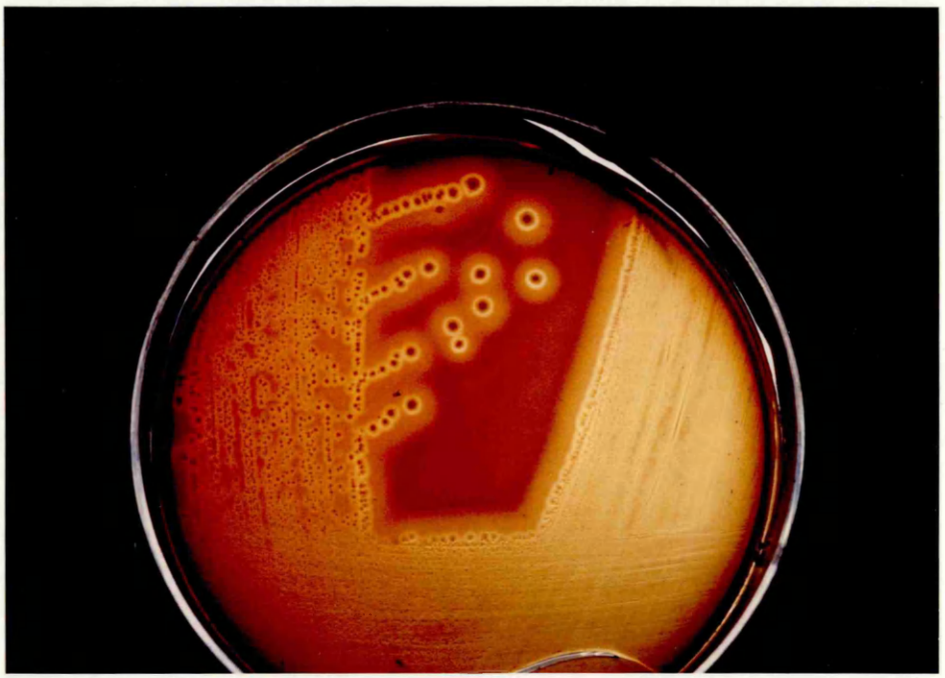

FIG. 10: Pure culture of C.perfringens type A (isolate 7) used for the experimental infection of hysterectomy-derived, colostrum-deprived piglets (Experiment 1). 24 hour culture on horse blood agar.
Note the double zone of haemolysis around the colonies.

(b) Results

(i) Clinical findings

Changes in faecal consistency were noted in all 3 infected piglets within 24 hours of inoculation. Softening of the faeces was observed. 48 hours post-infection piglet U5 developed diarrhoea which was profuse, creamy-yellowish in colour and stained the hindquarters of the piglet. Piglet U3 developed diarrhoea at 72 hours post-infection, whereas diarrhoea was not observed in piglet U4 until the 4th day post-infection. The faecal changes persisted in all the 3 piglets until the end of the observation period. The faecal changes are summarised in table 9.

The infected piglets were weak and in poor bodily condition. None of the clinical signs were observed in the control piglets.

(ii) Bacteriological examination of rectal swabs

C.perfringens type A was isolated in profuse culture from the rectal swabs of the infected animals from the first day after inoculation. The organism was isolated from the control U1 from the second day of experiment and from control U2 from the sixth day, but few colonies were detected in the cultures from faecal swabs of these animals (Table 9).

(iii) Pathological findings

Pathological changes were similar in all infected animals. The general bodily condition was poor. The eyes were sunken and the flanks hollowed. The perineal region was matted with dry crusts of faeces (Fig. 11).

The thoracic organs were normal. In the abdominal cavity, the liver was pale in all infected animals. Spleen and kidneys were normal. The stomachs of these piglets were distended with milk and some gas. The gastric mucosa of piglets U3 and U5 were slightly

TABLE 9

Faecal consistency changes of HDCD piglets following the inoculation of pure cultures of C.perfringens type A, and isolation of the organism from faecal swabs

Piglet No.	Infected	Day of experiment 0	1	2	3	4	5	6	7	8	9
U1	-	N	N	N	N	N	N	N	N	N	N
		-	-	+	+	+	+	+	+	+	+Sa
U2	-	N	N	N	N	N	N	N	N	N	N
		-	-	-	-	-	-	+	+	+	+Sa
U3	+	N	S	S	D	D	D	D	D	D	D
		-	+	+	+	+	+	+	+	+	+Sa
U4	+	N	S	S	S	D	D	D	D	D	D
		-	+	+	+	+	+	+	+	+	+Sa
U5	+	N	S	D	D	D	D	D	D	D	D
		-	+	+	+	+	+	+	+	+	+Sa

N = normal faeces
S = soft faeces
D = diarrhoea
- = no C.perfringens type A isolated
+ = C.perfringens type A isolated
Sa = sacrificed

congested while that of piglet U4 presented a normal appearance. The gastric serosal surface of piglet U5 and the lower portion of the duodenum to the mid-jejunum appeared reddened from the serosal surface; in piglet U4 the serosal surface of the lower jejunum to the mid-jejunum appeared pale. The duodenum of piglet U5 contained fluid with some gas and the mucosal surface was mildly congested. Piglet U3 did not have gas in the duodenum, its mucosa was slightly congested. The duodenal mucosal surface of piglet U4 appeared normal. Jejunal contents of piglets U3 and U5 were frothy and brownish in colour (Fig. 12). Necrotic debris could be seen on the mucosa which was congested. The jejunal mucosa of piglet U4 was slightly congested. The ileal contents of all 3 animals were fluid with some necrotic material, some mucoid tags were seen on the mucosa which was congested and inflamed. Pinpoint haemorrhages were present in some areas of the mucosal surface of the ileum of all animals.

The serosal surface of the large intestine in all infected piglets was reddened with congested areas (Fig. 13) which in piglet U3 were present throughout most of the entire large intestine (Fig. 14). The mucosa of the caecum and colon of all infected animals was extensively congested with some pinpoint haemorrhages; the contents were brownish and pasty with some mucoid tags. The mesenteric lymph nodes were enlarged and mildly congested.

Control piglets U1 and U2 were in good bodily condition, their gastrointestinal tract, mucosa and contents were all normal in appearance.

(iv) Bacteriological findings

Colonies of C.perfringens type A were isolated in large numbers from the mucosa of the stomach, duodenum, jejunum, ileum, caecum and colon of piglet U3. Isolations of the organism from stomach, colon and caecum of piglets U4 and U5 were fewer in number. Fewer colonies of the organism were isolated from the control piglets (Table 10).

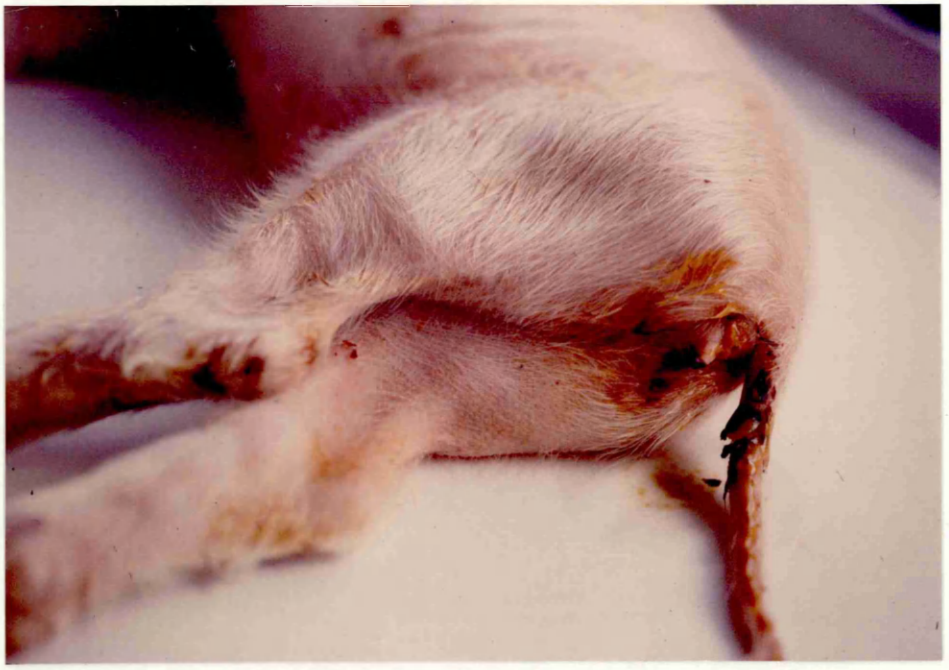

FIG. 11: Perineal region of piglet U3, 9 days post-inoculation with C.perfringens type A.
Note the soiling of the perineum with faeces and the poor condition of the piglet.

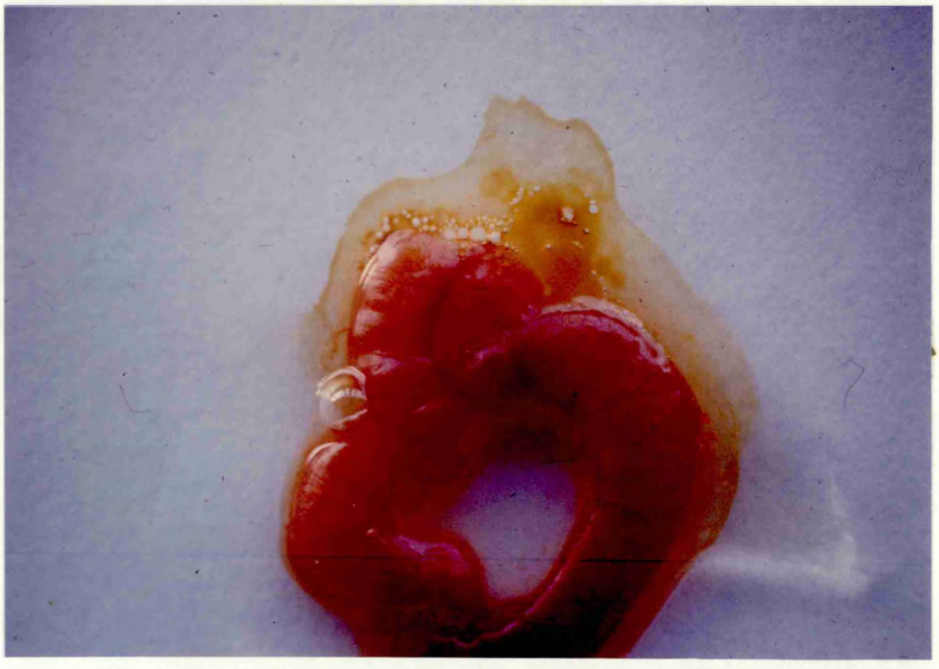

FIG. 12: Segment of jejunum and contents of piglet U5, 9 days post-inoculation with C.perfringens type A.
Note the congestion and the frothy contents.

FIG. 13: Macroscopic appearance of the serosal surface of the large intestine of piglet U5, 9 days post-inoculation with C.perfringens type A.
Note the congestion of the serosa (arrow).

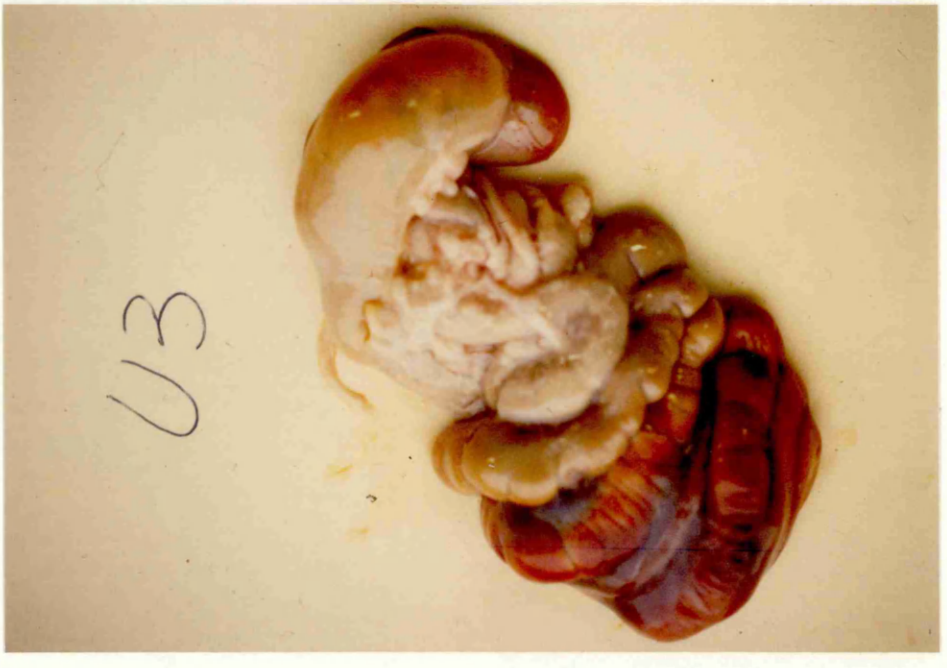

FIG. 14: Macroscopic appearance of the serosal surface of the large intestine of piglet U3, 9 days post-inoculation with C.perfringens type A.
Note the extensive areas of congestion.

TABLE 10

Sites from which C.perfringens type A was isolated from the HDCD piglets of Experiment 1 examined at post-mortem following inoculation with pure cultures of the organism

Piglet No.	Infected	Site of isolation					
		Stomach	Duodenum	Jejunum	Ileum	Caecum	Colon
U1	-	-	-	+	N.T.	N.T.	+
U2	-	-	-	-	N.T.	N.T.	+
U3	+	++	++	++	++	++	++
U4	+	+	+	++	++	+	+
U5	+	+	+	++	++	+	+

- = No colonies of C.perfringens type A isolated.

+ = Between 1 and 8 colonies of C.perfringens type A isolated.

++ = More than 8 colonies of C.perfringens type A isolated.

N.T. = Not tested.

Gram-stained smears prepared from the mucosa of these organs contained Gram-positive rods with no demonstrable spores, with morphology similar to that of C.perfringens type A. A few Gram-positive rods with this morphology were found in the smear from the jejunal mucosa of piglet U1; they were absent from the mucosal smears of other organs and in all organs of piglet U2.

Other bacteria were also isolated. They were non-haemolytic E.coli, Streptococcus spp. and Bacillus spp. isolated from different sites of both infected and control piglets.

(v) Virological and parasitological findings

No virus particles were demonstrated in negatively stained preparations from the intestinal contents of all animals when viewed by electron-microscopy.

No evidence of cryptosporidia was found in mucosal smears of different organs of infected and control piglets.

4. DISCUSSION

(a) The initial survey

(i) The limitations of the survey

The initial survey was not extensive. Only 17 submissions were examined comprising 60 animals or samples. The material was presented with little or no history. This meant that treatments given to the animals prior to sampling were not recorded and may have eliminated some Clostridia or perhaps other bacterial pathogens. The samples submitted were often submitted with no clear history of the length of time since they were taken or since the animal died. Most piglets submitted entire were from farrowing houses where the ambient temperature is high and decay can be rapid. It is known (Prevot et al., 1967) that C.perfringens can multiply rapidly after death and this type of delay may have affected the results obtained.

In this survey full examination for viruses and parasites was not carried out in every case. The examinations carried out for cryptosporidia would not have revealed coccidial oocysts and too little histology was carried out for accurate diagnosis to have been made by that means. No rotaviruses were demonstrated by the direct E.M. examination of faeces in this study but the detection of coronaviruses would have been unlikely using this method. Full characterisation of the E.coli isolates was not carried out as the study concentrated on C.perfringens. Even the isolation of this organism might have been improved by the use of specific media such as tryptone-sulphite-cycloserine agar.

(ii) C.perfringens isolations

In this study C.perfringens could be isolated from 30 of the 60 samples examined. The use of non-selective media such as horse blood agar inevitably leads to the masking of organisms present in small numbers by those in larger numbers and it is likely that C.perfringens is present more often than appears in the present survey. Thus, the lower than expected isolations of C.perfringens type A may in part be accounted for by the masking of these organisms in mixed populations. If suitable selective media, such as tryptone-sulphite-cycloserine agar as employed in the studies of Chapter 6, had been employed, it is likely that a higher rate of isolation would be achieved for C.perfringens. However, a relationship may exist between the numbers of C.perfringens present in the gastrointestinal tract and the number of colonies found in non-selective media such as horse blood agar. Thus, abnormally high numbers in the gut might be indicated by an increase in the number of colonies of the organism isolated on blood agar.

The 30 positive cases appeared to contain 3 cases of C.perfringens type C. All these cases were from the same submission and C.perfringens type C was not demonstrated in any of the other 27 positive cases. Of these 27 cases from which C.perfringens type A was isolated, 2 non-haemolytic isolates were identified and the remaining 25 were haemolytic. As haemolytic colonies are more obvious on isolation plates other non-haemolytic

isolates may have been present but would not have been detected if they were present in smaller numbers. Haemolytic colonies were much more obvious when present in low numbers. This finding corresponds with what is reported to be the case for C.perfringens type A isolated from humans in which haemolytic strains are far more frequently isolated than are non-haemolytic strains (Skjelkvale et al., 1979). There are no reports about the incidence of haemolytic and non-haemolytic strains from the pig.

The techniques used to confirm the identity of these organisms as C.perfringens type A were biochemical and cultural. No other toxins apart from alpha toxin were found in cooked meat filtrates and this technique was considered to confirm their identity as C.perfringens type A.

It is of interest that no C.perfringens spores were recorded from smears made in this study. Re-examination of those smears still available suggests that few if any were present. None of the cultures obtained were seen to sporulate at the time but in further studies using these isolates (Chapter 6) it was clear that some isolates were capable of sporulating.

(iii) Significance of the C.perfringens isolations

It has been reported that an increase in the numbers of C.perfringens type A in faeces is related to outbreaks of diarrhoea. In humans, faeces of patients suffering diarrhoea contained significantly higher numbers of C.perfringens type A than healthy individuals (Sutton, 1966). Neonatal diarrhoea of piglets was associated with an increase of the number of C.perfringens type A in intestinal contents of diseased animals (Secasiu, 1984). All the pigs in this series were diarrhoeic, but C.perfringens was isolated from only half the submissions. The possible reasons for this isolation from only half the submissions have been outlined above. In addition, organisms may have been present during disease prior to submission but have disappeared. No counts were carried out at this stage and only qualitative results are available.

The results obtained here (Table 7) resembled those obtained by Olubunmi (1982). As in his study, other bacteria such as E.coli and Campylobacter coli were identified and other pathogens might not have been detected. The clinical signs and post-mortem findings in the 28 cases apparently associated with the isolation of C.perfringens alone were described in the results above. They resemble those described by Olubunmi (1982) and Nabuurs et al. (1983). All the animals affected were young, but the majority of samples submitted were from pigs aged 1-3 weeks apart from 1 submission from 3-month old animals (Table 6) and this finding can not, therefore, be significant. The clinical signs and post-mortem findings associated with C.perfringens type A were distinct from those associated with C.perfringens type C and could not be confused with those of that disease. Especially noteworthy was the loss of condition and the low mortality in C.perfringens type A disease.

The post-mortem findings were described above but certain features were particularly prominent in all of the 10 animals in which, at post-mortem, C.perfringens type A was isolated in profuse to moderate amounts. The mucosa of the small intestine was congested to a variable extent. Lesions were more prominent at jejunal and ileal level. The changes were milder in the mucosa of the large intestine. In most cases the jejunum and ileum appeared distended with fluid contents and gas. Villi were usually reduced in height but not destroyed. The mucosa of the large intestine was usually congested. The changes were complicated by the rapid onset of decay in some of these animals.

Decay meant that few samples were taken for histopathology and in those few cases only mild changes were recorded, possibly because the changes develop rapidly (Olubunmi, 1982). In none of the samples examined here were the lesions as extreme as those he showed in experimentally-infected piglets killed in extremis. Some villous atrophy and congestion was noted, and villous atrophy was clearly present in gross specimens not processed for histology.

The C.perfringens type A was isolated from the intestines of 10 piglets and the distribution of the organism was shown in Table 8. C.perfringens type A was isolated from the stomach of 4 piglets and from the duodenum of 9 piglets. In humans, except under disease conditions, C.perfringens type A is not found in the stomach or duodenum, while it is commonly isolated from the ileum (Vince et al., 1972). C.perfringens type A is not present in the stomach and duodenum of the healthy piglet (Ducluzeau, 1985). The presence of the organism in these sites in piglets in this survey indicates abnormality and may be associated with diarrhoea. It is, however, possible that these results may have been affected by post-mortem change. Although this could have happened within the gut it clearly did not happen in the carcase as a whole for no C. perfringens type A was isolated from sites other than the gut in this series.

The isolation of C.perfringens type A from the samples examined in this survey suggests that, both on the evidence presented and that from the literature, C.perfringens type A was associated with both diarrhoea and enteric lesions in the animals surveyed.

(b) Experimental reproduction of C.perfringens type A enteritis in piglets

(i) The reproduction of clinical disease

C.perfringens type A isolate 7 appeared to cause a syndrome in the inoculated 8-day old hysterectomy-derived, colostrum-deprived piglets similar to that seen in the field and that described by Nabuurs et al. (1983). Profuse creamy diarrhoea was noted in the inoculated animals. All three infected piglets survived to the end of the period of observation but remained in poor condition with diarrhoea throughout this period. Their survival was in contrast to the findings of Olubunmi (1982) who used slightly younger piglets.

The incubation period appeared to be 24-48 hours (Table 9). Softening of the faeces was observed in all piglets 24 hours post-inoculation and frank diarrhoea developed in piglet U5 24 hours later; piglets U3 and U4 presented diarrhoea until 48 and 72 hours, respectively, after the appearance of first changes in faecal consistency. At the end of the period of observation the piglets were emaciated, with hairy coats and soiled hindquarters. The control piglets remained in good condition until killed and passed normal faeces, providing a marked contrast with the inoculated animals.

The duration of the diarrhoea (6-7 days) was similar to that found by Nabuurs et al. (1983) although the animals in this study were killed while still diarrhoeic.

(ii) The relationship between C.perfringens type A and the clinical signs

C.perfringens type A was recovered from the faeces of the infected piglets within 24 hours of inoculation (Table 9) and was found consistently throughout the period of observation denoting its establishment in the intestinal tract. The organism was isolated from the faeces of the uninfected controls, from control U1 from the second day of experiment and from control U2 from the sixth day. This fact may be due to an external contamination with the organism involving a different strain of C.perfringens type A rather than the one used in the experiment, or to a cross-contamination involving the same strain used for infection. Although precautions were taken to avoid contaminations, C.perfringens type A is so ubiquitous that the maintenance of animals free of the organism is only possible under gnotobiotic conditions. It is possible that the organism was already present in the intestine of all piglets prior to the start of the experiment, although in numbers undetectable by the techniques used. C.perfringens colonies were always detected in considerably fewer numbers in the faeces of control piglets than in the infected animals, and it may be that the numbers present were too low to cause disease.

(iii) The relationship between C.perfringens type A and the post-mortem findings

C.perfringens type A was isolated from the mucosa of the stomach and duodenum of all infected animals while the organism could not be isolated from these sites of uninfected controls (Table 10). From the jejunal mucosa of the inoculated piglets the organism was isolated in large numbers while no organisms were isolated at this level from control piglet U2 and control piglet U1 presented significantly fewer colonies of C.perfringens type A than the infected animals. As the assessments of the number of organisms present was merely qualitative, any association between lesions and numbers of organisms cannot be absolute. This is particularly the case since post-mortem examination was not carried out until 6-7 days after the onset of diarrhoea.

Gross pathological changes apparently attributable to C.perfringens type A infection were observed in all infected piglets. The lesions were confined to the gastrointestinal tract. A correlation between the number of colonies of C.perfringens type A isolated and the severity of lesions observed appeared to exist. Congestion of the stomach, small and large intestine was more apparent in piglet U3 from which larger numbers of colonies of the organism were isolated than from the other two infected animals. The mucosa of the small intestine, particularly that of the ileum, was congested with pinpoint haemorrhages in all infected piglets. The mucosa of caecum and colon presented also large areas of congestion and pinpoint haemorrhages. The uninfected control piglets had no pathological changes in their gastrointestinal tracts, in keeping with the low number of C perfringens isolated from them (Table 10).

Nabuurs et al. (1983) gave no account of gross pathological lesions in their study except the mention of abundant gas in the lumen of the small and large intestine. Olubunmi (1982) found more severe lesions, with congestion and necrosis in the small intestine as well as in the large intestine. His results may differ because

of the age of the piglets used and because the piglets died early in the disease. The lesions described here do not differ substantially from those in his surviving piglet.

(c) Conclusions

The results of this survey indicated that C.perfringens type A could be isolated in large numbers from cases of diarrhoea in piglets. The organism was associated with a syndrome in which diarrhoea, loss of condition and pasting of the perineum occurred. Mortality was low in contrast to infection with C.perfringens type C. The pathological changes found included congestion of the small intestine with villous atrophy and necrosis and similar inflammatory changes in the large intestine associated with the presence of large numbers of C.perfringens type A organisms. Twenty seven isolates of C.perfringens type A were stored for further examination and one of these, isolate 7, was shown to be pathogenic for piglets and to cause an enteritis similar to that found in piglets in the survey and described in the literature.

CHAPTER 4

STUDIES ON THE IMMUNISATION AND PROTECTION OF PIGS AGAINST INFECTION WITH C.PERFRINGENS TYPE A

1. INTRODUCTION

The results of the survey presented in Chapter 3 indicated that C.perfringens Type A was associated with enteric disease in piglets. This supposition was confirmed in Experiment 1 in which one of these C.perfringens type A isolates, isolate 7 was used to reproduce diarrhoea in hysterectomy-derived, colostrum-deprived piglets. The literature surveyed in Chapter 1 did not allow any clear idea to be formed about the possible mechanism of this diarrhoea. In an attempt to study the relevance of various fractions of the bacteria and its products, an immunisation study was set up and its results assessed by the protection of neonatal hysterectomy-derived, colostrum-deprived piglets using sera, colostra and milks produced from immunised animals.

This chapter describes the production and testing of the antigens in part 2, the assessment of the antibodies produced in sows in part 3 and the final protection test in part 4. The results are discussed in part 5.

2. PRODUCTION AND EVALUATION OF C. PERFRINGENS TYPE A TOXOID AND BACTERIN

(a) Production of antigens

(i) Alpha toxin toxoid

This was produced by the methods described in Chapter 2 from C.perfringens type A isolate 7 obtained in the survey and shown to be pathogenic for piglets in Experiment 1.

Assay of the culture supernatant indicated that its mouse lethal dose (MLD) was 16 MLD per ml. The toxin titre increased to 128 MLD per ml after concentration of the supernatant by ammonium sulphate precipitation and dialysis against polyethylene glycol.

The antigenicity of the toxin was tested in vitro by counterimmunoelectrophoresis using commercial C.perfringens type A antiserum. It showed a toxin titre of 1:8 which was increased to 1:64 by concentration. The concentrated toxin was tested for the progress of detoxification daily by the haemolysis test using sheep red blood cells described in Chapter 2. Complete absence of haemolysis was observed at the 12th day of incubation; 5 mice were then inoculated intraperitoneally with the detoxified material and kept under observation for 48 hours. This material did not cause any symptoms in the inoculated mice. This concentrated toxoid was then tested for sterility and found to be sterile.

The toxoid contained 22.9 mg of protein per ml and was combined with aluminium hydroxide as an adjuvant as described in Chapter 2. The sterility test was repeated and the toxoid was found to be sterile.

(ii) Whole culture bacterin

This was prepared by the methods described in Chapter 2. The clarified culture contained 3.5×10^7 colony-forming units per ml when counts were performed. Inactivation was carried out as described in Chapter 2 and no bacteria could be isolated by the 14th day after inactivation began. At this stage a safety test was performed in 5 mice all of which survived. Sterility tests were also carried out and the material found to be sterile. Its protein concentration was found to be 73.6 mg of protein per ml and it was then mixed with adjuvant as described in Chapter 2. The final adjuvanted whole culture bacterin was tested for sterility and found to be sterile.

(iii) Freeze-dried washed cell bacterin

This was prepared by the methods described in Chapter 2 and contained 6.0 x 10^9 colony forming units per ml after washing and before inactivation. The efficiency of inactivation was tested daily as for the whole culture bacterin and also found to be complete within 14 days of inactivation. It was both sterile and safe for all 5 mice inoculated. The protein concentration of the cell suspension was 96.7 mg protein per ml before freeze-drying.

(b) Potency testing of the 3 antigens

The potency of the freeze-dried bacterin was not tested but that of the crude toxoid and the whole culture vaccines was tested for potency in rabbits, mice and pigs using the methods and schedule described in Chapter 2. IgG, IgM and IgA antibody levels against whole C.perfringens cells were detected using the indirect immunofluorescence test described in Chapter 2. Serum or milk protein was tested at 5 dilutions, neat, 1:10, 1:100, 1:1,000 and 1:10,000. Antibody to alpha toxin was detected by counterimmuno-electrophoresis and the Nagler reaction methods also described in Chapter 2.

(c) Results of potency testing

(i) Mouse protection tests

Mouse protection tests indicated a 100 per cent protection against challenge by the immunisation of mice with a dilution of the adjuvanted toxoid up to 1:4 and up to 1:2 for the adjuvanted whole culture bacterin. Other dilutions of both immunogens offered different degrees of protection (Tables 11 and 12).

(ii) Serological responses to the antigens

Immunisation of mice and rabbits either with adjuvanted toxoid or adjuvanted whole culture bacterin, elicited a detectable serological response which was identical for both groups. IgG

TABLE 11

Potency testing of concentrated alpha toxin toxoid

Mouse protection test

Dilutions of toxoid	Immunisation dose (a)	Challenge toxin dose (MLD/Ml)	Number survived (b)
1:2	.5 ml	16	5/5
1:4	.5 ml	16	5/5
1:8	.5 ml	16	4/5
1:16	.5 ml	16	2/5
1:32	.5 ml	16	0.5
non-vaccinated controls	-	16	0/5

(a) Immunised twice subcutaneously at 14 day intervals.

(b) Dead/alive

TABLE 12

Potency testing of concentrated whole culture bacterin

Mouse protection test

Dilutions of bacterin	Immunisation dose (a)	Challenge toxin dose (MLD/Ml)	Number survived (b)
1:2	.5 ml	16	5/5
1:4	.5 ml	16	4/5
1:8	.5 ml	16	1/5
1:16	.5 ml	16	0/5
1:32	.5 ml	16	0/5
non-vaccinated controls	-	16	0/5

(a) Immunised twice subcutaneously at 14 days intervals.

(b) Dead/alive

titres to C.perfringens type A cells were greater than 1:1,000 (Fig. 15). Uninoculated control mouse sera were negative (Fig. 16) but antibody was detected in neat serum from the uninoculated control rabbit. Anti-alpha toxin antibodies were detected in both groups by counterimmunoelectrophoresis and by the Nagler reaction (Fig. 17); no attempts were made to determine the anti-alpha toxin titre (Table 13). No antibody was demonstrated in these tests in the uninoculated control sera. Immunisation of pigs with adjuvanted toxoid, adjuvanted whole culture bacterin and with toxoid-bacterin mixture elicited or enhanced an antibody response to C.perfringens type A cells and to alpha toxin. The pig immunised with a mixture of toxoid-bacterin and the non-immunised control pig were free from antibody to C.perfringens type A cells and alpha toxin prior to immunisation (Table 14). The pigs immunised with toxoid and whole culture bacterin had IgG and IGM titres of 1:10 against C.perfringens type A cells. No IgA titres were detected in the pre-immunisation sera. Only one pig, that immunised with whole culture bacterin, had antibody to alpha toxin (titre 1:4) in pre-immunisation serum (Table 15).

All immunised pigs showed production or increase of their titres to C.perfringens type A cells and to alpha toxin after the first immunisation (Tables 14 and 15). There was a further increase of antibody titres after the second immunisation. Slight variations in antibody titres among the immunised animals were found. The non-immunised pig control developed a low IgM titre to C.perfringens type A cells at the last bleeding. The Nagler reaction was less sensitive than counterimmunoelectrophoresis for the detection of antibodies to alpha-toxin.

3. IMMUNISATION OF PREGNANT SOWS WITH ADJUVANTED TOXOID AND BACTERIN AND WITH FREEZE-DRIED BACTERIN IN FEED

(a) Experimental design

Ten hybrid sows on Farm 2 were immunised with a subcutaneous dose of 5.0 ml of toxoid, bacterin or toxoid-bacterin combined up to eight weeks before farrowing, and a second 5.0 ml subcutaneous dose three weeks before farrowing (Table 16).

TABLE 13

Serological response of rabbits and mice immunised with adjuvanted toxoid and adjuvanted whole culture bacterin

Species	Immunisation	Reciprocal of IgG titre to *C.perfringens* type A cells	Anti-alpha toxin C.I.E.	Anti-alpha toxin Nagler reaction
Rabbit (1)	toxoid	1,000	+	+
Rabbit (1)	bacterin	1,000	+	+
Rabbit (1)	uninoculated control	1	-	-
Mice (6)	toxoid	1,000	+	+
Mice (6)	bacterin	1,000	+	+
Mice (6)	uninoculated control	-	-	-

- = absent

+ = present

TABLE 14

Serological response of pigs immunised with adjuvanted toxoid and adjuvanted whole culture bacterin

(a) Reciprocal of antibody titres to C.perfringens type A cells.

Antigen inoculated	Pre-immunisation			14 days after first immunisation			14 days after second immunisation		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
toxoid	1	10	-	10	10	1	100	1,000	1
bacterin	10	10	-	100	1,000	1	100	1,000	10
toxoid-bacterin	-	-	-	10	100	1	1,000	1,000	1
control	-	-	-	-	-	-	-	10	-

- = absent

TABLE 15

Serological response of pigs immunised with adjuvanted toxoid and adjuvanted whole culture bacterin

(b) Antibody titres to alpha toxin (a)

Antigen inoculated	Pre-immunisation		14 days after first immunisation		14 days after second immunisation	
	Titre	Nagler reaction	Titre	Nagler reaction	Titre	Nagler reaction
toxoid	-	-	1:4	-	1:16	±
bacterin	1:4	-	1:16	+	1:32	+
toxoid-bacterin	-	-	1:4	-	1:4	-
control	-	-	-	-	-	-

(a) detected by counterimmunoelectrophoresis.

- = absent
+ = positive

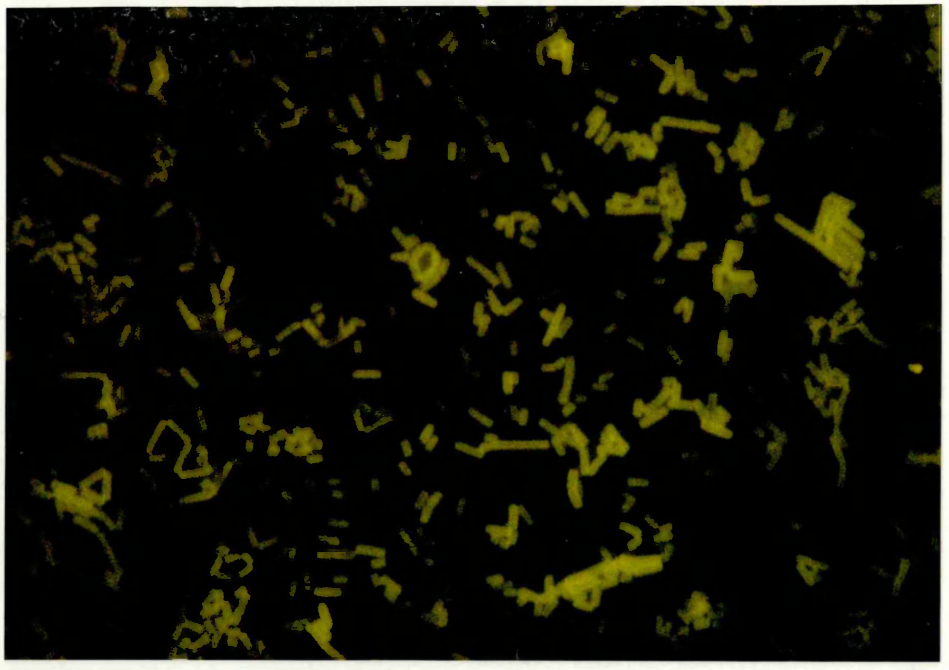

FIG. 15: C.perfringens type A fluorescent cells following treatment with serum from a mouse immunised with whole culture bacterin. Stained using fluorescent rabbit anti-mouse IgG.

(x1200)

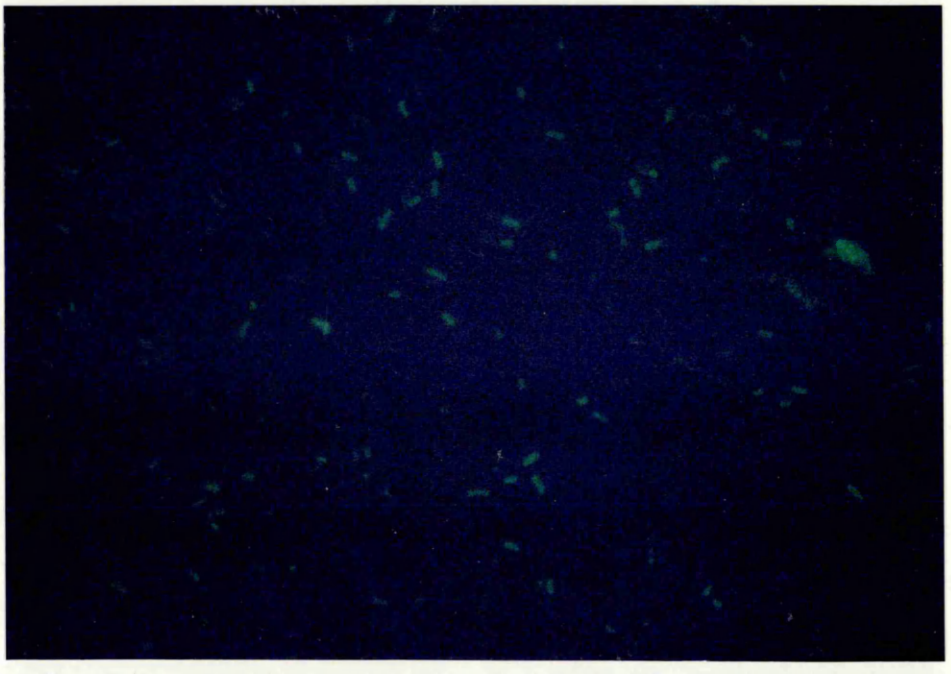

FIG. 16: Control preparation for Fig. 15 in which serum from a non-immunised mouse was used.

Compare the fluorescence in the two preparations.

(x1200)

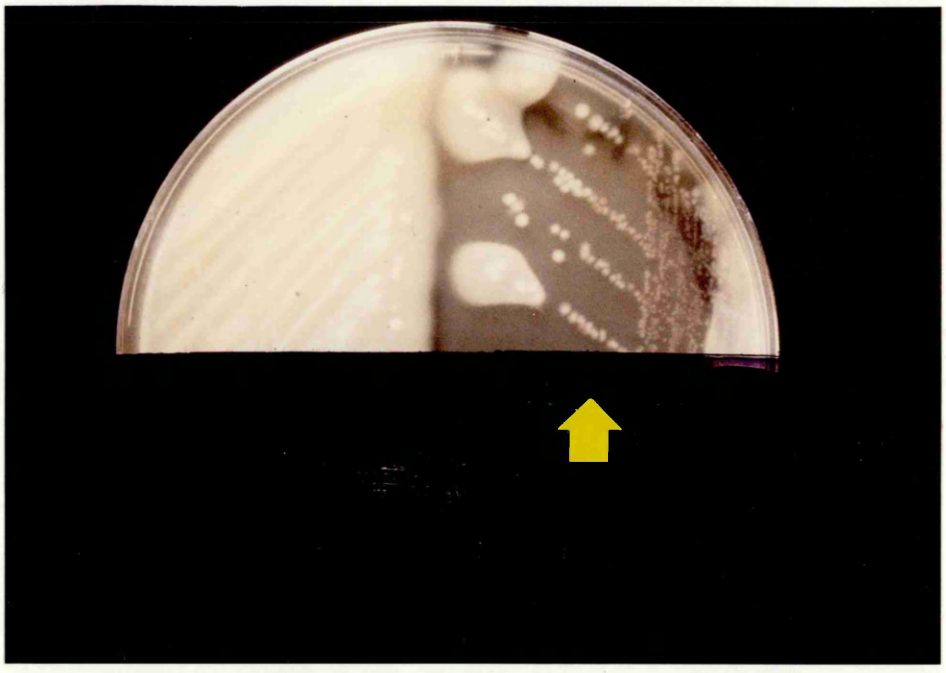

FIG. 17: Nagler reaction on egg yolk agar plate.
Note the absence of the opalescent zone from the half of the plate which had been treated previously with the serum of the rabbit immunised with C.perfringens type A toxoid (arrow).

Three of these sows were given freeze-dried bacterial cells which were previously mixed at a 10 per cent concentration with cornflour, this mixture was administered in the feed in a relation of 3.75g per 2.5kg of feed. The sows received this mixture daily from eight weeks before farrowing until delivery (Table 16).

Serum samples were taken from the sows before they were immunised, before second immunisation and at farrowing time. Samples of colostrum were taken from three sows just after farrowing. Milk samples were taken from eight sows. Colostra and milks were cleared of solids by the method described in Chapter 2 and stored at -20°C until required. Two milks from non-immunised sows were used as controls.

(b) Results

Immunisation of pregnant sows with adjuvanted toxoid and bacterin and with freeze-dried bacterin in feed elicited or enhanced an antibody response to C.perfringens type A cells and to alpha toxin. Sows 402 and 406 were devoid of antibodies to C.perfringens type A cells in their pre-immunisation sera whereas sows 36 and 93 showed low titres of IgM antibodies; these sows plus sow 405 were negative for antibodies to alpha toxin in their pre-immunisation sera. All other sows presented variable titres of antibodies to C.perfringens type A cells and to alpha toxin in their pre-immunisation sera. All immunised sows showed a rise in their antibody levels after the first immunisation. The second immunisation increased further the average titre of the immunised sows. The milks from two non-immunised sows showed lower titres of antibodies to C.perfringens type A cells than those of the immunised animals, and were negative to antibodies to alpha toxin.

Results are summarised in Tables 17 and 18.

TABLE 16

Immunisation of pregnant sows with adjuvanted toxoid and bacterin and with freeze-dried bacterin in feed

Sow No.	Immunisation	Days before farrowing	
		First immunisation	Second immunisation
295	Toxoid	38	23
93	Toxoid	39	24
403	Toxoid	48	33
36	Bacterin	40	25
292	Bacterin	43	28
401 (a)	Toxoid-bacterin	-	-
402	Toxoid-bacterin	36	21
406 *	Toxoid	57	42
40 *	Bacterin	59	44
45 *	Toxoid-bacterin	58	43

* Freeze-dried cells administered in feed from first immunisation to farrowing

(a) = Sow immunised but did not farrow.

TABLE 17 Serological response of sows immunised with adjuvanted toxoid, adjuvanted whole culture bacterin and freeze-dried bacterin in feed

a) Reciprocal of antibody titres to C.perfringens type A cells.

Sow No.	Immunisation	Pre-immunisation			Before second immunisation			At farrowing in serum			At farrowing in colostrum			In milk		
		IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
295	Toxoid	10	10	10	10	1000	10	100	1000	10	1000	1000	10	1000	10	1000
93	Toxoid	-	1		10	100	1	1000	1000	10	1000	1000	10	100	10	100
403	Toxoid	100	100	10	1000	1000	10	1000	1000	10	NT	NT	NT	1000	100	1000
36	Bacterin	-	1	-	10	100	1	1000	1000	10	NT	NT	NT	1000	1000	1000
292	Bacterin	100	100	1	1000	100	10	1000	1000	10	NT	NT	NT	1000	100	1000
401	Toxoid-bacterin	1	1	-	100	10	1	NT	NT	NT	NT	NT	NT	NT	NT	NT
402	Toxoid-bacterin	-	-	-	10	100	-	100	100	1	1000	100	10	1000	100	1000
406*	Toxoid	-	-	-	10	100	10	100	100	10	NT	NT	NT	100	100	1000
40*	Bacterin	10	10	1	100	100	10	100	100	10	NT	NT	NT	NT	NT	NT
405*	Toxoid-bacterin	1	1	-	10	100	1	100	1000	10	NT	NT	NT	100	100	1000
Control	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	10	1	10
Control	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	1	1	10

* Freeze-dried cells administered in feed from first immunisation to farrowing.

- = absent. NT = not tested.

TABLE 18

Serological response of sows immunised with adjuvanted toxoid, adjuvanted whole culture bacterin and freeze-dried bacterin in feed

b) Antibody titres to alpha toxin ([a])

Sow No.	Pre-immunisation	Before second immunisation	farrowing in serum	farrowing in colostrum	In milk
285	1:4	1:16	1:16	1:32	1:8
93	-	1:8	1:16	1:32	1:8
403	1:8	1:16	1:32	NT	1:8
36	-	1:2	1:4	NT	1:2
292	1:2	1:2	1:8	NT	1:2
401	1:2	1:8	NT	NT	NT
402	-	1:4	1:4	1:8	1:2
406	-	1:16	1:16	NT	1:4
40	1:4	1:8	1:16	NT	NT
405	-	1:8	1:32	NT	1:4
Control	NT	NT	NT	NT	-
Control	NT	NT	NT	NT	-

(a) detected by counterimmunoelectrophoresis.

(-) = absent. NT = not tested.

4. THE PROTECTION OF HYSTERECTOMY-DERIVED, COLOSTRUM-DEPRIVED PIGLETS AGAINST INFECTION WITH C.PERFRINGENS TYPE A (EXPERIMENT 2)

(a) Materials and Methods

(i) Immune products for passive protection

Sera from pigs immunised with alpha toxin toxoid (A) and with mixture toxoid-whole culture bacterin (B) described in Section 2, and the following pools of milks and colostra from immunised sows described in Section 3 were sterilized by gamma irradiation.

Pool C. Milks from sows immunised with toxoid (sows 93, 295, 403).

Pool D. Colostra from sows immunised with toxoid (sows 93, 295).

Pool E. Milks from sows immunised with bacterin (sows 36, 292).

Pool F. Milk and colostrum from sow immunised with mixture toxoid-bacterin (sow 402).

Pool G. Milks from sows which were given freeze-dried cells administered in feed (sows 405, 406) and toxoid-bacterin parenterally.

(ii) Piglets

Twenty-three hysterectomy-derived, colostrum-deprived piglets were obtained by the methods described in Chapter 2 and housed in the same pen in racks of cages individually or in pairs.

(iii) Experimental design

The piglets were passively immunised two hours after delivery by the oral or intraperitoneal routes or both according to the schedule given in Table 19.

TABLE 19

Protection of HDCD piglets against C.perfringens type A infection by passive immunisation with colostrum, milk or serum from immunised pigs
Immunisation schedule.

Number of piglets	Passive immunisation	Route and quantity	Infection
2	-	-	-
6	-	-	+
2	A	10 ml i/p 5 ml oral	+
2	B	10 ml i/p 5 ml oral	+
3	C	10 ml oral	+
2	D	10 ml oral	+
2	E	10 ml oral	+
2	F	10 ml oral	+
2	G	10 ml oral	+

A = serum from pig immunised with toxoid-bacterin.
B = serum from pig immunised with toxoid.
C = pool of milks from sows immunised with toxoid.
D = pool of colostra from sows immunised with toxoid.
E = pool of milks from sows immunised with bacterin.
F = pool of milk and colostrum from sow immunised with toxoid-bacterin.
G = pool of milks from sows immunised with freeze-dried bacterin in feed and parenteral toxoid-bacterin.

Two piglets were kept as non-immunised, non-infected controls, and 6 piglets as non-immunised, infected controls.

Twenty-two hours after delivery the piglets were challenged with the same C.perfringens type A (isolate 7) used for the experiment described in Chapter 3 and grown under the same conditions.

Five ml of inoculum containing approximately 1.6 x 10^7 organisms/ml were given to each piglet after being fasted for 12 hours.

All piglets were examined daily and their appearance, appetite and the consistency of their faeces were recorded. Rectal temperatures were taken from each piglet daily. Rectal swabs were taken daily from all animals and examined for the presence of C.perfringens type A and other bacteria by the methods described in Chapter 2.

Blood samples were taken from all piglets at slaughter and the sera collected and stored at -20°C. These sera were examined for the presence of antibodies to C.perfringens type A cells and anti-alpha toxin by the methods described in Chapter 2.

The piglets were sacrificed in the sequence shown in Table 20 by the methods described in Chapter 2, and post-mortem, histological and bacteriological examinations were also carried out using the methods described in Chapter 2.

(b) Results

(i) Clinical findings

All piglets were clinically normal and healthy during the period prior to infection with the exception of piglets CO+-2 and CO+-3 which were weak and underweight. Their faeces were normal (Table 20).

TABLE 20

Changes in faecal consistency in HDCD piglets passively immunised, following infection with pure cultures of C.perfringens type A and isolation of the organism

Piglet No.	Passive immunisation	Infection	Day of Experiment 0	1	2	3	4	5	6	7	8	9	10	11	12
CO- -1	-	-	N	N	N	N	N	NK							
			-	-	-	+	+	+							
CO- -2	-	-	N	N	N	N	N	N	N	N	N	NK			
			-	-	-	+	+	+	+	+	+	+			
CO+ -1	-	+	N	D	D	DK									
			-	+	+	+									
CO+ -2	-	+	N	D	D*										
			-	+	+										
CO+ -3	-	+	N	D*											
			-	+											
CO+ -4	-	+	N	D	D	D*									
			-	+	+	+									
CO+ -5	-	+	N	D	D*										
			-	+	+										

TABLE 20 (Continued)

Piglet No.	Passive immunisation	Infection	Day of Experiment												
			0	1	2	3	4	5	6	7	8	9	10	11	12
CO+ -6	-	+	N	D	D	D	D	S	N	N	N	NK			
			-	+	+	+	+	+	+	+	+	+			
A -1	A	+	N	N	N	N	N	NK							
			-	-	-	+	+	+							
A -2	A	+	N	S	N	N	NK								
			-	+	+	+	+	+							
B -1	B	+	N	N	N	N	N	N	N	N	N	N	NK		
			-	+	+	+	+	+	+	+	+	+	+		
B -2	B	+	N	N	N	S	N	S	N	N	N	S	SK		
			-	-	+	+	+	+	+	+	+	+	+		
C -1	C	+	N	N	N	N	N	N	N	N	N	SK			
			-	-	-	+	+	+	+	+	+	+			
C -2	C	+	N	N	N	N	N	N	N	N	N	N	N	NK	
			-	-	-	+	+	+	+	+	+	+	+	+	
C -3	C	+	N	N	N	N	N	NK							
			-	-	-	+	+	+							
D -1	D	+	N	N	N	N	N	NK							
			-	-	-	+	+	+							

TABLE 20 (Continued)

Piglet No.	Passive immunisation	Infection	Day of Experiment 0	1	2	3	4	5	6	7	8	9	10	11	12
D -2	D	+	N	N	N	S	N	N	N	N	NK				
			-	+	+	+	+	+	+	+	+				
E -1	E	+	N	D	D	N	N	N	NK						
			-	+	+	+	+	+	+						
E -2	E	+	N	D	D	S	N	N	S	N	N	N	S	DK	
			-	+	+	+	+	+	+	+	+	+	+	+	
F -1	F	+	N	S	S	S	N	N	NK						
			-	+	+	+	+	+	+						
F -2	F	+	N	N	S	N	N	N	S	N	N	N	S	D	DK
			-	-	+	+	+	+	+	+	+	+	+	+	+
G -1	G	+	N	N	N	N	N	N	N	N	N	N	N	N	NK
			-	-	-	+	+	+	+	+	+	+	+	+	+
G -2	G	+	N	N	N	N	N	N	N	N	N	N	N	N	NK
			-	-	-	+	+	+	+	+	+	+	+	+	+

N = normal faeces. * = died. + = C.perfringens isolated from faecal swabs.

S = soft faeces. K = killed. - = C.perfringens not isolated from faecal swabs.

For key to passive immunisation groups see Table 19.

Twelve hours after infection piglet CO+-3 presented diarrhoea and was moribund, dying a short time later. No clinical signs were seen in the rest of the animals at that time. Twenty-two hours after infection all infected, non-immunised control piglets developed a profuse diarrhoea, creamy in colour (Table 20). Less profuse but similar diarrhoea developed in immunised piglets E-1 and E-2 and the faeces of piglets A-2 and F-1 also became soft and yellowish in colour. All other animals, immunised and non-infected controls were healthy with normal faeces.

At 36 hours post-infection piglets CO+-2 and CO+-5 were found dead. Their hindquarters were soiled with a creamy diarrhoea and they were in poor bodily condition.

Forty-eight hours post-infection piglet CO+-4 was found dead and piglet CO+-1 was moribund and was sacrificed. Both piglets had hollowed flanks. Their coats were hairy, their eyes sunken and they were in poor bodily condition. Their perineums were soiled with diarrhoea. The remaining infected, non-immunised piglet CO+-6 appeared weak and depressed with a profuse diarrhoea. Immunised piglets E-1 and E-2 were diarrhoeic though not profusely so. Piglets F-1 and F-2 had soft faeces. All the rest of the animals were clinically normal.

Infected, non-immunised piglet CO+-6 presented diarrhoea until the fourth day post-infection being weak and depressed then at the fifth day post-infection the diarrhoea stopped but the animal remained feeble for the remainder of the period of observation. The immunised piglets as well as the controls remained healthy and passed normal faeces. The changes in the faecal consistency of the piglets are summarised in Table 20.

The rectal temperatures of all piglets remained within the normal range with the exception of the two non-immunised non-infected controls CO-1 and 2 (38°C, day 1) and infected non-immunised control piglet CO+-6 which had a rectal temperature of 38°C until Day 4.

(ii) Faecal bacteriology

Pre-inoculation faecal swabs were all negative for the presence of C.perfringens type A in all animals. The organism was isolated in profuse culture from the rectal swabs of all infected, non-immunised piglets from the first day post-infection. It was also isolated in profuse culture from rectal swabs of piglets A-2, E-1 and E-2. A few colonies of the organism were isolated from piglets B-1, D-2 and F-1. C.perfringens type A was not detected in the rectal swabs of the rest of the animals on the first day post-inoculation.

On the second day post-infection profuse cultures of C.perfringens type A could still be isolated from the rectal faeces of the infected non-immunised piglets and from immunised piglets E-1 and E-2. Fewer organisms were isolated from piglet A-2. Few colonies of C.perfringens type A were isolated from the remaining piglets shown to be infected in Table 20.

On the third day post-infection all animals including non-infected controls were infected with the organism (Table 20).

Non-haemolytic E.coli, Clostridium spp., Streptococcus spp. and Staphylococcus spp. were isolated from the rectal swabs of most of the piglets from the second day of the experiment.

β-haemolytic E.coli was isolated from the rectal swabs of piglets B-2, E-2 and F-2 in the tenth day of observation period.

(iii) Pathological findings

At post-mortem examination, infected, non-immunised piglets CO+-1, CO+ -2, CO+ -3, CO+ -4 and CO+ -5 were in poor bodily condition, their hindquarters soiled with diarrhoea, sunken eyes, hollowed flanks and hairy coats (Fig. 18). Gross changes were confined to the abdominal viscera. The livers of these animals were slightly pale and the mesenteric lymph nodes were congested.

Piglet CO+ -2 had an extensive necrotising peritonitis. The gastrointestinal tract of this piglet was severely congested with large areas of haemorrhage.

The stomach of piglets CO+ -1, CO+ -3, CO+ -4 and CO+ -5 appeared normal on their external surfaces though distended with milk and gas. Both the spleen and kidneys were normal. The jejunum and ileum in all these animals were distended with gas. The mesenteric vessels were congested. The large intestine was reddened from the serosal surface and distended.

The gastric mucosa of these piglets appeared slightly congested, except that of piglet CO+ -2 which was severely congested with large areas of haemorrhage, a feature that was present throughout the gastrointestinal tract in this piglet. The duodenal mucosa of piglets CO+ -1, CO+ -3, CO+ -4 and CO+ -5 was slightly congested. Necrotic debris was present on the mucosa of the jejunum and ileum which appeared distended and largely congested, with pinpoint haemorrhages in some areas. The jejunal and ileal contents were frothy and yellowish in colour (Fig. 19). The mucosa of caecum and colon was congested in large areas, the contents were brownish in colour with mucoid tags.

Examination of portions of the jejunum under the dissecting microscope showed shortened villi. In the case of piglet CO+-2 they were completely absent.

Infected, non-immunised piglet CO+ -6 was killed on day 9 of the observation and had clinical signs of diarrhoea at the time of death. The serosal surface of the jejunum, ileum and caecum were reddened but all other organs appeared normal. The gastric and duodenal mucosa was normal. The jejunal and ileal mucosa were congested with normal contents. The mucosa of the colon was slightly congested and the contents were solid. The mesenteric lymph nodes were enlarged and pale in appearance.

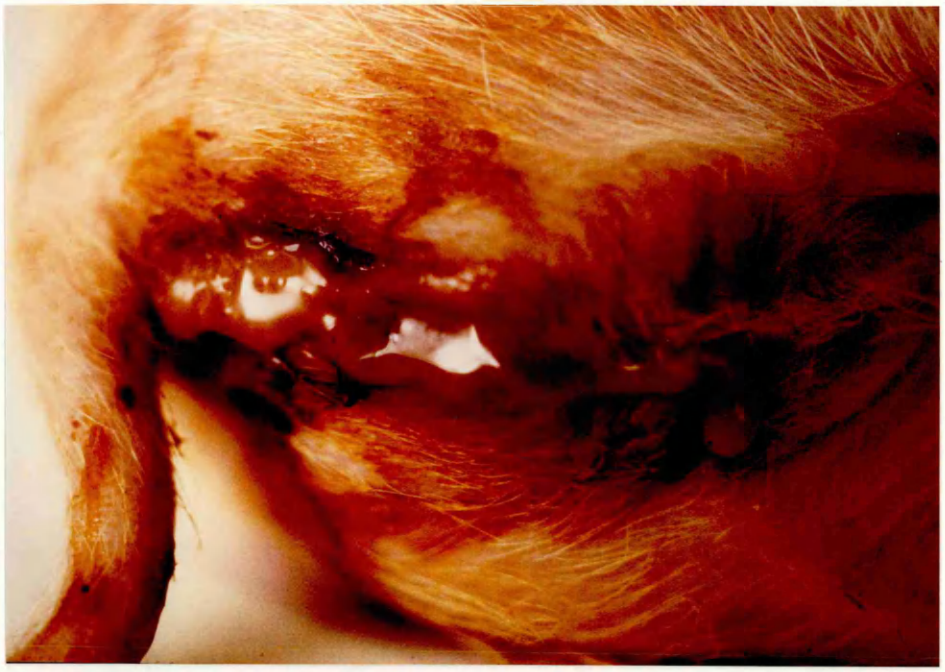

FIG. 18: Perineal region of piglet CO+-1, 3 days post-inoculation with C.perfringens type A.
Note the soiling of the perineum with diarrhoea.

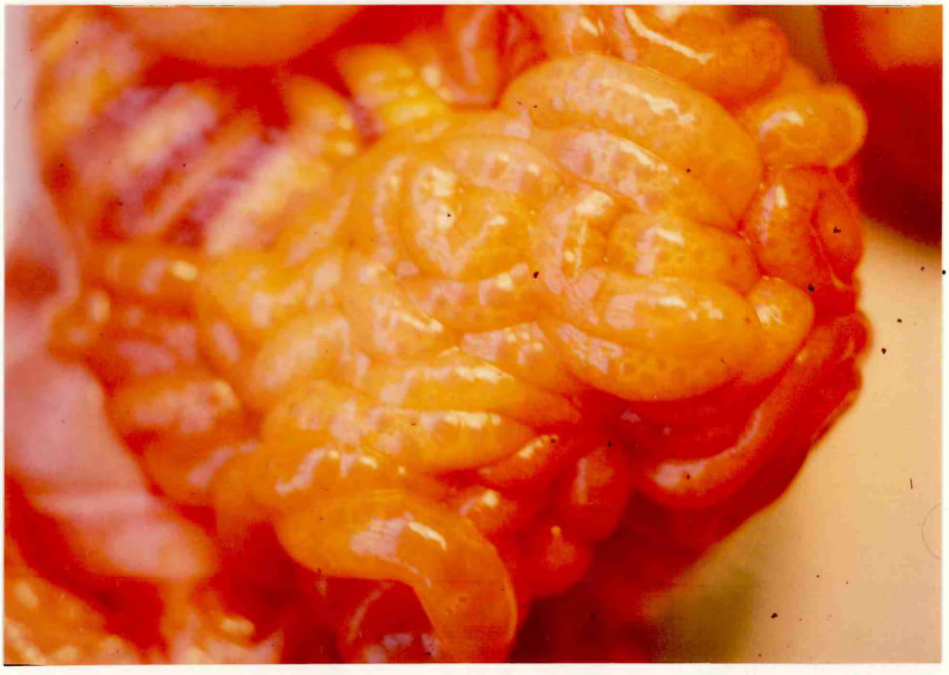

FIG. 19: Gross appearance of the small intestine of piglet CO+-1, 3 days post-inoculation with C.perfringens type A.
Note the distention of the intestine and the frothy contents.

With the exceptions listed below the rest of the infected, immunised animals as well as the non-infected controls, were in good bodily condition. No pathological changes were seen in the organs of these animals. The gastrointestinal tract, mucosa and contents were all normal in appearance.

Infected, immunised piglets A-2, E-2 and F-2 were in good bodily condition when sacrificed. The stomachs of these piglets were distended and filled with undigested milk. The duodenums were normal in appearance and the jejunums and ileums were distended and pale. The caecum and colon of piglets E-2 and F-2 were dilated and filled with fluid contents. The caecum and colon of piglet B-2 were empty and pale. The mucosal surface of the jejunum and ileum of these three piglets was congested and covered with flecks of undigested milk. The villi were of the normal height. The mucosal surface of the caecum and colon was slightly congested.

Piglet E-1 when killed at the 6th day of observation had slightly hollowed flanks but was otherwise in good bodily condition. The serosal surface of jejunum and ileum appeared reddened. All other parts of the gastrointestinal tract appeared normal. The mesenteric lymph nodes were enlarged and pale in colour. The mucosa of the jejunum and ileum was slightly congested. The villi were not reduced in height.

Histopathology was carried out on only two of the infected, non-immunised piglets, CO+ -1 and CO+ -6. The gastric mucosa of both piglets appeared normal. Piglet CO+ -1 had shortened villi and marked congestion in the lamina propria of the jejunum. Piglet CO+ -6 only had shortened villi at this level. Changes in the ileum of both piglets included shortening of the villi; marked congestion of the lamina propria, especially that of piglet CO+ -1; submucosal lymphoid hyperplasia and both eosinophilic and neutrophilic polymorphonuclear leucocytes were prominent in the lamina propria, free cells were observed in the lumen (Fig. 20). Piglet CO+ -6 had areas of localised oedema at lamina propria level.

The caecum of piglet CO+ -1 was congested at all levels, there was presence of oedematous fluid and cells in the lumen, and hypercellularity was evident in the lamina propria was evident (Fig. 21). Milder changes at this level were seen in piglet CO+ -6 in which congestion and the presence of inflammatory cells in the lamina propria. No changes were noted in the colon of piglet CO+ -6 whereas that of piglet CO+ -1 presented areas of extensive oedema, free cells in the lumen and a diffuse hypercellularity at mucosal and submucosal levels.

Some histological changes were noted in the gastrointestinal tract of piglet E-1. These included congestion of the lamina propria of the jejunum and ileum. No changes were seen in the stomach, caecum and colon. Similarly piglet B-2 had congestion of the lamina propria of the jejunum and ileum; inflammatory cells were present at this level. In the caecum and colon there was fluid in the lumen. Areas of localised oedema were seen in the lamina propria.

Histopathological changes were seen in both E-2 and F-2 which included a diffuse hypercellularity of the lamina propria of the jejunum, ileum, caecum and colon. The mucosa and submucosa of both caecum and colon were congested and diffuse oedema of the lamina propria was seen.

No histological changes were seen in the gastrointestinal tract of the other infected, immunised piglets or in the non-infected control animals (Fig. 22).

(iv) Bacteriological findings

Gram-stained smears prepared from the jejunal, ileal, caecal and colonic mucosa of all pigs contained C.perfringens-like bacteria with no spores. The numbers seen varied considerably and were least in the non-infected control piglets and greatest in the non-immunised, infected control piglets and in immunised piglets C-1, E-1, E-2, F-1 and F-2.

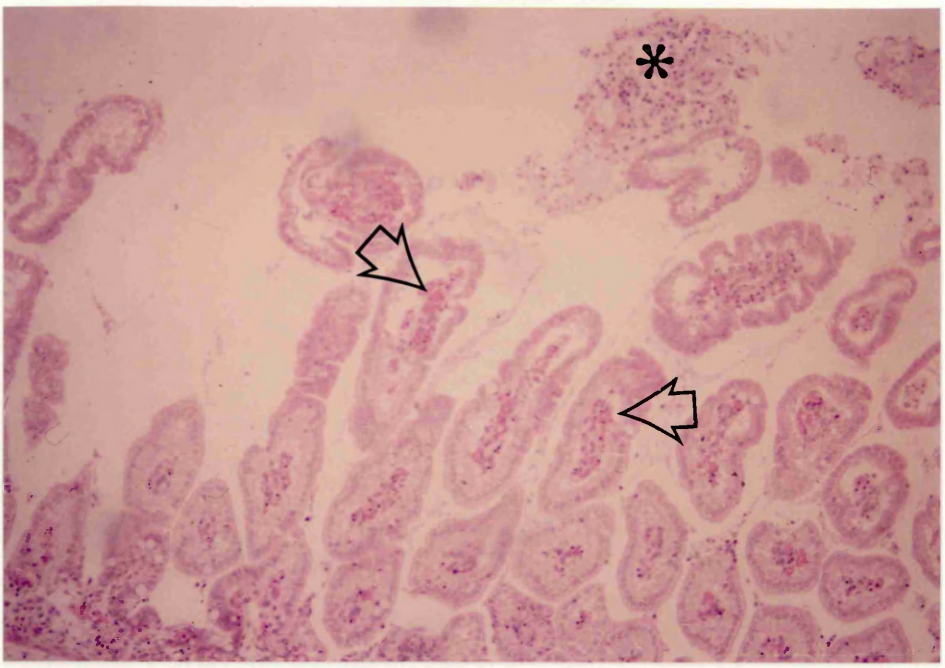

FIG. 20: Histological section of the ileal mucosa of piglet CO+-1, 3 days post-inoculation with C.perfringens type A.
Note the congestion of the lamina propria (arrow) and the presence of free cells in the lumen (star).

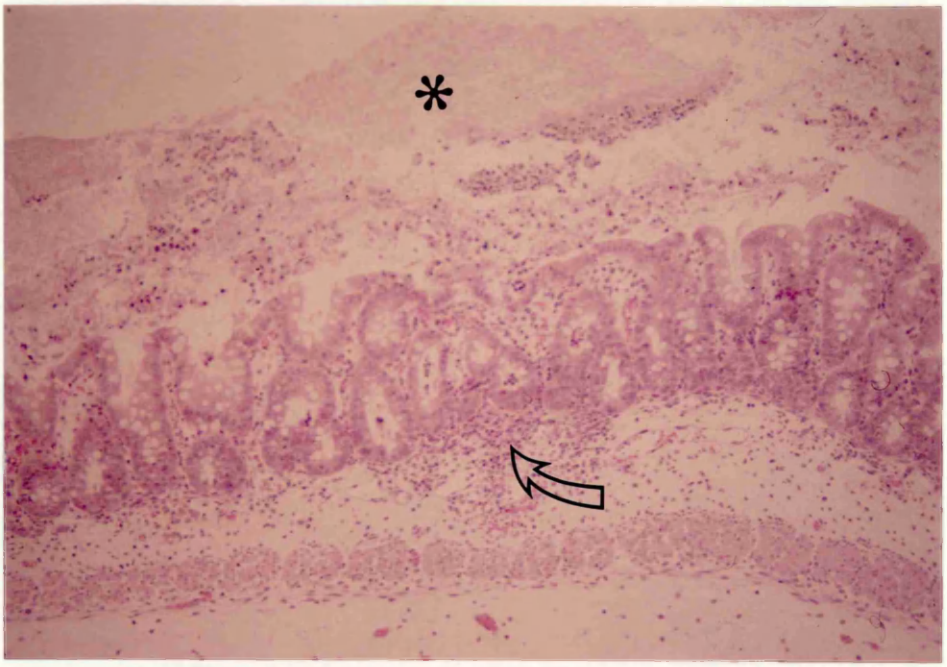

FIG. 21: Histological section of the caecal mucosa of piglet CO+-1, 3 days post-inoculation with C.perfringens type A.
Note the hypercellularity in the lamina propria (arrow) and the presence of free cells and oedematous fluid in the lumen (star).
(HE x 120)

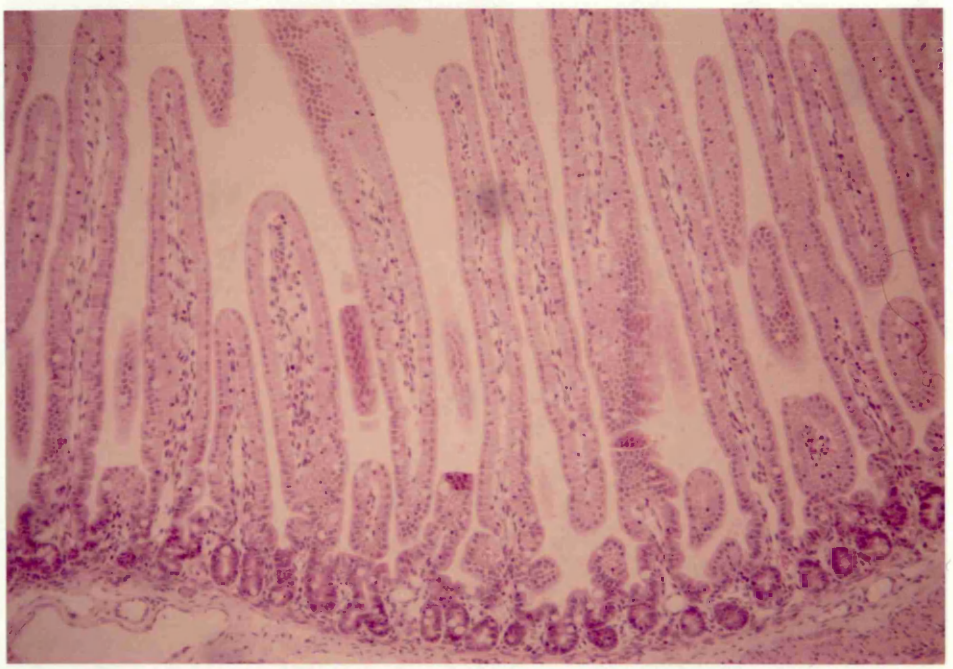

FIG. 22: Histological section of the ileal mucosa of immunised piglet A-1, 4 days post-inoculation with C.perfringens type A. Note the normal appearance of the villi and the lamina propria.

(HE x 120)

The recovery of C.perfringens type A from tissues sampled at post-mortem examination is shown in Table 21. Colonies of C.perfringens type A were isolated in large numbers from all the organs sampled, of all infected, non-immunised animals. C.perfringens type A was isolated from the stomach only in immunised piglets E-1 and E-2. C.perfringens type A was found in all other parts of the gastrointestinal tract regardless of the protection given or the length of time for which piglets had been infected.

In general, colonies of the organism isolated from immunised piglets and from non-infected controls were fewer in numbers than from infected, non-immunised animals.

Other bacteria were also isolated from the gastrointestinal tract of all piglets. They were non-haemolytic E.coli, Streptococcus spp., Staphylococcus spp., Clostridium spp., and Bacillus spp. β-haemolytic E.coli was isolated from the gastrointestinal tract of piglets D-2, E-2 and F-2.

(v) Serological findings

The serological findings are shown in Table 22. The sera of all passively immunised piglets contained antibodies to C.perfringens type A cells. IgG titres ranged from 1:10 in the serum of piglet E-2 to greater than 1:1000 in the sera of piglets A-1, A-2, B-1, B-2, C-3, D-1, D-2, F-1 and F-2. The infected, non-immunised piglet CO+ -6 showed a titre of 1:10. IgM titres ranged from 1:10 to greater than 1:1000 among the immunised animals. Piglet CO+-6 presented an IgM titre of 1:10. Titres for IgA antibodies varied from 1:1 to 1:10; no IgA antibodies to C.perfringens type A cells were detected in piglets E-2 and F-2 or in piglet CO+ -6. Titres of antibodies to alpha toxin ranged from 1:2 to 1:8 but were not detected in the sera of piglets E-1, E-2, F-2 and CO+ -6. Sera from piglet CO+ -1 and piglets non-infected controls CO+ -1 and CO+ -2 were negative to the presence of antibodies to C.perfringens type A cells and to alpha toxin.

TABLE 21

Sites from which C.perfringens type A was isolated from HDCD piglets passively immunised, following infection with pure cultures of the organism

Piglet No.	Passive immunisation	Infection	Stomach	Jejunum	Ileum	Caecum	Colon
CO- -1	-	-	-	+	+	+	+
CO- -2	-	-	-	+	+	+	+
CO+ -1	-	+	+	++	++	++	++
CO+ -2	-	+	++	++	++	++	++
CO+ -3	-	+	+	++	++	++	+
CO+ -4	-	+	++	++	++	++	++
CO+ -5	-	+	++	++	++	++	++
CO+ -6	-	+	+	++	++	++	++
A-1	A	+	-	++	+	+	+
A-2	A	+	-	+	+	+	+
B-1	B	+	-	+	+	+	+
B-2	B	+	-	+	+	+	+
C-1	C	+	-	++	++	+	+
C-2	C	+	-	+	+	+	+
C-3	C	+	-	+	++	++	++
D-1	D	+	-	+	++	+	+
D-2	D	+	-	+	++	++	++
E-1	E	+	+	++	++	+	+
E-2	E	+	+	++	++	+	+
F-1	F	+	-	++	++	++	++
F-2	F	+	-	++	++	+	+
G-1	G	+	-	+	+	+	+
G-2	G	+	-	+	+	+	+

- = no colonies of C.perfringens isolated

+ = between 1 and 8 colonies of C.perfringens isolated

++ = more than 8 colonies of C.perfringens isolated

TABLE 22

Serological findings in HDCD piglets passively immunised and infected with pure cultures of C. perfringens type A

Antibody titres to C.perfringens type A cells and to alpha toxin

Piglet No.	Passive immunisation	Infection	Collection of samples (days after immunisation)	Reciprocal titres to C.perfringens cells			Titres to alpha toxin
				IgG	IgM	IgA	
CO- -1	-	-	4	-	-	-	-
CO- -2	-	-	8	-	-	-	-
CO+ -1	-	+	2	-	-	-	-
CO+ -2	-	+	-	NT	NT	NT	NT
CO+ -3	-	+	-	NT	NT	NT	NT
CO+ -4	-	+	-	NT	NT	NT	NT
CO+ -5	-	+	-	NT	NT	NT	NT
CO+ -6	-	+	9	1	10	-	-
A-1	A	+	4	1,000	100	1	1:2
A-2	A	+	4	1,000	100	1	1:2
B-1	B	+	10	1,000	10	1	1:8
B-2	B	+	10	1,000	100	10	1:8

TABLE 22 (continued)

Serological findings in HDCD piglets passively immunised and infected with pure cultures of C. perfringens type A

Antibody titres to C.perfringens type A cells and to alpha toxin

Piglet No.	Passive immunisation	Infection	Collection of samples (days after immunisation)	Reciprocal titres to C.perfringens cells			Titres to alpha toxin
				IgG	IgM	IgA	
C-1	C	+	9	100	10	1	1:2
C-2	C	+	11	100	10	1	1:2
C-3	C	+	4	1,000	100	1	1:4
D-1	D	+	5	1,000	1,000	10	1:4
D-2	D	+	8	1,000	1,000	1	1:2
E-1	E	+	5	100	10	1	-
E-2	E	+	11	10	10	-	-
F-1	F	+	4	1,000	100	1	1:2
F-2	F	+	12	1,000	100	-	-
G-1	G	+	12	100	100	1	1:2
G-2	G	+	12	100	1,000	1	1:4

- = absent

NT = not tested

5. DISCUSSION

(a) Potency and safety of the toxoid and bacterins

(i) Toxoid

Previous reports (MacLennan, 1962; Yamamoto et al., 1970) mentioned the difficulties of producing an alpha-toxin toxoid. By following the method described by Ito (1970) in which detoxification was carried out by stringent control of the constituents of the medium, formalin concentration, temperature and incubation period, a complete detoxification was achieved.

This detoxification was demonstrated by the safety tests in mice and by the haemolysis test using sheep red blood cells. Antigenicity was still present and was demonstrated by the production of serum antibody in mice, rabbits and pigs capable of neutralising crude alpha toxin using Nagler reaction. Mice, rabbits and pigs immunised with the toxin all produced antibodies containing anti-alpha toxin demonstrated by counter-immunoelectrophoresis (Tables 13 and 15). Assay of the sera produced by immunisation of these animals using the crude alpha toxin toxoid showed that antibody to cells was also present (Tables 13 and 14). Pre-inoculation sera were not available from mice and rabbits where contemporary control animals were used. No antibody to whole cell antigen was demonstrated in the sera of control mice but in both rabbit and pig sera low levels of antibody to cells were found either pre-inoculation or in uninoculated animals (Tables 13 and 14). The consistent response to cell antigen following the use of toxoid suggests that the toxoid was contaminated with cellular antigens.

(ii) Whole culture inactivated bacterin

In the cell culture preparation produced in this study, both alpha toxin and whole cell antigenicities were preserved in spite of the problems reported by Yamamoto et al. (1970). Inactivation of the bacterin was achieved under different conditions using a higher

formalin concentration, higher temperature and longer incubation period but even so alpha toxin antigenicity was preserved (Tables 13 and 15). Inactivation was complete using this method and was confirmed by the mouse inoculation and the haemolysis test.

Antigenicity of the cells was also preserved. Antibody to cells was demonstrated in the sera of immunised mice, rabbits and pigs (Tables 13 and 14). Pre-inoculation levels of antibody to cells were found in the sera of pigs (Table 14) and in the sera of the control rabbit (Table 13) but in the mice and in the pig inoculated with whole killed culture there was no evidence for previous antibody and high levels of antibody were formed following immunisation. In the pigs, the high levels of IgM found suggest that this was a primary response.

The ubiquity of C.perfringens type A in the intestines and environment of all the animals used to assess antigenicity was a complicating factor in this part of the study. It must be assumed that animals with no obvious serum antibody response had been exposed to the organism at some stage. In the case of the control pig (Table 14) IgM antibody to whole cell antigen appeared during the period of immunisation of the remainder of the group. This may suggest that all the pigs were exposed to infection or environmental antigen during immunisation.

The inactivated whole culture bacterin was potent when used at 1:2 and 1:4 dilutions in mouse protection studies (Table 12). Protection was only 80% complete at 1:4, but was complete at 1:2. Estimation of potency for the two preparations differed less than would be expected on the grounds of their protein concentration alone, this may be due to a higher amount of detoxified alpha toxin present in the toxoid.

(iii) Combined toxoid-whole culture bacterin

As both toxoid and whole culture bacterin stimulated the production of antibody to whole cells and to alpha toxin the mixed

product also produced similar results. The mixture was assumed to be potent and was not tested by mouse immunisation.

(b) The Immunisation of pregnant sows

(i) Pre-immunisation antibody to C.perfringens

The sows were from the same farm as the pigs used to produce serum antibody and also had antibody to C.perfringens cells and sometimes to alpha toxin in pre-immunisation titres (Tables 17 and 18). Two sows (402 and 406) were completely free from such antibody pre-inoculation.

Low levels of antibody to C.perfringens cells but not to alpha toxin were found in the milks of the control animals.

(ii) Serological response to immunisation

All 3 products induced serum antibody in the sows in exactly the same way as in the immunised weaned pigs. In the majority of cases, post-immunisation serum antibody levels to whole cells increased (Table 17) and in all cases serum antibody levels to alpha toxin rose above pre-immunisation levels. IgA levels in serum remained low at 1:10 in exactly the same way as in the weaned pigs. The addition of the freeze-dried whole cells to the feed of sows 406, 40 and 405 appeared to have had no obvious effect on the levels of antibody produced. The reason for this is not clear but the response may have been masked by the effects of the priming injections or the antigen may have been destroyed in the gut.

(iii) The colostral and milk antibody response to immunisation

IgG and IgM antibodies to C.perfringens type A cells were found to be predominant in the colostra of immunised sows (Table 17). Higher titres of IgA antibody were found in milks than in colostra and sera being the immunoglobulin class which presented the highest

titres to the organism in milks. Naturally-occurring C.perfringens type A, a predominantly enteric organism, would be expected to evoke enhanced production of IgM and IgA, the two classes primarily associated with local immunity. Results from this experiment agree with the above prediction by demonstrating enhanced IgM and IgA titres in sera, colostra and milks, although IgG titres were also increased probably due to the systemic immunisation of the sows. Anti-alpha toxin titres were lower in milks than in colostra or sera probably due to a lower amount of IgG antibodies to alpha toxin in milks where the predominant, immunoglobulin is of IgA class. No anti-alpha toxin was demonstrated in the control milks, thus the anti-alpha toxin demonstrated was probably entirely due to immunisation. It is unfortunate that control colostra were not available for comparative purposes.

Only slight differences in antibody levels were found between groups and once again no obvious benefit from the oral feeding of freeze-dried cells was detected.

In all cases, colostra and milks obtained from immunised sows contained antibody to both cells and alpha toxoid and so might be considered protective. The evidence for this view comes indirectly from the work of Djurickovic et al. (1975) who found that immunising sows with C.perfringens type C toxoid at 4 and 2 weeks before parturition increased antitoxin titres in sera, colostra and milks when compared to non-immunised sows. Pre-farrowing immunisation of sows with toxoid and bacterin in the present study was also found to increase IgG, IgM and IgA titres in sera, colostra and milks. They concluded that immunisation of sows and allowing passive transfer of immunity to their progeny protected piglets against C.perfringens type C enterotoxaemia. Immunisation for enhancement of passive transfer should be directed toward stimulating maximum antibody production with subsequent elevated specific immunoglobulin levels in colostrum and milk. In the present experiment, the sows immunised 3-6 weeks prior to farrowing showed a significant increase in sera, colostra and milks and it was therefore assumed that these colostra, milks and sera would be protective.

(c) The efficacy of protection

(i) The validity of the controls

The immune sera, colostra and milks protected the hysterectomy-derived, colostrum-deprived piglets of Experiment 2 against death from C.perfringens type A challenge (Table 20). The results have been summarised here in Table 23 where it can be seen that the control infected piglets suffered from severe and rapidly fatal disease. The uninfected control animals remained healthy although they did become colonised by C.perfringens type A later in the study, either from the environment or by cross infection from the other animals. In ideal circumstances these controls would have been separated from the infected animals in another room or in an isolator.

The severe disease produced resembled that described by Olubunmi (1982) but was more severe than that described by Nabuurs et al. (1983) and that described in Experiment 1 (Chapter 3). These piglets were younger and more closely resembled those of Olubunmi. They therefore provided a perfect positive control and demonstrated that the protection shown was real. Unfortunately the numbers available did not allow the inclusion of protected control groups using serum, milk or colostra from non-immune animals.

(ii) Disease in the infected controls

The severity of the disease in the infected controls differed from that seen in Experiment 1 and therefore requires discussion. The piglets in this study were younger, the inoculum was, however, smaller (8×10^7 cells versus 1.5×10^9 cells per piglet) but the piglets were also smaller in body size. They had had no opportunity to form adequate levels of plasma protein, gastric acidity or protective intestinal flora (Tlaskalova et al., 1983) and piglets CO+2 and CO+3 were small and weak. The other 3 piglets which died were, however, strong animals. As in Experiment 1 the diarrhoea was creamy, became pasted over the hindquarters and was

accompanied by loss of bodily condition. This loss of condition continued in piglet CO+6 which recovered on day 4 but it remained weak.

C.perfringens type A was isolated from the faeces of all pigs of this control group within 24 hours of inoculation. The differences between this group and the others and their significance are discussed below.

The post-mortem lesions were restricted to the gastrointestinal tract in 4 of the piglets which died but peritonitis was seen in one (CO+2). As no protected piglets died, direct comparison on an age-matched basis is only possible with piglet CO+6 which recovered. The early lesions were most severe in piglet CO+2 in which all levels of the gastrointestinal tract were congested and where large areas of haemorrhages were seen in both small and large intestine as in the pigs described by Olubunmi (1982). Piglets CO+ -1, CO+ -3, CO+ -4 and CO+ -5 presented less dramatic pathological changes. The jejunums and ileums of these animals were distended with gas and slightly congested; the large intestines appeared to have large areas of congestion. Piglet CO+ -6 (recovered) had congestion of the jejunal and ileal mucosa; the mucosa of the large intestine was less congested than that of the other infected, non-immunised animals. It is with this animal that the pathological changes seen in the protected groups are best compared.

Histological changes were observed in infected, non-immunised piglets CO+ -1 and CO+ -6. Histology was not carried out in the other piglets of this group due to the time that had elapsed from death to the necropsy of these animals. No histological changes were seen in the stomach. Shortened villi were observed at jejunum and ileum level, a marked congestion of lamina propria was evident together with diffused hypercellularity. Changes in the large intestine included large areas of congestion, the presence of inflammatory cells at lamina propria, and, in the case of piglet CO+ -1 the presence of shed cells in the lumen with oedematous fluid. These changes resemble much more closely those described

by Olubunmi (1982) than those found in Experiment 1.

C.perfringens type A was present throughout the gastro-intestinal tracts of all 6 controls (Table 21).

(iii) Changes in the protected animals

Complete protection against challenge was given by Serum A (toxoid-immunised pig), Milk C (pooled sow milk; toxoid-immunised), Colostrum D (pooled sow colostrum; toxoid-immunised) and Milk G (pooled sow milk, toxoid-bacterin and feed immunised).

In these completely protected piglets there was no diarrhoea and faecal isolation of C.perfringens type A was delayed until days 2 (Group D) or 4 (Groups A, C and G). This late colonisation, complete absence of clinical signs and pathological changes was identical with the uninfected-unprotected controls (Table 23). Minor differences in the distribution of C.perfringens in the gastrointestinal tract were seen (Table 21). C.perfringens appeared to be more profuse in some regions of the gut of the infected-protected animals when compared with the uninfected controls This finding is only subjective. C.perfringens type A was absent from the stomach in all protected animals, unlike the situation in the animals of groups CO+ and E in which clinical disease occurred.

No deaths occurred in the remaining protected piglets but E.coli diarrhoea did occur on the last day of life in piglets B2, E2 and F2. This diarrhoea was separated in time from the diarrhoea seen in the challenged animals and was not considered to affect the results of the study. Clostridial diarrhoea did occur in the piglets of Group E (pooled milk, sows bacterin-immunised). In this group C.perfringens type A appeared in the faeces within 24 hours of infection but the diarrhoea only lasted 2 days (Table 20). Groups B and F were protected from clinical clostridial disease. The lesions seen in them at post-mortem examination may be

TABLE 23

Protection of HDCD piglets against C.perfringens type A infection by passive immunisation with colostrum, milk or serum from immunised pigs

Protection given (1)	Infected	Number of piglets	Summary of results: Clinical disease (2)	Clostridial* diarrhoea (2)	Deaths (2)	Mean colonisation days	Lesions (2)	Serum Ab (3)
None CO-	-	2	0	0	0	3	0	0
None CO+	+	6	6	6	5	1	6	1
Serum (A)	+	2	0	0	0	3	0	2
Serum (B)	+	2	0	0	0	2	1	2
Milk (C)	+	3	0	0	0	3	0	3
Colostrum (D)	+	2	0	0	0	2	0	2
Milk (E)	+	2	2	2	0	1	2	2
Milk-Colostrum (F)	+	2	0	0	0	2	1	2
Milk (G)	+	2	0	0	0	3	0	2

(1) = reference to Table 19. (2) = number of piglets affected.

(3) = number of piglets with antibodies to C.perfringens type A cells.

* = E.coli diarrhoea occurred in piglets B2, E2 and F2 d 10, day killed.

attributable to the E.coli infection identified in the piglets with lesions. This supposition is borne out by the distribution of C.perfringens in their gastrointestinal tracts which resembled that of the protected animals. In piglets from Group E, the distribution of C.perfringens type A found was that of infected untreated controls and the lesions in E1, killed before any E.coli infection could have been due to C.perfringens type A. Those in piglet E2 may have been due to E.coli.

(iv) Passive antibody levels in the piglets

As may be expected, the control uninfected animals had no trace of antibody either to cells or to alpha toxin (Tables 22 and 23). One of the two members of the infected control group, CO+-6, had low levels of IgG and IgM which probably resulted from active infection. No antibody to alpha toxin was detected.

IgG and IgM antibody to C.perfringens cells was present in all protected piglets and levels were in general highest in those animals which had received serum or colostrum. IgA was not found in the sera of piglets E1 and F2 and was low in most piglets. There is therefore no absolute relationship between levels of antibody to C.perfringens type A detected at slaughter and the occurrence of C.perfringens diarrhoea. There is, however, a stronger correlation between antibody to alpha toxin and protection. Neither piglet of Group E (pooled milk, sows bacterin immunised) had any and they developed diarrhoea. One of the Group F piglets also lacked detectable serum anti-alpha toxin but did not develop C.perfringens diarrhoea.

These antibody levels may have been affected in orally-protected animals by the retention of secretory IgA and some IgM in the gut lumen with consequent neutralisation of any initial clostridial products. Some serum antibody, once absorbed may also have been lost during challenge but there appears to be little relationship between levels of antibody and day of slaughter. This also applies to actively produced antibody which was demonstrated

in control piglet CO+ -6 killed on day 9. The beginnings of active immunity could, therefore, have contributed to the final levels found.

(v) Significance of the protection study

The results of the study reported in this chapter and discussed above, provide clear evidence that colostrum, milk and serum from animals immunised with toxoid prepared from crude C.perfringens alpha toxin and serum and colostrum from pigs immunised with a whole culture bacterin alone or in combination can protect piglets against challenge with C.perfringens type A of piglet origin. This protection included protection against clinical disease, death and lesions but not against colonisation which was merely delayed in protected piglets.

The actual reasons for this protection were not clear. Antibody to alpha toxin and to cells was produced by all the immunogens used and it cannot be said with any certainty which antigen gave rise to protective antibody although the poorest protection (against death only) was provided by milk from whole culture vaccinated sows giving low anti-alpha toxin levels in piglet serum. Neither alpha toxin or cell wall antigens may have been involved as unsought antigens such as minor toxins, hyaluronidase, collagenases or proteinases may have been present in the material used. The lack of a normal antibody-free serum, colostrum and milk controls mean that an element of uncertainty must remain about the specificity of the protection observed. Similarly, it is not completely clear which immunoglobulin class is responsible for protection, neither is it clear at which site protection was being provided. Toxins may have been neutralised in the gut lumen or systemically.

For this reason it was decided to concentrate on purified antigens and alpha toxin was chosen for the first study.

CHAPTER 5

STUDIES ON THE ALPHA TOXIN OF CLOSTRIDIUM PERFRINGENS TYPE A

1. INTRODUCTION

It appeared from the results of the studies described in Chapter 4 that toxoid prepared from crude C.perfringens type A alpha toxin would induce protective antibodies against challenge infections in hysterectomy-derived, colostrum-deprived piglets. The antisera, colostra and milks from pigs immunised with this toxoid possessed antibody both to alpha toxin and to cell antigens, thus providing no clear evidence for the protective value of anti-alpha toxin alone. For this reason the studies described here were undertaken in order to clarify the role of alpha toxin in C.perfringens Type A enteritis in the pig.

Alpha toxin was purified, used to produce specific antisera and used as an antigen in serological studies. The alpha toxin production of pig isolates of C.perfringens type A was studied and the effects of pure alpha toxin on the pig intestine were studied in gut loops.

2. THE PURIFICATION OF ALPHA TOXIN AND THE PRODUCTION OF ANTISERA TO IT

(a) Purification of alpha toxin

(i) Methods

The methods used were described in Chapter 2.

(ii) Results

The results of the different stages of purification are summarised in Table 24.

TABLE 24

Alpha toxin activity at different stages of purification

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity* (HU/ml)	Total Activity (HU)	Recovery (%)	Specific activity (HU/mg protein)
Growing medium supernatant	10000	2.6	26000	16	160000	100	6.15
0-50% ammonium sulphate precipitate	1000	11.7	11700	128	128000	80	10.94
Active fraction after chromatography on Ultrogel AcA-34 column	95	1.54	146.3	64	6080	3.8	41.56
Active fraction after isoelectric focusing	20	0.59	11.8	64	1280	0.8	108.5

* Reciprocal of highest dilution producing haemolysis of sheep red blood cells

50 per cent of ammonium sulphate saturation of the 10 litres culture supernatant resulted in 8-fold increase of alpha toxin activity/ml. This step gave a 80 per cent recovery of total alpha toxin activity. Ultrogel AcA-34 gel chromatography gave a recovery of 3.8 per cent from the original alpha toxin activity. The first peak after the void volume consisted of the bulk of protein since it had a high absorbance at 280nm and little alpha toxin activity. The highest haemolytic activity per ml eluted was found in the second peak (Fig. 23).

Alpha toxin which produced turbidity of saline extract of egg yolk was separated in a narrow peak with an isoelectric point of 5.6. Low alpha toxin activity was also found between pH 5.4-5.5. No alpha toxin activity was detected at other pH ranges (Figs. 24 and 25). The recovery of alpha toxin activity after isoelectric focusing was 0.8 per cent of original activity with a specific activity of 64 haemolytic units/ml and a protein concentration of 0.59 mg/ml.

Figure 26 shows the extent of homogeneity at each step as determined on polyacrylamide gels. After ammonium sulphate precipitation 6-8 bands were revealed. After gel chromatography 3 bands were identified. A single stained band was revealed after the last step of purification.

Purified alpha toxin gave a single line of identity when tested against the rabbit anti-crude alpha toxin serum described in Chapter 4 by double immudiffusion (Fig. 27). A single line was also identified when the purified alpha toxin was tested by immunoelectrophoresis (Fig. 28). The purified alpha toxin was stored at -20°C until required.

(b) The production of antiserum to pure alpha toxin

(i) Methods

Alpha toxin purified in part (a) above was used to inoculate

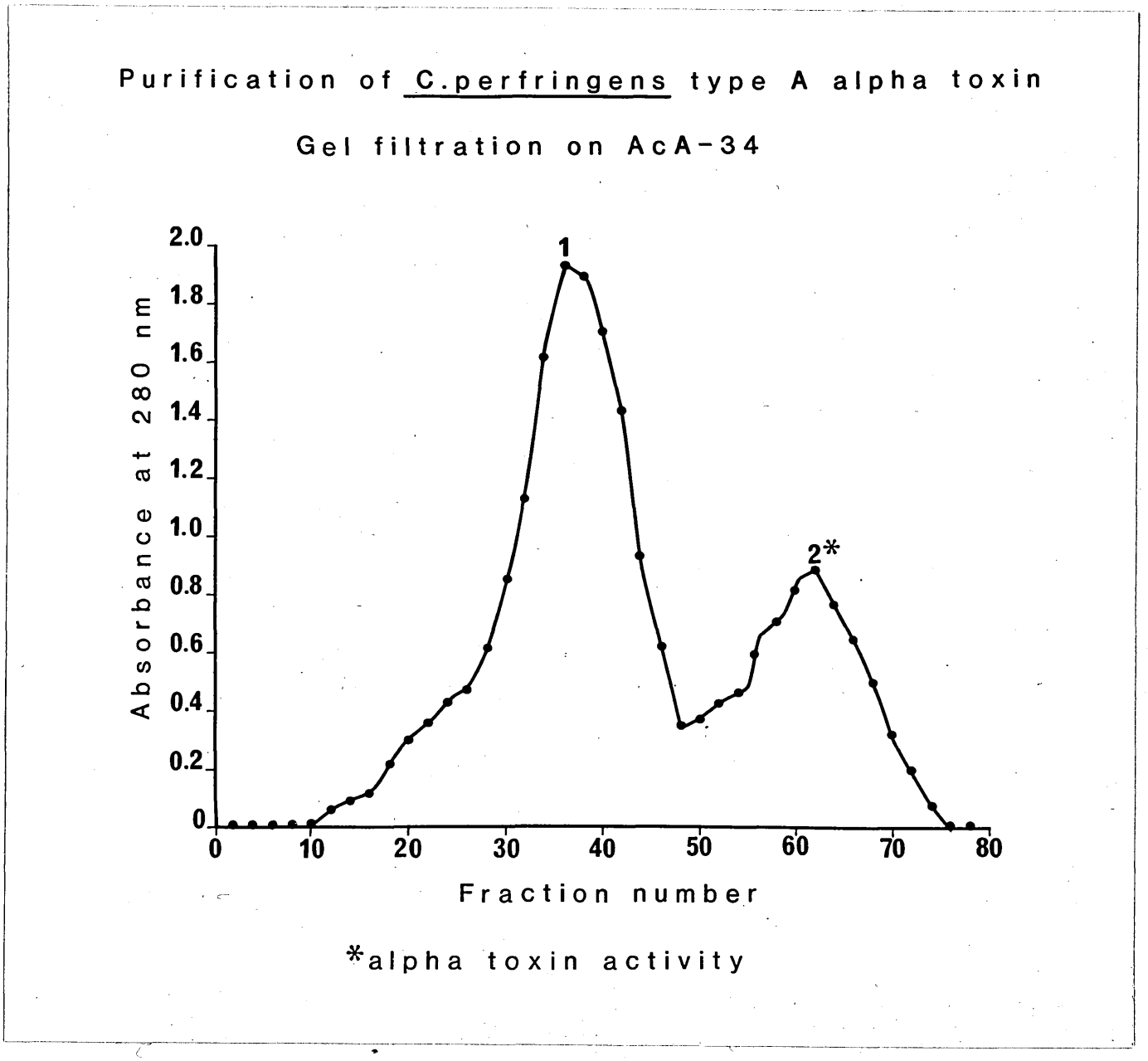

FIG. 23: Separation of alpha toxin by gel filtration chromatography.

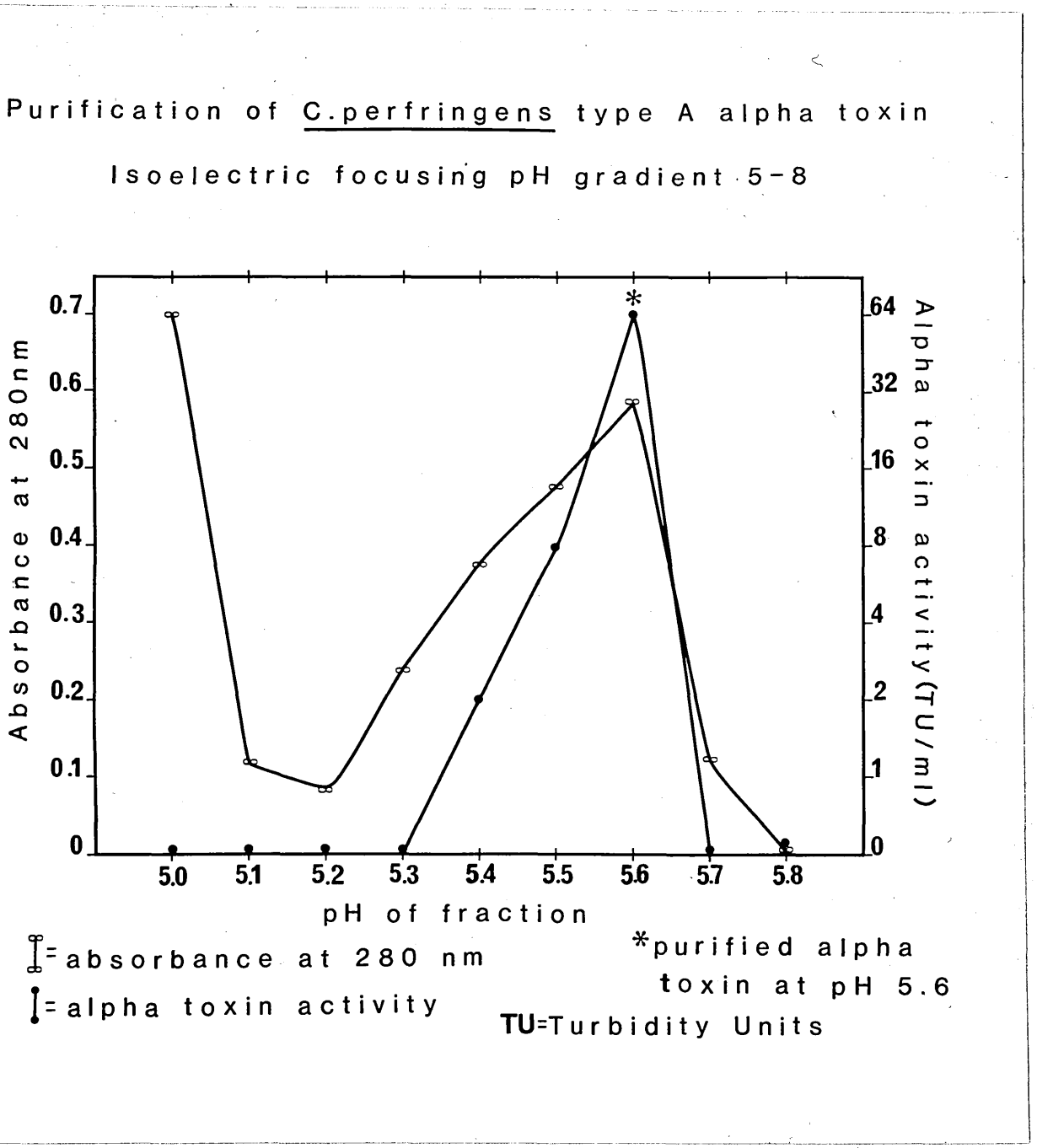

FIG. 24: Separation of alpha toxin by isoelectric focusing in a pH gradient 5-8.

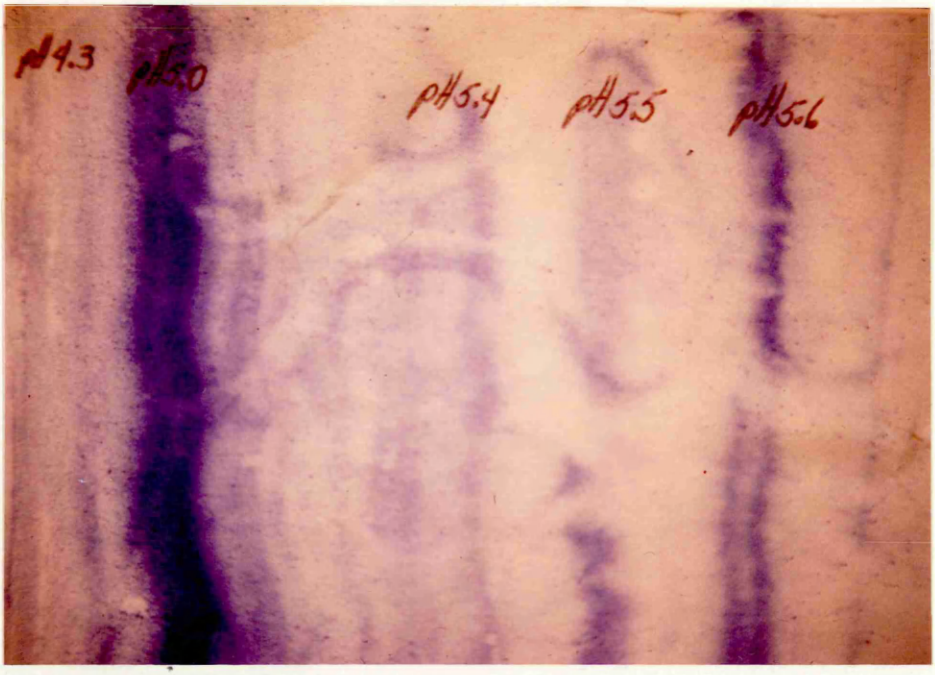

FIG. 25: Isoelectric focusing pattern of the electrophoresed proteins from peak 2 (Fig. 23).
The alpha toxin activity was contained at pH 5.6.

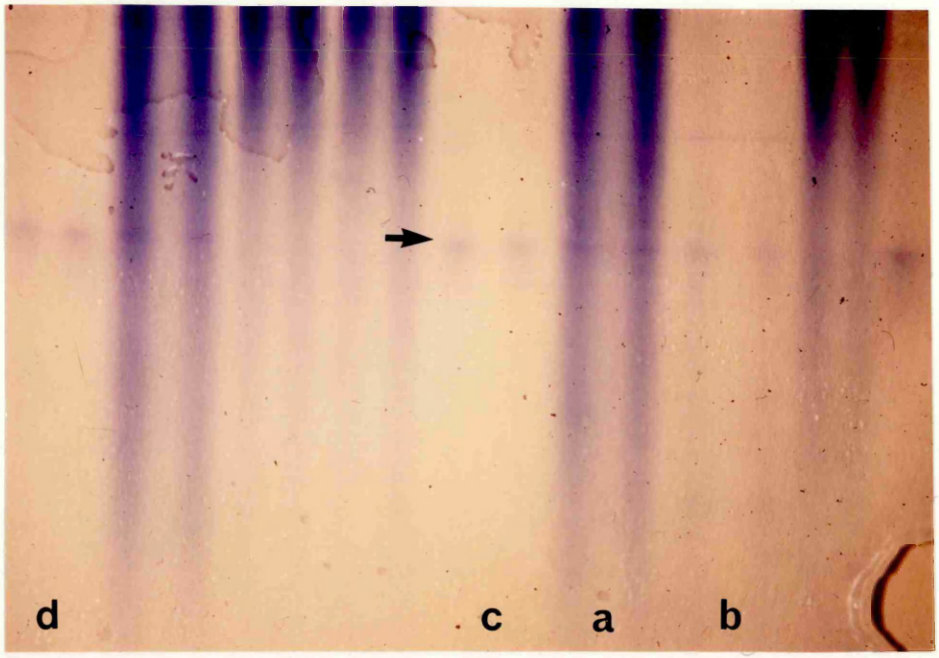

FIG. 26: Polyacrylamide gel electrophoresis pattern obtained after each step of purification of alpha toxin. (a) after ammonium sulphate precipitation; (b) after gel chromatography; (c) after isoelectric focusing electrophoresis; (d) Reference control Phospholipase C XII.
Note the alpha toxin bands at each step of purification (arrow).

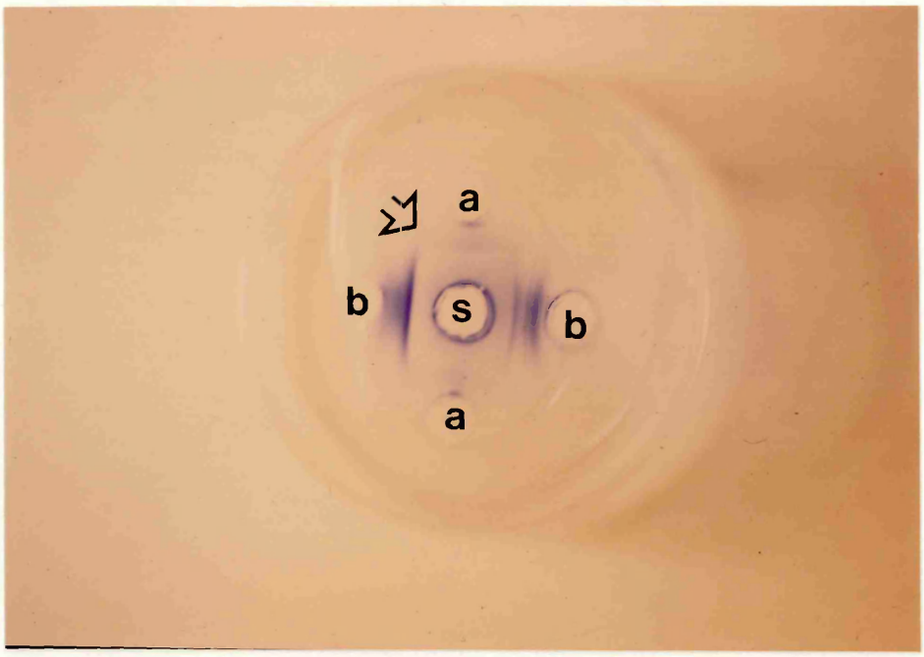

FIG. 27: Double immunodiffusion pattern obtained with purified alpha toxin (a), crude alpha toxin (b) and rabbit antiserum to crude alpha toxin (s).
Note the presence of only one line of identity between alpha toxin and antiserum to it (arrow).

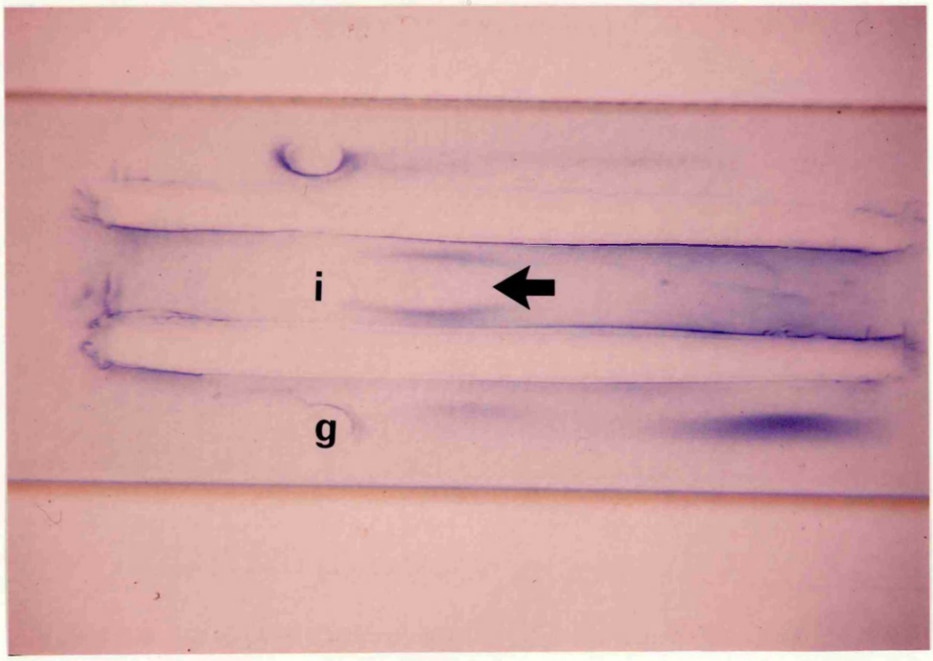

FIG. 28: Immunoelectrophoresis pattern obtained with alpha toxin after gel chromatography (g) and after further purification by isoelectric focusing (i). The troughs are filled with rabbit antiserum to crude alpha toxin.
Note the single line of precipitation produced by the alpha toxin (arrow).

two rabbits using the immunisation schedule described in Table 4. The antigens consisted of 1ml of pure alpha toxin containing 590µg protein per ml and an activity of 64 haemolytic units/ml emulsified with Freund's adjuvant.

Antibody was detected using counterimmunoelectrophoresis using the purified alpha toxin described above as an antigen (Fig. 29).

(ii) Results

The results are shown in in Table 25.

Preimmunisation sera of the two rabbits were negative for antibodies to alpha toxin. Titres of 1:4 were produced in the serum of both animals after the first immunisation. Further increases in the titre were obtained with each immunisation. After the fifth immunisation a titre of 1:128 was found in serum of rabbit 1, whereas serum of rabbit 2 possessed a titre of 1:64 to alpha toxin.

Figure 30 shows that only one precipitin line of partial identity was produced when the antisera were tested against crude alpha toxin by double immunodiffusion. Pure alpha toxin produced a single line against both antisera. Both antisera were able to neutralise 64 haemolytic units of purified alpha toxin to sheep red blood cells, at the following dilutions: 1:16 from rabbit 1, and 1:8 serum from rabbit 2, thus giving a specific neutralising activity of 1024 and 512 HU units/ml respectively.

3. ALPHA TOXIN PRODUCTION BY C.PERFRINGENS TYPE A ISOLATES FROM PIGS

(a) Materials and Methods

(i) Isolates

The isolates of C.perfringens Type A used in this study were those isolated in the surveys described in Chapters 3 and 6.

TABLE 25

Antibody titres* of rabbits immunised with purified alpha toxin

Rabbit No.	Preimmunisation	Titre 14 days after each immunisation No. of immunisations				
		1	2	3	4	5
1	-	1:4	1:16	1:32	1:64	1:128
2	-	1:4	1:16	1:16	1:64	1:64

* = detected by counterimmunoelectrophoresis

- = absent

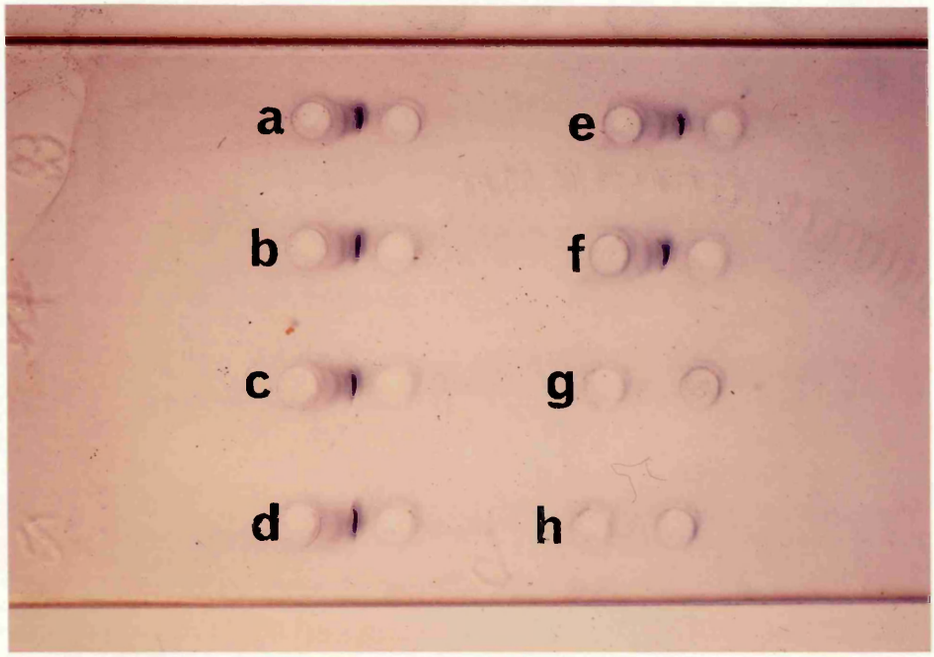

FIG. 29: Detection of antibodies to purified alpha toxin by counterimmunoelectrophoresis. Double dilutions of serum from rabbit 2 immunised with purified alpha toxin: (a) 1:2; (b) 1:4; (c) 1:8; (d) 1:16; (e) 1:32; (f) 1:64; (g) 1:128; and (h) 1:256.

Note the titre of 1:64.

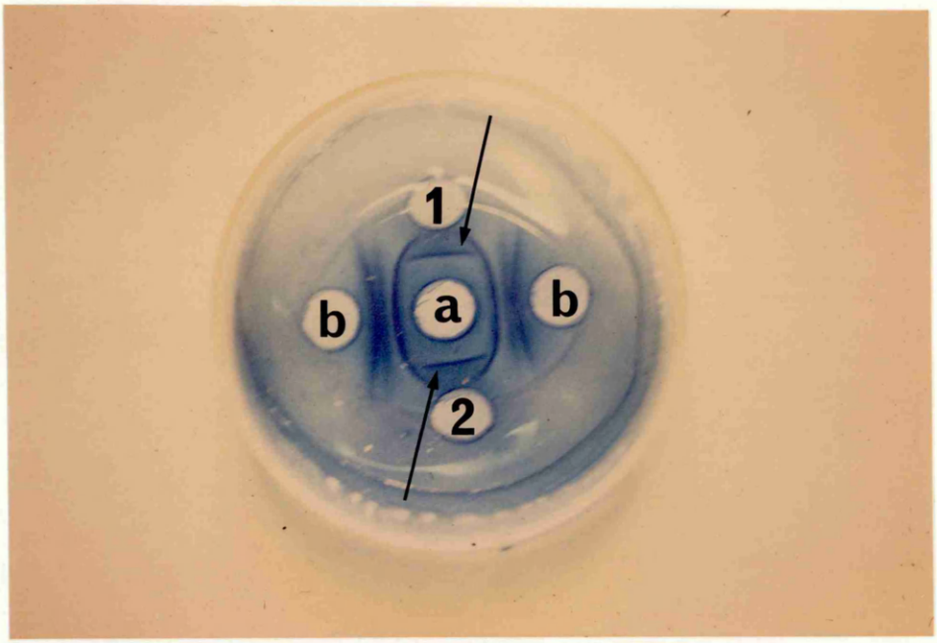

FIG. 30: Double immunodiffusion pattern obtained with crude alpha toxin (a) and antisera to purified alpha toxin from rabbit (1) and rabbit 2 (2). Commercial antiserum to C.perfringens type A (b). Note the line of partial identity between crude alpha toxin and the rabbit antisera to alpha toxin (arrow).

(ii) Procedures

Isolates stored in cooked meat medium were plated on horse blood agar plates and then grown in the peptone medium described by Takahashi et al. (1981). Samples were treated and tested as previously described. Isolates 5,6,14,20,21,29 and 34 were tested repeatedly.

Haemolysin activity was tested in all isolates, whereas turbidity in saline extract of egg yolk, toxicity for mice and alpha toxin titres detection by counterimmunoelectrophoresis were tested only in 22 of the 42 isolates.

(b) Results

The levels of alpha toxin produced by each isolate are shown in Table 26. The results for isolates 7, 8, 14, 21, 22, 29, 30 and 35 were the same on each single occasion and as reported in Table 26 when 22 of the isolates were tested for alpha toxin production by the haemolysis of sheep red blood cells (Fig. 31) the turbidity of saline extract of egg yolk, toxicity to mice and counterimmuno-electrophoresis. Good agreement was found between the results of the haemolysis test and the other tests. Alpha toxin levels were identical in all cases when tested by the haemolysis of sheep red blood cells and turbidity of saline extracts of egg yolk. Toxicity to mice proved to be slightly less sensitive as a method of detecting alpha toxin levels than the other tests as 13 isolates showed a lower level of toxin than that detected by the other methods. Counterimmunoelectrophoresis indicated the highest alpha toxin levels in 13 isolates, whereas in 9 isolates it produced an identical titre as the one detected by haemolysis of sheep red blood cells and turbidity of saline extract of egg yolk.

Alpha toxin was produced by 38 of the 42 isolates tested (90.4 per cent) 4 isolates (9.6 per cent) did not produce alpha toxin or did not produce sufficient for detection by the methods used. The remainder produced varying amounts of alpha toxin

TABLE 26

Alpha toxin production by 42 C.perfringens type A isolates in peptone medium

Submission number	Sample number	Amount of alpha toxin detected (HU/ml)[a]	(TU/ml)[b]	(MLD/ml)[c]	(CIE)[d]
2	5	16	16	16	32
	6	64	64	32	64
	7	16	16	16	32
	8	32	32	16	64
3	14	16	16	8	16
	17	16	16	16	32
4	20	16	16	8	32
	21	64	64	64	128
	22	8	8	4	16
6	29	-	-	-	-
	30	64	64	32	64
8	34	128	128	64	128
9	35	8	8	4	8
	36	16	16	8	32
	37	64	64	32	64
12	44	16	16	8	32
	45	16	16	16	32
	46	16	16	16	32
	47	16	16	8	16
15	53	4	4	4	8
	54	4	4	4	8
	55	4	4	2	4
16	56	16	NT	NT	NT
	57	16	NT	NT	NT
	58	16	NT	NT	NT
17	59	8	NT	NT	NT
	60	-	NT	NT	NT
19	62	256	NT	NT	NT
21	64	8	NT	NT	NT
	65	8	NT	NT	NT
25	74	8	NT	NT	NT
	75	32	NT	NT	NT
	76	32	NT	NT	NT
	77	16	NT	NT	NT
	78	32	NT	NT	NT

TABLE 26 (Cont'd)

Alpha toxin production by 42 C.perfringens type A isolates in peptone medium

Submission number	Sample number	Amount of alpha toxin detected (HU/ml)(a)	(TU/ml)(b)	(MLD/ml)(c)	(CIE)(d)
28	82	64	NT	NT	NT
29	83	16	NT	NT	NT
	84	16	NT	NT	NT
31	86	8	NT	NT	NT
	87	8	NT	NT	NT
32	88	-	NT	NT	NT
	89	-	NT	NT	NT

(a) = Reciprocal of highest dilution producing haemolysis of sheep red blood cells.

(b) = Reciprocal of highest dilution producing turbidity of saline extract of egg yolk.

(c) = Reciprocal of highest dilution causing death of mice in 24 hours.

(d) = Reciprocal of highest dilution detected by counterimmuno-electrophoresis.

- = absent
NT = not tested

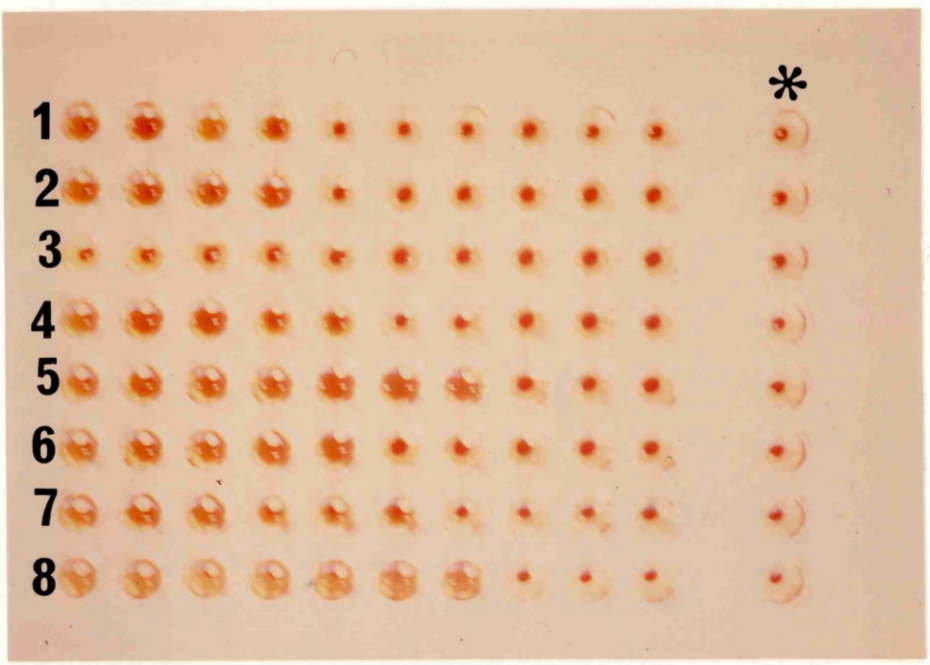

FIG. 31: Haemolysis test. Alpha toxin production by C.perfringens type A isolates: 22 (1), 35 (2), 29 (3), 7 (4), 21 (5), 14 (6), 8 (7) and 30 (8) producing 8,8,0,16,64,16,32 and 64 haemolytic units respectively. Neutralisation of haemolysis of undiluted samples achieved with rabbit antiserum to purified alpha toxin (star).

measured in haemolytic units (Table 26). 3 isolates (7.1 per cent) produced 4HU, 8 isolates (19 per cent) produced 8HU, 16 isolates (38 per cent) produced 16HU, 4 isolates (9.5 per cent) produced 32HU, 5 isolates (11.9 per cent) produced 64 HU, one isolate (2.3 per cent) produced 128HU and 1 isolate (2.3 per cent) produced 256HU.

4. ANTIBODY LEVELS TO ALPHA TOXIN IN PIG SERA

(a) Materials and Methods

106 serum samples from 4 Scottish farms were obtained and divided as previously described in Chapter 2. The sera were diluted two-fold with phosphate buffered saline, pH 7.2. The anti-alpha toxin titre was determined by counterimmunoelectrophoresis using a constant alpha toxin amount of 10µl containing 1.60 mg of protein/ml.

(b) Results

The results are summarised in Table 27. Of 106 sera examined, 45 (42.4 per cent) were found to possess antibodies to C.perfringens type A alpha toxin with titres ranging from 1:2 to 1:16.20 sera presented titres of 1:2; 14 showed a titre of 1:4; 8 sera showed a titre of 1:8, and 3 sera showed a titre of 1:16. No obvious differences in serum titres to alpha toxin were found in the 4 different pig populations tested.

5. THE EFFECTS OF ALPHA TOXIN IN PIGLET INTESTINAL LOOPS

(a) Materials and Methods

One conventionally born one day-old piglet was used. The ligated intestinal loop technique was carried out as described in Chapter 2. The animal was killed 12 hours after injection of the preparations.

TABLE 27

Distribution of serum antibody titres to C.perfringens type A alpha toxin in adult pigs

Alpha toxin CIE titre	No. of pigs Farm 1	Farm 2	Farm 3	Farm 4	Total	%
0	24	17	14	6	61	57.5
2	8	6	4	2	20	18.8
4	5	3	3	3	14	13.2
8	3	2	3	0	8	7.5
16	0	2	0	1	3	2.8
Total	40	30	24	12	106	100

13 intestinal loops were ligated. Three loops were inoculated with purified alpha toxin containing 0.60mg of protein per ml and an activity of 64 haemolytic units per ml. One loop was injected with the same alpha toxin previously incubated for 30 minutes at room temperature with 0.25 ml of rabbit anti-alpha toxin serum possessing a titre of 1024 anti-alpha toxin units. Two loops were inoculated with the filtered culture supernatant of isolate number 7, grown in peptone medium with meat particles, containing 2.60mg of protein per ml and an activity of 16 haemolytic units per ml. Another loop was injected with some filtered culture supernatant fluid previously incubated with the anti-alpha toxin serum. Two loops were inoculated with some culture supernatant concentrated by dialysis against polyethylene glycol 20,000 to a protein concentration of 11.7mg per ml and an activity of 64 haemolytic units per ml. One loop was injected with the same concentrated culture supernatant previously incubated with anti-alpha serum. Two loops were inoculated with saline solution and one with sterile peptone medium as controls. Accumulation of fluid, macroscopic congestion of mucosa and histopathological changes were recorded for each of the inoculated loops. Segments of the loops were kept frozen at -70°C for immunofluorescence studies.

(b) Results

Examination of the stomach of this piglet at post-mortem examination showed that it had ingested colostrum in spite of assurances from farm staff that it had not done so.

The macroscopic findings are indicated in Table 28. No differences were apparent among the inoculated loops. No accumulation of fluid, congestion of the mucosa or apparent lesions were seen in any of the inoculated loops (Fig. 32). Histological changes were observed only in loops 1,2,3,5,9 and 13 inoculated with purified alpha toxin and with concentrated culture supernatant. The changes consisted only of a mild congestion of the lamina propria and localised hypercellularity at lamina propria level (Fig. 33). No histological changes were noticed in loops

TABLE 28

Accumulation of fluid and congestion of the mucosa in ligated intestinal loops of a conventionally born piglet injected with purified alpha toxin and C.perfringens culture supernatant fluids

Loop No.	Preparation injected	Volume injected per loop (ml)	Protein (mg/loop)	Activity (HU/ml)	Rabbit anti-alpha toxin serum added (0.25ml/loop)	Accumulation of fluid	Microscopic congestion of mucosa
1	alpha toxin	0.5	0.30	64	-	-	+
2	culture supernatant concentrated	0.5	5.85	64	-	-	+
3	culture supernatant	0.5	1.30	16	-	-	+
4	0.85 per cent of NaCl solution	0.5	-	-	-	-	-
5	culture supernatant concentrated	0.5	5.85	64	-	-	+
6	culture supernatant concentrated	0.5	5.85	64	+	-	-
7	culture supernatant	0.5	1.30	16	+	-	-
8	0.85 per cent of NaCl solution	0.5	-	-	-	-	-
9	alpha toxin	0.5	0.30	64	-	-	+

TABLE 28 (Cont'd)

Accumulation of fluid and congestion of the mucosa in ligated intestinal loops of a conventionally born piglet injected with purified alpha toxin and C.perfringens culture supernatant fluids

Loop No.	Preparation injected	Volume injected per loop (ml)	Protein (mg/loop)	Activity (HU/ml)	Rabbit anti-alpha toxin serum added (0.25ml/loop)	Accumulation of fluid	Microscopic congestion of mucosa
10	alpha toxin	0.5	0.30	64	+	-	-
11	culture supernatant	0.5	1.30	16	-	-	-
12	sterile peptone medium	0.5	-	-	-	-	-
13	alpha toxin	0.5	0.30	64	-	-	+

- = negative
+ = positive

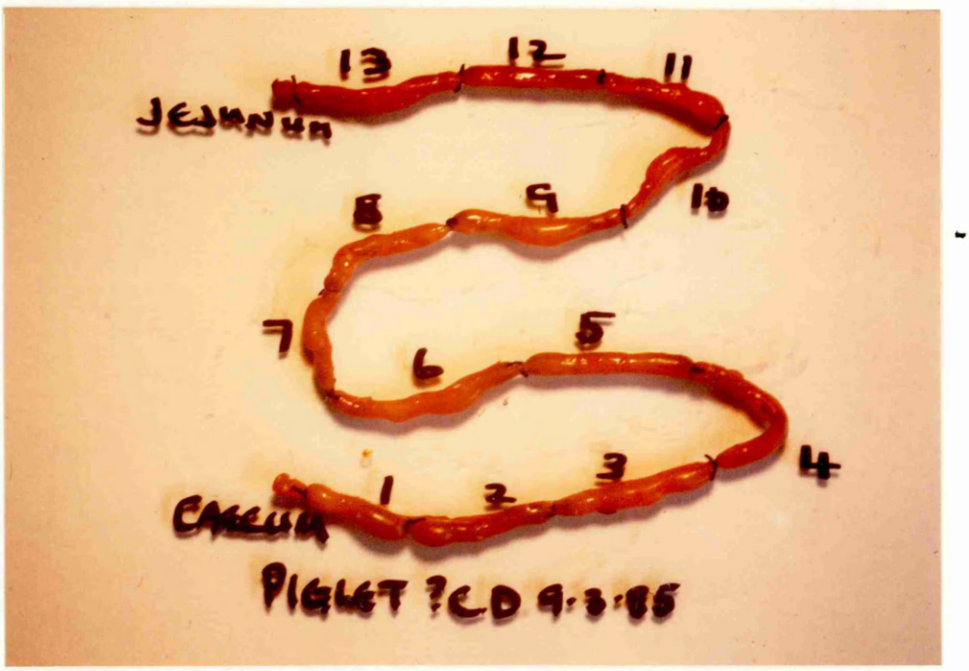

FIG. 32: Inoculation of purified alpha toxin into intestinal loops of conventionally born piglet.
Note the absence of apparent changes among the inoculated loops.

inoculated with alpha toxin and concentrated culture supernatant previously incubated with anti-alpha toxin serum. No histological changes were seen in loops injected with culture supernatant, sterile peptone medium and saline solution (Fig. 34).

No binding of alpha toxin to the intestinal epithelium could be demonstrated by indirect immunofluorescence.

6. DISCUSSION

(a) Purification

Alpha toxin was purified in three steps and was found to be devoid of contaminating antigens as shown by double immunodiffusion, immunoelectrophoresis and electrophoresis on a polyacrylamide gel. This purification was achieved by combination of the purification methods reported by Takahashi et al., (1981) and Mollby and Wadstrom (1973). The first authors purified the toxin by four steps involving affinity chromatography, ion exchange chromatography and gel filtration chromatography methods which followed an initial precipitation with ammonium sulphate. The second authors purified alpha toxin by ion exchange chromatography, affinity chromatography and isoelectric focusing following freeze-drying of the toxin as first concentration step. The purification procedure of Takahashi et al., (1981) yielded very active toxin with 1620 haemolytic units/mg of protein, whereas the procedure of Mollby and Wadstrom (1973) yielded 371 haemolytic units/mg of protein. In the present experiment the yield was of 108 haemolytic units/mg of protein, this low-yield was possibly due to the variations from the methods of purification reported or to differences in the strain of C.perfringens type A. Although the isolate used for purification of alpha toxin isolate 7 is not a high yield alpha toxin producer, it was employed because it was the isolate used in the previous experiment described in Chapter 3 and 4.

The first step of purification, 50 per cent saturation with

ammonium sulphate, produced a considerable increase in alpha toxin activity per ml of medium by concentrating the toxin and very little total activity was lost at this step, however, after gel chromatography 96.2 per cent of the original alpha toxin activity was lost. This effect was possibly due to the binding of the toxin to other proteins present in the medium or to a denaturation of the toxin. A further loss occurred after the isoelectric focusing technique when 0.8 per cent of the original alpha toxin activity was recovered. Separation of the toxin by this method was in agreement with Smyth and Arbuthnott (1973) who reported that most of the alpha toxin activity was found at pH 5.6 when isoelectric focusing was carried out. When the fraction collected from isoelectric focusing was examined for lecithinase, haemolytic and lethal activities, it was found to possess all three properties thus confirming the presence of alpha toxin. The purity of the toxin was considered satisfactorily high for immunological and biological use.

(b) Antibody production

Immunisation of rabbits with purified alpha toxin mixed with Freund's adjuvant showed a high immunogenicity of the toxin by eliciting and enhancing anti-alpha toxin titres after each immunisation. Alpha toxin has been considered to be a poor antigen (Oakley, 1970). Ito (1970) reported that detoxified alpha toxin was superior in immunogenicity to non detoxified toxin which induced a low titre anti-alpha toxin response; no adjuvants were used in this work. The good antigenicity of alpha toxin in the present study may be attributed to the use of adjuvants. Similar effects were obtained in rabbits using the crude toxoid and aluminium hydroxide adjuvants (Table 13, Chapter 4). The lower titres obtained in that study may have been due to the lower concentration of alpha toxin in the toxoid, the adjuvant or to the smaller number of injections.

(c) Alpha toxin production by porcine isolates of C. perfringens type A

The high percentage of alpha toxin producer isolates found in this study (Table 26) confirms the findings of Chapter 3 that most of C.perfringens type A strains were alpha toxin producers. The selection of colonies which were haemolytic on primary isolation plates were discussed at the end of Chapter 3.

Comparison of the alpha toxin levels (Table 26) in 22 C.perfringens strains demonstrated by the haemolysis test of sheep red blood cells and the turbidity test of saline extract of egg yolk, indicated that theta toxin was not produced in the peptone medium in which the isolates were grown, or if produced, it was in quantities that did not enhance the haemolytic activity of alpha toxin. This failure to detect the toxin in the supernatant is in agreement with Takahashi et al. (1981) who reported that the peptone medium employed impeded theta toxin production. It therefore seemed that the test used for the activity of alpha toxin in this study was specific.

It has been reported (Nakamura et al., 1969) in quantitative studies that alpha toxin production by C.perfringens type A is dependent upon the strain, the medium, the culture conditions, pH, temperature of incubation and the length of incubation. The isolates tested in this study were all cultured under the same conditions on the same occasion and it was noticed that the amount of alpha toxin produced by the isolates was always the same if the medium, pH, temperature and length of incubation did not change in the isolates when cultured repeatedly. These data indicate that alpha toxin production is associated with properties of the strains. No attempt was made in this study to measure the numbers of organisms inoculated or the optical density or bacterial counts achieved in each culture. These factors may introduce an element of uncertainty into the results described in Table 26.

The variation in alpha toxin production among isolates was

marked. Isolates 29,60,88 and 89 produced no detectable alpha toxin and isolate 62 produced 256 haemolytic units of alpha toxin per ml.

Variations in the alpha toxin production by C.perfringens type A isolated from animals with the same submission number (Submissions 2,4,6,9,17 and 25) were found, indicating that different C.perfringens type A strains may be isolated from related animals presenting a clinical disease. The possible association of these strains and their alpha toxin production with disease is not clear from the data obtained in this study. Isolate 7 was a proven pathogen (Experiments 1, Chapter 3 and 2, Chapter 4) but was only a moderate alpha toxin producer. The best producers of alpha toxin, isolates, 62 and 34 were neither of them isolated from cases in which they were present as the sole cause (62) or in large numbers (34). The other isolate of known pathogenicity isolate 29, (Experiment 3, Chapter 6) produced no alpha toxin and also produced an entirely different disease suggesting that there might be some relationship between disease and alpha toxin production.

(d) Serum antibody to alpha toxin in adult pigs

Of 106 pig sera examined 45 were found to possess antibodies to alpha toxin with titres ranging from 1:2 to 1:32. No obvious differences in serum titres were found among the pig populations of the four tested farms. It was known that all the animals tested for anti-alpha toxin antibodies were healthy at the time of being sampled. As the age of the animals sampled was not recorded it was not possible to say whether there was any effect of age or parity on the levels found.

The presence of anti-alpha toxin antibodies in the tested sera could possibly be due to prolonged absorption of alpha toxin in symptomless carriers possessing high numbers of C.perfringens type A, or, it may suggest that the indigenous intestinal C.perfringens flora produces sufficient toxin to elicit an immune response. It was unlikely that preformed alpha toxin would be ingested with the

feed or in the faeces of piglets. Any such ingested toxin would probably be destroyed in the stomach by the low pH or by the presence of antitoxic factors in feed. Finally there is the possibility of subclinical systemic exposure from local lesions, for example in the reproductive tract.

The finding that 42.5 per cent of all 106 sows surveyed had serum antibody to alpha toxin is of interest. These figures were not used in any statistical analysis as the sample was considered to be too small and unrepresentative, but the results suggest that antibody to C.perfringens alpha toxin is common in sows. Further evidence for this view comes from the findings in the pre-immunisation sera from the sows used in the protection studies described in Chapter 4 where 5 out of 10 sows sampled had circulating anti-alpha toxin antibody.

(e) The gut loop studies

The most important feature in assessing the significance of these studies was the finding that the piglet used had ingested colostrum and was therefore of unknown immune status. From the discussion in section d above, it is clear that antibody to alpha toxin can be demonstrated in the sera of 40 per cent of normal sows. Neither the serum antibody titre of the sow to alpha-toxin nor the passive antibody titre of the piglet used were determined in this study.

No accumulation of fluid or macroscopic lesions were observed in intestinal ligated loops receiving purified alpha toxin, culture supernatant or concentrated culture supernatant. These results are in agreement with Hauschild et al. (1968) and Duncan et al. (1968) who failed to produce an intestinal loop response when crude alpha toxin injected into loops prepared in lambs and rabbits respectively. The first authors, however, observed accumulation of fluid in intestinal loops when large amounts of alpha toxin were injected. 1000 MLD of alpha toxin per loop produced fluid accumulation which amplified several-fold when 8000 MLD were

inoculated into loops. Such large quantities of alpha toxin inducing accumulation of fluid provide no indication of the role of the toxin in C.perfringens enteritis because it is unlikely that such amount of alpha toxin would be produced in a natural case of C.perfringens type A infection. Such high levels of alpha toxin were not used in this study.

With the 64 haemolytic units of toxin used in this study, there were histological lesions of mild congestion and localised hypercellularity in loops injected with pure alpha toxin and concentrated culture supernatant (Table 28, Fig. 33). These changes were not seen in loops inoculated with unconcentrated culture supernatant containing 16 haemolytic units but were present when the culture supernatant was concentrated to contain 64 haemolytic units, the same as in the pure alpha toxin. These lesions may be due to the effects of the alpha toxin as they did not appear in controls. The changes may have been due to the effect of alpha toxin which stimulates histamine release from mast cells to cause inflammation. However, no globular leucocytes were seen in the sections and this mechanism may not be important in animals of this age.

No binding of alpha toxin to the intestinal epithelium could be demonstrated by indirect immunofluorescence. This result may be explained due to the enzymatic nature of alpha toxin which results in rapid release of the antigen from surfaces to which it fixes initially. The alpha toxin may also have been neutralised in the lumen by antibody from the milk. Analysis of the gut loop contents by the haemolysis test or by counterimmunoelectrophoresis would have resolved the question as to whether active alpha toxin or inactivated, but still antigenic, alpha toxin was still present. The lack of fluid in the loops at 12 hours post-inoculation would have made this procedure difficult but it might have been possible earlier, for example 3 hours after inoculation.

(f) Conclusions

The studies described in this chapter produced pure alpha toxin and antibody to it. Using these reagents it became clear that the majority of C.perfringens isolated from pig enteritis produced alpha toxin, although there was some doubt about the comparability of the results between isolates because of the lack of controls on culture density. The repeated studies suggested that there was little variability under the conditions used and that real variation in alpha toxin production was present.

Antibody to alpha toxin was widespread in adult pig sera (42.5 per cent of all pigs surveyed) and confirmed that exposure of animals to C.perfringens type A was common. These studies and those reported so far did not lead to any unequivocal association between alpha toxin production and pathogenicity. There is no doubt that isolates 7 and 29 were pathogenic but the diseases produced differed. It may be that the changes reported in Chapters 3 and 4 (Experiments 1 and 2) were largely due to alpha toxin, as isolate 29 was not an alpha toxin producer. One of these changes, congestion of the mucosa was reproduced in the piglet of the intestinal loop study reported here but, as already discussed, the ingestion of colostrum by this piglet rendered these results inconclusive. It seemed, therefore that although alpha toxin might be involved in piglet enteritis, it was not the only component and enterotoxin was selected for study in Chapter 6.

CHAPTER 6

STUDIES ON ENTEROTOXIN PRODUCTION BY PORCINE CLOSTRIDIUM PERFRINGENS TYPE A AND ITS ASSOCIATION WITH ENTERIC DISEASE IN THE PIG

1. INTRODUCTION

The studies described in this chapter were intended to assess the effects of enterotoxin in porcine enteric disease and fall into 7 parts. In the first part, pig faeces and intestinal contents were studied to determine whether or not sporulating C.perfringens were present. The C.perfringens isolates obtained were combined with those isolated earlier and all were tested for their ability to sporulate and produce enterotoxin. In the second part enterotoxin was purified from one of these isolates and antiserum to it was produced. These reagents were then used to study the incidence of serum antibody to enterotoxin in adult pigs in the third part and the presence of enterotoxin in pig faeces in the fourth part. The numbers of sporulating and vegetative cells present were also determined. The effects of enterotoxin on the piglet intestine were then assessed by gut loop studies in part 5, by intraluminal injection in part 6 and by infection and protection studies in piglets in part 7.

2. SPORULATION AND ENTEROTOXIN PRODUCTION BY PORCINE ISOLATES OF C.PERFRINGENS TYPE A

(a) Materials and Methods

(i) Isolates of C.perfringens type A used

42 isolates of C.perfringens type A were used. They comprised 27 isolates obtained in the survey described in Chapter 3 and 15 isolates obtained in a second survey carried out at Norwich Veterinary Investigation Centre and described in detail in section 5 of this chapter. They were the same as those used in Chapter 5 for studies on alpha toxin production.

(ii) Methods

The media and methods used to assess the ability of the organisms to sporulate have been described in Chapter 2. All three sporulation media (Duncan and Strong, Ellner and Tsai media) were used for each of the 42 isolates. The production of spores was recorded and enterotoxin production was determined by counter-immunoelectrophoresis using the antiserum to enterotoxin produced in part 2 of this chapter and confirmed by Vero cell assay.

(b) Results

The percentage of sporulation and the numbers of enterotoxin producing isolates are shown in Table 29. Enterotoxin was produced by 11 of the 42 (26.1 per cent) C.perfringens type A isolates. 13 isolates (30.9 per cent) produced spores at a frequency of 10 per cent or more. Spore production was recorded in 24 (57.1 per cent) of the isolates. The titres of enterotoxin produced ranged from 1:2 to 1:64. Of the 11 enterotoxin producing isolates 3 were non alpha toxin producers of which one produced the highest percentage of spores and the largest amount of enterotoxin giving a titre of 1:64. Among the 9 alpha toxin producing isolates only 2 produced a frequency of sporulation of 50 per cent or more and titres of 1:8 and 1:16 for enterotoxin; relatively low levels of sporulation and enterotoxin production were detected in the other 7 alpha toxin producing isolates. No enterotoxin was detected in 4 isolates which had a percentage of sporulation between 5 to 15 per cent, these isolates were also negative using Vero cell assay. One non alpha toxin producing isolate failed to sporulate or to produce enterotoxin.

The degree of sporulation and enterotoxin production differed in the three media used. Duncan and Strong medium was best with 23 isolates sporulating in this medium, followed by Tsai medium (19 isolates) and lastly Ellner's with 17 isolates. The variation in spore production in the three media is given in Table 29.

TABLE 29

Sporulation and enterotoxin production by 42 C.perfringens type A isolates in Duncan and Strong, Ellner and Tsai media

Submission number	Sample number	Percentage sporulation in media			Enterotoxin titre (a,b)	Alpha toxin titre (c)
		Duncan and Strong	Ellner	Tsai		
2	5	-	-	-	-	16
	6	-	-	-	-	64
	7	<1	<1	-	-	16
	8	-	<1	-	-	32
3	14	5	<1	<1	-	16
	17	<1	<1	-	-	16
4	20	-	-	-	-	16
	21	1	-	-	-	64
	22	-	-	-	-	8
6	29	95	<1	20	64	-
	30	5	30	<1	8	64
8	34	5	5	10	4	128
9	35	-	-	-	-	8
	36	-	-	-	-	16
	37	-	-	-	-	64

TABLE 29 (Cont'd)

Sporulation and enterotoxin production by 42 C.perfringens type A isolates in Duncan and Strong, Ellner and Tsai media

Submission number	Sample number	Percentage sporulation in media			Enterotoxin titre (a,b)	Alpha toxin titre (c)
		Duncan and Strong	Ellner	Tsai		
12	44	-	-	-	-	16
	45	-	-	-	-	16
	46	5	<1	5	-	16
	47	<1	-	<1	-	16
15	53	-	-	-	-	4
	55	-	-	-	-	4
16	56	15	1	5	-	16
	57	<1	<1	<1	-	16
	58	1	-	<1	-	16
17	59	50	-	<1	8	8
	60	-	-	-	-	-
19	62	10	<1	1	-	256
21	64	10	<1	65	8	8
	65	5	10	35	2	8

TABLE 29 (Cont'd)

Sporulation and enterotoxin production by 42 C.perfringens type A isolates in Duncan and Strong, Ellner and Tsai media

Submission number	Sample number	Percentage sporulation in media			Enterotoxin titre (a,b)	Alpha toxin titre (c)
		Duncan and Strong	Ellner	Tsai		
25	74	-	-	-	-	8
	75	-	-	-	-	32
	76	-	-	-	-	32
	77	1	-	-	-	16
	78	1	-	<1	-	32
28	82	85	<1	<1	16	64
29	83	-	-	-	-	16
	84	-	-	-	-	16
31	86	1	10	<1	2	8
	87	<1	25	<1	8	8
32	88	15	<1	<1	2	-
	89	15	-	5	2	-

(a) = Enterotoxin was tested from the medium with the highest percentage of sporulation

(b) = Reciprocal of highest dilution detected by counterimmunoelectrophoresis

(c) = Haemolytic units (described in Chapter 5)

- = absent < = less than

3. THE PRODUCTION OF ENTEROTOXIN AND THE PRODUCTION OF ANTISERUM TO IT

(a) Materials and Methods

(i) Organisms

Isolate number 29 which produced 95 per cent of spores in Duncan and Strong medium (Fig. 35) but no alpha toxin (Table 29) was used as a source of enterotoxin.

(ii) The production and purification of enterotoxin

The methods used in production and purification are described in Chapter 2.

(iii) The production of specific antiserum

Specific antiserum was produced in rabbits using the adjuvants described in Chapter 2 and the schedule described in Table 4. Antibody levels to enterotoxin were assessed by counterimmuno-electrophoresis as were those to alpha toxin. The protective properties of the antiserum were assessed by the mouse protection test and in Vero cells. The antiserum was examined for its activity against enterotoxin produced by the reference strain of C.perfringens type A.

(b) Results

(i) The purification of enterotoxin

High yields of enterotoxin activity were obtained when the percentage of sporulated cells was higher than 90 per cent of the total cellular count. The result on the different stages of purification are summarised in Table 30.

The three-step purification procedure gave a homogeneous protein with a recovery of 25 per cent of the initial enterotoxin activity. The results from the gel filtration chromatography are

TABLE 30

Enterotoxin activity at different stages of purification

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity* (TU/ml)	Total Activity (TU)	Recovery (Percentage)	Specific activity (TU/mg protein)
Cell sonicate	400	3.6	1440	64	25600	100	18
0-40 per cent ammonium sulphate precipitate	100	8	800	128	12800	50	16
0-15 per cent ammonium sulphate precipitate	30	6.4	192	256	7680	30	40
Active fraction after chromatography on Sephadex G-200 column	25	5.7	142.5	256	6400	25	45

* Reciprocal of highest dilution producing a morphological effect on Vero cells

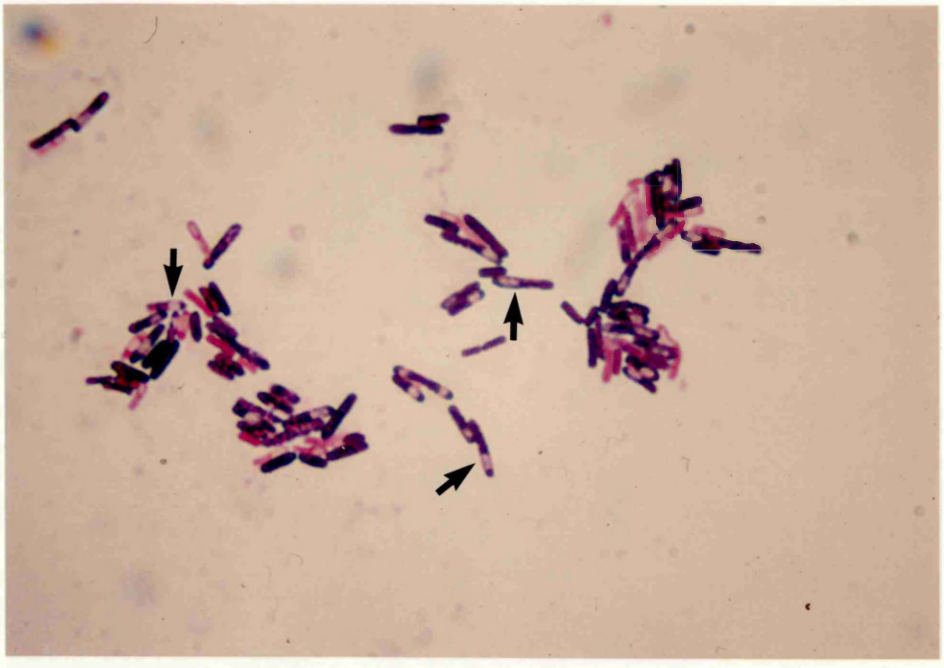

FIG. 35: Smear of C.perfringens type A isolate 29. 8 hour culture in Duncan and Strong medium.
Note the sporulated organisms (arrow).

(Gram x 1200)

given in Figure 36. The first peak after the void volume consisted mainly of nucleic acids since it had a much higher absorbance at 260nm than at 280nm. All the enterotoxin activity was found in the second peak of elution.

Figure 37 shows the extent of homogeneity at each step as determined on polyacrylamide gels. After the second ammonium sulphate precipitation the enterotoxin appeared nearly homogeneous. This figure also shows the results of electrophoresis of a 0-40 per cent ammonium sulphate precipitate of a cell sonicate of a culture with 40 per cent of sporulated cells. A weak band with the electrophoretic mobility of enterotoxin was detected by comparing it with purified enterotoxin from a culture with a higher percentage of sporulation.

The purified enterotoxin had a biological activity of 64 MLD/ml when injected intraperitoneally to mice, and 256 TU/ml on Vero cells.

(ii) Antiserum production

The titres of antibodies to enterotoxin, in immunised pigs and rabbits, detected by counterimmunoelectrophoresis using a constant purified enterotoxin concentration of 500µg/ml are shown in Table 31.

Preimmunisation sera of the two pigs showed titres to enterotoxin of 1:4 and 1:2 respectively, whereas both pre-immunisation sera from rabbits were negative to the presence of antibodies to enterotoxin. Titres were increased or produced after a first immunisation and a further increase was seen after a second immunisation (Fig. 38).

Figure 39 shows that only one precipitation line of identity appeared with the antisera produced and purified enterotoxin when tested by double immunodiffusion. Lines of identity appeared when supernatants from the reference strain were tested.

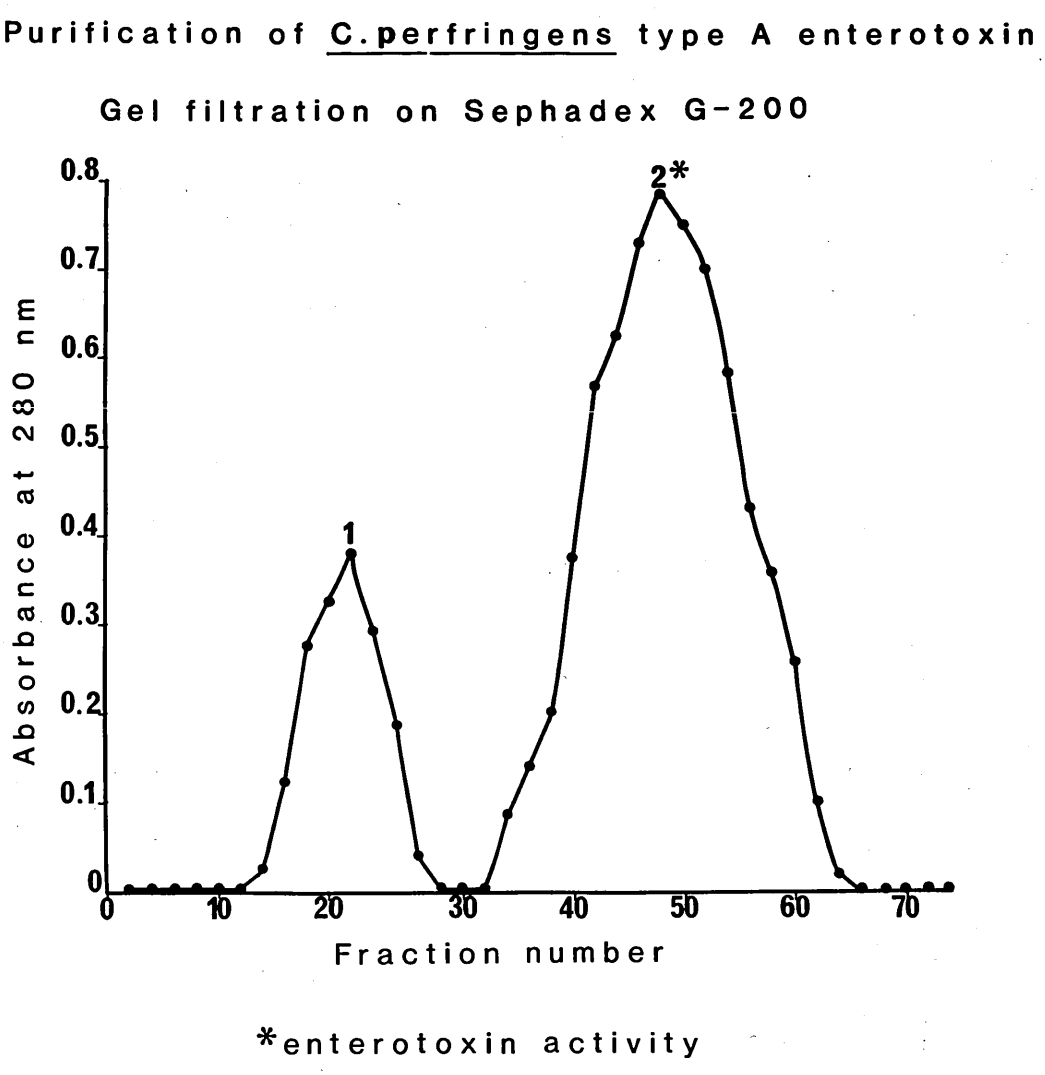

FIG. 36: Separation of enterotoxin by gel filtration chromatography.

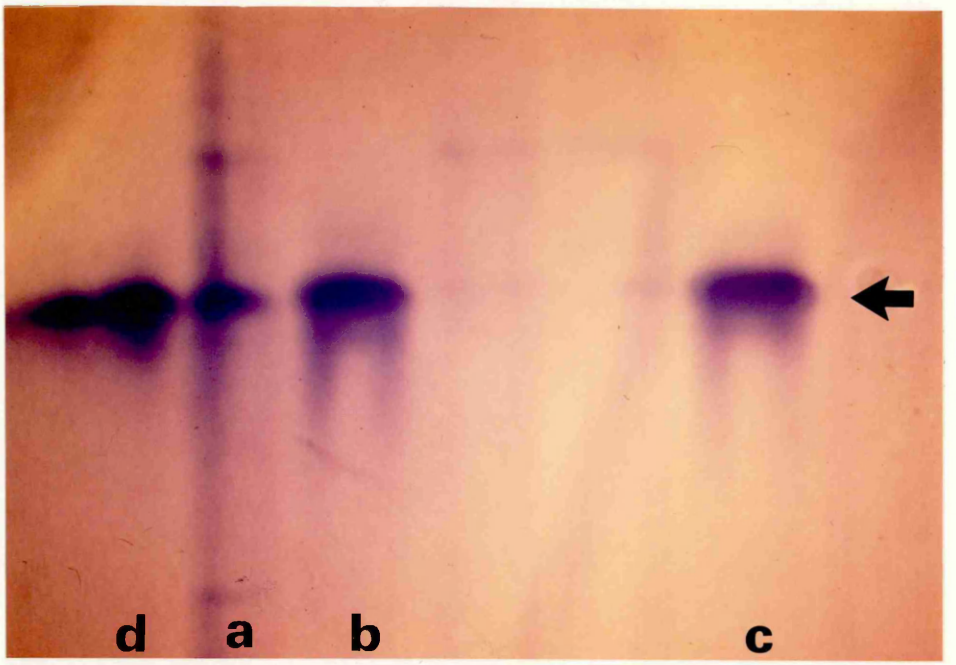

FIG. 37: Polyacrylamide gel electrophoresis pattern obtained after each step of purification of enterotoxin. (a) after 0-40 per cent ammonium sulphate precipitation; (b) after 0-15 per cent ammonium sulphate precipitation; (c) after gel chromatography; (d) reference control enterotoxin.
Note the enterotoxin bands at each step of purification (arrow).

TABLE 31

Antibody titres* of pigs and rabbits immunised with purified enterotoxin

Animal	Preimmunisation	21 days after first immunisation	21 days after second immunisation
Pig 1	1:4	1:16	1:32
Pig 2	1:2	1:8	1:16
Rabbit 1	-	1:4	1:8
Rabbit 2	-	1:4	1:16

* = detected by counterimmunoelectrophoresis

- = absent

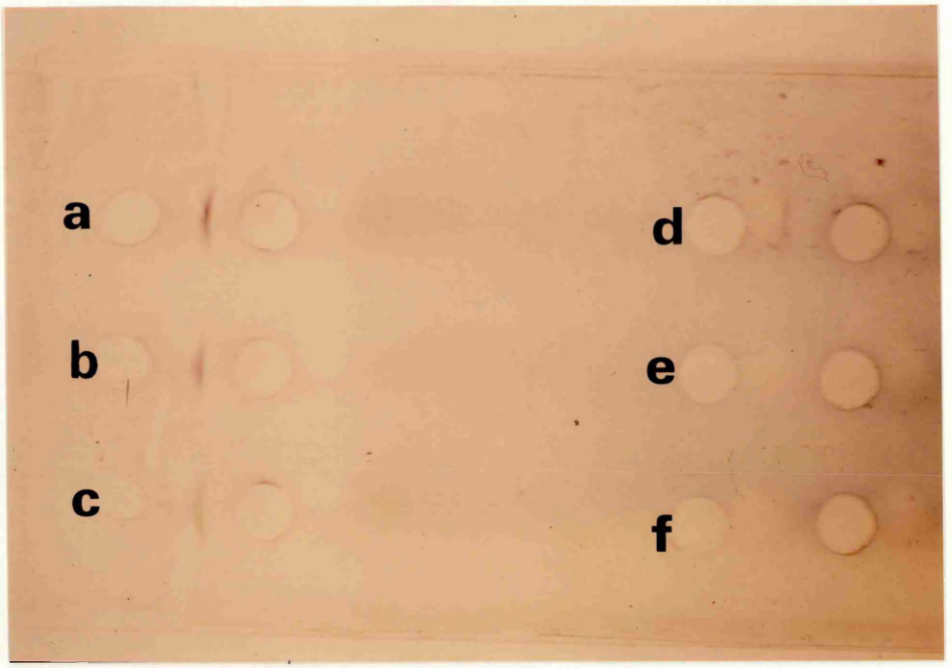

FIG. 38: Detection of antibodies to purified enterotoxin by counterimmunoelectrophoresis. Double dilutions of serum from rabbit 2 immunised with purified enterotoxin: (a) 1:2; (b) 1:4, (c) 1:8; (d) 1:16; (e) 1:32 and (f) 1:64.
Note the titre of 1:16.

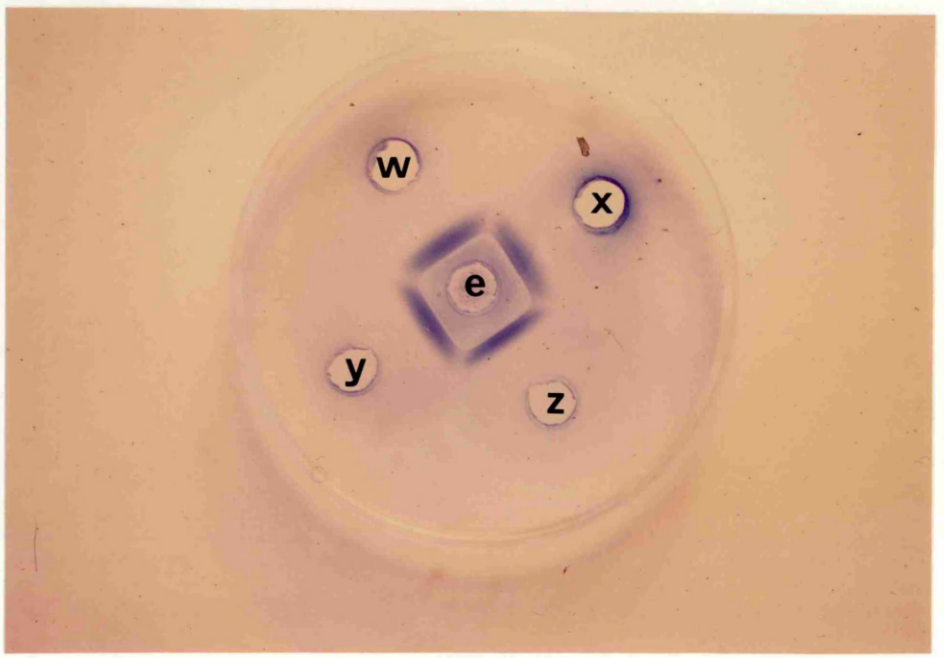

FIG. 39: Double immunodiffusion pattern obtained with purified enterotoxin (E) and antisera to enterotoxin from pig 1 (W), pig 2 (X), rabbit 1 (Y) and rabbit 2 (Z).
Note the presence of only one line of precipitation between purified enterotoxin and the antisera to it.

The antisera produced were able to neutralize 8 MLD of purified enterotoxin when injected intraperitoneally into mice, at the following dilutions: 1:8 (pig 1) 1:4 (pig 2), 1:2 (rabbit 1) and 1:4 (rabbit 2), thus giving a specific activity of 64, 32, 16 and 32 neutralising MLD units/ml respectively.

The sera neutralised the toxicity of 32 TU of purified enterotoxin to Vero cells at the following dilutions: 1:4 (pig 1) 1:2 (pig 2), 1:1 (rabbit 1) and 1:2 (rabbit 2) giving a specific activity of 128, 64, 32 and 64 neutralising TU/ml respectively.

4. SURVEY OF ANTIBODY TO ENTEROTOXIN IN ADULT PIG SERA

(a) Materials and Methods

106 serum samples from sows on 4 Scottish farms were used in this study as described in Chapter 2. They were the same as those used in Chapter 5 for the study of antibody to alpha toxin. Prior to testing the sera were diluted two fold in phosphate buffered saline pH 7.2. The level of antibody to enterotoxin was determined by counterimmunoelectrophoresis using a constant volume of 10µl of enterotoxin containing 5.7mg protein per ml.

(b) Results

Results are summarised in Table 32. Of 106 sera examined, 98 were found to possess serum antibodies to C.perfringens type A enterotoxin with titres ranging from 1:2 to 1:64. No obvious differences in serum titres were found in the pig population of the four farms tested.

5. LEVELS OF VEGETATIVE C.PERFRINGENS TYPE A CELLS, SPORES AND THE PRESENCE OF ENTEROTOXIN IN PIG FAECES

(a) Materials and Methods

(i) Origin of the faecal samples

A total of 33 faecal samples was examined. 29 samples were

TABLE 32

Distribution of serum titres to C.perfringens type A enterotoxin in adult pigs

Enterotoxin CIE * titre	Farm 1	Farm 2	Farm 3	Farm 4	Total	percentage
0	3	2	3	0	8	7.5
2	5	8	6	1	20	18.8
4	9	7	8	4	28	26.4
8	17	8	5	2	32	30.1
16	4	3	0	2	9	8.4
32	2	1	2	3	8	7.5
64	0	1	0	0	1	0.9
Total	40	30	24	12	106	100

* CIE = Counterimmunoelectrophoresis

obtained at Norwich V.I. Centre and 4 were part of the diagnostic material submitted to Glasgow University Veterinary School (Chapter 2). 23 of the samples were from diarrhoeic pigs and 10 were from non diarrhoeic animals.

(ii) Examinations carried out

The numbers of vegetative cells and spores of C.perfringens were determined using tryptone sulphite agar medium (Fig. 40) as described in Chapter 2. The presence of enterotoxin was determined using Vero cell assay (Figs. 41, 42 and 43), the specificity of which was confirmed using neutralisation of toxicity by anti-enterotoxin and by indirect immunofluorescence on Vero cells (Figs. 44 and 45). The other bacteria and enteric pathogens present were studied using the methods outlined in Chapter 2 and the results recorded. C.perfringens type A colonies isolated from these samples were stored for use in the alpha toxin determination described in Chapter 5 and the sporulation and enterotoxin studies described in section 2 of this chapter.

(b) Results

The results are shown in detail in Table 33.

C. perfringens type A colonies were detected in horse blood agar plates in 15 samples from diarrhoeic animals; colonies were not detected in samples from non-diarrhoeic animals, but some diarrhoeic samples failed to yield C.perfringens type A on horse blood agar.

C.perfringens vegetative cells were, however, detected in all the samples with viable counts ranging from 2.5×10^2 cells per gram of intestinal material to 3×10^7 cells per gram of faeces, on tryptone sulphite agar.

C.perfringens spores were detected in 25 out of the total of 33 samples with ranges from 2×10^2 cells per gram of intestinal material to 5×10^6 cells per gram of faeces. Higher viable counts of spores than vegetative cells were present in samples 64,65,82,86

TABLE 33

Viable counts of C.perfringens type A vegetative cells and spores and the presence of enterotoxin and other microorganisms in pig faecal samples

Submission number, age of animal and type of sample	Sample number and consistency	Isolated in horse blood agar	C.perfringens Viable counts Vegetative	C.perfringens Viable counts Spores	Enterotoxin detected	Other agents
18 (16w,F)	61 (N)	-	6.5×10^3	5×10^2	-	B,C,E,G
19 (2d,C)	62 (D)	+	7×10^5	3×10^3	-	B,C,E,O
20 (12w,F)	63 (N)	-	3.5×10^3	-	-	B,C,E,H
21 (7d,C)	64 (D)	++	6×10^4	4×10^6	+	B,D,G,H
	65 (D)	+	1.5×10^3	3.5×10^6	+	B,C,H,I
22 (14d,C)	66 (D)	-	3×10^4	2×10^2	-	B,E,H,I,P
	67 (D)	-	2.5×10^2	-	-	B,E,F,L,P
	68 (D)	-	4.5×10^2	-	-	B,C,E,K,P
23 (4-8w,F)	69 (N)	-	5×10^3	3×10^2	-	B,E,H
	70 (N)	-	3×10^3	-	-	B,C,H
	71 (N)	-	8.5×10^3	-	-	B,E,H,L
	72 (N)	-	6×10^3	-	-	B,E,I,L

TABLE 33 (Cont'd)

Viable counts of C.perfringens type A vegetative cells and spores and the presence of enterotoxin and other microorganisms in pig faecal samples

Submission number, age of animal and type of sample	Sample number and consistency	Isolated in horse blood agar	C.perfringens Viable counts Vegetative	Spores	Enterotoxin detected	Other agents
24 (12w,C)	73 (N)	-	$8x10^5$	$3x10^3$	-	B,C,G,J
25 (12-16w,F)	74 (D)	+	$2x10^3$	$4.5x10^2$	-	B,C,E,H,K
	75 (D)	+	$8x10^5$	$6.5x10^3$	-	B,C,E,H
	76 (D)	+	$2.5x10^4$	$8x10^2$	-	B,J,L
	77 (D)	++	$7x10^4$	$3.5x10^2$	-	B,C,E,G,H,I,L
	78 (D)	++	$3x10^7$	$7x10^3$	-	B,C,E,K
26 (8w,F)	79 (N)	-	$7x10^2$	-	-	B,E,J,L
27 (16w,F)	80 (N)	-	$7.5x10^5$	$2x10^4$	-	B,E,G,L
	81 (N)	-	$6x10^4$	$5.5x10^2$	-	B,E,G
28 (3w,F)	82 (D)	++	$4x10^3$	$2.5x10^4$	+	A,B,C,I
29 (3d,C)	83 (D)	+	$1.5x10^6$	$6x10^4$	-	B,D,E,I,J,O
	84 (D)	+	$8.5x10^4$	$3x10^3$	-	B,D,E,I,J,O
30 (10w,C)	85 (D)	-	$4.5x10^6$	$2x10^4$	-	B,E,N

TABLE 33 (Cont'd)

Viable counts of C.perfringens type A vegetative cells and spores and the presence of enterotoxin and other microorganisms in pig faecal samples

Submission number, age of animal and type of sample	Sample number and consistency	Isolated in horse blood agar	C.perfringens Viable counts Vegetative	Spores	Enterotoxin detected	Other agents
31 (12w,F)	86 (D)	++	$3x10^3$	$4.5x10^5$	+	B,C,J
	87 (D)	++	$7.5x10^4$	$5x10^6$	+	B,C,E,J
32 (12w,F)	88 (D)	++	$8.5x10^4$	$3x10^3$	-	B,K
	89 (D)	+	$4.5x10^6$	$2x10^4$	-	B,E,K
33 (4-6w,F)	90 (D)	-	$4x10^6$	$2x10^2$	-	B,E,L,H
	91 (D)	-	$8x10^5$	$4x10^2$	-	B,C,E,L
	92 (D)	-	$2.5x10^6$	-	-	B,C,E,G,M
	93 (D)	-	$5x10^6$	$5x10^2$	-	B,C,H,M

(F) = faeces
(C) = carcase

Number of C.perfringens type A colonies in horse blood agar plates
+ = 1-8 colonies

TABLE 33 (Key)

(N) = normal faeces ++ = more than 8 colonies

(D) = diarrhoea

(w) = weeks + = positive

(d) = days - = negative

A = β-haemolytic E.coli

B = Non-haemolytic E.coli

C = Streptococcus spp.

D = Peptostreptococcus spp.

E = Bacillus spp.

F = Corynebacterium spp.

G = Proteus spp.

H = Bacteroides spp.

I = Fusobacterium spp.

J = Lactobacillus spp.

K = Staphylococcus spp.

L = Pseudomonas spp.

M = Campylobacter spp.

N = Salmonella typhimurium

O = Transmissible Gastroenteritis Virus

P = Porcine Epidemic Diarrhoea Virus

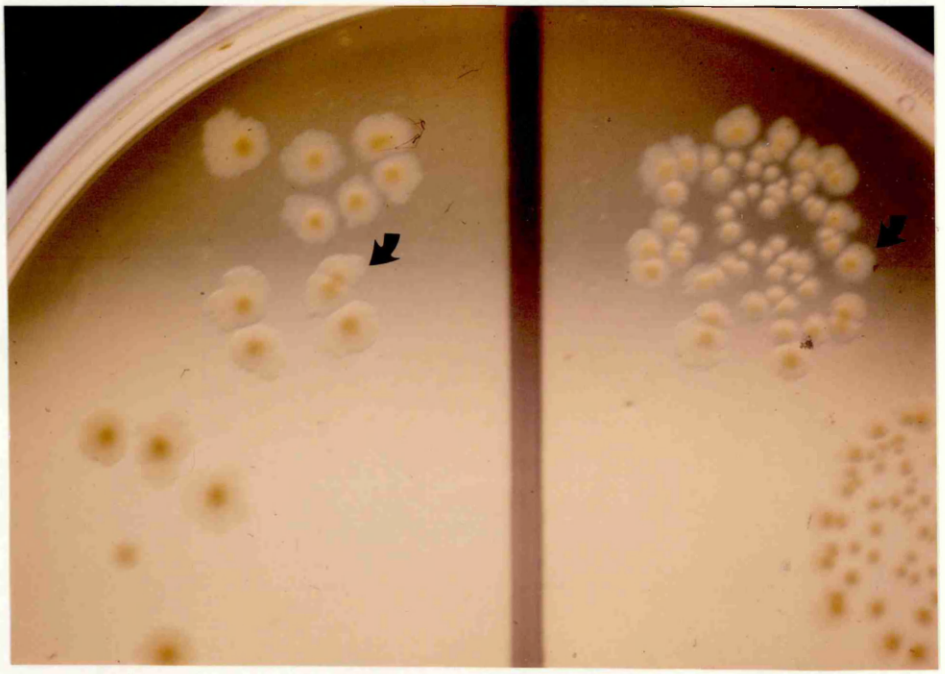

FIG. 40: C.perfringens type A spore counts. Heat-shocked dilution showing colonies of the organism. 24 hour culture on tryptone-sulphite agar.
Note the colour and morphology of the colonies (arrow).

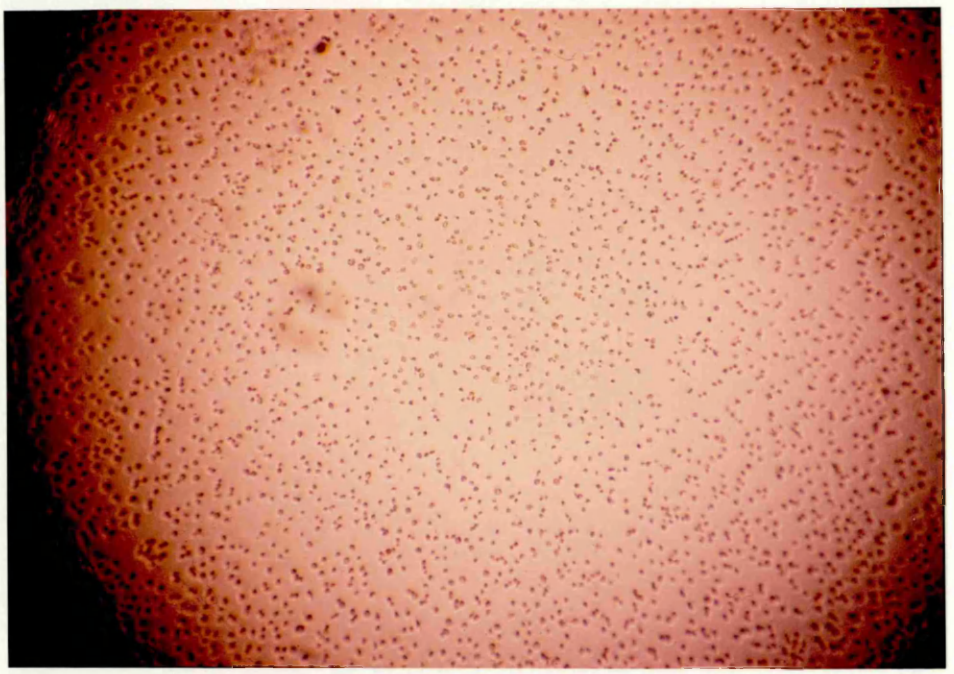

FIG. 41: Demonstration of enterotoxin by its effects on Vero cells. Vero cells after 60 minutes exposure to a sample containing 0.25MLD/ml of enterotoxin.
Note the spherical morphology of the cells.
(Transmitted light x 40)

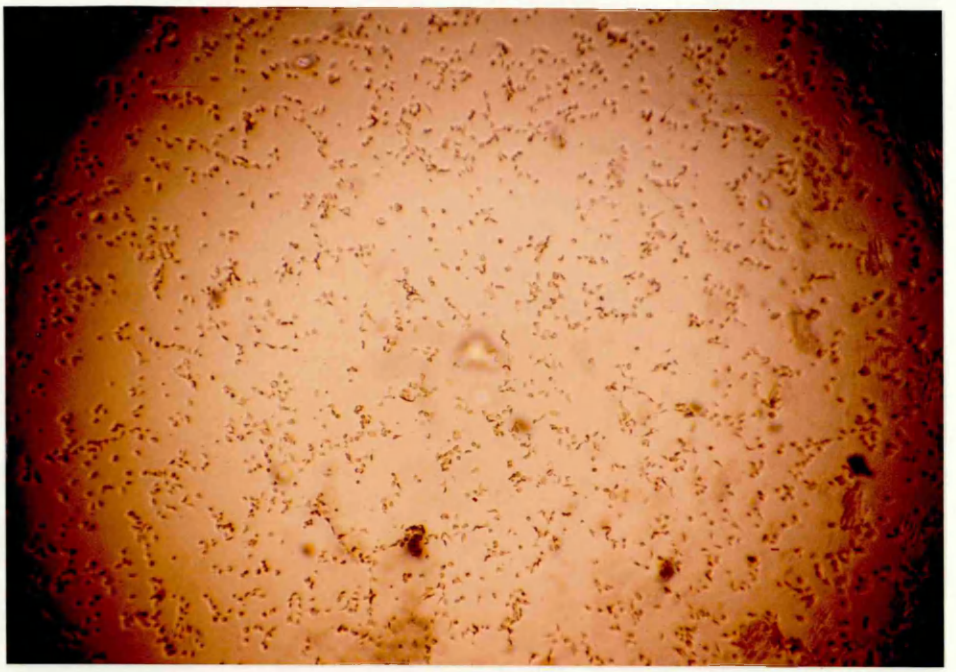

FIG. 42: Demonstration of enterotoxin by its effects on Vero cells. Vero cells after 60 minutes exposure to a sample containing 1 MLD/ml of enterotoxin.
Note the detachment of the cells.

(Transmitted light x 40)

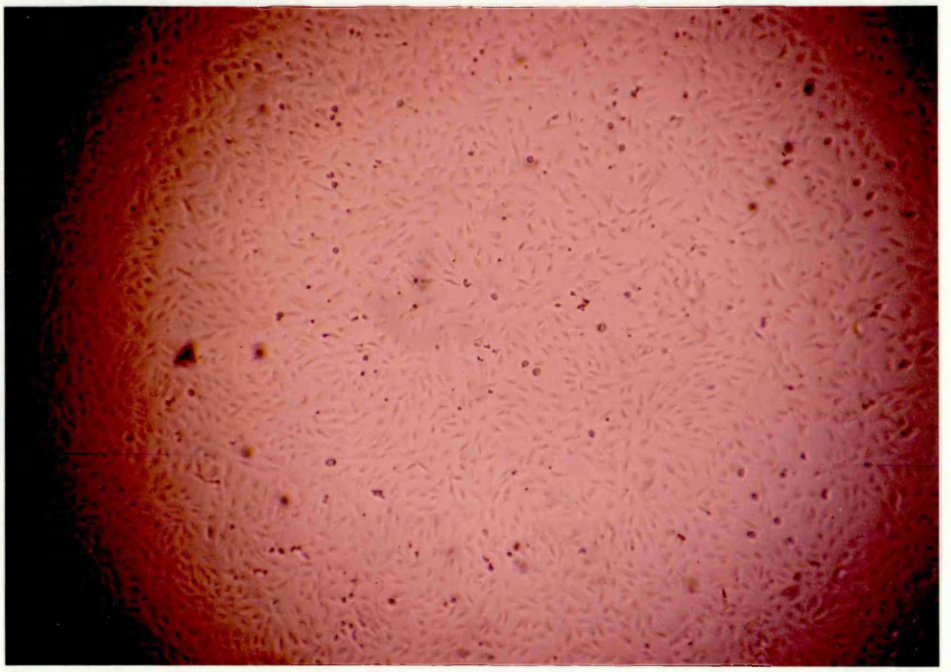

FIG. 43: Negative sample to enterotoxin on Vero cells.
Note the typical Vero cell morphology.

(Transmitted light x 40)

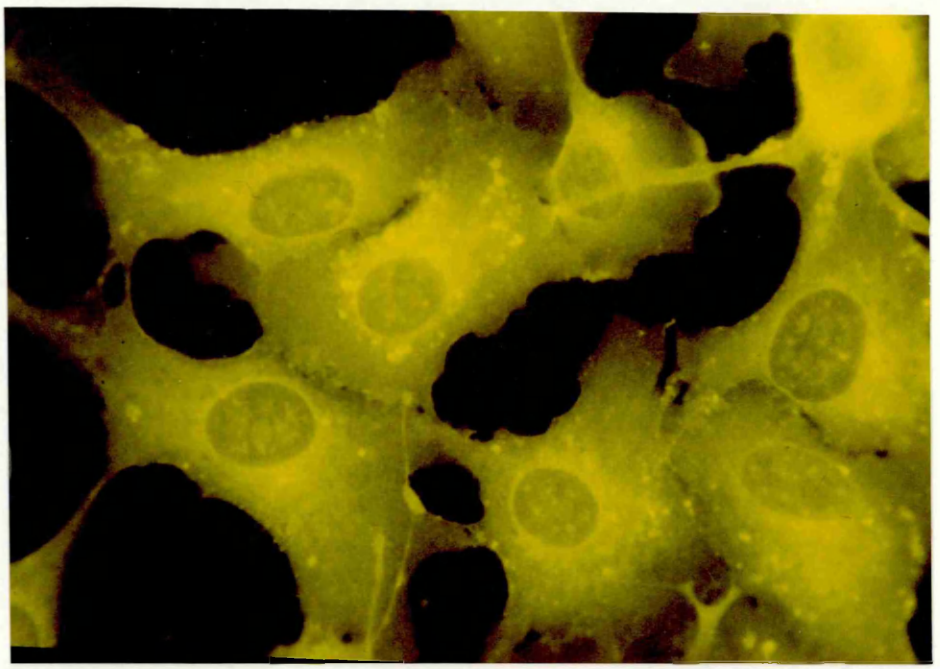

FIG. 44: Demonstration of enterotoxin adsorbed onto Vero cells by indirect immunofluorescence. Sample positive for enterotoxin. Note the fluorescence concentrated in the peripheral cytoplasm of the cells.

(x 1200)

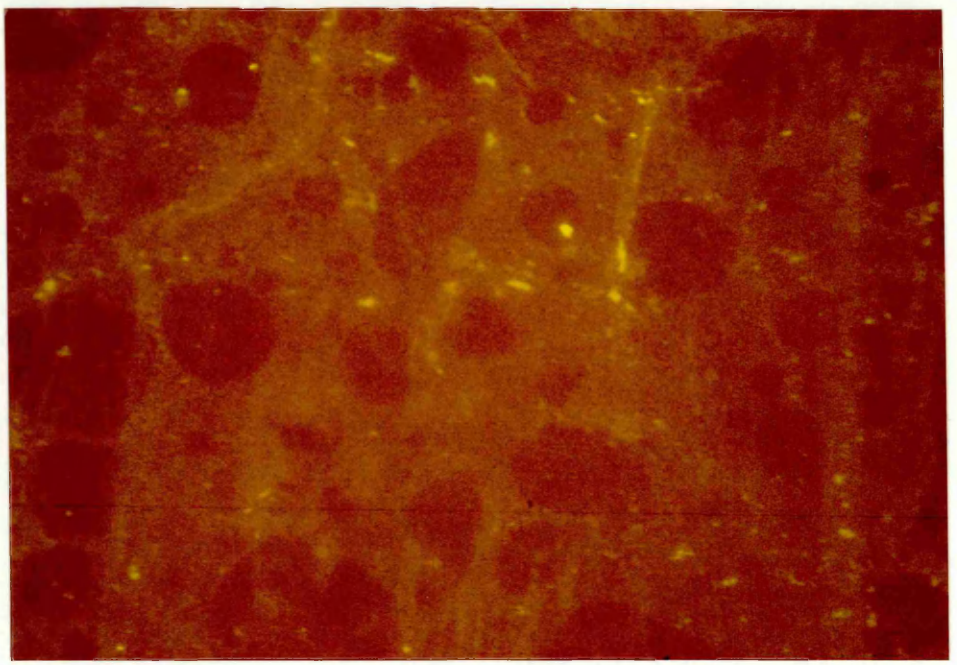

FIG. 45: Sample negative for enterotoxin.
Note the absence of fluorescence and the integrity of the cell sheet.

(Fluorescent antibody x 1200)

and 87. These same samples were the only ones in which enterotoxin could be detected. These samples were from the submissions, 21,28 and 31. The clinical signs associated with these samples are given below.

Submission 21 consisted of carcases from 2 seven day old piglets from a herd with 20 per cent losses from birth to weaning at 3-10 days of age. The diarrhoea and death occurred only in gilt litters. Gilts were housed separately from sows. The carcases were in poor condition with a yellowish creamy diarrhoea. The small intestines were mildly congested and empty and the large intestines were filled with creamy or pasty yellowish contents. No gross lesions were seen in other organs. C.perfringens was isolated in profuse culture on horse blood agar from the duodenum, jejunum, ileum and large intestine. Submission 28 consisted of diarrhoeic faeces from a 3 week old piglet submitted with no history. The β-haemolytic E.coli isolated was untypable. Submission 31 consisted of 2 diarrhoeic faeces samples from 12-week old pigs with mucoid diarrhoea in a pen of 8 pigs none of which had died. Swine dysentery was absent.

6. THE EFFECTS OF ENTEROTOXIN ON PIGLET INTESTINAL LOOPS

(a) Materials and Methods

(i) Animals

Two hysterectomy-derived, colostrum-deprived piglets and one conventionally born, colostrum-deprived piglet were used.

(ii) Methods

The ligated intestinal loop technique was performed as previously described in Chapter 2. The animals were killed 12 hours after injection of the preparations.

Eleven intestinal loops were prepared in the conventionally born, colostrum-deprived piglet as shown in Table 34. Two loops were inoculated with purified enterotoxin containing 1.68mg of protein per ml with an activity of 512 toxic units to Vero cells

per ml. One loop was injected with the same enterotoxin after incubation for 30 minutes at room temperature with 0.25ml of rabbit anti-enterotoxin serum containing 16 units of antibody to enterotoxin when tested by counterimmunoelectrophoresis. Two loops were inoculated with the culture supernatant of isolate number 34 grown in Duncan and Strong medium, centrifuged and filtered through a filter of APD 0.22µm. Another loop consisted of the same culture supernatant fluid previously incubated with anti-enterotoxin serum for 30 minutes at room temperature. Two loops were injected with the culture supernatant fluid of isolate 29. One loop was inoculated with this culture supernatant incubated with anti-enterotoxin serum. Two control loops received sterile Duncan and Strong medium. Accumulation of fluid, macroscopic congestion of mucosa, and histo-pathological changes were recorded for each of the inoculated loops.

Ten intestinal loops were utilised in hysterectomy-derived, colostrum-deprived piglet according to the schedule in Table 35. Five loops were injected with dilutions of purified enterotoxin ranging from a concentration of protein per loop of 0.04mg to 1.14mg. The activity of the inoculated dilutions of enterotoxin ranged from 4 to 256 toxic units per ml. Two loops were inoculated with dilutions of purified enterotoxin containing 16 and 64 toxic units per ml, respectively which were previously incubated with 0.25ml of pig anti-enterotoxin serum possessing a titre of 32 anti-enterotoxin units when tested by counterimmunoelectrophoresis. One loop was injected with the whole culture of isolate 29 grown in Duncan and Strong medium, and contained 95 per cent of sporulated cells (total number of $3x10^8$ cells per ml). One control loop was injected with saline solution. Accumulation of fluid and macroscopic congestion of the mucosa were recorded for each segment. Histopathological and immunofluorescence studies were carried out on the mucosa of the loops.

Eleven intestinal loops were utilised in hysterectomy-derived colostrum deprived piglet 2 inoculated at the same time as piglet 1 with a complementary series of C.perfringens products. Loops were inoculated as follows: five loops were injected with dilutions of purified enterotoxin ranging from a concentration of

protein per loop of 0.04mg to 1.14mg. One loop received a dilution of purified enterotoxin containing 32 toxic units per ml which was previously incubated with 0.25ml of pig anti-enterotoxin serum described above. One loop was inoculated with whole culture of isolate 29 grown in cooked meat medium. Another loop received vegetative cells (isolate 29) grown in the same cooked meat medium and washed by centrifugation in saline solution. Two loop controls were incubated with saline solution and one loop control received sterile cooked meat medium.

(b) Results

(i) Conventionally born, colostrum-deprived piglet

Ligated intestinal loops prepared in the conventionally born, colostrum deprived piglet showed accumulation of fluid when purified enterotoxin was injected, (Table 34, Fig. 46) one of these loops (loop 9) was found to have burst when post-mortem examination was carried out and congestion of the mucosa was evident. The effect of the enterotoxin was neutralised by the rabbit anti-enterotoxin serum preventing accumulation of fluid and congestion of the mucosa in the inoculated intestinal loop (loop 5). No evidence of accumulation of fluid was noted in loops injected with culture supernatant alone, although congestion of the mucosa was present in the 4 loops (loops 2,3,10 and 11) inoculated with these preparations. Loops inoculated with culture supernatants previously incubated with rabbit anti-enterotoxin serum did not show evidence of congestion of the mucosa (loops 6 and 7). Loops injected with sterile Duncan and Strong medium did not show fluid accumulation or congestion of the mucosa (Table 34).

Intestinal loops inoculated with enterotoxin showed marked histological changes consisting of massive destruction of the villous architecture (Fig. 47). Necrosis and inflammation of the mucosa was evident. Large quantities of cell debris were present in the lumen. Congestion of the mucosa and lamina propria was observed. The loop inoculated with enterotoxin mixed with anti-enterotoxin serum presented an increased hypercellularity in lamina propria but no other histological changes were denoted in this

TABLE 34

Accumulation of fluid and congestion of the mucosa in ligated intestinal loops of conventionally born, colostrum-deprived piglet injected with purified enterotoxin and C.perfringens culture supernatant fluids

Loop No.	Preparation injected	Volume injected per loop (ml.)	Protein (mg/loop)	Activity (TU/ml)	Rabbit anti-enterotoxin serum added (0.25ml/loop)	Accumulation of fluid	Microscopic congestion of mucosa
1	enterotoxin	0.5	0.84	512	-	+	+
2	culture supernatant-1	0.5	NT	NT	-	-	+
3	culture supernatant-2	0.5	NT	NT	-	-	+
4	sterile Duncan and Strong medium	0.5	-	-	-	-	-
5	enterotoxin	0.5	0.84	512	+	-	-
6	culture supernatant-1	0.5	NT	NT	+	-	-
7	culture supernatant-2	0.5	NT	NT	+	-	-
8	sterile Duncan and Strong medium	0.5	-	-	-	-	-
9	enterotoxin	0.5	0.84	512	-	+	+

TABLE 34 (Cont'd)

Accumulation of fluid and congestion of the mucosa in ligated intestinal loops of conventionally born, colostrum-deprived piglet injected with purified enterotoxin and C.perfringens culture supernatant fluids

Loop No.	Preparation injected	Volume injected per loop (ml)	Protein (mg/loop)	Activity (TU/ml)	Rabbit anti-enterotoxin serum added (0.25ml/loop)	Accumulation of fluid	Microscopic congestion of mucosa
10	culture supernatant-1	0.5	NT	NT	-	-	+
11	culture supernatant-2	0.5	NT	NT	-	-	+

NT = not tested

\- = negative

\+ = positive

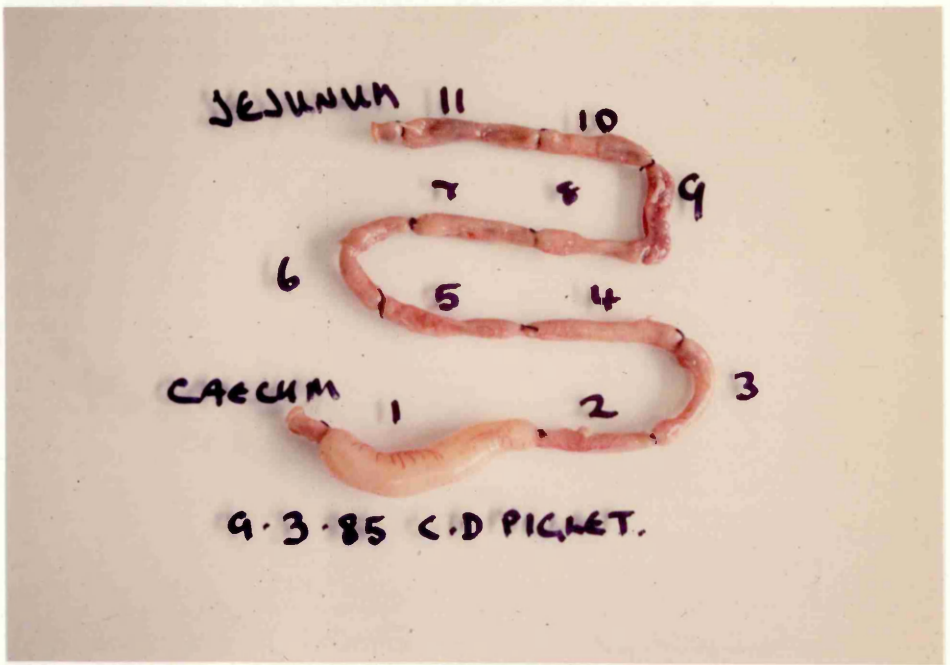

FIG. 46: Inoculation of purified enterotoxin and C.perfringens type A culture supernatant fluids into intestinal loops of conventionally-born, colostrum-deprived piglet. Loops 1,9 and 5 were inoculated with concentrated purified enterotoxin. Loop 1 is distended with fluid, loop 9 has burst and in loop 5 the toxin was neutralised by rabbit antiserum.

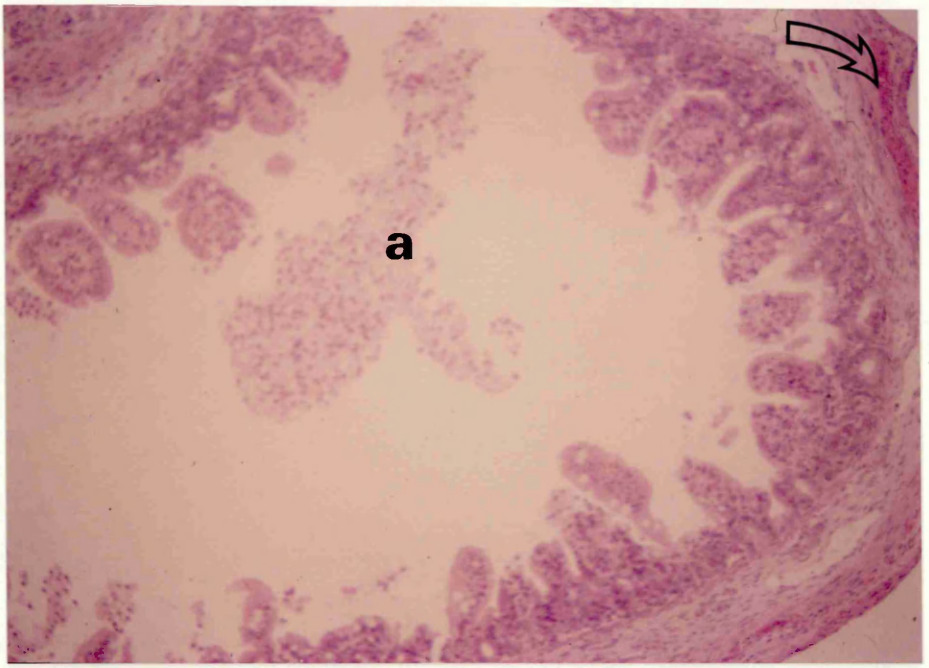

FIG. 47: Histological section of the ileal mucosa in loop 1 of conventionally born, colostrum-deprived piglet, inoculated with purified enterotoxin.
Note the destruction of the villous architecture, the cell debris in the lumen (a), and areas of congestion of the lamina propria.

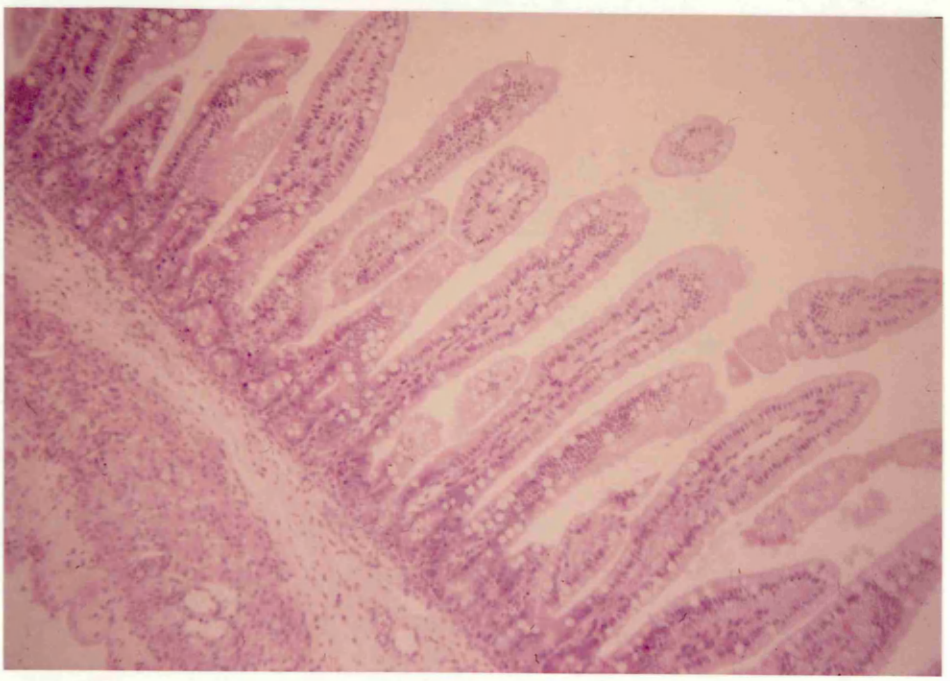

FIG. 48: Histological section of the ileal mucosa of control loop 4 of conventionally born, colostrum-deprived piglet, inoculated with sterile Duncan and Strong medium.
Note the normal appearance of the villi and the lamina propria.
(HE x 120)

loop. Loops inoculated with culture supernatants had congestion of the mucosa and lamina propria but no desquamation of villous epithelium was observed in these loops. Loops injected with culture supernatants mixed with anti-enterotoxin serum and control loops inoculated with sterile Duncan and Strong medium did not show any histopathological changes (Fig. 48).

No immunofluorescence studies were carried out on this animal.

(ii) Hysterectomy-derived, colostrum-deprived piglet

Ligated intestinal loops prepared in the hysterectomy-derived, colostrum-deprived piglet showed accumulation of fluid when doses of 1.14 (loop 1), 0.85 (loop 10) and 0.35mg (loop 4) of protein were injected (Table 35, Fig. 49). The degree of fluid accumulation was correlated with the amount of enterotoxin inoculated, the highest dose produced a larger accumulation of fluid. Enterotoxin doses of 0.08 and 0.04mg of protein did not induce accumulation of fluid in the injected loops 8 and 9. Accumulation of fluid was not noted in loops inoculated with 0.71 and 0.17mg of enterotoxin mixed with pig anti-enterotoxin serum (loops 3 and 7). Accumulation of fluid was absent in loops inoculated with sporulated cells, saline solution and sterile Duncan and Strong medium. Macroscopic congestion of the mucosa was evident in loops 1,10 and 4 inoculated with enterotoxin doses of 1.14, 0.85 and 0.35mg, as well as in loop 3 inoculated with 0.71mg of enterotoxin mixed with pig anti-enterotoxin serum.

Intestinal loops inoculated with doses of enterotoxin of 1.14, 0.85 and 0.35mg presented a massive destruction of the intestinal epithelium. Necrosis and inflammation of the mucosa were evident. Cell debris was present in the lumen. Large haemorrhages were seen in loops with doses of enterotoxin of 1.14 and 0.85mg (Fig. 50). Congestion of the mucosa and lamina propria was present in loops inoculated with 1.14, 0.85 and 0.35mg of enterotoxin as well as in loop inoculated with 0.71mg of enterotoxin mixed with pig anti-enterotoxin serum. Loop inoculated with sporulated cells had shortened villi and areas of haemorrhage at lamina propria level. Loops inoculated with doses of enterotoxin of 0.08 and 0.04mg

TABLE 35

Accumulation of fluid and congestion of the mucosa in ligated intestinal loops of hysterectomy-derived, colostrum-deprived piglet 1 injected with purified enterotoxin and C.perfringens sporulated cells

Loop No.	Preparation injected	Volume injected per loop (ml)	Protein (mg/loop)	Activity (TU/ml)	No cells/ loop	Pig anti-enterotoxin serum added (0.25ml/loop)	Accumulation of fluid	Microscopic congestion of mucosa
1	enterotoxin	0.2	1.14	256	-	-	+	+
2	0.85per cent NaCl solution	0.5	-	-	-	-	-	-
3	enterotoxin	0.5	0.71	64	-	+	-	+
4	enterotoxin	0.5	0.35	32	-	-	+	+
5	Sterile Duncan and Strong medium	0.5	-	-	-	-	-	-
6	Sporulated cells	0.5	-	-	1.5×10^8	-	-	+
7	enterotoxin	0.5	0.17	16	-	+	-	-
8	enterotoxin	0.5	0.08	8	-	-	-	-
9	enterotoxin	0.5	0.04	4	-	-	-	-
10	enterotoxin	0.3	0.85	128	-	-	+	+

- = negative

+ = positive

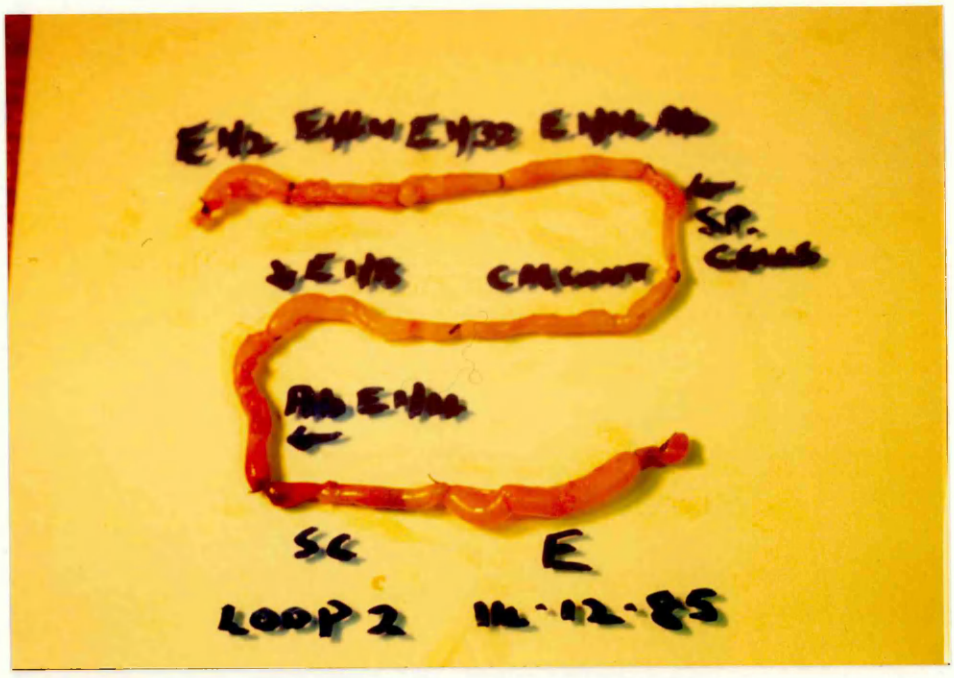

FIG. 49: Inoculation of purified enterotoxin and C.perfringens type A sporulated cells into intestinal loop of hysterectomy-derived, colostrum-deprived piglet 1.
Note the accumulation of fluid in loops 1 (E), 4 (E 1/8) and 10 (E 1/2) which were inoculated with dilutions of purified enterotoxin. The other loops did not show accumulation of fluid. The enterotoxin effect was neutralised by rabbit antiserum in loop 3 (Ab E 1/4).

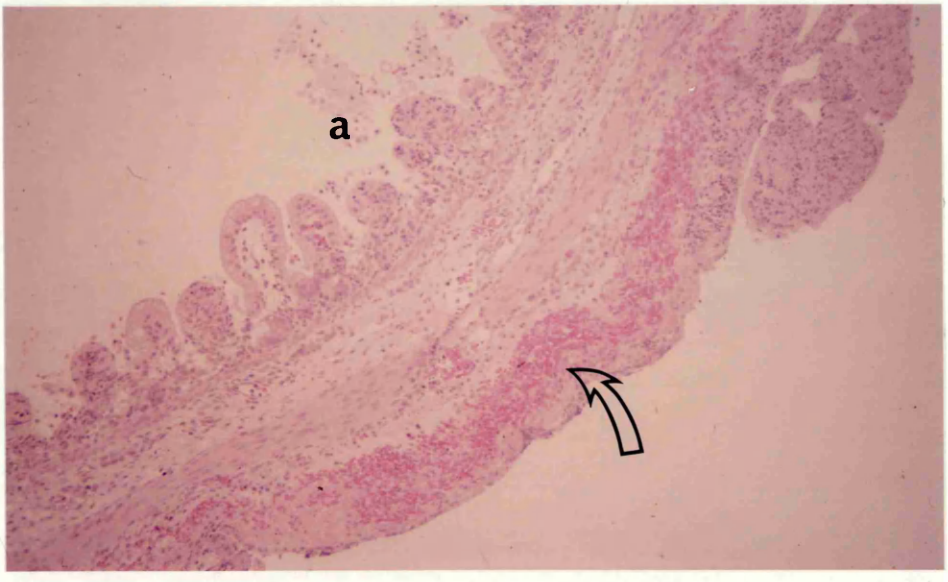

FIG. 50: Histological section of the ileal mucosa of loop 10 of hysterectomy-derived, colostrum-deprived piglet 1, inoculated with purified enterotoxin.
Note the destruction of the villous architecture, the cell debris in the lumen (a) and the haemorrhages in the lamina propria (arrow).

(HE x 120)

showed a mild congestion in the lamina propria. Loops injected with saline solution and sterile Duncan and Strong medium did not show histological changes.

Tracing of enterotoxin with its specific antiserum by indirect immunofluorescence in frozen sections of intestinal loops revealed its presence at the surface of the villous epithelium. Doses of 1.14 and 0.85mg of enterotoxin produced an almost solid fluorescent outline on the epithelium (Fig.51) and complete bright fluorescence of individual epithelial cells. Strongly-fluorescent desquamated epithelial cells were found in the lumen (Fig.52). Enterotoxin could be detected in the epithelium of loops inoculated with doses of 0.08 and 0.04mg but the fluorescence seen was weaker than with higher levels of enterotoxin (Fig. 53) and to be concentrated in the peripheral cytoplasm of the cells.

Fluorescence could be demonstrated also in the epithelium of the loop inoculated with sporulated cells and loop 3 inoculated with enterotoxin and antiserum, fluorescence in these segments was similar to that found with low doses of enterotoxin in intestinal loops.

Fluorescence could not be demonstrated in the epithelium of loops inoculated with saline solution and sterile Duncan and Strong medium (Fig. 54).

Piglet 2 died 2 hours after operation and there were no obvious changes in the loops (Table 36). No further examination was carried out on this animal.

7. THE INTESTINAL RESPONSE TO THE INTRALUMINAL INJECTION OF C.PERFRINGENS ENTEROTOXIN

(a) Materials and Methods

Two hysterectomy-derived, colostrum-deprived piglets were used. The operative procedure for the intraluminal injection of enterotoxin was carried out as previously described in Chapter 2. The animals were injected with a 2ml dose of purified enterotoxin

TABLE 36

Accumulation of fluid and congestion of the mucosa in ligated intestinal loops of hysterectomy-derived, colostrum-deprived piglet 2 injected with purified enterotoxin and C.perfringens vegetative cells

Loop No.	Preparation injected	Volume injected per loop (ml)	Protein (mg/loop)	Activity (TU/ml)	No cells/ loop	Pig anti-enterotoxin serum added (0.25ml/loop)	Accumulation of fluid	Microscopic congestion of mucosa
1	enterotoxin	0.5	1.14	256	-	-	-	+
2	0.85 per cent NaCl solution	0.5	-	-	-	-	-	NT
3	enterotoxin	0.5	0.71	64	-	-	-	NT
4	sterile cooked meat medium	0.5	-	-	-	-	-	NT
5	vegetative cells	0.5	-	-	$7x10^7$	-	-	NT
6	enterotoxin	0.5	0.35	32	-	+	-	NT
7	enterotoxin	0.5	0.17	16	-	-	-	NT
8	washed vegetative cells	0.5	-	-	$5x10^9$	-	-	NT
9	enterotoxin	0.5	0.04	4	-	-	-	NT
10	0.85 per cent NaCl solution	0.5	-	-	-	-	-	NT
11	enterotoxin	0.5	0.85	128	-	-	-	+

- = negative + = positive NT = not tested

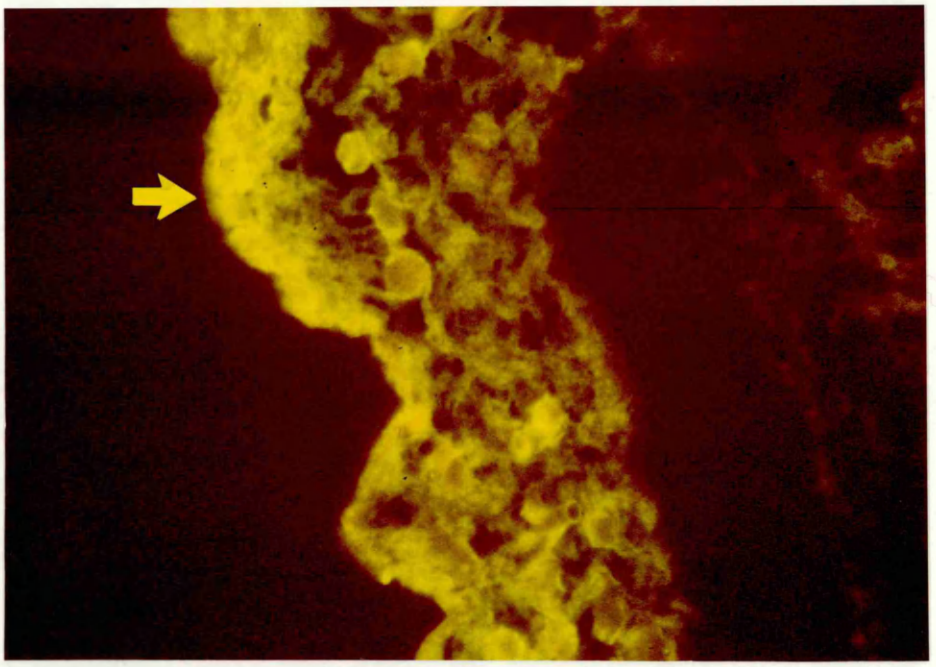

FIG. 51: Demonstration of enterotoxin by indirect immuno-fluorescence on the mucosal epithelium of loop 10 of hysterectomy-derived, colostrum-deprived piglet 1 inoculated with purified enterotoxin.
Note the solid fluorescent outline of the epithelium (arrow).

(x 300)

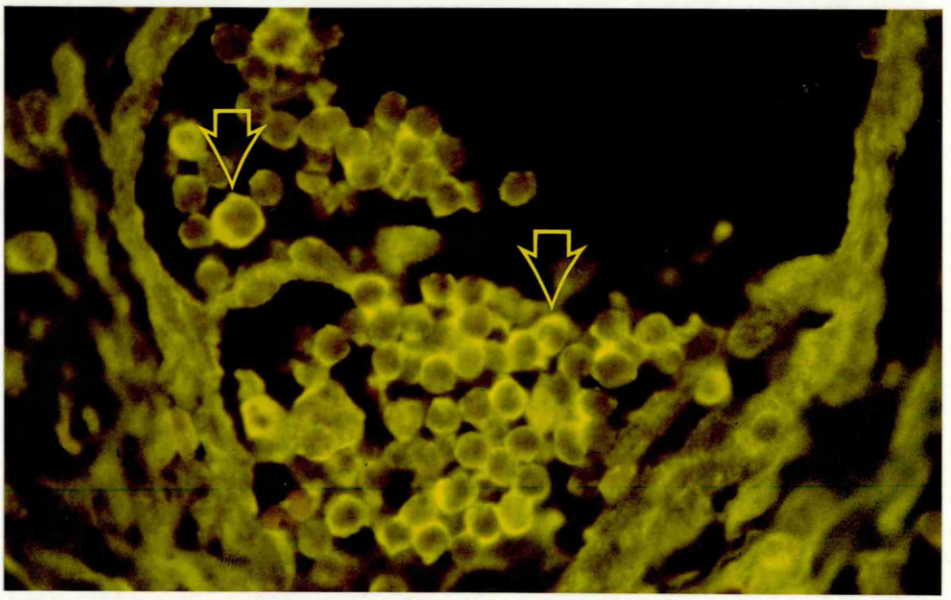

FIG. 52: Demonstration of enterotoxin by indirect immuno-fluorescence on desquamated epithelial cells in the ileal lumen of loop 10 of hysterectomy-derived, colostrum-deprived piglet 1 inoculated with purified enterotoxin.
Note the bright fluorescence of individual epithelial cells (arrow).

(x 400)

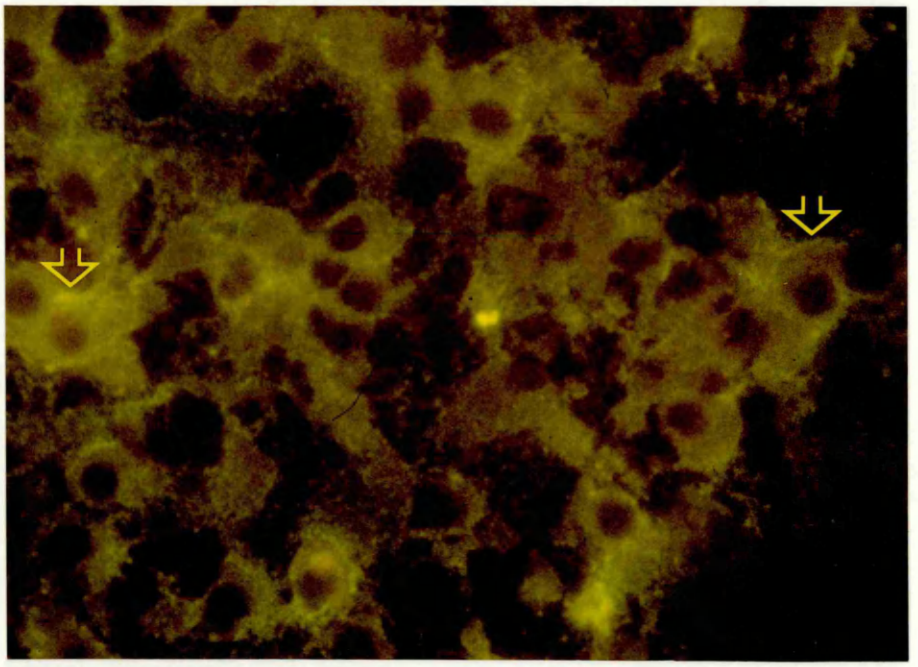

FIG. 53: Demonstration of enterotoxin by indirect immunofluorescence on the mucosal epithelium of loop 9 of hysterectomy-derived, colostrum-deprived piglet 1 inoculated with purified enterotoxin.

Note the weaker fluorescence (arrow) than in loops inoculated with higher levels of enterotoxin (Figs. 51 and 52).

(x 400)

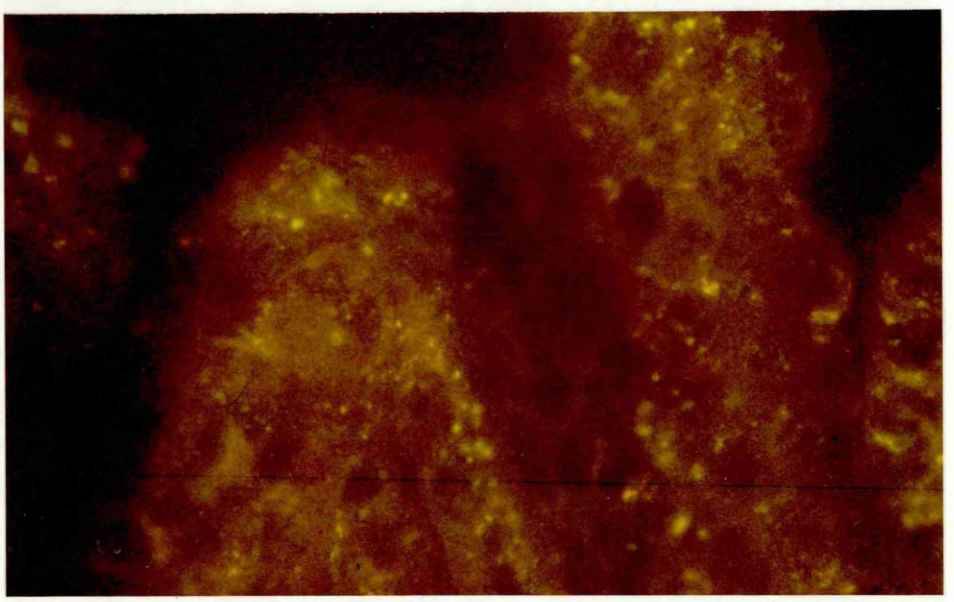

FIG. 54: Control loop 5 of hysterectomy-derived, colostrum-deprived piglet 1 inoculated with sterile Duncan and Strong medium.

Note the absence of fluorescence.

(Fluorescent antibody x 400)

containing 5.7mg of protein per ml and an activity of 256 toxic units per ml to Vero cells into what was intended to be the jejunum.

The injection site was marked with dye in one piglet and by a suture in the other. After laparotomy, the animals were observed at intervals for the occurrence of diarrhoea and killed 12 hours after the enterotoxin injection. The whole intestine of the piglets was extended and observed for the presence of fluid accumulation and macroscopic lesions. Segments of duodenum, jejunum, ileum, caecum and colon were taken for histology and immunofluorescence studies and processed by the methods described in Chapter 2.

(b) Results

(i) Clinical

A watery-mucoid diarrhoea which stained the perineum developed in both piglets within one hour post-inoculation (Fig. 55) followed by return to an apparently normal state at 6 to 8 hours.

(ii) Pathological

At post-mortem examination the site of injection could not be identified unequivocally in the animal marked with dye but was thought to be 30cm anterior to the caecocolic junction. In the sutured animal it was found to be in the mid-ileum 30cm anterior to the ileocaecocolic junction.

The duodenum, and jejunum of both animals appeared normal with no excess of fluid. From the site of enterotoxin injection, at mid-ileum, to the terminal ileum, a mild congestion of the mucosa was noticed in both piglets. The colon of one of the piglets was filled with a watery mucoid fluid, in the other animal fluid was also present although in smaller quantities. The colonic mucosa of both piglets was mildly congested.

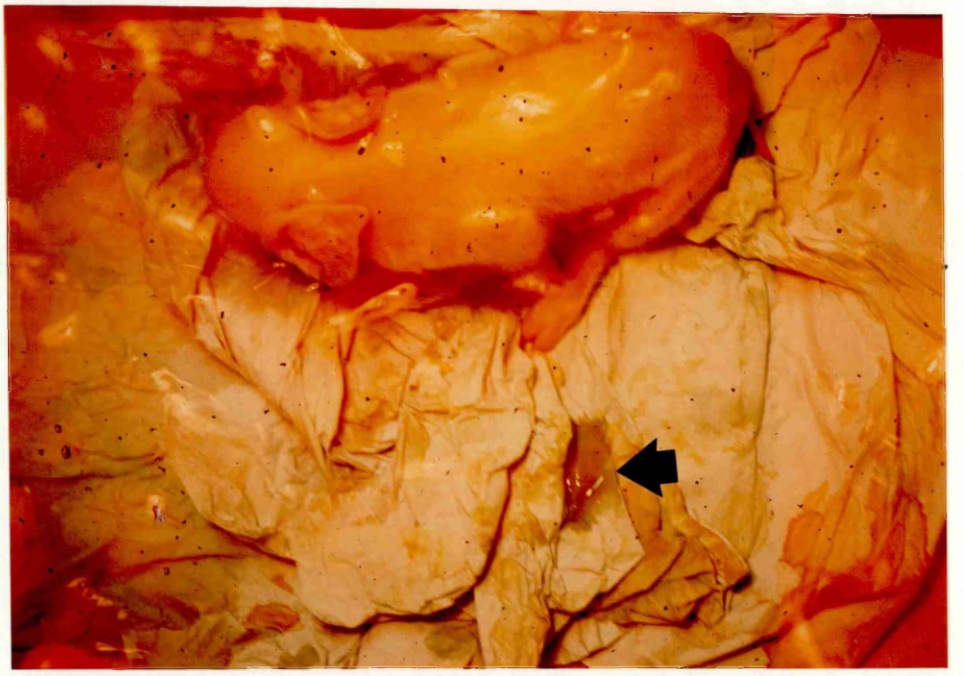

FIG. 55 Hysterectomy-derived, colostrum-deprived piglet 1 with diarrhoea, one hour post-intraluminal inoculation of purified enterotoxin.

Note the watery mucoid diarrhoea (arrow).

Histologically, the duodenum and jejunum of both animals did not show pathological changes. The upper ileum, anterior to the site of injection appeared to have areas of congestion in the lamina propria in both animals. The epithelial cells were normal at this level. The caudal region of the ileum, posterior to the site of injection showed a shortening of the villi to about two-thirds from the normal length in both piglets. At this level one of the animals had areas of congestion in submucosa, muscular layer and serosa; the lamina propria between the crypts and in the cores of the villi contained inflammatory cells mainly mononuclear cells, and haemorrhages were present at lamina propria level. Desquamated epithelial cells were observed in the lumen (Fig. 56). Less severe lesions were observed in the second piglet at this level of the ileum where a mild congestion of lamina propria was observed; areas of localised oedema were also noted at lamina propria level in this animal. The caecal and colonic lesions of both animals were identical.

Areas of haemorrhage were seen in the lamina propria. Inflammatory cells were present in large numbers mainly in the submucosa. The surface epithelium was uneven and covered with oedematous fluid. Desquamated epithelial cells were found in the lumen. There was local cytoplasmic irregularity of the lumenal epithelium of caecum and colon (Fig. 57).

Enterotoxin was demonstrated by indirect immunofluorescence in the intestinal sections of first piglet from the terminal jejunum where a weak fluorescence was observed to the colon. Intestinal sections of the second piglet showed fluorescence from the mid-ileum to the colon. Sections of upper duodenum, lower duodenum and mid-jejunum were negative for enterotoxin as well as section of terminal jejunum of the second piglet (Table 37). Enterotoxin could be demonstrated in the epithelial cells of the intestinal sections and when desquamated epithelial cells were present in the lumen of terminal ileum, caecum and colon, fluorescence was demonstrated on them (Fig. 58).

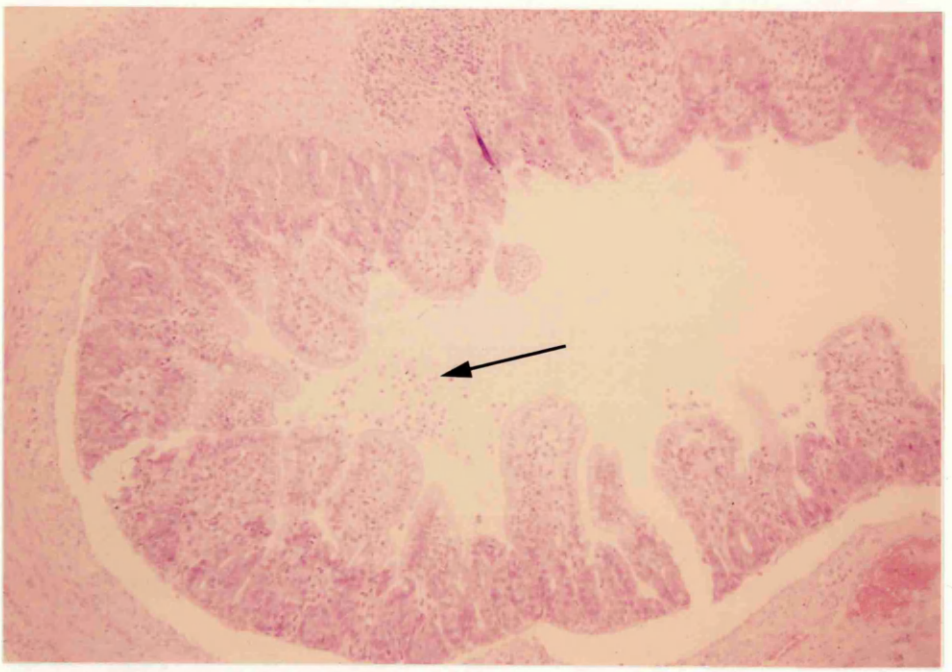

FIG. 56: Histological section of the ileal mucosa of hysterectomy-derived, colostrum-deprived piglet 1 intraluminally inoculated with purified enterotoxin.
Note the shortening of the villi and the cell debris in the lumen (arrow).

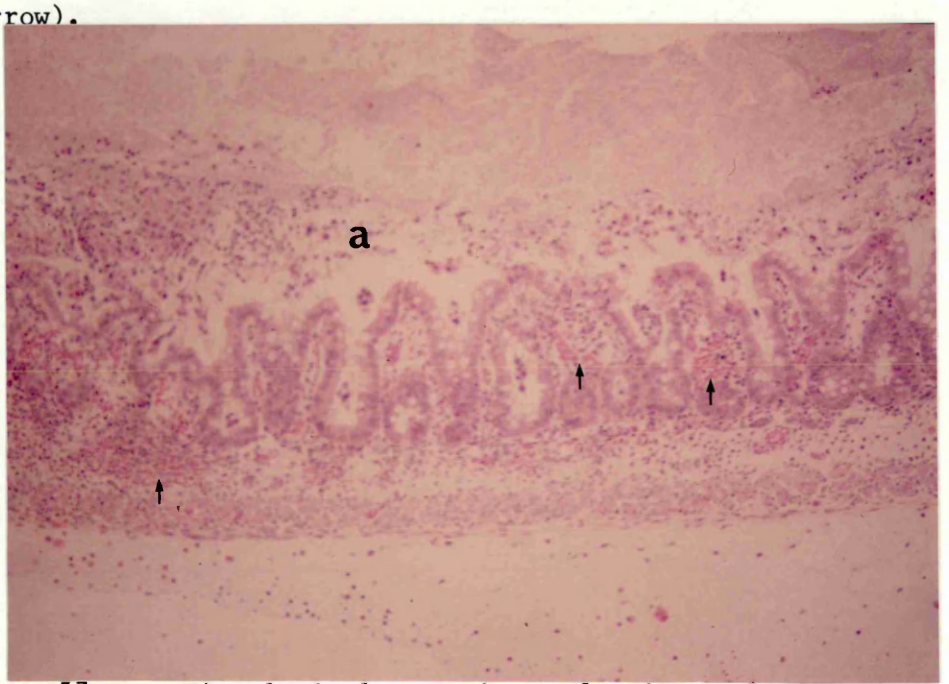

FIG. 57: Histological section of the colonic mucosa of hysterectomy-derived, colostrum-deprived piglet 1 intraluminally inoculated with purified enterotoxin.
Note the cell debris in the lumen (a) and the haemorrhages at lamina propria level (arrow).

(HE x 120)

TABLE 37

Demonstration of enterotoxin antigen by indirect immunofluorescence on intestinal sections of piglets injected intraluminally with purified enterotoxin

	Intestinal section							
	Upper duodenum	Lower duodenum	Mid-jejunum	Terminal jejunum	Mid-ileum	Terminal ileum	Caecum	Colon
Piglet 1	-	-	-	+	++	+++	++	++
Piglet 2	-	-	-	-	+	+++	++	++

Results graded + to +++ on amount of fluorescence observed

- = negative

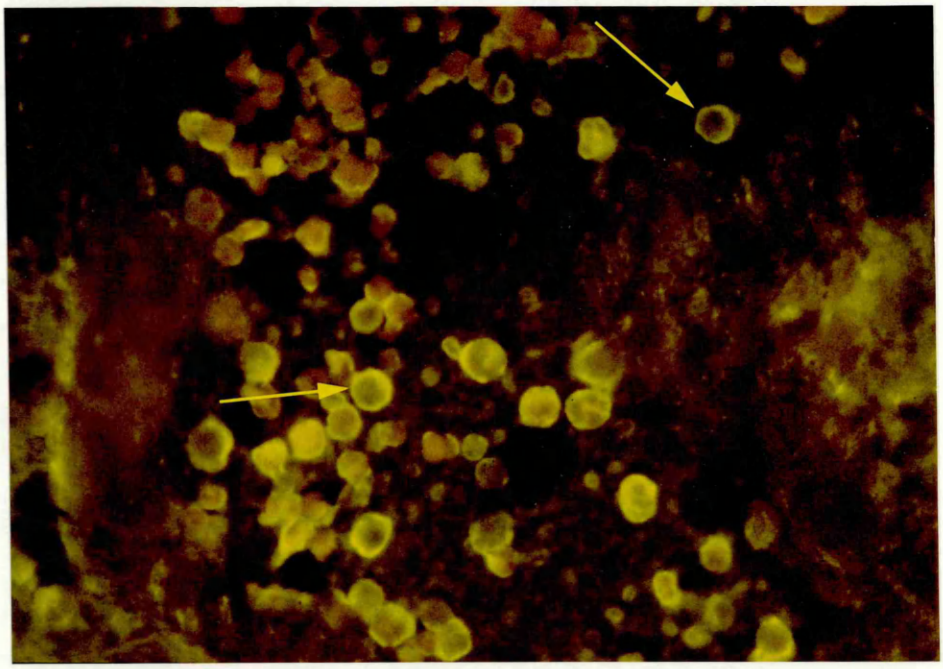

FIG. 58: Demonstration of enterotoxin by indirect immuno-fluorescence on desquamated epithelial cells in the ileal lumen of hysterectomy-derived, colostrum-deprived piglet 1 intraluminally inoculated with purified enterotoxin.
Note the bright fluorescence of individual epithelial cells (arrow).

(x 400)

8. EXPERIMENTAL INFECTION OF PIGLETS WITH AN ENTEROTOXIN PRODUCING ISOLATE OF C.PERFRINGENS TYPE A AND PROTECTION AGAINST IT (EXPERIMENT 3)

(a) Materials and Methods

(i) Animals

Ten hysterectomy-derived, colostrum-deprived piglets were produced by the methods described in Chapter 2, and housed in the accomodation used in the earlier experiments. Two of the piglets were found to have atresia ani after allocation to groups and inoculation had been carried out.

(ii) Procedures

The animals were divided into pairs forming 5 groups (Table 38). The first group was formed of non-infected, non-immunised controls. The second group comprised non-immunised animals infected with sporulated cells of C.perfringens type A. The third group consisted of passively immunised animals infected with sporulated cells. A fourth group was formed of non-immunised piglets infected with vegetative cells of C.perfringens type A. The fifth group consisted of passively immunised animals infected with vegetative cells.

Anti-enterotoxin serum from pig 1 described in Part 3 above containing 128 neutralising TU/ml was absorbed with washed C.perfringens type A cells (isolate 29) and filtered through a membrane filter of APD 0.22µm. Passive immunisation was carried out by giving 5ml orally and 5ml intraperitoneally of this anti-enterotoxin serum to each of the piglets described above one hour after delivery.

Two hours after delivery the piglets were challenged with C.perfringens type A (Isolate 29, Chapter 3, and part 2 above) which was an enterotoxin producer but which did not produce alpha toxin.

5ml of inoculum containing approximately $3x10^8$ C.perfringens type A sporulated cells/ml were given to four piglets, whereas other four animals received 5ml of inoculum containing approximately $4x10^7$ C.perfringens type A vegetative cells/ml (Table 38).

All piglets were examined daily and their appearance, appetite and the consistency of their faeces were recorded. Rectal swabs were taken daily from all animals and examined for the presence of C.perfringens type A and other bacteria by the methods described in Chapter 2.

Viable counts of C.perfringens type A vegetative cells and spores were determined on tryptone sulphite agar medium as previously described. The viable counts were determined in faecal samples taken 36 hours after infection and in intestinal fluids collected from the piglets at post-mortem. The presence of enterotoxin in these samples was detected using Vero cell assay and confirmed using anti-enterotoxin serum by the methods described in Chapter 2.

The piglets were sacrificed in the sequence shown in Table 37 and post-mortem examination was carried out as previously described in Chapter 3.

Mucosal samples from jejunum, ileum and colon of each piglet were examined for the presence of C.perfringens type A and other bacteria by the methods previously described. The percentages of spores seen in intestinal smears were recorded. Histological, immunofluorescence and bacteriological examinations were all carried out on sections of intestine. Blood samples were taken from all piglets at slaughter and the sera collected and stored at -20°C. These sera were examined for the presence of antibodies to enterotoxin by counterimmunoelectrophoresis.

(b) Results

(i) Clinical findings

All piglets were clinically normal and healthy during the period prior to infection with the exception of piglets 1 and 5 which had atresia ani, this condition was noticed after the groups had been formed.

The clinical effects of infection are shown in Table 38. 24 hours after infection piglets 3,4,7 and 9 presented a profuse milky diarrhoea. No clinical signs were noted in the rest of the animals at that time.

At 36 hours post infection piglets 6,8 and 10 developed the same kind of diarrhoea, thus, all infected piglets presented diarrhoea at that time. Piglet control 2 was healthy, passing normal faeces.

48 hours after infection diarrhoea stopped in all infected piglets except in piglet 7 which presented a mild creamy diarrhoea. 6 hours later diarrhoea stopped in this piglet. At that time piglets 8 and 10 presented soft faeces. The rest of the animals were clinically normal.

At 60 hours post-infection all piglets were clinically normal and passing normal faeces.

96 hours post-infection piglet 3 developed a mild creamy diarrhoea which lasted until slaughter on the fifth day of observation.

All the rest of the piglets remained healthy and had normal faeces. The changes in the faecal consistency of the piglets are summarised in Table 38.

(ii) Cultural findings from faecal swabs

Pre-infection faecal swabs were all negative for the presence of C.perfringens type A in all infected piglets. The organism was isolated in pure culture from the rectal swabs of all infected

TABLE 38

Changes in faecal consistency in HDCD piglets following infection with pure cultures of C.perfringens type A enterotoxin-producer strain and isolation of the organism from faecal swabs

Piglet No.	Passively immunised	Infection	Day of Experiment 0	1	2	3	4	5	6
1	-	-	*	*	*				
					K				
2	-	-	N	N	N	N	N	N	NK
			-	-	-	-	$+^2$	$+^2$	$+^2$
3	-	A	N	D	N	N	D	DK	
			-	$+^1$	$+^1$	$+^{1,2}$	$+^2$	$+^2$	
4	-	A	N	D	N	N	N	NK	
			-	$+^1$	$+^1$	$+^{1,2}$	$+^2$	$+^2$	
5	+	A	*	*	*				
					K				
6	+	A	N	D	N	N	N	NK	
			-	$+^1$	$+^1$	$+^{1,2}$	$+^2$	$+^2$	
7	-	B	N	D	D	N	N	N	NK
			-	$+^1$	$+^1$	$+^1$	$+^{1,2}$	$+^2$	$+^2$
8	-	B	N	D	S	N	NK		
			-	$+^1$	$+^1$	$+^1$	$+^{1,2}$		
9	+	B	N	D	N	N	N	N	NK
			-	$+^1$	$+^1$	$+^1$	$+^2$	$+^2$	$+^2$
10	+	B	N	D	S	N	NK		
			-	$+^1$	$+^1$	$+^1$	$+^2$		

A = 5ml inoculum containing $3x10^8$/ml C.perfringens sporulated cells

B = 5ml inoculum containing $4x10^7$/ml C.perfringens vegetative cells

* Piglets with atresia ani

N = normal faeces D = diarrhoea

S = soft faeces K = killed

+ = 1 - C.perfringens non-haemolytic isolated from faecal swabs

2 - C.perfringens haemolytic isolated from faecal swabs

- = C.perfringens not isolated from faecal swabs

animals on the first day post-inoculation (Table 38). The isolated C.perfringens type A corresponded in all cases to the non-haemolytic strain used for the infection. Control piglet 2 did not show presence of any bacteria at this time.

On the second day of experiment profuse cultures of the same non-haemolytic C.perfringens type A were obtained from faecal swabs of all infected piglets, although, only in piglets 9 and 10 did it appear as a pure culture. Staphylococcus spp. was isolated from faecal swabs of the rest of the animals including control 2 which remained negative for C.perfringens type A. On the third day post-inoculation faecal swabs from piglets 3,4 and 6 contained mixed colonies of haemolytic and non-haemolytic C.perfringens type A. Plates from faecal swabs of piglets 7,8,9 and 10 showed only the presence of non-haemolytic colonies of the organism. Control piglet 2 remained negative to the presence of the organism.

On the fourth day of the experiment, faecal swabs from piglets 3,4,6,9 and 10 revealed only the presence of haemolytic C.perfringens type A, whereas infected animals 7 and 8 still had mixed colonies of haemolytic and non haemolytic C.perfringens type A. Control piglet 2 had C.perfringens type A haemolytic colonies only. Other bacteria identified in the faecal swabs of all animals on this day were non-haemolytic E.coli, Streptococcus spp. and Staphylococcus spp.

On the fifth day post-infection, only haemolytic C.perfringens type A colonies were detected in faecal samples of piglets 2,3,4,6,7 and 9.

On the sixth day of the experiment the remaining animals 7,9 and control 2 presented colonies of C.perfringens type A haemolytic apart from colonies of other bacteria which included non-haemolytic E.coli, Clostridium spp. Staphylococcus spp. and Streptococcus spp.

In all cases, when haemolytic C. perfringens type A was isolated, was not present in profuse culture, and, generally few colonies of the organism were detected in each case.

(iii) Viable counts of C.perfringens type A cells and enterotoxin detection

The results of the viable counts carried out on faeces are shown in Table 39. At 36 hours post-infection sporulating cells were present at levels of up to 4.5×10^6 per gram. None were found in the faeces of the uninoculated controls. Higher levels of sporulating cells were identified than of vegetative cells.

Enterotoxin was present at levels of 1:4 to 1:32 in the faeces of all inoculated piglets (Table 39). None was detected in the faeces of control piglet 2.

(iv) Post-mortem findings

The results of the post-mortem examinations are described below:

Piglets 1 and 2, uninfected controls killed on days 2 and 6

Piglet 1 had atresia ani but the findings in both piglets were essentially similar. Both piglets had grossly normal gastrointestinal tracts with normal contents which in the case of piglet 1 consisted of meconium in the colon. No histological abnormalities were detected and no enterotoxin was found (Table 40) in immunofluorescence studies.

C.perfringens cells were seen in mucosal smears from both piglets (Table 41) but no spores were seen. C.perfringens type A of the haemolytic colony type was isolated in small numbers from both piglets from the sites shown in Table 42. The small numbers seen were confirmed by the counts of vegetative cells and spores shown in Table 43. No spores were detected and low levels of vegetative cells (10^2-10^3/gram) were present. No enterotoxin was identified and no antibody to enterotoxin was found in their sera (Table 43).

Piglets 3 and 4, infected with sporulated cells but non-immunised and killed on day 5. Piglet 3 was found to have congestion of the jejunum and ileum with oedema of the large

TABLE 39

Viable counts of C.perfringens type A before and after heat-shocking at 80°C for 10 minutes and enterotoxin titres in faecal samples from HDCD piglets 36 hours post-infection with C.perfringens type A enterotoxin-producer strain

Piglet No.	Passively immunised	Infection	Viable Count C.perfringens/g)		Enterotoxin (a) (level (TU/ml)
			Before heating	After heating	
1	-	-	NT	NT	NT
2	-	-	-	-	-
3	-	A	$3x10^3$	$7x10^5$	8
4	-	A	$1.5x10^3$	$5.5x10^5$	8
5	+	A	NT	NT	NT
6	+	A	$4x10^2$	$6x10^4$	8
7	-	B	$6x10^4$	$3.5x10^6$	32
8	-	B	$7x10^3$	$4.5x10^6$	16
9	+	B	$3x10^3$	$4x10^3$	16
10	+	B	$7.5x10^3$	$5x10^3$	4

- = absent

NT = not tested

(a) Reciprocal of highest dilution producing a morphological effect on Vero cells

intestinal wall and pinpoint haemorrhages of the mucosa. The contents were fluid but no mucus, blood or necrotic material was seen in them. Piglet 4 was grossly normal. The major feature of the histological findings were congestion of the lamina propria with no apparent abnormalities of the villous structure or epithelium. Cellular accumulations consisting largely of lymphocytes and neutrophils were seen in the lamina propria of the ileum. Haemorrhages, inflammatory cell infiltrates and oedema were present in the lamina propria of the colon (Fig. 59). Mild congestion of the jejunal mucosa and inflammatory cell infiltration of the lamina propria were the only changes seen in the gastro-intestinal tract of piglet 4.

Enterotoxin was only demonstrated in sections of the colon of piglet 3 (Table 40) and was located in the epithelium. Both vegetative and sporulating cells of C.perfringens were seen in direct smears of the mucosa of both pigs (Table 41). Appreciable numbers of sporulating cells (5 to 10 per cent) were seen only in the colon. C.perfringens type A was isolated from both piglets and from the sites shown in Table 42. Non-haemolytic C.perfringens type A was only detected in the colonic contents of piglet 3. Sporulating cells were, however, present in the intestinal contents of both piglets at levels of 10^3 organisms per ml (Table 43) but no enterotoxin was demonstrated in these contents. No antibody to enterotoxin was demonstrated in the sera of either piglet (Table 44).

Piglets 5 and 6, infected with sporulated cells, passively immunised and killed on days 2 and 5. Piglet 5 had atresia ani, the intestines were dilated with fluid contents and the walls of the intestine were thin. The gastrointestinal tract of piglet 6 was normal. The only histological abnormality seen in piglet 5 was accumulation of lymphocytes and neutrophils in the lamina propria of the jejunum. In piglet 6, however, shortening of the ileal villi and accumulations of inflammatory cells in the lamina prorpia were seen but no other microscopic changes were detected. Enterotoxin was detected in the epithelium of the jejunum, ileum and colon (Table 40) but none was seen in the gut of piglet 6.

C.perfringens was seen in direct smears of the mucosa of both piglets (Table 41) but was present in greatest numbers and with 30 per cent of spores in piglet 5 (Fig. 60). These findings were confirmed by culture (Table 42) and all of the C.perfringens type A isolated from piglet 5 were found to be non-haemolytic. In contrast those from piglet 6 were all haemolytic. The viable counts (Table 43) also confirmed the results of the smears and showed that high levels of both vegetative cells ($4x10^8$ per gram) and spores ($6.5x10^4$ per gram) were present in the intestinal contents of piglet 5. Enterotoxin was also present in these contents. Antibody to enterotoxin was present in the sera of both piglets (Table 44).

Piglets 7 and 8, infected with vegetative cells but not passively immunised and killed on days 6 and 4. There was mild congestion of the jejunal and ileal serosa and mucosa of both piglets and patchy congestion of the colonic mucosa of piglet 8. The contents were normal in both piglets. Histological changes included oedema and congestion of the lamina propria of the jejunum and ileum of piglet 8. The apparent congestion seen in the intestines of piglet 7 could not be identified in histological sections. Enterotoxin was detected by immunofluorescence in the colons of both animals and in the ileum of piglet 7 (Table 40).

Vegetative C.perfringens cells were seen in all regions of the gut in gram stained smears (Table 41) and large percentages of spores (10 per cent-piglet 7; 25 per cent-piglet 8) were present in the colonic mucosa of both piglets. The presence of C.perfringens type A was confirmed by culture (Table 42) when a mixture of both haemolytic and non-haemolytic C.perfringens type A was isolated from the colons of both piglets and from the jejunum and ileum of piglet 7. Few non-haemolytic colonies were isolated from these sites. The colony counts confirmed that both sporulating and vegetative cells were present (Table 43) but no enterotoxin was detected in the gut contents of either piglet. No antibody to enterotoxin was present in their sera (Table 44).

TABLE 40

Demonstration of enterotoxin antigen by indirect immunofluorescence on intestinal sections of HDCD piglets following infection with pure cultures of C.perfringens type A enterotoxin-producer strain

Piglet No.	Passively immunised	Infection	Intestinal section		
			Jejunum	Ileum	Colon
1	-	-	-	-	-
2	-	-	-	-	-
3	-	A	-	-	++
4	-	A	-	-	-
5	+	A	++	+++	++
6	+	A	-	-	-
7	-	B	-	+	+
8	-	B	-	-	+
9	+	B	-	-	-
10	+	B	-	-	-

Results graded + to +++ on amount of fluorescence observed

- = negative

TABLE 41

Detection of vegetative cells and spores of C.perfringens in direct mucosal smears from HDCD piglets following infection with pure cultures of C.perfringens type A enterotoxin-producer strain

Piglet No.	Passively immunised	Infection	Mucosal smear		
			Jejunum	Ileum	Colon
1	-	-	-	-	+
2	-	-	+(0)	+(0)	+(0)
3	-	A	++(◁1)	++(◁1)	++(5)
4	-	A	++(0)	++(◁1)	++(10)
5	+	A	+++(30)	+++(30)	+++(30)
6	+	A	+(0)	++(◁1)	++(◁1)
7	-	B	++(◁1)	++(◁1)	++(10)
8	-	B	++(◁1)	++(◁1)	++(25)
9	+	B	++(◁1)	++(0)	++(◁1)
10	+	B	++(0)	++(◁1)	++(◁1)

\- = no C.perfringens detected in smear

\+ = few of the bacteria observed corresponding to C.perfringens

++ = most of the bacteria observed corresponding to C.perfringens

+++ = only C.perfringens observed in smear

(◁) = percentage of spores from total number of C.perfringens cells

◁ = less than

TABLE 42

Sites from which C.perfringens type A was isolated from HDCD piglets following infection with pure cultures of C.perfringens type A enterotoxin-producer strain

Piglet No.	Passively immunised	Infection	Jejunum	Ileum	Colon
1	-	-	-	-	-
2	-	-	+	+	-
3	-	A	++	+	+*
4	-	A	++	+	+
5	+	A	++*	++*	++*
6	+	A	+	+	-
7	-	B	++*	++*	++*
8	-	B	++	++	++*
9	+	B	++	++	+
10	+	B	++	++	+*

- = no colonies of C.perfringens isolated

+ = between 1 and 8 colonies of C.perfringens isolated

++ = more than 8 colonies of C.perfringens isolated

* = Non-haemolytic colonies of C.perfringens detected

TABLE 43

Viable counts of C.perfringens type A before and after heat-shocking at 80°C for 10 minutes and enterotoxin titres in intestinal fluids from HDCD piglets at post-mortem, following infection with pure cultures of C.perfringens type A enterotoxin-producer strains

Piglet No.	Infection	Collection of samples (days after infection)	Viable Count (C.perfringens/g) Before heating	After heating	Enterotoxin (a) (TU/ml)
1	-	2	2.5×10^2	-	-
2	-	6	7.5×10^3	-	-
3	A	5	5.5×10^4	7×10^3	-
4	A	5	4×10^4	3.5×10^3	-
5	A	2	4×10^8	6.5×10^4	4
6	A	5	8×10^3	6.5×10^2	-
7	B	6	4×10^4	3×10^3	-
8	B	4	3.5×10^5	4×10^3	-
9	B	6	3.5×10^4	5.5×10^2	-
10	B	4	6×10^4	2×10^2	-

- = absent

(a) Reciprocal of highest dilution producing a morphological effect on Vero cells

TABLE 44

Antibody titres to enterotoxin in sera of HDCD piglets passively immunised, following infection with pure cultures of C.perfringens type A enterotoxin-producer strain

Piglet No.	Passively immunised	Infection	Collection of samples (days after immunisation)	CIE titres to enterotoxin
1	-	-	2	-
2	-	-	6	-
3	-	A	5	-
4	-	A	5	-
5	+	A	2	1:32
6	+	A	5	1:16
7	-	B	6	-
8	-	B	4	-
9	+	B	6	1:16
10	+	B	4	1:32

- = absent

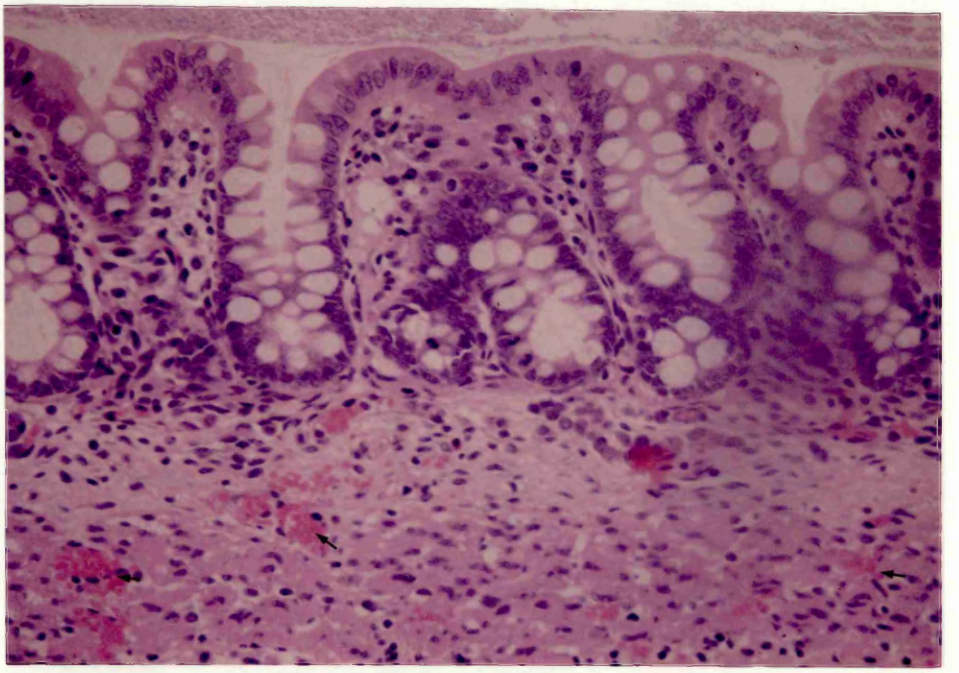

FIG. 59: Histological section of the colonic mucosa of hysterectomy-derived, colostrum-deprived piglet 3, 5 days post-inoculation with C.perfringens type A enterotoxin-producer isolate 29.

Note the haemorrhages at the lamina propria level (arrow).

(HE x 400)

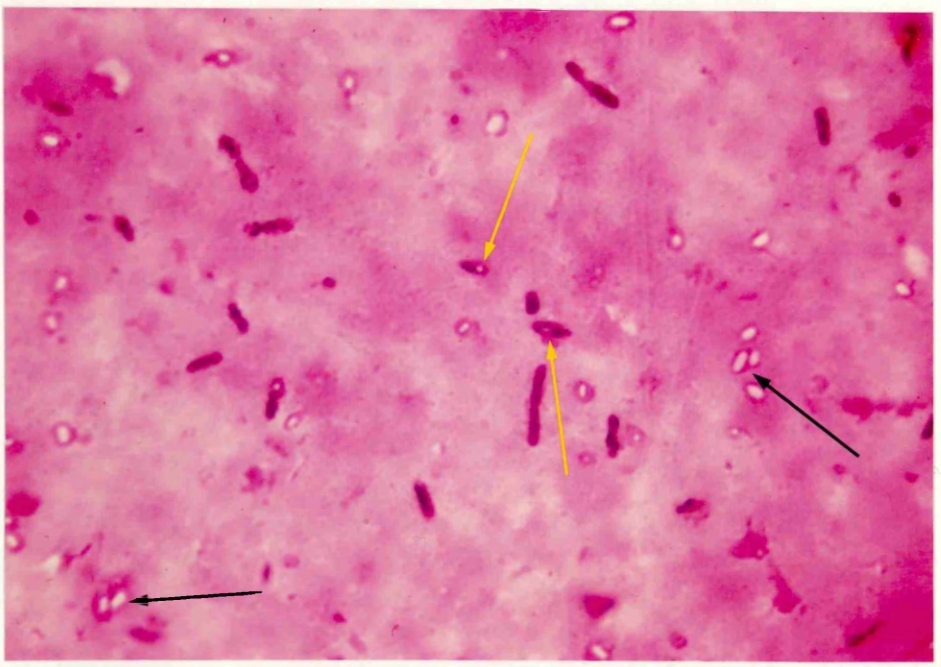

FIG. 60: Smear of the colonic mucosa of hysterectomy-derived, colostrum-deprived piglet 5, 3 days post-inoculation with C.perfringens type A enterotoxin-producer isolate 29.
Note the sporulated C.perfringens type A organisms (yellow arrow) and the free spores (black arrow).

(Gram x 1200)

Piglets 9 and 10, infected with vegetative cells, passively immunised and killed on days 6 and 4. Piglet 4 was normal apart from mild congestion of the jejunum, ileum and colon. In contrast, piglet 10 was moribund when killed and had marked distension of the small intestine with gas, mild congestion of the jejunum and ileum and marked oedema of the meso-colon. The contents of the large intestine were normal. In histological sections of piglet 9 there was congestion of the jejunal lamina propria, congestion and inflammatory cell infiltrates were present in the wall of the ileum and epithelial cell shedding but no villous atrophy was seen. The colonic mucosa appeared to be normal. Piglet 10 appeared to be histologically normal apart from the presence of colonic oedema. No enterotoxin was detected by immunofluorescence in either piglet (Table 40).

Vegetative cells of C.perfringens with few spores were seen in direct smears from the mucosa of all levels of the intestine (Table 41) and this was confirmed by isolation (Table 42). A few non-haemolytic C.perfringens cells were isolated from the colon of piglet 10. Spores were only present in low levels (10^2 per gram; Table 43) and vegetative cells were present in greater numbers (10^4 per gram). No enterotoxin was detected in the contents but serum antibody to it was found in both piglets in sera taken at slaughter (Table 44).

9. DISCUSSION

(a) Sporulation and enterotoxin production by porcine isolates of C.perfringens

The studies on the ability to sporulate and produce enterotoxin by 42 C.perfringens type A isolates revealed that 24 were able to sporulate to a lesser or greater degree as shown in table 29. In general, all isolates sporulated better in Duncan and Strong medium than in Ellner or Tsai media, this result differs slightly from Tsai et al. (1974) who reported that from a total of 65 strains, a better sporulation was produced in the medium developed by them than in Duncan and Strong and Ellner media. However, two isolates from the same submission (64 and 65) produced

a larger number of spores in Tsai medium than in the others.

The difficulty of getting C.perfringens to sporulate in most laboratory media is widely reported (Sacks and Thompson, 1978; Labbe, 1981). A number of sporulation media and techniques have been developed to sporulate C.perfringens but production of a significant number of spores has been a continuing problem in many strains.

High levels of spores were produced by 3 isolates in Duncan and Strong medium. The best was isolate 29 (95 per cent sporulation) while isolate 59 produced 50 per cent of spores and isolate 82, 85 per cent of spores. Isolates such as 35, 36, 37, 53, 54 and 55 produced no spores and it is of interest that these were all from individual submissions. In other submissions such as submission 12 both sporulating and non-sporulating isolates were found. The significance of this finding is not clear but pigs from a common background are likely to be infected with similar organisms. This difference of sporulation percentage is not associated with selection for spores at isolation as all these isolates were taken from horse blood agar plates. The occurrence of sporulation in strains selected from spores might be much higher.

The ability of C.perfringens type A to produce enterotoxin has been shown to be directly related to the ability of the organism to sporulate, thus, non-sporulating strains are non-enterotoxin producers. The results shown in table 29 are in agreement with this statement, thus, all enterotoxin producing isolates were found to produce spores and in all cases the percentages of spores obtained were 10 per cent or higher. However, 2 isolates (56 and 62) formed a relatively high percentage of spores but were found to be non-enterotoxin producers. Sporulating strains of C.perfringens type A have been found to be non-enterotoxin producers possibly because of a mutation of the gene that codifies enterotoxin production (Ando et al, 1985).

A total of 11 of the 42 C.perfringens type A isolates tested (26 per cent) was capable of producing enterotoxin under the

conditions used in this study. This result suggests that enterotoxin-producing isolates are relatively common in enteric disease in pigs. The selection of isolates from heat-treated cultures (Table 33) would probably reveal an even higher percentage of enterotoxin producers. Three of the eleven enterotoxin producer-isolates (29,88,89) were found to be non-alpha toxin producers. Tsai et al (1974) reported that a higher proportion of enterotoxigenic strains were non-alpha toxin producers; however, as 8 of the enterotoxin-producer isolates produced alpha toxin in different amounts, it is not possible to relate enterotoxin production to alpha toxin production.

(b) The purification of enterotoxin

On the purification of enterotoxin, a 3-step purification procedure gave a homogeneous protein with a recovery of 25 per cent of the initial activity (Table 30). The 0-15 per cent ammonium sulphate precipitate obtained appeared nearly homogeneous on polyacrylamide gel electrophoresis (Fig. 37). This result agrees with that of Bartholomew and Stringer (1983) who described the purification of enterotoxin by the same method. The chromatography on Sephadex G-200 did not significantly increase the yield based on protein content. The enterotoxin produced appeared to have all the properties of enterotoxin produced from reference strains of C.perfringens type A and this was confirmed by immunodiffusion studies using the antiserum and enterotoxin from the reference strain.

(c) Production of antibody to enterotoxin

Preimmunisation samples of pig serum (Table 31) contained antibody to enterotoxin but none was found in the rabbit sera. The greater increase in antibody levels in the pig sera when compared with those from the rabbits was probably due to the previous exposure of the pigs. The initial levels present in the pig sera suggested that the animals used had been previously exposed to enterotoxin, possibly from an enteric infection. The absence of such antibody from the rabbits used may reflect a species difference or a difference in the quality of housing.

The low levels of antibody obtained (maximum 1:32 in pigs and 1:16 in rabbits) agree with those produced in rabbits immunised with purified enterotoxin by Bartholomew and Stringer (1983) who pointed out some of the difficulties for the obtainment of high titres of antibodies to purified enterotoxin.

The specificity of the antiserum produced was confirmed by the results of the double immunodiffusion tests (Fig. 39) and the neutralisation tests. The use of a reference strain confirmed its relationship to enterotoxin produced by human food poisoning strains.

(d) Antibody to enterotoxin in adult pig sera

98 of the 106 adult pig sera examined were found to possess antibody to enterotoxin with titres ranging from 1:2 to 1:64 (Table 32). This finding suggests that antibody to enterotoxin is common in adult pig sera in Scotland and therefore its presence in the sera of the two pigs hyperimmunised for antibody production was not unusual.

The high prevalence of antibodies to enterotoxin in these pig sera may suggest that the exposure to C.perfringens enterotoxin-producer strains is common and that this organism sporulates and produces enterotoxin in the intestinal tract of pigs. The antibody production might be induced during an enterotoxaemia of C.perfringens type A enterotoxin-producer, but could possibly also be due to a prolonged absorption of enterotoxin in symptomless carriers who harbor C.perfringens enterotoxin-producing strains. The occurrence of disease in these sows was not known and their previous exposure to enterotoxin was therefore unknown. It is of interest to note that only 45 of the same sera were positive for antibody to alpha toxin (Table 27, Chapter 5) suggesting that enterotoxin exposure may be more common or that enterotoxin binds more closely to the gut or is absorbed more readily than alpha toxin. The ready inactivation of alpha toxin by gut contents may also contribute to this difference in exposure.

(e) The survey of pig faeces for vegetative and sporulating C.perfringens type A and enterotoxin

C.perfringens (Table 33) type A vegetative cells were detected in all 33 samples when inoculated onto tryptone sulphite agar containing D-Cycloserine. The viable counts ranged from $2.5x10^2$ cells per gram of intestinal material to $3x10^7$ cells per gram of faeces. C.perfringens type A colonies in horse blood agar plates were detected in only 15 of the 33 samples indicating the effect discussed in Chapter 3, in which the isolation of C.perfringens may be masked by the presence of other organisms when non-selective media such as horse blood agar are used. The presence of C.perfringens type A vegetative cells in all the samples counted confirms the statement that C.perfringens type A is always present in the intestine of all animals as part of the normal intestinal microflora (McDonel 1980). The reasons for the failure to isolate C.perfringens type A on horse blood agar when they are clearly present in large numbers in the sample is not obvious. Sample 93 contained $5x10^6$ cells of C.perfringens type A in total and $5x10^2$ spores yet the organism was not detected on horse blood agar. It may be that inhibitory substances such as antibody or antibiotic are not sufficiently or uniformly diluted out when streaked on plates. In the Miles and Misra technique this would always occur. The lowest level of detection of vegetative cells by streaking on horse blood agar was in sample 74 which contained only $2x10^3$ vegetative cells per gram.

Vegetative cell levels could not be related to diarrhoea in this survey in contrast to the situation found in Chapter 3 when much of the material was from neonatal pigs.

C.perfringens spores were detected in 25 out of the 33 samples (Table 33) with ranges from $2x10^2$ cells per gram of intestinal material to $5x10^6$ cells per gram of faeces.

Higher viable counts of spores than vegetative cells were present in samples 64,65,82,86 and 87; this effect has been reported (Sutton et al., 1971) in cases of human food poisoning in which higher counts of spores than vegetative cells are observed

when heat-shock is carried out. Samples from cases other than C.perfringens type A food poisoning do not show the activation of spores and vegetative cell counts are always higher than spore cell counts. The ability of C.perfringens to sporulate in faecal samples is not known but it is not considered likely.

In the study spores were present in only 5 of the 10 normal faeces samples at levels varying from $3x10^2$ to $2x10^4$ (mean count $4.8x10^3$ spores per gram). In contrast spores were found in 20 of the 23 diarrhoeic samples at levels varying between $2x10^2$ to $5x10^6$ spores per gram (mean count $6.5x10^5$ per gram). This association of spores with diarrhoea took no account of the presence of other infectious agents. In three of the animals with diarrhoea and no faecal spores other agents were present (Epidemic diarrhoea, 67 and 68; Campylobacter, 92).

Spores were present in the faeces of pigs of all ages examined (2 days - 16 weeks).

Enterotoxin was identified in 5 samples of pig faeces. All were from diarrhoeic pigs and in all samples more spores were counted than vegetative cells. The levels of enterotoxin present were not determined in this study. Samples in which enterotoxin was demonstrated were related to the history and to the other bacteria present (Table 33). Enterotoxin was present in piglets of seven days of age, 3 weeks of age (with enteropathogenic E.coli) and in mucoid diarrhoea from 12 week old pigs. There was no common feature of the submissions other than the association with C.perfringens spores. It is possible that C.perfringens sporulating strains and enterotoxin could be associated with the three distinct syndromes seen here: creamy diarrhoea at 7 days of age, post-weaning diarrhoea at weaning and mucoid diarrhoea in slightly older pigs.

It was clear from the studies discussed above that C.perfringens type A strains in pigs could sporulate and produce enterotoxin in vitro and in vivo and that enteritis might be associated with sporulating organisms in vivo. The actual relationship between the clinical and pathological signs seen in

the few field cases were studied in the experiments described in sections 6,7 and 8.

(f) Enterotoxin from C.perfringens type A in porcine intestinal loops

This study demonstrated that C.perfringens type A enterotoxin of porcine origin (from isolate 29) could cause intestinal lesions in gut loops in contrast to the absence of evidence found for alpha toxin and discussed in Chapter 5.

Doses of purified enterotoxin of 32 to 512 TU/ml (Table 34 and 35) produced accumulation of fluid in the inoculated loops. Lower doses of purified enterotoxin (4 to 16 TU/ml) failed to produce a detectable response in the hysterectomy-derived, colostrum-deprived piglet 1 (Table 35). Culture supernatants 1 and 2 of unknown toxic activity failed to produce fluid accumulation in the conventionally born, colostrum-deprived piglet (Table 34) but, congestion of the mucosa was observed in the loops inoculated with these preparations, possibly indicating a mild enterotoxic activity.

In loops which contained fluid, the mucosa was disrupted and the villous architecture destroyed (Fig. 47). When immunofluorescence was carried out (hysterectomy-derived piglet 1) enterotoxin was present in large amounts on the epithelium (Fig. 51). The changes found resembled those seen grossly in piglets which had died from natural C.perfringens type A associated diarrhoea (Survey 1, Chapter 3) or experimental infections (Chapter 4) but were more severe than those seen microscopically in moribund animals, the only ones to be examined histologically in these chapters.

The lesions appeared to be dose dependent, as described above but enterotoxin was detectable in the epithelium of loops inoculated with all levels of enterotoxin tested. It was also present to some extent in loop 3 piglet 1 in which enterotoxin had been neutralised by antiserum and in loop 6 inoculated with sporulated cells. The congestion of the mucosa seen in these loops (Table 35) may, therefore have been caused by enterotoxin but is

unlikely to have been due to levels of enterotoxin similar to those seen in loops 7 and 9 in which weak fluorescence but no congestion were seen. The changes may therefore have been due to incomplete neutralisation of the enterotoxin or to dissociation of the toxin-antibody complexes in the gut. A similar quantity of antiserum had been found to protect Vero cell against damage (Section 2b above). Similar changes were seen and enterotoxin was demonstrable when sporulated cells were inoculated into loop 6. It is possible that more than 8 toxic units but less than 32 toxic units of enterotoxin was produced by this culture or was present in it at inoculation. The loss of piglet 2, intended to provide a full control for this point is to be regretted. Duncan and Strong medium alone caused no such lesions (loop 5). No counts, cultural studies or enterotoxin determinations were carried out on the contents of this loop. Had they been done the possibility of multiplication of C.perfringens, sporulation and enterotoxin production in vivo in the loop might have been determined. Duncan et al.(1968) considered that enterotoxin was not present in washed cells but could be produced when these were suspended in a milk substrate not used here.

The loss of piglet 2 meant the loss not only of the 16 toxic unit enterotoxin loop but also the vegetative cell control intended to resolve this point.

The only information to be gained from piglet 2 was that fluid accumulation took more than 2 hours to become apparent in loops.

(g) The intraluminal inoculation of piglet intestine

This study was largely successful. It was clear that intestinal marking with dye was unsuccessful but the point of inoculation could be determined in both cases, by immuno-fluorescence in piglet 2 (Table 37) and, by inference the injection site was the terminal jejunum. Enterotoxin was only found attached to the epithelium distal to the point of injection.

The effects of the enterotoxin were both clinical and histopathological. Clinical signs consisting of a transient watery mucoid diarrhoea lasting 6-8 hours resulted from the inoculation of

enterotoxin. Lesions consisting of villous shortening, mild congestion and the presence of inflammatory cells were seen at sites where enterotoxin was identified. The absence of a saline inoculated control may reduce the value of these results but the relatively distal portion of the ileum inoculated in piglet 2 provides a histological control for the jejunum and ileum of piglet 1.

It was clear from this study that clinical signs could result from enterotoxin administration to piglets and that histological changes resulted, thus confirming the results of the gut loop studies.

(h) The experimental infection of piglets with sporulating isolate 29 of C.perfringens type A and protection using anti-enterotoxin serum

The results of this study were marred to some extent by the presence of two piglets with atresia ani. Both piglets did, however, provide valuable information. A transient diarrhoea resulted from the inoculation of the two piglets given washed sporulated cells. It was creamy, contained no blood or mucus and did not occur until 36 hours after inoculation. The reason for the later diarrhoea seen in piglet 3 (Table 38) was not clear. No diarrhoea occurred in the controls. A similar but slightly longer lasting diarrhoea occurred in the two piglets inoculated with vegetative cells and it must be assumed that this diarrhoea was associated with the inoculation of C.perfringens type A isolate 29.

Colonies with the characters of this organism were isolated from all infected piglets on days 1-3 but another organism with different haemolysis characters appeared on that day and was present in all piglets by day 4. The changes seen on day 4 and subsequently could have been affected by the organism. In the control animal (piglet 2) neither clinical nor pathological changes were seen and the haemolytic organism remained at low levels in faeces and in the gut. These findings provide additional evidence for the view that the early changes seen were associated with isolate 29.

The studies of the faeces shown in Table 39 confirmed the presence of vegetative cells at $4x10^2$ to $6x10^4$ in the faeces of all inoculated piglets and the presence of larger numbers of spores in most cases. Enterotoxin was present in the faeces at levels of 4TU/ml - 32TU/ml (piglet 7). It must be assumed that both spores and toxin found in the faeces were produced in the intestine as no ready-formed enterotoxin should have been present in either inoculum and no spores were present in inoculum B (vegetative cells).

Passive protection by a combination of oral and parenteral serum containing antibody capable of neutralising 128TU of enterotoxin per ml was unsuccessful. Neither group immunised remained unaffected to challenge and the diarrhoea which resulted was of similar duration and intensity to that in unprotected controls. Serum antibody to enterotoxin was still present at slaughter (Table 44) and little if any of the antibody given could have been lost in spite of exposure to enterotoxin which was present in gut during the disease. It seems from this study that the failure of anti-enterotoxin to neutralise completely the effects of enterotoxin in the gut loop studies also occurred here. The failure was presumably also associated with the type of antibody given - largely IgG and IgM with little or no secretory IgA antibody.

No gross or microscopic lesions were found in either control animal. The lesions found at post-mortem examination in the infected animals may, therefore, have been associated with isolate 29 infection and almost certainly were in the case of piglet 5 killed with atresia ani. The presence of haemolytic C.perfringens type A and the time which elapsed between the end of the diarrhoea and post-mortem examination could have affected the changes seen. In all piglets there was some congestion of the intestinal mucosa and in piglet 6 there was shortening of the villi. Oedema of the mesocolon was seen in piglet 10. A variety of changes including some accumulations of inflammatory cells and congestion of the lamina propria were seen but these could not be associated with passive protection or with inoculation with vegetative or sporulated cells.

More marked changes associated with C.perfringens type A of the inocular type were seen in piglet 5 with atresia ani. Enterotoxin was demonstrated in its jejunum, ileum and colon (Table 40) and 30 per cent of the C.perfringens seen in smears were sporulated (Table 41). This enterotoxin demonstration could be due to the early slaughtering of the piglet and/or by the trapping of the enterotoxin by the atresia ani condition of the animal.

This piglet had dilated intestines with fluid contents and the presence of inflammatory cells. No villous atrophy or cell shedding was seen unlike the situation in gut loop studies where fluid had accumulated. The only other piece of small intestine in which enterotoxin was demonstrated was the ileum of piglet 7 in which there was mild congestion. This region of the gut contained many non-haemolytic C.perfringens type A (Table 42) but few spores (Table 41).

More sporulating organisms, enterotoxin and non-haemolytic organisms were found in the colons (Tables 41,42 and 43) but of the piglets 3,5,7 and 8 in which enterotoxin was demonstrated, the most severe changes were seen in piglets 3 and 5 which had high levels of enterotoxin attached to the wall. Changes were also seen in piglet 8 which had 25 per cent spores and enterotoxin in the colonic mucosa but none were seen in that of piglet 7 with 10 per cent spores and enterotoxin. The absence of enterotoxin from the intestinal contents suggests that any enterotoxin present was fixed and not free and that the absence of enterotoxin in the faeces does not exclude the presence of fixed enterotoxin and enterotoxin-related changes, at least in the large intestine.

(i) Conclusions

The studies described in this chapter and discussed above suggest that sporulating C.perfringens type A occurs in the pig and that enterotoxin can be produced by porcine isolates of the organism both in vivo and in vitro. Both enterotoxin produced from one of these isolates and the organism itself were capable of producing lesions in gut loops and clinical signs in experimental pigs. The significance of these findings is discussed further in relation to the literature in Chapter 7.

CHAPTER 7

GENERAL DISCUSSION

1. INTRODUCTION

In this chapter, the results of Chapters 3, 4, 5 and 6 are discussed in the context of C.perfringens type A in porcine enteric disease and in enteric disease in general. The results of the individual studies and their validity have already been discussed in the appropriate chapters and, as the work falls into 2 main parts, this discussion has been organised in a similar manner.

In the first section (Section 2), the results of the survey and the role of C.perfringens type A in porcine enteric disease in the field are discussed with relation to the literature reviewed in Chapter 1 and material which is considered relevant in view of those findings.

In the second section (Section 3), the experimental studies are discussed under four subheadings. The results obtained from animal experiments are discussed. The roles of alpha toxin and enterotoxin in clostridial enteric disease are examined in terms of the results presented above and with relation to the general literature. Lastly the role of immunity in revealing the pathogenesis of C.perfringens type A infections and in protection from them is discussed.

The whole is put into context by a brief conclusion.

2. C.PERFRINGENS TYPE A IN FIELD CASES OF PORCINE ENTERIC DISEASE

(a) Bacteriological results from survey material

The examination of field cases in the surveys described in

Chapters 3 and 6 showed that a wide variety of bacteria (which included C.perfringens type A) could be isolated from the rectal swabs, faecal material and carcases submitted. Other agents such as T.G.E. and Porcine Epidemic Diarrhoea were demonstrated and agents such as rotavirus, coccidia and cryptosporidia may have been present in some samples but were not recorded owing to the limitations in technique and sample quality discussed in Chapters 3 and 6.

More than one species of bacterium was always found in the samples. In some cases, the bacteria isolated are known to cause enteric lesions in pigs e.g. β-haemolytic E.coli, C.perfringens type C, Salmonella typhimurium and Campylobacter coli, but others have been considered to be normal inhabitants of the alimentary tract e.g. non-haemolytic E.coli, Streptococcus spp., Clostridium spp., Fusobacterium spp., and Bacteroides spp. (Ducluzeau, 1983). In general, bacteria which have been reported to be present in the intestinal tract of the pig as part of the normal intestinal flora were isolated in the surveys. C.perfringens type A has been generally considered to be a normal inhabitant of the gastroenteric tract of the pig (Ducluzeau, 1983) and of other species of animal (Niilo, 1980). The evidence obtained from the surveys suggested that it was involved in enteric disease and this view was supported by a growing body of observations from other workers (Olubunmi, 1982; Nabuurs et al., 1983; Secasiu, 1984 and Jestin et al., 1985).

The evidence that suggests C.perfringens type A was involved in gastrointestinal disease was presented in Tables 7 (Chapter 3) and 33 (Chapter 6). C.perfringens type A was isolated in profuse culture from the jejunum, ileum and caecum of 16 diarrhoeic piglets (Table 7) and from 2 diarrhoeic piglets in the second study (Table 33).

The organism was less commonly isolated from the gastric, duodenal and colonic mucosa. These results differ slightly from the findings reported by Olubunmi (1982) who recovered the organism in profuse cultures from the mucosa of the duodenum, jejunum and ileum and less frequently from the mucosa of the stomach, caecum

and colon. C.perfringens type A was isolated from the stomach of four piglets and from the duodenum of nine animals (Table 8) and from both duodenums in the second study. In humans, except under disease conditions, C.perfringens type A is not found in the stomach and duodenum, while it is commonly isolated from the ileum (Vince et al., 1972). C.perfringens type A is normally absent from the stomach and duodenum of the healthy piglet (Ducluzeau, 1985). Thus, the presence of the organism at these levels in the sampled piglets may indicate the involvement of C.perfringens type A in the diarrhoeic syndrome observed in the animals.

Additional evidence for the involvement of C.perfringens type A in piglet diarrhoea came from its identification from the faeces of 14 animals in study 1 (Table 7, Chapter 3). This result conflicts with the statement that C.perfringens type A is normally present in the intestinal tract of healthy animals (Niilo, 1980). However in this survey, non-selective media were used and this may have led to the masking of C.perfringens type A by other organisms present in larger numbers. When a selective medium, tryptone-sulphite-cycloserine agar, was used in the studies described in Chapter 6, C.perfringens type A was identified in every sample examined thus, an agreement with the above mentioned statement was found. However, as already discussed in Chapters 3 and 6, a relationship may exist between the numbers of C.perfringens type A present in the gastrointestinal tract and the number of colonies found in non-selective media. The results of the second survey described in Chapter 6 and discussed there suggest that, although there is a general relationship between numbers of C.perfringens present and detection on blood agar, this relationship is not absolute. The results in the two surveys tend to confirm those of Secasiu (1984) who associated an increase in the number of C.perfringens type A in the intestines of diseased animals with clostridial disease.

The diarrhoeic animals in both surveys on which post-mortem examination was carried out and from which C.perfringens type A was isolated in the absence of any other known pathogens, showed

pathological lesions similar to those reported by Nabuurs et al. (1983) who described a diarrhoeic syndrome produced experimentally in piglets one to three weeks old with C.perfringens type A. At post-mortem the jejunum and ileum of these animals were distended with abundant gas. Histologically the villi were slightly reduced in length, and infiltration of inflammatory cells was found in the lamina propria. Olubunmi (1982) reported similar lesions in field and experimental cases of neonatal diarrhoea associated with C.perfringens type A, although some of the animals described by this author showed more severe lesions than those found in the surveys in Chapters 3 and 6; large haemorrhages and sloughing of the villi were described.

The results discussed above link findings concerning vegetative cells with diarrhoea and enteritis but there are no accounts of any link between sporulating organisms and diarrhoea in the pig or even the presence of C.perfringens type A spores in pig faeces. The results of the spore counts described in Chapter 6 (Table 33) provide completely new information for the pig. In the pig and in other species studies which describe the presence of enterotoxin or enterotoxigenic C.perfringens type A infer the presence of sporulating organisms. Spore counts and the association of sporulating C.perfringens type A with diarrhoea are best documented in man (Sutton et al., 1971) where food-borne intoxication and the ingestion of both spores and preformed enterotoxin is most commonly considered to be the source. However, the 7-day old piglets (submission 21, Table 33) with high faecal spore counts could not have ingested preformed toxin or large numbers of spores as piglets of this age ingest milk direct from the sow. This finding suggests that sporulation is taking place in the gut of the diarrhoeic pig. The survey described in Chapter 6 (Table 33), suggested that spores were present in the faeces of pigs of all ages, that few or low numbers of spores were present in normal faeces (range $3 \times 10^2 - 2 \times 10^4$ per g) and that high numbers were present in diarrhoeic faeces ($2 \times 10^2 - 5 \times 10^6$ per g). As spores form an indication of the possible presence of enterotoxin, the relationship between diarrhoea and the presence of spores is discussed under that heading below.

(b) Serological studies

Antibody to alpha toxin and enterotoxin was sought in the sera of sows and the results described in Chapters 5 and 6 and discussed there, suggested that a high proportion (42.3 per cent) contained antibody to alpha toxin and to enterotoxin (92.4 per cent). The significance of these results is discussed below with the discussion of the individual toxins and their role in pathogenicity.

3. EXPERIMENTAL STUDIES WITH C.PERFRINGENS TYPE A

(a) Introduction

As C.perfringens type A was present in mixed culture in most of the samples examined and as post-mortem changes and treatment had affected the animals submitted for examination, it was decided to use animal and in vitro experiments to study the disease further. An isolate of C.perfringens type A (isolate 7) was considered typical of vegetative cells and was selected for study of the disease. Experimental infections with this isolate are described in Experiments 1 and 2, Chapters 3 and 4. Another isolate (isolate 29) considered typical of enterotoxin-producing sporulating cells was also used in Experiment 3 (Chapter 6).

The remainder of these studies involved the study of alpha toxin, enterotoxin and immunity to them in the pathogenesis of the disease. The results of each type of study provided new information about C.perfringens type A disease in the pig and the significance of these results is discussed below in the context of the existing literature.

(b) Animal experiments

Both isolate 7 (vegetative cells) and isolate 29 (sporulating, enterotoxin producer) of C.perfringens type A produced clinical disease in hysterectomy-derived, colostrum-deprived piglets. Both

types of organism produced diarrhoea in the inoculated piglets and colonised the gastrointestinal tract. The results in each experiment were different and differed also to some extent from previously described experimental infections in similar piglets (Olubunmi, 1982 and Nabuurs et al., 1983). The infection of 8 day-old HDCD piglets with isolate 7 in Experiment 1 produced a clinical disease less severe than that produced in 1 day-old HDCD piglets where most of the infected animals died within 48 hours post-infection (Experiment 2). The 8 day-old HDCD piglets developed a profuse creamy diarrhoea after infection which lasted throughout the period of observation (Table 9). The infection was not fatal for these animals although a marked loss of bodily condition was observed. On the other hand, when non-immune one day-old HDCD piglets were infected, the infection appeared to be fatal for most animals. The difference in the severity of the infection for these two groups of piglets may be due to the establishment of the microbial flora in the 8 day-old piglets which could have competed with C. perfringens type A lessening its pathogenic effects (Tlaskalova et al., 1983). Increasing maturity of the gastro-intestinal tract, increased ability to control fluids and electrolytes and possibly the beginnings of an immune response to natural C. perfringens type A contaminants may also have been involved. The results of Experiment 1 resembled most closely those of Nabuurs et al. (1983) who used slightly older piglets and those of Experiment 2 resembled most closely those of Olubunmi (1982) who infected neonates as was the case here.

The main site of the infection was at jejunal and ileal level and inflammatory and necrotic changes appeared to result from the infection of piglets. In the 8 day-old HDCD piglets, fluid intestinal contents and congestion appeared to be the most consistent findings. In the one-day old HDCD piglets, the distention of the small intestine at jejunal and ileal levels with the presence of gas and frothy contents and congestion of the mucosa were marked features. The lesions seen in natural cases described in the survey resembled those seen in the experimental infections and confirmed that the disease produced was similar to

that seen in the field. The lesions resembled those found by Nabuurs et al. (1983) but were less severe than those described by Olubumni (1982).

The experiment described in Chapter 6 in which one day-old HDCD piglets were infected with a non-haemolytic, enterotoxin-producer strain (isolate 29) showed that another different diarrhoeic syndrome was produced from those described above. This study, which will be discussed in the section d of this chapter, appears to represent the first account of the isolation of C.perfringens type A enterotoxin-producer from a case of diarrhoea in pigs and its relation to a specific diarrhoeic syndrome in infected piglets by experimental reproduction. Other reports of disease associated with enterotoxin-production in pigs (Popoff and Jestin, 1985; Jestin et al., 1985) did not consider the isolation of the organism or the reproduction of the disease. The disease produced by isolate 29 was milder, of shorter duration and was non-fatal in neonatal piglets. Few lesions were seen in the intestinal tract (Chapter 6).

The clinical signs and lesions observed in the piglets infected with C.perfringens type A did not resemble those of C.perfringens type C. The massive haemorrhage reported in that disease was absent. In C.perfringens type C infections, bloody diarrhoea, haemorrhagic intestines and advancing zone of necrosis are reported to occur (Bergeland, 1981) involving the mucosa, submucosa and the tunica muscularis. When necrotic lesions were observed in C.perfringens type A (Experiment 2) they were limited to the mucosal layer. It is clear from the studies described in Chapters 3, 4 and 6 that the mode of action of C.perfringens type A in causing enteric disease in pigs appears to be very different from that of C.perfringens type C.

The observations discussed above indicate that C.perfringens type A may be isolated in large numbers in cases of diarrhoea in piglets and is capable of producing a diarrhoeic syndrome and pathological changes when given orally to hysterectomy-derived,

colostrum-deprived piglets. The syndrome resembles that produced by other workers and is distinct from that produced by C.perfringens type C. The way in which C.perfringens type A produces the changes seen is discussed below.

(c) The role of alpha toxin in porcine enteric disease

C.perfringens type A alpha toxin has been incriminated in gastro-intestinal disease in several animal species. Necrotic enteritis of chickens has been reported (Al-Sheikhly and Truscott, 1977) to be caused largely by alpha toxin, and also, it has been found (Niilo, 1978) that C.perfringens type A strains associated with necrotic enteritis in chickens were strong alpha toxin producers. In this thesis, the high percentage of alpha toxin producer isolates (Table 26) confirmed the fact that most of C.perfringens type A strains isolated from enteric disease in pigs are alpha toxin producers, and the isolation of strong alpha toxin-producer strains (haemolytic units of 16 or more) may indicate a relationship with the enteritis observed in the animals from which these C.perfringens type A strains were isolated.

Skjelkvale et al. (1979) reported that from cases of food poisoning in humans, only 55 per cent of C.perfringens type A isolated were alpha toxin producers, indicating that the distribution of alpha toxin producers and non-producer strains may vary according to the environment or syndrome from which C.perfringens type A strains are isolated.

It was not possible to correlate the porcine diarrhoeic syndromes studied in the surveys in this thesis with the alpha toxin production of the C.perfringens type A isolated. The role of alpha toxin production by C.perfringens type A strains in gastro-intestinal disease remains unclear and requires further investigations. However, there were differences in the syndromes produced in Experiments 2 and 3 in neonatal piglets. It may be that these reflect the fact that isolate 7 was a moderate alpha toxin producer and produced obvious haemolysis while isolate 29

produced no observable changes on blood agar and no alpha toxin (Table 26). It produced less severe disease and this reduced severity might be associated with the absence of haemolytic products especially alpha toxin.

When the effect of alpha toxin was tested in intestinal ligated loops (Table 28), no accumulation of fluid or macroscopic lesions were observed in intestinal loops receiving purified alpha toxin, culture supernatant or concentrated culture supernatant. These results are in agreement with those of Hauschild et al. (1968) and Duncan et al. (1968) who failed to produce an intestinal loop response when crude alpha toxin was injected into loops prepared in lambs and rabbits respectively. The first authors, however, observed accumulation of fluid in intestinal loops when large amounts of alpha toxin were injected. 1000 MLD of alpha toxin per loop produced fluid accumulation which was amplified several-fold when 8000 MLD were inoculated into loops. Such large quantities of alpha toxin inducing accumulation of fluid provide no indication of the role of the toxin in C.perfringens type A enteritis because it is unlikely that such amount of alpha toxin would be produced in a natural case of C.perfringens type A infection.

Microscopically, the intestinal loops injected with purified alpha toxin and with concentrated culture supernatant showed a mild congestion and localised hypercellularity at lamina propria (Chapter 5). These lesions may be due to the effect of alpha toxin which stimulates histamine release from platelets causing an increased cellularity (Ohsaka et al., 1978). These changes were also reported by Hauschild et al. (1968) in his lamb studies. This represents the first report of this type of change in the pig.

The specificity of these changes was confirmed by the neutralisation studies performed in the gut loops using rabbit antiserum to pure alpha toxin. Similar protection was also found to the effects of crude culture supernate. This protection was also found in vivo in Experiment 2 when colostrum and milk from

hyperimmunised sows was used to protect against challenge with isolate 7. The milk from sows immunised with bacterin alone was least protective and had the lowest titres against alpha toxin (Table 18). Unfortunately the presence of other antibodies (Discussion Chapter 4) meant that the protection observed could not be attributed exclusively to anti-alpha toxin.

It may be suggested that with the multiplication of C.perfringens type A in the intestine, the organism produces specific enzymes which could play a role in the pathogenesis by altering the permeability of the intestinal mucosa to alpha toxin and the enhancement of its effects. In this particular role, some of the minor toxins with activities of hyaluronidases, collagenases and proteinases produced by C.perfringens type A could be of significance in the pathogenesis by giving the opportunity to other toxins, such as alpha toxin, to act at intestinal level. Nothing is known about the activity at intestinal level of most of the C.perfringens type A toxins and this requires further study.

It is also possible that close attachment to the epithelium might also enhance the effects of alpha toxin. Attachment is known to occur early in C.perfringens type C infections both in pigs (Arbuckle, 1972) and in man (Walker, 1985). Comparable studies of early infections have not been carried out in the pig with C.perfringens type A.

(d) The role of enterotoxin in porcine enteric disease

C.perfringens type A enterotoxin has been identified as the factor responsible for diarrhoea experimentally induced in several animal species, and for cases of human food-poisoning.

Enterotoxin production differs from strain to strain, most C.perfringens type A strains isolated from faecal samples obtained from cases of food-poisoning in humans produce enterotoxin, but few strains from other sources are enterotoxigenic. In this thesis, 26 per cent of the C.perfringens type A isolates were found to produce

detectable levels of enterotoxin (Table 29). Higher numbers of enterotoxin-producer strains were isolated by Yasukawa et al. (1975) who reported enterotoxin production by 51 (77 per cent) of 66 strains associated with outbreaks of human food-poisoning. However, the same authors found that the percentage frequency of enterotoxin-producer strains from healthy individuals was low (less than 1 per cent). Similar results were obtained by Skjelkvale et al. (1979) who found that enterotoxin was produced by 56 of 65 (86 per cent) of food-poisoning strains, but that 94 strains isolated from other sources were negative for enterotoxin. The isolation of enterotoxin-producing C.perfringens type A strains (Table 29) indicates the presence of such strains in pig populations. The diarrhoeic syndrome present in the animals from which enterotoxin-producer strains were isolated may have been produced by the action of enterotoxin at intestinal level, producing a syndrome resembling human food-poisoning, and in some cases, this syndrome might have been exacerbated by the presence of other pathogens.

A correlation between alpha toxin and enterotoxin has been reported (Harmon and Kautter, 1976) suggesting that enterotoxin levels produced by a strain could be estimated by measuring its alpha toxin production. The results indicated in Table 29 do not show this correlation. Some of the enterotoxin-producer isolates were weak alpha toxin producers, and three of the isolates did not produce alpha toxin. These results are in agreement with those of Skjelkvale et al. (1979) who reported that some strains of alpha toxin negative C.perfringens type A strains can produce large amounts of enterotoxin.

Enterotoxin was detected in five faecal samples in which increased spore counts in relation to vegetative cells were demonstrated (Table 33). These findings resemble cases of human food-poisoning in which increased counts can be demonstrated before and after heating the faeces: faeces from healthy individuals do not show increased C.perfringens counts after heating (Sutton et al., 1971). The detection of enterotoxin in faeces has been proved to be a diagnostic test of C.perfringens type A human food-

poisoning. Faecal isolates from cases of food-poisoning reveal the presence of enterotoxin, whereas faeces of healthy individuals do not show presence of enterotoxin (Itoh et al., 1979). In pigs, Jestin et al. (1985) demonstrated the presence of enterotoxin in the faeces of diarrhoeic pigs in which no other pathogen could be demonstrated, and enterotoxin was not detected in the faeces of healthy pigs. In the studies described in Chapter 6, the increased spore counts, the enterotoxin detection and the absence of other pathogens, excepting sample 82 (Table 33) from which β-haemolytic E.coli was isolated, provide strong evidence that the diarrhoeic syndrome in the pigs was provoked by the action of the enterotoxin at intestinal level.

No previous reports of the presence of enterotoxin in diarrhoeic piglet faeces have been published. Those of Jestin et al. (1985) refer only to weaned animals. Enterotoxin was demonstrated in the faeces of both weaned and sucking piglets in this study.

Purified enterotoxin was prepared from isolate 29 and inoculated into intestinal ligated loops (Tables 34, 35 and 36) where it induced the accumulation of fluid and pathological changes which were shown to be enterotoxin dose-dependent, thus, doses of enterotoxin from 32 to 512 TU/ml produced increasing amounts of accumulation of fluid and respectively more severe lesions which ranged from slight congestion of the mucosa to complete desquamation of the epithelium with severe oedema, haemorrhage and infiltration of inflammatory cells (Figs. 47 and 50). The results are in agreement with studies on the effect of enterotoxin in intestinal loops in other animal species (Duncan and Strong, 1969; Hauschild et al., 1970; McDonel, 1974; Yamamoto et al., 1979). By immunofluorescence, enterotoxin was found to be bound to the epithelial cells of the intestine (Figs. 51 and 52). It was observed that a stronger fluorescence was correlated with higher enterotoxin doses. This finding indicates that binding of the enterotoxin is essential for its biological activity. McDonel (1980) reported the binding of the enterotoxin molecule in the

rabbit intestine and that this binding was not reversible, thus, the enterotoxin molecule binds to a single cell and consequently the effects produced by the enterotoxin at intestinal level are dose dependent. The specificity of the effects was confirmed by the neutralisation of the effects using anti-enterotoxin.

The injection of purified enterotoxin into the intestinal lumen of HDCD piglets indicated that good correlation exists between the ability of the enterotoxin to produce accumulation of fluid in intestinal loops and production of diarrhoea after inoculation into the ileum of the piglets. The time of onset and duration of diarrhoea in the piglets was comparable to experimentally induced diarrhoea by intraluminal inoculation of enterotoxin in rats (McDonel, 1974) and rabbits (McDonel and Demers, 1982). The watery transient diarrhoea produced in these unfed, colostrum-deprived pigs did not resemble that seen in field cases of C.perfringens type A diarrhoea.

The enterotoxin was found to be bound to the epithelial cells of the intestine from the site of inoculation to the colon, with the strongest fluorescence at the level of the terminal ileum (Table 37). This finding is in agreement with the results reported by Hauschild et al. (1973) who found that enterotoxin is most active in the ileum, mildly active in the jejunum, and nearly inactive in the duodenum. As in this thesis, the inoculations of the enterotoxin were carried out in the terminal jejunum in piglet 1, and in the ileum in piglet 2, further studies are required to evaluate the activity of the enterotoxin in the duodenum and jejunum of the pig.

The infection of HDCD piglets with vegetative and sporulated cells induced a diarrhoea which was similar in its onset and duration to experimentally produced diarrhoea with C.perfringens type A enterotoxin-producer strains in lambs (Niilo, 1971), calves (Niilo, 1973a), chickens (Niilo, 1974), monkeys (Hauschild et al., 1971) and humans (Strong et al., 1971).

Enterotoxin was detected in the diarrhoeic faeces of all infected animals, and the faeces showed increased spore counts in relation to vegetative cells (Table 39). These findings indicate that C.perfringens type A sporulated in the intestine of the piglets releasing enterotoxin. Enterotoxin was detected only in the intestinal contents of piglet number 5 which was sacrificed two days after infection (Table 39). This result indicates that enterotoxin can be detected only for a short period after infection and is in agreement with Dowell et al. (1975) who reported that enterotoxin can not be detected in the faeces collected from individuals after the disappearance of the symptoms provoked by a C.perfringens food poisoning outbreak. These authors observed that enterotoxin could only be detected in the faeces of individuals with active diarrhoea. It is clear, however, that fixed enterotoxin can be detected on cells of the ileum and colon in pigs which still have moderate or high counts of C.perfringens type A spores (Table 40). This evidence for the continued production and fixation of enterotoxin in the gut is of interest. It suggests that the assay method used here (Vero cell assay) may not be sufficiently sensitive to detect all faecal enterotoxin and that it might have been detected using ELISA tests (Table 3). The intestines of piglets naturally affected with C.perfringens diarrhoea have not been tested in this way and it is not known how widespread this phenomenon of fixed enterotoxin may be. The rate at which fixed toxin is lost was not determined in these studies. McDonel (1980) showed that enterotoxin is not released from cells to which it binds and the persistence of toxin-bearing colonic cells may reflect the slower turnover of colonic epithelium when compared with other regions of the gut rather than continued production of toxin.

Fixation of enterotoxin to the colonic cells may be of little importance in diarrhoeal disease as McDonel and Demers (1982) showed that it had no effect on colonic function in the rabbit. Fixation in the ileum was more important and it may be that the presence of fixed toxin in the ileum in this study did cause fluid production but that colonic compensation prevented diarrhoea. The

numbers of enterotoxin-producing cells necessary in the ileum for diarrhoea to be produced has not been determined for any species. The studies described in Chapter 6 indicate that C.perfringens type A entero-toxin-producer strains can be isolated from naturally occurring diarrhoeas in the pig and that C.perfringens type A enterotoxin induces an experimental diarrhoeic response in the pig similar to the one reported in other animal species. Further investigations are required to know the incidence of the disease and its effect on productivity in pig populations.

(e) Immunity to C.pefringens type A cells and toxins

Studies on the immunity to C.perfringens cells and toxins are the basis of the investigations described in Chapter 4 and some information was obtained from results described in Chapters 5 and 6.

The studies carried out in Chapter 4 demonstrated that a toxoid and a bacterin prepared from cultures of C.perfringens type A could be completely detoxified and inactivated respectively. Both preparations were found to elicit antibodies to alpha toxin and to C.perfringens type A cells in parenterally immunised animals as discussed in Chapter 4.

Few reports exist in the literature about the production of toxoids and bacterins involving C.perfringens type A cells or alpha toxin. This situation may be due to the fact that alpha toxin had been considered until a recent past, to be a poor antigen (Oakley, 1970) and also, it was reported (MacLennan, 1962) that it was impossible to convert C.perfringens alpha toxin to a toxoid. Also, the lack of literature related to the subject may be due to the decreasing importance of gas gangrene as a cause of disease with the widespread use of antibiotics in the prophylaxis and treatment of the disease, particularly in human beings. Also, as C.perfringens type A has been considered to be part of the normal intestinal flora and studies are still being carried out to elucidate its role in enteric disease, few attempts to produce

bacterins from the organism have been described. Bousicaux et al. (1974) immunised sheep with a multi-component bacterin containing whole cultures of C.perfringens types A, B, C and D and, antibodies to alpha, beta and epsilon toxins were demonstrated in the immunised animals and the presence of these antibodies were considered to be protective in field trials. However, it is not possible to evaluate from this study the role played by C.perfringens type A cells and toxins in the protection achieved because other types of C.perfringens were also present in the bacterin.

In this thesis, the immunisation of sows with crude C.perfringens type A toxoid and bacterins and the demonstration of antibodies to alpha toxin and C.perfringens type A cells with the subsequent passive protection offered by these antibodies against challenge, appears to be the first account in the literature. The use of C.perfringens type C toxoids to immunise sows and to provide passive protection for their litters has been known for some years (Djurickovic et al., 1975). Antibodies to beta toxin have been detected in their milk and colostrum and the transfer of these antibodies to the progeny protects against the disease. The mechanism of sow vacination with clostridial products for the protection of the litter against clostridial disease is, therefore, well established. What was new, however, was the attempt to protect against C.perfringens type A disease.

Examination of the antibodies present in the colostrum and milk from immunised sows showed that neither the toxoid nor the bacterin had stimulated the production of antibodies to alpha toxin or to cells alone (Experiment 2, Chapter 4). In contrast, the protection experiment carried out with antibody to enterotoxin (Experiment 3) used relatively specific porcine antiserum to enterotoxin (Chapter 6). The results of the two protection studies differed markedly and have been compared below.

The antibodies from sows immunised with toxoid and bacterin, when passively transferred to hysterectomy-derived, colostrum-

deprived piglets, were able to protect the animals against challenge with C. perfringens type A isolate number 7.

In contrast, protection against disease was not observed when hysterectomy-derived, colostrum-deprived piglets were passively immunised with anti-enterotoxin serum and then challenged with C.perfringens type A isolate 29 (Experiment 3, Chapter 6). In this experiment there was no difference between passively immunised piglets and non-immunised, infected animals. There was no difference either in the recovery rate of C.perfringens type A and the post-mortem bacteriological findings in the gastrointestinal tract were identical in both immunised and non-immunised piglets. On the other hand, in Experiment 2, the recovery of C.perfringens type A from rectal swabs of immunised piglets was less profuse than from infected, non-immunised controls, and this difference in recovery rate was present not merely in the initial stages but throughout the infection period as observed in the case of piglet C+6. At post-mortem there was also a considerable decrease in recovery of C. perfringens type A from the gastrointestinal tract of immunised piglets when compared with infected, non-immunised controls (Table 21).

The macroscopic and microscopic findings also showed differences in the two experiments. In Experiment 3, the pathological changes in both immunised and non-immunised animals were similar. In contrast, the pathological changes in the gastrointestinal tract of immunised piglets in Experiment 2 were much less severe when compared to those found in non-immunised, infected animals; in particular the marked congestion so characteristic of the disease seen in infected, non-immunised controls was not evident in the immunised piglets.

In Experiment 2, passively immunised piglets were completely protected against challenge with isolate 7 by serum, colostrum or milk containing antibody to alpha toxin and somatic antigens. The exact component giving this protection was not clear. Antibody to pure alpha toxin was able to prevent the slight changes noted in

the gut loops inoculated with alpha toxin in Chapter 5 and it is possible that this antibody alone was completely protective. Protection studies with pure porcine antibody to alpha toxin were not carried out. The reasons for the failure of anti-enterotoxin to protect in Experiment 3 are not entirely clear. The antiserum was administered both parenterally and orally as with the sera in Experiment 2 but failed to protect. Antibody levels in the piglets were similar to those in the serum given and transfer of anti-enterotoxin was successful. These findings are not unexpected. Niilo et al. (1971) used a bacterin prepared from sporulating cultures of an enteropathogenic strain of C.perfringens type A and produced serum antibody to enterotoxin. No protection occurred when the lambs were subsequently challenged with the C.perfringens by the intraduodenal route. Similar results have been recorded in man by Skjelkvale and Uemura (1977) who showed that volunteers with serum antibody to enterotoxin were susceptible to oral challenge.

Since the antibody used neutralised the effects of enterotoxin in gut loops (Chapter 6) it appears that systemic antibody to enterotoxin can not neutralise the enterotoxin in the gut. As the organism and enterotoxin are restricted to the gut lumen it may be that local immunity, especially IgA, is important in preventing infection. No studies have been carried out on the neutraliation of enterotoxin by IgA antibody and local immunity in the gut has never been reported. The presence of antibody to enterotoxin in the colostrum or milk has not been reported in any species.

The examination of pig sera for the presence of antibodies to alpha toxin (Table 27) and to enterotoxin (Table 32) revealed that 42.3 per cent of the sera possessed antibodies to alpha toxin with titres ranging from 1:2 to 1:16, and these same sera, 92.4 per cent possessed antibodies to enterotoxin with titres ranging from 1:2 to 1:64. The prevalence of antibodies to these toxins indicates that pig populations are commonly exposed to these toxins. The higher percentage of animals with antibody titres to enterotoxin than to alpha toxin may indicate a better immunogenicity of enterotoxin than alpha toxin at intestinal level, possibly due to the

resistance of enterotoxin to degradation by the intestinal contents. The demonstration of antibodies to alpha toxin in pig populations does not appear to be reported in the literature. The results of studies on the presence of antibodies to enterotoxin differed markedly from those reported by Niilo and Bainborough (1980) who found that of 620 pig sera, only 10 possessed antibody titres to enterotoxin. The same authors reported that of 345 cattle sera, 53 possessed antibodies to enterotoxin; of 768 horse sera, 89 were positive for antibodies to enterotoxin, and of 244 human sera, 101 possessed antibody titres to enterotoxin. On the other hand, Brant et al. (1978) reported that from 141 human sera and 151 cattle sera, all were positive to antibodies to enterotoxin. Ochoa and Velandia (1978) reported also that of 150 horse sera, all were positive for antibodies to enterotoxin. These differences in antibody titres may indicate variations of C.perfringens type A enterotoxin-producer strains among populations, or they may be due to variations on the techniques used for the detection of antibodies to enterotoxin. Whether or not the presence of antibodies to C.perfringens type A cells, alpha toxin and enterotoxin in sows have an effect on protection and productivity when passively transferred to their progeny in field conditions remains to be investigated. The results obtained here suggest that exposure to C.perfringens type A and its toxins is widespread in the pig population and that infection may be important.

CONCLUSIONS

In conclusion, the studies carried out in this thesis have shown that C.perfringens type A may be isolated from cases of diarrhoea, particularly in young piglets and the disease may be reproduced experimentally by infection of hysterectomy-derived, colostrum-deprived piglets.

Two different diarrhoeic syndromes were observed to be produced by C.perfringens type A, one that might involve alpha

toxin in its pathogenesis and the other that clearly involves enterotoxin.

The first diarrhoeic syndrome was characterised by a long lasting diarrhoea in 8 day-old HDCD infected piglets and by diarrhoea and death when one day-old HDCD piglets were infected with the organism. The disease could be prevented in these last animals by passive immunisation with sera, colostra or milks from immunised animals with C.perfringens type A bacterin or toxoid. Further investigations are required to elucidate the pathogenesis and mechanisms of protection of this syndrome. Protection studies with specific antibody to pure alpha toxin should be included.

The second diarrhoeic syndrome was characterised by a short lasting diarrhoea in one day-old HDCD infected piglets. C.perfringens type A was found to sporulate and to produce enterotoxin at intestinal level; the disease produced may be entirely attributed to the enterotoxin. The disease could not be prevented by passive immunisation with serum from an animal immunised with purified enterotoxin. This syndrome resembles closely the syndrome of food-poisoning in humans and the diarrhoeas produced experimentally in other animal species. This study appears to represent the first account of the isolation of enterotoxin producing C.perfringens type A from diarrhoea in pigs and its relation to a specific diarrhoeic syndrome in infected piglets by experimental infection. The relationship of this infection to effects on productivity requires further study and experimental infections in weaned pigs should also be carried out.

The relationship between both types of C. perfringens type A infections and other bacteria, viruses and parasites in the gut also requires further investigation.

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