Investigation into the role of *Clostridium novyi* in sudden death in pigs

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by

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ABSTRACT

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The review of the necropsies carried out on sows that died suddenly between 1986 and 1991 revealed that almost 35% of dead sows examined were diagnosed as *Clostridium novyi* sudden death (clostridial hepatopathy). This study was undertaken to survey sow mortality in intensive indoor breeding herds and to determine the epidemiology, predisposing factors, pathological presentation and microbiology of *Clostridium novyi* sudden death. In the three year investigation into mortality on large pig breeding herds clostridial hepatopathy was diagnosed 17 times (16.7% of all submissions). Abdominal accidents or torsion of abdominal organs (17.6%) were the most frequently diagnosed cause of death. Cases of cystitis and pyelonephritis (11.8%) and perforated or bleeding gastric ulcers (9.8%) also accounted for a large proportion of sow mortality. Overall, the mortality rate increased during the period of study, principally due to an increased number of sows culled on the farm. The death rate remained at around 8%, with an average herd inventory of 610 sows. The mean parity at the time of death was 4.3 $\pm$ 0.3 (SEM) litters, with a quarter of all deaths occurring before the second litter. Thirty percent of the deaths on these farms were during the summer, but submissions for necropsy were lowest at that time. Predominantly, sows that died during lactation were submitted for necropsy.

Seventeen Clostridial hepatopathy cases were diagnosed during the study. All the cases presented generalised oedema and subcutaneous infiltration with bubbles and foul smelling bloody fluid in pericardial, pleural and abdominal cavities. The liver was enlarged, dark and the parenchyma was uniformly infiltrated with gas bubbles giving a spongy appearance. All cases were highly positive to the FAT and C. *novyi* type A (4 occasions) and type B (5 occasions) were isolated. Older sows (mean parity 5.6 litters), in good body condition and during gestation were more likely to die suddenly due to *Clostridium novyi*. The spring months saw more cases submitted for necropsy. The liver smears taken from other sudden death sows examined were negative to the FAT.

Examination of healthy slaughterhouse sows and cull sows on the farm revealed that the liver contained dormant C. *novyi* spores. In cold weather sows left unopened for 48 hours after death gave a positive result to the fluorescent antibody test.

Studies with *Clostridium novyi* confirmed the sensitivity to atmospheric oxygen, the requirement for fresh, pre-reduced, supplemented media for culture on plates. Techniques for selective isolation were evaluated and the growth curve established. The sensitivity of culture collection isolates of C. *novyi* to growth promoting antibiotics was established.

Molecular biology techniques were employed to detect the phospholipase C gene of C. *novyi* using PCR on bacterial DNA and to determine the aminoacid sequence of the gene. Identification of the different types within C. *novyi* could be developed.

I would like to dedicate this thesis to my Grandfather, Frederick Lewis Norman, who introduced me to farm animals, politics and quotations and inspired me to become a Veterinary Surgeon.

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1. INTRODUCTION

".....my life has been a bit like jazz, which is probably why I like jazz. All that is certain is a begining and an end."

Studs Terkel, writer and broadcaster, The Observer (1995).

1.1. The anaerobic world

Anthon van Leeuwenhoek in 1683, way ahead of his time, described finding minute "animalcules" in the oral cavity of cases of "stinking mouth" (cited by Finegold, 1977a). Not only was this the first recorded description of microorganisms, but Leeuwenhoek also linked these living organisms with disease and found they could survive without air. These findings were largely ignored for two centuries.

Diseases caused by anaerobes had been described long before his account. In ancient Greece, as early as the fourth century B.C., Hippocrates described the clinical signs and terrifying deaths due to tetanus (cited by Willis, 1989). The Greek historian Xenophon (430 B.C.) recorded an outbreak of oral disease in soldiers which presented foul smelling breath and sore mouths, probably acute necrotising ulcerative gingivitis, an infection likely to be caused by anaerobes (cited by Willis, 1989).

The discovery of anaerobiosis has been credited to Louis Pasteur who in a paper presented to the Academie Francaise in 1861, reported seeing "little infusorial animals" that under the microscope were motile in the absence of air, but became motionless and ceased to ferment substrates after being in contact with air (cited by Sebald and Hauser, 1995). Pasteur, at this time, also determined that oxygen in the atmosphere was the inhibiting factor.

Few people, at the time, believed that life could exist without oxygen and Smith and Williams (1984a) suggested, in a review of anaerobes and the toxic effect of oxygen, that this belies the fact that man as an obligate aerobe, finds it inconvenient to work with obligate anaerobes.

Early workers in the field were dogged by technical problems in providing an appropriate anaerobic environment and by massive confusion in nomenclature that arose from studying mixed type cultures.

The advent of the first world war accelerated the impetus of anaerobic bacteriology, particularly the study of the histotoxic clostridia of gas gangrene (MacLennan, 1962). Willis (1989) described the events in the battlefields around the Ypres, Loos and the Somme as one of the great anaerobic tragedies in history. The incidence of wound sepsis in this conflict was high and a reported 12% of the wounded soldiers contracted gas gangrene, with a mortality rate of 20%.

The discovery by McIntosh and Fildes (1916) of the anaerobic jar provided the environment needed for the isolation of most pathogenic anaerobic bacteria. The original jars used a heated palladium catalyst fitted on the jar's lid plus a supply of hydrogen from a cylinder. Later cold catalysts replaced the heated catalyst providing a much safer method, which has continued to be used by microbiologists, with minor modifications, to the present day (Willis, 1977).

The non clostridial anaerobic infections received little attention until the first half of this century when Altemeier (1940) determined that the foul smelling pus found in cases of tuboovarian abscesses was not "sterile", but in fact contained anaerobic streptococci. During this period purulent appendicitis and peritonitis was believed to be caused by single organisms, but Altemeier (1938) dispelled this notion by proving mixed infections, including anaerobes, were found in these cases. At this time the endogenus origin of these and other bacterial infections due to anaerobes was established.

Eggerth (1935) and later studies by Finegold, Sutter and Mathiesen (1983) who cultured human faeces using selective media found *Bacteroides* spp to be an important part of the intestinal flora of humans. Finegold (1995) stated that anaerobes outnumber aerobes by a ratio of ten to one in the oral and vaginal flora and by a factor of 1000 to 1 in the colon of humans. Gram negative rods and particularly the *Bacteroides fragilis* group are considered the most commonly encountered anaerobes in human infections (Finegold, 1995).

Difficulties arise when trying to define anaerobic bacteria because these organisms comprise such a wide range of sensitivities to atmospheric oxygen, from the relatively aerotolerant *Clostridium perfringens*, that will survive several hours left on the bench, to *C. haemolyticum*, inhibited by concentrations of gaseous oxygen of 2%, or even the more strict anaerobic oral spirochaetes that were found not to tolerate an oxygen concentration in air of 0.02% (Rosebury, 1966 cited by Smith, 1975b). A working definition, which must reflect the quantitative nature of the effect of oxygen was suggested by Smith and Williams (1984a): "Anaerobes are organisms that grow better in the absence of air than they do in its presence". Finegold (1989) proposed a set of practical definitions to be used in the laboratory:

- An **anaerobe** is an organism that requires a reduced oxygen tension for growth and fails to grow on the surface of solid media in the presence of 10% CO_2 in air (21% oxygen).
- **facultative anaerobes** can grow both in the presence or absence of air.
- **microaerophilic** organisms are those that grow better with a reduced oxygen tension, provided in an atmosphere with 10% CO_2, over either aerobic or anaerobic conditions.
- **aerotolerant organisms** are ones that can tolerate the presence of oxygen on the surface of freshly prepared media.

Two mechanisms are thought to be responsible for the inhibitory effect of oxygen on anaerobes:

Firstly, direct inhibition of growth in the presence of oxygen, which presents a wide variation between anaerobic species, combined with an inability to incorporate gaseous oxygen into the cytoplasm . Anaerobes lack oxidases and oxygenases and are unable to use oxygen as an electron acceptor, therefore depend on fermentation to generate energy for their metabolism (Sebald and Hauser, 1995).

Secondly, anaerobes can be inhibited by an indirect, more serious effect, due to superoxide anions and hydrogen peroxide. These products of organic metabolism occur when O_2 is reduced by one electron (superoxide anion), two electrons (hydrogen peroxide) or gains 3 electrons (hydroxyl radical). These compounds have recently been named reactive oxygen species (ROS) (Sies, 1991). All aerobic organisms have enzymes that protect the cell against the superoxide free radical, principally superoxide dismutases (SOD) that catalyse the reaction between two superoxide anions to form molecular oxygen and hydrogen peroxide. Many obligate anaerobes do not posses SOD, but all pathogenic anaerobes do (Tally, Jacobus, Goldin and Gorbach, 1975). Most anaerobes do not produce catalase, but several species of *Bacteroides* produced small quantities when grown in certain media (Smith and Williams, 1984a). On the other hand, some facultative anaerobic bacteria appear to be catalase negative .

The third factor involved in the growth of anaerobes, the oxidation-reduction potential (redox potential or E_h) becomes critical in some cases, as demonstrated by the fact that some anaerobic bacteria could be grown in broth whilst a stream of air was passed though the media by lowering the redox potential employing electrical means (Finegold, 1977a).

Low redox potentials in bacteriological media are necessary for the initiation of growth of anaerobes from a small inoculum (Smith and Williams 1984a).

The redox potential is measured in microbiology using dyes that decolorise when they are reduced. Resazurin and methylene blue have frequently been used for this purpose (Willis, 1977).

Reducing agents may be added to bacteriological media to allow growth of fastidious anaerobes. Moore (1968) formulated a complex medium for the isolation of *Clostridiium novyi* and included cysteine and dithioerythritol, which supported good growth of this fastidious organism on solid media. Using Moore's medium on blood agar plates and conventional anaerobic jar procedures, Watt, Collee and Brown (1974) reported good recoveries of *Clostridium novyi* and *C. tetani* and total bacterial counts from faecal samples equivalent to those achieved using an anaerobic cabinet.

Recently, a further explanation for the sensitivity of anaerobes to the effect of oxygen has been postulated by Sebald and Hauser (1995). These authors suggested that anaerobes lack the ability to repair proteins following oxidation. An oxygen reducing membrane fraction from *E. coli* , the Oxyrase Enzyme System, has been shown to remove oxygen from the media, enhanced growth of anaerobes and promoted septation in an *E. coli lon* mutant (Adler, Carrasco, Crow and Gill, 1981). The product from the gene responsible (*lon*) has been characterised and encoded an ATP-dependant protease that catalysed the repair of damaged proteins in *E. coli* (Phillips, Van Bogelen and Neidhart, 1994). The suggestion that damaged proteins in the bacterial cell wall affected the viability of anaerobes was supported in recent findings regarding the variability of Gram staining of some anaerobes (Johnson, Thatcher and Cox, 1995). These authors demonstrated consistent staining of Gram positive anaerobes when fixing and staining was carried out in the anaerobic cabinet. In conclusion, this work suggested that cell wall integrity was compromised by a combination of toxic effects of oxygen by-products with the damage from fixatives and reagents, causing Gram-positive bacteria to appear Gram-negative .

1.2. Anaerobes in Veterinary Medicine

Finegold (1995), in his overview of anaerobic infections in humans and in the preface to the landmark text "Anaerobic bacteria in human disease" (Finegold, 1977a) suggested anaerobic infections were common, but had been the most frequently overlooked of the bacterial infections. The main reason for this oversight, Finegold believed, was lack of awareness of anaerobic infections by bacteriologists and clinicians, the difficulties arising from collection and transport of samples and the cumbersome culture and identification of anaerobes. To a great extent the same problems have been encountered in veterinary bacteriology. Osbaldiston and Stowe (1971) remarked that with the exception of the genus *Clostridium* the role of anaerobic bacteria as aetiological agents of veterinary importance had been poorly established. Furthermore, the reasons for this were thought to be; difficulties with isolation, problems stemming from pathogenic anaerobic bacteria being part of the normal flora of mucosal surfaces, the intestine or the skin and finally, difficulties fulfilling Koch's postulates. As the culture and identification techniques became more widely available to veterinary laboratories, surveys of anaerobic infections in animals have beeen published (Prescott and Barnum, 1977; Berkhoff and Redenbarger, 1977; Berg, Fales and Scanlan, 1979; Hirsh, Biberstein and Jang, 1979; Prescott, 1979; Walker, Richardson, Bryant and Draper, 1983; Hirsh, Indiveri, Jang and Biberstein, 1985; Hariharan, Lamey and Heaney, 1995). The prevalence of certain genera of bacteria from anaerobic infections in animals were found to differ when compared with anaerobes in human infections, as was to be expected considering the distinct normal flora. Comparison between different laboratories, encompassing different animal species and differing techniques can lead to misconceptions, but to serve as a guide some of the findings from these surveys are summarised (see table 1).

Table 1.1. Comparison of different anaerobic bacteria isolated in surveys of animal and human infections (%).

	Infection in companion animals Berg *et al* (1979)	**Infection in all domestic animals** Berkhoff and Redenbarger (1977)	**Infection in all domestic animals** Hirsh *et al* (1985)	**Human infections** Finegold and Sutter (1977)
Total number of isolates	187	78	1596	890
Gram +ve sporeforming bacilli				
TOTAL	30.5	50	10	8
(C. perfringens)	(19.2)	(17.9)	(5)	
Gram +ve non-sporeforming bacilli				
Actinomyces spp.	9.1	10.3	-	
Propionibacterium spp.	3.7	6.4	-	
Eubacterium spp.	3.2	2.6	-	
Bifidobacterium spp.	-	1.2	-	
other G+ve rods	-	9.0	-	
TOTAL	16	29.5	8	19
Gram +ve cocci				
Peptostreptococcus	7.5	1.2	12	
other G+ve cocci	4.3	-	-	
TOTAL	11.8	1.2	12	22
Gram -ve bacilli				
Bacteroides fragilis	3.7	5.2	7	15
B. melaninogenicus	13.4	3.8	8	
Bacteroides spp.	13.9	-	29	
TOTAL	31	12.8	44	
Fusobacterium spp.	5.3	1.2	3	
Fusobacterium necrophorum	5.3	5.2	18	
TOTAL Gram -ve bacilli	41.6	19.2	65	44

Early surveys of veterinary anaerobic isolates found that *Clostridium* was the genus most commonly involved in infections, particularly *C. perfringens*. Berkhoff and Redenbarger (1977) remarked that 50% of their isolates belonged to the genus *Clostridium*, four times the rate usually found in human surveys (Finegold and Sutter, 1977). Later studies reported much lower isolation rates for *Clostridium* spp, around 10%, closer to the proportion found in human anaerobic infections (Hirsh *et al*, 1979; Prescott, 1979; Hirsh *et al*, 1985).

The Gram negative rod *Bacteroides fragilis*, which accounted for a large proportion of anaerobic bacterial isolates in human disease (Finegold, 1995) and forms part of intestinal flora of man and animals (Hentges, 1989) was not as prevalent in veterinary laboratory isolations (Berg *et al*, 1979; Hirsh *et al*, 1979). This was perhaps because many human isolates originated from blood cultures of hospitalised immunocompromised patients, an uncommon event in veterinary hospitals. On the other hand, enterotoxigenic *Bacteroides fragilis* was recently implicated in outbreaks of acute diarrhoea in calves, lambs, foals and young pigs (Myers, Firehammer, Shoops and Border, 1984; Border, Firehammer, Shoops and Myers, 1985; Myers and Shoop, 1987; Myers, Shoop and Byars, 1987). *B. fragilis* was also isolated from mastitis in cattle (Du Preez, 1989), osteomyelitis in domestic animals (Walker *et al*, 1983; Muir and Johnson, 1992) and from pleural fluid in horses with pneumonia or pleuropneumonia (Sweeney, Holcombe, Barningham and Beech, 1991).
Recent studies on the prevalence and susceptibility of anaerobic bacteria from clinical veterinary practice revealed disturbing findings (Hirsh *et al*, 1985). The isolation rate of *Bacteroides fragilis* showed a sevenfold increase in the isolation rate compared with the same laboratory 4 years earlier. The authors believed this data represented a true increase in incidence.

Also worrying was the fact that 20% of the specimens were found to contain an isolate resistant to penicillin, ampicillin and cephalothin. All of these antibiotic resistant isolates belonged to the genus *Bacteroides*, principally *B. fragilis*, which commonly was also resistant to tetracycline.

Similar findings were reported from Canada (Hariharan *et al*, 1995). These workers identified as *Bacteroides* spp. 51.5% of all anaerobic isolates and *B. fragilis* was the single most isolated species.

Bacteroides melaninogenicus was commonly isolated from animals (Prescott and Barnum, 1977; Berg *et al*, 1979) and described in mixed infection with *Fusobacterium necrophorum* as an specific cause of Foot-rot in cattle and sheep. *Actinomyces* spp. and *F. necrophorum* also appeared to be more commonly involved in animal infections in comparison to those found in human anaerobic infections (Berg *et al*, 1979; Hirsh *et al*, 1985). *Fusobacterium necrophorum* in particular was commonly isolated from cases of liver abscessation in cattle (Prescott, 1979).

Mixed cultures were a common finding in anaerobic infections of man (Finegold, 1989), and isolation of both aerobic and anaerobic organisms was reported in the majority of the specimens submitted to veterinary laboratories (Berkhoff and Redenbarger, 1977; Berg *et al*, 1979; Prescott, 1979: Hirsh *et al*,1985). More than one anaerobe was frequently isolated, Prescott (1979) reported an average of 3.1 anaerobic species per specimen and Hirsh *et al* (1985) a mean number of 1.9 anaerobic species per sample.

1.3. AIMS OF THE STUDY

Epidemiological studies.

1. To analyse the causes of sow mortality in large breeding herds.
2. To determine the presence of spores or vegetative cells of *Clostridium novyi* in the liver of healthy sows and the influence of *post mortem* invasion from the gut.
3. To investigate the incidence of *C. novyi* sudden death in pig breeding herds by examining past necropsy records and carrying out a prospective study of sudden deaths submitted for *post mortem* examination.
4. To determine if *C. novyi* sudden death is more likely to occur in particular age groups, a period of the production cycle or time of the year.
5. To establish the influence of concurrent disease or any predisposing factors in the incidence of *C novyi* sudden death.

Microbiological investigations with *C. novyi*.

1. To become proficient in the manipulation of anaerobic bacteria.
2. To study the cultural, biochemical and toxicological properties of *C. novyi* and other species of *Clostridium.*
3. To achieve consistent isolation of *C. novyi,* by developing selective and enrichment procedures for bacterial culture.
4. To study the molecular genetic control of the toxins of *C. novyi* .

2. Literature review

"The anaerobist working on bibliography is surely a creature to be pitied and is not to be blamed for failing to follow the work of others conscientiously and thoroughly."

Hilda Heller (1922) quoted by Smith (1977a)

2.1. Genus *Clostridium*

Clostridia were defined as obligate anaerobic sporeforming Gram positive bacilli (Cato, George and Finegold, 1986; Hatheway, 1990; Willis, 1983). Gram staining was found to be variable unless the examination was carried out in the early stages of bacterial growth. Some species were found to grow, but not sporulate in the presence of air (Cato *et al*, 1986). The genus *Clostridium* was differentiated from Gram positive aerobic sporeforming *Bacillus* by their lack of catalase production (Smith and Williams, 1984), although trace amounts have been detected in some strains (Cato *et al*, 1986). None of the clostridia formed oxidase or reduced sulphate (Willis, 1983).

Most species grew better at a pH of 6.5 to 7.0 and at temperatures between 30°C and 37°C, but some species were found to be thermophilic whilst others, like *Clostridium putrefaciens*, required temperatures of 22°C to 25°C for adequate growth (Cato *et al*, 1986).

The majority of members from this genus were described as motile by peritrichate flagella and lacking a capsule, with the notable exception of *Clostridium perfringens* (Willis, 1983). Clostridia in the sporing stage were resistant to heat, drying and disinfectants, but wide variation between the different species was noted (Hobbs, Williams and Willis, 1971).

Many *Clostridium* species could be infected by bacteriophages (Smith, 1959; Ackermann, 1974; Ogata and Hongo, 1979), which were shown in some species to confer pathogenicity after infecting non-toxigenic strains or a different species (Eklund, Poysky, Peterson and Meyers, 1976).

Clostridium butyricum is the type species of the genus *Clostridium.*

Over 100 species have been recognised for this genus, with a wide variation in phenotypes (Cato, Hash, Holdeman and Moore, 1982; Lawson, Llop-Perez, Hutson, Hippe and Collins, 1993). Furthermore, the G + C content of chromosomal DNA of clostridia was found to range from 22 to 55% (Cato *et al*, 1986). These authors and also work by Johnson and Francis (1975), who examined rRNA homology, suggested the division of *Clostridium* into two genera, one containing those species with a mol % G+C between 22 and 34 and another genus including species with chromosomal G+ C of 40 to 55 %.

Recently, Lawson *et al* (1993) proposed a change in the phylogeny of clostridia based on the 16S rRNA sequences. These authors concluded the genus was extremely heterogeneous and that many non spore-forming bacteria were closely intermixed with *Clostridium* species. Further work by this group (Collins, Lawson, Willems, Cordoba, Fernandez-Garayzabal, Garcia, Cai, Hippe and Farrow, 1994) confirmed the previous findings and proposed the division of the genus into five new genera and eleven new species combinations, with the majority of the clinically important clostridia in a redefined genus including *C. butyricum* and its relatives.

Clostridia were described as ubiquitous in nature and have been found in soil, sewage, marine sediments, in the intestinal tract of man and animals and isolated from decaying animal and plant products (Cato *et al*, 1986). Willis (1983) believed that soil was the main habitat for clostridia and that some species were ingested on vegetable matter by animals and humans, becoming permanently or temporarily adapted to life in the intestinal canal.

Only 20 species, listed by Hatheway (1990), were considered pathogenic and could be encountered in disease or infections of man and animals. Most if not all of the pathogenic clostridia were found to produce toxins; biologically active proteins that were antigenic and could be neutralized by appropriate antisera (Hatheway, 1990). The molecular mass of these proteins was found to range between 22 to 600 kilodaltons (kDa). Toxins have been detected by their effect on animals and cell cultures, lethality and by observation of biochemical reactions (Hatheway, 1990). Greek letters were assigned to these toxins according to their time of discovery, regardless of activity. This may lead to confusion as distinct toxins of different species were given the same name.

Investigation of clostridial toxins, in particular those produced by *Clostridium perfringens* and the neurotoxins of *Clostridium tetani* and *Clostridium botulinum* has led to detection of the genes responsible. A superb and up to date review of toxigenic clostridia has recently been published by Hatheway (1990) who compiled extensive information on the activities, mechanisms and genetics of clostridial toxins. The toxins produced by the different clostridial species can be seen summarised in table 2.1.

Table 2.1. Toxigenic clostridia and their toxins.

Species	Toxins	Size of molecule (kDa)	Activity/disease
C. botulinum	Neurotoxin	150	Botulism
	C2 (binary):		Permease
	component I	50	ADP-ribosylation
	component II	105	Binding
	C3	25	ADP-ribosylation
C. tetani	Neurotoxin	150	Tetanus
	Tetanolysin	48	Oxygen-labile haemolysin
C. perfringens	Alpha	43	Phospholipase C
	Beta	40	Lethal, necrotic/enterotoxaemia
	Epsilon	34	Lethal, permease/enterotoxaemia
	Iota (binary):		Enterotoxaemia
	component a	48	ADP-ribosylation
	component b	72	Binding
	Enterotoxin	35	Foodborne diarrrhoea
C difficile	Toxin A	400-500	Enterotoxins/Antibiotic
	Toxin B	360-470	associated pseudomembranous colitis
	CDT	43	ADP-ribosylation
C. sordelii or	Alpha	43	Phospholipase C
C. bifermentans	Beta:		Lethal
	HT	525	~ to *C. difficile* toxin A
	LT	250	~ to *C. difficile* toxin B
	Haemolysin	43	oxygen labile haemolysin
C. novyi and	Alpha	200	Lethal, dermonecrotic
C. haemolyticum	Beta	32	Phospholipase C
	Gamma	30	Phospholipase C
	Delta	ND[a]	Oxygen labile haemolysin
	Epsilon	ND	Lipase
C. chauvoei	Alpha	48	Lethal, necrotising
and	Beta	45	DNase
C. septicum	Gamma	ND	Hyaluronidase
	Delta	ND	oxygen labile haemolysin
C. histolyticum	Alpha	ND	Necrotizing
	Beta:		Collagenases
	Class I	various	
	Class II	various	
	Gamma	50	Proteinase
	Delta	>10, <50	Proteinase
	Epsilon	ND	Oxygen labile haemolysin
C. spiroforme	Iota (binary):		Diarrhoea in rabbits
	component a	43-47	ADP-ribosylation
	component b	ND	Binding

a **ND,** Not determined

(Adapted from Hatheway, 1990)

2. 2. Toxigenic clostridia and their role in veterinary medicine

The subject to be covered is very vast and with the exception of *C. novyi* and *C. haemolyticum*, which are directly relevant to this thesis, brief descriptions of the important organisms will be carried out. Particular emphasis will be given to species that have important effects on pig health.

The toxigenic clostridia can be broadly divided into two groups according to their mechanism of action:

Firstly, largely non-invasive species that produce extremely potent neurotoxins, comprising *Clostridium botulinum* and *Clostridium tetani*, plus the less well studied *Clostridium argentinense* (*C. botulinum* type G) and neurotoxin producing strains of *Clostridium baratii* and *Clostridium butyricum.*

Secondly, a group of clostridia that can invade tissues, the histotoxic clostridia, including *Clostridium perfringens, C. chauvoei, C. novyi, C. septicum* or affect the intestine, the so called enterotoxigenic clostridia, like *C. perfringens* and *Clostridium spiroforme.*

2.2.1. *Clostridium botulinum*

The various types of *C. botulinum* were found to have different cultural and serological properties and varied in their normal habitats (Smith, 1977). Cultural characteristics and toxigenicity changed during isolation and subculture in the laboratory (Willis, 1977). The susceptibility of animal species to the different botulinum toxins was also shown to present a great diversity, regardless of the route of administration (Smith, 1977). Botulism was described as a rapidly fatal flaccid motor paralysis after ingestion (in most cases) of a pre-formed *C. botulinum* toxin (Blood, Radostits, Arundel and Gay, 1989).

The organism. *C. botulinum* was described as a large, Gram positive bacilli of around 4 x 1 μm in size (Cato *et al*, 1986). The morphology was not at all characteristic, but the rods had straight walls, with rounded ends and prominent oval terminal or subterminal spores. The fluorescent antibody test was used to identify the different strains of the organism (Walker and Batty, 1964). Variation in cultural characteristics between the different types and even between strains of a type have been described (Willis, 1977). The species has been divided, according to the toxin produced, into 7 types (A-G). Determination of the presence and type of toxin was considered the only reliable way of identifying *C. botulinum* (Levett, 1991a).

C. botulinum was classified as a strict anaerobe (Smith, 1977). On solid media the colonies were found to be large, transparent with a granular surface and a lobular or indefinite edge, with a tendency to spread over the agar surface (Cato *et al*, 1986). Usually strains caused haemolysis on blood agar and all types except Type G (*Clostridium argentinense*) produced areas of opalescence and a pearly layer on egg yolk agar due to lipolysis (Willis, 1977). Acetic acid was the major metabolic product of all types (Holdeman, Cato and Moore, 1977).

Further distinction between members of the species *botulinum* have been based on their ability to digest egg proteins, leading to the creation of four groups within the species (Smith, 1977; Hatheway, 1990). These groups were designated by Roman numerals; Group I included proteolytic strains of toxin types A, B and F. Group II included non proteolytic strains of type B and F plus all strains belonging to toxin type E. Group III, perhaps the most important from a veterinary point of view, included strains of types C and D involved in avian and animal botulism. Group IV contained asaccharolytic organisms that produce type G toxin (*C. argentinenese*).

Some non-neurotoxin producing clostridia were antigenically, phenotypically and genetically closely related to *C. botulinum* (Poxton, 1984; Collins *et al*, 1994). *C. sporogenes* isolates differed from proteolytic *C. botulinum* strains only in their toxigenicity. *C. novyi* could only be distinguished from group III isolates by comparison of the toxins produced. Furthermore, it has been demonstrated that interspecies conversion could be achieved by infecting *C. botulinum* type C with a phage from *C. novyi* (Eklund, Poysky, Meyers and Pelroy, 1974).

The disease: In human botulism various forms have been described, most commonly foodborne intoxication (Smith, 1977), but also wound botulism affecting chronic drug abusers. Infant botulism occured when toxin was produced by toxigenic organisms that colonized the intestine of infants under one year of age (Hatheway, 1990). This intestinal colonization has been described in adults suffering from a serious disruption of the bowel (McCroskey and Hatheway, 1988).

Conversely, botulism in animals was almost invariably caused by ingestion of preformed botulinum toxin from decomposing animal carcasses (Blood *et al*, 1989; Jones, 1991).

The disease was judged of major economic importance in four animal species; cattle, sheep, horses and mink (Smith, 1977) and has been reported very frequently in waterfowl (Hariharan and Mitchell, 1977; Quinn, Carter, Markey and Carter,1994) and was common in domestic chicken (Smith, 1977). On the other hand, pigs, dogs and cats appeared to be largely resistant, but susceptible to intoxication (Smith, 1977; Sterne,1981; Blood *et al*, 1989).

The distribution of *C. botulinum* spores in the soil has been studied (Smith, 1975 and 1977). Type A seemed to favour neutral or alkaline soils, whilst Type C appeared to proliferate in acid soils.

C. botulinum type B and type E were prevalent in wet soils in the USA (Blood *et al*, 1989).

Cattle in areas where pasture was deficient in phosphorous were more likely to develop "Pica", or depraved appetite, leading to ingestion of carcasses, particularly chewing on bones. When *C. botulinum* spores were abundant in the soil, the digestive tract and therefore the carcass of a dead animal would also be heavily contaminated with the organism and the toxin. Smith (1977) suggested up to 10^6 mouse LD per gram could be found in these carcasses. Considering that the lethal dose for cattle was estimated at five hundred thousand mouse LD of type C toxin given orally (Muller, 1963), even a very small amount of material could prove fatal to cattle. Outbreaks in cattle have also been linked to the use of contaminated poultry waste (faeces, litter, carcases) as bedding, feed or pasture fertiliser (Blood *et al*, 1989; Jones, 1991). Horses suffered the so called "Forage botulism" when toxin was ingested with big bale silage (Ricketts, Greet, Glyn, Ginnett, McAllister *et al*, 1984) or hay contaminated by dead rodents (Kelly, Jones, Gillick and Sims, 1984).

Pigs appeared to be highly resistant to botulinum toxins ingested by mouth (Smith, Davis and Libke, 1971; Taylor and Bergeland, 1992), possibly due to poor intestinal permeability of the toxins. On the other hand, pigs were moderately susceptible to type B neurotoxin when infused intravenously. Few outbreaks have been reported in pigs: Beiers and Simmons (1967) diagnosed the disease in five adult pigs that contracted the disease from ingestion of fish carcasses. The fish had died after a lagoon dried-up during a drought. *Clostridium botulinum* type C was thought to be the culprit.

Pathogenesis: The botulinum toxins were thought to exert their pharmacological activity on the neuromuscular motor synapse by blocking the release of acetylcholine (Hambleton, 1993) resulting in flaccid paralysis.

Botulism paralysis was believed to occur in three steps: 1) recognition of a receptor site on the pre-synaptic nerve surface, followed by binding of the toxin molecule; 2) internalisation, when the toxin crossed the plasmalemma entering the nerve; 3) the toxin fragment prevented acetylcholine release (Hatheway, 1990).

Recently, both botulinum B and tetanus neurotoxins were proven to be enzymatic in nature (Schiavo, Rossetto, Santucci, Das Gupta and Montecucco, 1992). These neurotoxins were believed to have zinc endopeptidase activity, cleaving the protein components of the synaptic vesicle membrane therefore disrupting the release of neurotransmitters.

Diagnosis: A presumptive diagnosis has generally been made on the basis of the clinical signs. The incubation period and severity of an outbreak were believed to be dependant on the amount of toxin available (Blood *et al*, 1989). Horses and cattle tended to die suddenly or showed a very rapid generalised muscle weakness and paralysis (Smith, 1977). In most cases, botulism presented in a subacute form in these species (Kelly *et al*, 1984; Jones, 1991). Restlessness, incoordination and ataxia were followed by an inability to rise or to lift the head. The tongue became paralysed and hung outside the mouth, combined with an inability to masticate and excessive salivation. In some outbreaks no impairment of mastication or swallowing was reported (Jones, 1991). Respiration became abdominal and laboured due to paralysis of the intercostal muscles. Death usually followed 1 to 4 days after the begining of the illness, with the animal conscious and afebrile. Clinical pathology and necropsy findings did not aid the diagnosis of botulism (Blood *et al*, 1989, Jones, 1991).

In experimental pigs, signs began one to three days after consumption of toxic material (Smith *et al*, 1971). Initially weakness was found in the hind limbs and the lumbar region. The pupils remained dilated. Appetite and drinking

ceased. General paralysis followed soon after, with death due to respiratory failure in one to seven days after the beginning of clinical signs (Smith *et al*, 1971). Adult pigs in a field case presented other clinical signs that included vomiting, weakness, lordosis, dilated pupils with partial or complete blindness, anorexia, excessive salivation and, in the terminal stages, laboured breathing (Beiers and Simmons, 1967). Death occurred between 19 and 52 hours after ingestion of the decomposing fish carcases. In this outbreak two animals that presented with incoordination and unsteady gait recovered after treatment with skimmed milk containing magnesium sulphate.

Confirmation of the diagnosis of botulism was deemed to require the detection of the neurotoxin in the serum or intestinal tract of affected cases and in the suspected source of the poisoning (Lyerly and Wilkins, 1991). Mice, inoculated with extracts of serum, liver and gut contents from suspected botulism cases were observed for clinical signs of botulism. Confirmation of the diagnosis was carried out by neutralisation of the toxin with specific antitoxin. This technique was found to be valuable in birds, but gave false negatives when used to diagnose cattle and horse botulism (Thomas, 1991).

Investigations to reduce the use of laboratory animals by developing an ELISA test to detect botulinum toxin concluded that these assays were less sensitive than mouse inoculation (Dezfulian, Hatheway, Yolken and Bartlett, 1984: Thomas, 1991).

Isolation of the organism from the faeces or intestinal contents of affected animals was considered to be of little diagnostic value (Blood *et al*, 1989). Serum detection of botulinum toxins appeared not to be diagnostic in the pig (Smith *et al* , 1971). In suspected porcine botulism, Taylor and Bergeland (1992) believed isolation and identification of the organism was diagnostic.

On the other hand, work from Japan suggested that pigs in some areas could be silent carriers of *C. botulinum* type C (Yamakawa, Kamiya, Yoshimura and Nakamura, 1991). These researchers isolated toxigenic *C. botulinum* from the livers of healthy swine, but not from healthy cattle. This investigation also found that in *C. botulinum* positive herds the farm environment and faecal samples revealed extremely high isolation rates for the organism without evidence of clinical botulism. These findings differed from those of Muller (1963) who found healthy cattle and pigs rarely carrried toxigenic *C. botulinum* type C.

2.2.2. *Clostridium tetani*

Tetanus, the disease caused by *Clostridium tetani*, was decribed by Hatheway (1990) as a highly fatal hypertonia of the striated muscles commmonly accompanied by clonic paroxysmal muscular spasms or contractions. The disease in humans has become rare in the Western World (Furste, Aguirre and Knoepfler, 1989), but has devastating consequences in developing nations. The World Health Organisation reported 600 thousand deaths worldwide due to neonatal tetanus in 1990, invariably after infection of the umbilical stump. The disease in animals was considered to affect primarily unvaccinated horses after penetrating wounds and other animals after surgical or obstetrical procedures with inadequate antisepsis (Blood *et al*, 1989).

The organism: *C. tetani* , a strict anaerobe, was rapidly killed upon exposure to oxygen (Cato *et al*, 1986). Bacilli were filamentous and Gram positive in young cultures, but developed the characteristic "drum stick" appearance due to the protruding terminal endospores after 48 hours incubation (Willis, 1977). Older cultures became Gram negative, but identification was possible using a fluorescent labelled antibody (Batty and Walker, 1964).

In culture on solid media *C. tetani* tended to spread over the surface of the plate as a fine rhizoidal film with filamentous margins, particularly on moist agar. In mixed cultures the thin swarming growth could be overlooked. Stiff agar media with 3-4% agar allowed for individual colonies to be seen (Willis, 1977), particularly if half tetanus antitoxin plates were used. On blood agar *C. tetani* exibited β-haemolysis around flat translucent or gray colonies with a matte surface (Cato *et al*, 1986). *C. tetani* was asaccharolytic, did not digest proteins and was lecithinase and lipase negative (Holdeman *et al*, 1977). Indole was produced and gelatin liquified slowly. Gas liquid chromatography showed butanol, acetic, propionic and butyric acid as metabolites in peptone yeast extract broth cultures (Holdeman *et al*, 1977).
The structural gene of the tetanus toxin was found to be plasmid borne (Finn, Silver, Habig, Hardegree, Zon and Garon, 1984) and the complete amino acid and nucleotide sequences for this gene have been determined (Fairweather and Lyness, 1986).

The disease: Tetanus, characterised by tonic spasms of voluntary muscles, required infection with *C. tetani* and the presence of a reduced oxidation-reduction potential in tissues, below that found in healthy muscle, for production of toxin (Furste *et al*, 1989). Wounds usually became infected by spores. Spores of *C. tetani* were found to be highly persistent in soil and resisted steam heat at 100°C for 30 to 60 minutes and other disinfection procedures, but were destroyed by autoclaving (Willis, 1983). The spores were thought to be particularly numerous in the soil of pastures and cultivated land contaminated by animal faeces (Willis, 1983; Blood *et al*, 1989; Taylor and Bergeland, 1992). Deep infection was required for production of the neurotoxin.

The toxin, tetanospasmin, acted by blocking inhibitory synapses of the spinal chord motorneurons (Hatheway, 1990). The tetanospasmin was thought to spread from the infected site either by diffusing into adjacent muscle tissue (localised tetanus) or by transport via lymphatics to the blood and nerves (generalised tetanus). The toxin bound to gangliosides in nerve tissue and then travelled along nerves by retrograde axonal transport (Schwab, Suda and Thoenen, 1979). Finally, tetanospasmin was demonstrated to become attached to the nerve ending, thus preventing the release of the inhibitory mediators gamma-aminobutyric acid and glycine (Osborne and Bradford, 1973). This induced uncontrolled spasms of voluntary muscles.

Ascending tetanus occurred when toxin travelled through a regional motor nerve in the limb, causing tetanus in those peripheral muscles. Henceforth the toxin spread upward affecting the contralateral limb and the trunk (Timoney, Gillespie, Scott and Barlough, 1988). On the other hand the form normally seen in humans and horses, descending tetanus, was thought to initially involve circulating toxin in blood and lymphatics. This systemic circulation would affect the most susceptible motor endings of the neck and head first, followed by the forelimbs, the trunk and finally the hindlegs (Timoney *et al* 1988).

Clinical signs could take between 3 days to several months after infection to develop. All animal species presented a similar clinical picture (Sterne, 1981; Blood *et al*, 1989; Quinn *et al*, 1994). The earliest sign detected was a stiffened gait, followed by rapid progression to difficulty walking, erect ears, extended tail and slightly elevated head. The protusion or prolapse of the nictitating membrane was a very useful differential clinical sign in animals. Ruminants presented severe bloat and all species were constipated and had difficulties urinating, as the normal posture of micturition could not be achieved.

In the final stages of tetanus the animal remained in lateral recumbency, with both thoracic and pelvic limbs rigidly extended, in opisthotonus. Noise or any sensory stimulation induced severe tetanic spasms. Rectal temperature and heart rate, initially within normal range, became elevated. Death usually occured due to respiratory failure resulting from severe skeletal muscle spasms (Taylor and Bergeland, 1992).

Contamination of castration wounds was the most frequently reported cause of tetanus in pigs , but umbilical infection and wounds due to unclipped canine teeth were also implicated (Taylor and Bergeland, 1992).

2.2.3.*Clostridium perfringens*

During the nineteenth century *C. perfringens* was known by a variety of names, but the present day nomenclature arose from the description by Veillon and Zuber in 1898 of *Bacillus perfringens* (cited by Smith and Williams, 1984b). Some workers, particularly in Britain, used the synonym *Clostridium welchii*. The species was divided into five types, A to E, according to the production of the major lethal toxins (see table 2.2.) (Sterne and Warrack, 1964). Recently, typing of *C. perfringens* by *in vitro* amplification of toxin genes using the Polymerase Chain Reaction (PCR) has been described (Daube, China, Simon, Hvala and Mainil, 1994).

Clostridium perfringens has been found widely distributed in nature; Smith and Williams (1984b) described Type A as part of the microflora of the soil and the intestinal tract of man and animals, whilst the other types were thought to be obligate parasites of domestic animals and occasionally part of the microflora of humans.

The organism: Cells appeared as Gram-positive, atrichous, nonmotile straight rods with blunt ends (Cato *et al*, 1986). Young cultures in broth appeared coccoid or cubical and could occur in pairs (Willis, 1977).

Capsules were occasionally seen *in vivo*, but were not be observed *in vitro* (Willis, 1977). Size has been found to vary between types and strains, usually measuring between 0.6-2.4 μm by 1.3-19 μm (Cato *et al*, 1986). Spores, not usually apparent in vivo or when grown in routine laboratory media were detected by culture in special sporulation media for *C. perfringens*. Various sporulation media have been proposed; Ellner medium (Ellner, 1956), Duncan and Strong medium (Duncan and Strong, 1968), Tsai medium (Tsai, Torres and Riemann, 1974) and, more recently, a quinoline medium (Phillips, 1987). Strains of *C. perfringens* synthesised the enterotoxin responsible for food poisoning outbreaks in humans during sporulation (Estrada Correa and Taylor, 1989; Van Damme-Jongsten, Rodhouse, Gilbert and Notermans, 1990). Johnson (1989) suggested that sporulation and enterotoxin production were coincidental events that occur under similar environmental conditions.

C. perfringens has been grown in the presence of small amounts of oxygen (Smith and Williams, 1984b). Growth on the surface of plates became apparent, on occasion, after 4 to 6 hours incubation, making *C. perfringens* one of the most rapidly growing anaerobes (Willis, 1977). Colonies were described as circular, convex, semitranslucent or grey, with an entire edge and 2 to 4 mm in diameter. Rough colonies and other colonial characterisitics have been described (Cato *et al*, 1986).

On blood agar plates colonies were surrounded by the characteristic double area of haemolysis (target haemolysis), narrow and complete due to theta toxin and a wider partial haemolysis due to the alpha toxin.

Types D and C strains produced a wide area of haemolysis due to the delta toxin. Variation in these effects was seen on blood from different species (Cato *et al*, 1986). The CAMP phenomenon, a synergistic haemolysis, has been described with co-cultures of *C. perfringens* and *Streptococcus agalactiae.* (Quinn *et al*, 1994).

The optimum temperature for growth of types A, D and E was 45°C, whilst types B and C grew at the same rate at 37°C or 45°C (Smith and Williams, 1984b).

The pH range over which growth takes place was reported to be between 5.5 and 8.0, and growth was not inhibitied by 20% bile or 2% NaCl (Cato *et al*, 1986). Lecithinase, produced by 95% of *C. perfringens* strains can be demonstrated as a diffuse area of opalescence on egg yolk agar plates. Half antitoxin plates to detect the inhibition of the α toxin, a phospholipase C, have been used for identification of *C. perfringens*. Lactose was fermented presenting as a 'stormy clot' reaction in milk. The main products of fermentation detected by gas liquid chromatography were acetic and butyric acids and sometimes butanol (Holdeman *et al*, 1977).

Bacteriophages of this species have been recognised for some time (Smith, 1959) and the presence of plasmids conferred strains with antibiotic resistance (Brefort, Magot, Ionesco and Sebald, 1977; Rood, Buddle, Wales and Sidhu, 1985) and allowed bacteriocin production (Ionesco, Bieth, Dauget and Bouanchaud, 1976).

The pathogenicity of *C. perfringens* depends on the production of toxins which appear summarised in table 2.2.. Apart from toxins involved in diseases other antigens with various effects have been described (Table 2.3).

Major lethal toxins:

Alpha toxin. Produced in large amounts by type A strains and by all the other *C. perfringens* types. Alpha toxin, a phospholipase C (EC 3.1.4.3.) that hydrolysed lecithin and sphyngomyelin (Mollby, 1978; Hatheway, 1990), has been classed as a zinc metallophospholipase consisting of a single polypeptide chain with an estimated relative molecular weight of 43 kDa (Krug and Kent, 1984, Titball, 1993).

Table 2.2. Distribution of Major Lethal toxins among the types of *Clostridium perfringens*.

	TOXIN			
Type	Alpha	Beta	Epsilon	Iota
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Table 2.3. Minor toxins produced by *C. perfringens*.

Toxins	Biological activity
Gamma	Not defined, may not exist
Delta	Haemolysin
Eta	Not defined, existence questionable
Theta	Haemolysin (Oxygen labile), Cytolysin
Kappa	Collagenase, gelatinase
Lambda	Protease
Mu	Hyaluronidase
Nu	DNase
Neuraminidase	N-Acetylneuraminic acid glycohydrolase

Hatheway, 1990

Various researchers have published the nucleotide sequence encoding the alpha toxin (*cpa* or *plc*) gene (Leslie, Fairweather, Pickard, Dougan and Kehoe, 1989; Saint-Joanis, Garnier and Cole, 1989; Titball, Hunter, Martin, Morris, Shuttleworth, Rubidge, Anderson and Kelly, 1989; Tso and Siebel, 1989).

The toxin was thought to be the factor responsible for myonecrosis after *C. perfringens* infection, although the pathogenesis has yet to be fully elucidated (Titball, 1992). The effects of the alpha toxin of *C. perfringens* have been described; increased vascular permeability (Sugahawa, 1977), cytotoxicity (Sato, Chiba and Sato, 1989; Titball, 1992) and activation of the arachidonic acid cascade leading to the release of thromboxane A, an inflammatory mediator (Fujii and Sakurai, 1989). Recently, Williamson and Titball (1993) demonstrated that mice were protected against gas gangrene by a genetically engineered alpha toxin vaccine.

Beta toxin. Produced by both type B and C strains of *C. perfringens* the beta toxin has been held responsible for necrotic enteritis in animals and Pig-bel and Darmbrand in humans (Lawrence and Cooke, 1980). The toxin has been purified and was found to have a molecular mass of 40 kDa and an isoelectric pH of 5.6 (Sakurai and Fujii, 1987). The nucleotide sequence for the beta toxin gene (*cpb*) has been determined and was found to have homology with the alpha toxin gene of *Staphylococcus aureus* (Hunter, Brown, Oyston, Sakurai and Titball, 1993). The increased blood pressure demonstrated in experimental rats was thought to be induced by chatecholamine release (Hatheway, 1990). Lawrence and Cook (1980) demonstrated that beta toxin required a protease inhibitor to produce necrotic lesions in the small intestine of guinea pigs. The toxin was believed to be highly sensitive to trypsin in the intestine.

Epsilon toxin. Both type B and D *C. perfringens* strains produced epsilon toxin in the form of a protoxin. It was thought that these protoxins were

activated by proteolytic enzymes produced by the organism itself or by the addition of trypsin to the media (Hatheway, 1990).

The gene encoding for the epsilon toxin (*etx*) of *C. perfringens* , a protein of 38 kDa (Habeeb,1975), has been sequenced and expressed in *E. coli* (Hunter, Clarke, Kelly and Titball, 1993). Some differences between the nucleotide sequences of epsilon toxin genes from *C. perfringens* type B and type D have been reported (Havard, Hunter and Titball, 1992).

The epsilon toxin was found to increase the permeability of the intestinal mucosa to other toxins and enhance its own absorption (Buxton, 1978). Once in the circulation the toxin caused foci of necrosis, perivascular oedema and haemorrhages, particularly in the meninges (Buxton, Linklater, Dyson, 1978). Griner (1961) found microscopic foci of liquefaction necrosis in the basal ganglia, thalamus, internal capsule, substantia nigra, subcortical white matter and cerebellum of lambs with acute intoxication. The mechanism of action was thought to be related to the breakdown of intercellular junctions which allowed fluid to escape (Buxton and Morgan, 1976). These changes were manifested by pericardial and peritoneal effusions and pulmonary oedema. Hyperaemic and degenerative changes were found on the kidney cortex, making it soft and friable (pulpy kidney), in addition to rapid *post-mortem* autolysis (Timoney *et al*, 1988; Buxton and Donachie, 1991). All the effects of the toxin were due to the damage to the vascular epithelium (Smith, 1979). Buxton (1978) proposed that the epsilon toxin altered the adenylcyclase system in affected cells.

Iota toxin. This toxin has only been produced by strains of *C. perfringens* type E, which was isolated from calves with enterotoxaemia in Australia (Hart and Hooper, 1967). The toxin required trypsin for activation and had lethal, dermonecrotic and vascular permeability effects (Smith, 1979). The Iota toxin of *C. perfringens* type E was similar to iota toxin of *Clostridium spiroforme* in serological, enzymatic and biological activities (Hatheway, 1990).

C. spiroforme caused enterotoxaemia in rabbits after treatment with clindamycin (Carman and Borriello, 1984).

Enterotoxin. This virulent factor has been studied mainly in *C. perfringens* type A strains, but types D and C also produced enterotoxin (Hatheway, 1990). The enterotoxin was described as a single polypeptide of 35 kDa with a unique amino acid sequence (McClane, 1992). The complete gene encoding the enterotoxin (*cpe*) has been cloned, sequenced and expressed in *E. coli* (Czeczulin, Hanna and McClane, 1993). Previously only overlapping DNA fragments spanning the complete *cpe* gene had been obtained (Van Damme-Jongsten, Wernars and Notermans, 1989).

The toxin has been involved in food-poisoning illness in humans (Johnson, 1989; McClane, 1992) and enterotoxaemia in piglets (Taylor and Olubunmi, 1982; Taylor, Estrada-Correa and Al-Mashat, 1987; Collins, Bergeland, Bouley, Ducommun, Francis and Yeske, 1989).

The action of the enterotoxin was recently reviewed by McClane (1992) and the pathogenesis involved; i) specific binding of the enterotoxin to proteinaceous receptors on the membranes of intestinal epithelial cells and other mammalian cell types, ii) insertion of the toxin into plasma membranes, iii) formation of a complex with host membrane proteins, iv) alteration of the membrane permeability to ions, v) induction of secondary effects that caused cell death.

In vitro and in the infected host it was thought that sporulation of the *C. perfringens* type A strains was required for production of the enterotoxin (Estrada-Correa and Taylor, 1989). Czeczulin *et al* (1993) found that vegetative *C. perfringens* cultures and recombinant *E. coli* clones carrying the *cpe* gene were able to produce enterotoxin, although sporulation did facilitate expression of the gene. The appearance of enterotoxin during sporulation was thought to represent a derepression of an incidental gene but not expression of a sporulation specific gene (Johnson, 1989).

Diseases caused by the different toxin types of *C. perfringens* :

The species was divided into 5 types according to the ability to produce the major lethal toxins (Table 2.2.). These toxins were antigenic and typing was carried out by neutralisation with type specific antisera in laboratory animals (Sterne and Batty, 1975), using an ELISA (Naylor, Martin and Sharpe, 1987; Martin, Naylor and Sharpe, 1988; Holdsworth and Parrat, 1994), by the latex agglutination test (Martin and Naylor, 1994) and by detection of the toxin genes using PCR (Havard *et al*, 1992; Daube *et al*, 1994). The diseases caused by strains of the different toxin types can be seen summarised in table 2.4..

Two enterotoxaemic syndromes due to *C. perfringens* have been described in pigs. The most frequently diagnosed form was due to the beta toxin of*C. perfringens* type C (MacKinnon, 1989; Johnson, Fitzgerald, Welter and Welter, 1992; Taylor and Bergeland, 1992). The affected pigs became ill during the first or second day of life. Death almost invariably occurred 12 to 24 hours after onset of clinical signs. Profuse diarrhoea, often blood red was detected, sometimes soiling the perineum of weak collapsed piglets. Sudden death was commonly seen. Necropsy examination of affected piglets revealed a swollen, dark red jejunum. The contents were either blood stained or vey watery. The intestinal mucosa presented a diphtheritic layer and haemorrhages. Histologically, the intestinal mucosa presented necrosis, desquamation of the villi and large numbers of Gram positive rods in the crypts. Bergeland (1981) reported more chronic forms of the disease that caused wasting and ill thrift from 10 days to weaning.

Recent reports have described less severe outbreaks in piglets caused by the enterotoxin of type A strains (Amtsberg, Bisping, Sukhon, Matthiesen, Krabisch and El, 1976; Taylor and Olubunmi, 1982; Estrada-Correa and Taylor, 1989; Collins *et al*, 1989; Okewole, Itodo, Oyetunde, Chima, Irokanulo and Ocholi, 1991).

Table 2.4. Diseases caused by *C. perfringens*.

Toxin type	Diseases
A	Gas gangrene (myonecrosis) and foodborne illness and infectious diarrhoea in humans.
	Enterotoxaemia in lambs ("the yellows"), calves, piglets, cattle, sheep and others.
	Necrotic enteritis in fowl.
	Equine intestinal clostridiosis.
B	Lamb dysentery.
	Sheep, goat and foal enterotoxaemia.
C	Darbrand (Germany) and Pig-bel (Papua-New Guinea) in humans.
	"Struck" in sheep.
	Enterotoxaemia in lambs and pigs.
	Necrotic enteritis in chickens.
D	Enterotoxaemia in lambs ("Pulpy kidney"), adult sheep, calves, cattle and camels.
E	Enteritis in rabbits and rarely in calves.

Based on Niilo (1980), Sterne (1981), Blood *et al* (1989) and Hatheway (1990)

2.2.4. Histotoxic clostridia

This group of Clostridia includes the species involved in "gas gangrene", "malignant oedema" and "clostridial myositis". These diseases occurred when infection of muscle tissue with histotoxic clostridia became established causing a severe necrosis, with gas formation, oedema and toxaemia (MacLennan, 1962; Sterne, 1981). These infections were believed to be more accurately described as clostridial myonecrosis which was unlikely to substitute the numerous terms found in clinical texts (MacLennan, 1962; Finegold, 1995). A summary of diseases in man and animals caused by these organisms is presented in table 2.5. Williams (1977) isolated *C. chauvoei* alone in 43% of cattle myonecrosis submissions. *C. novyi* was found as a sole agent in 31% of cases submitted, whilst *C. septicum* was found generally as part of mixed infections. The two species more commonly isolated in animal myonecrosis were *C. chauvoei* in ruminants and *C. septicum* in other species (Blood *et al*, 1989).

***Clostridium chauvoei*:** This Gram positive rod presented rounded ends, was sometimes pleomorphic, with oval subterminal spores which could swell the cell giving a lemon shaped appearance (Sterne, 1981). In tissue smears the organism occurred singly or in short chains whilst *C. septicum* and other wound isolates appeared as long chains (Sterne and Batty, 1975; Timoney *et al*, 1988). Identification with *C.chauvoei* specific fluorescent antibody has been suggested (Batty and Walker, 1963) and could differentiate between this species and *C. septicum*. Isolation of *C. chauvoei* required rapid processing of the tissue samples and a strict anaerobic atmosphere, preferably on supplemented media (Sterne and Batty, 1975). On blood agar a faint haemolysis occured and cooked meat broth appeared pinkish and cloudy. A characteristic odour of rancid butter has been used by experienced workers to tentatively identify *C. chauvoei* (Sterne and Batty, 1975).

Table 2.5. Myonecrosis and Gas Gangrene caused by clostridia.

Organism	Associated disease	Animals affected
C. chauvoei	Blackleg	Even toed ungulates, rarely pigs
	Wound infection, contaminated needles	Cattle and sheep
C. septicum	Malignant oedema and blackleg	Ruminants
	Blackleg	Pigs
	Braxy or Bradsot	Sheep
C. novyi type A	"Big head"	Rams
	Gas gangrene	Man and animals
C. novyi type B	Gas gangrene	Cattle
C. perfringens type A	Gas gangrene	Man
	Mixed infections	Animals
	Fatal metritis after induced abortions	Man
C. sordelli	Blackleg/malignant oedema	Cattle
C. hystolyticum	Gas gangrene	Man (rare)

Adapted from Sterne (1981)

The pathogenesis of blackleg in cattle has not been completely elucidated. Young, fast growing animals on a high plane of nutrition seemed to be more susceptible to the disease. Infection was believed to enter via the alimentary mucosa as pasture contamination and isolation of *C. chauvoei* from liver, spleen and alimentary tract from normal cattle were common (Kerry, 1964; Blood *et al*, 1989). Spores in muscle tissue could have been activated by unknown conditions which allowed toxin production, proliferation of the organism locally and generalised toxaemia. Blackleg progressed rapidly with death occuring 12 to 36 hours after the first clinical signs (Williams, 1977). Affected muscles presented a very dark red colour with localized oedema and gas bubbles.

Sheep became infected by contaminated injections, wounds due to fighting, docking, castration or parturition (Sterne, 1981). Valberg and McKinnon (1984) reported cases of clostridial cellulitis in horses and identified *C. chauvoei* in two occasions, whilst Sterne (1981) concluded that equidae and man were completely resistant to this species.

Blackleg in pigs has rarely been reported, although one well documented outbreak diagnosed cases in swine kept in a knackers yard after wounds became infected (Sterne and Edwards, 1955). Hygiene standards were poor and young cattle previously housed in that yard had recently suffered an outbreak of the disease. Another outbreak occurred when pigs were fed a dead calf. Calves kept at this farm recently suffered an outbreak of blackleg. The investigator described severe swelling and oedema around the face and throat of the affected pigs (Eggleston, 1950).

Clostridium septicum . Morphologically *C. septicum* was found to be similar to *C. chauvoei*, in fact some authors believed they were members of the same species (MacLennan, 1962). The two organisms were distinguished by the history, the diseases they caused and *post mortem* findings (Sterne and Thomson, 1963).

Malignant oedema, caused by *C. septicum*, presented more generalised lesions; subcutaneous oedema, fibrino-haemorrhagic peritonitis, lung oedema and amber fluid in the pleural cavity and pericardium. Usually a wound or source of entry for the organism could be found. The localised lesions presented pitting oedema and gas was not necessarily present.

C. septicum was described as a Gram positive rod of 0.6 to 0.8 μm by 3 to 8 μm long, straight with rounded ends (Cato *et al*, 1986). Spores were oval, subterminal and swell the cell. This species grew readily on ordinary media in anaerobic conditions, and often outgrew other pathogenic clostridia in clinical samples (Sterne, 1981). Haemolysis surrounded the colonies on blood agar plates, due to the haemolytic, lethal and necrotising alpha toxin (Ballard, Bryant, Stevens and Tweten, 1992). The alpha toxin has been purified and was found to be a basic protein of 48 kDa (Ballard *et al*, 1992). Antibodies raised against the *C. septicum* alpha toxin did not react with culture supernatants of *C. chauvoei* or other toxigenic clostridia.

C. septicum was considered the most common aetiological agent of clostridial myositis in pigs (Taylor and Bergeland, 1992). It also caused "Braxy" in sheep, by invading the mucosa and submucosa of the abomasum damaged by frozen grass. The invasion was followed by a rapidly fatal bacteraemia and toxaemia (Blood *et al*, 1989).

2.2.5. Other toxigenic clostridia

Clostrium difficile; Until the late 1970's *C. difficile* was considered a normal inhabitant of the intestinal tract of infants (Burdon, 1992). Later, Bartlett, Onderdonk, Cisneros and Kasper (1977) demonstrated antibiotic induced enterocolitis in hamsters after treatment with clindamycin and isolated a toxigenic clostridia. *C. difficile* was identified as the cause of pseudomembranous colitis by Burdon and George (1978).

The gut of infants was thought to be the main habitat of C. *difficile*, but spores could be found in soil and the environment of hospital wards (Princewill, 1985; Levett, 1986). Domestic animals were known to carry the organism as part of their flora; 40% of cats and dogs sampled in a veterinary hospital (Riley, Adams, O'Neill and Bowman, 1991), horses, a donkey, cattle, a camel, a seal, goose, duck, hamster and a snake, even a laboratory locust (Levett, 1986) have been confirmed as carriers. Riley *et al* (1991) suggested domestic pets should be considered as potential reservoirs of infection. Antibiotic associated colitis has been described in rabbits (Rehg and Shoung, 1981), guinea pigs (Lowe, Fox and Bartlett, 1980), foals (Timoney *et al*, 1988) and pigs (Jones and Hunter, 1983).

Clostridium colinum caused ulcerative enteritis or "quail disease" in quail, pheasant and chickens (Berkhoff, 1985). Orally infected birds suffered 0.1 to 2 mm discrete ulcers in the lower third of the intestine (Berkhoff and Campbell, 1974). C. *colinum* entered the liver via the intestinal wall and portal circulation causing diffuse liver necrosis.

2.2.6. *Clostridium novyi and Clostridium haemolyticum*

Like many other clostridia the early history of C. *novyi* was clouded in difficulties regarding classification and identification. The organism was first isolated and named *Bacillus oedematiens maligni* from guinea pigs that had been inoculated with casein and suffered malignant oedema (Novy, 1894 cited by Willis, 1989), but the name was changed to *Bacillus novyi* (Migula, 1900 cited by Smith and Williams, 1984c). Weinberg and Seguin (1915, cited by MacLennan, 1962) demonstrated *Bacillus oedematiens* from cases of gas gangrene during the first World War. These isolates were reclassified as *Clostridium oedematiens/novyi* (Bergey, Breed, Murray and Hitchen, 1939).

The first isolation from a case of necrotic hepatitis in sheep was made by Albiston (1927), by which time the nameC. *oedematiens* was used, but German workers isolated an organism from necrotic hepatitis and named it *Bacillus gigas* (Zeissler and Rassfeld, 1929 cited by Smith and Williams, 1984c) complicating matters further. At this time Kraneveld (1934, cited by Prevot, 1965), described a similar bacillus from cases of osteomyelitis in water buffalo, which Prevot (1965) named *Clostridium bubalorum*. These strains later became the non-pathogenic *C. novyi* type C. The name *C. oedematiens* remained in frequent use in the veterinary literature until the adoption of *C. novyi* (Cato *et al*, 1986). Prevot (1965) in his nomenclature of the genus, suggested a subgenus within *Clostridium*, that included 9 species of these *oedematiens* strains based on their morphological, cultural and pathogenic characteristics. Other authors (Scott, Turner and Vawter, 1934) divided *C. oedematiens* into three types on that basis, A, B and C. Studies on the toxin production by strains from those 3 types confirmed their findings and established the use of toxins for the classification of this species (Oakley, Warrack and Clarke, 1947). Previous attempts by Turner and Eales (1943) to group the different strains according to their "H" and "O" antigens were not practical. In some texts, *Clostridium haemolyticum* has been classified as a separate species because the presentation of the disease it causes in cattle, bacillary haemoglobinuria, was so different to the pathology induced by the otherC. *novyi* types (Cato *et al*, 1986). Other workers described *C. haemolyticum* as *C novyi* type D (Willis, 1977). This classification was justified because type D strains were found to share somatic antigens with *C. novyi* type A and B (Poxton, 1984), had high levels of rRNA homology with other *C. novyi* strains (Johnson and Francis, 1975), presented DNA-DNA homology of 90 to 100% (Nakamura, Kimura, Yamakawa and Nishida, 1983), produced beta toxin indistinguishable from *C. novyi* type B (Oakley and Warrack, 1959) and resembled *C. novyi* culturally and morphologically.

1. Morphology and staining characteristics

C. novyi was described as a large Gram positive rod, motile by means of peritrichous flagellae (Smith and Williams, 1984c). The size varied between toxin types (Cato *et al*, 1986). Type A strains measured 1-1.5 μm by 1.6-10μm, whilst type B isolates were larger, 3.3-22.5 μm in length. *C. novyi* sporulated readily and spores were oval, central or subterminal, swelling the cell.

2. Cultural characteristics

C. novyi was described as a strict anaerobe, particularly *C. novyi* type D (*haemolyticum*) which Willis (1977) considered to be one of the most fastidious clostridia known. Type A strains grew readily on freshly prepared pre-reduced blood agar, but strains belonging to types B and D required a supplemented media for luxuriant growth (Collee *et al*, 1971). Good growth on solid media was obtained with Moore's neopeptone glucose blood agar medium (Moore, 1968). Moore's medium included cysteine and dithiothreitol as reducing agents, whilst other workers achieved improved growth simply by sprinkling iron filings over the surface of plates (Collee, Rutter and Watt, 1971).

Sterne and Batty (1975) warned against placing too much reliance on colonial morphology for identification of *C. novyi*. The appearance of individual colonies varied between strains and depending on the medium used for isolation (Smith and Williams, 1984c). Cultures on blood agar were β haemolytic, with the exception of the non haemolytic type C strains. *C. novyi* formed irregularly circular colonies, 2-3 mm in diameter, semitranslucent or grayish, with a finely lobate or crenated edge, after 48 hours incubation on blood agar plates. The surface appeared granulated in Type A strains, whilst the *C. novyi* type B strains presented a more glossy colony. Particularly on wet plates, some strains may have been difficult to detect as the organism swarmed finely over the medium.

Further characterisation of this species was demonstrated on egg yolk agar plates (Nakamura *et al*, 1983). *C. novyi* type A strains produced a diffuse opalescence and pearly layer, due to lipolysis, coextensive with the colony. Both *C. novyi* type B and D only produced the diffuse opalescence. Strains of *C. novyi* Type D produced extensive haemolysis and diffuse opalescence due to abundant production of beta toxin.

The optimum temperature for growth was determined. Ideal growth was demonstrated at 45°C, but growth at 37°C was generally as good (Cato *et al*, 1986).

Spores of *C. novyi* survived heating at 100°C for 5 minutes (Willis, 1977), but Nishida and Nakagawara (1965) suggested that sporulating potency and toxigenicity were inversely related. This was believed to preclude the use of heat shock for selective isolation of toxigenic *C. novyi*.

Cooked meat broth supported good growth with slight gas production, generally after 48 hours (Collee *et al*, 1971).

3. Biochemical characteristics

All strains of *C. novyi* fermented glucose and liquified gelatin, milk and cooked meat were weakly digested by type B strains but *C. novyi* type A strains only produced slight acidification (Nord, Wadstrom, Dornbusch and Wretlind, 1975; Holdeman *et al*, 1977; Cato *et al*, 1986). Other carbohydrate fermentations were variable depending on the laboratory and the medium used, but mannose was not fermented by strains of *C. novyi* type A (Rutter, 1970; Roberts, Guven and Worral, 1970a; Nord *et al*,1975; Holdeman *et al*, 1977). Indole was produced in large amounts by *C. haemolyticum*, but other *C. novyi* types could be indole negative, particularly strains of type A (Smith and Williams, 1984c). Copious hydrogen peroxide was produced by strains of all types, a fact that was thought to explain the extreme sensitivity of this clostridial species to growth in air (Smith and Williams, 1984a).

Major products of metabolism detected by gas liquid chromatography included propionic and butyric acid, with small amounts of acetic and valeric acid produced (Holdeman *et al*, 1977).

4. Antigenic structure

Initial studies on the "H" and "O" antigens of *C. novyi* were carried out by Turner and Eales (1943). These researchers found that isolates from the various strains loosely classified in the *oedematiens* group shared two "O" antigens in various combinations, but presented variation in respect of the "H" antigens. Batty and Walker (1964) prepared specific antisera to the "O" antigens of *C. novyi* type B. These antibodies were labelled with fluorescein and used to detect the organism and the other *C. novyi* types in tissue and culture smears (Bagadi, 1973).

Poxton (1984) and Poxton and Byrne (1984) demonstrated shared antigens between *C. novyi* type A and *C. botulinum* types C and D by ELISA. Both whole cells and EDTA extracted cell wall material were used as antigens.

5. Toxins

The classification of the species *C. novyi* was based on work carried out by Oakley, Warrack and Clarke (1947) and Oakley and Warrack (1959). These authors examined the various toxins in culture fluids of different isolates with similar phenotypes (see table 2.6.). Considering the main toxins, type A produced alpha toxin only, whilst type B produced both alpha and beta toxins. Large amounts of beta toxin, but no alpha toxin were found in culture filtrates of *C. novyi* type D (*haemolyticum*), whilst type C isolates were considered non toxigenic and non pathogenic.

Table 2.6. Toxins of *Clostridium novyi*.

Toxin	Activity	*C. novyi*			
		Type A	Type B	Type C	Type D
Alpha	Necrotizing, lethal	+	+	-	-
Beta	Lecithinase; necrotizing; lethal; haemolytic	-	+	-	+
Gamma	Lecithinase; necrotizing; haemolytic	+	-	-	-
Delta	oxygen labile haemolysin	+	-	-	-
Epsilon	Lipase (pearly layer)	+	-	-	-
Zeta	Haemolysin	-	+	-	-
Eta	Tropomyosinase	-	+	-	+
Theta	Opalescence in egg yolk	-	traces	-	+

Adapted from Oakley and Warrack (1959) and Smith and Williams (1984c).

Problems regarding toxin typing were reported by various workers, particularly trying to differentiate types B and D (Rutter and Collee, 1969; Roberts *et al*, 1970a; Nakamura, Takematsu and Nishida, 1975). Recently, an ELISA for the detection of alpha toxin, that was less labour intensive and eliminated the use of large numbers of laboratory animals has been developed (Pietrzykowski, Cox, Zachariou and MacGregor, 1991).

Mounting evidence has suggested that the ability to produce toxins by the closely related *C. botulinum* type C and type D, *C. novyi* and *C. novyi* type D (*haemolyticum*) was linked to infection with specific bacteriophages (Schallehn and Lenz, 1975; Schallehn andKramer, 1981). Eklund *et al*, (1974) converted a non toxigenic *C. botulinum* to *C. novyi* type A using a bacteriophage. Further work by this group (Eklund, Poysky, Peterson and Meyers, 1976) studied the relationship between specific bacteriophages and the production of the lethal alpha toxin in *C. novyi* types A and B. They proved that the presence of specific bacteriophages was required for the production of alpha toxin. Type B strains cured of their tox^+ phage became indistinguishable from *C. novyi* type D. To confirm these findings Schallehn and Eklund (1980) converted a *C. novyi* type D isolate into an alpha toxin producing type A, by infecting it with bacteriophages from donor type A strains. The close taxonomic relationship between these *Clostridium* species was later confirmed by DNA-DNA homology studies (Nakamura *et al*, 1983).

Alpha toxin. This toxin was believed to be responsible for gas gangrene in humans and animals (Smith and Williams, 1984c) and infectious necrotic hepatitis in sheep (Bagadi, 1974). Phillips and Batty (1963) and Phillips, Batty and Wood (1970) first described attempts to purify the alpha toxin from *C. novyi* type B using gel filtration, absorption onto alumina and ultracentrifugation.

The molecular weight of the purified toxin was estimated to be 132.000 and because of the high molecular weight thought unlikely to be a single polypeptide chain (Phillips *et al*, 1970). Further studies by Izumi, Kondo, Ohishi and Sakaguchi (1983a) purified alpha toxin from *Clostridium novyi* type A. The purified toxin was found on sodium dodecyl sulphate polyacrylamide gel electropheris (SDS-PAGE) and gel filtration on columns to be a single peptide with a molecular weight between 260 and 280.000. The purified toxin was found to be responsible for the lethal, vascular permeability and oedematizing effects, but not the haemolysis or the lecithinase activities (Izumi *et al*, 1983a; Izumi, Niiro and Kondo, 1983b; Izumi, Tokumaru and Kondo, 1983c).

More recent work suggested a single, large polypeptide (200 kD) for the alpha toxin of *C. novyi* type A (Bette, Frevert, Mauler, Suttorp and Habermann, 1989; Bette, Oksche, Mauler, Eichel-Streiber, Popoff and Habermann, 1991; Ball, Van Tassell, Roberts, Hahn, Lyerly and Wilkins, 1993) and similar values for the alpha toxin produced by type B isolates (Pietrzykowsky *et al*, 1991; Schranner, Erhard, Kaltner and Losch, 1992).

Ball *et al* (1993) determined that the alpha toxin was an acidic protein stable at 4^{o}C and following exposure to buffers with a pH between 4 to 7. The isoelectric point was determined at 5.8 to 6.0. The toxin was inactivated by proteases, oxidizing agents and harsh detergents.

Schranner *et al* (1992) cleaved the purified alpha toxin and examined the immunogenicity and lethality of the peptide fragments. Immunogenic peptide fragments appeared to be in different locations in the polypeptide chain to the lethal fragment. This raised the possibility of preparing a highly specific, non toxigenic vaccine to protect against gas gangrene and necrotic hepatitis.

The alpha toxin produced byC. *novyi* type A and type B strains was antigenically indistinguishable (Oakley *et al*, 1947) and had approximately the same size (Pietrzykowsky *et al*, 1991; Ball *et al*, 1993).

However, Schranner *et al* (1992) determined the N-terminal amino acid sequence (Arg-Phe-Pro-Ala) of an alpha toxin from a C. *novyi* type B strain, that differed completely from the amino acid sequence (Met-Leu-Ile-Thr-Glu-Gln-Leu-Met-Lys) reported by Ball *et al* (1993) for the type A alpha toxin.

The action of C. *novyi* alpha toxin was first studied by Elder and Miles (1957) and Aikat and Dible (1960). Both groups used crude culture filtrates and experimental animals to study the copious oedema seen in clinical cases of clostridial myonecrosis. They concluded that the toxin increased capillary permeability for an unexpectedly long time (96 hours), that intramuscular injection led to disruptive oedema of connective and muscle tissue with only a mild inflammatory reaction and that these changes could be neutralised by specific antitoxins. Lethality (Izumi, Tokumaru, Amano, Niiro and Kondo, 1979; Izumi *et al*, 1983b) and dermonecrotic effects (Sterne and Batty, 1975) were demonstrated. Cotran (1967) confirmed these findings by inducing a delayed, long lasting increase in vascular permeability after injection of sub-necrotizing doses of alpha toxin. Histologically, intercellular gaps were seen in the endothelium accompanied by cellular changes that indicated increased activity. Bette *et al* (1989) attempted to quantify these changes in vivo using rat paw oedema. Volumetric measurement were made after inoculation of known concentrations of toxin.

Further studies on the mechanism of action of the alpha toxin used cultured endothelial cells to the study the effect on cellular morphology (Bette *et al*, 1989; Bette, Mauler, Mohr and Habermann, 1990; Bette *et al*, 1991; Muller, von Eichel-Streiber and Habermann, 1992; Oksche, Nakov and Habermann, 1992).

This work with endothelial cell culture confirmed earlier findings and determined that; i) a latency period dependant on concentration of the alpha toxin was seen, ii) the effects on endothelial cells were long lasting and not reversible by removing the cells from toxin containing medium or by microinjection of alpha antitoxin, iii) the cell rounding, retraction and loss of the coblestone appearance of cultured pulmonary artery endothelial cells was due to damage to the microfilament system. Other structural changes observed in the nucleus were secondary, iv) the enlarged mitochondria and endoplasmic reticulum suggested increased cellular metabolism, v) the target for the alpha toxin was within the cytoplasmic compartment. Initial binding to a receptor on the cell wall was thought necessary, vi) the toxin did not ADP-ribosylate actin.

Bette *et al*, (1991) and Ball *et al*, (1993) suggested that the alpha toxin of C. *novyi*, toxin B of C. *difficile* and C. *sordelli* lethal toxin should be arranged into a common group of large cytotoxins that perturb the microfilament system.

Beta toxin. Oakley *et al*, (1947) discovered that lecithinases from C. *oedematiens* type A strains (gamma toxin) and from type B strains (beta toxin), with a similar enzymatic effect, were in fact different proteins. Oakley and Warrack (1959) demonstrated that the haemolytic, necrotizing and lethal activities of C. *haemolyticum* (C. *novyi* type D) were due to a lecithinase, the beta toxin. The phospholipase C activity of culture filtrates of C. *novyi* type B and C. *haemolyticum* was neutralised byC. *novyi* type B antiserum, but not by C. *novyi* type A or C. *perfringens* antisera (Oackley *et al*, 1947; Macfarlane, 1950). More beta toxin was produced by C. *novyi* type D isolates when compared to C. *novyi* type B strains (Nakamura *et al*, 1975). Nakamura *et al* (1983) determined that serologically beta toxin from C. *novyi* strains was also comparable to a lecithinase produced by C. *botulinum* type C.

The beta toxin from *C. haemolyticum* was purified by gel filtration chromatography and the molecular weight estimated at 32.000 (Darakhshan and Lauerman, 1981). The toxin was labile at 60°C and inactivated by trypsin.

Detection of the lecithinase activity was carried out by detecting opalescence in the lecithovitellin (LV) preparations inoculated with culture filtrates (Rutter and Collee, 1970; Willis, 1977). Appropriate neutralization with antiserum inhibited the reactions.

Gamma toxin. The gamma toxin, a phospholipase C, was demonstrated in culture filtrates of *C. novyi* type A and was serologically distinct from other phospholipases produced by clostridia (Macfarlane, 1948). The gamma toxin was purified by Taguchi and Ikezawa (1975) using gel filtration chromatography. The estimated molecular weight was 30 kDa and the enzyme hydrolysed lecithin and also catalyzed the hydrolysis of phosphatidylinositol and phosphatidylglycerol.

Delta toxin. The delta toxin was described as an oxygen labile haemolysin, produced by type A strains only (Willis, 1977). Serologically it appeared to be related to the haemolysin of *C. tetani* and theta toxin of *C. perfringens* (Hatheway, 1991).

Zeta toxin. This haemolysin was only produced by type B strains (Willis, 1977), was not oxygen sensitive or related to other oxygen labile haemolysins (Smith and Williams, 1984c).

Epsilon toxin. The epsilon toxin was used by Rutter and Collee (1969) to identify *C. novyi* type A strains by demonstrating the "pearly layer" on egg-yolk agar plates.

The soluble antigen was found to be a lipase that decomposed tryglycerides and dyglycerides in the medium. *C. botulinum* type C and D strains also produced epsilon toxin (Rutter and Collee, 1969).

Eta toxin. Macfarlane (1955) described a sulphydryl-activated enzyme that decomposed tropomyosin. The toxin was only found in culture filtrates of *C. oedematiens* (*novyi*) type B and *C. haemolyticum.*

Other biologically active substances produced in culture by *C. novyi* were demonstrated: theta toxin, a lipase, was found in *C. haemolyticum* (Oakley and Warrack, 1959), but was not responsible for opalescence on egg yolk agar. Hyaluronidase was found in culture filtrates of *C. novyi* type B (Smith and Williams, 1984).

6. Distribution in nature

C. novyi has been isolated from soil, sea sediments and forms part of the normal intestinal flora of animals (Smith and Williams, 1984c). *C. novyi* spores were identified in the liver of healthy sheep, rabbits, cattle and one crow grazing in Black disease (infectious necrotic hepatitis) pastures by Jamieson (1949). Other workers confirmed these findings in cattle and sheep (Corbould, 1966; Niilo, Dorward and Avery, 1969; Roberts *et al*, 1970b; Bagadi and Sewell, 1973a). Nishida and Nakagawara (1964) isolated toxigenic *C. novyi* type A from all 62 soil samples collected from different areas in Japan. *C. haemolyticum* was also isolated from liver and kidney of normal cattle (Smith and Jasmin, 1956) and a relationship to swampy, wet, alkaline soils was suggested by Van Ness and Erickson (1964).

2.2.6.1. Clostridium novyi infection in animals and man

Myonecrosis (Gas gangrene, malignant oedema) in man.

During World War I and World War II, investigators found *C. novyi* , then known as *C. oedematiens* in 30% to 40% of gas gangrene cases (MacLennan, 1943; MacLennan, 1962). In his review of histotoxic clostridial infections MacLennan (1962) described *C. novyi* myonecrosis. The average time from wounding to the onset of disease was 5.25 days. The first symptom, preceeding pain, was a sense of increased weight on the affected part. Once pain was felt the course of the diseae was acute, with rapid development of toxaemia and a very profuse golden yellow fluid discharging from the wound. Gas and odour were rarely a feature of these infections, the skin and muscle usually showed pallor and only ocassionally haemorrhages. Later the muscles became dark red, purple and soft. The patient's demeanour was apathetic and delirious approaching death. The infections were characterised by oedema and a generalised toxaemia that was not in proportion to the area of infected tissue.

For clostridial myonecrosis to develop some important factors were thought to be necessary; i) a devitalising injury and deep laceration to large muscle masses (gluteal, thigh), ii) contamination with foreign bodies, iii) impaired blood supply and, iv) delay in surgical treatment (Smith, 1977).

Infectious necrotic hepatitis ("Black disease").

Infectious necrotic hepatitis was described by Sewell (1975) as an acute toxaemic disease caused by the elaboration in necrotic liver tissue of alpha toxin of *C. novyi* type B.

Infectious necrotic hepatitis has been described, mainly in sheep, in Australia (Albiston, 1927; Osborne, 1958), the U.K. (Jamieson, Thomson and Brotherston, 1948; Williams, 1962; Bagadi and Sewell, 1973a), New Zeland (Wallace, 1966), Africa (Abu Samra, El Sanusi, Idris, Bagadi, Salam, Ali and Musa, 1984), Iran (Ardehali and Darakhshan, 1979), South America (Gonzalez, De Lucia and Rodriguez, 1981; Moreira and Salamanco, 1991) and India (Jayaraman and Harbola, 1971). Cattle dying from infectious necrotic hepatitis were reported less commonly by Williams (1964) in Wales, Niilo *et al* (1969) and Kelch, Coles, Reynolds and Bailey (1977) in the U.S.A. and by Samad and Haque (1987) in Bangladesh. The disease has been rarely reported in other species; in the horse (Hollingsworth and Green, 1978; Gay, Lording, McNeil and Richards, 1980), the goat (Hamid, Mohamed, Abu Samra and Hamad, 1991) and the pig (Wise and Munday, 1964).

The aetiology and pathogenesis of infectious necrotic hepatitis were explained, firstly by the work of Turner (1939) and Jamieson (1949) and later by the work of Bagadi and Sewell (1974a). Albiston (1927) was the first author to associate *C. novyi* with cases of "black disease" in sheep. Turner (1939) established that latent spores of *C. novyi* were present in the body of healthy sheep. Jamieson (1949) published the first experimental confirmation of the need for latent *C. novyi* type B spores in the liver to be activated by migrating immature liver fluke. This, alas, was demonstrated in guinea pigs and rabbits after infection with spores by the intravenous or intraperitoneal routes. Bagadi and Sewell (1973b) induced the disease in sheep by previous infection with metacercariae of *Fasciola heopatica* followed by oral infection with spores two weeks later. Sheep that presented serological and parasitological evidence of severe liver damage by flukes appeared more likely to die from infectious necrotic hepatitis. Death occurred 6 to 8 weeks after infection with *F. hepatica*, when maximal parenchymal damage to the liver had been demonstrated (Bagadi, 1974).

Spores of *C. novyi* were isolated from soil (Nishida and Nakagawara, 1964) and found in the liver of sheep from "black disease" areas (Bagadi and Sewell, 1973a), but the route of dissemination of ingested spores was unknown. Experimental studies showed that the spores could be recovered from the mesenteric lymph nodes by 12 hours post ingestion and the liver by 24 hours after administration (Bagadi and Sewell, 1974a). At no stage were *C. novyi* isolated from heart blood, portal circulation or kidneys. On one occasion the organism was recovered from a peritoneal wash in a guinea pig. The authors suggested dissemination occurred via the lymphatic system.

Other parasites that cause hepatic injury, like *Cysticercus tenuicollis* and *Echinococcus granulosus* predisposed to infectious necrotic hepatitis (Sewell, 1975). Damage by hepatotoxic chemicals (Jamieson, 1949) and liver biopsy (Duncan, 1984) also allowed latent spores to germinate, production of alpha toxin by the multiplying *C. novyi* and acute toxaemia.

The disease, well described only in sheep, was striking because sick animals were rarely seen and the period between first appearance of symptoms and death was usually only a few hours (Bagadi, 1974). Sheep were found dead or recumbent, on their briskett (Jamieson *et al*, 1948). When still alive the animals lagged behind the herd, lay on their brisket, with rapid, shallow breathing and an elevated rectal temperature. The rectal temperature dropped to subnormal levels shortly before death (Williams, 1962). The sheep on occasion presented neurological signs; hyperaesthesia, twitching of head and ears, but usually died without a struggle (Jamieson *et al*, 1948).

The most characteristic *post mortem* changes occurred in the liver (Sewell, 1975). The whole organ appeared congested with a dark brown or blueish colour and a distended gall-bladder (Jamieson, 1949). Usually, haemorrhagic or necrotic tracts caused by migrating liver flukes could be seen on the capsule and parenchyma.

One or more sharply demarcated, yellow, necrotic areas of various sizes, surrounded by a well defined area of congestion were described by Albiston (1927), Jamieson (1949) and Williams (1962).

These changes when viewed under the microscope were regarded as pathognomonic (Bagadi,1974). On histological examination the lesions showed a central area of coagulative necrosis surrounded by a thin rim of leukocytes, mostly polymorphonuclear cells, outside of which was an area of intense congestion and necrotic hepatic parenchyma. Numerous large Gram positive rods with subterminal spores were seen in the inflammatory border. Elsewhere in the carcass frothy discharge from the mouth and nostrils, extensive dark ventral subcutaneous haemorrhage and oedema could be seen. Body cavities were generally filled with moderate amounts of clear, straw coloured fluid.

Bacillary haemoglobinuria in cattle.

This acute, highly fatal toxaemia of cattle and rarely sheep, was characterized clinically by high fever, haemoglobinuria and jaundice, and the presence of necrotic infarcts in the liver (Stogdale and Booth, 1984). The disease, caused by the beta toxin of *Clostridium haemolyticum* (*C. novyi* type D) was first described in the U.S.A. (Meyer, 1916) and the organism first isolated by Records and Vawter (1945 cited by Williams, 1964). The disease has been reported around the world (Marshall, 1959; Niilo *et al*, 1969; Wellington and Perceval, 1966) and was first reported in the U.K. by Soltys and Jennings (1950) at the Liverpool Veterinary School.

C. haemolyitcum was found mainly in soil and water (Stogdale and Booth, 1984), was recovered from bones of fallen cattle (Jasmin, 1947) and isolated from the liver and kidneys of healthy cattle (Smith and Jasmin, 1956).

Williams (1964) postulated that thrombosis of a subterminal branch of the portal vein lead to an infarct in which latent *C. haemolyticum* spores could germinate. The disease was caused experimentally by inducing liver damage with a liver biopsy (Olander, Hughes and Biberstein, 1966) or by direct liver inoculation of spores suspended in calcium chloride (Erwin, 1977). In the field, liver flukes were thought to play an important part in the pathogenesis of bacillary haemoglobinuria (Erwin, 1978), but attempts to confirm this hypothesis were not succesful. Blood *et al* (1989) suggested other hepatic injuries like necrobacillosis caused by *Fusobacterium necrophorum*, as possible precipitating factors. Batty *et al* (1964) believed fatty liver, a metabolic disturbance resulting in reduced liver perfusion, was implicated in cases in beef cattle. Once the appropriate reduced conditions were present in the liver parenchyma the *C. haemolyticum* spores harboured within the Kupffer's cells germinated (Stogdale and Booth, 1984). According to these authors the beta toxin was then produced, causing lysis of the cytoplasmic membrane and releasing vegetative bacteria. Local lesions self-propagated in the liver parenchyma until lethal amounts of the toxin were produced. Erythrocytes were lysed by the increasing concentration of toxin, giving rise to the clinical signs of toxaemia, haemolytic anaemia and haemoglobinuria.

Clinically, a good description of bacillary haemoglobinuria was provided by Williams (1964). In these cases haemoglobinuria was not always a feature of the disease as described by others (Wellington and Perceval, 1966). Sudden death was found in one third of cases. Others showed acute cessation of appetite, rumination and gut movements, arched back and an elevated rectal temperature that later dropped below normal. Blood stained faeces or frank dysentery were noted. Mucous membranes became jaundiced and pale. Death usually occurred around 36 hours after the initial clinical signs.

Williams (1964) also recorded brisket, conjunctival and submandibular oedema. Two suspected cows investigated by this author recovered after treatment with penicillin. Serum beta antitoxin titres measured 4 weeks after clinical disease had risen by almost 20 times in the recovered cases.

At necropsy typical findings included generalised jaundice, subcutaneous oedema, blood stained fluid in thoracic, pericardial and peritoneal cavities. Widespread subcutaneous, serosal and mucosal haemorrhages, including the intestines and abomasum, which contained bloody contents. The liver was mahogany coloured with one or two anaemic infarcts of between 5 and 20 cm in diameter, surrounded by a zone of hyperaemia (Stogdale and Booth, 1984). Many cases presented petechiation of the kidney and discoloured urine in the bladder and kidney. Other cases appeared like infectious necrotic hepatitis without distinctive pathological lesions and isolation of *C. haemolyticum* instead of *C. novyi* type B (Williams, 1964).

Bacillary haemoglobinuria has rarely been described in sheep (Williams, 1962) and one case with large, pale liver infarcts was described in the pig, confirmed by isolation of a toxigenic *C. haemolyticum* (Records and Huber, 1931).

Other infections caused by *C. novyi*.

Isolation of *C. novyi* was reported in the literature sporadically in other animal species and conditions.

"Bighead" in rams was described by Smith and Williams (1984c). The disease was characterized by marked swelling of the neck and head. Young males were the only group affected. The disease was thought to occur after fighting caused bruising and battered subcutaneous tissues, ideal for the proliferation of *C. novyi*.

Wallace (1966) examined sheep that had died unexpectedly after lambing. Fluorescent labelled antibody indicated a large proportion of these ewes had evidence of *C. novyi* in uterine smears. No description of lesions in the uterus from these cases was put forward by the author.

In companion animal practice rare isolations of the bacterium have been published; in dogs *C, novyi* was demonstrated as a complication in a case of splenic torsion, with concurrent multifocal necrosis of the liver (Moreau and Henley, 1981). Another case reported isolation of *C. novyi* from liver lesions in a dog suffering from pancreatic acinar carcinoma (Love, Maddison, Finnimore and Rothwell, 1981). In the cat, two reports isolated *C. novyi*, one from a case of gas gangrene after aortic embolism (Schiffer and Miller, 1982), the other in case of disseminated osteomyelitis (Dunn, Farrow and Doige, 1983). The origin of the bacterium in all these clinical cases was not discovered, although the authors assumed an endogenous origin for the clostridia.

In one horse out of a series of five cases of equine clostridial cellulitis *C. novyi* was isolated in mixed culture (Valberg and McKinnon, 1984). The authors concluded *C. novyi* was not a common cause of cellulitis or myonecrosis in the horse.

In broiler chicken, *C. novyi* type A was isolated from the hock and other leg lesions that led to severe lameness, posibly initiated by trauma (Peterson, 1964). There was severe arthritis in the hock joint, oedema, haemorrhages and sometimes gas in subcutaneous tissues. Clostridia were also isolated from the liver, spleen, heart and kidney.

The review of published literature concerning *C. novyi* sudden death cases in pigs can be found in section 2.6.e.

2.3. Treatment of clostridial infections and the use of vaccines in the prevention of clostridial diseases

Early work concentrated on the treatment and prevention of tetanus, firstly with antitoxin for protection of horses (Mac Farland and Ranck, 1900), which was put to invaluable use during the First World War (Willis, 1989). Ramon and Zoeller (1927, cited by Willis, 1989) developed the formol inactivated toxoid for active immunisation of human subjects, that consequently saved many lives during the Second World War. Tetanus immunisation has been routine in horses for some time (Sterne, 1981) and has formed an essential part of multicomponent vaccines used in livestock (Hjerpe, 1990).

Since the pioneering work of Mason, Dalling and Gordon (1930, cited by Ripley and Gush, 1983) the importance of protecting breeding livestock against clostridial diseases and the passive transfer of protection via the colostrum to the dam's offspring have been fully realised.

Sterne, Batty, Thomson and Robertson (1962) developed the first multicomponent clostridial vaccine for sheep. Immunisation required large volumes of the alum precipitated antigens and repeated inoculations.

Improvements were proposed with the preparation of a septavalent vaccine that contained *C. perfringens* beta and epsilon toxoids, *C. septicum* alpha toxoid, *C. novyi* alpha toxoid, *C. tetani* toxoid and *C. chauvoei* antigen, emulsified in oil (Thomson, Batty, Thomson, Kerry, Epps and Foster, 1969). Unacceptable tissue reactions were found after intramuscular inoculation, so the authors suggested an initial intraperitoneal injection followed by a water based subcutaneous booster inoculation. This protocol assured excellent immunity and good passive protection to lambs.

At around this time the prevention of gas gangrene after contaminated bullet wounds was investigated using experimental sheep (Boyd, Thomson and Walker, 1972). Active and passive immunisation was compared to prevention by prophylactic use of penicillin. Sheep immunised with a mixed vaccine containing toxoids of *C. perfringens, C. septicum* and *C. novyi* demonstrated an almost complete protection against gas gangrene.

Early problems with production of commercial vaccines concerned the potency testing of the various antigens (Frerichs and Gray, 1975) in particular *C. novyi* toxoids (Macheak, 1975). Webster and Frank (1985) suggested sheep rather than experimental animals produced a more reliable measure of efficacy and immune response to administered clostridial vaccines. Harbola and Verma (1988) found *C. novyi* type B toxoids highly immunogenic and protective in sheep. Recently, Pietrzykowski *et al* (1991) developed an enzyme immunoassay for the detection of *Clostridium novyi* type B alpha toxin that eliminated the need to test toxoids and vaccines *in vivo*.

Various improvements to clostridial vaccines have ensued over the years (Kerry and Craig, 1979; Sterne, 1981) and currently numerous commercial adjuvanted multivalent vaccines for the protection of sheep, cattle, goats and pigs can be used (Debuf, 1994). Walker (1992) reviewed the use of clostridial vaccines in livestock and concluded that highly efficatious, crude antigen preparations had proven highly successful. The economic benefits of clostridial vaccination in cattle kept in feedlots was assessed by Knott, Erwin and Classick (1985), who found huge savings in reduced mortality alone and a gain of $ 10.30 per head of cattle.

In pigs, vaccination was recommended mainly to control necrotic enteritis due to *C. perfringens* type C in neonatal piglets (Taylor and Bergeland, 1992).

A protocol that consisted of vaccinating gilts at mating and three weeks before farrowing suggested by Ripley and Gush (1983) was highly effective in preventing necrotic enteritis. The importance of vaccination against clostridial necrotic enteritis in outdoor pig production was stressed by MacKinnon (1989) and Waddilove and Wilkinson (1994). Sudden death due to gastric torsion andC. *novyi* infection in adult pigs was controlled by vaccination with a multivalent clostridial vaccine in conjuction with other measures by Blackburn *et al* (1974), and was the recommended method of control for this disease in problem farms (Taylor and Bergeland, 1992).

Some exciting developments in clostridial vaccines have recently been reported; Schranner *et al* (1992) proposed a C. *novyi* alpha toxin vaccine using only the immunogenic portion of the large toxin polypeptide, and Williamson and Titball (1993) reported a genetically engineered vaccine that protected mice against gas gangrene caused by the C. *perfringens* alpha toxin.

Treatment of clostridial infections in animals was considered of little value except in blackleg infections in cattle, when antibiotics if used early enough could have some beneficial effect (Sterne, 1981).
In the face of an outbreak of enteritis in lambs, calves or piglets due to C. *perfringens* types B, C and D, specific antitoxins have been used (Blood *et al*, 1989). Taylor and Bergeland (1992) described the use of beta antitoxin in necrotic enteritis outbreaks in piglets on farms where the sows had not been vaccinated. Parenteral injection of antitoxin as soon as possible after birth and concomitant use of oral antimicrobials were advocated. Prophylaxis by passive immunisation was considered much more valuable as long as the piglets ingested sufficient colostrum (Ripley and Gush, 1983).

The use of antibiotics for control of clostridial infections has been almost exclusively confined to intensive broiler chicken production. Growth promoting and therapeutic antimicrobials have been used to treat and prevent necrotic enteritis in poultry (Blaxland, Cullen, Gordon and Jordan, 1982).

Schultz, Rock, Cue and Schultz (1986) suggested the use of bacitracin methylene disalicylate in sow feed during late pregnancy and lactation for the prevention of *C. perfringens* type C enteritis in suckling pigs. The authors believed the carriage rate of this organism in the sows intestinal tract was reduced, but advocated the continued used of vaccination and hygiene for the control of this disease in piglets.

Dutta, Devriese and Van Assche (1983) examined the susceptibility of various clostridial species isolated from the caeca of pigs, cattle and poultry to 21 antimicrobials, including some growth promoting agents. Chloramphenicol was the most active *in vitro* followed by Penicillin G, the lincosamines and tetracycline. *C. butyricum* was found to be resistant to penicillin G thanks to the production of a β lactamase. Most of the growth-enhancing drugs with the exception of flavomycin were effective against clostridia. The continued susceptibility of *C. perfringens* to zinc bacitracin was described by Walton (1990). Recently, Devriese, Daube, Hommez and Haesebrouck (1993) evaluated the susceptibility of *Clostridium perfringens* isolated from farm animals to growth-enhancing antibiotics. All isolates were resistant to 64 g/ml of flavomycin and susceptible to avoparcin (MIC_{90} 0.25 μg/ml), avilamycin (MIC_{90} 0.5 μg/ml and salinomycin ($MIC_{90} \leq 0.12$ μg/ml). Some poultry and calf isolates were resistant to bacitracin and resistance to tylosin and virginiamycin was seen in strains from all species. No changes in susceptibility compared to the findings of a previous survey (Dutta *et al*, 1983) were noted.

Concern over the development of antibiotic resistance in *C. perfringens*, after the use of therapeutic and growth promoting antimicrobials in pigs were expressed by various authors (Rood, Maher, Somers, Campos and Duncan, 1978; Rood *et al*, 1985). These workers reported that farms which used antimicrobials had a higher percentage of isolates resistant to tetracyclines or macrolide antibiotics compared to a piggery that never used antimicrobials. Tetracycline resistant plasmids were reported from *C. perfringens* isolates from these farms.

Earlier work, by Brefort *et al* (1977) demonstrated the existance of two transferable plasmids in clinical isolates of *C. perfringens*. One was found to confer resistance to tetracycline and clindamycin, the other to chloramphenicol and erythromycin.

The majority of anaerobes examined were found to be sensitive to metronidazole *in vitro* (Marrie, Haldane, Swantee and Kerr, 1981), but by mutagenesis experiments *C. perfringens* strains resistant to this antimicrobial agent were isolated (Britz, 1981). Clinical isolates of *C. perfringens* appeared to show increased resistance to penicillin in one study (Marrie *et al*, 1981). A more recent review of antibiotic resistance in anaerobes found all strains of *C. perfringens* tested to be sensitive to penicillin G (Finegold and Wexler, 1988).

2.4. Anaerobic microflora of pigs

The characterization of the flora of the large bowel of the pig has only recently been undertaken (see table 2) (Salanitro, Blake and Muirhead, 1977; Russell, 1979; Robinson, Whipp, Bucklin and Allison, 1984; Moore, Moore, Cato, Wilkins and Kornegay, 1987). Earlier research had only identified aerobic organisms from the intestinal microflora (Kenworthy, 1973), but acknowledged the omission of anaerobes, particularly from the large intestine would create a distorted picture of the normal flora in the pig. Both earlier studies and subsequent work have striven to determine the relationship between the normal bacterial population and productivity or the resistance to intestinal disease. Allison (1989) reviewing the current literature related to the anaerobic flora of the large bowel of pigs stated that determination of the species involved, the effects of these organisms and how these anaerobes carry out those actions, have yet to be accomplished. The flora of the large intestine of the pig appears to be comparable to that of the rumen, but cannot be used as a model for colonic populations in humans, as gross differences at the species level were found between the two floras (Moore *et al*, 1987).

In studies of porcine large intestinal flora wide variation between populations, even at the genus level, have been described (see table 2). Various studies found streptococci as the most frequently isolated genus (Moore *et al*, 1987, Robinson *et al*, 1984) and often *Streptococcus intestinalis*. This species had not previously been described, and was characterised by the production of urease. In some studies of the large intestinal microflora only a small percentage of isolates conformed to described species, so generally they were characterised only to the genus level. *Clostridium* isolates formed between 0 and 7 % of the isolated genera.

Table 2.7. Predominant genera (%) of the large bowel microflora of pigs.

Genus	Moore *et al*, 1987	Salanitro *et al*, 1977	Robinson *et al*, 1984	Russell 1979
Streptococcus	29	44	47	15
Lactobacillus	14	< 8	8	21
Eubacterium	12	36	6	0
Fusobacterium	12	0	14	26
Bacteroides	8	< 8	22	5
Peptostreptococcus	5	0	0	17
Bifidobacterium	4	0	0	2
Selenomonas	4	< 8	0	0
Clostridium	3	7	0	4

Data from Allison (1989)

The flora of the large bowel may change depending upon the effect of diet and in association with disease (Allison, 1989). The study of these factors and the protection of the bowel from colonization by pathogenic organisms (colonization resistance) like *Salmonella* in humans or *E. coli* in pigs will benefit from knowledge of normal flora.

In humans, diet was thought not to have a major influence on the distribution of major species in the faecal flora (Drasar and Hill, 1974), although diets rich in polyunsaturated fats appeared to reduce the aerobe:anaerobe ratio (Hentges, 1989). Moore *et al* (1987) on the other hand found that feeding a high fibre diet to pigs significantly altered the faecal flora, when compared to the flora of pigs on basal and oil-rich diets. These findings echoed those of Varel, Pond and Yen (1984), who found the numbers of cellulolytic and xyalolytic bacteria isolated from faeces increased when high amounts of alfalfa fibre were fed to pigs. An interesting study attempted to induce arthritis in pigs that had been fed high concentrations of fish meal (Peltonen, Eerola, Suomi, Aho, Kuusisto and Toivanen, 1993). The hypothesis these researchers formulated was that the fish meal in the diet would increase the number of *Clostridium perfringens* in the large intestine of the pigs and increase the incidence of arthritis. These investigators found that the bacterial flora was significantly changed, with 10 to 40 times as many *C. perfringens* in the treatment pigs. No effect on the joints was linked to these changes.

Diarrhoea in the pig, when caused by a gastrointestinal pathogen, will be characterised by a change in the faecal flora and increased isolation of that pathogen (i.e. enterobacteriae) (Kenworthy, 1973).

Swine Dysentery, the most important disease of the large intestine in pigs, caused by the anaerobic spirochaete *Serpulina hyodysenteriae* has enhanced colonization and increased lesions to the colonic mucosa in the presence of indigenous anaerobic bacteria (Harris and Lysons, 1992). Infection with *Serpulina hyodysenteriae* markedly changed the population adherent to colonic mucosa, with *Acetivibrio ethanolgignes* becoming the most common isolate followed by *Selenomonas* and *Escherichia* (Robinson *et al*, 1984). In contrast, healthy pigs had Streptococcus, Bacteroides and Lactobacillus species as the most prevalent isolates.

Recently, the anaerobic microflora of the pars oesophagea of the stomach in pigs has been investigated to determine any possible links with gastric ulceration (McGillivery and Cranwell, 1992). Unlike the disease in humans the pars oesophagea of the stomach was found overwhelmingly to be the area affected by gastric ulceration in the pig (Taylor, 1995b). These workers found that *Lactobacillus, Clostridium* and *Eubacterium* species predominated in healthy pigs and postulated that these anaerobes perhaps exerted a protective effect by preventing colonization by other microorganisms. Unless the pigs were suffering from diarrhoea, when enterobacteria became more frequent, a small number of facultative anaerobic bacteria were isolated. Most clostridial isolates were *Clostridium perfringens*, which has also been consistently reported in the upper gastrointestinal tract in healthy and diseased pigs (Smith, 1965).

2.5. Sow mortality in breeding herds

In 1967 Jones remarked that " the nature and extent of disease amongst adult stock..... is much less clear for there have been few recorded investigations of sow losses in this country", and although the systems of production have changed a great deal since the sixties, the published analysis of the causes of sow death as an assessment of sow health still receive little attention. In more recent papers dealing with this subject, the lack of investigation of sow deaths and culling, particularly compared to investigation of mortality in lactating and growing pigs, has been highlighted by Straw (1984) and Madec (1984). D'Allaire and Drolet (1992) indicated that producers were often unaware of the extent of sow losses and pointed out the importance of determining the most frequent causes of death in severely affected herds.

Early studies on sow mortality were carried out in the United Kingdom (U.K.) by Perry (1956) and Pomeroy (1960). These authors studied death and culling in breeding herds and concluded that the average life expectancy of breeding sows was short (3.2 and 3.75 litters respectively). These figures were similar to those found by Jones (1967) in his survey of an intensive 475 sow herd. Subsequently, Jones (1968) carried out a more extensive 12 month analysis of deaths in 105 herds in Essex and found the average parity of dead sows was even lower (2.5 litters) and the annual mortality rate amongst 2488 sows from these herds was 3.9%.

Jones (1968) also found that 58% of these deaths occurred during the winter months when the sows were housed indoors and that the most frequent causes of death were complications at the time of parturition, followed by cystitis/nephritis.

Beynon (1978), analysing the cost of disease to the pig industry, cited an identical mortality rate of 3.9% for sows and boars during the period between 1961 to 1964 in the South west of England. This compared favorably with a rate of 4.1% a decade later, when the average herd size had increased almost three-fold. Still, the total number of sow and boar deaths in the U.K. for the period between 1973-74 was estimated to be 43,000.

This mortality rate was similar to the value reported in a Danish study from small herds (average size 66 sows) during a period of 4 years that found a mortality rate of 4.7%, mainly due to polyarthritis and osteomyelitis (Svendsen, Nielsen, Bille and Riising, 1975).

Further surveys of sow mortality in the U.K. were published by Ward and Walton (1980) who studied a recently established 600 sow herd and found a mortality rate of 6%. This value was higher than that accepted as normal by Muirhead (1976), between 2 to 3%. Mortality was also above values of 3.7% reported in breeding herds in Brittany (Madec, 1984) and an average death rate of 3.3% recorded in Quebec herds (Chagnon, D'Allaire and Drolet, 1991). On the other hand, in a study investigating the reasons for removal, including culling and death, in Minnesota breeding herds D'Allaire, Stein and Leman (1987) found an average death rate of 5.7%, with individual farms suffering losses as high as 14.3 %. Straw (1984) reviewed the causes and control of sow losses and suggested targets of 3% sow mortality for herds with 150 sows or less and up to 5% for herds above 200 sows.

The peripartum period was particularly propitious for unexpected death. Madec (1984) reported 26% of the animals examined died during this short period of the reproductive cycle and Chagnon and colleagues in Quebec reported over 40% of deaths to occurred in the space of three days before and after farrowing (Chagnon *et al*, 1991).

The advent of widespread use of computerised recording of herd data in many commercial farms has allowed the culling and mortality rate figures to be readily accessible to clinicians and researchers (Stein, Dijkhuizen, D'Allaire and Morris, 1990). The study of large populations allowed for improved statistical analysis, but could conceal individual variation from farm to farm and relied on farmer compliance with regards to recording and diagnosis of causes of death.

Figures available for analysis from the U.K. National Pig Survey for the years 1992-93 (Sheppard, 1994) involving around 5% of all pigs in the U.K., indicated a sow mortality of 5.3% for the herds recorded (average herd size 141 sows), with the bottom third of producers averaging losses of 6.7%.

Recently, figures from the Pig Management Yearbook (Easicare, 1994) were published. Compiling data from over 100,000 sows in 368 herds, this data represented approximately 12.5% of the U.K. national herd (see table 2.8.). The average herd size recorded was 315 sows and gilts, a figure above that published by the National Pig Survey, but presenting a similar mortality figure (5.7%). A comparative analysis between indoor and outdoor production systems included in this survey reported lower sow mortality in outdoor rearing systems, particularly in the more profitable herds.

Table 2.8. Mortality figures for U.K. herds (1992-1993).

	Av. number of sows/gilts per herd	Sow Mortality (%)	
		All herds	Top 10 %
Total	315	5.7	6.3
Indoor herds	301	6.2	6.1
Outdoor herds	361	4.5	3.7

Data from Easicare (1994)

This apparent rise in sow mortality during the past two decades was noted by Muirhead (1990) in particular from herds suffering from urinary tract infections, a fact also reflected by various workers (Madec,1984; Smith 1984; Carr, 1990). Smith (1984) stated that the increased incidence of ascending urinary tract infections was linked to the confinement of sows and gilts in slatted stalls and tethers.

Another factor implicated in the rise of the mortality rate may be the growing number of cases diagnosed as torsion and accidents of the abdominal organs (Morin, Sauvageau, Phaneuf, Teuscher, Beauregard and Lagace, 1984; Sanford, Josephson and Rehmtulla,1994; Ward and Walton 1980). Surveys carried out during the sixties and seventies did not include these problems as a major cause of losses in sows (Jones 1968; Svendsen *et al*, 1975). More recent surveys in the U.K. and Canada found that torsion and accidents of abdominal organs were the second most common cause of death, with 12 to 15 % of the total cases examined (Chagnon *et al*, 1991; Smith, 1984). Morin *et al* (1984) indicated that some management practices, feeding once a day, excitement during or after feeding, certain types of diet or the total volume of feed appeared to be involved in the pathogenesis of these deaths.

Increased mortality figures since 1990 may have been influenced by changes in the regulations in relation to the transport of sick and injured animals in the U.K. (HMSO, 1977) because more sows were being culled on farm (Blowey 1992, 1994). Figures for euthanised sows were often recorded in the data as deaths, possibly leading to some confusion when interpreting mortality data.

2.6. Causes of death in sows

The most common causes of death in sows, accounting for over half of all mortality in recent surveys (Chagnon *et al*, 1991; D,Allaire *et al*, 1991; Madec, 1984; Smith, 1984), were: Torsion and accidents of abdominal organs, cystitis and pyelonephritis and heart failure.

a. Torsion and accidents of the abdominal organs, mainly gastric dilatation and torsion, but including intestinal, splenic and liver lobe torsions, plus intestinal rupture.

Most detailed descriptions concentrated on gastric dilatation followed by torsion, which were accompanied by a splenic torsion in around two thirds of cases (see below). Blackburn *et al* (1974) investigated the increase in sow mortality in a breeding unit and found that most deaths ocurred between 6 and 24 hours after feeding, presented a grossly distended somach and were housed in confinement stalls with slatted floors. The affected sows' ration had recently been reduced, a practice commonly carried out in early pregnancy and late lactation. *Post mortem* examination of the cases revealed that sows presented a 180^{o} or 360^{o} clockwise torsion of the stomach and sometimes the spleen. Rupture of the twisted spleen with subsequent haemorrhage was also described. The stomach was heavily distended by fluid, partly digested food and gas. The authors suggested that the cause of these deaths was extreme agitation of the sows at feeding time. Sows were observed plunging at the food trough, rearing up, salivating profusely and chewing at the stall bars, whilst waiting to be fed. Once feeding began the sow would rapidly ingest a large volume of wet, sloppy food. Interestingly, *Clostridium novyi* was identified in the liver of 3 gastric torsion cases and from the damaged abdominal wall of another.

Crossman (1978) described a similar situation in a larger breeding herd and agreed that a managemental reason was responsible for the increased mortality due to abdominal accidents in pregnant sows fed once a day.

More recent studies refering to Canadian and German herds described similar pathological and epidemiological findings (Bilkei, 1987; Morin *et al*,, 1984; Sanford, Waters and Josephson, 1984). Bilkei (1987) suggested a genetic predisposition for these deaths after tracing back the history of dead sows and finding that all were the progeny of one Landrace boar and the subsequent cessation of deaths after the removal of this boar. The epidemiological data available appeared to suggest thinner, hungrier sows at peak production, after the third pregnancy, were perhaps predisposed to torsion and other accidents of abdominal organs (Morin *et al* , 1984; Sanford *et al*, 1994). Ward and Walton (1980) reported gastric torsion or distension in sows during all stages of production including a gilt in quarantine.

Torsion of the spleen without gastric involvement, which often became engorged with blood, ruptured and haemorrhaged into the abdomen was less frequently reported. Splenic torsion was thought to occur with relative ease due to the loose, long gastro-splenic ligament found in pigs (Morin *et al*, 1984).

Liver lobe torsion has been described by Blackburn *et al*, (1974), in 21% of accidents of the abdominal organs by D'Allaire *et al*, (1991) and by Morin *et al* (1984) in a similar proportion of cases.

Intestinal volvulus and obstruction appeared to be more common before intensive indoor production became widespread (Jones, 1967) and in growing pigs, frequently reported as a cause of unexpected death (Casteel, Schwartz, Bailey and Camp, 1987).

Sporadically, sows with torsion of the mesentery around a heterotopic (ectopic) bone have been described (Sanford and Rehmtulla, 1994).

Forsyth (1987) described various cases in young boars and hypothesised that ectopic bone formation on the mesentery was precipitated by stretching of these ligaments, furthermore, Forsyth believed these ectopic osseous formations then perforated the intestine, caused obstruction and interfered with peristalsis. Intestinal rupture has been described in sows fed whey, after excessive fermentation in the intestine caused overdistension and rupture (Hani, Zimmermann, Huber, Schmidt, 1993; McCausland and Southgate, 1980).

b. Cystitis and Pyelonephritis.

Most surveys have found cystitis and pyelonephritis to be one of the principal causes of death in sows, with incidence varying between 7 and 40% of all deaths recorded in various surveys (Chagnon *et al*, 1991; Jones, 1968; Madec, 1984; Smith, 1984).

As early as 1873, Kitt described a disease characterised by cystitis and pyelonephritis in pigs (Soltys and Spratling, 1957), but it was not until the early 20th century that bacteria were investigated in the pathogenesis of the disease by Degen (1907 cited by Soltys and Spratling, 1957). These authors were the first to isolate the Gram positive anaerobic bacillus *Eubacterium (Corynebacterium)suis* from cases of unexpected death in sows.

The disease has become well characterised in recent years (Jones, 1992; Carr, Walton and Done, 1995), as an ascending bacterial infection causing haemorrhagic ulcerated cystitis, changes to the ureterovesical junction, ureteric dilatation and ulcerated necrotising ureteritis with haemorrhages plus a severe pyelonephritis.

The aetiological agents most frequently isolated in cases of cystitis and pyelonephritis were *Escherichia coli, Proteus* spp. and *Eubacterium (Corynebacterium) suis,* but other bacteria like *Klebsiella* and *Enterococcus faecalis* were also be isolated from cases (Carr, 1990; Jones, 1992).

Various predisposing factors are thought to allow bacterial colonisation and subsequent ascending infection; damage to urethra during mating, reduced water intake, infrequent micturition with incomplete emptying and high urinary pH (Carr *et al,* 1995). The boar has been implicated as a source of infection to sows, and *Eubacterium (Corynebacterium) suis* has been isolated from the preputial diverticulum of boars (Jones and Dagnall, 1984; Elliot, 1987) and the environment of service pens (Carr and Walton, 1990).

To aid in the diagnosis of this disease after death, Drolet, D'Allaire and Chagnon (1990) investigated the value of analysing the aqueous humor taken from dead sows and found that there was a good correlation between *ante mortem* serum and *post mortem* aqueous humor concentration of urea. These authors recorded a mean aqueous humor urea concentration of 52.3 mmol/l ± 19.0 (SEM) in cases of cystitis-pyelonephritis, compared to 9.9 ± 1.5 (SEM) in sows that had died due to other causes, a statistically significant difference (Chagnon *et al,* 1991).

D'Allaire *et al,* (1991) found the disease more prevalent in older sows, on average after more than 5 pregnancies, and did not report the condition in gilts. Akkermann (1984) in a study of 1445 sow carcasses also found a direct correlation between old age and urinary tract infection.

The recognition of an adequate supply and intake of water for sows as a valuable preventative measure has reduced the incidence of urinary tract disorders in the U.K. (Carr *et al,* 1995), but in many countries cystitis and pyelonephritis continues to be a major cause of death in sows.

c. Heart Failure.

Cardiac failure has beeen reported as the most common cause of sow death in Canadian herds (Drolet, D'Allaire and Chagnon, 1992) and in approximately 12% of sows examined in a Scottish survey of sow mortality (Smith, 1984).

Death usually followed events that increased the demands on the cardiovascular system, such as mating, transport, fighting or parturition. Other environmental and management factors that were believed to predispose to cardiac failure were; high ambient temperature, halothane positive stock, lack of cardiovascular fitness and obesity (Drolet *et al*, 1992). Also reported by these authors, in their very elegant paper on cardiac failure, was the fact that more than 60% of these deaths occurred in the peripartum period, and this time was even more dangerous for sows when combined with heat stress. Age did not appear to have a direct correlation with increased risk of cardiac failure, but sows that died from cardiac failure were heavier, fatter and had a smaller heart weight to bodyweight ratio than those dead of other causes.

Cardiac failure could only be diagnosed when every other possible cause of death had been excluded and a suitable clinical history of fighting or other stressful event inmediately before sudden death was available. Drolet *et al* (1992), mentioned transudate in pericardial, thoracic and abdominal cavities, dilation of cardiac chambers, pulmonary oedema and passive congestion of internal organs as lesions indicative of cardiac failure.

d. Other causes of death

Other reasons generally not found as a major cause of death in surveys of sow mortality included: Endometritis, uterine prolapse, gastric ulceration of the pars oesophagea with severe haemorrhage, proliferative haemorrhagic enteropathy and pneumonia (Chagnon *et al*, 1991; Smith, 1984).

e. Sow deaths due to *Clostridium (oedematiens) novyi* infection.

Few reports were found in the literature and certainly the condition was not mentioned in the surveys carried out by Jones (1968) on mortality in English farms, Smith (1984) in Scotland or by Chagnon *et al*, (1991) in their survey of mortality in Quebec. On the other hand Madec (1984) examining death losses from herds in Brittany found over 10% of sows presented pronounced degeneration of the liver, but concluded that these changes were difficult to evaluate objectively, particularly if a long delay between death and examination of the carcass had occurred.

The importance of carrying out a *post mortem* examination and collection of samples for the diagnosis of clostridial diseases as soon as possible after death was highlighted by Batty, Kerry and Walker (1967) and Bagadi and Sewell (1974b). These investigators concluded that demonstration of C. *novyi* in the liver of sheep dead for more than 24 hours did not alone constitute sufficient evidence of infectious necrotic hepatitis.

The first report of sudden death in pigs due to C. *novyi* , at that time known as *Clostridium oedematiens*, described a single case in a sow kept outdoors, that was found recumbent, followed by rapid deterioration and death (Wise and Munday, 1964).

Post mortem examination revealed copious pleural and peritoneal exudates, nodular enlargement of the spleen and a circular necrotic area of 6 cm on the diaphragmatic surface of the liver. The centre of the necrotic area contained a tapeworm cyst. Confirmation of the diagnosis of *C. novyi* infection was based on the Gram stain and inoculation of macerated liver tissue into guinea pigs. Shortly afterwards, Batty, Buntain and Walker (1964) described cases of sudden death in sheep, cattle and pigs in which the Fluorescent Antibody Test (FAT) was used to confirm the diagnosis. The porcine cases originated from various areas in England and presented a remarkable degeneration of the liver, regardless of the time elapsed between death and examination of the carcass. Liver smears taken from 7 rapidly decomposing livers were positive to *C. novyi* antigen using the FAT. The pathogenesis in these cases of sudden death in pigs was was not discussed, but the authors remarked that cattle often died in herds, not infested with liver fluke, feed on a high barley diet and that the developement of fatty liver may have presented ideal conditions for proliferation of *C. novyi*. Early descriptions of *C. novyi* sudden death, confirmed by the newly developed fluorescent antibody test, remarked on the similarity between the gross pathology seen in these cases and the findings in deaths caused by anthrax (Bourne and Kerry, 1965; Corbould and Munday, 1966). Neither of these authors described lesions of parasite or larval migration in the liver, and indeed did not describe necrotic or haemorrhagic foci on the liver.

After the impetus in reported cases during the nineteen sixties, provided by the widespread use of the fluorescent antibody techniques developed at the Welcome Research Laboratories (Batty and Walker, 1964), publication of case descriptions and research in pigs was not found.

The first reported isolation from a case of "gas gangrene" in the pig has recently been published (Kita, Hamaoka and Minato, 1987). These authors found large numbers of rod shaped organisms on Gram stained liver and spleen smears from a single case of sudden death in a sow. Presumptive diagnosis was made by gas liquid chromatography, when propionic, butyric and acetic acids were detected from tissue samples. The bacterial isolates from the liver and spleen had a similar Volatile Fatty Acid pattern, which was consistent with *C. novyi.* Subsequently the isolate was proven to be lecithinase positive and lipase negative, classified as type B or type D, but was not typed using specific antitoxins. Also in Japan, the occurrence of *C. novyi* infection in gilts was reported by Itoh, Uchida, Sugiura, Oguso and Yamakawa (1987), but in this case the isolate was thought to be type A. These authors remarked on the sponge-like appearance of the liver parenchyma and bubbles on the surface of this organ. Again in 1987, Sanford reported the acute death of four sows with clostridial myositis, presenting with swollen, hard and purple ventrocaudal abdomen and rump (Sanford, 1987). There was subcutaneous oedema, emphysema plus haemorrhage and the semitendinosus muscle had a "cooked" appearance. The abdominal lymph nodes were enlarged and dark red. Histology revealed a cellulitis and myositis characterised by oedema and coagulation necrosis. The muscle fibres were infiltrated with neutrophils, mononuclear cells and large, Gram positive rods. *C. novyi* was isolated and identified by FAT, but not typed.

More recently, Walton and Duran (1992) reported cases of *C. novyi* sudden death in intensive pig breeding units, and diagnosis was based upon the absence of any other detectable cause of death, the rapid decomposition and tympany of the carcass and confirmed by the fluorescent antibody test. The authors suggested there may be a link between hepatosis dietetica and this disease. Many *C. novyi* deaths also presented lesions of cystitis and pyelonephritis.

3. GENERAL MATERIALS AND METHODS

"To make a good salad is to be a brilliant diplomatist- the problem is exactly the same in both cases. To know how much oil one must put with one's vinegar." Oscar Wilde (1883), in The Nihilists.

3.1. PROTOCOL FOR THE ISOLATION OF *Clostridium novyi* .

3.1.1. Cases of sudden death in sows and sows euthanased and submitted for *post mortem* examination at Leahurst

Submissions were classed into three groups depending upon the macroscopic findings at necropsy; suspected *C. novyi* deaths, sudden death due to other reasons and culled sows. In sows that had died from "abdominal catastrophes", such as a ruptured stomach, perforated gastric ulcer, intestinal rupture or sows that had been dead for over 24 hours, bacteriological culture and isolation were not carried out. Early cases of contamination with intestinal contents in which bacterial isolation was attempted indicated that tissues yielded Gram negative bacteria and other *post mortem* invaders, mainly *C. perfringens* and *C. septicum* , as indicated by Sterne and Batty (1975), Walker, Harris and Moore, (1971) and Williams (1962) when carrying out bacteriology from sheep carcasses.

Swabs and transport media were not used for collection of specimens, as Levett (1991a) suggested that anaerobes were better protected from oxygen in tissues and recovery of organisms on cotton wool swabs was likely to be reduced by a factor of 90% due to bacterial adherence to the swab. Furthermore, complicated transport systems were considered expensive.

All media used was prepared fresh and pre reduced. Incubation was under anaerobic conditions (see below) for 48 hours at 37°C unless stated otherwise.

Protocol used in suspected *C. novyi* deaths

1. Specimens of approximately 1 cm^3 from the liver and spleen were collected for bacterial culture in clean, non sterile glass jars after making an impression smear on a glass microscope slide. Other organs and muscle tissue were collected and impression smears were also prepared. Heart blood, peritoneal and pericardial fluid were collected in sterile bijoux bottles or with a disposable sterile syringe.
2. The tissues collected were taken to the laboratory for immediate processing.
3. Triplicate smears of the fluid specimens were made, air dried and fixed for 10 minutes in anhydrous acetone for the direct fluorescent antibody test or heat fixed for Gram and polychrome methylene blue staining.
4. The surface of tissue specimens was seared on all sides.
5. A sterile scalpel-blade was used to divide the tissue in half, the freshly cut surface was sampled with a flamed platinum loop and used to inoculate a fastidious anaerobe agar (FAA) plate and a Neomycin FAA and also to prepare a Gram stain. Later, neomycin at the concentration of 70 µg/ml recommended by Willis and Phillips (1983) and Levett (1991a) was discovered to be inhibitory for *C. novyi.* type B strains. This observation was supported by previous work (Rutter, 1968).

One half of the tissue sample was macerated with a pestle and mortar and emulsified in 2 ml of sterile distilled water. Two drops of the emulsion were inoculated without selective treatment in cooked meat fastidious anaerobe broth (CMFAB), usually incubated for 24 hours at 37°C.

The tissue emulsions were alcohol and heat shocked (see below) and incubated at 37°C in CMFAB for 48 hours or until turbid growth was visible.

Alcohol shock method for selective isolation of spore forming organisms

a) Specimens were alcohol shocked by adding 1 ml of 70% ethanol to 1 ml of the macerated tissue suspension .

b) The suspension was then vortex mixed and kept on the bench for 1 hour at room temperature. The emulsion was inverted repeatedly to ensure good mixing.

c) Finally the emulsion was centrifuged at 2500 R.P.M. for 10 min., the supernatant was discarded and the deposit was inoculated into CMFAB.

Heat shock method for the isolation of spore forming organisms

Heat shock was carried out by inoculating a pre-reduced Universal bottle with CMFAB and 4 drops of the macerated tissue sample and heating in a thermostatically controlled water bath at 80°C for ten minutes.

The second half of the tissue was stored in a sterile , labelled plastic bag at -20°C for future use.

6. Purity plating of all positive broths and plates was carried out on fastidious anaerobe agar with a 5 μg metronidazole disk (Mast Laboratories). Blood agar plates were incubated aerobically and in a microaerophilic atmosphere to exclude facultative anaerobes and those that prefer a CO_2 rich atmosphere. Egg yolk agar was also inoculated to detect the lecithinase reactions of the isolates.

Possible *C. novyi* isolates were detected initially by their Gram reaction, FAT and colonial appearance. Isolates were characterised further when the following criteria were met:

a) microscopically, large Gram positive rods with oval subterminal spores although variable Gram staining has been noted, particularly in older cultures,

b) positive in smears to the FAT,

c) sparse growth on solid media and none at all in aerobic and microaerophilic conditions,

d) isolates sensitive to metronidazole, β-haemolytic on blood agar and showing a positive lecithinase reaction.

Protocol used when the cause of sudden death was not *Clostridium novyi* and to sample euthanased sows

Tissue samples were only collected from the liver. The specimens were processed in the same way as suspected *C. novyi* sudden deaths.

3.1.2. Field samples (submitted by Veterinary Surgeons in pig practice)

1. Practitioners were asked to collect liver samples from sows that had died unexpectedly. Field *post-mortem* examinations were carried out by the veterinarian. If no other cause of death was apparent and macroscopic findings were consistent with *C. novyi* infection, 1 cm cubes from the affected liver were collected, preferably ensuring that contamination with intestinal contents was avoided.
2. If possible searing of the external surfaces of the tissues was carried out.
3. Universal bottles containing 20 ml of cooked meat fastidious anaerobe broth (CMFAB) were supplied. The practice laboratory was instructed to remove oxygen from the broth by partially submerging the universal bottles in a boiling water bath for 10 minutes, keeping the lid loosely closed. Immediately afterwards, bottles containing the broth had the lid tightly shut and were allowed to cool. Whole seared cubes of liver were used to inoculate the pre-reduced broth.
4. The samples were either mailed immediately or incubated for 24 hours at 37°C in the practice laboratory before being sent to the laboratory at Leahurst.
5. Samples received from the field in this way were accompanied by a case history sheet (Appendix I) supplied for this purpose.

6. Inoculated CMFAB bottles were firstly examined by the Fluorescent Antibody Test (*q.v.*) to confirm the presence of vegetative forms of *C. novyi.* All samples received were subcultured as follows:

a) Pre-reduced CMFAB

b) FAA plates

c) Neomycin FAA plates (later discontinued)

d) 1 ml of broth was selectively cultured after alcohol shock and another 1 ml was heat shocked, then incubated in pre-reduced CMFAB for at least 48 hours.

e) purity plating was carried out on FAA plates with metronidazole disks and on egg yolk agar plates. Isolates were selected for further identification with the same criteria as with Leahurst isolates.

3.2. ANAEROBIC CULTURE METHODS

Anaerobic jars

Before an anaerobic incubator became available, BBL Gas Pak system jars were used with 1 g of a 0.5% palladium catalyst (bioMerieux) in a wire mesh capsule held under the lid of the jar and an anaerobic gas generator (Generbox anaer, bioMerieux). This system was based on hydrogen and carbon dioxide being generated inside an envelope divided into two compartments; one contained citric acid plus sodium bicarbonate, another filled with sodium borohydride and cobalt chloride. This reaction was activated by adding water immediately before closure of the GasPak jar.

A resazurin anaerobic indicator (Oxoid) was included to confirm anaerobiosis. Maintenance of the catalyst pellets was important, particularly ensuring that moisture was removed after use by placing in a drying oven at 160°C for two hours. Another reason for catalyst failure could be poisoning

by hydrogen sulphide- if this occurred or after 3 to 5 uses the catalyst pellets were discarded. The main disadvantages of the GasPak systems were;

a) the cost of setting up numerous jars,

b) more importantly, the fact that anaerobiosis was not achieved for at least 3 or 4 hours after setting up the jars, sufficient exposure for oxygen to kill vegetative forms of *C. novyi*.

Micro environment systems- Generbag anaer or Generbag microaer (bioMerieux)

These novel disposable systems were used on occasion according to the manufacturers instructions.

Anaerobic cabinet

Initially a Gallenkamp anaerobic incubator was borrowed, but its use was discontinued after discovering it provided inadequate and unreliable anaerobiosis and presented numerous gas leaks.

Subsequently, a Mark II Whitley anaerobic work station (Don Whitley Scientific Ltd) was used for all anaerobic incubations. This work station allowed anaerobic manipulations using bare hands with latex rubber sleeves, rather than cumbersome rubber gloves. Anaerobiosis was maintained by an anaerobic growth mixture of 10% hydrogen, 10% carbon dioxide and 80% nitrogen (BOC special gases Ltd). Removal of traces of oxygen was effected by the use of a Deoxo catalyst and hydrogen sulphide and Volatile Fatty Acids (VFA's) were removed by Anotox pellets (from Don Whitley Scientific Ltd). Humidity and temperature were automatically controlled. Excess moisture was collected by self indicating Silica gel, that changed from bright blue to a pink colour after absorbing water. Resazurin as an anaerobic indicator (see below) was included at all times. Pink colour in the solution indicated a concentration of oxygen above 300 ppm.

Frequent changes of indicator were required because CO_2 turned the solution yellow when it became alkaline.

Resazurin solution

0.1 g of resazurin (Sigma Chemicals) was dissolved in 100 ml of distilled water.

The following were then dissolved in 19 ml of distilled water

Tris (hydroxymethyl) aminomethane	4 g
Glucose	0.2 g
0.1% (w/v) aqueous resazurin	1ml

The solution was boiled in a universal bottle until decolorised, the cap was tightened and rapidly transferred to the anaerobic incubator.

3.3 PROTOCOL FOR THE IDENTIFICATION OF CLOSTRIDIAL ISOLATES

Forms for recording the results (see appendix II) were adapted from those used by the Virginia Polytechnic Institute (V.P.I.) in their Anaerobe Laboratory Manual (Holdeman *et al;* 1977). The procedures for identification and the diagnostic characteristics were based on those described by Willis (1977), Holdeman *et al* (1977) and Levett (1991b).

3.3.1. Initial examination

1. Colonial morphology; haemolysis and swarming are useful features commonly found in clinically important clostridia.
2. Atmospheric requirements; *C. novyi* of all 4 types are strict anaerobes. Growth in air or under microaerophilic (air + 10% CO_2) conditions can be used to discount isolates as possible *C. novyi*..

3. Gram stain; following the method in the Manual of Veterinary investigation laboratory techniques (MAFF, 1984).

4. Malachite green spore stain; occasionally used to detect spores from cultures if not obvious on a Gram stain.

Method: A heat fixed film was flooded with 5% malachite green (Prolab Diagnostics) and heated for approximately 1 minute or until the solution was bubbling. After a wash with water the smear was counter stained with carbol fuschin for one minute, washed in water, dried and examined under oil immersion.

5. Polychrome methylene blue; for suspected Anthrax cases a smear of the peritoneal fluid was made on a glass microscope slide. The slide was flooded with polychrome methylene blue for two minutes, followed by washing. Using this method *Bacillus anthracis* appears as a blue, square ended rod surrounded by a capsule that should stain a pinkish-red colour (Mac Fadyan's reaction).

6. Fluorescent Antibody Test (FAT); first described by Batty and Walker (1964)

Reagents and apparatus

-Anti-*Clostridium novyi* antibody labelled with fluorescein isothionate from Welcome Diagnostics initially, subsequently purchased from Pragma.

-Anhydrous acetone, Phosphate buffered saline (PBS) 10x pH 7.6, stored in the refrigerator and then diluted in distilled water before use.

-Mounting fluid; Glycerol saline ($NaHCO_3$ 0.0715 g, Na_2CO_3, 0.016 g, distilled water 10 ml, Glycerol 100 ml) was initially used. Subsequently, Fluorescence mounting fluid was used. Thus enabling long examination of smears without appreciable fading.

The fluid was prepared according to Johnson and Nogueira Araujo (1981) as follows: 10 ml PBS, 100 mg p-phenylenediamine and 90 ml glycerol. The mounting fluid was stored in the dark at 2°C.

-Microscope slides and cover slips

-Humidified sandwich boxes, with a soaked sponge in the base

-Slide staining rack for washing slides

-Fluorescent microscope with an Orthomat microscope camera (Leitz).

Procedure

a. The suspected tissue or culture was smeared on a microscope slide and allowed to dry.

b. The slide was fixed by immersion in acetone for 10 minutes.

c. One drop of the FITC- labelled immunoglobulin was placed on the smear and spread evenly.

d. The slide was incubated in the moist chamber for 30 minutes at room temperature.

e. The labelled globulin was rinsed with a stream of PBS followed by immersion in PBS for at least 10 minutes and gently blotted dry.

f. The preparations were mounted using a cover slip, then viewed under the U.V. microscope.

3.3.2. Commercial identification systems

Two miniaturised systems were tried;

API 20 A (bioMerieux) was used initially. This system relied on the conventional biochemical reactions, mainly fermentation of carbohydrates, but was found to be of little use for *C. novyi* as the organism failed to grow even after 48 hours incubation. Rapid ID 32A (bioMerieux) which has superseded the previous method, detects pre-formed enzymes in cultures of anaerobes. Four hours aerobic incubation will provide an identification.

Comparative studies carried out to asses the accuracy of these systems (Marler, Siders, Wolters, Pettigrew, Skitt and Allen, 1991, Levett 1991b) suggested correct identification of *Clostridium* to the species level in over 70% of isolates and were even more accurate with additional test e.g. egg yolk agar. The main advantages were speed and convenience. Unfortunately this miniaturised system did not include C. *novyi* identification data in the software. This system was used according to the manufacturers instructions.

3.3.3. Agar plate identification method

Biochemical test were carried out on solid media following the method first described by Phillips (1976), for non-sporing anaerobes, as adapted by the Anaerobe Reference Unit, PHLS, Cardiff. The tables and identification were based on the 4th V.P.I. Anaerobe Laboratory Manual (Holdeman *et al*, 1977).

Method for carbohydrate fermentation reactions:

1. Universal bottles with 20 ml of 20% (w/v) solutions of the desired substrates were prepared in sterile distilled water.

The substrates useful in identification of *Clostridium* species were: Aesculin, Fructose, Glucose, Lactose, Maltose, Mannitol, Mannose, Melibiose, Ribose, Sucrose, and Xylose. Aesculin solution was prepared with aesculin (2% w/v) and ferric citrate (1% w/v). All the chemicals were purchased from BDH Merk Ltd with the exception of Ferric citrate which was supplied by Sigma Chemicals Ltd.

2. Solutions were sterilised by steaming at 100°C for 10 minutes and stored at 2° to 4°C.

3. Freshly prepared anaerobic identification media (A.I.M.'s) plates with 6% sheep's blood were used (see Appendix III.) for the inoculations.

Firstly, plates were dried in the oven a 40°C., this was followed by spreading 1 ml of the different sterile 20% carbohydrate solutions over the surface of the plates, which were labelled and allowed to dry. Some solutions needed to be heated in a water bath at 56°C to allow crystals to dissolve. One plate without substrate was used as a control.

4. The plates were divided, with a sterile scalpel blade, into 5 segments, sufficient to accommodate 4 isolates to be identified plus an uninoculated control portion.

5. Each isolate was spot inoculated into a separate segment of the plate.

6. Plates were incubated at 37°C for 48 hours or until good growth had occurred.

7. The results were read by removing a plug of agar with a rubber teat and a plastic drinking straw, these plugs were then placed into the wells of a microtitre tray (Dynatech microtiter). Finally 20 µl of 0.004% aqueous bromothymol blue (BDH) was added to each plug . Aesculin hydrolysis was indicated by blackening of the medium around the bacterial growth.

8. The colour change was observed. dark green coloration- negative
yellow- positive
pale green- weak reaction

The control plugs should all remain dark green.

The following were also used to characterise *Clostridium* isolates;

- egg yolk agar plates (see Appendix III) were used to determine whether the isolates produced lecithinase and/or lipase. The reactions of various anaerobes on egg yolk agar can be summarised as in table 3.1.

Lecithinase C produces intense opacity in egg yolk medium surrounding the colonies. On the other hand lipase activity is confined to the medium underlying the bacterial colony. Sometimes the so called "pearly layer" can be seen underlying the bacterial colony.

This reaction can be difficult to read in species that present both the lecithinase and lipase activities e.g. *Clostridium novyi* type A. The pearly layer is made out of insoluble fatty acids which will float to the surface of a drop of water added to the surface of the plate.

Table 3.1. Reactions of various anaerobes on egg yolk media.

Organism	Opalescence		
	Diffuse lecithinase C	Restricted lipase	Pearly layer lipase
C. perfringens A-E	+	-	-
C. barati	+	-	-
C. bifermentans- sordelli	+	-	-
C. botulinum A-F	-	+	+
C. sporogenes	-	+	+
C. novyi			
Type A	+	+	-
Type B	+	-	-
Type C	-	-	-
Type D	+	-	-
Fusobacterium necrophorum	-	+	+

- Starch hydrolysis and fermentation were determined on starch agar plates (see Appendix III). Fermentation was determined in a similar way as the sugar fermentations, by removing a plug of agar from an area of bacterial growth and adding bromothymol blue. The rest of the plate was flooded with Lugol's iodine. Zones of clearance around colonies indicated starch hydrolysis.

- Gelatin hydrolysis was observed by incubating the organism on a gelatin agar plate (see media preparation) After 48 hours the plate was flooded with 15% mercuric chloride (BDH Merk) in 1 molar HCl. Positive reactions were seen after a few minutes when zones of clearing appeared around growth of proteolytic organisms.

-Catalase was detected by adding a drop of hydrogen peroxide to the growth on a plate, immediate bubbling of the bacterial colony indicated a catalase positive reaction. Catalase is not generally produced by bacteria of the genus *Clostridium*.

-Spot Indole was used as a rapid way of determining production of indole using a 1% (v/v) solution of p-dimethylaminocinnamaldehyde (Bactident indole, BDH Merk).

Filter paper was soaked with the solution and placed in the base of a Petri dish. Using platinum loop, colonies of the organism grown in blood agar were smeared on the soaked filter paper. Positive indole reactions presented a blue or green colour after a few seconds.

3.3.4. Gas Liquid Chromatography

Volatile Fatty Acid (VFA) detection using Gas-liquid chromatography (GLC) was considered essential for the definitive identification of anaerobic bacteria (Levett, 1991b). The media used for cultivation of the anaerobes was known to alter the composition of the end products of bacterial metabolism. Strains to be identified were grown for 48 hours either on solid media (FAA) or CMFAB.

The production of VFA's was compared to the profiles of a previously run standard solutions.

Equipment used in GLC measurements:

Pye Unicam series 104 chromatograph, 1 ml pipettes, 15 ml conical centrifuge tubes and bungs, 10 µl graduated micropipette (Phase Separations Ltd).

Preparation of VFA standards: The following volume of volatile acids were dissolved in 100 ml of distilled water.

Acetic acid	5.7 ml
Propionic acid	7.5 ml
Isobutyric acid	9.2 ml
Butyric acid	9.1 ml
Isovaleric acid	12.7 ml
Valeric acid	12.5 ml
Isocaproic acid	12.6 ml
Caproic acid	12.5 ml

Also required: diethyl ether and 50% H_2SO_4.

Protocol

1. 1 ml of spent culture broth was pipetted into a centrifuge tube
2. 0.2 ml of 50% aqueous H_2SO_4 were added
3. 1ml of diethyl ether was also added, followed by vortexing for 10-15 min
4. Centrifugation was used to break the water/ether emulsion
5. The aqueous and ether layers were allowed to separate.
6. Rapidly 1 µl of the top layer (ether) was injected into the GLC.

When solid media was used a plug of Fastidious Anaerobe agar was removed from beneath the area of growth. The plug was placed in a well of a microtitre plate. Two drops of sterile distilled water were added and left for 15-30 minutes. 1 µl was collected and injected into the chromatograph.

4. STUDIES WITH *Clostridium novyi*

"In space nobody can hear you scream." Alien (1979)

4.1. Introduction

Difficulties obtaining reliable growth of the strictly anaerobic Gram positive bacillus *Clostridium novyi* (*oedematiens*) have been reported by many workers. Early attempts to isolate the organism from infected sheep livers on solid media (Albiston, 1927; Keppie, 1944) were unsuccessful. Williams (1962) reported successful isolation on freshly poured sheep blood agar plates, but described frequent failure to grow pure cultures from infectious necrotic hepatitis cases. *C. novyi* was overgrown by less fastidious clostridia, mainly *C. perfringens* and *C. septicum* . The factors limiting the recovery of *C. novyi* on solid media were not properly addressed until the work of bacteriologists at the University of Edinburgh Medical School in the early 1970's (Collee *et al*, 1971; Watt, 1972; Watt, 1973). The description of Moore's medium, containing neopeptone plus cysteine and dithiothreitol as reducing agents, allowed workers to consistently achieve luxuriant surface growth on solid media (Moore, 1968). Still, Willis (1977) stated that isolation of *C. novyi* type B was more difficult than strains of type A and that strains of type D were amongst the most fastidious clostridia known.

C. novyi was differentiated into 4 toxin types according to the major soluble antigens produced by the different strains (Oakley *et al*, 1947); *C. novyi* type A, produced the lethal and necrotizing alpha toxin and the epsilon and gamma toxins, but not the beta toxin. *C. novyi* type B, isolated from cases of infectious necrotic hepatitis, produced the lethal alpha toxin, but also the beta toxin, a lecithinase.

Strains classified as C. *novyi* type D (C. *haemolyticum*), comprised isolates mainly from bacillary haemoglobinuria in cattle or occasionally sheep. Culture filtrates of these strains were found to contain only the beta toxin (Oakley and Warrack, 1959).

C. *novyi* type C included non toxigenic strains.

The initial purpose of this work was to become familiar with the morphology, cultural characteristics, growth requirements and identification of the strict anaerobic bacterium *Clostridium novyi* using strains from various culture collections. Other pathogenic clostridia were cultured for comparison and to recognise other species likely to be isolated from clinical specimens.

Work was carried out to ensure the correct identification of *Clostridium novyi* isolates, by cultural and morphological appearance, biochemical properties and fluorescent antibody staining. The production of the different toxins by the 3 toxigenic types was studied.

Finally, the C. *novyi* culture collection strains were also used to determine the ideal culture medium both for selective and enrichment purposes. The culture methods developed would be applied to isolation protocols from clinical material.

4.2. Bacterial strains.

The following culture collection strains were used for the various experiments. The alpha toxin producing *Clostridium perfringens* type A (NCTC8237), *Clostridium novyi* type A (NCTC538, NCTC6737, NCTC6738), *Clostridium novyi* type B (NCTC9691), *Clostridium novyi* type D (*haemolyticum*) (NCTC8145, NCTC9692), *Clostridium botulinum* type C (NCTC3732), *Clostridium sporogenes* (NCTC532), *Clostridium chauvoei* (NCTC8070) *Clostridium septicum* (NCTC547) and *Clostridium bifermentans* (NCTC506 and NCTC8780) received from the National Collection of Type Cultures, PHLS.

Clostridium novyi type B (NCIMB10626) was supplied by the National Collection of Industrial and Marine Bacteria, Aberdeen.

Three *Clostridium novyi* type B isolates and one type A strain (mprl 2537,2538,2543 and mprl 2531) from the Scottish Anaerobe Reference Unit collection, were donated by Dr. Ian Poxton, Department of Microbiology, University of Edinburgh. A further C. *novyi* type B (strain R4669) was kindly donated by Professor Duerden from the collection of the Anaerobe Reference Unit, Cardiff.

The *C. novyi* strains were labelled in the laboratory using a simplified working code shown in table 4.1.

Table 4.1. Culture collection strains of *Clostridium novyi*.

Working identification	Identification and source
***Clostridium novyi* type A**	
N1A	NCTC538, National Collection of Type Cultures
N2A	NCTC6738, National Collection of Type Cultures
N3A	NCTC6737, National Collection of Type Cultures
N4A	mprl2531, Dept. of Microbiology Univ. of Edinburgh
***Clostridium novyi* type B**	
N1B	R4669, Anaerobe Reference Laboratory
N2B	NCTC9691, National Collection of Type Cultures
N3B	m2537, Dept. of Microbiology Univ. of Edinburgh
N4B	m2538, Dept. of Microbiology Univ. of Edinburgh
N5B	m2543, Dept. of Microbiology Univ. of Edinburgh
N6B	NCIB10626, National Collection of Industrial Bacteria
Clostridium haemolyticum	**(type D)**
N1D	NCTC9692, National Collection of Type Cultures
N2D	NCTC8145, National Collection of Type Cultures

4.3. Identification of *Clostridium novyi*

Morphology; All strains were Gram positive in young culture, but became Gram variable after longer incubation. Sometimes bipolar staining could be seen. The morphology was not characteristic, and considerable variation was noted between *C. novyi* strains of the same toxin type and depending on the time and medium used for incubation. The bacilli appeared as large rods, with parallel sides and rounded ends. Occasionally filamentous forms were seen after repeated subculture. After a 48 hour incubation numerous spores could be seen free or as large, oval, subterminal spores that distended the organism. The appearance of *C. novyi* in Gram stain preparations can be seen in figures 4.1. and 4.2.

Cultural characteristics; Growth from laboratory strains of *C. novyi* was in the form of well defined colonies on solid media, which differed from the spreading colonies expected from clinical specimens (Willis, 1977). On sheep blood fastidious anaerobe agar a zone of beta haemolysis following the contour of the colonies was seen with strains of type A and B (see figures 4.3 and 4.4.), whilst *C. novyi* type D (*C. haemolyticum*) colonies produced a wider area of haemolysis (figure 4.5.). After repeated subculture on solid media, strains of type A and B lost the zone of partial haemolysis, prompting worries regarding the purity of the isolates (FAT was positive, see conclusions).
Colonies of all types were circular, semitranslucent or grey in older cultures, and presented a crenated or lobulated edge. Cultures of *C. novyi* had a tendency to spread if there was excessive surface humidity or after prolonged culture. Colonies of *C. novyi* type A (2-3 mm in diameter) tended to be larger and grow more rapidly than strains of type B. *C. novyi* type D strains presented smaller colonies, hardly visible after 24 hours incubation. All *C. novyi* strains were sensitive to metronidazole (5 μg), presenting a wide zone of clearing around the antibiotic disc.

Further characterisation was possible after subculture on egg yolk fastidious anaerobe agar. Strains of all three pathogenic types produce lecithinases, visible on egg yolk plates as zones of opalescence around the colonies. *C. novyi* type D strains, in particular, could be distinguished by the wider diffuse zone of opalescence. Additionally, strains of *C. novyi* type A presented a restricted pearly layer due to lipolysis by the epsilon toxin. The pearly layer required oblique illumination to be detected. Figure 4.6. illustrates the lecithinase and lipase reactions of *C. novyi* type A.

Fluoresecent antibody test; The various *Clostridium* isolates were incubated on solid media and in broths for 48 hours at 37°C. Smears were prepared after emulsion in sterile saline and stained following the protocol described in the previous chapter (section 3.3.1.). The fluorescein labelled anti-*Clostridium novyi* antisera only gave specific fluorescence on smears prepared with all strains of *C. novyi* (see figure 4.7.) and with *C. botulinum* type C. This result was expected after the work of Poxton (1984), which demonstrated common antigens for isolates of *C. novyi* and type C and D of *C. botulinum*.

Biochemical characterisitics; All the clostridial isolates examined were catalase negative. Culture collection isolates were identified following the protocol described in chapter 3. The results of sugar fermentation can be seen in table 4.2. Weak acidification of the medium was considered as a positive result in the table. Growth was often scant on AIMS and the results varied for the same isolate in different identifications. Strains of both *C. novyi* type A and B were indole negative, but both isolates of type D were positive to the spot indole test. All strains tested produced gelatinase when tested on gelatin agar, but starch was hydrolysed by 1 from 4 strains of *C. novyi* type A, but neither type B nor type D strains presented areas of clearing surrounding colonies on starch agar plates flooded with Lugol's iodine.

Table 4.2. Sugar fermentation results for *Clostridium novyi* strains.

Type	Aes	Fru	Glu	Lac	Mal	Ma	Man	Mel	Rib	Suc	Xyl
A	0/4*	4/4	4/4	0/4	4/4	0/4	1/4	1/4	2/4	0/4	0/4
B	0/6	2/6	6/6	0/6	5/6	1/6	5/6	1/6	1/6	0/6	0/6
D	0/2	0/2	2/2	0/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2

Aes; Aesculin. **Fru;** Fructose. **Glu;** Glucose. **Lac;** Lactose. **Mal;** Maltose. **Ma;** Mannitol. **Man;** Mannose. **Mel;** Melibiose. **Rib;** Ribose. **Suc;** Sucrose. **Xyl;** Xylose.

* The first number indicates a positive fermentation, the second indicates the total number of strains tested.

Analysis of volatile fatty acid production; The gas liquid chromatograph originally available stopped working. Some isolates were examined at the Anaerobe Reference Unit, P.H.L.S., Cardiff. The principal products of metabolism detected were propionic and butyric acids with a small amount of acetic acid detected as a minor peak.

Alpha toxin detection; During the study a monoclonal antibody raised against the purified alpha toxin of *C. novyi* type B became available (Commonwealth Serum Laboratories. Ltd, Parkville, Australia). Work was commenced using culture collection isolates incubated in fastidious anaerobe broth at 37°C for 72 hours. After centrifugation of the broth at 4°C for 1 hour at 4000*g* the supernatant was filtered through a 0.45µm membrane. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a dot blot test were performed with the culture filtrates of *C. novyi* type A and B strains plus *C. perfringens* and broth filtrate as negative controls. The dot blot test only gave a positive result with *C. perfringens* filtrate and the SDS-PAGE did not give clear bands. Further work with this monoclonal antibody is required.

Figure 4.1. Overnight broth culture of *Clostridium novyi* type B. Gram Stain (100x).

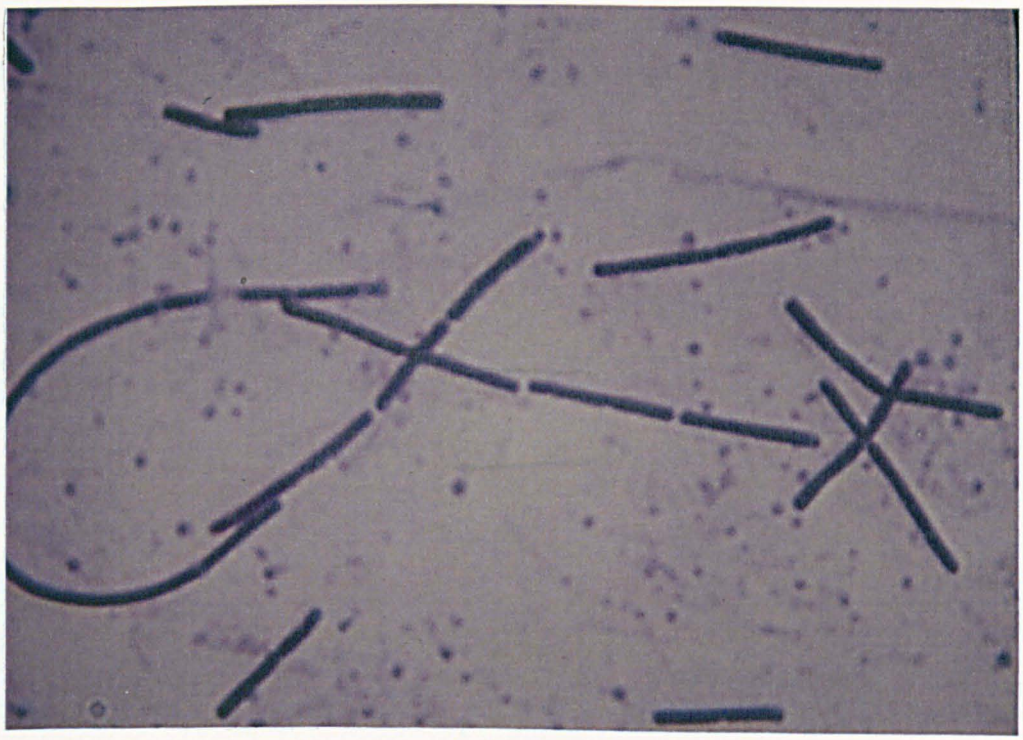


Figure 4.2. *Clostridium novy* type B smear prepared from a 48 hour culture on fastidious anaerobe agar. Gram stain (100x).

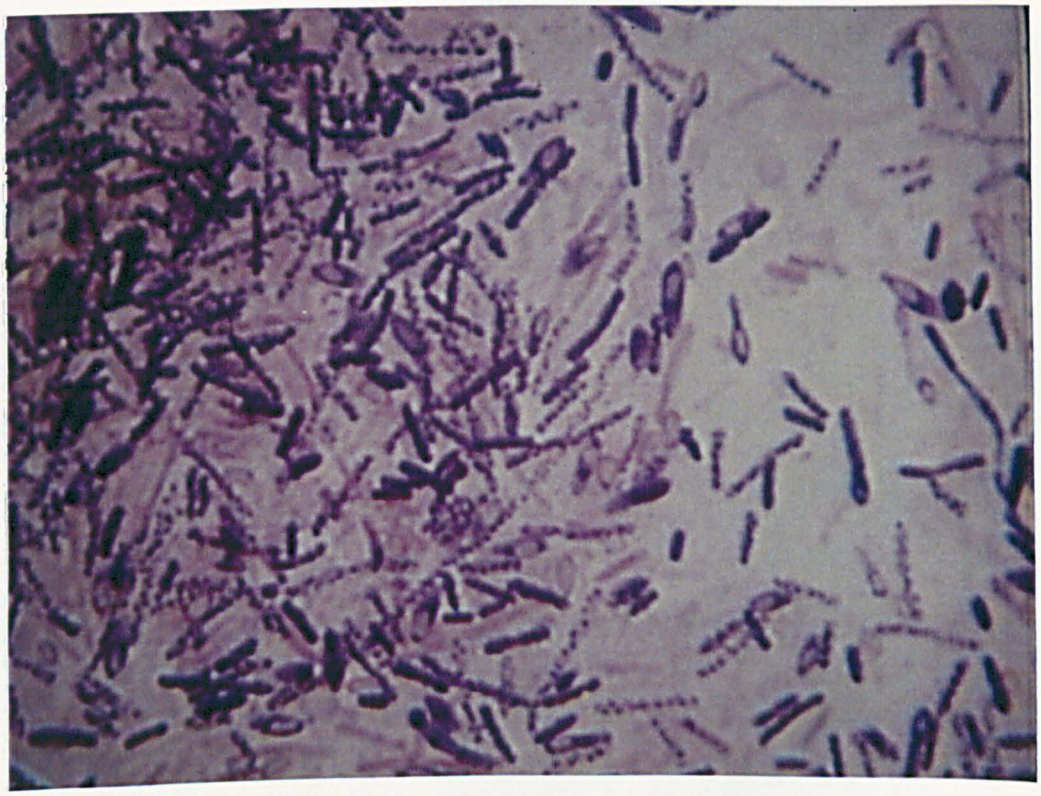


Figure 4.3. *Clostridium novyi* type A. Sheep blood fastidious anaerobe agar, incubated for 24 hours at 37°C.

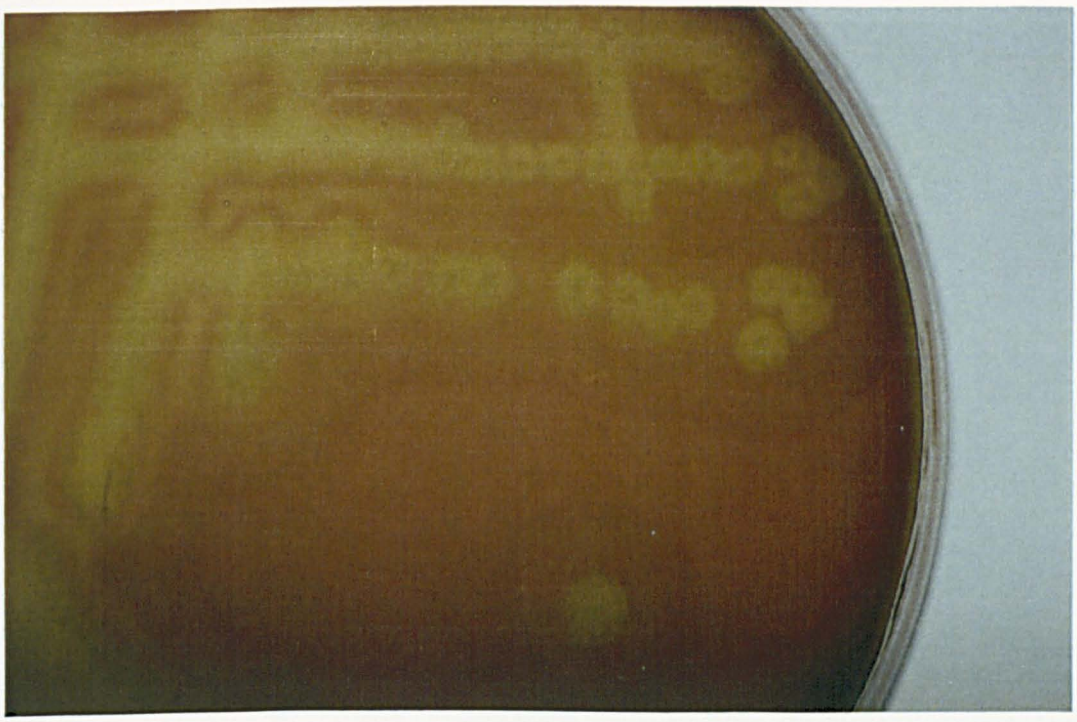


Figure 4.4. *Clostridium novyi* type B. Sheep blood fastidious anaerobe agar, incubated for 24 hours at 37°C.

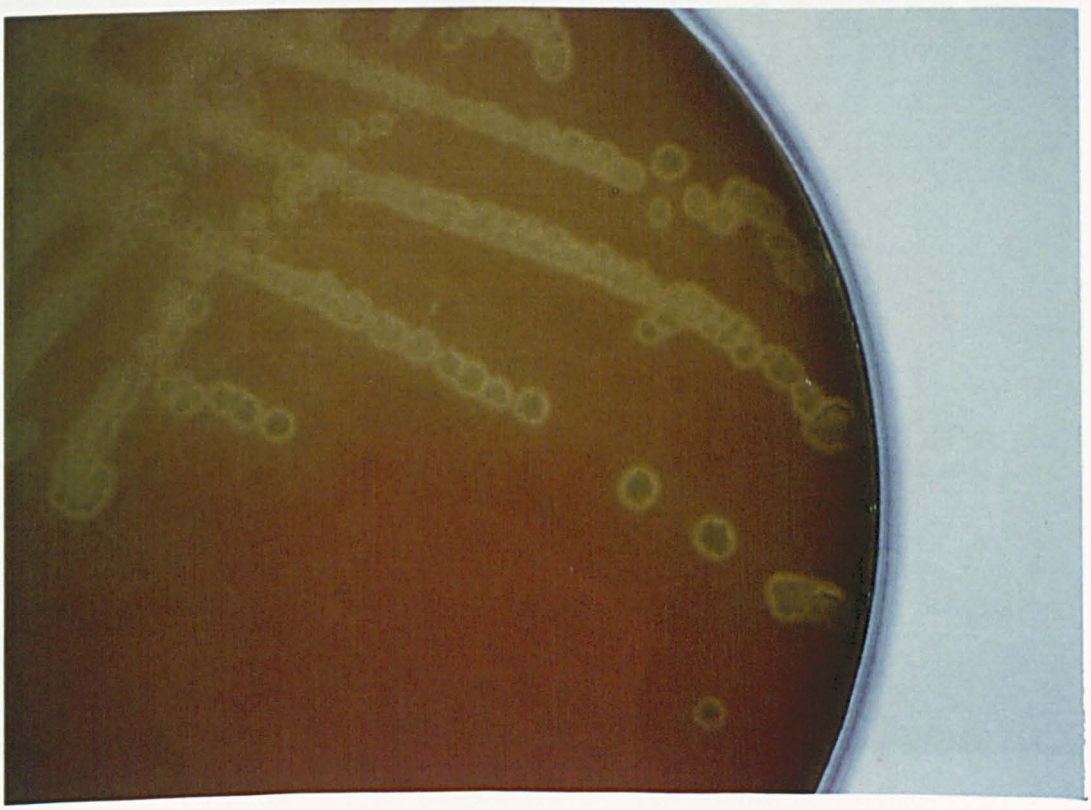


Figure 4.5. *Clostridium novyi* type D (*C. haemolyticum*). Sheep blood fastidious anaerobe agar, incubated for 24 hours at 37°C.

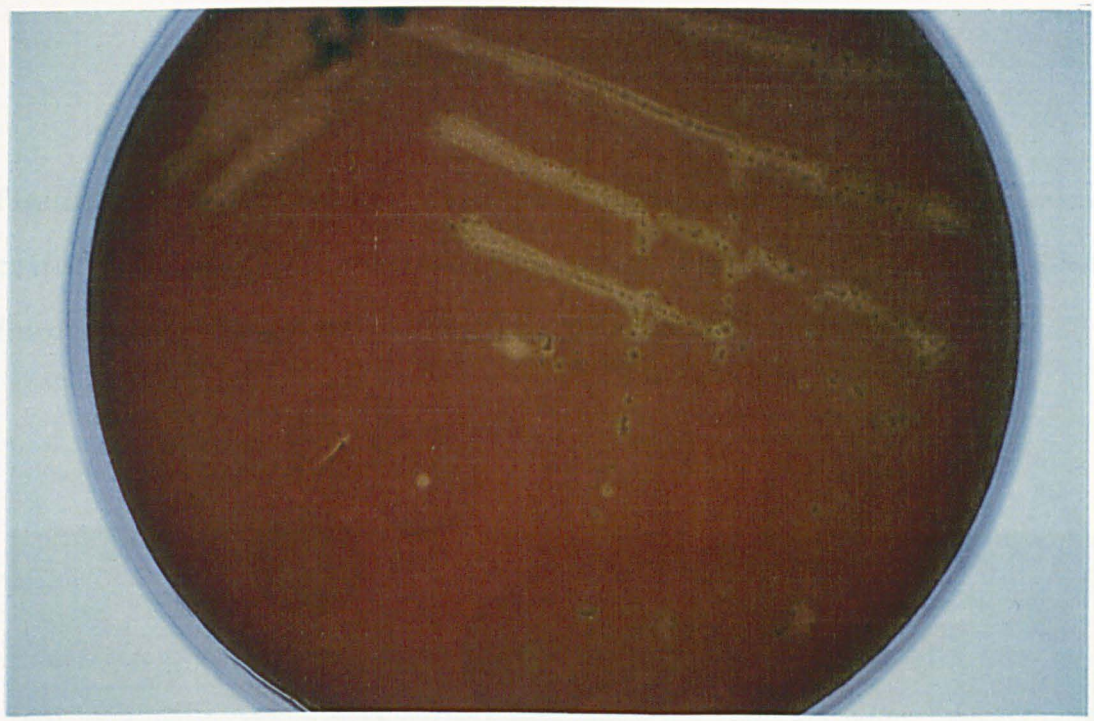


Figure 4.6. *Clostridium novyi* type A. Egg yolk fastidious anaerobe agar incubated for 24 hours at 37°C. Note both lecithinase and lipase reactions.

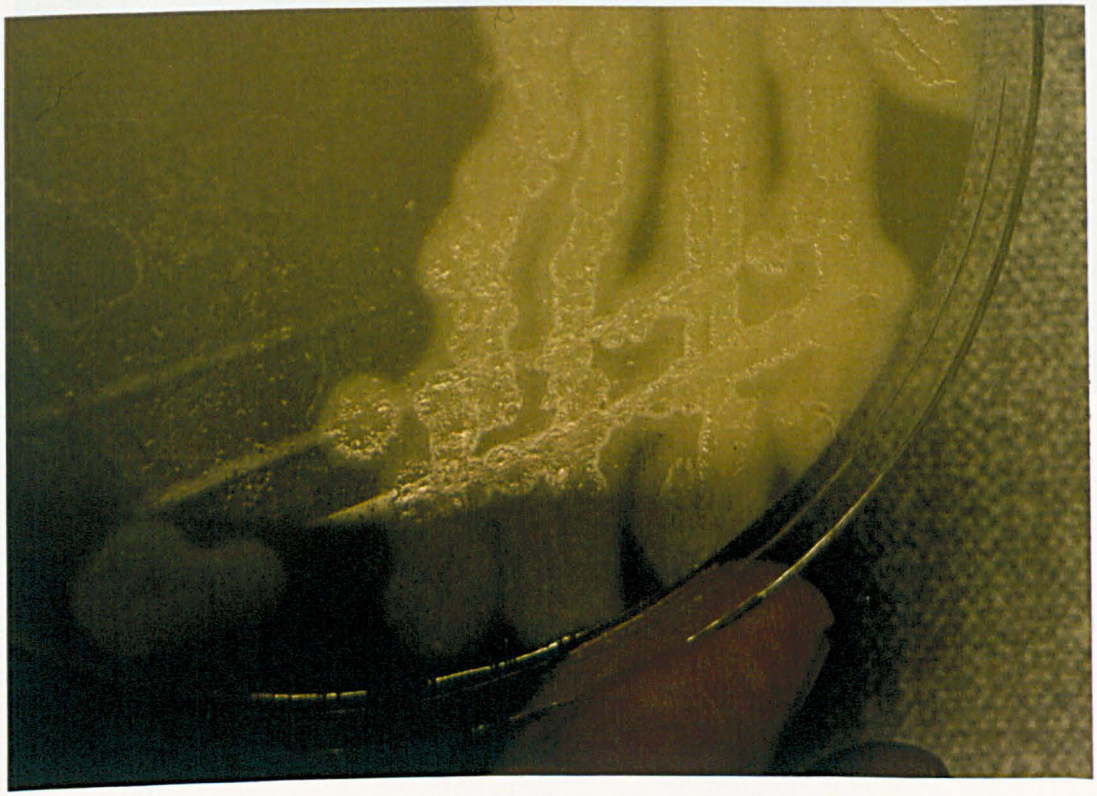
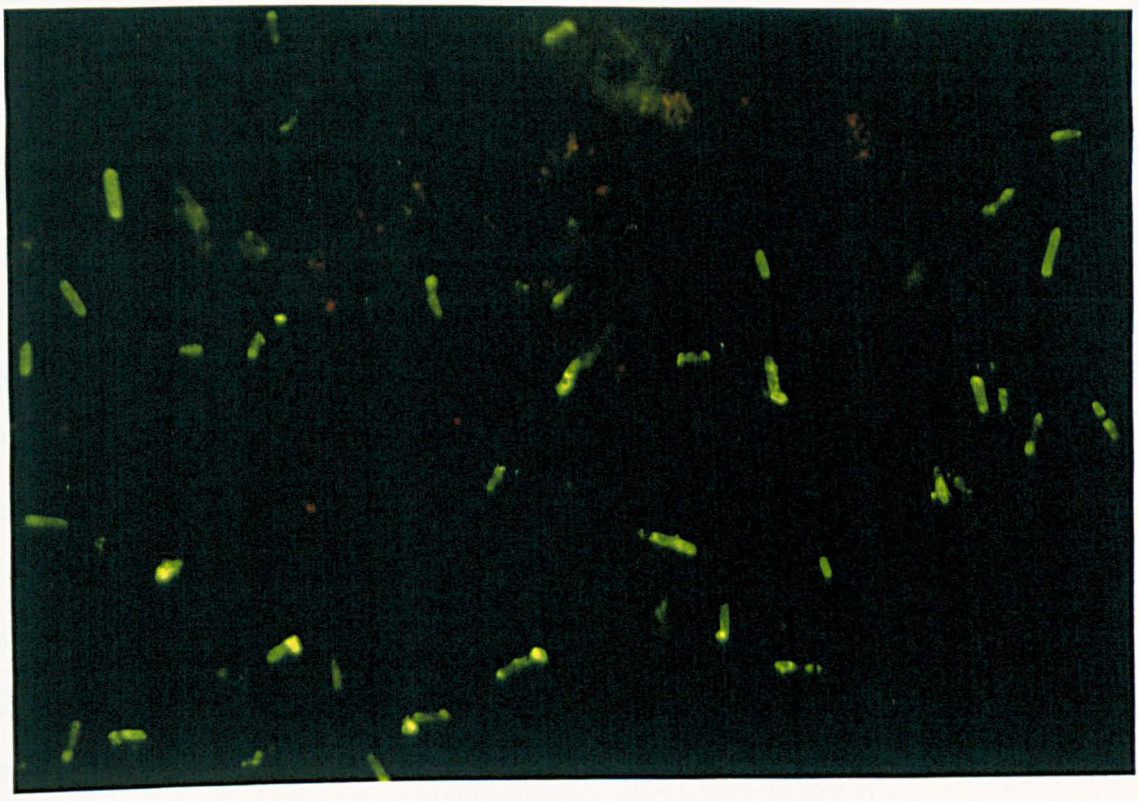


Figure 4.7. *Clostridium novyi* type B. Smear prepared from a 48 hour culture on sheep blood fastidious anaerobe agar and stained with fluorescein isothiocyanate labelled *C. novyi* specific antibody.



4.4. Anaerobiosis

C. novyi was found to be highly sensitive to atmospheric oxygen. Attempts to carry out subculture from broth cultures onto freshly prepared blood agar plates followed by incubation in anaerobic jars with a cold catalyst were unsuccessful when using 24 hour cultures. Subculture from older broth cultures (48 hours) of *C. novyi* type A strains gave scant growth on blood agar plates after 48 hour incubation. Strains of *C. novyi* type B and D incubated in cooked meat broth for 48 hours did not grow on blood agar plates after subculture and 5 days incubation. Anaerobic indicator strips detected oxygen for 4 to 6 hours after the anaerobic jars had been set up.

Subculture on solid media using freshly prepared or pre-reduced fastidious anaerobe agar when performed on the bench gave scant, inconsistent growth with strains of *C. novyi* type B and D. Isolates of *C. novyi* type A grew adequately employing these methods.

The availability of an anaerobic incubator allowed the manipulation of cultures in an anaerobic environment, ensuring adequate conditions for growth of these strict anaerobes. To ensure bacterial isolation of *C. novyi* from clinical specimens all manipulations were performed inside the anaerobic cabinet.

4.5. Growth medium

Growth of isolates of *C. novyi* on liquid media was achieved in a variety of media: thioglycollate medium USP, brain heart infusion broth, cooked meat broth and Wilkins-Chalgren medium (from Oxoid), and fastidious anaerobe broth (FAB, LabM, Amersham). Excellent results were achieved using cooked meat particles supplemented with fastidious anaerobe broth (CMFAB). Slight gas production was noted. This medium was used routinely for reviving cryopreserved cultures, isolation from clinical material and storage.

Fastidious anaerobe broth alone was used for dilution and growing up isolates for DNA extraction. With all liquid media it proved important for consistent luxuriant growth to use broths that were either freshly prepared or pre-reduced by driving off the oxygen by boiling.

Surface culture, particularly with strains of *C. novyi* type B proved difficult, even under anaerobic conditions. Only freshly prepared fastidious anaerobe agar plates (FAA, LabM, Amersham) pre-reduced in the anaerobic cabinet gave a consistent, luxurious growth. Sheep blood and egg yolk was added to improve detection. From a CMFAB inoculum, well defined non spreading colonies were detected after overnight culture on FAA. The appearance of culture collection isolates of all *C. novyi* types on FAA can be seen in section 4.3.

4.6. Growth curve of *Clostridium novyi*

To investigate the kinetics of *C. novyi* in broth culture the growth curve was plotted. Three or four individual colonies from a 24 hour culture of a *C. novyi* type B strain (N1B) on FAA+sheep blood was used to inoculate 100 ml of pre-reduced FAB. Measurements of the optical density (OD) were carried out hourly during the first 18 hours then at 24 and 48 hours after inoculation of the broth (see table 4.1). Sterile plastic cuvettes (1 ml) and a spectrophotometer were used to measure the absorbance at 550 nm. An uninoculated broth was used as a blank. Manipulations were carried out anaerobically, but the broth removed for spectrophotometry was taken out of the anaerobic cabinet and measured immediately. At this time 10-fold dilutions of the broth were made. FAA plates were inoculated with duplicate 20µl aliquots of each dilution for colony counting using the method of Miles and Misra (1938). The results of the growth curve determination can be seen in table 4.3. and figure 4.8.

Figure 4.8. Growth curve of *Clostridium novyi* type B (N1B).

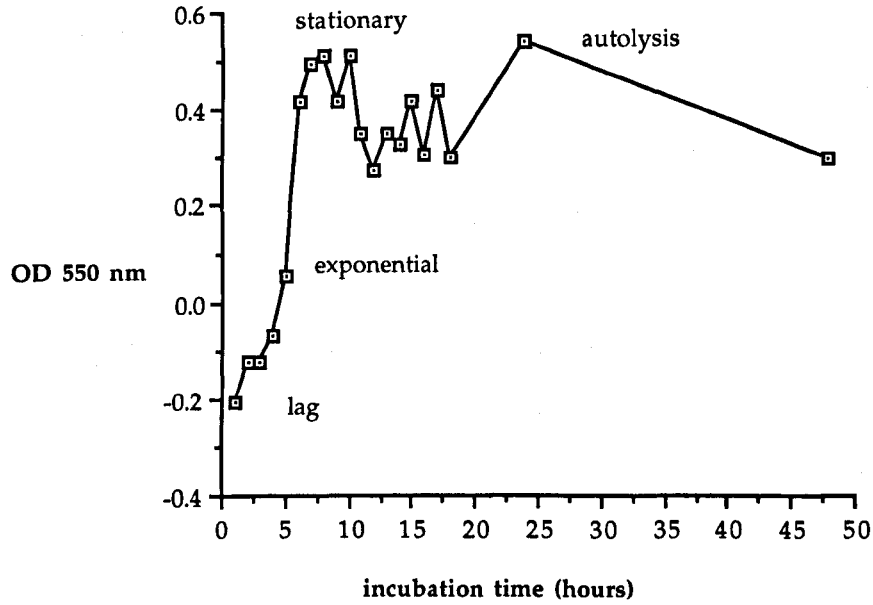


Table 4.2. Optical density (OD 550nm) of a fastidious anaerobe broth culture of *Clostridium novyi* (N1B) over a 48 hour period.

Incubation time (hours)	OD A	OD B	mean OD
1	0.75	0.5	0.625
2	1.0	0.5	0.750
3	0.75	0.75	0.750
4	1.2	0.5	0.850
5	1.75	0.5	1.125
6	3.0	2.25	2.650
7	3.0	3.25	3.125
8	4.25	2.25	3.250
9	3.0	2.25	2.625
10	4.25	2.25	3.250
11	2.25	2.25	1.875
12	2.25	2.25	2.250
13	2.5	2.0	2.250
14	2.25	2.0	2.125
15	3.0	2.25	2.625
16	2.25	1.8	2.025
17	2.75	2.75	2.750
18	2.5	1.5	2.0
24	3.75	3.2	3.475
48	2.0	2.0	2.0

Samples were inoculated on sheep blood agar and incubated aerobically to ensure broth purity. Purity and identity checks were also carried out at the end of the experiment.

The number of colony forming units could not be determined because cultures had spread over the surface of the plate in a confluent growth. The surface of the agar plates had become wet in the anaerobic incubator during pre-reduction and the duration of the experiment, because numerous plates and operator perspiration overwhelmed the desiccant. In a different experiment, colony counts were calculated at intervals of 12, 24, 48 and 72 hours, giving 3 $x10^5$, 3.5 $x10^6$, 3.25 $x10^6$ and 1.75 $x10^6$ cfu/ml respectively.

4.7. Selective isolation

Experiments to establish the value of selective isolation methods were carried out. Ethanol and heat treatment were applied to 48 hour cultures of *C. novyi* type B (N2B) in CMFAB as follows: 1ml of the culture was mixed with 1ml of 70% ethanol for one hour at room temperature or heated in a 80°C waterbath for 10 minutes. The broth was diluted to $1/10^6$ by preparing 10-fold dilutions in FAB. The colony forming units were determined using the method of Miles and Misra (1938). The dilutions and plating were carried out in an anaerobic incubator. FAA plates were prepared fresh and dried thoroughly before being introduced into an anaerobic cabinet for pre-reduction. The pre-shock count of the CMFAB was $9.5x10^6$ cfu/ml. Two protocols were followed after alcohol shock; one sample (A) was centrifuged at 2500 r.p.m. for 10 minutes to remove the ethanol and the pellet was resuspended in 1 ml of pre-reduced FAB. The other sample (B) was plated directly. The plates were incubated for 24 hours, then counted. Method A yielded 3.75 $x10^5$ cfu/ml, whilst method B only gave a count of 10^2 cfu/ml. The ethanol treated samples (0.5 ml) were also inoculated into CMFAB and incubated for 24 hours.

The colony count was carried out as before. Sample A presented 5.75 $x10^6$ cfu/ml, similar to the pre-shock count. The colony count from sample B revealed 7 $x10^5$ cfu/ml.

The heat shock sample yielded a colony count of 4.3 $x10^5$ cfu/ml.

Neomycin was also examined as a selective agent for culture on solid media. Firstly, the culture collection isolates were tested using 70µg/ml of neomycin sulphate in poured plates of FAA with blood. This was the concentration suggested for selective culture of clostridia (Levett, 1991a).

All the strains of *C. novyi* type A tested grew well after subculture from an overnight broth culture. On the other hand, the recovery of type B strains was not achieved. Strains of *C. novyi* type B and D did not grow on the neomycin fastidious anaerobe blood agar after inoculation.

This finding prompted the determination of the minimum inhibitory concentration (MIC) for neomycin against the various culture collection strains. The MIC was calculated on microtitre plates using the broth microdilution method (see below). Doubling dilutions in Wilkins-Chalgren medium starting at a concentration of 70 µg/ml were employed, with both positive (no antibiotic) and negative (uninoculated) control wells. All strains of *C. novyi* type A were resistant to neomycin at 70µg/ml, whilst the MIC value for *C. novyi* type B strains was 35µg/ml for N1B, N2B, N3B, N5B and N6B and 70µg/ml for N4B.

4.8. *In vitro* susceptibility of *Clostridium novyi* to growth enhancing antibiotics.

Minimum inhibitory concentration (MIC) determinations were carried out with 7 growth promoting antibiotics against the *C. novyi* culture collection strains of type A and B.

The broth microdilution method employed was described by Rosenblatt (1991).

1) Sterile Wilkins-Chalgren (W-C) broth was prepared and doubling dilutions of the antimicrobials were carried out to achieve final concentrations between 0.125 and 64 μg/ml. Seven antibiotics were used; avilamycin (Elanco) was dissolved in acetone, Zinc bacitracin (Apothekernes Laboratorium) and avoparcin (Cyanamid) were dissolved in 0.1M NaOH and chlortetracycline (Pfizer), salinomycin (Hoescht), tylosin (Elanco) and virginiamycin (SmithKline Beecham) were disolved in water. The antibiotic dilutions were dispensed in volumes of 0.1ml per well of a plastic microtitre plate, with a lid to prevent evaporation. Two wells were left without antibiotic to serve as a positive and negative (uninoculated) controls. The microtitre plates were stored in plastic bags at -70°C.

2) Portions of 5 single colonies from an overnight sheep blood FAA culture of *C. novyi* strains, N1A, N2A, N3A, N4A, N1B, N2B, N3B, N4B, N5B AND N6B were incubated for 24 hours in W-C broth.

3) The frozen microtitre plates were thawed and reduced in the anaerobic cabinet for 4 hours.

4) The turbidity of the inoculum was adjusted to the 0.5 McFarland standard. After 24 hours incubation the count was approximately 10^6 cfu/ml in W-C broth. As 0.01ml were inoculated in 0.1ml of broth the final inoculum per well was $1x10^5$ cfu/ml.

5) The microtitre plates were incubated anaerobically for 48 hours at 37°C.

6) The MIC was read as the lowest concentration of antimicrobial that inhibited growth.

The results of the experiment can be seen in table 4.4.

Table 4.4. MIC's (μg/ml) of 7 growth enhancing antibiotics against *Clostridium novyi* culture collection isolates.

Clostridium novyi type A strains

Compound	N1A	N2A	N3A	N4A
Avilamycin	2	1	2	0.5
Avoparcin	0.125	0.125	0.125	0.125
CTC	0.125	0.125	0.125	0.125
Salinomycin	0.125	0.125	0.125	0.125
Tylosin	0.25	0.125	0.125	0.125
Virginiamycin	0.25	0.125	0.25	0.25
Zinc bacitracin	0.125	0.125	0.125	0.125

Clostridium novyi type B strains

Compounds	N1B	N2B	N3B	N4B	N5B	N6B
Avilamycin	4	2	64	2	0.25	0.125
Avoparcin	0.125	0.125	0.125	0.125	0.125	0.125
CTC	0.125	0.5	0.25	0.125	0.25	0.125
Salinomycin	0.125	0.125	0.125	0.125	0.125	0.125
Tylosin	0.125	0.125	0.25	0.125	0.125	0.25
Virginiamycin	0.25	0.5	0.5	4	0.25	0.125
Zinc bacitracin	0.125	16	0.125	0.125	0.125	0.125

4.9. Conclusions

C. novyi has proven difficult to study in pure culture in the past and the work in this chapter examined the identification, growth requirements and selective isolation of this group of organisms.

Identification of *C. novyi* of the three types; A, B and D was performed by traditional methods. Variation in the Gram stain presentation, colony appearance and results of carbohydrate fermentation was noted within the same strain. The growth medium and time of incubation of cultures were responsible for great variation. Difficulties with inconsistent growth on the anaerobe identification media were experienced and an alternative would be to perform the tests in broth medium. The haemolysis detected on sheep blood agar plates changed after repeated subculture of *C. novyi* types A and B, with loss of the wider area of haemolysis. Repeated subculture was proven to cure *C. novyi* and *C. botulinum* of toxigenic phages (Eklund, Poysky and Habig, 1989), and this could be occurring in some cases.

The strict anaerobic nature of *Clostridium novyi* strains was confirmed in this study, requiring pre-reduced growth media and handling of isolates in the anaerobic cabinet to ensure consistent heavy growth. The nutritional needs of the organism were not addressed directly, but good growth was achieved with existing broth and agar media, available commercially.

Growth of *C. novyi* type B strain (N1B) was studied. By 12 hours the culture had ended the exponential phase and between 24 and 48 hours autolysis had begun. Spores were detected in Gram stains after 24 hours incubation.

Selective isolation employing heat shock at 80°C, alcohol shock and neomycin in solid media was assessed. Both heat and ethanol were useful in recovery of *C. novyi* , but neomycin at 70 µg/ml was deleterious to strains of *C. novyi* type B. The MIC of 5 strains tested was 35 µg/ml, whilst one strain was resistant at 70 µg/ml.

In the future a selective method of isolation using antibodies raised against the whole *C. novyi* cell should be examined. The method consists of coating immunomagnetic beads (Dynabeads, Dynal) with the antibodies and mixing them with a broth culture or tissue macerate. The complex formed by antigen (*C. novyi*), the antibody and the magnetic bead can be retained by using a magnet and washing the unbound material. Removing the magnet releases the beads which can be used to inoculate a broth or plate. A similar affinity technique was employed to isolate *C. perfringens* type C in man and pigs (Lawrence, Brown, Bates, Saul, Davis, Spark and Anian, 1984).

Finally, the *in vitro* susceptibility of *C. novyi* type A and B strains to growth enhancing antibiotics was determined. All the type A strains tested were highly sensitive to avoparcin, chlortetracycline, salinomycin, tylosin, virginiamycin and Zinc bacitracin, but presented a higher MIC for avilamycin.

C. novyi type B strains were similarly sensitive to avoparcin, chlortetracycline, salinomycin and tylosin. Zinc bacitracin had a low MIC with most strains except one (N2B). Avilamycin proved to be less effective against 4 *C. novyi* type B strains. These findings differed from some results in the only other report of antibiotic sensitivity testing of *C. novyi* (Singh, Bhat and Singh, 1994). One isolate was examined and resistance to bacitracin at 30 µg/ml was reported and the MIC for neomycin was set at 15 µg/ml.

Future work would require the examination of field strains of *C. novyi* to determine the efficacy of the growth promoters in prevention of clostridial hepatopathy in problem herds.

5. MOLECULAR STUDIES FOR THE DETECTION OF TOXIN GENES OF *Clostridium novyi.*

"PCR can actually detect a needle in a haystack and then make a haystack of needles."

Klingeborn (1992)

5.1. Introduction

Strains of *Clostridium novyi* produce two different phospholipases C; the gamma and beta toxins (Hatheway, 1990). Only strains of *C. novyi* type A produces the gamma toxin, whilst both type B and D isolates produce the beta toxin (Oakley and Warrack, 1959). The beta toxin from strains of both types was shown to be serologically indistinguishable (Nakamura *et al*, 1983). The various phospholipase C enzymes produced by bacteria (see table 5.1.), even within the genus *Clostridium* and possibly between strains of one species, appear to be different proteins (Titball, 1992). The variation in biochemical, toxicological and immunological properties of these enzymes would support this suggestion.

Following the successful cloning and sequencing of the phospholipase C gene (*plc* gene) from *C. perfringens* NCTC 8237 (Titball *et al*, 1989), a probe derived from this gene was prepared by these investigators to analyse other phospholipase producing clostridia. Using the probe, homologous fragments were detected in DNA isolated from various *Clostridium* strains, including *C. novyi* type A (NCTC 6737).

At this time, the nucleotide sequence of phospholipase C gene from the related *C. bifermentans* was also described (Tso and Siebel, 1989). The nucleotide sequence was found to be 64% homologous when compared with the *plc* gene of *C. perfringens* . Furthermore, Leslie *et al* (1989) concluded that the deduced *C. perfringens* alpha toxin sequence shared significant structural homology with the phospholipase C of *Bacillus cereus*.

These findings prompted the author's idea of using the known sequence of the *plc* gene to detect the hitherto not studied phospholipase genes in DNA extracted from *C. novyi*. Highly degenerate primers were constructed based on the published aminoacid sequence encoded by the *plc* gene. After preparation of *C. novyi* DNA as a template, *in vitro* amplification using the polymerase chain reaction (PCR) with a thermostable DNA polymerase was used. The amplified DNA products were then cloned and the product was sequenced using the chain termination method of Sanger, Nicklen and Coulson (1977).

The DNA from *C. novyi* type A, B and D was used as a template for the amplification experiments. Once a portion of a gene was detected, specific primers to amplify a further portion of genome could be used. DNA probes prepared from transformed plasmid DNA could be employed for hybridisation with DNA from strains of the different types of *C. novyi* or other clostridia. A molecular method to differentiate *C. novyi* types A and B would complement the use of the ELISA to detect the alpha toxin, therefore avoiding the use of toxin neutralisation tests in laboratory animals.

Table 5.1. Phospholipases of *Clostridium* and *Bacillus* spp.

Enzyme	Substrates hydrolysed	Gene cloned	Properties
C. perfringens			
alpha toxin	PC, Sph, PS	Yes	Toxic, haemolytic
B. cereus			
PC-PLC	PC, PE, PS	Yes	All non toxic and non
Sphingomyelinase	Sph	Yes	haemolytic
PI-PLC	PI	Yes	
C. bifermentans			
Phospholipase C	N.R.	Yes	Weakly toxic, weakly haemolytic
C. novyi type A			
gamma toxin	PC, Sph, PS, PE, PI	No	Toxic, haemolytic
C. novyi type B and type D			
beta toxin	PC, Sph	No	Toxic, haemolytic
C. absonum			
Phospholipase C	N.R.	No	Non toxic, non haemolytic
C. barati			
Phospholipase C	N.R.	No	Non toxic, non haemolytic

Adapted from Titball (1993)

Pc, phosphatidylcholine; Pe, phosphatidyethanolamine; Ps, phosphatidylserine; Sph, sphingomyelin; Pi, phosphatidylinositol; N.R., not reported

5.2. Materials and methods

i) Bacterial strains and growth conditions

The following culture collection strains were used for the various experiments. An alpha toxin producing *Clostridium perfringens* type A (NCTC 8237), *Clostridium novyi* type A (NCTC538 and NCTC6738), *Clostridium novyi* type B (NCTC9691), *Clostridium novyi* type D (*haemolyticum*) (NCTC8145 and NCTC9692) and *Clostridium bifermentans* (NCTC506 and NCTC8780) received from the National Collection of Type Cultures, PHLS. A further *C. novyi* type B (strain R4669) was kindly donated by Professor Duerden from the collection of the Anaerobe Reference Unit, Cardiff.

All isolates were grown either from frozen beads or from cooked meat fastidious anaerobe broth working stocks. Isolates for DNA extraction were taken from single colonies incubated anaerobically on fastidious anaerobe agar plates overnight and subcultured in 2 ml of freshly prepared fastidious anaerobe broth (Lab M, Amersham). All cultural procedures were carried out in the anaerobic incubator (MK2 anaerobic work station, Don Whitley Scientific). Initially overnight culture at 37°C was chosen to ensure high yields of bacteria. Later, shorter incubation periods were prefered to prevent inhibition of the *taq* polymerase and other possible negative effects of the soluble antigens dissolved in the broth media. Good growth of all strains was achieved in liquid media after 8 hours incubation at 37°C.

ii) Isolation of bacterial DNA

A volume of 1.5 ml of bacterial culture was transfered into a microcentrifuge tube (Eppendorf), followed by centrifugation for 3 minutes at 13,000 *g* . After removing the supernatant, the precipitated pellet was resuspended in 200 µl of cell resuspend solution (50 mM tris HCl pH 7.5, 10mM EDTA, 100 µg/ml RNase A). Next, 200 µl of lysis solution (0.2 M NaOH, 1% SDS) were added

to the solution that was then mixed by inversion until it cleared. The total volume was centrifuged at 13,000*g* for 5 minutes at room temperature.

The supernatant was then transferred to a new Eppendorf and the DNA was extracted by the phenol-chloroform method (Maniatis, Fritsch and Sambrook, 1982). Equal volumes of supernatant and phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) were centrifuged for 5 minutes at room temperature. The aqueous layer (top) was carefully collected and the procedure repeated if contamination with the interface had occurred. This step was followed by chloroform extraction.

DNA precipitation was achieved by adding 1/10 (v/v) 3M sodium acetate pH 5.2 and 2 volumes of ice cold absolute ethanol to the solution and mixed by inverting the microcentrifuge tube. The solution was allowed to precipitate either in the -20°C freezer overnight or at - 80°C for at least 1 hour. The DNA precipitate was centrifuged for 5 minutes at room temperature, sometimes allowing a whitish pellet to become visible. The traces of solute were washed with ice cold 70% ethanol and dried in a vacuum dessicator or in an incubator at 37°C. The DNA was resuspended in 50 µl of deionised water or in TE buffer (10 mM tris-HCl pH 8, 1 mM EDTA). The DNA was stored in screw top Eppendorf tubes at - 20°C.

DNA concentration was initially measured using the ethidium bromide assay and comparison with known concentrations of DNA. Later, a spectrophotometer (Gene Quant II, Pharmacia Biotech), was used to measure absorption at 260 and 280 nm. The protocol recommended by the manufacturer was followed. The 260:280 absorbance ratio allowed the protein contamination of the DNA sample to be determined.

iii) *In vitro* DNA amplification

Degenerate primers were derived from the published *plc* gene sequence (Leslie *et al*, 1989) shown in figure 5.1. Back-translating from the peptide sequence, two highly degenerate synthetic oligonucleotides (see below) were designed. The oligonucleotides were synthesized at the Molecular Medicine Unit, King's College School of Medicine & Dentistry.

Primers; NOV 1 (23 base pairs); concentration 0.86µg/µl

5'	ATG	AAA	AGA	AAG	ATA	TGT	AAA	GC	3'
		G C	T	A	T	C	G		
			G		C				
			C						

384 degeneracies

NOV 2 (21 base pairs); concentration 0.76 µg/µl

5'	CTC	AAA	CTT	AAC	GTG	ACC	AGC	3'
	T	G	T	T	A	T	T	
				G		G	G	
				C		C	C	

1024 degeneracies

The PCR reactions were performed using a DNA thermal cycler (Hybaid). The following components, to make up a total 50 µl of the reaction mix, were added to a 0.5 ml sterile Eppendorf tube: 5 µl of 10X PCR buffer (100mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin), 5 µl of 2 mM deoxynucleotides triphosphate (dNTP) solution, 0.1 to 0.5 µg of template DNA, 1 unit of *taq* polymerase from *Thermus aquaticus* (Applied Biosystems). The procedure used was as follows: 3 min at 94°C followed by 40 cycles consisting of 1 min at 94°C, 1 min at 37°C and 3 min at 72°C, finally 5 min at 72°C to complete the DNA synthesis.

The PCR product was analysed by electrophoresis in a 1.5% agarose gel including 1 µg/µl ethidium bromide, followed by viewing with ultraviolet transillumination.

Figure 5.1. DNA and aminoacid sequence of the *Clostridium perfringens* phospholipase C (*plc*) gene (Lesley *et al* , 1989). The primers Nov 1 and Nov 2 can be seen underlined.

```
Cl Perf plc  X13608 [785 to 1981] -> 1-phase Translation

DNA  sequence    2034 b.p.   GAATTCCAAGAC ... AAATAAAAGCTT   linear

785  /    1                                    815  /   11
atg aaa aga aag att tgt aag gcg ctt att tgt gcc gcg cta gca act agc cta tgg gct
met lys arg lys ile cys lys ala leu ile cys ala ala leu ala thr ser leu trp ala
845  /   21                                   875  /   31
ggg gca tca act aaa gtc tac gct tgg gat gga aag att gat gga aca gga act cat gct
gly ala ser thr lys val tyr ala trp asp gly lys ile asp gly thr gly thr his ala
905  /   41                                   935  /   51
atg att gta act caa ggg gtt tca atc tta gaa aat gat ctg tcc aaa aat gaa cca gaa
met ile val thr gln gly val ser ile leu glu asn asp leu ser lys asn glu pro glu
965  /   61                                   995  /   71
agt gta aga aaa aac tta gag att tta aaa gag aac atg cat gag ctt caa tta ggt tct
ser val arg lys asn leu glu ile leu lys glu asn met his glu leu gln leu gly ser
1025 /   81                                   1055 /   91
act tat cca gat tat gat aag aat gca tat gat cta tat caa gat cat ttc tgg gat cct
thr tyr pro asp tyr asp lys asn ala tyr asp leu tyr gln asp his phe trp asp pro
1085 /  101                                   1115 /  111
gat aca gat aat aat ttc tca aag gat aat agt tgg tat tta gct tat tct ata cct gac
asp thr asp asn asn phe ser lys asp asn ser trp tyr leu ala tyr ser ile pro asp
1145 /  121                                   1175 /  131
aca ggg gaa tca caa ata aga aaa ttt tca gca tta gct aga tat gaa tgg caa aga gga
thr gly glu ser gln ile arg lys phe ser ala leu ala arg tyr glu trp gln arg gly
1205 /  141                                   1235 /  151
aac tat aaa caa gct aca ttc tat ctt gga gag gct atg cac tat ttt gga gat ata gat
asn tyr lys gln ala thr phe tyr leu gly glu ala met his tyr phe gly asp ile asp
1265 /  161                                   1295 /  171
act cca tat cat cct gct aat gtt act gcc gtt gat agc gca gga cat gtt aag ttt gag
thr pro tyr his pro ala asn val thr ala val asp ser ala gly his val lys phe glu
1325 /  181                                   1355 /  191
act ttt gca gag gaa aga aaa gaa cag tat aaa ata aac aca gca ggt tgc aaa act aat
thr phe ala glu glu arg lys glu gln tyr lys ile asn thr ala gly cys lys thr asn
1385 /  201                                   1415 /  211
gag gct ttt tat act gat atc tta aaa aac aaa gat ttt aat gca tgg tca aaa gaa tat
glu ala phe tyr thr asp ile leu lys asn lys asp phe asn ala trp ser lys glu tyr
1445 /  221                                   1475 /  231
gca aga ggt ttt gct aaa aca gga aaa tca ata tac tat agt cat gct agc atg agt cat
ala arg gly phe ala lys thr gly lys ser ile tyr tyr ser his ala ser met ser his
1505 /  241                                   1535 /  251
agt tgg gat gat tgg gat tat gca gca aag gta act tta gct aac tct caa aaa gga aca
ser trp asp asp trp asp tyr ala ala lys val thr leu ala asn ser gln lys gly thr
1565 /  261                                   1595 /  271
gcg gga tat att tat aga ttc tta cac gat gta tca gag ggt aat gat cca tca gtt gga
ala gly tyr ile tyr arg phe leu his asp val ser glu gly asn asp pro ser val gly
1625 /  281                                   1655 /  291
aag aat gta aaa gaa cta gta gct tac ata tca act agt ggt gag aaa gat gct gga aca
lys asn val lys glu leu val ala tyr ile ser thr ser gly glu lys asp ala gly thr
1685 /  301                                   1715 /  311
gat gac tac atg tat ttt gga atc aaa aca aag gat gga aaa act caa gaa tgg gaa atg
asp asp tyr met tyr phe gly ile lys thr lys asp gly lys thr gln glu trp glu met
1745 /  321                                   1775 /  331
gac aac cca gga aat gat ttt atg act gga agt aaa gac act tat act ttc aaa tta aaa
asp asn pro gly asn asp phe met thr gly ser lys asp thr tyr thr phe lys leu lys
1805 /  341                                   1835 /  351
gat gaa aat cta aaa att gat gat ata caa aat atg tgg att aga aaa aga aaa tat aca
asp glu asn leu lys ile asp asp ile gln asn met trp ile arg lys arg lys tyr thr
1865 /  361                                   1895 /  371
gca ttc tca gat gct tat aag cca gaa aac ata aag ata ata gca aat gga aaa gtt gta
ala phe ser asp ala tyr lys pro glu asn ile lys ile ile ala asn gly lys val val
1925 /  381                                   1955 /  391
gtg gac aaa gat ata aac gag tgg att tca gga aat tca act tat aat ata aaa taa
val asp lys asp ile asn glu trp ile ser gly asn ser thr tyr asn ile lys OCH
```

v) Gene cloning procedures

The TA cloning system (Invitrogen) was used for PCR products following the manufacturer's instructions (see appendix VII). The plasmid used for the transformation of *E. coli* competent cells was pCRII.

The pCRII plasmid has the advantage of carrying an ampicillin resistance gene plus the *lacZ'* gene that codes for part of the enzyme β galactosidase. The latter property allows identification of recombinants by their inability to synthesize the enzyme. For this purpose recombinant *E. coli* clones were cultured on LB agar plates (Maniatis *et al*, 1982) with 50 μg/μl of ampicillin and 25 μl of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase, 40 mg/ml stock in dimethylformamide). Bluo-gal (5-bromoindole-β-D-galactopyranoside, Gibco-BRL) was a cheaper alternative substrate. White colonies isolated from the agar did not express galactosidase activity and therefore contained the recombinant plasmid DNA. Transformed cells (individual white colonies) were subcultured in LB broth with ampicillin at 37°C in a shaker bath for 6 hours and stored at -80°C in LB glycerol. Small scale purification of plasmid DNA was accomplished with a special purification resin and minicolumn supplied in a kit (Promega Wizard Minipreps) following the manufacturer's instructions (see appendix VI).

To verify the presence of an insert of the correct size in the recombinant plasmids, restriction enzyme digestion of purified plasmid DNA was carried out using *Eco* RI and other restriction enzymes (Boehringer-Mannheim) as needed.

vi) Sequencing

Double-stranded DNA sequencing was carried out by the chain termination method (Sanger, Nicklen and Coulson, 1977). The methodology, including the double stranded DNA purification, sequencing reactions and gel preparation are detailed in appendix VIII.

5.3. Results

i) *In vitro* amplification using PCR

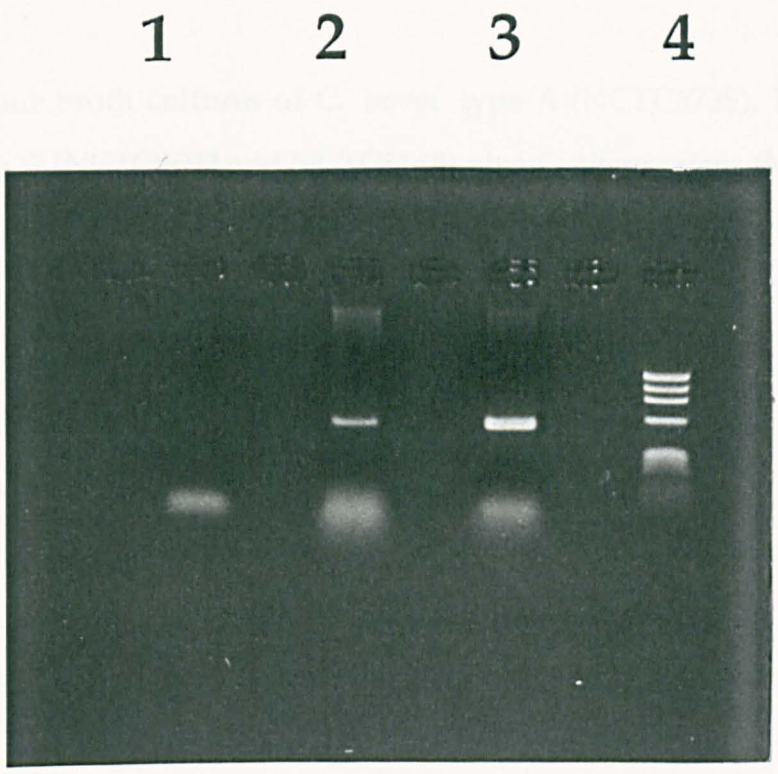
Initial work was carried out with *C. perfringens* DNA as a template for the amplification reactions. Two methods for DNA extraction were used in the first instance; one method using guanadinium thiocyanate/citrate /sarcosyl/β-mercaptoethanol (GT/C/Sarc/ME, appendix IX) was discontinued, the other (see methods above) was adopted for the remaining experiments.

The degenerate primers Nov 1 and Nov 2 were diluted for use in the PCR at a concentration of 2μM, and stored at -20°C as working solutions. The reaction amplified a fragment estimated to be approximately 500 bp in size, when compared with the molecular weight marker (ϕx174 Hae III digest, Sigma) on a 1.5% agarose gel as illustrated in figure 5.2. The fragment size corresponded with the expected size (510 bp) deduced from the *plc* gene sequence. The PCR product and the template from this reaction were subsequently used as positive controls in further amplifications. Both uninoculated broth and double distilled water were used as negative control templates for the PCR.

DNA template from *C. novyi* type A and type B cultures was prepared next. The PCR failed to amplify a fragment from the *C. novyi* template, but was successful with the positive control DNA, suggesting correct conditions for the polymerase reaction.

Also included in this PCR was a purified overnight broth culture of intestinal contents collected from a horse with anterior enteritis thought to be caused by *C. perfringens*. The 510 bp fragment amplified would indicate the presence of the *plc* gene in this broth, and presumably toxigenic *C. perfringens*. Unexpectedly, anaerobic bacterial culture from this sample did not yield any clostridia.

Figure 5.2. PCR products separated by electrophoresis on a 1.5% agarose gel. The band amplified with primers Nov 1 and Nov 2 was 510 bp in size.



1. Negative control. Template prepared from uninoculated broth.

2. *Clostridium perfringens* DNA extracted with GT/C/Sarc/ME.

3. *Clostridium perfringens* DNA (extraction using SDS method).

4. DNA molecular weight marker, Φ X174 *Hae* III digest.

Following this apparent failure, fresh DNA template from C. *novyi* types A, B and D and *Clostridium bifermentans* was prepared for amplification using primers Nov 1 and Nov 2. The amplification was not succesful.

This lack of success with the PCR prompted a review of the methodology. Firstly, the possibility of diluting the DNA template to decrease the concentration of possible *Taq* polymerase inhibitors was considered. Secondly, the primer concentration was investigated and finally different Mg^{++} ion concentrations were to be tried.

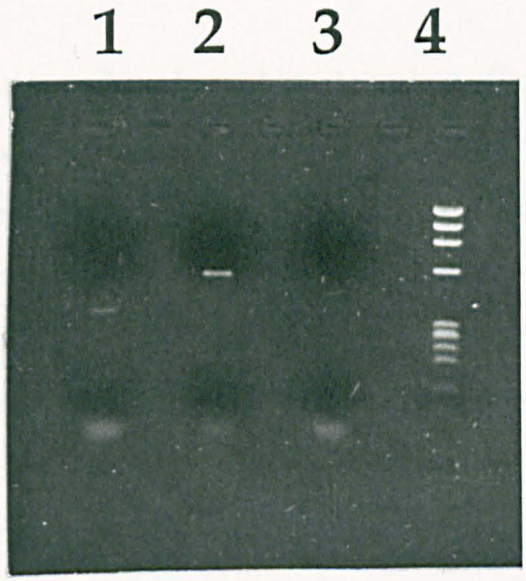
Eight hour broth cultures of C. *novyi* type A (NCTC6738), Type B (R4669) and type D (NCTC9692 and NCTC8145) plus C. *bifermentans* (NCTC506) were used to prepare the template. Two phenol:chloroform extractions were required to produce a clear aqueous layer and care was taken to avoid phenol contamination of the template. The PCR was carried out following the previously described protocol, but the templates were used undiluted and diluted with double distilled water at 1/10 and 1/100.

The band of amplified C. *novyi* DNA was smaller than the 510 bp C. *perfringens* product on the ethidium bromide stained gel. The band was estimated to be approximately 400 bp in length (figure 5.3.). The PCR product was used directly for the cloning experiments (*q.v.*).

Unfortunately, the PCR did not work consistently and on occasion multiple bands appeared on agarose gel electrophoresis after amplification.

Further refinements were considered; the template was prepared from resuspended culture on solid media and the template DNA concentration was calculated to find the optimum target molecules for PCR. These efforts did not improve the reliability or amount of PCR product.

Figure 5.3. PCR products separated by electrophoresis on a 1.5% agarose gel. Primers Nov1 and Nov 2 (2μM) were used. The amplified products were 400 bp and 500 bp in size.



Lane 1. *Clostridium novyi* type B DNA template.

Lane 2. *Clostridium perfringens* DNA. template

Lane 3. Negative control. Template prepared from uninoculated broth.

Lane 4. DNA molecular weight marker, Φ X174 *Hae* III digest.

The concentration of primers used was investigated; Nov 1 and 2 were used at the working dilution (2μM), undiluted (0.86 μg/μl and 0.76 μg/μl respectively) and diluted 1/10 and 1/100 with distilled water.

At the same time primers α1 (CCTGCTAATGTTACTGCCG) and α2 (GTCATGCTAGCATGAG), highly specific to the *C. perfringens* alpha toxin gene (*plc* gene), were used to verify that the reaction mix was working correctly. Fragments of 224 bp were expected to be amplified from the *C. perfringens* DNA template with these primers.

The template for these reactions was extracted from *C. novyi* type A (NCTC538), type B (NCTC9691 and R4469) with *C. perfringens* DNA as a positive control.

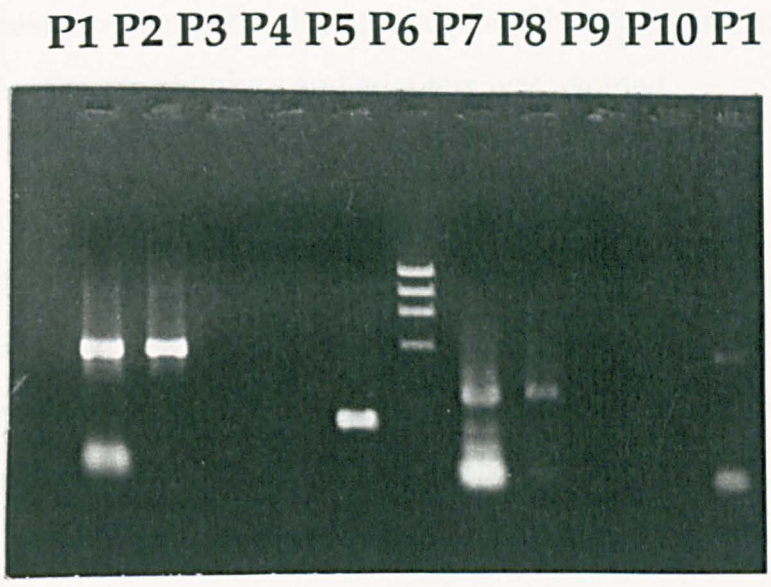
The degenerate primers Nov 1 and Nov 2 worked efficiently undiluted and at a 1/10 dilution, giving a strong band of approximately 400 bp with the *C. novyi* type B template (see figure 5.4.). The PCR products were ligated to pCRII and transformed in *E. coli* for further work. Nothing was amplified in the negative control reactions, discounting the possibility of contamination.

Some results were unexpected, particularly regarding the successful amplification of a fragment of ≃ 200 bp from *C novyi* type A with α1 and α2. The reaction using the alpha toxin specific primers also amplified a larger fragment (500 bp approximately) from *C. novyi* type B DNA.

Finally, the Ampliwax ™ PCR gem kit (Perkin Elmer) was tried in an attempt to use the Hot Start technique. This novel method has been used to prevent the mixing of the PCR reactants to avoid non specific annealing of primers to non target DNA.

The method consisted of separating the primers, deoxynucleotides and buffer from the template and polymerase by means of a wax pellet that becomes molten at 75-80°C. This technique did not improve the yield of the amplification when using the degenerate primers Nov 1 and Nov 2.

Figure 5.4. 1.5% Agarose gel electrophoresis of the PCR products amplified from *C. perfringens* and *C. novyi* type B DNA.



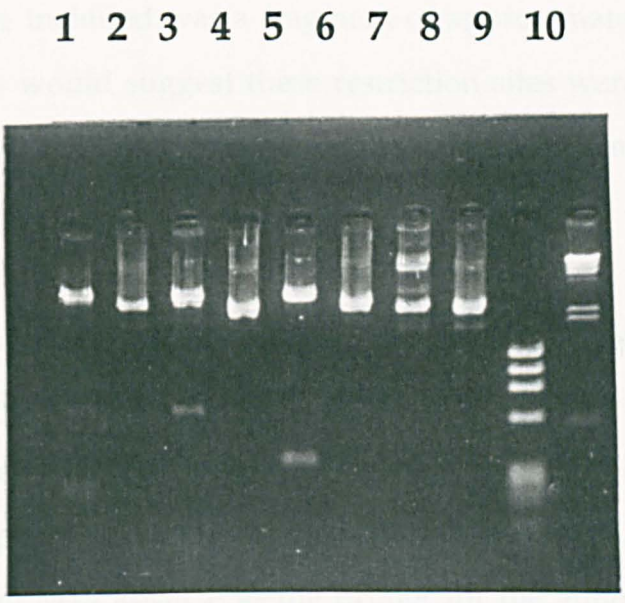
P1. *C. perfringens* DNA and primers Nov1 and Nov2 undiluted.
P2 *C. perfringens* DNA and primers Nov1 and Nov2 diluted 1/10.
P3. *C. perfringens* DNA and primers Nov1 and Nov2 diluted 1/100.
P4. Negative control.
P5. *C. perfringens* DNA and specific primers α1 and α2.
P6. DNA molecular weight marker, Φ X174 *Hae* III digest.
P7. *C. novyi* type B DNA and primers Nov1 and Nov2 undiluted.
P8. *C. novyi* type B DNA and primers Nov1 and Nov2 diluted 1/10.
P9. *C. novyi* type B DNA and primers Nov1 and Nov2 diluted 1/100.
P10. Negative control.
P11. *C. novyi* type B DNA and specific primers α1 and α2.

ii) Cloning experiments and sequencing of the PCR products

The technique employed for preparation of recombinants was based on the activity of thermostable polymerases used in PCR that are known to add a single deoxyadenosine to the 3' ends of duplex molecules, regardless of the template used. The 3' A overhangs were used to insert the PCR product from freshly performed amplifications into a pCRII plasmid vector which contains 3' T overhangs at the insertion site (335 on the graphic map). The vector was supplied in linear form and also included a fragment of the M13 bacteriophage, useful for sequencing double stranded DNA. By using this method restriction enzyme digestion of the DNA fragment and the plasmid vector plus the use of linkers and adapters was avoided.

After plating-out on ampicillin bluo-gal LB agar for detection of recombinant clones, 62 clones were selected. Of these, 42 were clones with putative inserts derived from PCR amplifications of *C. novyi* template. The other 20 were recombinants in which PCR product from *C. perfringens* template was cloned. As the expected inserts were small in size (400 bp and 510 bp) pale blue colonies on the agar plates were also selected, as small deletions in the *Lac Z'* gene may not completely prevent galactosidase activity. To confirm the presence of the insert in the clones, plasmid DNA was extracted and cut using *Eco* RI, as restriction sites occur upstream and downstream of the insertion site. Problems with unsuccessful enzyme digestion occurred. Some "false positives" were detected, white colonies that were found not to contain an insert after restriction enzyme digestion. Some of these clones were chosen for sequencing to ensure that a small insert was not being missed on the agarose gel electrophoresis.

Figure 5.5. Restriction enzyme digestion with *Eco*RI of plasmid DNA. 1.5% agarose gel electrophoresis.



Lane 1. Plasmid pCRII D21 cut. Two fragments of 500 and 230 bp.

Lane 2. Plasmid pCRII D21 uncut.

Lane 3. Plasmid pCRII C21 cut. 500 bp fragment.

Lane 4. Plasmid pCRII C21 uncut.

Lane 5. Plasmid pCRII D27 cut. 400 bp fragment.

Lane 6. Plasmid pCRII D27 uncut.

Lanes 7 and 8. Plasmid pCRII D57 cut and uncut. No insert detected.

Lane 9. DNA molecular weight marker, Φ X174 *Hae* III digest.

Lane 10. DNA molecular weight marker, λ*Hind*III digest.

Seven clones appeared to contain the insert, in three cases of approximately 600 bp and in the other 4 occasions a smaller 400 bp fragment, after the plasmid DNA had been digested as illustrated in figure 5.5. These clones were selected for sequencing (*q.v.*).

On other occasions very small fragments, less than 200 bp long, were seen on agarose gel electrophoresis. Other restriction enzymes were employed to ascertain that these smaller cloned fragments had not been cleaved by the presence of an *Eco* RI restriction site within the cloned DNA. Plasmid DNA was incubated with *Bam* H1, *Eco* RV alone and with both restriction endonucleases simultaneously for two hours at 37°C. Only when both enzymes were included was a fragment of approximately 250 bp in length cleaved. This would suggest these restriction sites were not present in the insert and that the cloned fragment was probably smaller than the PCR product.

Sequencing by the Chain Termination method of Sanger *et al*, (1977) was carried out, firstly on clones containing *C. novyi* DNA inserts. The initial attempts at determining the sequence were marred by blurred bands on the autoradiograph, particularly the column used for the A nucleotides. Later attempts using M13 Reverse as the primer for the annealing reaction were more successful allowing the plasmid sequence to the insertion site to be clearly readable. The Nov 2 primer sequence was obtained indicating that the insert was present. Unfortunately the sequence contained a high number of A bands which became very close, uneven and did not allow accurate, repeatable reading of the sequence.

Consistently, the clone pCRII L16 using the M13R primer gave a 69 bp sequence that was readable from the Nov 2 primer end (in lower case):

ttc gaa ttt aac gtg acc ggc TAG AGT TAA AAG TCT AGT CAA ATA TAA TTG ATA AGA TCC TGT CTC A

This sequence did not present an open reading frame. A non gradient polyacrylamide sequencing gel was used for some later experiments with disappointing results.

In a further attempt to sequence a longer portion of the insert, plasmid DNA from one clone (pCRII L16) was purified using the methods described in Appendix VIII and submitted for automatic sequencing using dye terminator chemistry (Applied Biosystems) with the M13 universal and reverse primers. The sequence revealed that many bases could not be determined accurately. Neither of the primer sequences were found and the plasmid sequence was also difficult to read, although the *Eco* RI restriction site and the M13 primer of the plasmid were identified. Furthermore, when the sequence was loaded into the DNA Strider computer program for analysis, it was found to contain an insert of only 215 bp in length, which was smaller than the DNA amplified by the PCR.

Clones containing the insert from amplified *C. perfringens* DNA, were also sequenced, to determine that the methodology employed was working correctly. Clone pCRII C21 was used. This recombinant *E. coli* was known to contain an insert of approximately 560 bp after plasmid DNA was cut with *Eco*RI. Plasmid purification was carried out using an improved Wizard miniprep (Promega). A high purity DNA was achieved (141 µg/ml, 260/280 ratio 1.836). Good sequences were achieved using the chain termination method with both M13 universal and reverse as the annealing primers. The 93 nucleotide sequence derived with M13 universal was compared to other known sequences using GenBank sequence data and the GCG (Genetics Computer Group, Winsconsin package) software and found to be identical to that of the *Clostridium perfringens plc* gene which was used to design the primers. M13 reverse provided 72 nucleotides of readable sequence with an 80% identity to *C. perfringens* phospholipase C gene over a 74 bp overlap.

Figure 5.6. Comparison of the published sequences of the *plc* gene of *C. perfringens* (lines 1 to 4) with the sequence derived from C21pCRII (5th line) and a consensus sequence.

```
                    1                                                  50
 pileup_187.msf{CLOPLC} ATGAAAAGAA AGATTTGTAA GGCGCTTATT TGTGCCGCGC TAGCAACTAG
    pileup_187.msf{CPA} ATGAAAAGAA AGATTTGTAA GGCGCTTATT TGTGCCGCGC TAGCAACTAG
pileup_187.msf{CLOPLCA} ATGAAAAGAA AGATTTGTAA GGCACTTATT TGTCGTACGC TAGCAACTAG
 pileup_187.msf{CPPLCG} ATGAAAAGAA AGATTTGTAA GGCGCTTGTT TGTGCCACGC TAGTAACTAG
pileup_187.msf{c21CRII} atgaaaagga aaatatgcaa ggcgcttatt tgtgccgcgc tagcaactag
             Consensus  ATGAAAAG-A A-AT-TG-AA GGC-CTT-TT TGT----CGC TAG-AACTAG

                   51                                                 100
 pileup_187.msf{CLOPLC} CCTATGGGCT GGGGCATCAA CTAAAGTCTA CGCTTGGGAT GGAAAGATTG
    pileup_187.msf{CPA} CCTATGGGCT GGGGCATCAA CTAAAGTCTA CGCTTGGGAT GGAAAGATTG
pileup_187.msf{CLOPLCA} CCTATGGGCT GGGGCATCAA CTAAAGTCTA CGCTTGGGAT GGAAAAATTG
 pileup_187.msf{CPPLCG} CCTATGGGCT GGGGTATCAA CTAAAGTCTA CGCTTGGGAT GGAAAAATTG
pileup_187.msf{c21CRII} cctatgggct ggggcatcaa ctaaagtcta cgcttgggat ggaaagattg
             Consensus  CCTATGGGCT GGGG-ATCAA CTAAAGTCTA CGCTTGGGAT GGAAA-ATTG

                  101                                                 150
 pileup_187.msf{CLOPLC} ATGGAACAGG AACTCATGCT ATGATTGTAA CTCAAGGGGT TTCAATCTTA
    pileup_187.msf{CPA} ATGGAACAGG AACTCATGCT ATGATTGTAA CTCAAGGGGT TTCAATCTTA
pileup_187.msf{CLOPLCA} ATGGAACAGG AACTCATGCT ATGATTGTAA CTCAAGGGGT TTCAATCTTA
 pileup_187.msf{CPPLCG} ATGGAACAGG AACTCATGCT ATGATTGTAA CTCAAGGTGT TTCAATCTTA
pileup_187.msf{c21CRII} atggaacagg aactcatgct atgattgtaa ctcaaggggt ttcaatctta
             Consensus  ATGGAACAGG AACTCATGCT ATGATTGTAA CTCAAGG-GT TTCAATCTTA

                  151                                                 200
 pileup_187.msf{CLOPLC} GAAAATGATC TGTCCAAAAA TGAACCAGAA AGTGTAAGAA AAAACTTAGA
    pileup_187.msf{CPA} GAAAATGATC TGTCCAAAAA TGAACCAGAA AGTGTAAGAA AAAACTTAGA
pileup_187.msf{CLOPLCA} GAAAATGATC TGTCCAAAAA TGAACCAGAA AGTGTAAGAA AAAACTTAGA
 pileup_187.msf{CPPLCG} GAAAATGATA TGTCCAAAAA TGAACCAGAA AGTGTAAGAA AAAACTTAGA
pileup_187.msf{c21CRII} gaaaatgatc tgtccaaaaa tgaaccagaa agtgtaagaa aaaacttaga
             Consensus  GAAAATGAT- TGTCCAAAAA TGAACCAGAA AGTGTAAGAA AAAACTTAGA

                  201                                                 250
 pileup_187.msf{CLOPLC} GATTTTAAAA GAGAACATGC ATGAGCTTCA ATTAGGTTCT ACTTATCCAG
    pileup_187.msf{CPA} GATTTTAAAA GAGAACATGC ATGAGCTTCA ATTAGGTTCT ACTTATCCAG
pileup_187.msf{CLOPLCA} GATTTTAAAA GAGAACATGC ATGAGCTTCA ATTAGGTTCT ACTTATCCAG
 pileup_187.msf{CPPLCG} GATTTTAAAA GATAACATGC ATGAGCTTCA ATTAGGTTCT ACTTATCCAG
pileup_187.msf{c21CRII} gattttaaaa gagaacatgc atgagcttca attaggttct acttatccag
             Consensus  GATTTTAAAA GA-AACATGC ATGAGCTTCA ATTAGGTTCT ACTTATCCAG

                  251                                                 300
 pileup_187.msf{CLOPLC} ATTATGATAA GAATGCATAT GATCTATATC AAGATCATTT CTGGGATCCT
    pileup_187.msf{CPA} ATTATGATAA GAATGCATAT GATCTATATC AAGATCATTT CTGGGATCCT
pileup_187.msf{CLOPLCA} ATTATGATAA GAATGCATAT GATCTATATC AAGATCATTT CTGGGATCCT
 pileup_187.msf{CPPLCG} ATTATGATAA GAATGCATAT GATCTATATC AAGATCATTT CTGGGATCCT
pileup_187.msf{c21CRII} attatgataa gaatgcatat gatctatatc aagaccacct ctgggatcct
             Consensus  ATTATGATAA GAATGCATAT GATCTATATC AAGA-CA--T CTGGGATCCT

                  301                                                 350
 pileup_187.msf{CLOPLC} GATACAGATA ATAATTTCTC AAAGGATAAT AGTTGGTATT TAGCTTATTC
    pileup_187.msf{CPA} GATACAGATA ATAATTTCTC AAAGGATAAT AGTTGGTATT TAGCTTATTC
pileup_187.msf{CLOPLCA} GATACAGATA ATAATTTCTC AAAGGATAAT AGTTGGTATT TAGCTTATTC
 pileup_187.msf{CPPLCG} GATACAAATA ATAATTTCTC AAAGGATAAT AGTTGGTATT TAGCTTATTC
pileup_187.msf{c21CRII} gatacagat. atgatttctc gaaggataat agttggtatt tagcttat..
             Consensus  GATACA-AT- AT-ATTTCTC -AAGGATAAT AGTTGGTATT TAGCTTAT--

                  351                                                 400
 pileup_187.msf{CLOPLC} TATACCTGAC ACAGGGGAAT CACAAATAAG AAAATTTTCA GCATTAGCTA
    pileup_187.msf{CPA} TATACCTGAC ACAGGGGAAT CACAAATAAG AAAATTTTCA GCATTAGCTA
pileup_187.msf{CLOPLCA} TATACCTGAC ACAGGGGAAT CACAAATAAG AAAATTTTCA GCATTAGCTA
 pileup_187.msf{CPPLCG} TATACCTGAC ACAGGGGAAT CACAAATAAG AAAATTTTCA GCATTAGCTA
pileup_187.msf{c21CRII} catacctgac acaggggaat cacaaataag cga..ttttc agcttagcta
             Consensus  -ATACCTGAC ACAGGGGAAT CACAAATAAG --A--TTT-- ---TTAGCTA

                  401                                                 450
 pileup_187.msf{CLOPLC} GATATGAATG GCAAAGAGGA AACTATAAAC AAGCTACATT CTATCTTGGA
    pileup_187.msf{CPA} GATATGAATG GCAAAGAGGA AACTATAAAC AAGCTACATT CTATCTTGGA
pileup_187.msf{CLOPLCA} GATATGAATG GCAAAGAGGA AACTATAAAC AAGCTACATT CTATCTTGGA
 pileup_187.msf{CPPLCG} GATATGAATG GCAAAGAGGA AATTATAAAC AAGCTACATT CTATCTTGGA
pileup_187.msf{c21CRII} gatatg..tg acaaagaggc ccctat.aac aagctacatt ctatc.tgga
             Consensus  GATATG--TG -CAAAGAGG- ---TAT-AAC AAGCTACATT CTATC-TGGA

                  451                                                 500
 pileup_187.msf{CLOPLC} GAGGCTATGC ACTATTTTGG AGATATAGAT ACTCCATATC ATCCTGCTAA
    pileup_187.msf{CPA} GAGGCTATGC ACTATTTTGG AGATATAGAT ACTCCATATC ATCCTGCTAA
pileup_187.msf{CLOPLCA} GAGGCTATGC ACTATTTTGG AGATATAGAT ACTCCATATC ATCCTGCTAA
 pileup_187.msf{CPPLCG} GAAGCTATGC ACTATTTTGG AGATATAGAT ACTCCATATC ATCCTGCTAA
pileup_187.msf{c21CRII} gaggctat.c actattttgg agatat..ga actccatatc atcctgctaa
             Consensus  GA-GCTAT-C ACTATTTTGG AGATAT---- ACTCCATATC ATCCTGCTAA

                  501                                                541
 pileup_187.msf{CLOPLC} TGTTACTGCC GTTGATAGCG CAGGACATGT TAAGTTTGAG A
    pileup_187.msf{CPA} TGTTACTGCC GTTGATAGCG CAGGACATGT TAAGTTTGAG A
pileup_187.msf{CLOPLCA} TGTTACTGCC GTTGATAGCG CAGGACATGT TAAGTTTGAG A
 pileup_187.msf{CPPLCG} TGTTACTGCC GTTGATAGCG CAGGACATGT TAAGTTTGAG A
pileup_187.msf{c21CRII} tgttactgcc gttgatagcg ctggtcccgt taaattcgag a
             Consensus  TGTTACTGCC GTTGATAGCG C-GG-C--GT TAA-TT-GAG A
```

The chain termination nucleotide data were confirmed by automatic sequencing using M13 universal and reverse primers and dye termination chemistry.

Further recombinant clones that putatively contained amplified *C. novyi* type B DNA were screened. After extraction of the plasmid DNA and restriction enzyme digestion with *Eco*RI, clone pCRII D27 was found to contain an insert of around 400 bp in length (figure 5.5). Plasmid DNA was extracted using an improved Promega miniprep (Appendix VIII) and sequenced by dye termination chemistry (see figures 5.7 and 5.8.). Both M13U and M13R were used as annealing primers.

Good quality sequence data was determined (figure 5.9.). The correct fragment had been sequenced as, excluding the primers (43 bases), 301 nucleotides of insert sequence were available for analysis. The sequence appeared to contain an open reading frame.

Figure 5.7. Dye termination sequencing of pCRII D27 with M13U primer.

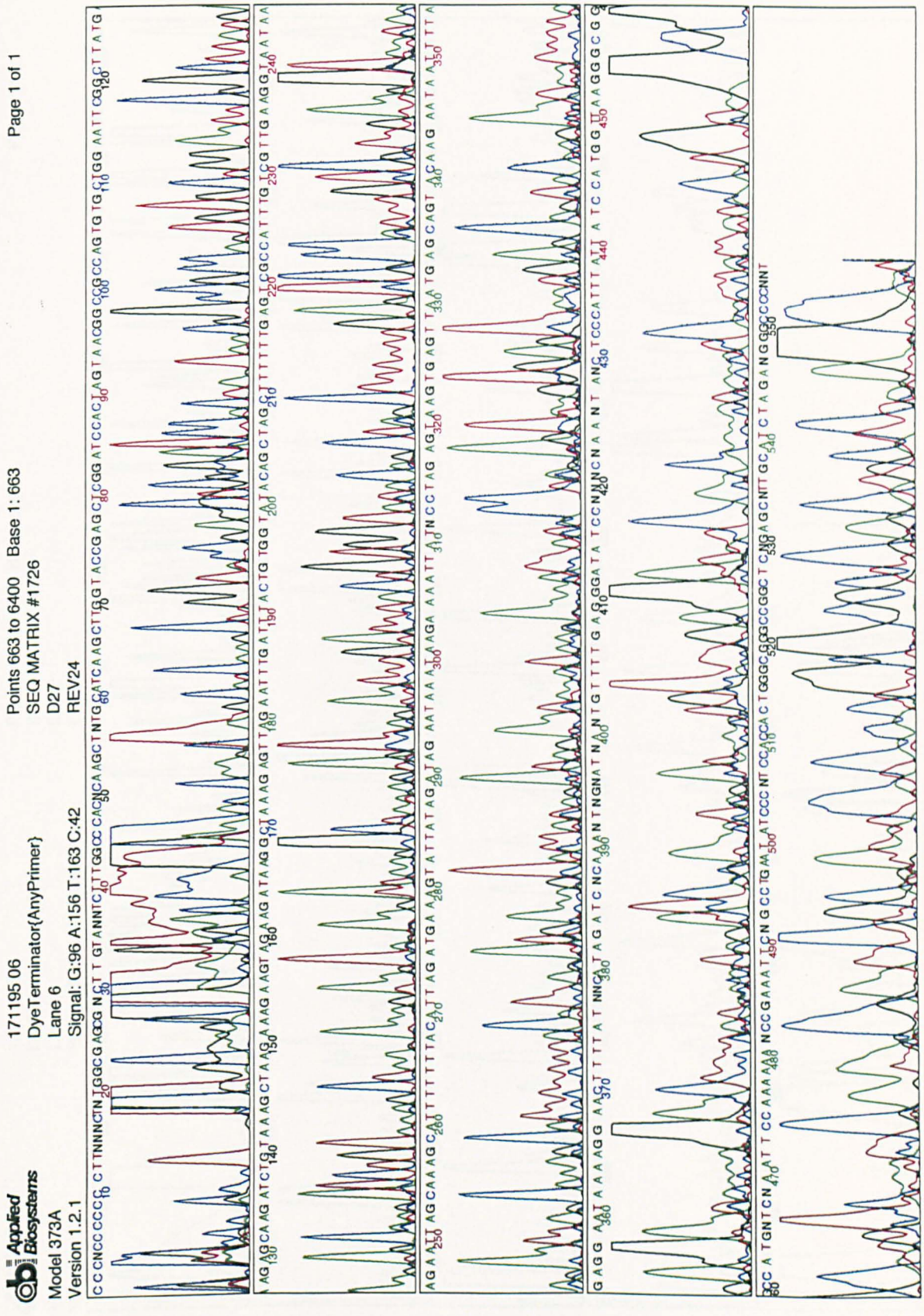


Figure 5.8. Dye termination sequencing of pCRII D27 with M13R primer.

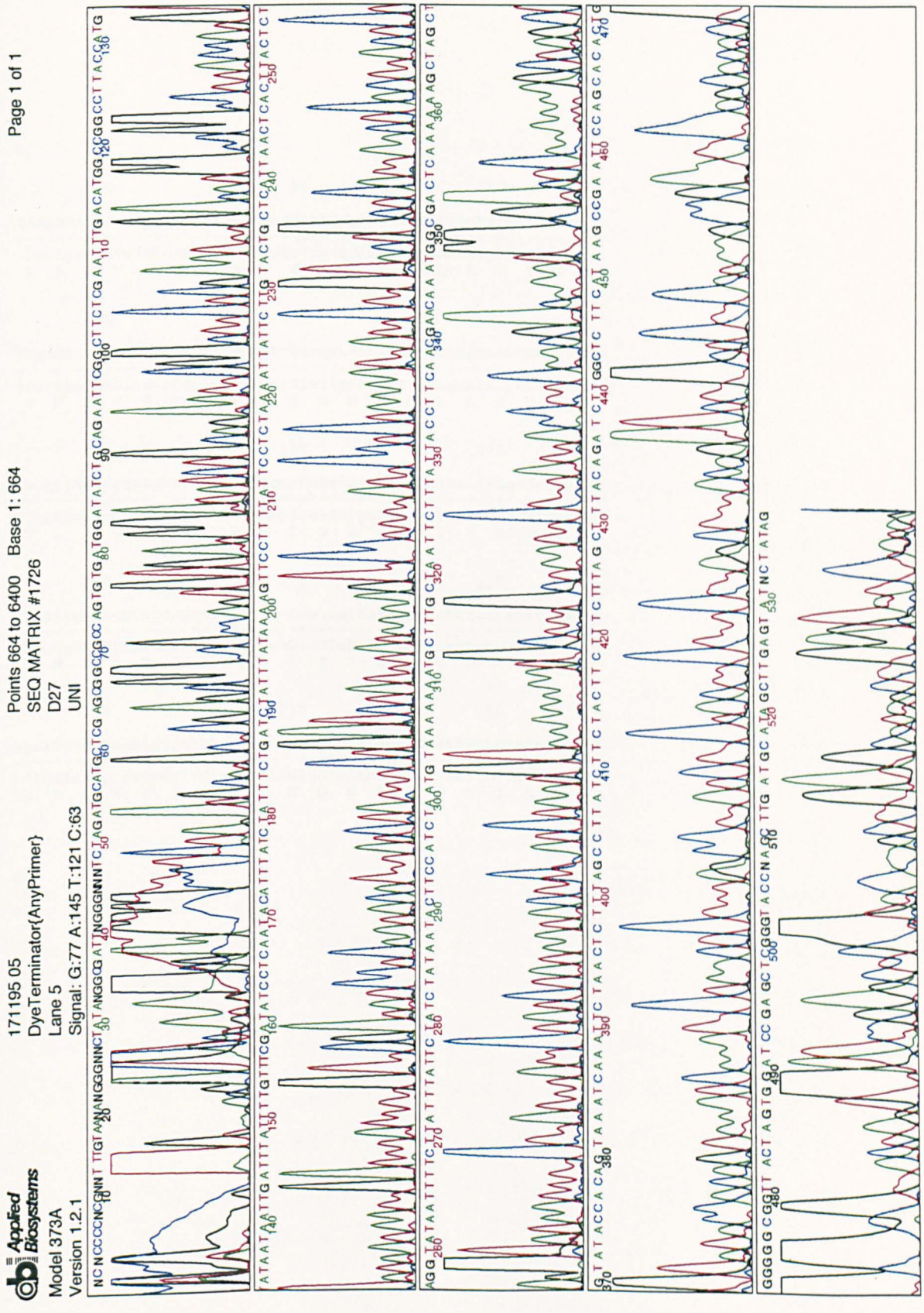


Figure 5.9. Nucleotide sequence (301 bases) of the amplified *C. novyi* fragment inserted into the plasmid pCRII D27. The aminoacid and protein translation can be seen below. The open reading frame ended at site 214.

```
        10                  30                  50
         .         .         .         .         .         .
taagaaagaagtagaagataaggctaaagagttagaatttgatttactgtggtatacagc
---------+---------+---------+---------+---------+---------+
 LysLysGluValGluAspLysAlaLysGluLeuGluPheAspLeuLeuTrpTyrThrAl
 K  K  E  V  E  D  K  A  K  E  L  E  F  D  L  L  W  Y  T  A

        70                  90                  110
         .         .         .         .         .         .
tagctttttgagtcgccatttgtcgttgaaggtaataaagaattagcaaagcatttttt
---------+---------+---------+---------+---------+---------+
aSerPhePheGluSerProPheValValGluGlyAsnLysGluLeuAlaLysHisPhePh
 S  F  F  E  S  P  F  V  V  E  G  N  K  E  L  A  K  H  F  F

       130                 150                 170
         .         .         .         .         .         .
tacattagatggaagtattatagatagaataaataagaaaattatacctagagatagtga
---------+---------+---------+---------+---------+---------+
eThrLeuAspGlySerIleIleAspArgIleAsnLysLysIleIleProArgAspSerGl
 T  L  D  G  S  I  I  D  R  I  N  K  K  I  I  P  R  D  S  E

       190                 210                 230
         .         .         .         .         .         .
gttaatgagcagtacaagaataatttttagaggaataaaaggaacttttataaaatagaatc
---------+---------+---------+---------+---------+---------+
uLeuMetSerSerThrArgIleIleLeuGluGluEndLysGluLeuLeuEndIleGluSe
 L  M  S  S  T  R  I  I  L  E  E  *  K  E  L  L  *  I  E  S

       250                 270                 290
         .         .         .         .         .         .
agaaatagataaatgtattgaggatatcgaacaaaataaatcaattattatacatggtaa
---------+---------+---------+---------+---------+---------+
rGluIleAspLysCysIleGluAspIleGluGlnAsnLysSerIleIleIleHisGlyLy
 E  I  D  K  C  I  E  D  I  E  Q  N  K  S  I  I  I  H  G  K

g
-
s
```

5.4. Discussion

The phospholipase C gene (*plc* gene) which encodes for the cytolytic alpha toxin from *Clostridium perfringens* strain NCTC8237 was cloned and sequenced by different groups in 1989 (Leslie *et al*, 1989; Okabe, Shimizu and Hayashi, 1989; Tso and Siebel, 1989; Titball *et al*, 1989). The DNA sequences were virtually identical in these reports. Furthermore, the cloned *plc* gene product in transformed *E. coli* possessed both phospholipase and haemolytic activities and was proven to be biochemically and immunologically identical to protein produced by *C. perfringens* (Titball *et al*, 1989; Tso and Siebel, 1989).

Various *Clostridium* species and other bacteria have been reported to produce phospholipase C enzymes (Titball, 1993). The *Bacillus cereus* PC-PLC, *C. perfringens* alpha-toxin, *C. bifermentans* PLC, *Listeria monocytogenes* PCL-B, the phospholipases C produced by *C. absonum* and *C. barati* and *C. novyi* gamma toxin formed a group of closely related enzymes according to Titball (1993). These enzymes, the Zinc metallophospholipases, contained essential zinc ions and were reversibly inactivated by EDTA or o-phenanthroline (Titball, 1993).

Some of these enzymes have been wholly or partially neutralised by *C. perfringens* alpha toxin antiserum in laboratory animal protection assays (Willis, 1977; Mollby, 1978; Titball, 1992). On the other hand, early work by Oakley *et al*, (1947) and Macfarlane (1950) demonstrated that neither the gamma toxin of type A strains of *C. novyi* or the beta toxin of *C. novyi* types B and D were neutralised by antiserum raised against the alpha toxin of *C. perfringens*. Titball (1992) believed the various biochemical, antigenic and toxicological properties of the zinc-metalloproteinases indicated that these enzymes were probably different proteins.

The alignment of the deduced aminoacid sequence of the phospholipase gene from *C. perfringens* and *C. bifermentans* revealed extensive homology between the two proteins (Tso and Siebel, 1989). In particular, the N-termini was found to be 60% homologous between alpha toxin and the *C. bifermentans* enzyme. Titball (1992) showed that the phosphatidylcholine hydrolysing enzyme (PC-PLC) produced by *Bacillus cereus* had some sequence homology with the first two thirds of the *plc* gene.

The toxin genes of strains of *C. novyi*, encoding two different phospholipases C, the beta toxin (type B and D) and the gamma toxin (type A) were examined in this study.

The primers Nov1 and Nov2 were chosen from the first 250 residues of the deduced aminoacid sequence of the *C. perfringens plc* gene (N-terminal domain) that encode for the phospholipase C activity. The experiment was based on the assumption that the *C. novyi* genes has homologous domains with the *plc* gene. Nov1 and Nov2 were highly degenerate primers, which probably explained the need to use them at high concentrations in the PCR.

The PCR successfully amplified a fragment of the *plc* gene when *C. perfringens* was used as a template, which suggested the methodology was carried out correctly. On the other hand difficulties arose when the PCR was applied to *C. novyi* template DNA. Various improvements in the techniques allowed the successful amplification of an approximately 400 nucleotide fragment. The amplified product was cloned and sequenced. The derived sequence was analysed using GCG software and compared with other known sequence data from EMBL/GenBank. An area upstream of the *plc* gene of *C. perfringens* was found to have 63% homology with the Nov sequence over 107 nucleotides. Further work needs to be carried out analysing the data and confirming the reliability of the sequencing data.

The identity of the PCR product can then be confirmed by preparing a homologous hybridization probe from the cloned insert to detect the gene in

C. novyi DNA (McPherson, Jones and Gurr, 1992). Other clostridia and genomic *C. novyi* clones could also be screened to detect corresponding cDNA or inserted fragments. Alternatively, specific primers could be prepared from the derived sequence and used to amplify portions of the gene or in a molecular method of toxin typing of *Clostridium novyi* isolates. The latter technique has been applied to *C. perfringens* typing (Daube *et al*, 1994; Fach and Guillou, 1993).

To sequence the phospholipase C genes of *C. novyi* an approach based on detection of lecithinase activity of recombinant clones may yield good results. This method was used by workers sequencing the *plc* gene of *C. perfringens* (Leslie *et al*, 1989).

New primers, named Nov3 and Nov 4, were designed based on the N-terminal domain of the known *plc* gene sequence for future use. These primers contained fewer degeneracies and improved PCR results could be achieved.

Nov3 5' TAT CAA GAT CAT TTC TGG GAT 3'
C G C C T C

Nov4 5' ATT AAA ATC TTT ATT TTT 3'
G G C C G C

The work carried out provided the opportunity to become proficient in molecular biology techniques. A region of the *Clostridium novyi* DNA was amplified using *taq* polymerase, degenerate primers derived from the *plc* gene sequence and PCR. The amplified fragment, approximately 400 bp in size, was successfully cloned in *E. coli* and the nucleotide sequence was determined. Some homology with other clostridial genes at the nucleotide level was detected by comparison with sequence data in EMBL/GenBank.

6. CAUSES OF DEATH IN SOWS: A RETROSPECTIVE STUDY.

"it's not that I'm afraid to die, I just don't want to be there when it happens."
Woody Allen, Without Feathers (1972)

6.1. Introduction

The different causes of sow mortality have been investigated by authors in the U.K. (Jones, 1968; Ward and Walton, 1980; Smith, 1984) and in other countries (Svendsen *et al* , 1975; Madec, 1984; Chagnon *et al*, 1991). The purpose of this study was to review the necropsy results from sows examined at the University of Liverpool Veterinary Field Station, Leahurst, between January 1986 and October 1991, to determine the incidence of the various causes of sow mortality. In particular to determine the incidence of clostridial hepatopathy (infectious necrotic hepatitis) in four indoor pig breeding herds where cases had been described in the past (Carr, 1989; Walton and Duran, 1992).

C. novyi (oedematiens) type B, the organism thought to be implicated in this disease, was described as a large motile Gram positive pleomorphic rod, with oval subterminal spores that usually distended the bacterium (Cato *et al*, 1986). *C. novyi* was defined as a fastidious anaerobe. The organism was isolated from soil, mud from sea beds and from wounds in animals and man. In sheep (Bagadi and Sewell 1973a), cattle (Niilo *et al* , 1969), goats (Hamid *et al*, 1991) and horses (Gay *et al* , 1980) *C. novyi* type B caused infectious necrotic hepatitis ('Black disease').

The *C. novyi* sudden death has been described in pigs (Wise and Munday, 1964; Batty *et al*, 1964; Bourne and Kerry, 1965; Corbould and Munday, 1966; Walton and Duran , 1992), and *C. novyi* was isolated from cases in Japan (Itoh *et al* , 1987; Kita *et al* , 1987). The term clostridial hepatopathy was adopted for sow cases in this study (Baker, Personal communication, 1991).

6.2. Material and methods

The records of necropsies performed between 1986 and 1991 were collated from the archives of the Department of Veterinary Pathology, University of Liverpool. Unfortunately the data concerning the age of the sows, stage of the reproductive cycle or the condition score was sometimes unavailable.

The different diagnoses were classed in thirteen categories, based on the likely lethal cause. The categories comprised: clostridial hepatopathy, cystitis/pyelonephritis, abdominal catastrophes, which included torsion of the intestines, hepatic lobe torsion, gastric torsion and rupture, gastric dilatation and other accidents involving the abdominal organs.
Other categories included were: complications of parturition (retained piglets, torn vagina, recto-vaginal fistula, prolapsed uterus), perforated or haemorrhagic gastro-oesophageal ulceration, splenic torsion, heart failure, pneumonia, peritonitis, alimentary or generalised lymphosarcoma, endometritis and others which referred to conditions seen less frequently, like mastitis, porcine stress syndrome, proliferative haemorrhagic enteropathy or infectious arthritis.
All sows examined came from 4 nearby farms and were kept indoors in confinement stalls on partially slatted floors. The four herds comprised a total inventory of 1900 sows. Management practices varied between the farms, but the genetic stock was similar.

Two farms were stocked mainly with pure breed Large White sows, whilst the other two were commercial herds with F1 Large White-Landrace crosses. All farms predominantly or exclusively used boar mating. One herd was free from enzootic pneumonia, streptococcal meningitis, atrophic rhinitis, swine dysentery, transmissible gastro-enteritis, internal parasites and sarcoptic mange. Two other herds had a minimal disease status, but presented evidence on lung checks and clinical signs of enzootic pneumonia (*Mycoplasma hyopneumoniae* infection). The fourth farm was not minimal disease and various diseases were endemic.

The *post mortem* examination of the sows submitted by the farmers was carried out by members of the Department of Pathology.

Submission of samples for bacterial culture, histopathology and analysis of the urea concentration in aqueous humor were carried out, when necessary. Liver smears, from cases which were suggestive of *Clostridium novyi* infection, were examined using the Fluorescent Antibody Test to confirm the diagnosis. Heat fixed smears from the spleen or abdominal fluids, stained with polychrome methylene blue, were used to exclude the possibility of Anthrax.

6.3. Results

A total of 170 sow casualties were included in the study. The number of submissions per year varied during the period analysed: 26, 30, 45, 24, 27, 18 for the years between 1986 and 1991.

The different diagnoses were classed in thirteen categories, based on the likely lethal cause (see table 6.1). The three most common diagnoses, clostridial hepatopathy, cystitis and pyelonephritis and abdominal catastrophes, comprised over 65% of all submissions.

Pneumonia was diagnosed as a cause of death in 5 occasions (2.9%), mostly affecting maiden gilts (3 cases).

Table 6.1. Distribution of the cause of death amongst 170 sows examined at Leahurst (1986-1991)

Cause of death	Number	%
Clostridial hepatopathy	59	34.7
Cystitis/pyelonephritis	39	22.9
Abdominal catastrophes (1)	17	10
Complications of parturition	8	4.7
Perforated/haemorrhagic gastric ulcer	3	1.8
Splenic torsion	4	2.3
Heart failure	5	2.9
Pneumonia	5	2.9
Peritonitis	2	1.2
Lymphosarcoma	5	2.9
Endometritis	1	0.6
Others (2)	13	7.6
Not diagnosed	9	5.3
TOTAL	170	100

(1) Abdominal catastrophes included gastric dilatation and torsion, gastric rupture (excluding perforated ulcers), torsion of a lobe of the liver, ruptures and torsion of small and large intestine.

(2) Others included conditions seen less frequently on these farms, like Proliferative Haemorrhagic Enteropathy, trauma or acute mastitis.

Urinary tract disease was the cause of death in over 22% of sows examined, mainly a combination of severe cystitis, ureteritis and pyelonephritis, reflected in an elevated concentration of urea in the aqueous humor. Urinary tract disease became increasingly frequent, peaking in 1988, followed by a gradual reduction during the next three years, that was parallel to the reduction in total number examined (see table 6.2.).

The other frequent causes of death were diagnosed at similar rates during the period of study (see fig. 6.1). The number of deaths examined annually appeared to reflect the number of urinary tract and *C. novyi* deaths.

Generalised and alimentary lymphosarcoma was seen in 5 occasions (2.9%) mainly in young animals (below parity 3).

The category "others" included 3 cases of porcine haemorrhagic enteropathy, two deaths due to the porcine stress syndrome, 3 died due to severe complicated arthritis and osteomyelitis and the other 13 cases (7.6%) incorporated conditions that were seen only once (mastitis, septicaemia, trauma, lung oedema, multiple abscessation).

A diagnosis was not reached in 9 cases (5.3%), sometimes due to extreme autolytic changes, others because a clear single cause of death was not apparent.

Sow deaths caused by Clostridial hepatopathy were diagnosed in 59 occasions, representing 34.7% of all the carcasses examined.

The monthly and seasonal distribution of clostridial hepatopathy cases can be seen in table 6.3. and in figure 6.2. Cases were diagnosed more frequently during the winter months with 34% of the submissions diagnosed as *C. novyi* sudden death occurring from December to February. The monthly distribution of *C. novyi* cases when compared with other causes of death did not reveal a consistent pattern or correlation.

Figure 6.1 Total number examined annually compared with the major causes of death.

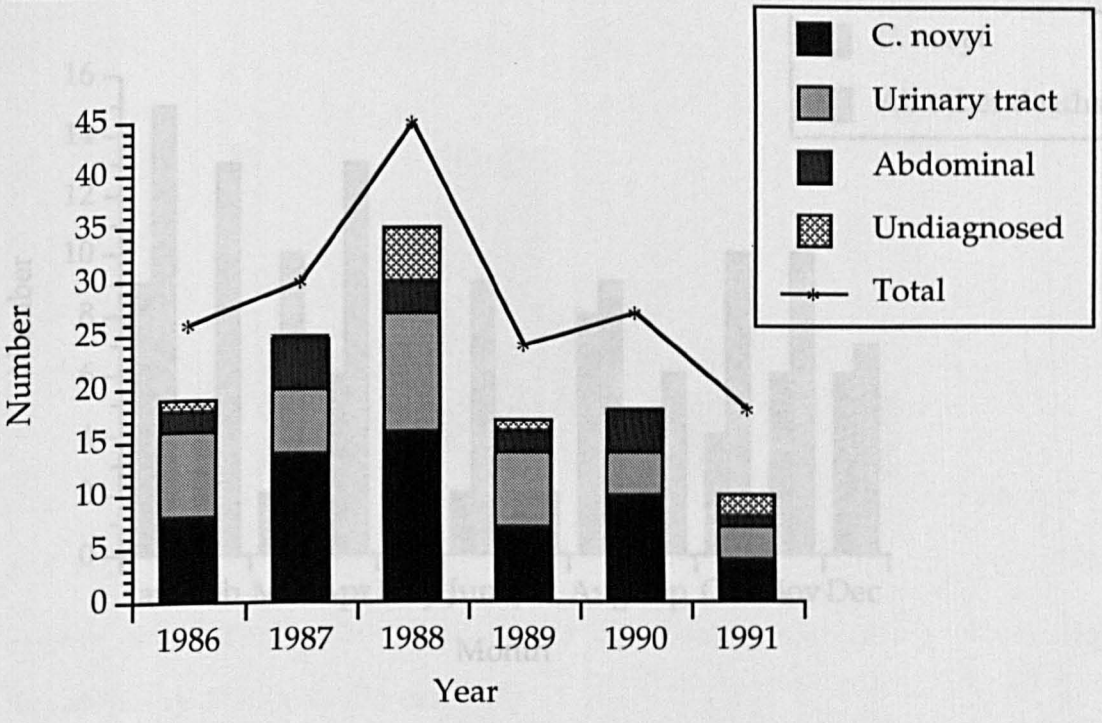


Table 6.2. Number of submissions per year of the major causes of death. (170 deaths)

Year	Total	*C. novyi*	Urinary	Abdominal	Farrowing	Undiagnosed
1986	26	8	8	2	1	2
1987	30	14	6	5	0	0
1988	45	16	11	3	1	4
1989	24	7	7	2	1	5
1990	27	10	4	4	4	2
1990	18	4	3	1	1	0

C. novyi refers to deaths caused by this organism, **urinary** includes the diagnosis of cystitis and pyelonephritis, **abdominal** refers to accidents involving the abdominal organs (torsion, dilation), **farrowing** alludes to complications at the time of parturition (retained piglets, uterine tears).

Figure 6.2. Comparison of the distribution of *C. novyi* and other deaths by month between 1986 and 1992 (170 cases).

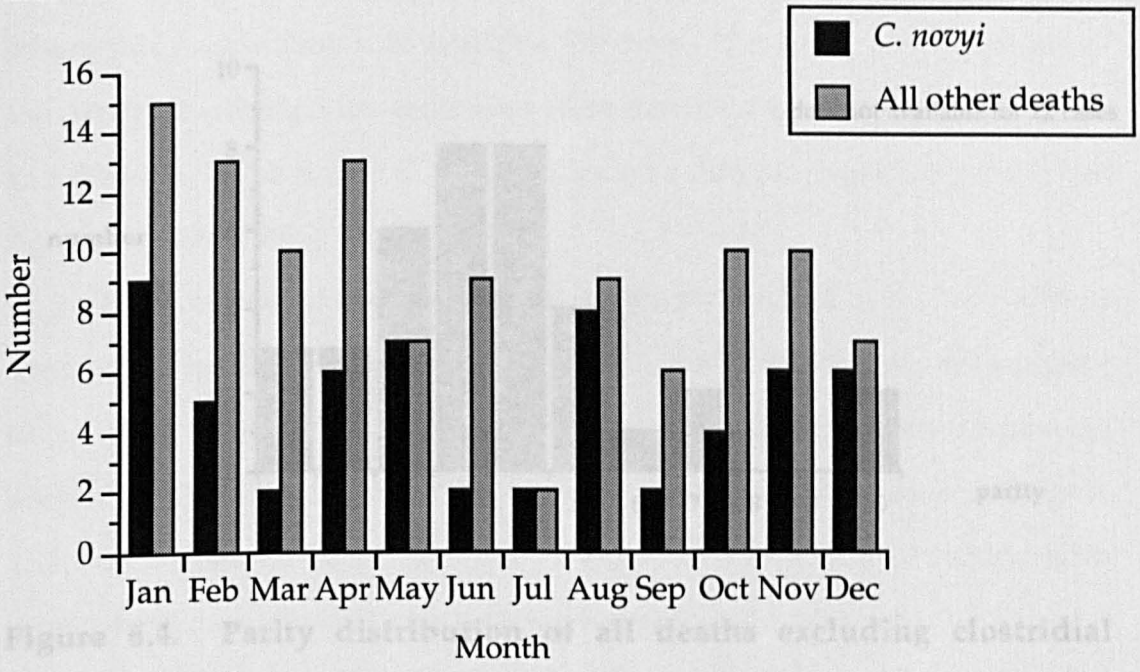


Table 6.3. Distribution of all deaths by month between 1986 and 1991.

Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
24	18	12	19	14	11	4	17	8	14	16	13

Table 6.4. Incidence of clostridial hepatopathy (1986-1991)

Month	Numbers	Season	TOTAL
December	6		
January	9	WINTER	20
February	5		
March	2		
April	6	SPRING	15
May	7		
June	2		
July	2	SUMMER	12
August	8		
September	2		
October	4	AUTUMN	12
November	6		
TOTAL	59		

Figure 6.3. Parity distribution of deaths due to *C. novyi* 1986-1991 (37 cases).

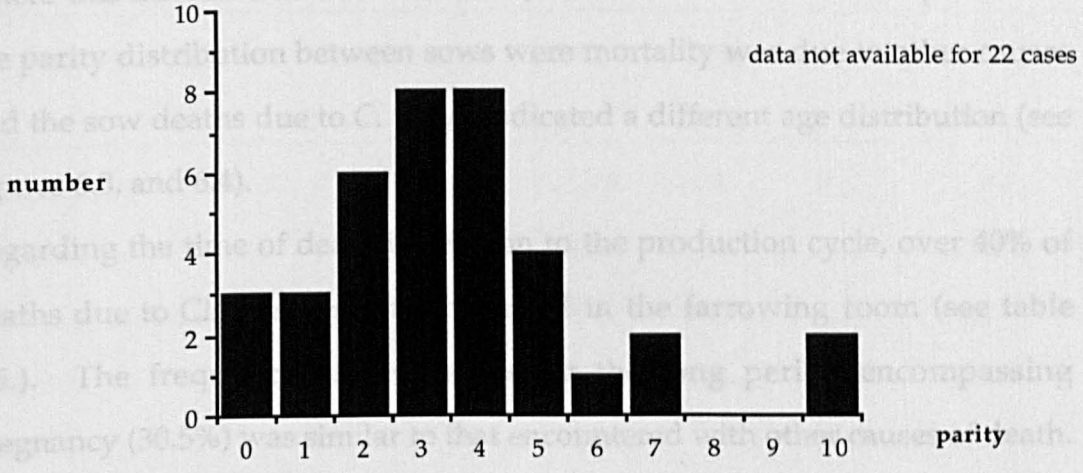


Figure 6.4. Parity distribution of all deaths excluding clostridial hepatopathy 1986-1991 (85 cases).

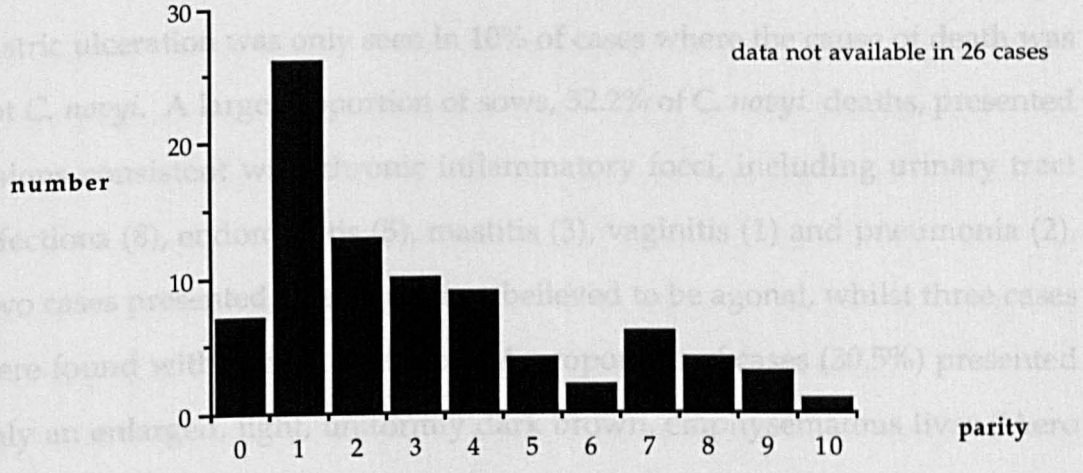


Table 6.5. Parity distribution of sows submitted for necropsy 1986-1991 (170 cases).

Parity	0	1	2	3	4	5	6	7	8	9	10	Not available
C. novyi	3	3	6	8	8	4	1	2	0	0	2	22
Others	7	26	13	10	9	4	2	6	4	3	1	26

The mean age of the clostridial hepatopathy cases where data referring to parity was available (37 cases) was 3.54 litters. The mean parity for all deaths where this information was available (85 cases) was 3.07. A comparison of the parity distribution between sows were mortality was due to other causes and the sow deaths due to *C. novyi* indicated a different age distribution (see figures 6.3. and 6.4).

Regarding the time of death in relation to the production cycle, over 40% of deaths due to *Clostridium novyi* occurred in the farrowing room (see table 6.5.). The frequency of deaths during the long period encompassing pregnancy (30.5%) was similar to that encountered with other causes of death. The times when *C. novyi* was diagnosed more frequently coincided with times of elevated stress (Parturition, post-weaning and lactation).

A majority of the *post mortem* examinations concerning clostridial hepatopathy deaths revealed other pathological lesions. Oesophago-gastric ulceration was seen in 27.1% of all *C. novyi* cases (16). On the other hand, gastric ulceration was only seen in 10% of cases where the cause of death was not *C. novyi*. A large proportion of sows, 32.2% of *C. novyi* deaths, presented lesions consistent with chronic inflammatory focci, including urinary tract infections (8), endometritis (5), mastitis (3), vaginitis (1) and pneumonia (2). Two cases presented splenic torsion, believed to be agonal, whilst three cases were found with gastric dilatation. A proportion of cases (30.5%) presented only an enlarged, light, uniformly dark brown, emphysematous liver ('Aero chocolate liver') with copious abdominal, pleural and pericardial fluid. Inguinal and neck areas presented severe subcutaneous gas and oedema. Muscle tissues presented a gas gangrene appearance and petechial haemorrhages. No other gross pathology was described.

Table 6.6. Stage of the reproductive cycle in 59 deaths due to Clostridium *novyi*.

Stage in cycle	Number of *C. novyi* deaths	%	Other causes	%	All deaths (%)
Peripartum	10	16.9	4	3.6	**8.2**
Lactation	15	25.4	16	14.4	**18.2**
Weaning and service	5	8.5	2	1.8	**4.1**
Pregnancy	17	28.8	36	32.4	**31.2**
Not in pig	3	5.1	25	22.5	**16.5**
Data not available	9	15.2	28	25.2	**21.2**
TOTAL	59	100	111	100	**100**

6.4. Discussion

In a limited survey of sow mortality, between 1986 and 1991, using material from Cheshire farms, it was found that 34.7% of deaths examined were thought to be due to *C. novyi*. This condition had not been reported in previous surveys, except for Madec (1984). This author reported that 11.3% of dead sows in a mortality investigation were found to present an advanced liver degeneration, similar to that found in the cases in this series.

The proportion of Cystitis/pyelonephritis cases (22.9%) was similar to that reported by Smith (1984). The incidence of urinary tract disorders declined in these herds after 1988, when control measures, mainly an improved water supply and intake were instituted (Walton, personal communication, 1991).

In other surveys around 10% of deaths due to cystitis and pyelonephritis were reported (Svendsen *et al*, 1975; D'Allaire *et al*, 1991 and Chagnon *et al*, 1991). A higher incidence (40%) of severe pyelonephritis/cystitis was cited by Madec (1984).

Death arising from accidents involving abdominal organs were infrequent in early surveys of mortality (Jones, 1968; Svendsen *et al*, 1975). Ward and Walton (1980) described the mortality in one of the farms included in this study and found gastric torsion and distension to be a major cause of death. Accidents involving the abdominal organs continued to be a problem on these farms as 10% of cases examined presented this finding as the cause of death. The proportion of these accidents was very similar to that described by Smith (1984), but smaller than the figures reported in Canadian surveys (D'Allaire *et al*, 1991; Chagnon *et al*, 1991).

Cardiac failure (2.9%) was under-reported in comparison with most previous studies, perhaps due to the fact that stockmen recognised the clinical presentation of these deaths and did not submit these cases for examination.

The *C. novyi* deaths were more common (42.3%) in the farrowing room, during the peripartum period and lactation. In comparison only 28.8% clostridium hepatopathy cases died during pregnancy. This was in agreement with previous clinical observations (Walton, personal communication 1991). In general, compared with deaths by other causes, stressful periods appeared to be propitious for clostridial hepatopathy. This finding was reported by Drolet *et al*, (1992) in relation to sow deaths due to cardiac failure.

Clostridial sudden deaths were diagnosed more frequently in winter and spring. These times coincided with months when more sows were submitted for examination. This latter finding was completely at odds with the data compiled by Chagnon *et al* (1991), who reported that the number of deaths in Canadian herds was significantly higher during the summer months. Total submissions for *post mortem* examination may have been reduced in the summer due to the fact that carcasses quickly became too decomposed.

The fact that incidence was not elevated during the warmer summer months would suggest that *C. novyi* deaths were not merely evidence of rapid *post mortem* autolysis after invasion of the liver by intestinal clostridia.

Clostridial hepatopathy was diagnosed in maiden gilts and older sows (10 litters), but the mean number of pregnancies before death for this group was 3.54. This result must be viewed with caution as data from 22 cases was unavailable. This figure was higher than the average parity of sows that died for other reasons; 3.30 litters, or 3.35 after excluding maiden gilts. Chagnon *et al* (1991) found a higher average age for deaths in Canadian herds (4.2 litters), when only served females were included in their study.

Interestingly, a high proportion of deaths due to *C. novyi* toxaemia (27.1%) also presented lesions of oesophago-gastric ulceration not considered severe enough to cause death. This finding could indicate a link between the two conditions as only 10% of the sows that died from other causes presented ulceration. Similarly, chronic inflammation, for example urinary tract infection, mastitis, or endometritis, was seen in 32.2% of clostridial hepatopathy cases and could also play a part in the pathogenesis of this condition. Taylor (1995a) suggested that chronic pneumonia or enteritis leading to decreased oxygen tension in the liver was followed by germination of *C. novyi* spores in this organ. In 30.5% of *C. novyi* deaths the carcasses presented only liver lesions (emphysema) and those related to the toxaemia that followed the proliferation of the bacterium in the liver. The reason for the activation of the *C. novyi* spores, putatively present in the liver, was not apparent.

Diagnosis of infectious necrotic hepatitis or clostridial hepatopathy in sows can be difficult in particular when sows have been dead for more than eight hours, during the summer months or in animals that have died in a warm farrowing room.

Further work must gather more data on the epidemiology of this condition, to comfirm the findings concerning predisposing lesions, seasonal distribution, stage of the reproductive cycle and age.

Particularly important must be the *post mortem* examination of cases shortly after death. A better understanding of clostridial hepatopathy will also require improved methods of bacterial isolation and identification of the organism and the toxins produced.

7. SURVEY OF SOW MORTALITY IN 3 LARGE PIG BREEDING HERDS.

"There are only two certainties in life ; death and taxes"
Franklin D. Roosevelt.

7.1. Introduction

Sow mortality has been increasing in the U.K. pig breeding herds during the last few years according to data from national computerised recording schemes (Easicare, 1994, Sheppard, 1994). This may be due in part to recent welfare regulations that determined lame animals should be euthanased on farm rather than transported to the slaughter-house, therefore increasing the recorded death rate. Lameness accounts for a high proportion of removals from a pig breeding herd (Stein *et al*, 1990; Blowey, 1992). Some pig clinicians have suggested infection with the porcine reproductive and respiratory syndrome virus (P.R.R.S.v.), which has affected a large number of the U.K. herds, may be responsible for higher mortality figures in breeding sows (White, 1993 personal communication).
The sow mortality on the units included in this study has been analysed before; Ward and Walton (1980) described high mortality due to gastric dilatation and torsion. Carr (1990) examined the incidence of cystitis and pyelonephritis in these herds. Walton and Duran (1992) and Duran (1993) examined the prevalence of *Clostridium novyi* hepatopathy as a cause of death.

The purpose of this study was to gather information on the mortality of sows housed in confinement, to closely examine necropsy cases and to detect possible deaths due to *C. novyi*.

The data collected was then compared to findings of other surveys, used to determine epidemiological information and to confirm the previously reported (Chapter 6) high incidence of clostridial hepatopathy as a cause of death in these herds.

7.2. Materials and methods

The sows received for necropsy were supplied by 3 nearby units, with a total inventory of 1830 sows. All farms housed the sows in insulated buildings, with confinement stalls on partially slatted floors. The 3 units, A, B and C, had 1100, 650 and 80 sows respectively. Natural service and, with increasing frequency, artificial insemination were used on these units. Lactation was on average 24-28 days long. Computerised records were available from all farms.

Farms A and B fed compound feed following the same formulation and kept pure breed Large White and Landrace sows from the same genetic stock. Both these units bred replacement gilts for sale to commercial breeders.

Farm C, on the other hand, reared pigs for slaughter, bred from hybrid female replacements supplied by farm A. Commercially prepared compound feed was used.

Farm B had a minimal disease health status, free from enzootic pneumonia (*Mycoplasma hyopneumoniae*), P.R.R.S. virus, swine influenza virus, porcine corona virus, atrophic rhinitis, streptococcal meningitis, swine dysentery (*Serpulina hyodysenteriae*) and internal and external parasites. Both farms A and C were free from all of the above except enzootic pneumonia, swine influenza and P.R.R.S. virus. Routine vaccination to protect against *Erysipelothrix rhusiopathiae*, parvovirus and *E. coli* infection were used on all farms. Farm A used Gletvax 5 (Pitman-Moore) a combined *E. coli* and *C. perfringens* vaccine to protect piglets against neonatal diarrhoea.

Sows submitted for necropsy were examined by the author following a similar protocol to that outlined by Wells (1978) and described in appendix V. Sow history data, the estimated time of death, parity, reproductive status, any clinical signs detected or treatments carried out were submitted with the carcass.

For the purpose of classification the peripartum period included three days before and three days after parturition. Lactation extended from three days after farrowing to the time of weaning. The wean to service period encompassed the days between weaning and first service. Sows found to be non gravid at the time of the *post mortem* examination and maiden gilts were classed as not in pig (NIP).

The macroscopic pathology findings were supported by bacteriological examination, measurement of the aqueous humor urea concentration (Drolet, D'Allaire and Chagnon, 1990), anthrax smears and Fluorescent antibody testing with a specific *C. novyi* antibody. Histological examination of tissues was only carried out occasionally. Samples for bacterial isolation were cultured aerobically on sheep blood agar and McConkey agar plates and anaerobically on fastidious anaerobe agar (LabM, Amersham). Samples for isolation of *C. novyi* were processed as detailed in chapter 3.

Computerised farm records were examined to complement the necropsy results, to gain epidemiological data and to assess the relevance of the results. Mortality rate was defined as the number of sows and gilts that died or were culled on the farm during one year divided by the average herd number. The death rate was compiled in a similar fashion, but excluding cull sows and gilts.

7.3. Results

7.3.1. Sows submitted for necropsy

A total of 102 sows were submitted for necropsy during the period of study between January 1992 and December 1994. The annual number of sows received for *post mortem* examination remained almost constant, with 36, 31 and 35 for the years 1992, 1993 and 1994, respectively. Not all the sows that died during the period of study were submitted for examination, because carcasses were too decomposed, the stockmen made a diagnosis or problems with carcass delivery, mainly due to farm staff shortages and herd health security.

The seasonal influence on the number of submissions per month over the three year study was noted (see table 7.1 and figure 7.1). Higher numbers were submitted for necropsy during the spring and autumn, with fewer sows examined during the summer months.

Data concerning the age of the sows submitted revealed a mean parity of 4.3 $\pm$ 0.3 (SEM), with 50% of cases having produced 4 litters or less. The distribution amongst the different parities can be seen in figure 7.2.

Almost 55% of all deaths occured during the four to five week period between farrowing and weaning, compared to 42% from the gestation period (table 7.2). Many deaths (14) from the gestation (dry) houses from these farms were found to be non pregnant. Four non pregnant animals were maiden gilts.

A majority of all submissions (72.5%) were reported by the stockmen to have died without presenting clinical signs before sudden and unexpected death.

The number of carcasses submitted for examination was: 64 from farm B, 29 from farm A and 9 from farm C. Only the submissions from farm C represented all the deaths on this unit during the period of the study. The cause of death diagnosed in 102 sows can be seen on table 7.4.

In 16 cases, 15.7% of all submissions, a definite diagnosis could not be established. This was due to advanced autolysis of the carcass (6 cases), on four occasions the macroscopic lesions did not allow a definite diagnosis of cardiac failure. The only pathological changes these cases presented was transudate in the pericardial cavity, which was considered insufficient alone to diagnose cardiac failure. A definite diagnosis was not reached in a further two submissions that exibited an enlarged, emphysematous, dark green liver because of autolytic changes elsewhere in the carcass, elevated ambient temperatures at the time of death and because more than 18 hours had elapsed between death and necropsy. *Clostridium novyi* was demonstrated in large numbers on tissue smears. On two occassions a single cause of death could not be determined. Two submissions presented no observable macroscopic pathology

Mortality due to accidents involving abdominal organs accounted for 17.6% of all submissions, almost 23% if the cases of splenic torsion and rupture were added to this group. Gastric dilation and rupture was diagnosed in 9 occasions, gastric torsion in 2, intestinal tears, strangulation and torsion in 6 cases and oesophageal obstruction in one case only. Liver lobe torsion was only seen in conjunction with a gastric torsion. The average parity for these cases was 3.9 pregnancies, and a majority of deaths occurred during lactation (33%). All gastric dilation cases occurred during lactation, one day post farrowing or after weaning.

Splenic torsion was predominantly seen in older sows, never before producing 3 litters, and was diagnosed at an average parity of 6.6. Deaths occurred evenly at all stages of production.

Clostridial hepatopathy, accounted for 17 deaths (16.7%) during the three years, with death after 5.6 pregnancies on average. Further details from these cases can be seen in chapter 9 (*q.v.*).

Urinary tract disease was responsible for 12 deaths, generally acute or chronic cystitis with ascending ureteritis and pyelonephritis. Mixed infections with *Escherichia coli, Proteus* spp. and *Eubacterium* (*Corynebacterium*) *suis* were demonstrated, unless antibiotic treatment had been attempted before death. The average parity was 2.4 pregnancies and almost half of all the deaths had only one litter before dying. Death occurred most frequently during pregnancy (50% of cases). Clinical signs, including haemorrhagic/muco-purulent vulval discharge, loss of appetite and pain were detected by the stockmen in 67% of cases. In addition, urinary tract infections, mainly cystitis alone, was seen in 14 other sows that were believed to have died due to other causes.

Complications of parturition, generally retained piglets, but also uterine prolapse, were seen in almost 5% of all submissions, in sows of more than 3 pregnancies (mean parity 6.6).
Endometritis alone as a cause of death was seen in less than 2% of the submissions, but was frequently seen in conjunction with other conditions, mainly clostridial hepatopathy (5 cases), urinary tract infections (3) and in four other occassions.

Severe oesophagogastric ulceration with massive haemorrhage into the stomach or perforation of the gastric wall was diagnosed in almost 10% of all cases. The mean parity was 3.7 and most cases occurred during lactation (60%). Gastric ulceration was seen in another 10% of sows that had died for other reasons.

Cardiac failure was diagnosed in 4 cases, all deaths occurred after stressful times in the productive cycle, 2 during service, one in the peripartum period and one during lactation in the summer. The average parity was 4.2.

Pneumonia was diagnosed as the cause of death on 4 occasions, mainly in gilts from farm A (3 cases). Deaths occurred during the acute onset of the PRRS virus infection accompanied by severe secondary bacterial infection with *Pasteurella multocida*. One older sow (parity 7) was found with multiple lung abscessation, *Streptococcus* spp. were isolated in pure culture.

Lymphosarcoma and peritonitis were infrequently diagnosed (one case of each) and other diagnoses included mastitis (2), internal haemorrhage (1), trauma (1), haemorrhagic enteropathy of unknown aetiology and two "downer sows" that were euthanased *in extremis* or were found dead after becoming recumbent.

Table 7.1. Submissions for necropsy by month (1992-1994)

Month	Numbers	Season	TOTAL	%
December	5			
January	11	WINTER	23	22.5
February	7			
March	11			
April	11	SPRING	31	30.4
May	9			
June	8			
July	6	SUMMER	21	20.6
August	7			
September	11			
October	7	AUTUMN	27	26.5
November	9			
TOTAL	102			

Figure 7.1. Monthly distribution of submissions for necropsy (1992-1994)

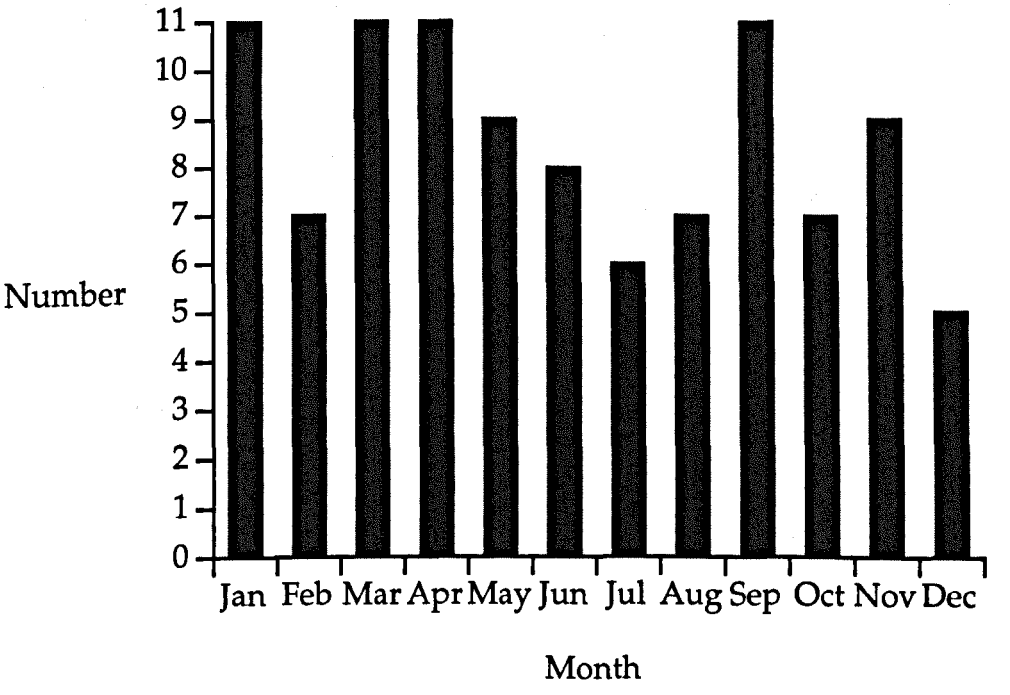
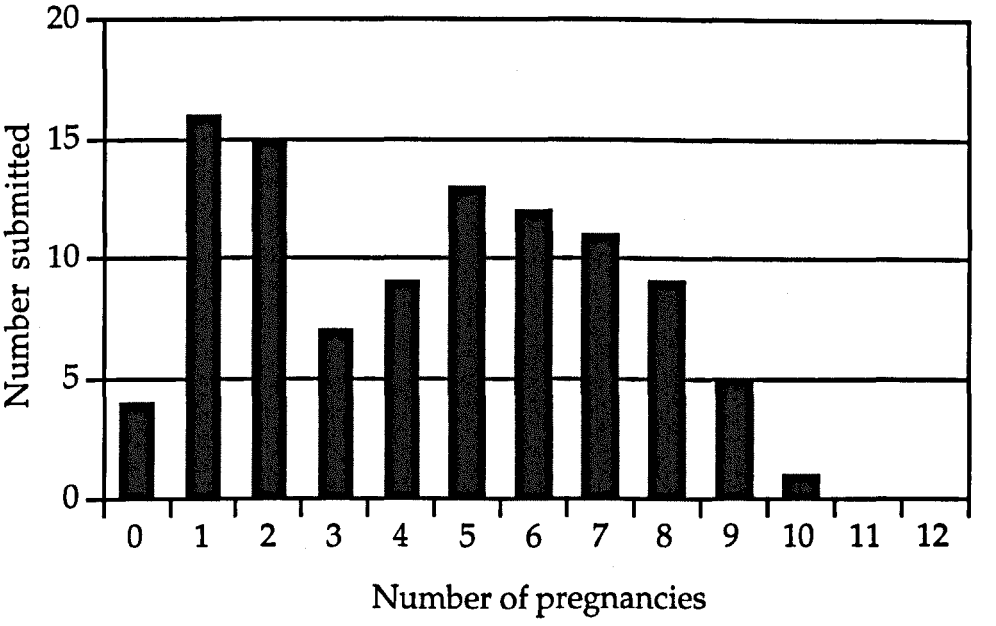


Figure 7.2. Parity distribution of 102 sow deaths examined (1992-1994)



Submissions	4	16	15	7	9	13	12	11	9	5	1	0	0

Table 7.2. Stage of the reproductive cycle in 102 deaths submitted for necropsy (1992-1994)

Stage in cycle	Number	%
Peripartum	16	15.7
Lactation	30	29.4
Post-weaning and service	9	8.8
Pregnancy	29	28.4
Not in pig	18	17.6
TOTAL	102	100

Figure 7.3. Annual diagnosis of the most frequent causes of death (1992-1994).

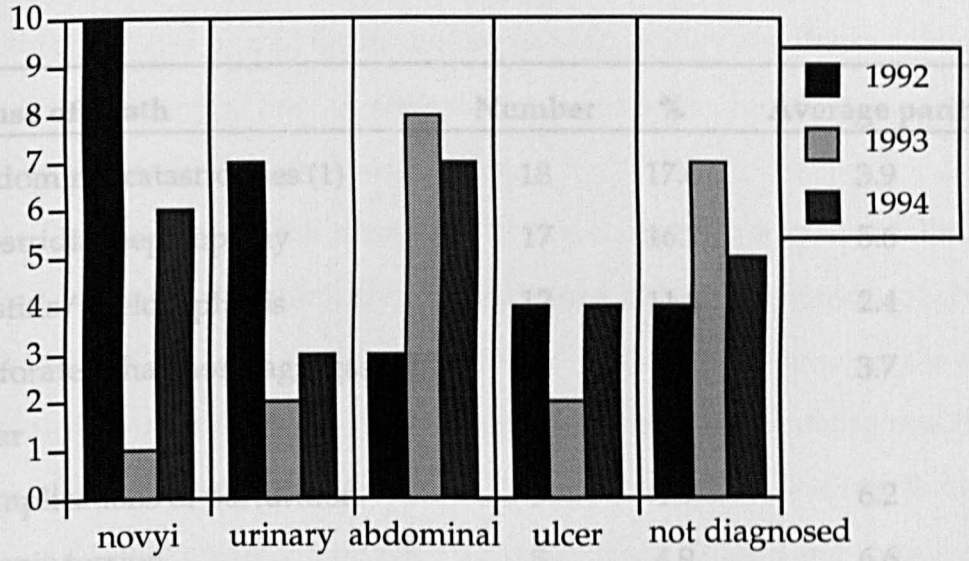


Table 7.3. Most frequent causes of death by year.

	novyi	urinary	abdominal	ulcer	not diagnosed
1992	10	7	3	4	4
1993	1	2	8	2	7
1994	6	3	7	4	5

novyi; sudden death due to*Clostridium novyi*, urinary; deaths involving the urinary tract, abdominal; includes death due to accidents involving abdominal organs, ulcer; includes death after perforated or haemorrhaging oesophago-gastric ulceration.

Not diagnosed; comprises sow deaths were a conclusive diagnosis was not reached.

Table 7.4. Distribution of the cause of death and the average parity amongst 102 sows examined at Leahurst (1992-1994).

Cause of death	Number	%	Average parity
Abdominal catastrophes (1)	18	17.6	3.9
Clostridial hepatopathy	17	16.7	5.6
Cystitis/pyelonephritis	12	11.8	2.4
Perforated/haemorrhagic gastric ulcer	10	9.8	3.7
Complications of parturition	5	4.9	6.2
Splenic torsion	5	4.9	6.6
Heart failure	4	3.9	4.2
Pneumonia	4	3.9	2
Endometritis	2	1.9	
Peritonitis	1	1	
Lymphosarcoma	1	1	
Others (2)	7	6.9	4.7
Not diagnosed	16	15.7	3.9
TOTAL	102	100	4.3

(1) Abdominal catastrophes included gastric dilatation and torsion, gastric rupture (excluding perforated ulcers), torsion of a lobe of the liver, ruptures and torsion of small and large intestine.

(2) Others included conditions seen less frequently, like mastitis, haemorrhagic enteropathy, "downer sows" or trauma.

7.3.2. Farm mortality data

Information from each farm was recorded using computerised records and provided in printed format for the author to analyse the data. Records for the year 1992 pertaining to farm A were destroyed in a fire, but data concerning number and month of deaths was available.

An initial observation of the mortality rate can be seen illustrated in table 7.5. On farms A and B the mortality rate increased during the period of study from 9.5% to 10.1% and 16.4% respectively. Both herds increased the sow inventory during the study. Farm C recorded a rise in the mortality rate from 2.5% to 3.7% during the period of study, but remained at a lower rate than the bigger herds. The mean herd size for the study was 610 sows and the average mortality rate rose during the study from 8.9% in 1992 to 10.1% in 1993 and 12.2% during 1994. These figures included sows culled on the farm due to lameness or other locomotor problems. The annual death rate for all three herds was 8%, 7.6% and 8.2% during 1992, 1993 and 1994 respectively.

Table 7.5. Mortality and culling rate in three indoor herds (560 deaths).

Year	Deaths (%)	Culls (%)	Total (%)	Herd size	Farm
1992	n.a.	n.a.	88 (9.5)	929	
1993	85 (7.7)	24 (2.2)	109 (9.9)	1100	Farm A
1994	82 (7.5)	29 (2.6)	111(10.1)	1100	
1992	43 (6.6)	15 (2.3)	58 (9.5)	650	
1993	53 (8.2)	21 (3.2)	74 (11.4)	650	Farm B
1994	67 (9.9)	44 (6.5)	111 (16.4)	675	
1992	2 (2.5)	none	2 (2.5)	80	
1993	2 (2.5)	none	2 (2.5)	80	Farm C
1994	3 (3.7)	2 (2.5)	5 (6.2)	80	

Culls: describes sows euthanased on farm; **n.a.:** data not available

The distribution of 418 deaths by month and season did not reveal a clear pattern of mortality (see table 7.6. and figures 7.4. and 7.5.). Deaths were more frequent during the summer months , with 29.4% of all mortality taking place during these months (123 deaths). During June 1994 an unexpected number of deaths (16) occurred in farm B. The cases examined from this period revealed haemorrhagic oesophago-gastric ulceration causing mortality in lactating sows. Almost a quarter of all deaths (104) occurred during autumn months, whilst 101 (24.2%) and 90 (21.5%) took place during spring and winter respectively.

The mean number of litters before death in herd A was 4 ± 0.2 (SEM), 4.1 ± 0.2 (SEM) in herd B and 5.6 ± 0.6 (SEM) in farm C. Table 7.7. and figures 7.6. and 7.7. illustrate the distribution of the deaths in the various age groups. The values do not follow a normal distribution.

Table 7.6. Deaths by month in the three farms investigated (418 deaths).

	Farm A			Farm B			Farm C
Month	**1992**	**1993**	**1994**	**1992**	**1993**	**1994**	**1992-94**
Jan	4	8	2	5	4	6	2
Feb	15	2	3	6	3	4	1
Mar	5	4	4	2	3	6	0
Apr	7	9	5	6	5	5	1
May	12	3	10	5	3	6	0
Jun	12	9	9	3	3	16	0
Jul	6	5	7	4	7	5	0
Aug	4	7	9	2	7	8	0
Sep	9	12	6	3	3	1	3
Oct	4	10	7	0	5	2	0
Nov	4	9	10	3	8	4	1
Dec	6	7	10	4	2	4	1

Figure 7.4. Monthly distribution of mortality on Farm A (255 deaths).

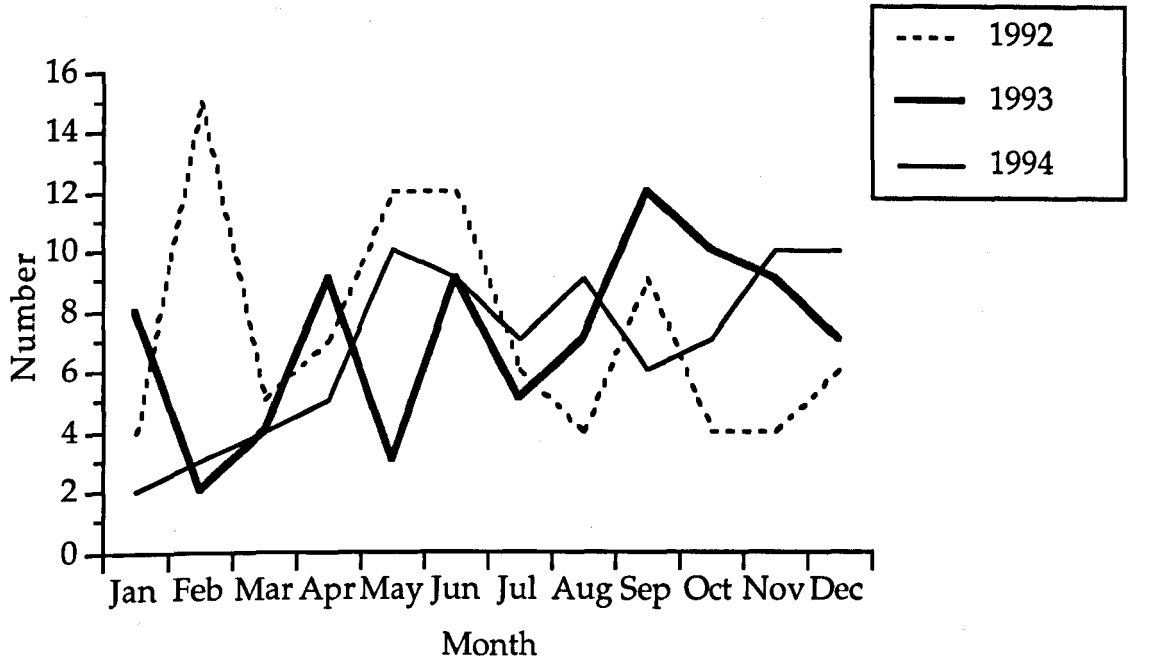


Figure 7.6. Monthly distribution of mortality in Farm B (163 deaths).

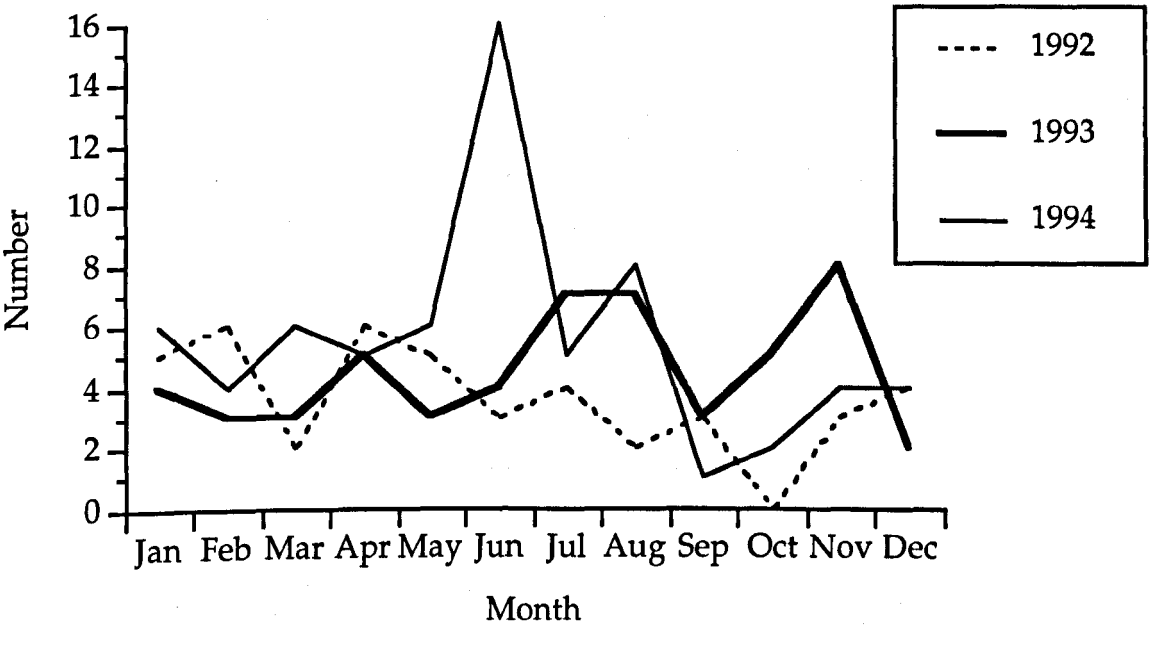


Table 7.7. Parity distribution of all deaths (including on farm cull sows).

Parity	Farm A	Farm B	Farm C	All deaths
0	20	11	0	31
1	43	43	0	86
2	24	40	0	64
3	20	25	0	45
4	18	17	4	39
5	28	28	1	57
6	18	21	1	40
7	18	21	2	41
8	15	20	0	35
9	7	14	1	22
10	6	3	0	9
11	1	1	0	2
12	2	0	0	2

Figure 7.6. Parity distribution of mortality on Farm A (220 deaths).

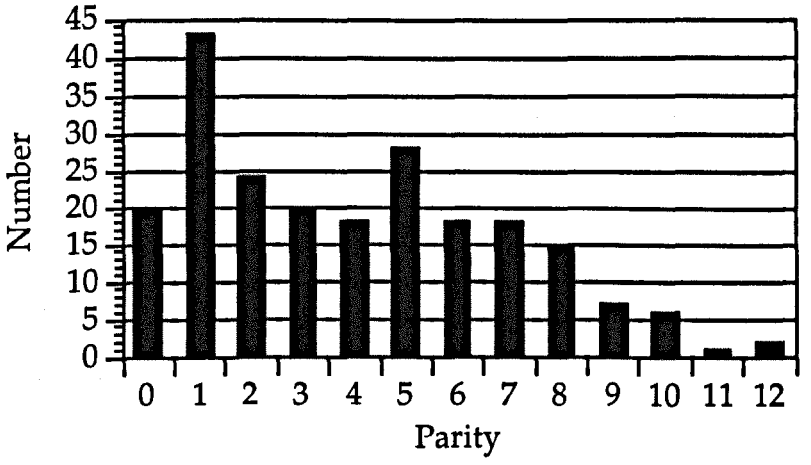


Figure 7.7. Parity distribution of mortality on Farm B (243 deaths).

Data concerning the stage of the production cycle when death occurred was available in 339 cases (see table 7.8.). The figures relating to deaths during 1992 on farm A were not available. Overall, almost half the deaths took place during pregnancy, but a large variation between farms was noted. The mortality rate around the time of farrowing was similar for all farms. During lactation farm B suffered a higher than average number of losses, particularly towards late lactation (14 days post farrowing). The number that died at the time of weaning or during service (22.2%) on farm C was also above the percentage for the other two farms. Non pregnant sows (7.7%) included mostly maiden gilts (21 out of 26).

Table 7.8. Stage of the production cycle of 339 deaths in three pig breeding herds (%).

	Peripartum	Lactation	Weaning	Pregnancy	N.I.P.	Total
Farm A	21 (12.6)	37 (22.2)	6 (3.6)	87 (52.1)	16 (9.6)	167
Farm B	19 (11.7)	53 (32.5)	13 (8)	70 (43)	8 (5)	163
Farm C	1 (11.1)	1 (11.1)	2 (22.2)	3 (33.3)	2 (22.2)	9
Total	41 (12.1)	91 (26.8)	21 (6.2)	160 (47.2)	26 (7.7)	339

Peripartum: Includes deaths that occurred three days before and three days after parturition; **Lactation:** Mortality during lactation, between 21 and 28 days in length; **Weaning:** Deaths at the time of weaning or during service; **Pregnancy:** Mortality during pregnancy; **N.I.P.:** not in pig, including sows and maiden gilts that were not pregnant.

7.4. Discussion

Recent surveys analysing breeding pig mortality have been published for Canadian herds (Chagnon *et al*, 1991; D'Allaire *et al*, 1991) and for herds in the USA (D'Allaire *et al*, 1987; Stein *et al*, 1990). European studies examining the most frequent causes of death in herds of intensively housed sows were published some time ago (Jones, 1968; Madec, 1984; Smith, 1984).

The variation in mortality rates and main causes of death between the different surveys reflected the differences in management, housing, geography, climate, diet, genotype and disease status according to Chagnon *et al* (1991). Hence individual farm records and *post mortem* examination of deaths should be used to evaluate the different causes responsible for an increased mortality in sows as suggested by Straw (1984). Nevertheless, the value of surveys of sow mortality was stressed by various authors (Madec, 1984; Smith, 1984; Chagnon *et al*, 1991).

The average mortality rate in the farms included in this study rose from 8.9% in 1992 to 12.2% in 1994. This increase was mainly due to a higher number of sows being culled on the farm rather than sold as casualty animals, but the average death rate remained high at around 8%. The average herd size during the period of study was 610 sows. Straw (1984) believed that the mortality rate rose as the herd size increased and quoted 5% as a reasonable target for herds with more than 200 sows. Chagnon *et al* (1991) reported the average herd death rate in 24 Canadian swine breeding herds was 3.3% and Madec (1984) reported a similar death rate for breeding herds in Brittany (3.7%). In both surveys there was considerable variation between farms, ranging from 0 to 10%. The average herd inventory for these surveys was below 200 breeding females, smaller than in this study.

The mean parity at the time of death was 4.3 ± 0.3 (SEM) for the sows submitted for necropsy and 4.6 according to farm records. These values were similar to the average of 4.2 litters reported in Canada (Chagnon *et al*, 1991) and 3.9 in 18 North American herds (Stein *et al*, 1990), but higher than an average parity of 3.4 in surveys by Madec (1984) and D'Allaire *et al* (1987). The parity distribution of deaths submitted for necropsy and data from the total mortality presented similar curves (see figures 7.2., 7.6. and 7.7.). A quarter of all mortality in these herds occurred before the second litter and over half of the deaths were before parity 4.

Chagnon *et al* (1991) stated that a significantly higher number of deaths in Canadian herds were recorded during the summer months. This was supported by farm record data from this study as almost 30% of all deaths occurred in the summer. On the other hand, submissions for necropsy in the present study were lowest in the summer, perhaps because carcasses became autolysed and were deemed unsuitable for examination. Sanford *et al* (1994) also reported fewer submissions during the summer in a limited survey of mortality.

There were differences between the results of this investigation and published data regarding the stage at which deaths were more likely to occur. Madec (1984) reported that 26% of the sows examined died in the period around farrowing. Chagnon *et al* (1991) concluded that as many as 42% of all the deaths in the farms surveyed occurred during the peripartum period. Mostly these were cases of heart failure and vaginal or uterine prolapse. Data from the sows examined in this study indicated a large proportion of sows died during the lactatation (29.4%) or pregnancy (28.4%). Data from death losses on the three farms suggested that mortality was more likely to occur during pregnancy (47.2%). Mortality during lactation was higher than reported in other mortality surveys (Madec, 1984; Chagnon *et al*, 1991). Variation between the farms was marked. In particular, farm B which had older

buildings, smaller farrowing crates and more stressful farrowing accomodation presented very high mortality during the lactation and weaning period. Mortality during lactation often involved oesophagogastric ulceration or gastric dilation. Marco (1995) reported elevated death losses during lactation in a large breeding herd. The small number of deaths on farm C makes analysis of the data unwise.

The cause of death was determined in 86 sows submitted for necropsy. Abdominal catastrophes, clostridial hepatopathy, cystitis and pyelonephritis and oesophago-gastric ulceration were diagnosed in more than half of all submissions.

Torsion and accidents involving abdominal organs, in particular gastric dilation, was the most frequently diagnosed cause of death. These cases accounted for 22.6% of all submissions, a large increase in comparison with submissions from these units in the past (see chapter 6). This diagnosis was the second most important cause of death in other studies (Chagnon *et al* 1991; Smith, 1984) and caused serious mortality in some herds (Morin *et al*, 1984; Sanford *et al*, 1984; Bilkei, 1987). Ward and Walton (1980) investigated mortality in farm B and found gastric dilation was a major cause of death.

Splenic torsion tended to occur in older sows (6.6 litters on average) whilst other abdominal accidents affected younger sows at the peak of production. Other studies found these deaths occured in older sows, sometimes during pregnancy (Morin *et al*, 1984) or at various stages of the reproductive cycle (Chagnon *et al*, 1991). Factors believed to increase the incidence of torsion and accidents of abdominal organs were once a day feeding, disruptions in the feeding routine, genetic predisposition and type of food (Blackburn *et al*, 1974; Morin *et al*, 1984; Bilkei, 1987; Chagnon*et al*, 1991). All the cases of gastric dilation and the majority of other accidents were seen either during lactation or after weaning, times when high feed intakes coincided with stressful events. No other predisposing factor could be determined.

The second most frequently diagnosed cause of death was clostridial hepatopathy, which accounted for 16.7% of mortality. This represented a decrease in diagnosed cases compared with 34.7% of all submissions diagnosed as clostridial hepatopathy between 1986 and 1991. *Clostridium novyi* deaths were more likely to be diagnosed in older sows, with an average parity of 5.6. The incidence of clostridial hepatopathy submissions appeared to be linked to that of urinary tract disorders (see figure 7.3.). As cystitis and pyelonephritis deaths decreased so did the number of *C. novyi* sudden deaths. No other survey with the exception of Madec (1984) reported this syndrome as a cause of death in sows. Madec found 11% of the sows necropsied presented a pronounced degeneration of the liver, but was unwilling to interpret this finding. Mortality caused by *C. novyi* accounted for over 46% of deaths in a herd in north-western Spain (Marco, 1995). Most of these deaths were during lactation.

The reduction in cystitis and pyelonephritis cases detected during the retrospective study (see chapter 6), was reflected in a further decrease in the percentage of submissions between 1992 to 1994. Almost twelve percent of the sows presented severe cystitis and pyelonephritis, compared with almost 23% in the period between 1986-1991. The current proportion of deaths compared favorably with those reported from Brittany (40%) by Madec (1984) and Scotland (22.9%) by Smith (1984). Herds in Canada presented lower rates of 8% (Chagnon *et al*, 1991).

The average parity of the cystitis and pyelonephritis cases submitted for necropsy was 2.4. Even a first parity gilt had died with severe pyelonephritis. This finding differs from the observation that cystitis and pyelonephritis was more likely in older sows (Jones, 1967; Madec, 1984; Chagnon *et al*, 1991). The majority of the sows died during early to mid pregnancy a fact confirmed by other workers (Carr, 1990; Chagnon *et al*, 1991).

An elevated urea concentration in the aqueous humor measured *post mortem* aided in the diagnosis of severe urinary tract disease as reported (Drolet *et al*, 1990; Chagnon *et al*, 1991). Bacteriology was useful only if the sows had not received previous antibiotic treatment. The reduction in mortality due to cystitis and pyelonephritis seen during this study was probably due to increased use of artificial insemination over boar mating, improved water availability for the sows and increased stockmen awareness of the disease.

A remarkable increase in diagnosed haemorrhagic gastric ulcers was seen in comparison with the retrospective study (chapter 6). The percentage of submissions, 9.8%, was much higher than that reported in other studies (Jones, 1967; Svendsen *et al*, 1975; Smith, 1984; Madec, 1984; Chagnon *et al*, 1991). Only Sanford *et al* (1994) in a limited survey of sudden death found a higher proportion of deaths caused by gastric ulcers (18.4%). Marco (1995) described elevated mortality due to gastric ulceration in a large breeding herd. In the discussion the author related ulceration to the composition of the diet, in particular the high inclusion levels of wheat and tapioca in the diet.

Acute heart failure, on the other hand, was diagnosed infrequently in this series (3.9%). This finding was in disagreement with both Chagnon *et al* (1991) and Smith (1984), who reported heart failure as the first and second most frequent cause of death. Some cases may have not been submitted for necropsy because the stockmen recognised sudden death of sows during service, with discolouration of the skin and collapse as heart failure. Farm recording of mortality suggested that between 3 and 8% of deaths seen annually were cases of heart failure. Also, as mentioned by Drolet *et al* (1992), the diagnosis of acute cardiac failure was difficult, particularly if other lesions were present in the carcass.

Vulval discharges and problems during parturition were infrequently seen on these farms. This was reflected in a low number of submissions with retained piglets, endometritis, torn vaginas or uterine prolapses.

The main reason for this was that, even in the large herds, stockmanship was of a high standard.

The present survey, carried out on three breeding herds in Cheshire was flawed for two reasons: firstly, not all sow deaths from the farms surveyed were made available for necropsy. The herd biosecurity measures on farms A and B caused difficulties in submission of carcasses, particularly at week-ends when farm staff was limited. Perhaps the importance of examining all deaths was not conveyed by the author. The unpredictability of deaths in the field and the availability of one person to carry out the examination made necropsy of all deaths impossible. The results may be biased as submissions were likely to reflect areas or individuals of concern to the stockmen. This failing was amended by the analysis of farm mortality data.

Secondly, diagnosis relied mainly on recognition of macroscopic lesions and bacteriology, without histo-pathology in most cases. This may not be essential for an accurate diagnosis. Madec (1984) found that in 90% of the sows examined in a mortality study, necropsy alone allowed at least a plausible cause of death to be determined. The percentage of submissions in the present survey of sow deaths without a definite diagnosis was 15.7%, similar to the figure of 14.6% described by Chagnon *et al* (1991). In that study many of the deaths classed as "not known" were either too decomposed or had not been submitted. Smith (1984) reported that in 7.87% of submissions a diagnosis was not reached. The retrospective analysis of sow necropsy records described in chapter 6 indicated that a diagnosis was not established in 5.3% of all sows submitted.

7.5. Summary

In summary, the pig breeding herd mortality in housed sows kept in confinement was examined during three years. The average mortality rate increased between 1992 and 1994, mostly because more sows were being culled on the farm rather than sold as casualty animals. The mortality rate rose from 8.9% to 12.2%. The death rate remained stable at around 8% even though the herds increased the sow inventory.

More deaths were recorded during the summer months, but the seasonal variation in mortality was not marked. The majority of sow deaths occurred during gestation (47.2%), but submissions for necropsy were mainly lactating sows. Younger sows were more likely to die in these herds, with a mean parity of 4.6 litters.

The principal reasons for death were: Torsion or accident of abdominal organs (17.6%), clostridial hepatopathy (16.7%), cystitis and pyelonephritis (11.8%) and haemorrhagic oesophago-gastric ulceration (9.8%).

The continued high mortality due to *Clostridium novyi* sudden death remains a major cause of death in the herds investigated.

The diagnosis of sudden death still remained difficult particularly when multiple lesions or degeneration of the carcass was present. This was reflected by the fact that in 15.7% of submissions a definite diagnosis could not be reached.

8. INVESTIGATION INTO THE PRESENCE OF *Clostridium novyi* IN THE LIVER OF CULL SOWS ON FARM AND AT THE SLAUGHTERHOUSE AND IN SOWS SUBMITTED FOR NECROPSY.

"Poetry is like fish: if it's fresh, it's good; if it's stale, it's bad; and if you are not certain, try it on the cat." Osbert Sitwell.

8.1 Introduction

The presence of *Clostridium novyi* in the liver of healthy sheep, grazing in infectious necrotic hepatitis areas, has been known for some time (Jamieson, 1949; Corbould, 1966; Roberts *et al*, 1970b; Bagadi and Sewell, 1973a; Worrall, Moekti and Lubis, 1987). Other species have also been reported to carry latent *C. novyi* spores, particularly in the liver of cattle (Corbould, 1966; Batty *et al*, 1967; Niilo *et al*, 1969). Furthermore, when examining sheep, cattle and pigs submitted for necropsy there appeared to be a high percentage of animals carrying *C. novyi* in the liver (Corbould, 1966; Thomson, Barnum and Ide, 1968). Narayan (1968) reported isolating *C. novyi* type A from the kidney and muscle of two out of 100 healthy pigs at slaughter. This author also isolated *C. novyi* type B on 4 occasions from the spleen, kidney and muscle, but not from the liver of slaughtered pigs. Thomson *et al* (1968) examined 100 slaughter pigs, but failed to demonstrate *C. novyi, Clostridium chauvoei* or *Clostridium septicum* in samples of liver tissue.

The aims of this work were to determine if vegetative cells or latent spores of *C. novyi* were present in the liver of healthy sows by examining samples collected from the abattoir and cull sows euthanased on the farm because of lameness or traumatic injury.

The validity of the FA test in the diagnosis of sudden death due to C. *novyi*, was tested. Sows submitted for necropsy and diagnosed as having died for a different reason were examined using the FAT.

Finally, culled sows were allowed to stand at room temperature for 12, 24 and 48 hours before the necropsy to determine the influence of *post mortem* autolysis on the diagnosis of clostridial hepatopathy.

8.2. Materials and methods

i) Examination of cull sows from the herds under investiation.

Sows euthanased on the farms were examined to investigate the possibility of sows carrying vegetative C. *novyi* bacteria in the liver. The *post mortem* examination was carried out immediately after euthanasia. A full necropsy was carried out and any abnormalities of the internal organs were noted. Liver samples were collected and an impression film was prepared for examination with the C. *novyi* specific fluorescein labelled antibody as described earlier.

ii). Examination of the liver in 3 culled sows after delaying the necropsy for 12, 24 and 48 hours.

This experiment was set up to determine the effect of delaying the *post mortem* examination on the presence of vegetative C. *novyi* in the liver.

The sows were stunned with a Cash Special captive bolt pistol and pithed. The reproductive and health data were collected from farm records and the sow's condition score was determined following the scale suggested by Straw and Meuten (1992). The carcasses were kept at ambient temperature and a necropsy was carried out, but only the liver was examined. Impression smears were stained using fluorescent anti-C. *novyi* specific antibodies. Portions of the liver were cultured anaerobically as in the general methods.

iii) Sampling of livers from healthy sows at the slaughterhouse.

An abattoir in the south-east of England especialising in adult female pigs was used to collect random samples of liver tissue. The following protocol was used: First, the sow was identified using the slap mark. Second, an examination of internal organs was made to determine whether any lesions suggestive of systemic disease were present. Third, the liver was examined and any abnormalities, parasite migration scars, necrotic or inflamed areas were recorded.

The surface of the right central lobe was seared on the visceral side using a red hot scalpel blade, then with another sterile scalpel blade a 4x1x1 cm cube was removed. The cube of liver tissue was placed in a numbered sterile cell culture dish. With a fresh sterile scalpel blade the sample was divided in two portions, half remaining in the dish and the other half sealed in a labelled polythene bag. The sample bags were immediately placed on dry ice in a cooler box. These were then stored at -20^{o}C for bacterial culture and isolation of *C. novyi*. The sterile incision site of the liver was used to make thin smears. The glass slides were labelled and allowed to air-dry in a covered slide holder. After allowing the smears to dry they were fixed in acetone for 10 minutes, ready for fluorescent antibody staining.

The other portion of liver remained in the dish, which was securely closed with cellotape and placed into a bag. Once the Generbag AnO_2 (bioMerieux) was filled with petri dishes, anaerobiosis was rapidly achieved by adding 3 ml of water to the central orifice and a drop to humidify the anaerobic indicator. The bags were sealed. The samples were placed in the anaerobic incubator at 37^{o}C immediately after arrival at the laboratory. Serial examinations using the FA test were carried out on liver smears at 8, 24 and 48 hours after death. Fluorescein labelled *C. novyi* polyclonal antibody (Pragma) was used according to the protocol in chapter 3. Slides were examined under a 40x magnification with a fluorescent microscope (Leitz).

Initially a rapid scan of the whole slide was made, followed by systematic examination and counting of cells in positive smears.

Samples that gave a positive reaction with the specific antibody and had been stored in the freezer could then be thawed and cultured anaerobically, as described in chapter 3. A portion of the liver sample was allowed to stand in a sterile petri dish in anaerobic conditions for 48 hours to allow germination of the bacterial spores, before inoculating pre-reduced FAA plates. The other half was macerated in a sterile pestle and mortar in 1 ml of sterile distilled water. The macerate (0.5 ml) was heat and alcohol shocked and allowed to incubate for 48 hours anaerobically.

iv) Detection of *C. novyi* in the liver of sows submitted for necropsy that had died for reasons other than clostridial hepatopathy.

From January 1993 the liver of all sows was tested using the FA test, regardless of the diagnosis. Samples were not collected when the abdominal cavity was contaminated by gastric or intestinal contents.

8.3. Results

8.3.1. Sows culled on the farm.

Necropsies were carried out on 8 sows culled for lameness. Two sows had fractures (distal epicondyle and mid shaft of the humerus respectively), 1 was euthanased after "doing the splits" and presented severe haemorrhages in the inguinal area, but no other macroscopic lesions. The other 5 sows were culled after developing chronic intractable septic laminitis. Therapy using injectable lincomycin had been used.

None of these sows presented lesions indicating systemic illness. Impression smears made from the liver of these sows was negative to the FA test in all eight cases.

8.3.2. Examination of liver smears from carcasses left unopened 12, 24 and 48 hours, using the FAT.

The month of March was chosen for the experiments to avoid extremes of temperature. The temperature during the experiment remained between 8 and 14°C in the building.

Three weaned sows culled for lameness were used in the experiment. Sow A, was condition score 2 and had reared 8 litters. She was culled first and left unopened for 48 hours. Two further sows B (parity 7) and C (parity 1) were culled the next day and allowed 12 and 24 hours before necropsy. Both sows were condition score 3.

None of the carcasses when opened had marked *post mortem* change. Sow A presented a greenish discoloration and moderate distension of the abdomen. There was no generalised emphysema or gas formation in the muscles, although the latter were pale with a cooked chicken appearance and some pinkish subcutaneous fluid was seen. The internal organs were autolysed, soft and discoloured. The liver presented a pale brown colour, was infiltrated by gas and was friable. The lobes around the gall bladder were stained a bright orange colour. Impression smears made from the liver demonstrated the presence of *C. novyi*. The carcasses of sows B and C presented very slight *post mortem* autolysis. The liver in both cases presented patchy dark discoloured areas on the surface and a more friable parenchyma, but no generalised gas infiltration. The fluorescent antibody test did not detect *Clostridium novyi*, but Gram stained smears did show numerous Gram positive rods. *C. perfringens* was frequently isolated.

Isolation of *C. novyi*, from cultured liver samples was unsuccessful.

8.3.3. Results from the examination of slaughterhouse livers.

Liver samples were collected from 30 sows from a large outdoor herd. The sows had travelled at least 5 hours and had spent some time in the lairage.

No gross lesions were seen on inspection of the carcasses. Examination of the livers did not reveal areas of parasite migration, necrosis or inflammation.

The impression smears collected at the slaughterhouse (0 hours) were all negative with the FA test. On ten of these impression smears, small round fluorescent structures resembling free spores were seen, but were possibly an artifact. Three smears were not examined, because of poor fixation due to excessively thick smears. After 8 hours anaerobic incubation, vegetative rod shaped fluorescent bacteria could not be detected. Gram and malachite green stained smears did not indicate the presence of spores in the liver impression smears. Both Gram positive rods and cocci and Gram negative bacilli could be seen.

Two liver samples appeared to be positive after 24 hours incubation. The microscopic image was not expected. Numerous cell like structures fluoresced brightly, particularly a central area surrounded by less fluorescent hazy areas delineated by a brighter wall. These findings have not been described before as typical of positive results. After the 48 hour incubation was over, 5 samples presented extremely high numbers of large fluorescent bacilli (+++), which frequently presented straight parallel sides, rounded ends and a subterminal spore (figure 8.1.). A further 3 slides were moderately positive (++) using the FA test and 6 slides had between 5 and 90 fluorescent bacilli per slide (+). The rest of the samples were negative for *C. novyi*. Five negative samples had become dry after 48 hours incubation, reducing the quality of the smears, which may have affected the sensitivity of the test.

C. novyi type A was isolated from one sample. The isolate grew poorly on solid media, with flat, grey, small (24 hours) colonies with a rhyzoid or crenated edge. A double area of haemolysis around the colonies was visible and on egg yolk agar both a lecithinase and a lipase reaction were present. The Gram stain showed a large Gram positive/variable bacilli with oval subterminal spores deforming the cell. The FAT was positive.

Figure 8.1. Impression smear from a liver incubated for 48 hours. *Clostridium novyi* bacilli can be seen fluorescing (FAT+++).

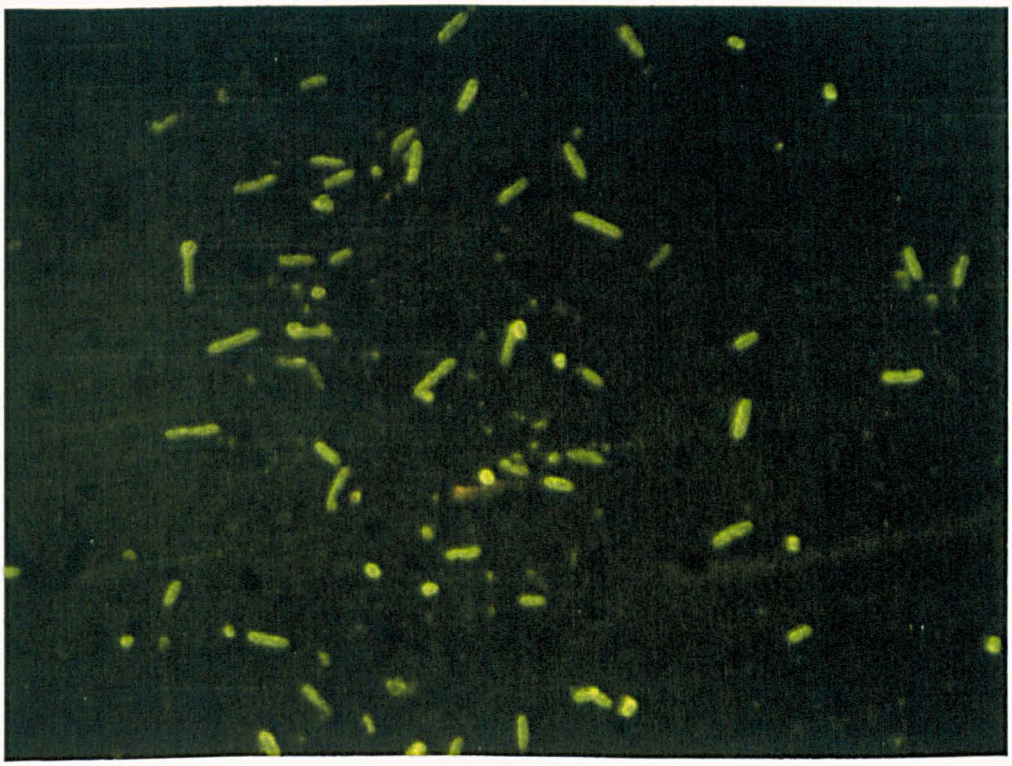
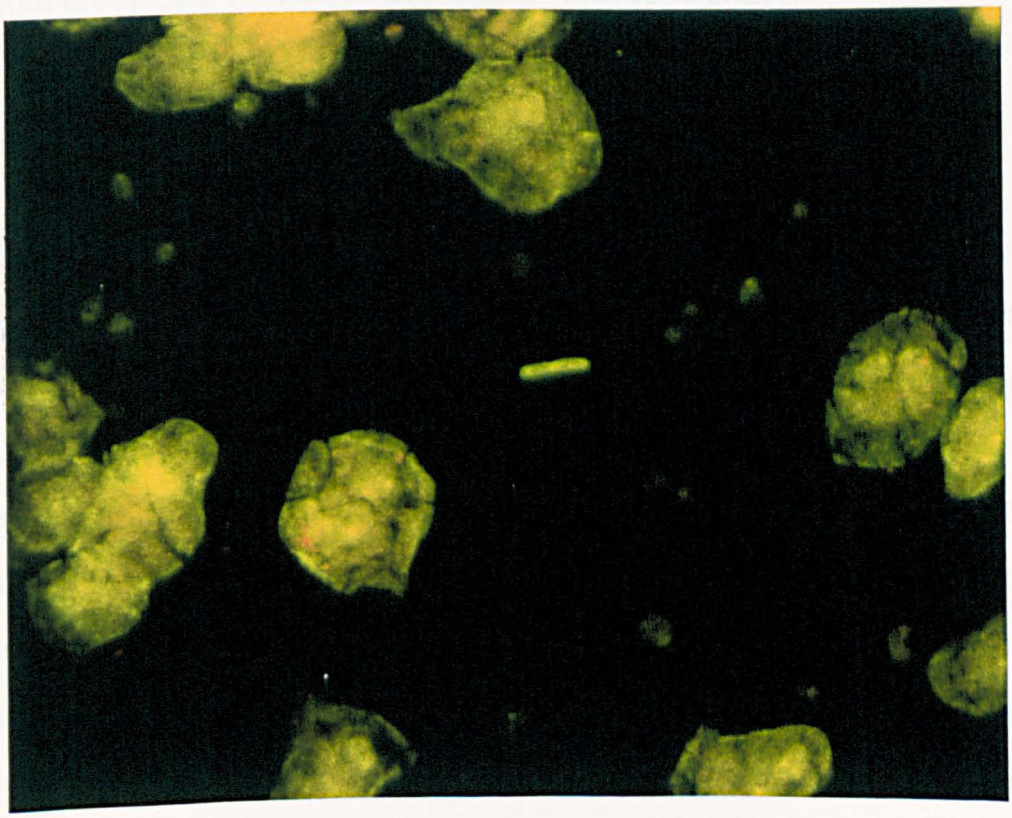


Figure 8.2. Liver smear stained with fluorescein labelled anti-*Clostridium novyi* antibody. Only one bacilli detected per field. Note the non specific binding to liver cells.



8.3.4. Fluorescent antibody test results from sows submitted for necropsy and not diagnosed as *Clostridium novyi* sudden death.

Impression smears of the liver were collected from 39 sows during two years. Thirty of the liver smears were negative when examined with the *C. novyi* fluorescent antibody.

On 9 occasions (23%) the FAT was positive, but the death was not diagnosed as a clostridial hepatopathy. Three of these cases had only one or two organisms per field (+) even though death had occurred more than 24 hours before examination and were classified as "not diagnosed" (see figure 8.2.). The cause of death was not determined in one sow presenting lesions suggestive of cardiac failure and very few fluorescent *C. novyi*. Another liver impression smear classified as a + was collected from a sow with cystitis and pyelonephritis. The rest of the cases (4) presented highly positive results after examination of the smears. More than 24 hours had elapsed before necropsy was carried out in three of these submissions and death was caused by haemorrhagic enteropathy, retained piglets and splenic torsion. Finally a case of gastric dilatation presented a highly positive FAT on a smear collected from the spleen, whilst two liver smears proved negative.

8.4. Discussion

The findings of this study suggest that *C. novyi* spores, but not vegetative bacteria, can be present in the liver tissue of healthy sows. The evidence was provided by the presence of *C. novyi* in the liver of almost 50% of healthy sows examined. This fact has been well documented in sheep, cattle, crows and rabbits (Jamieson, 1949; Niilo *et al*, 1969; Bagadi and Sewell, 1973a). Berkoglu (1989) observed that cattle were at risk of bacillary haemoglobinuria and infectious necrotic hepatitis because 60% of the cattle livers examined were positive in smears to the *C. novyi* FAT. Previous studies examining the

liver of slaughter pigs failed to detect *C. novyi* from this tissue (Narayan, 1968; Thomson *et al*, 1968). The current study only comprised a small number of samples collected from sows raised outdoors. Furthermore, isolation was only successful on one occasion. The isolate was classified as *Clostridium novyi* type A due to the production of both diffuse opalescence (lecithinase) and the pearly layer (lipase) on egg yolk agar.

The positive fluorescence detected after 48 hours incubation may in fact prove the presence of *Clostridium botulinum*. *C. botulinum* type C was found to share somatic antigens with *C. novyi* (Poxton, 1984), gave positive fluorescence with the anti-*C. novyi* antibody (see chapter 4) and has been found in healthy swine liver tissue (Yamakawa *et al*, 1992).

The temperature during the experiment examining liver smears at different time periods was below 14°C and perhaps delayed the germination of spores in the liver. Niilo *et al*, (1969) investigating the presence of *C. novyi* in the liver of cattle could not decide whether true liver germination occurred or if the liver was seeded from the intestine during the agonal stages or rapidly after death. This study and others by Corbould (1966) and Bagadi and Sewell (1973a) found that *C. novyi* spores were detectable in livers removed from the carcass immediately after death. Courbould (1966) commented on the difficulties in isolating the organism from liver samples and the fact that isolates were often weakly toxigenic. In this study isolation from comfirmed positive liver tissue was mostly unsuccessful. The reasons could be that freezing of the samples prior to isolation destroyed the dormant spores, perhaps few spores were present in the tissues or that the spore was not the significant viable particle, as Collee *et al*, (1971) suggested.

Further studies examining more samples from the abattoir, including both indoor and outdoor herds are required.

The danger of relying exclusively on liver smears and the FA test for diagnosis of infectious necrotic hepatitis in sheep was demonstrated by Batty *et al* (1967) and Bagadi and Sewell (1974b), particularly if 24 hours had elapsed since death. The current work supports this finding because *C. novyi* was detected in cull sows left for 48 hours before performing a necropsy and in sow deaths were necropsy had been delayed. Bagadi and Sewell (1974b) suggested that large numbers of vegetative *C. novyi* in the spleen would indicate *post mortem* contamination.

Examination of liver smears prepared from sows where another diagnosis had been reached revealed that 23% of cases were FAT positive. Similar complications to diagnosis were described by previous papers (Courbould, 1966; Niilo *et al,* 1969; Roberts *et al,* 1970b). The "false positives" presented plausible explanations and highlighted the importance of collecting the liver samples as soon as possible after death, excluding other causes of mortality and determining the presence of numerous *C. novyi* per field in the liver smears.

9. Sudden death in sows caused by infection with *Clostridium novyi*.

"When people are least sure, they are often most dogmatic"
J.K. Galbraith, 1955.

9.1. Introduction

The anaerobic Gram positive bacillus *Clostridium novyi* (*oedematiens*) was first described by Novyi (1894, cited by Willis, 1989) after an experimental inoculation of guinea pigs with casein precipitated a lethal myonecrosis. The bacterium has been isolated widely from soil (Nishida and Nakagarawa, 1964), was found in the liver of healthy ruminants, other animals and birds (Jamieson, 1949; Bagadi and Sewell, 1973a) and forming part of the normal gut flora of animals (Smith and Williams, 1984c). *C. novyi* causes myonecrosis in man and animals (MacLennan, 1962), infectious necrotic hepatitis in ruminants, horses and pigs (Sewell, 1975; Gay *et al*, 1980; Wise and Munday, 1964) and bacillary haemoglobinuria in cattle (Stogdale and Booth, 1984).

C. novyi as a cause of sudden death in pigs has been recorded infrequently in the literature. Batty *et al*, (1964) first diagnosed various individual cases in sows from different areas in Britain.

The diagnosis was confirmed by the newly developed fluorescent antibody test (FAT). Other workers from Britain (Bourne and Kerry, 1965) and Australia (Corbould and Munday, 1966) followed with further reports of cases in breeding sows and in fattening pigs. These reports emphasised the similarity between the clinical presentation of *C. novyi* sudden death cases and some forms of anthrax.

More recently, two cases from Japan have been published (Itoh *et al*, 1987; Kita *et al*, 1987). Succesful bacterial isolation from the liver samples

confirmed the positive fluorescent antibody results. Itoh *et al* (1987) isolated 10^4-10^8 *C. novyi* type A organisms from all the organs sampled. Kita *et al* (1987) on the other hand identified their isolate as a type B or D strain, according to biochemical results and GLC analysis.

Walton and Duran (1992) reported the occurrence of *C. novyi* sudden death in intensive pig breeding herds. The diagnosis was based on the elimination of other causes of death, rapid distension and autolysis of the carcass and the characteristic emphysematous liver. Liver smears were tested with the fluorescent antibody to confirm the diagnosis.

Baker (Personal communication, 1991) coined the term clostridial hepatopathy to define the *C. novyi* sudden death cases, a term that has been adopted in the text.

The following work details the epidemiological, pathological and bacteriological findings from 17 cases of *C. novyi* sudden death in breeding sows submitted for necropsy during the period of study (1992-94).

9.2. Materials and methods

The cases described were submitted for *post mortem* examination as part of the study described in chapter 7 and the methodology employed to perform the necropsy (see appendix V) and subsequent culture, isolation and identification techniques (see chapter 3) have been described elsewhere. The sows were submitted from 3 pig breeding herds. Farms A, B and C had an inventory of 1100, 650 and 80 sows respectively. More details on the units can be found in Chaper 7, section 7.2. The body condition of the sows examined was scored from thin to fat according to a subjective scale of 1 to 6 as described by Straw and Meuten (1992).

Three further cases were submitted by veterinary practitioners and processed as described in chapter 3.

9.3. Results

9.3.1. Epidemiological data

Seventeen cases of clostridial hepatopathy were diagnosed in the course of the study of mortality in the three herds. These deaths represented almost 17% of all submissions. *C. novyi* sudden death cases were diagnosed 10, 1 and 6 times during 1992, 1993 and 1994 respectively. A seasonal distribution of the diagnosed cases during the 3 years revealed that 8 deaths (47%) due to *C. novyi* occurred during the spring months. The winter and autumn had similar diagnosis rates, 4 (23.5%) and 3 (17.6%), respectively. During the summer only 2 cases (11.8%) were diagnosed. The seasonal distribution of the *C. novyi* sudden deaths and other causes of death is summarised in table 9.1 and illustrated in figure 9.1.

The mean number of litters for clostridial hepatopathy cases 5.6 ± 0.5 (SEM), was higher than 4.3 ± 0.3 (SEM), the mean parity for other causes of death submitted in this survey. During the study, *C. novyi* sudden death was not diagnosed in maiden gilts or first pregnancy sows, and 88% of sows classified as *C. novyi* deaths were parity 4 or higher. Data compiled from the mortality survey in chapter 7 was examined alongside these findings as illustrated in figure 9.2.

The majority of the clostridial deaths, 52.9%, occurred in the gestation stalls in the dry sow houses. Three sows from this group (17.6%) were found to be non pregnant when examined. Both these figures were higher than for all other deaths, where 41.2% of the submissions died in the gestation stalls and 12.5% were found to be non pregnant.

Deaths during lactation appeared with similar frequency for clostridial hepatopathy and other causes of death (see table 9.2).

Nine clostridial hepatopathy cases were diagnosed from farm A, five from farm B and three from farm C.

Table 9.1. Seasonal distribution of *C. novyi* sudden death compared with all other causes of death (1991-1994).

Month	Number	Season	Total *C. novyi*	% *C. novyi*	Total all causes	% all causes
Dec	1					
Jan	1	Winter	4	23.5	19	22.3
Feb	2					
Mar	3					
Apr	4	Spring	8	47	23	27
May	1					
Jun	1					
Jul	0	Summer	2	11.8	19	22.3
Aug	1					
Sep	1					
Oct	2	Autumn	3	17.6	24	28.2
Nov	0					

Figure 9.1. Distribution of *Clostridium novyi* and other deaths by month (1992-1994).

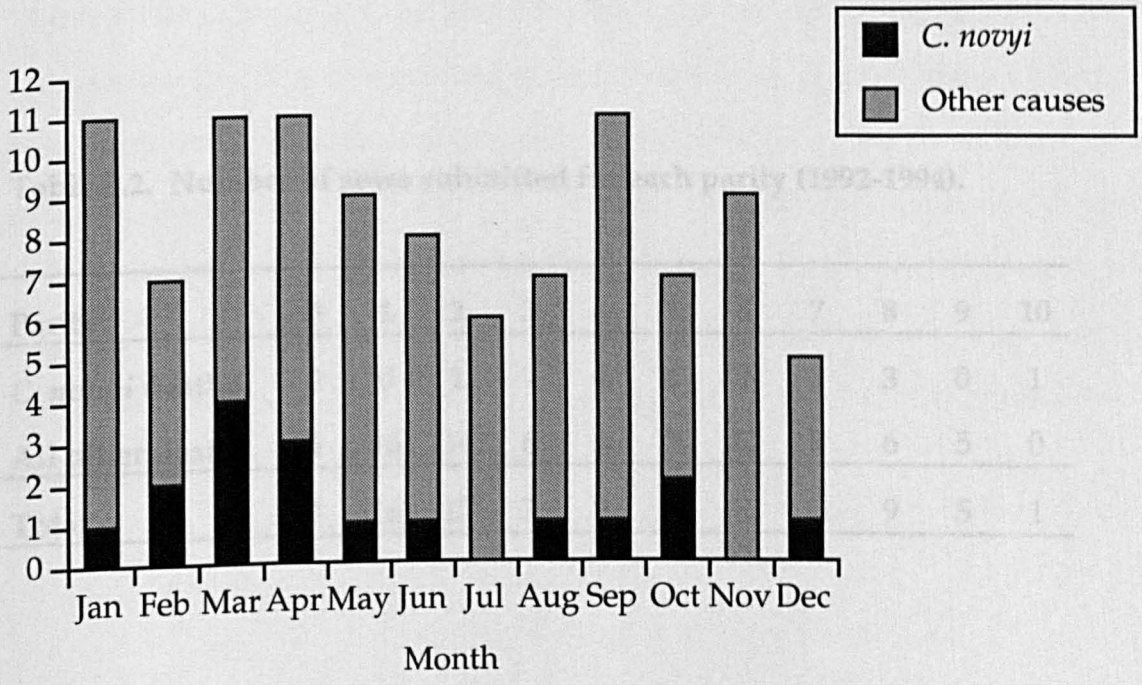


Figure 9.2. Parity distribution of *Clostridium novyi* cases compared with the distribution for other causes of death.

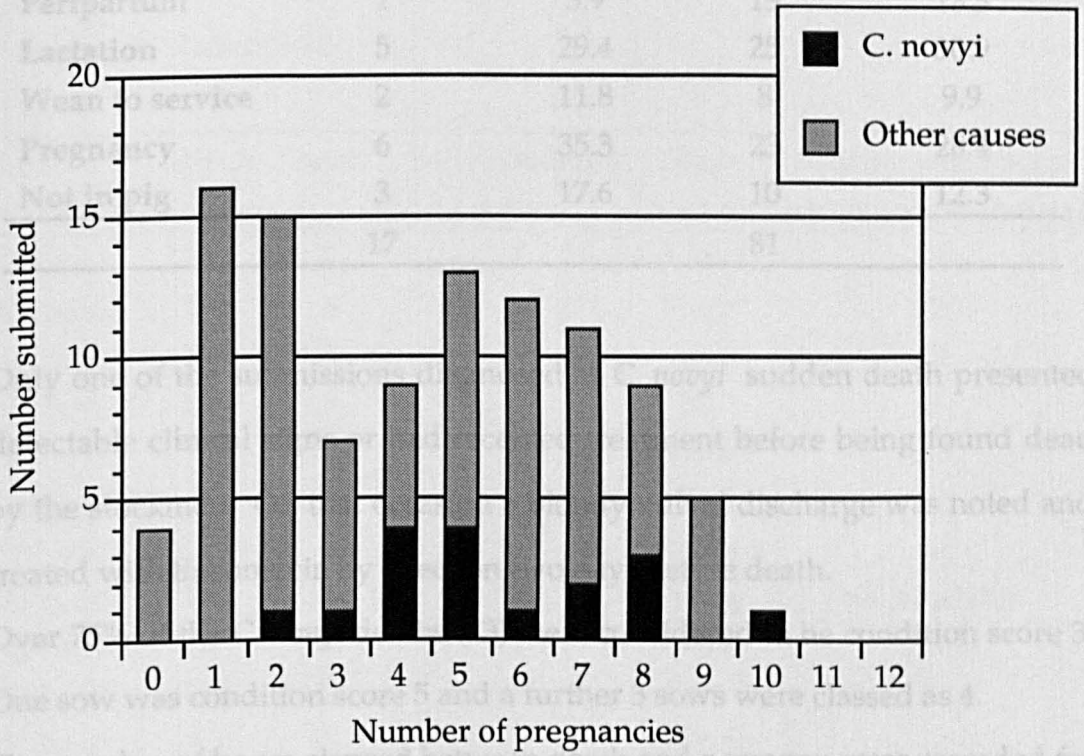


Table 9.2. Number of sows submitted for each parity (1992-1994).

Parity	0	1	2	3	4	5	6	7	8	9	10
C. novyi deaths	0	0	1	1	4	4	1	2	3	0	1
All other deaths	4	16	14	6	5	9	11	9	6	5	0
Total	4	16	15	7	9	13	12	11	9	5	1

Table 9.3. Distribution of *Clostridium novyi* sudden deaths and sows dead of other causes within the different stages of production (excluding data for 4 maiden gilts) between 1992 and 1994.

Stage of cycle	*C. novyi* cases	*C. novyi* %	All deaths	All deaths %
Peripartum	1	5.9	15	18.5
Lactation	5	29.4	25	30.9
Wean to service	2	11.8	8	9.9
Pregnancy	6	35.3	23	28.4
Not in pig	3	17.6	10	12.3
	17		81	

Only one of the submissions diagnosed as *C. novyi* sudden death presented detectable clinical signs or had received treatment before being found dead by the stockmen. On that occasion a bloody vulval discharge was noted and treated with lincomycin by injection two days before death.

Over 75% of the *C. novyi* deaths (13) were considered to be condition score 3. One sow was condition score 5 and a further 3 sows were classed as 4.

The number of hours elapsed between death and necropsy were recorded for all submissions. These figures depended on detection and reporting by farm staff, and when deaths occurred overnight could only be considered an estimate, particularly from Farm A. The time of death was not available for two cases. The mean number of hours between death and *post mortem* examination was 10.7 ± 1.13 (SEM) hours for 15 clostridial hepatopathy cases. Other sows submitted were examined a mean of 9.9 ± 0.9 (SEM) hours after death. Table 9.4. summarises these and other data.

Table 9.4. Historical data compiled from 17 cases of *C. novyi* sudden death between 1992 and 1994.

Case number	Month	Parity	Body condition score	Stage in cycle	Time between death and necropsy (hours)
92/nov1	Jan	4	3	Lact [a]	12
92/nov2	Feb	5	5	Preg [b]	12
92/nov3	Feb	8	3	NIP [c]	12
92/nov4	Mar	3	4	NIP	8
92/nov5	Apr	5	3	Wean [d]	12
92/nov6	Apr	8	3	Lact	8
92/nov7	May	4	3	Preg	6
92/nov8	Oct	6	3	Preg	not available
92/nov9	Oct	4	3	Wean	8
92/nov10	Dec	10	3	Part [e]	24
93/nov3	Aug	8	3	Lact	12
94/nov1	Mar	2	4	Preg	not available
94/nov2	Mar	7	3	Preg	12
94/nov3	Apr	4	3	NIP	12
94/nov4	Apr	5	4	Lact	8
94/nov5	Jun	7	3	Lact	8
94/nov6	Sep	5	3	NIP	6

a - lactatation, from 3 days after parturition until weaning; **b** - Pregnancy;

c - Not In Pig, not pregnant; **d** - Period between weaning and service;

e - Peripartum period, three days before and after farrowing.

9.3.2. Necropsy findings in the *Clostridium novyi* sudden death sow

General body condition and external lesions- Degeration of the carcass was very advanced in all cases regardless of season, ambient temperature or time between death and necropsy. The abdomen was timpanitic and widespread subcutaneous emphysema plus discoloration of the skin in ventral areas was seen in all seventeen *C. novyi* sudden death cases (figure 9.3.). Particular alteration of the normal body shape was apparent in the submandibular, neck, axillae and inguinal areas, which were swollen and crepitant (figure 9.4.). Greenish discoloration was seen on 4 occasions. External injuries and fighting wounds were not detected. On three occasions damage to skin had been caused when trying to remove dead sows from the stalls. The mammary glands presented a purple colour and oedema seven times, six in sows dead during lactation. A bloody serous exudate, occasionally blood stained froth from the nostrils, was seen escaping from body orifices in 15 out 17 carcasses examined. In six cases the rectum was prolapsed and congested. The vulva presented a purplish discoloration in three carcasses from this series. The mucosal membranes were unremarkable, except one sow with pale membranes (an oesophago-gastric ulcer was found) and one cyanotic case. The ocular globe was prominent in two cases.

Subcutaneous tissues- After incising the skin, pale pink or clear oedema fluid oozed out from many areas, in particular the submandibular and inguinal regions. The tissues surrounding the jugular vein were often congested. These changes also involved the submandibular and superficial inguinal lymph nodes, oedematous in all 17 cases. Subcutaneous ecchymotic haemorrhages were found in two carcasses.

Figure 9.3. *Clostridium novyi* sudden death. Note distension of the carcass, purplish ventral discoloration and bloody nasal discharge.

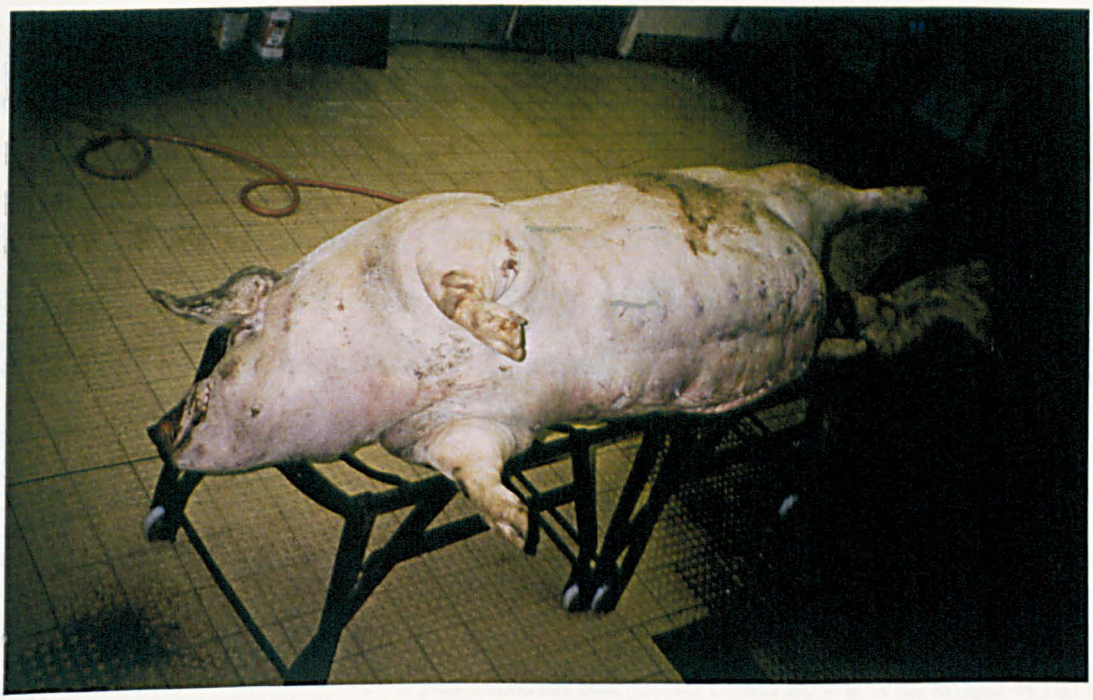
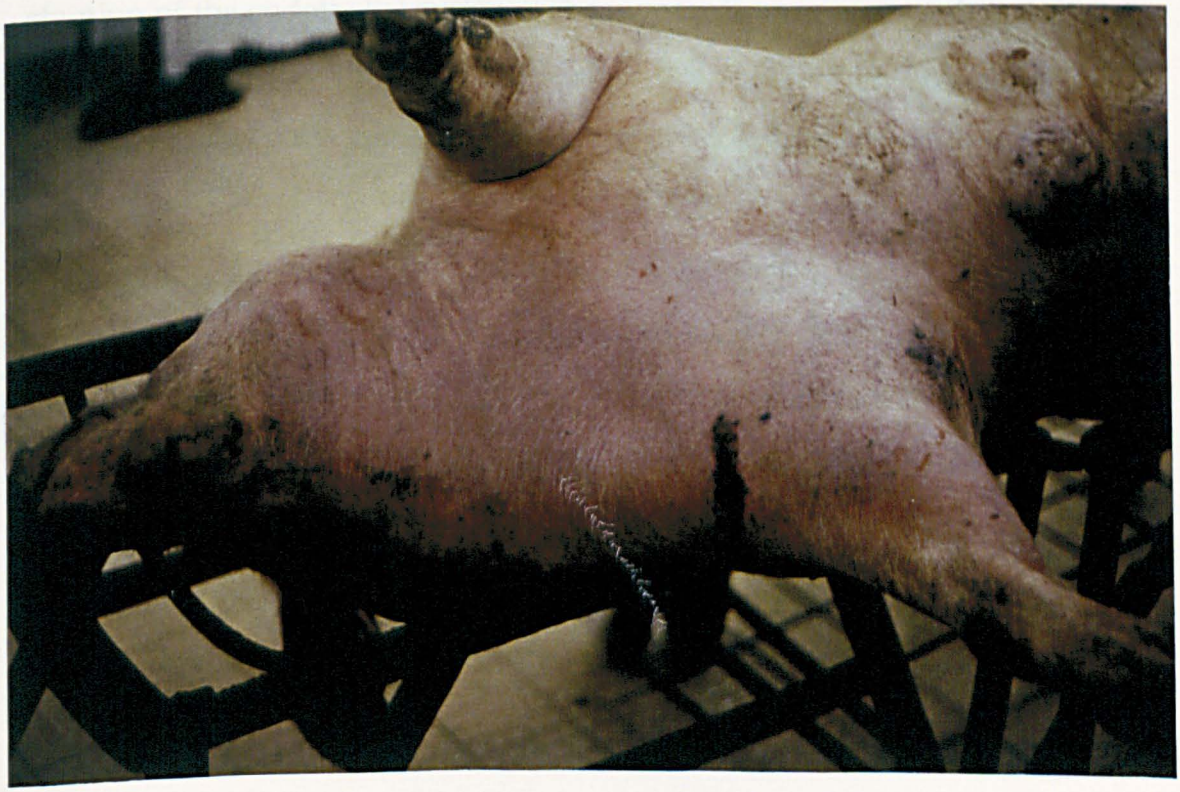


Figure 9.4. Close-up of the axilla and neck area of a clostridial hepatopathy case. Gross distension and discoloration of subcutaneous tissues.



Respiratory system- The larynx and trachea were often surrounded by congestion, haemorrhages and oedema. The trachea contained pinkish or white froth in 7 cases, and the mucosa was stained a red colour in 5 necropsies. These pathological changes extended down to the bronchial tree. The lungs were always congested, oedematous on 7 occasions, and severely autolysed in 8 cases. Small areas of pneumonic consolidation on the cranial lobes were seen once. Small fibrin tags covering the surface of the lungs were detected in a separate death. The bronchial lymph nodes were frequently congested and oedematous. The pleural cavity was filled with excessive serosanguinous fluid in all 17 *C. novyi* deaths. The quantity of fluid was variable, it was foul smelling, presented a shiny fatty appearance and did not contain blood clots. The pleural wall was often stained red.

Cardiovascular system- The pericardial sac in 15 dead sows was filled with a sanguinous effusion. The heart appeared flaccid, discoloured and infiltrated by gas bubbles. The cardiac chambers were often empty. Valvular abnormalities were not detected, but the major vessels presented a pink staining of the endothelial surface. The myocardium was severely autolysed in 11 cases and infiltrated with visible gas bubbles on two occasions. Haemorrhages on the surface of the heart were not detected.

Alimentary system- Abnormalities of the mouth, tongue, tonsils and oesophagus were not found in the 17 *C. novyi* sudden death cases examined. The stomach presented a red staining on the visceral surface in 15 cases. The stomach was very full of undigested food on 8 occasions, full on 6 and gas filled in two cases. One sow had suffered a gastric rupture and another a 180^{o} torsion of the stomach, but lack of haemorrhage or vascular changes suggested these events were *post mortem* or occurred shortly before death.

Gastric ulcers located in the *pars oesophagea* were seen in 5 sows, plus one with yellowish keratinisation. The ulcer lesions had not caused haemorrhages into the gastric lumen and appeared to be healing.

The small and large intestines were distended by gas in 13 of the seventeen carcasses examined. Overall the guts were remarkably fresh in appearance in comparison with the other viscera. Six sows presented patchy congestion or petechiation on the serosal surface. The mucosa was autolysed in most cases, but no inflammatory changes were detected and the intestinal contents frequently had a greyish, or green, watery appearance. The mesenteric lymph nodes were oedematous on one occasion.

The peritoneal cavity was always filled with a foul smelling, serosanguinous effusion. On one occasion, after the stomach had ruptured, a small amount of gastric content was also present. Fibrin tags covering the abdominal viscera were seen once.

Liver- All cases presented an enlarged, friable, light, emphysematous liver. The colour of this organ was either dark brown/black or green/black (figure 9.5.). Twice the liver was a pale brown colour. The diaphragmatic surface of all the lobes was covered in small bubbles. An incision into the liver parenchyma revealed a honeycomb appearance with a generalised gas bubble infiltration, the so called "aero chocolate" liver (figure 9.6.). A pinkish exudate was visible escaping the cut surface and blood vessels.

The gall bladder and bile duct were unremarkable. Localised areas of necrosis, haemorrhage, infarction, parasite migration or other damage were not found.

Histopathology from one liver sample (94/nov6) presented an autolysed organ, with centrilobular congestion, heavily infiltrated with rod shaped bacteria, but no inflammatory cell infiltration.

Figure 9.5. Liver from a sow that had died 2 hours earlier. The case was confirmed as a *Clostridium novyi* sudden death. The liver was congested emphysematous and enlarged.

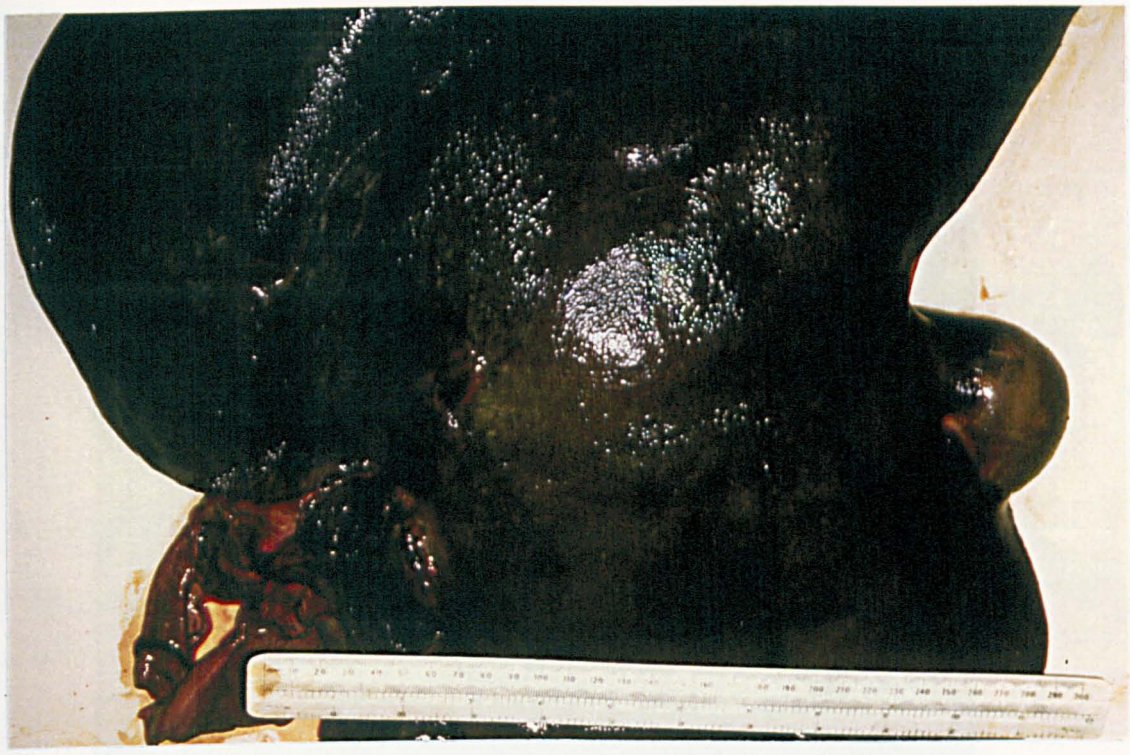
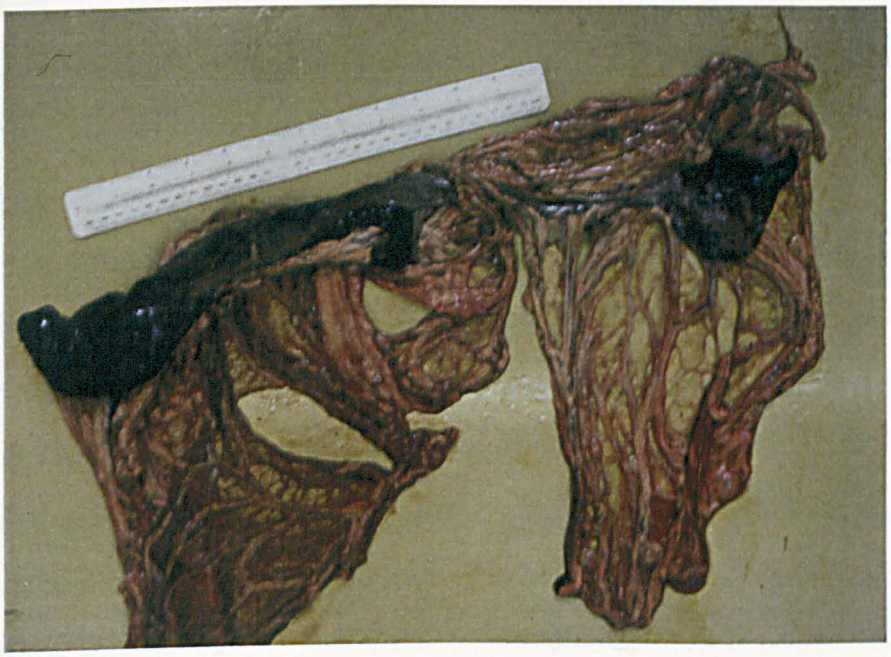


Figure 9.6. Cut surface of the liver parenchyma of a clostridial hepatopathy case. Aero chocolate liver, with emphysema and destruction of the normal architecture of the liver.

Figure 9.7. Spleen removed from a clostridial hepatopathy case. The severe atrophy of 2/3 of this organ could not be explained.



Spleen- In 14 cases the spleen was enlarged, soft, dark, and blackish in colour, suggesting acute congestion. Emphysema was detected in 5 cases. Two sows presented a 180º torsion, were enlarged without major dilatation of the splenic vein, suggesting the torsion occurred shortly before death, perhaps during the agonal struggle. Three cases presented a remarkable atrophy of the spleen, which was reduced in size, with a shrivelled capsule and a leathery consistency (figure 9.7.). Two of these sows were mature (5 and 7 litters). Another spleen was necrotic, after the ventral half had twisted on its own axis and had been strangulated by the omentum.

Urinary system- On three occasions changes consistent with chronic cystitis, ureteritis and pyelonephritis were detected. The values of the aqueous humor urea concentration taken from the sows did not suggest these changes alone had been enough to cause death.

Cystitis alone was seen in a further two cases, one with a greenish, thick, firm purulent ball occupying the bladder. The kidney was deformed and friable, the cortex infiltrated by gas, in 8 *C. novyi* deaths. Haemorrhages and congestion on the kidney surface were seen twice.

Genital tract- Endometritis was present in the uterus of 5 sows diagnosed as dead due to *C. novyi* proliferation. Another sow had vaginitis. Two sows presented an emphysematous, oedematous uterus. On one occasion the gravid womb contained piglets that were severely autolysed.

9.3.3. Samples collected from sudden death cases by practising veterinary surgeons.

During 1993 three samples and appropriate history were submitted by practising veterinary surgeons. All cases were from units in the north east of England. A summary of the case histories can be seen in table 9.5.

The necropsy findings in cases 93/nov2 and 93/nov3, were typical of those described above: Serosanguinous fluid in all body cavities, foetid gas and oedema in subcutaneous tissues, and an emphysematous, dark brown liver. The spleen was also emphysematous in one case. These findings were present in carcasses necropsied shortly after death.

The third sample submitted came from a herd where sudden death due to clostridia had been a problem for some time.

Deaths in this herd occurred during late lactation, with rapid decomposition of the carcass and severe oedema and gas in subcutaneous tissues. Changes were particularly pronounced in the mammary gland. This particular case did not present a typical "aero chocolate" liver.

Samples were submitted in pre-reduced fastidious anaerobe meat broth. Initial confirmation of the diagnosis using the C. *novyi* specific immunofluorescent antibody gave strong positive results in all cases.

Table 9.5. Details concerning 3 samples submitted by Veterinary practitioners.

	93/nov1	93/nov2	93/nov4
Farm data	Indoor (yard+stalls)	Outdoor	Indoor (stalls)
Herd size	135	200	500
Sow mortality	5-10%	5-10%	5-10%
Clostridial vaccination	No.	Yes.	Yes.
Sow history			
Parity	3	1	3
Condition score	3.5	4.5	4
Stage of cycle	Pregnancy	Pregnancy	Weaning
Sudden death	Yes.	Yes.	Yes.
Time between death and necropsy(hours)	6	4	3

9.3.4. Bacteriological findings

Tissue smears, particularly taken from the liver, were highly positive (+++) to the *C. novyi* specific fluorescein labelled antibody in all cases. Anthrax smears prepared from the peritoneal fluid were negative in all 17 cases.

During 1992, before the anaerobic incubator was available and pre-reduced fastidious anaerobe agar was employed routinely, isolation of *C. novyi* was not achieved. *C. perfringens* was isolated in eight out of ten cases during 1992. Subsequently, both *C. novyi* type A (4 occasions) and *C. novyi* type B (5 occasions) were isolated in pure culture after repeated purity plating. The type B strains often grew poorly on solid media after 24 hours, but appeared as finely spreading haemolytic colonies after 48 hours. *C. novyi* type A strains grew a little faster, but colonies were often small. Mixed cultures including *C. perfringens*, *Clostridium septicum* (twice) and *Bacillus* species were common. Egg yolk fastidious anaerobe agar proved a very useful culture medium, as individual colonies tended be detectable, surrounded by lecithinase areas of opalescence. The pearly layer due to the epsilon toxin of type A strains was detected, once the cultures were pure. Testing of carbohydrate fermentation was complicated by poor growth on the anaerobe identification media, even when identification procedures were carried out inside the anaerobic cabinet. All strains were catalase and indole negative and sensitive to metronidazole (5μg). FA test carried out on films of the pure cultures was positive in all cases. Detection of the alpha toxin was not carried out. Case number 94/nov6 was interesting because both type B and A were isolated from the different organs sampled. The pericardial fluid yielded *C. novyi* type B whilst type A was isolated from the liver sample.

A summary of the findings can be seen in table 9.6.

Table 9.6. Bacteriology findings for 17 cases of *C. novyi* sudden death between 1992 and 1994.

Case number	Month	FAT results	Culture	Anthrax smear	Time between death and necropsy (hours)
92/nov1	Jan	L ++ S +	not successful	-ve	12
92/nov2	Feb	L +++	not successful	-ve	12
92/nov3	Feb	L ++	not successful	-ve	12
92/nov4	Mar	L ++	not successful	-ve	8
92/nov5	Apr	L +++	not successful	-ve	12
92/nov6	Apr	L +++	No	-ve	8
92/nov7	May	L +++	not successful	-ve	6
92/nov8	Oct	L +++	No	-ve	not available
92/nov9	Oct	L +++	not successful	-ve	8
92/nov10	Dec	L +++ Pc +++			
		Pt ++ pg ++ M -	not successful	-ve	24
93/nov1	Jun	L +++	*C.novyi* type B	-ve	6
93/nov2	Jul	L +++	not successful	-ve	4
93/nov3	Aug	L +++ S ++	*C.novyi* type A	-ve	12
93/nov4	Sep	L +++	*C.novyi* type B	-ve	3
94/nov1	Mar	L +++	*C.novyi* typeA	-ve	not available
94/nov2	Mar	L +++ S +++	*C.novyi* type B		
		Br+++ I ++ H ++		-ve	12
94/nov3	Apr	L +++	*C.novyi* type B	-ve	12
94/nov4	Apr	L +++	not successful	-ve	8
94/nov5	Jun	L +++	*C.novyi* type A	-ve	8
94/nov6	Sep	L +++ Pc +++ Pt +++	*C.novyi* type A *C.novyi* type B	-ve	6

L - liver; **S** - spleen; **Pt** - peritoneal fluid: **Pc** - pericardial fluid; **Pg** - piglet liver; **M** - muscle; **Br** - bonchial lymph node; **I** - superficial inguinal lymph node; **H** - heart blood.

+++ More than 10 organisms per field; **++** Between 5 and 10 organisms per field; **+** Less than 5 organisms per field.

9.4. Discussion

The epidemiology, gross pathology and bacteriology findings from a series of 17 sudden deaths attributed to *C. novyi* have been described. This diagnosis represented the second most common cause of death in the three farms investigated during the period of study. The diagnosis of clostridial hepatopathy still remains complex for various reasons.

The rapid decomposition characteristic of these cases meant that histopathological examination was seldom carried out, a fact that Walton and Duran (1992) remarked earlier. Some authors argued that the presentation of these deaths was only a reflection of *post mortem* changes due to invasion of intestinal bacteria (Sanford, personal communication, 1992). Decomposition of the liver was believed to occur rapidly in sows, particularly if the body condition was good (Christensen, Vraa-Andersen and Mousing, 1995).

On the other hand, *C. novyi* sudden death was accompanied by severe pathological lesions that suggested a fulminant toxaemia, similar to that seen in mortality due to gas gangrene or clostridial myositis in other species (Hulland, 1993). In particular, widespread congestion and toxaemic degeneration of parenchymatous organs, extensive gas formation and subcutaneous oedema were characteristic findings. Extensive oedema was reported to be due to the specific vascular effects of the alpha toxin of *C. novyi* (MacLennan, 1962).

The pathological lesions seen in the 17 cases were similar to those described previously (Batty *et al*, 1964; Bourne and Kerry, 1965; Itoh *et al*, 1987; Carr, 1989). None of these authors detected focal lesions of necrosis or haemorrhage on the liver. Carr (1989) remarked that the degeneration and emphysema was often more severe on the left side, but this could not be confirmed in these cases.

Pathology of the urinary tract was noted in 5 cases in this study, a finding reflected by previous reports (Bourne and Kerry, 1965; Carr, 1989). Other inflammatory processes were detected in a number of *C. novyi* deaths, namely endometritis (5) and vaginitis (1). The influence of inflammatory focci as predisposing factors in the occurrence of *C. novyi* sudden death was suggested by Taylor and Bergeland (1992).

Gastric changes were also frequent in this series, oesophagogastric ulceration was present in 6 cases and the stomach was distended by large amounts of food in 14 occasions. Marco (1995) did not believe the incidence of gastric ulceration was correlated to *C. novyi* sudden death. Blackburn *et al* (1974) found cases of sudden death due to gastric dilation and torsion were positive when liver smears were examined using the FAT. The possibility that vitamin E variability in the ration affected the incidence of clostridial hepatopathy was explored by Carr (1989) and Walton and Duran (1992).

Difficulties isolating the causal organism, the strict anaerobe *C. novyi* complicated the confirmation of cases. The fluorescent antibody test was used in this study to confirm the diagnosis initially. Problems with relying too heavily on this technique were noted by Batty *et al*, (1964), Niilo *et al*, (1969) and Bagadi and Sewell (1974b). The specific anti-*Clostridium novyi* antibody was proven to also detect *C. botulinum* type C strains (chapter 4.) and this species shared somatic antibodies with *C. novyi* (Poxton, 1984).

C. novyi type A and B were isolated from 8 cases. These findings correspond with the previous isolation of *C. novyi* in pigs. Itoh *et al* (1987) isolated 10^4 to 10^8 organisms from the liver and other organs of a single case. The isolate was classified as type A. Kita*et al* (1987) isolated *C. novyi* type B from the liver and spleen of a single case in an adult pig.

The latter workers did not test for the production of alpha toxin, therefore could not rule out the possibility of the isolate actually being *C. novyi* type D. For complete identification of the isolates, detection of the alpha toxin must be carried out. This depends on further work with the alpha toxin monoclonal antibody, already initiated. Gas liquid chromatography of the 9 isolates would also improve the certainty of correct identification.

Failure to isolate the organism from some cases could be due to manipulation of the samples or the fact that the tissues contained mainly vegetative forms which were killed by the selective culture procedures. Collee *et al* (1971) when carrying out subculture from broths suggested that the spores of *C. novyi* were not the most significant viable particle .
Isolation of pure cultures was problematic because other bacteria present in the tissues often outgrew *C. novyi.*

The epidemiological findings relating to a series of *C. novyi* sudden death cases have never previously been published. In this study older sows were more likely to be affected as 88% of sows had over 4 litters before death and the average parity in 17 cases was 5.6 litters. Other reports, including the retrospective study described in chapter 6, differed from this fact, and diagnosed deaths in young sows and even first parity gilts (Itoh *et al*, 1987; Carr, 1989) was detailed.
Previous experience suggested clostridial hepatopathy was confined mainly to lactating sows, a fact that was not confirmed by the elevated incidence of deaths observed during gestation in this study (R. Evans, personal communication, 1993 ; Marco, 1995).

The condition score in cases of *C. novyi* sudden death was 3 in 75% of the cases submitted. The mean time after death before examination was 10.7 hours which was only slightly longer than the mean for all other subnmissions for necropsy. Both these facts would suggest that the *C. novyi* diagnosis was not likely to be *post mortem* degeneration. Furthermore, the majority of cases occurred during the spring rather than during the warmer summer months. The monthly distribution of the clostridial hepatopathy cases was compared to that of other submissions not diagnosed as *C. novyi* sudden death. The distribution was non parametric, so a Spearman rank correlation coefficient (Shott, 1990) was calculated. There was no correlation between the two groups suggesting that the increased incidence during spring was significant.

The number of *C. novyi* deaths per farm was disproportionate for farm C, which had three cases diagnosed out of a total mortality of nine (33.3%), compared with 14.1% and 17.2% for farms A and B. No reason for this bias was apparent.

10. GENERAL DISCUSSION AND FUTURE STUDIES

Firstly, the epidemiological findings and importance of *Clostridium novyi* as a cause of sudden death in breeding herds will be considered. Secondly, a discussion of the pathological presentation of clostridial hepatopathy cases and difficulties in diagnosis will be discussed. In the third instance, the bacteriological findings will be debated, in particular the isolation, identification and difficulties with the culture of *C. novyi*. Also, the results of studying the genes controlling the production of the phospholipase C toxins in *C. novyi* will be analysed. Fourthly, the treatment and prevention of *C. novyi* sudden death in sow herds will be reviewed and improvements for the future suggested. Finally, a deliberation on the pathogenesis and predisposing factors involved in the presentation of clostridial hepatopathy will be carried out.

10.1. Epidemiological considerations in relation to sudden death in sows caused by *Clostridium novyi*

The mortality in housed, stalled sows was examined over a three year period. The importance of regular surveys of mortality was highlighted by Chagnon *et al* (1991). These studies provide information on the different causes of death, determine the most prevalent reasons for sudden death and gives an indication of the predisposing husbandry or management factors. The variations from herd to herd and between rearing systems can also be compared to determine the effect of these factors on sow mortality. The last survey of sow mortality in Britain was published in 1984 by Smith.

The average mortality rate in the farms studied increased between 1992 and 1994, mostly because more sows were being culled on the farm rather than sold as casualty animals, rising from 8.9% in 1992 to 12.2% at the end of the study. The death rate, a figure reached after excluding culled sows, remained stable at around 8%, even though the herds increased the total sow inventory. The average herd size was 610 sows.

A total of 102 sows were submitted for necropsy during the period of study between January 1992 and December 1994. The annual number of sows received for *post mortem* examination was 36, 31 and 35 for the years 1992, 1993 and 1994, respectively. Not all the sows that died during the period of study were submitted for examination by the farmers.

The epidemiological, pathological and bacteriological findings from a series of 17 sudden deaths attributed to *C. novyi* (clostridial hepatopathy) have been described. This diagnosis represented the second most common cause of death in the three farms investigated during the period of study. The most common reasons for death were: Torsion or accident of the abdominal organs (17.6%), clostridial hepatopathy (16.7%), cystitis and pyelonephritis (11.8%) and haemorrhagic oesophago-gastric ulceration (9.8%).

These results confirmed the findings of the retrospective study of mortality, reported in chapter 6., which indicated that clostridial hepatopathy was frequently diagnosed in the cases of sudden death submitted. The relative importance of the disease in these studies must be tempered, in terms of the U.K. national pig herd and the situation in other pig rearing countries, by the lack of submissions from veterinary practitioners and the paucity of reported cases in the literature. Most British pig specialists contacted during the study felt that *C. novyi* sudden death was a sporadic problem in individual herds and in the majority of pig farms the condition was rarely diagnosed.

During the period of study samples from only three cases were submitted by veterinarians for bacteriological confirmation. On the other hand, VIDA, the Veterinary Investigation Diagnosis Analysis report published by the Ministry of Agriculture, Fisheries and Food, made available figures relating to the incidence of *C. novyi* deaths since 1981 (Anon, 1988; Anon, 1994). The number of cases diagnosed by the Veterinary Investigation Centres increased during the 1980's with a maximum 59 cases diagnosed during 1990. During 1994, a total of 35 cases were submitted, the majority during spring and winter.

In other countries the prevalence of clostridial hepatopathy has not been widely reported. Marco (1995) presented details regarding a large pig breeding herd in northwestern Spain after elevated mortality was caused by *C. novyi* and gastric ulceration. Mortality caused by *C. novyi* accounted for over 46% of deaths in the herd and most of these deaths occurred during the lactation period. Madec (1984) found 11% of the sows necropsied presented a pronounced degeneration of the liver, but was unwilling to interpret this finding. A recently published survey of sow mortality carried out in Denmark did not recognise any clostridial hepatopathy cases from 598 sows submitted for examination (Christensen *et al*, 1995). North American surveys of mortality did not include *C. novyi* amongst the causes of sow deaths (Stein *et al*, 1990; Chagnon *et al*, 1991; D'Allaire *et al*, 1991; Sanford *et al*, 1994).

The epidemiological findings relating to a series of *C. novyi* sudden death cases have not previously been published. In this study older sows were most affected, as 88% of the clostridial hepatopathy submissions had over 4 litters before death. No cases below parity 2 were submitted during this study. The average parity in the 17 cases was 5.6 litters, higher than for other necropsy submissions, 4.3 ± 0.3 (SEM) and than the mean parity of all deaths in the three herds investigated (4.6 litters).

Other reports and the retrospective survey of mortality (chapter 6) found *C. novyi* deaths occurred in younger sows and gilts (Itoh *et al*, 1987; Carr, 1989). Previous reports (Marco, 1995) stated that clostridial hepatopathy was confined to lactating sows, a fact that was not confirmed in this study. The prospective study, in chapter 9 of this thesis, found deaths were more frequent during gestation, but this conflicted with the results of the retrospective analysis, where *C. novyi* deaths occurred mainly in the farrowing room.

The condition score of the majority of the 17 *C. novyi* sudden death cases was 3, and only one sow was considered overweight. The average time between death and the necropsy for the clostridial hepatopathy cases was 10.7 hours ± 1.13 (SEM), compared with 9.9 ± 0.9 (SEM) for other submissions. The data relating to the condition score and the mean time between death and necropsy would suggest that the *C. novyi* diagnosis was not likely to be *post mortem* degeneration as suggested by Sanford (personal communication, 1992). Furthermore, the majority of cases occurred during the spring rather than during the warmer summer months when degeneration of the carcass would be more rapid. Certainly, care must be exercised diagnosing *C. novyi* sudden death in sows that have been dead for more than 18 hours, during warm weather and when deaths occur in the farrowing room. Seventeen percent of all submissions in a large Danish survey of sow mortality carried out in the winter presented severe decomposition of the liver and kidneys (Christensen *et al*, 1995). These authors mostly examined the carcasses two to three days *post mortem*.

The percentage of submissions without a definite diagnosis in the present survey of sow deaths was 15.7%, similar to the figure of 14.6% described by Chagnon *et al* (1991). In that study many of the deaths classed as "not known" were either too decomposed or had not been submitted.

During a limited survey of sow mortality Smith (1984) reported that a diagnosis was not reached in only 7.87% of submissions. Christensen *et al* (1995) determined that when the cause of death was assessed entirely on the necropsy findings, the reason for death could not be estimated in 21.8% of the cases. Madec (1984), on the other hand, found that after necropsy a plausible cause of death could be decided upon in 90% of cases.

The limitations of the present study were the inability to carry out necropsies on all the deaths that occurred during the period of investigation, reliance on macroscopic examination for diagnosis of the cause of death and the small number of herds examined. The results regarding the *C. novyi* deaths were derived from 17 deaths, therefore caution should be exercised when viewing the conclusions.

10.2. Discussion regarding the pathological findings in cases of clostridial hepatopathy in sows

The rapid decomposition of the sow carcass characteristic of clostridial hepatopathy cases increased the likelihood of submissions not being examined or even submitted for necropsy. Walton and Duran (1992) remarked that this fact may have explained the dearth of reports of clostridial hepatopathy in the literature. The pathological lesions presented in this series of 17 cases of *C. novyi* sudden death were similar to those described previously (Batty *et al*, 1964; Bourne and Kerry, 1965; Itoh *et al*, 1987; Carr, 1989). All the previous descriptions, corroborated by this case series, presented a remarkable degeneration, emphysema and enlargement of the liver, regardless of the time elapsed between death and examination of the carcass. Foci of necrosis, haemorrhage or evidence of parasite or larval migration were never detected on the liver surface.

Wise and Munday (1964) reported a necrotic area of around 6 cm in diameter on the diaphragmatic surface of the liver in one sow. The necrotic parenchyma was surrounded by inflammatory cells with a small tapeworm cyst in the centre. This description was similar to reports of infectious necrotic hepatitis in sheep (Williams, 1962; Sewell, 1975), but differed from other pig cases reported in the literature.

Early descriptions of *C. novyi* sudden death in pigs, confirmed by the fluorescent antibody test, remarked on the similarity between the gross pathology seen in these cases and the findings in certain forms of anthrax (Bourne and Kerry, 1965; Corbould and Munday, 1966). All the clostridial hepatopathy cases in this series were examined by polychrome methylene blue staining to discount the possibility of anthrax. The sponge-like appearance of the liver parenchyma and bubbles on the surface of this organ were characteristic in all cases. Discoloration of the liver was not remarked upon by other authors, but in many cases examined in this work the liver presented a very dark brown almost black discoloration.

C. novyi sudden death was accompanied by severe systemic lesions elsewhere in the carcass. In particular, widespread congestion and toxaemic degeneration of parenchymatous organs, extensive gas formation and subcutaneous oedema were typical findings. The extensive oedema seen in these cases and others may be due to the prolonged effects on the vascular permeability exhibited by the alpha toxin of *C. novyi* (Elder and Miles, 1957; Cotran, 1967; Muller *et al*, 1992).

Carcasses were grossly bloated and became more decomposed as the necropsy progressed. The superficial lymph nodes in the inguinal, cervical and axilla regions were often enlarged, haemorrhagic and oedematous.

Another striking finding, reported previously (Batty *et al*, 1964; Bourne and Kerry, 1965; Itoh *et al*, 1987; Carr, 1989) was the presence of blood stained fluid in body cavities and escaping from the mouth or the snout. These findings were very characteristic and would suggest a toxaemia similar to that seen in gas gangrene or clostridial myositis in other animal species (Jubb *et al*, 1985; Blood *et al*, 1989).

Sanford (1987) reported the acute death of four sows with clostridial myositis, and isolated *C. novyi* from various organs. The pathological presentation and the case history were different to that of the clostridial hepatopathy cases described here. The sows examined presented a swollen, hard and purple ventrocaudal abdomen and rump. There was subcutaneous oedema, emphysema plus haemorrhage and the semitendinosus muscle had a "cooked" appearance. The abdominal lymph nodes were enlarged and dark red. Histology revealed a cellulitis and myositis characterised by oedema and coagulation necrosis. The muscle fibres were infiltrated with neutrophils, mononuclear cells and large, Gram positive rods. In the seventeen cases diagnosed in this study puncture wounds or gross muscle pathology were not detected. The connective tissues appeared to be more affected in the areas around the large muscles.

The clostridial hepatopathy cases examined frequently presented other gross lesions, which were considered secondary to the principal cause of death. Gastric changes were frequent in this series; oesophagogastric ulceration was present in 6 cases and the stomach was distended by large amounts of food and gas in 14 occasions.

Cystitis and pyelonephritis or cystitis alone were noted in 5 *C. novyi* deaths in this study, a finding reflected in the reports by Bourne and Kerry (1965) and Carr (1989). Other inflammatory processes, namely endometritis (five times) and vaginitis (once) were also detected in clostridial hepatopathy deaths.

The significance of these secondary inflammatory lesions will be discussed later (*vide infra*).

Histopathological examination was seldom carried out because of the rapid degeneration of the liver and other organs in *C. novyi* deaths. A liver sample from the present series was examined in one case. Histology revealed an autolysed liver, with centrilobular congestion and infiltration with numerous inflammatory cells and rod shaped bacteria.

Finally, the possibility of an incorrect diagnosis of *C. novyi* sudden death must be addressed. Particularly when other pathological processes were present, deciding on *C. novyi* as the primary cause of death depended on confirmation by a strongly positive fluorescent antibody test, a short period of time having elapsed between death and necropsy and the exclusion of other causes. Other causes of death that could present with some features of *C. novyi* sudden death are: cardiac failure, porcine stress syndrome and septicaemia. The difficulties experienced in the diagnosis of cardiac failure in sows were mentioned by Drolet *et al* (1992). The number of unexplained sudden death in sows was believed to be increasing (Taylor, personal communication, 1994).

The protracted nature of mortality studies plus the cost of a *post mortem* examination in sows, especially when carrying out detailed microbiology, toxicology and histology, means that only farms with a high mortality rate tend to be studied in detail by investigators. Other areas of sow mortality, in particular the high proportion of sows culled for lameness need to be addressed urgently. Lameness in farm animals has become a subject of considerable welfare and financial importance (Dewey, Friendship and Wilson, 1993; MAFF, 1992).

10.3. Examination of the bacteriological investigations

Difficulties isolating the strict anaerobe *C. novyi* in pure culture were experienced. The presence of spores and vegetative cells was confirmed using the specific fluorescein labelled anti-*Clostridium novyi* antibody. Why did isolation fail?

Four main reasons need to be addressed; firstly the presence of other sporeforming bacteria in the tisue samples collected. Numerous reports have examined the carriage rate of *Clostridium* species in healthy farm animals (Thomson *et al*, 1968; Niilo *et al*, 1969; Bagadi and Sewell, 1974b; Partsvaniya, 1978; Bauer, Carpenter and Reagan, 1981). In this study *C. perfringens* and *C. septicum* were found outgrowing cultures of *C. novyi* particularly after plate inoculation with broth cultures. Bauer *et al*, (1981) found that *C. perfringens* could be isolated from the liver parenchyma of sows in 21% of animals examined, whilst the surface of the organ and the peritoneum only yielded *C. perfringens* in 12% of cases. The age of the pigs examined was not detailed in the report.

Secondly, failure to isolate *C. novyi* from sows that died suddenly could be due to failure in the selective methods employed. The significant viable particle was assumed to be the spore, so destruction of clostridial and facultative anaerobic vegetative cells was thought to make isolation of *C. novyi* more likely as suggested by Koransky, Allen and Dowell, (1978) and Levett (1991a). Heat shock and ethanol were used as selective isolation techniques. These procedures were tested on culture collection isolates with apparent success (see chapter 4.). Perhaps the procedures used were inappropriate for the less culture adapted strains found in field samples. The areas that possibly reduced the likelihood of isolation were the emulsion and maceration of the tissue sample in distilled water that would cause lysis of the vegetative cells. Manipulation at this stage was carried out on the bench,

therefore the samples were exposed to oxygen. The spores, visible on tissue smears from samples of the affected liver, should have survived this treatment. The suggestion then may be that spores of *C. novyi* do not provide an adequate inoculum for isolation as suggested by Collee *et al* (1971). These authors believed the spore was not the most significant viable particle, as more prolific growth was achieved from an inoculum of vegetative *C. novyi* in ideal conditions and culture media. Alternatively, the germination conditions were perhaps not provided for in cooked meat fastidious anaerobe broth, the medium used routinely by this and other laboratories for reviving spore forming organisms (Brazier, personal communication, 1993).

Perhaps collecting a larger sample volume would have improved the chances of isolation, by increasing the number of particles inoculated. Large fragments of affected tissue were used for inoculation from sheep cases diagnosed as infectious necrotic hepatitis (Williams, 1962; Bagadi and Sewell, 1974b). Pre-incubation of the tissue samples for 48 to 56 hours to ensure adequate sporulation occurs should also improve the isolation rate.

Neomycin (70 µg/ml) has been advocated as a useful selective agent for isolation of clostridia from faecal or other very mixed samples (Willis, 1977; Levett, 1991a). Growth of culture collection isolates of *C. novyi* type B in solid and liquid media with neomycin sulphate (70 µg/ml) was proven to be inhibited at that concentration (see chapter 4.). Surely this fact foiled some early attempts at isolation of *C. novyi* from early field cases of clostridial hepatopathy.

Recently, a description of new selective media for isolation of clostridia from faecal specimens has been published (Fujisawa, Namba, Hirayama, Lee and Mitsuoka, 1995). One medium contains novobiocin and colistin (NCA medium), the other colistin and crystal violet both employing Eggerth-Grannon agar as the basal medium. More clostridial species were isolated on these plates compared to heat treatment and plating on unselective medium.

Both these media supported growth of *C. novyi*, but also of many of the other clostridia that can overgrow this organism.

The importance of using freshly prepared plates and pre-reducing before subculture was quickly realised, particularly with *C. novyi* type B.

Egg yolk fastidious anaerobe agar has proven invaluable in the detection of the lecithinase producing *C. novyi*.

An area that could provide fruitful results, which should be investigated in the future, involves the use of specific somatic antibodies raised in rabbits against the washed whole cell of *C. novyi*. This technique was employed by Lawrence *et al* (1984) to selectively isolate *C. perfringens* type C from man and pigs in Papua New Guinea in outbreaks of necrotic enteritis (Pig bel). Immunomagnetic beads (Dynabeads, Dynal) coated with mouse anti-rabbit antibodies could bind with rabbit *C. novyi* antibodies. The antigen, when present in a broth culture or tissue macerate, could then become attached to the beads. The complex formed by antigen (*C. novyi*), the antibody and the magnetic bead can be retained by using a magnet while the unbound material is washed away. Removing the magnetic field releases the beads, which can be used to inoculate a broth or solid medium. This method has already been successfully employed in the isolation of Gram negative enteric bacteria from faeces (Lund, Hellemann and Vartdal, 1988). Hyperimmune *C. novyi* rabbit antisera has been raised and titrated by dot blot and ELISA. Further work is required.

Identification of *C. novyi* strains was complicated by variation in the Gram stain presentation, colony appearance and different carbohydrate fermentation results even for the same strain. The growth medium and time of incubation of cultures can be responsible for great variation. The haemolysis detected on sheep blood agar plates also changed after repeated subculture of *C. novyi* types A and B, with loss of the wider area of

haemolysis. Repeated subculture was proven to cure *C. novyi* and *C. botulinum* of toxigenic phages (Eklund *et al*, 1989), and this could be occurring in some cases.

Difficulties with inconsistent growth on the anaerobe identification media (Phillips, 1976) were experienced and an alternative would be to perform the tests in broth medium (Brown, personal communication, 1995).

The specific anti-*Clostridium novyi* antibody used for the FAT was proven to also detect *C. botulinum* type C strains (chapter 4.), but was very useful to examine broth cultures, emulsified bacterial colonies and tissue smears.

C. novyi type A or B were isolated from 8 cases of sudden death, presenting a typical clostridial hepatopathy pathological appearance. Failure to isolate the organism from early cases was experienced primarily before specialist anaerobic bacteriology training had been received by the author. The lack of an appropiate anaerobic environment for cultivation of *C. novyi* also interfered with consistent isolation. Collee (1980) did not believe an anaerobic cabinet or more specialised pre-reduced anaerobically sterilized roll tube techniques were required for isolation of clinically important clostridia. Instead this author advocated good anaerobic bacteriology practice and adequate anaerobic jar management. The operation of jars employing internal gas generation (GasPak system) did not allow consistent cultivation of strains of *C. novyi* type B.

The first reported isolation from a case of "gas gangrene" in the pig was published by Kita *et al*, (1987). These authors found a large number of rod shaped organisms on Gram stained liver and spleen smears from a single case of sudden death in a sow. Propionic, butyric and acetic acids were detected from tissue samples using gas liquid chromatography. The bacterial isolates from the liver and spleen had a similar Volatile Fatty Acid pattern, which was consistent with *C. novyi*.

The isolates were lecithinase positive and lipase negative, but differentiation between type B or type D was not possible. Itoh *et al* (1987), on the other hand isolated *C. novyi* type A from cases of sudden death in gilts with characteristic spongy liver lesions. Sanford (1987) isolated *C. novyi* from lesions of gas gangrene, which were identified by FAT, but not typed.

Both *C. novyi* type A and B were isolated from cases in this investigation, but as with the previous reports the inability to detect alpha toxin precludes a definite differentiation between strains of type B and D. The temporary classification of lecithinase positive, lipase negative, indole negative isolates as *C. novyi* type B, would seem more likely in view of the clinical presentation, as suggested by Williams (1964).

For complete identification of the isolates, detection of the alpha toxin should be carried out. Further work with an alpha toxin monoclonal antibody used in an ELISA will allow rapid detection. Workers have suggested the peroxidase-antiperoxidase (PAP) stain could be used to detect *C. novyi* type D (*C. haemolyticum*) directly from tissue samples and as a means of differentiating these strains from beta toxin producing type B strains (Uzal, Belak, Rivera, Robles and Feinstein, 1992). Gas liquid chromatography of the 9 isolates would also improve the certainty of correct identification.

The toxin genes of strains of *C. novyi*, encoding two different phospholipases C, the beta toxin (type B and D) and the gamma toxin (type A) were examined in this study. The work involved the amplification of the phospholipase C gene from *C. novyi.*

The experiment was based on the assumption that the *C. novyi* genes have homologous domains with the phospholipase C (*plc*) gene of *C. perfringens.* Nov1 and Nov2 were highly degenerate primers, chosen from the first 250 residues of the deduced aminoacid sequence (N-terminal domain) where an area of homology would be expected.

The high degeneracy of the primers probably explained the need to use high primer concentrations in the polymerase chain reaction (PCR) mix.

The PCR successfully amplified a fragment of the *plc* gene when C. *perfringens* was used as a template, which suggested the methodology was carried out correctly. Successful amplification of an approximately 400 nucleotide fragment was achieved with C. *novyi* type B template. The amplified product was cloned and sequenced. The derived aminoacid sequence was analysed using GCG software (Winsconsin Package) and compared with other known gene sequence data from EMBL/GenBank. An area upstream of the *plc* gene of C. *perfringens* was found to have 63% homology with the Nov sequence over 107 nucleotides. Some homology with the sequence of the C. *botulinum* phage C mutant gene for neurotoxin type C1 was also found, although the match was not included in the top 100 sequences with homologous portions. This finding generated considerable excitement as the close relationship between strains of the species C. *novyi* and C. *botulinum* type C and D has been well documented (Eklund *et al*, 1974; Nakamura *et al*, 1983; Poxton, 1984 and Collins *et al*, 1994). Further work needs to be carried out analysing the data and confirming the reliability of the sequencing data.

Further work confirming the identity of the PCR product must be carried out by preparing a homologous hybridization probe from the cloned insert to detect the gene in C. *novyi* DNA (McPherson *et al*, 1992). Alternatively, specific primers could be prepared from the derived sequence and used to amplify portions of the gene or in a molecular method of toxin typing of C. *novyi* isolates. The latter technique has been applied to C. *perfringens* typing (Daube *et al*, 1994, Fach and Guillou, 1993).

To sequence the entire phospholipase C genes of *C. novyi* an approach based on detection of lecithinase activity of recombinant clones may yield good results. This method was used by workers sequencing the *plc* gene of *C. perfringens* (Leslie *et al*, 1989).

New primers, named Nov3 and Nov 4, were designed based on the N-terminal domain of the known *plc* gene sequence for future use. These primers contained fewer degeneracies and improved PCR results could be achieved.

10.4. Prevention of *Clostridium novyi* deaths in pig breeding herds

Sudden death due to *C. novyi* infection in adult pigs was controlled by vaccination with a multivalent clostridial vaccine in conjuction with other preventive measures to reduce the incidence of gastric dilatation (Blackburn *et al*, 1974). Vaccination was the recommended method of control for this disease in problem farms according to Taylor and Bergeland (1992) and in outdoor herds (Waddilove and Wilkinson, 1994).

Various improvements to clostridial vaccines have ensued over the years (Kerry and Craig, 1979; Sterne, 1981) and currently numerous commercial adjuvanted multivalent vaccines for the protection of sheep, cattle, goats and pigs can be used (Debuf, 1994). Walker (1992) reviewed the use of clostridial vaccines in livestock and concluded that highly efficacious, crude antigen preparations had proven highly successful. These vaccines have included a toxoided *C. novyi* supernatant, that has been very successful in the control of infectious necrotic hepatitis in sheep (Brown, Parizek and Stewart, 1992).

Harbola and Verma (1988) found *C. novyi* type B toxoids highly immunogenic and protective in sheep. Recently, Pietrzykowski *et al* (1991) developed an enzyme immunoassay for the detection of *C. novyi* type B alpha toxin that eliminated the need to test toxoids and vaccines *in vivo*.

Failure of multicomponent clostridial vaccines in the control of *C. novyi* sudden death has been reported by veterinary practitioners (Walton and Duran, 1992; Evans, personal communication, 1993; White, personal communication, 1993; Marco, 1995). Various reasons for the inability to control clostridial hepatopathy can be put forward. Firstly, a correct diagnosis must be determined and other causes of sudden death must be excluded. Secondly, the immunogenicity of the multicomponent clostridial vaccines regarding the alpha toxin has not been tested in pigs, data for licensing purposes was derived from sheep instead. The antibody response to the *C. novyi* toxoid may be negligible in sows under the current vaccination regimes. This fact must be assessed in the field to ensure high antibody titres with the protocols used at present. The production of a strong antibody response may not necessarily indicate a protective immunity. A model for experimental challenge is not available at present. Another potential reason for vaccine failure could be the fact that different strains of *C. novyi* have been isolated from sudden death cases. The alpha toxin has been considered the principal pathogenic factor in infectious necrotic hepatitis, therefore vaccines are prepared using culture supernatant of type B only, and the response is titrated against alpha toxin (Pietrzykowski *et al*, 1991). The effect of the other potent *C. novyi* type A toxins on sows have not been determined.

Some exciting developments in clostridial vaccines have recently been reported; Schranner *et al* (1992) proposed a *C. novyi* alpha toxin vaccine using only the immunogenic portion of the large toxin polypeptide, and Williamson and Titball (1993) reported a genetically engineered vaccine that protected mice against gas gangrene caused by the *C. perfringens* alpha toxin.

These advances may improve the protection available in herds presenting a high proportion of clostridial hepatopathy deaths at an affordable price.

The economic benefits of clostridial vaccination in pigs have not been assessed, but in cattle kept in feedlots Knott *et al*, (1985), described huge savings by reducing mortality alone and a gain of $ 10.30 per head of cattle. In pigs, vaccination has been recommended mainly to control necrotic enteritis due to *C. perfringens* type C in neonatal piglets (Taylor and Bergeland, 1992), and has been proven clinically to be very beneficial. The sporadic or rare occurrence of *C. novyi* sudden death would not warrant widespread vaccination of all herds and the cost of developing and licensing a new vaccine would not be financially viable.

These problems with vaccination have led clinicians to employ other control methods. Walton and Duran (1992), Evans (Personal communication, 1993) and Marco (1995) introduced in feed medication with growth promoting antibiotics to all sows at risk in herds with severe clostridial hepatopathy problems. Marco (1995) prescribed Zinc bacitracin (Albac, AL, Oslo) at 200 ppm to sows in the lactation ration with a dramatic reduction in mortality due to *C. novyi*. Evans (1993) on the other hand used salynomycin sodium (Salocin 120, Hoechst) for lactating sows with good results. Neither of these antimicrobials are yet licensed for inclusion in sow rations. Schultz *et al*, (1986) suggested the use of bacitracin methylene disalicylate in sow feed during late pregnancy and lactation for the prevention of *C. perfringens* type C enteritis in suckling pigs. The authors believed the carriage rate of this organism in the sows intestinal tract was reduced, but advocated the continued used of vaccination and hygiene for the control of this disease in piglets. A similar effect may have taken place when antibiotics were used to control *C. novyi*.

Concern over the development of antibiotic resistance to *C. perfringens*, after the use of therapeutic and growth promoting antimicrobials in pigs have been expressed (Rood *et al*, 1985), but much of the work investigating growth enhancing antibiotics in farm animals does not support these concerns (Dutta *et al*, 1983; Walton, 1990; Devriese *et al*, 1993). Most of the growth promoters with the exception of flavomycin were effective against clostridia, but *C. novyi* isolates were not specifically examined in these studies.

The *in vitro* susceptibility of *C. novyi* type A and B culture collection strains to growth enhancing antibiotics was determined in this study (see chapter 4). All the type A strains tested were highly sensitive to avoparcin, chlortetracycline, salinomycin, tylosin, virginiamycin and Zinc bacitracin, but a higher Minimum Inhibitory Concentration (MIC) was deterrmined for avilamycin.

C. novyi type B strains were similarly sensitive to avoparcin, chlortetracycline, salinomycin and tylosin. Zinc bacitracin had a low MIC with most strains except one (N2B). Avilamycin proved to be less effective against 4 *C. novyi* type B strains. These findings differed from some results in the only other report of antibiotic sensitivity testing of *C. novyi* (Singh *et al*, 1994), when only one isolate was examined and resistance to bacitracin at 30 μg/ml was reported.

These findings coupled with the problems related to vaccination indicate medication as a viable control measure for clostridial hepatopathy in pig breeding herds with frequent deaths caused by *C. novyi*.

Future work will be required; particularly the examination of field strains of *Clostridium novyi* to determine the efficacy of the growth promoters in the prevention of clostridial hepatopathy in problem herds.

10.5. Considerations regarding the pathogenesis of sudden death caused by *Clostridium novyi* in pigs

The studies carried out during the examination of mortality in three large pig breeding herds has not proven a clear predisposing factor or particularly propitious time in the breeding cycle for sudden death due to C. *novyi*. At least, theories can be put forward for future examination. The study had to centre on developing the neccesary tools to enable further investigation into the disease. Examination of the pathogenesis of infectious necrotic hepatitis and other conditions caused by clostridia in man and animals may provide some insight.

Turner (1939) established that latent spores of C. *novyi* were present in the body of healthy sheep. Jamieson (1949) published the first experimental confirmation of the need for latent C. *novyi* type B spores in the liver to be activated by migrating immature liver fluke. Bagadi and Sewell (1973b) induced the disease in sheep by previous infection with metacercariae of *Fasciola hepatica* followed by oral infection with spores two weeks later. Sheep that presented serological and parasitological evidence of severe liver damage by flukes appeared more likely to die from infectious necrotic hepatitis. Death occurred 6 to 8 weeks after infection with *F. hepatica*, when maximal parenchymal damage to the liver had been demonstrated (Bagadi, 1974). Other parasites that cause hepatic injury, like *Cysticercus tenuicollis* and *Echinococcus granulosus* predisposed to infectious necrotic hepatitis (Sewell, 1975). Damage by hepatotoxic chemicals (Jamieson, 1948) and liver biopsy (Duncan, 1984) also allowed latent spores to germinate in sheep.

Spores of C. *novyi* have been isolated from soil (Nishida and Nakagawara, 1964) and were found in the liver of sheep from "black disease" areas (Bagadi and Sewell, 1973b), so the assumption was that ruminants ingested spores that migrated to the liver where, unless the appropriate conditions for

germination occurred, the spores remained dormant. The route of dissemination of ingested spores was unknown. Experimental studies showed that the spores could be recovered from the mesenteric lymph nodes by 12 hours post ingestion and the liver by 24 hours after administration (Bagadi and Sewell, 1974a). At no stage was C. *novyi* isolated from heart blood, portal circulation or kidneys. On one occasion the organism was recovered from a peritoneal wash in a guinea pig given large quantities of spores orally. The authors suggested dissemination occurred via the lymphatic system.

The presence of dormant C. *novyi* spores in sows has been demonstrated (chapter 8.), confirming the findings of Parsvaniya (1978) and the situation in other species (Thomson *et al*, 1968; Niilo *et al*, 1969). Certainly practitioners report that C. *novyi* sudden death can be highly prevalent in outdoor pig breeding herds, where sows have access to soil borne spores (Wilkinson and Waddilove, 1994).

The pathogenesis in the cases of sudden death in pigs reported by Batty *et al*, (1964) was not discussed, but the authors remarked that cattle often died in herds not infested with liver fluke, fed on a high barley diet and suggested that the development of fatty liver may have presented ideal conditions for proliferation of C. *novyi*. Chauhan, Kulshreshtha and Kaushik (1985) reported a case of sudden death in a camel and both C. *perfringens* and C. *novyi* type A were isolated. The authors felt the death was related to the consumption of vast quantities of freshly harvested grain. Problems with fatty liver have not been described in pigs.

Carr (1989) suggested there may be a link between hepatosis dietetica and C. *novyi* proliferation in the liver. In the herd investigated a reduction in the sow mortality was achieved after correct levels of vitamin E and Selenium were included in the diet. Many C. *novyi* deaths in Carr's report also presented lesions of cystitis and pyelonephritis.

Damage to the liver due to parasite migration was not detected in this study. The possibility of hepatotoxic damage due to mycotoxins was not directly investigated in this study. Various toxigenic fungi, mainly belonging to the genera *Aspergillus* or *Penicillium*, have been associated with hepatotoxicity in domestic animals (Adam, 1974).

The physiological effects of aflatoxin B1 contaminated feed in growing pigs included liver damage characterised by enlargement, elevated liver enzymes in blood and impaired protein synthesis (Schell, Lindeman, Kornegay and Blodgett, 1993). Histologically, the liver presented necrosis of parenchymal cells, hyperplasia of the bile duct epithelium and circulatory effects (Adam, 1974). Gross lesions of aflatoxicosis included a pale, tan coloured liver with centrilobular haemorrhages, petechiation and subecchymotic subserosal haemorrhages, sometimes also involving the intestine (Osweiler, 1992). Chronically affected livers were described as hard, fibrosed with exaggerated lobular pattern. Icterus became visible in the serosal and mucosal surfaces. Aflatoxin toxicity was considered time and dose dependent and also influenced by age (Osweiler, 1992). Young swine were considered much more sensitive than finishing or adult pigs. Sows fed aflatoxin at 500 and 700 ppm had normal pregnancies and litters, but the chronic effects on the liver were not described (McKnight, Armstrong, Hagler and Jones, 1983).

Recently, fumonisin B1 produced by *Fusarium moniliforme* growing in corn, has been shown to cause sudden death in swine associated with an acute interstitial pulmonary oedema (Osweiler, Ross, Wilson, Nelson, Witte, Carson, Rice and Nelson, 1992; Done, 1995). The affected pigs that survived the initial pulmonary oedema developed liver lesions of hepatocellular necrosis, hepatomegalocytosis and increased mitotic figures (Osweiler *et al*, 1992; Hascheck, Motelin, Ness, Harlin, Hall, Vesonder, Peterson and Beasley, 1992). Even sows developed acute disease accompanied by abortions two days after the outbreak had begun. Gilts fed fumonisin B1 developed chronic

toxicity, mainly in the form of hyperplastic liver nodules or hepatopathy (Casteel, Turk, Cowart and Rottinghaus, 1993).

Initial hepatic damage from toxins could potentially allow the *C. novyi* spores lying dormant in the liver to germinate, but seems unlikely in these herds, as deleterious effects on growth or health of the pigs were not detected. Corn and poorly dried and stored cereals associated with mycotoxin production were closely monitored in these herds. Feed bins, hoppers and other feed containers were checked and cleaned out regularly, with no problems with mouldy feed detected during the years of the study.

The influence of inflammatory focci in the occurrence of *C. novyi* sudden death was suggested by Taylor and Bergeland (1992). Certainly in this study sudden death cases presented inflammatory focci for example cystitis alone, cystitis and pyelonephritis, endometritis and vaginitis. Perhaps the production of toxins from bacteria invading these tissues induced liver necrosis. This has been demonstrated experimentally by injecting piglets with *Pasteurella multocida* toxin (Cheville, Rimler and Thurston, 1988).

In an investigation of sudden death in sheep shortly after assisted lambing numerous deaths were caused by massive uterine invasion with *C. novyi* (Wallace, 1966). This condition presented similarities with puerperal sepsis caused by *C. perfringens* or *C. sordellii* after instrumented abortion in humans (Hogan and Ireland, 1989; Dylewsky, Wiesenfeld and Latour, 1989). Some cases examined in this study presented a very degenerate soft, haemorrhagic uterus, but isolation of the bacterium from these tissues could not be achieved. *C. perfringens* was isolated from one such uterus, perhaps with some significance.

Evans (Personal communication, 1993) believed that correction of supply and availability of water to the sows inexplicably reduced mortality due to C. *novyi*. Perhaps an incorrect diagnosis was made and acute cystitis and pyelonephritis was being controlled by improving the overall urinary tract health.

Many sows diagnosed as being cases of C. *novyi* in both the retrospective study and in the survey of mortality had oesophagogastric ulceration. Marco (1995) did not believe the incidence of gastric ulceration was correlated to C. *novyi* sudden death, but clostridial hepatopathy deaths were more likely to present ulcer lesions than other submissions in this study. Blackburn *et al* (1974) found cases of sudden death due to gastric dilation and torsion were positive to C. *novyi* when liver smears were examined using the FAT. Eight C. *novyi* deaths described in this thesis presented with gastric dilatation at the time of death and gastric dilatation was a frequent cause of death in these herds. The splenic torsion seen in three cases of C. *novyi* death were thought to be due to the agonal struggle, although in sheep and humans dying from infectious necrotic hepatitis or malignant oedema the demeanour was often described as apathetic and resigned, without excessive struggle (Williams, 1962; MacLennan, 1962).

The pathological presentation, pathogenesis, predisposing factors and epidemiology of C. *novyi* sudden death have not previously being studied in depth. This study has provided evidence of numerous C. *novyi* sudden death cases in three indoor pig breeding herds. A detailed description of the pathological and microbiological findings has excluded other causes of death and has indicated areas of further investigation. Dormant C. *novyi* spores were demonstrated in the liver of healthy sows and therefore care must be exerted when employing the fluorescent antibody test for diagnosis if the time of death cannot be ascertained or in warm weather conditions. Excluding the other causes of unexpected death must be considered carefully.

Both rapid decomposition of the carcass, which may have discouraged necropsies being carried out, plus the difficulties in the culture of *C. novyi*, explain the paucity of reports and studies of this condition. On the other hand, many veterinarians only reported rare or sporadic cases and detailed sow mortality surveys from other countries failed to describe any cases (Chagnon *et al*, 1991, Christensen *et al*, 1995). Other causes of death, for example, urinary tract disease, gastric ulceration or abdominal accidents require more attention. Further studies examining more pig breeding herds may determine areas of concern on a more general scale.

Before further work on the pathogenesis of clostridial hepatopathy continues, better diagnostic techniques are required. The reliable and rapid detection of the lethal, haemolytic and oedema inducing alpha toxin is particularly important. An ELISA using monoclonal antibodies raised against the alpha toxin as developed by Pietrzykowski *et al* (1991) would allow the diagnosis of field cases of *C. novyi* sudden death, identify toxigenic *C. novyi* strains and detect antibody response in vaccinated sows.

Culture and identification of *C. novyi* using traditional methods has been complemented by identification of putative toxin genes using PCR and sequencing. Future studies concerning the control and location of the phospholipase genes and other toxin genes of *C. novyi* will be of value in the detection of toxigenic strains and the development of toxin specific genetically engineered vaccines.

"And all my endeavours are unlucky explorers
come back, abandoning the expedition;
the specimens, the lilies of ambition
still spring in their climate, still unpicked;
to find them, as the great collectors before me."

Keith Douglas (1944)

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TERMS AND ABREVIATIONS

Aero-chocolate liver Descriptive term for the liver seen in *Clostridium novyi* death cases; dark brown, spongy with a honey-combe surface.
Broiler chicken- A chicken reared for meat.
Clostridial hepatopathy- Term used to describe the pathological presentation of sow deaths due to *Clostridium novyi.*
Cull- Refers to euthanasia in farm animals (ie- after lameness or other injury).
Culling rate- The number of animals culled divided by the total herd number and expressed as a percentage.
Death rate- The number of animals that have died on the farm divided by the total herd number in percentage terms.
Farrowing- the process of parturition in sows.
Fasciola hepatica- liver fluke.
Gilt- Primiparous female pig.
Mortality rate- The sum of the culling and death rates, sometimes refered to as the removal rate.
Parity- Number of successful pregnancies, a measure of sow age in breeding herds.

Abreviations:

AIMS - Anaerobic identification Media.
c.f.u.- colony forming units.
DNA- Deoxyribonucleic acid.
ELISA- Enzyme Linked Immunoassay.
FAT- Fluorescent Antibody Test.
G.L.C- Gas Liquid Chromatography.
kDa- Kilo dalton
L.D. -Lethal Dose.
M.I.C.- Minimum Inhibitory Concentration.
NIP- Not in pig, not pregnant.
PBS- Phosphate buffered saline.
PCR- Polymerase Chain Reaction.
P.H.L.S.- Public Health Laboratory Service.
plc- phospholipase gene.
SEM- standard error of the mean.
SDS-PAGE- Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis.
V.F.A.- Volatile fatty acid.

APPENDIX I. History form supplied for field case submissions.

Clostridium novyi (oedematiens) **SUDDEN DEATH IN SOWS.**
CASE HISTORY

Name and address of veterinary surgeon:

PLEASE TICK BOXES.

Farm history: OUTDOOR HERD

INDOOR STRAW YARD
SOW CRATES

HERD SIZE: SMALL (< 50 SOWS)
MEDIUM (50-250 SOWS)
LARGE (OVER 250 SOWS)

SOW MORTALITY: HIGH (>10%)
LOW (5-10%)

VERY LOW (<5%)

CLOSTRIDIAL VACCINATION: YES NO

Sow history: PARITY:

CONDITION SCORE:

TIME OF DEATH: AROUND SERVICE
DURING PREGNANCY
FARROWING PRE-PARTUM
LACTATION
AROUND WEANING

ANY CLINICAL SIGNS BEFORE DEATH:
YES NO

TIME BETWEEN DEATH AND PM EXAMINATION

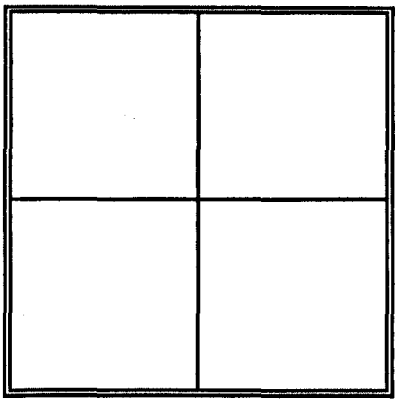
Brief description of PM findings:

Comments:

APPENDIX II. Anaerobic isolate identification report.

DATE: **Number:**
Identification:

Stains



Anaerobic growth:
Colony colour:
Colony size:
Colony elevation:
Colony edge:

Pitting:
Haemolysis:
Metro 5 µg
Swarming:
Spreading:

CO_2

Aero.

Presumptive identification:

Odour:
U.V. Fluorescence:
Spot Indole:
Oxidase:
Catalase:
Motility:

GLC

Spore stain:
Spore position:

Aes	F	G	Lac	Ml	Ma	Mn	Mel	Rib	Su	Xyl	Stc	StH	Gel	Lec	Lip	Ind

APPENDIX III.

CULTURE MEDIA

Fastidious anaerobe broth (FAB) LAB 090-A (Lab M, Amersham)

Fastidious anaerobe agar (FAA) LAB 071-A (Lab M, Amersham)

Cooked meat fastidious anaerobe broth (CMFAB)- 2g of cooked meat medium (CM81, Oxoid) were suspended in 20ml of FAB and sterilised by autoclaving at 121°C for 15 minutes.

Neomycin FAA plates- The final concentration of neomycin was 70 µg/ml. A stock solution of 70,000 µg/ml was prepared by adding 0.5 mg of neomycin sulphate (Sigma) to 5ml of sterile distilled water. 2ml of this solution were further diluted in 6ml of distilled water, giving a working solution of 17,500 µg/ml. 1ml of working solution was added to 250ml of molten FAA.

Egg yolk agar- prepared according to protocol of Levett (1990), the egg yolk of a clean, fresh egg was separated from the white after cracking. The yolk was then mixed with 20ml of sterile phosphate buffered saline (approx. 50% solution). 25ml of the emulsion were added to 250ml of sterilised molten FAA at 55°C. Thick plates were poured into Petri dishes.

Anaerobic identification media (AIM'S plates)- AIM base was prepared by adding 4g of beef extract, 16g of peptone mix and 15g of agar base no. 2 (all LabM, Amersham) with 5g of sodium chloride in 1 litre of distilled water, adjusting the pH to 7.2. After dissolving well, the solution was sterilised at 115°C for 15 mins. Plates with 5% sheep blood were poured and used fresh.

Starch agar plates- prepared by adding 5g of soluble starch (BDH Merk) and 23g of fastidious anaerobe agar base to 500ml of distilled water followed by autoclaving at 115°C for 5 minutes.

Gelatin agar plates- prepared as for the starch plates, but using 2g of gelatin (BDH Merk).

APPENDIX IV.

STORAGE OF ISOLATES

Maintenance of anaerobic isolates on glass beads at -70°C described by Jones, Pell and Sneath (1984) was used.

1. Glass embroidery beads were washed with detergent, dilute HCl and rinsed thoroughly in tap water. The beads (20 approx.) were dried and dispensed into 2ml screw-cap vials, sterilised by autoclaving at 121°C for 15 minutes.

2. Suspending medium was prepared:

Tryptone (Oxoid)	5g
NaCl	2.5g
Beef extract (Oxoid)	1.5g
Yeast extract (Difco)	2.5g
Cysteine hydrochloride	0.2g
Glucose	0.5g
$NaHPO_4$	2g
Glycerol	75ml
Distilled water	500ml

Dispensed in 10ml aliquots in Universal bottles. Sterilised by autoclaving.

APPENDIX V.

Outline of the technique for *post mortem* examination of sows.

1. The identification number, age (number of pregnancies or parity), breed and weight (200 Kg max.) were recorded.
2. Superficial lesions, discoloration of extremities, the presence of any discharge from the rectum, vulva, oral and nasal orifices and the body condition score were recorded. The degree of autolytic change was assessed by observation of the anterior chamber of the eye (white, cloudy discoloration would suggest death was more than 12-24 hours ago).
3. The sows were placed in lateral recumbency and a midline skin incision was made, starting between the mandibular symphysis, down the neck, chest and abdomen.
4. The muscles supporting the scapula were cut allowing the uppermost fore limb to be reflected. Likewise, the muscles in the inguinal region were cut followed by disarticulation of the hip joint.
5. The skin and superficial muscle layers were reflected, exposing the rib cage and the abdominal musculature. At this point the mammary glands were examined.
6. The abdomen was then opened by a midline incision and separation from the last rib, reflecting the muscles and peritoneum away from the midline. The presence of abdominal fluid, fibrin strands or displacement/discoloration of abdominal organs was recorded.
7. This was followed by opening the rib cage with two parallel cuts through the ribs, exposing the thoracic cavity.
8. The gastro-intestinal tract, together with the spleen, was removed by severing the rectum, dissection of the dorsal attachments and cutting the descending aorta, oesophagus and diaphragm.

9. The pluck (the tongue, pharynx, trachea both lungs and the heart) could then be removed, complete, by dissecting the tongue and hyoid bones, freeing the larynx, pharynx, the trachea and oesophagus. The thyroid gland and retropharyngeal lymph nodes were examined.

10. The uro-genital tract was also removed, occasionally the pubic bone was cut to ensure that the bladder neck and the vagina could be removed complete.

11. Examination of the different organs followed, without forgetting examination of superficial and deep lymph nodes.

12. The joints, by disarticulation, and the nervous system were examined when the clinical or pathological presentation suggested this would assist in the final diagnosis.

APPENDIX VI.

Small scale purification of plasmid DNA (miniprep) protocol using the Promega Wizard minipreps.

1. Sample preparation.

1.1. 3 ml of overnight culture of *E. coli* with the appropriate plasmid were pelleted by centrifugation for 5 minutes and the supernatant discarded. The pellet was resuspended in 200 µl of cell resuspend solution and transfered to an Eppendorf.

1.2. 200µl of cell lysis solution were added and mixed by inverting the tube several times. The solution cleared almost immediately.

1.3. 200 µl of neutralisation solution were added and mixed by inversion.

1.4. The solution was centrifuged for 5 minutes in a microcentrifuge at 13000 *g* for 5 minutes.

1.5 The clear supernatant was transfered to a new microcentrifuge tube.

2. Plasmid purification.

2.1. One disposable 3 ml luer-lock syringe was used for each miniprep. A minicolumn was prepared by removing the plunger from the syringe and attaching the barrel of the syringe to a minicolumn provided by the kit.

2.2. 1 ml of Wizard miniprep DNA purification resin was added to the supernatant from step 1.5. and mixed by inversion. The resin was thouroughly mixed before use to ensure an even distribution.

2.3. The resin/DNA mix was pipetted into the syringe barrel and the plungers were reconected. The mix was slowly pushed through the minicolumn.

2.4. The syringe was removed from the minicolumn, the plunger was pulled out and the syringe barrel re-attached to the minicolumn. 2 ml of column wash solution was pippetted and the pushed through the minicolumn.

2.5. The syringe was discarded and the minicolumn attached to a 1.5 ml microcentrifuge tube. This was spun for 2 minutes in a microcentrifuge to dry the resin.

2.6. The microcentrifuge was transferred to a new Eppendorf tube. 50 µl of double distilled water and left for at least one minute. To elute the DNA the minicolumn was spun for 20 seconds.

2.7. The minicolumn was removed and discarded. The plasmid DNA was stored at -20°C or used in restriction enzyme digests immediately.

3. Buffers and solutions.

Cell resuspension solution:	50mM Tris-HCl, pH 7.5
	10mM EDTA
	100 µg/ml RNase A
Cell lysis solution:	0.2M NaOH
	1% SDS
Neutralisation solution:	1.32M Potassium acetate, pH 4.8
Column wash solution:	200mM Na Cl
	20mM Tris-HCl, pH 7.5
	5mM EDTA
	Dilute with 100ml of 95% ethanol to a total volume of 240 ml.

APPENDIX VII.

CLONING OF PCR PRODUCTS DIRECTLY INTO A PLASMID VECTOR (pCR II) USING THE TA CLONING^{R} KIT (INVITROGEN)

This technique takes advantage of the non-template dependent activity of thermostable polymerases used in PCR, that add a single deoxyadenosine to the 3' ends of duplex molecules. The 3' A overhangs are used to insert the PCR product into a vector which contains 3' T overhangs at the insertion site.

TA cloning ^{R} Ligation

The PCR product, directly after the final amplification cycle, was used fresh. The ligations with the pCR vector were set up as a 1:1 molar ratio vector:PCR product. The PCR product concentration was estimated by comparing the band brightness on a gell with known concentrations of molecular weight marker. The amount of product used in the ligation for maximum efficiency was calculated following the formula:

$$\text{X ng PCR product} = \frac{(\text{Y bp PCR product})\,(50 \text{ ng pCR vector})}{(\text{size in bp of pCR vector})}$$

"X"ng= the amount of PCR product of "Y" base pairs to be ligated for a 1:1 molar ratio.

Ligation reaction:

5µl sterile water
1µl 10X ligation buffer
2µl resuspended pCR vector (25ng/µl)
1µl PCR product
1µl T4 DNA ligase

The ligation reaction was incubated overnight at 14^{o}C.

TA cloning^{R} transformation

The competent cells were thawed on ice, and mixing was done by gently tapping the tubes to avoid damage to the cells.

Materials required: 42°C water bath, LB agar plates with 50 µg/ml ampicillin, X-Gal (40mg/ml) and an ice bucket.

(1) One vial of SOC medium was warmed to room temperature.

(2) The LB agar plates with 50 µg/ml ampicillin were dried in the incubator. Two plates per ligation were used.

(3) The Eppendorf tubes containing the overnight ligation reaction were centrifuged briefly and placed on ice.

(4) 0.5M β-mercaptoethanol and one 50 µl vial of frozen Oneshot ™ competent cells per transformation were thawed on ice.

(5) 2µl of the 0.5M β-mercaptoethanol were pipetted into each vial and mixed gently.

(6) 1µl of the ligation reaction were pipetted directly into the vial with the competent cells and tapped gently. The remainder of the ligation reaction mixture was stored at -20°C.

(7) The mixture was incubated on ice for 30 mins.

(8) The tubes were removed from the ice and incubated in the water bath for 45 seconds at 42°C.

(9) After removing from the water bath the vials were rapidly placed for 2 mins on ice.

(10) 450 µl of pre-warmed SOC medium were added to each vial aseptically. The Eppendorf tubes were incubated in a shaker water bath at 37°C for 1 hour.

Transformation (cont)

(11) Bluogal (40µl) was spread evenly on the surface of the LB agar plates with L-shaped glass spreaders and allowed to soak into the agar for one hour. IPTG was not necessary as the OneShot cells do not express the *lac* repressor.

(12) The transformed vials were placed on ice.

(13) Transformed cells were spread on the surface of the LB agar with a glass spreader. 25µl of 100µl were spread on each plate.

(14) Overnight incubation at 37°C was used.

(15) The white colonies were selected for plasmid isolation, restriction analysis or sequencing. Sometimes pale blue colonies were selected as they could contain the insert (small PCR products > 500 bp).

APPENDIX VIII.

Double stranded DNA sequencing by the chain termination method (Sanger, Nicklen and Coulson, 1977).

Template preparation

(1) Bacterial plasmid host was grown up in 2 ml LB broth with 50 µg/ml ampicillin overnight at 37°C with shaking.

(2) The culture was centrifuged in bench centrifuge for 5 minutes. The supernatant was discarded.

(3) Bacteria were resuspended (vortexing) in 100µl of ice cold solution I and transferred to an Eppendorf.

(4) 200µl of solution II were added and mixed by inversion. The tubes were kept on ice.

(5) 150µl of ice-cold solution III were added, mixed for 10 seconds and stored on ice for a few minutes.

(6) The tubes were centrifuged for 5 minutes and the supernatant was transferred to a clean Eppendorf.

(7) Phenol-chloroform extraction with 0.5ml **acid phenol** (equilibrated to pH 4.0 with 50mM Sodium Acetate pH 4.0) was used instead of phenol-chloroform .

(8) After centrifugation for 5 minutes the supernatant was transferred to a clean Eppendorf.

(9) Chloroform extraction with 0.5ml chloroform:isoamyl alcohol (25:1) was carried out.

(10) After centrifugation for 2 minutes the supernatant was transferred to a clean Eppendorf.

(11) 800µl of absolute ethanol (room temperature) were added, vortexed and immediately centrifuged for 5 minutes.

Template preparation for sequencing (cont)

(12) The pellets were drained and dried at 65°C or under vacuum (carefully, not to overdry the DNA).

(13) The pellet was dissolved in 36µl TE pH 7.5, 4µl RNase A (5mg/ml) was added and incubated for at least 20 minutes at 37°C.

(14) 10µl of 1M NaOH, 1mM EDTA (made freshly by mixing equal volumes of 2M NaOH and 2mM EDTA) were added and incubated for a further 15 minutes at 37°C.

(15) The reaction was neutralised by adding 5µl 2M ammonium acetate pH 4.5.

(16) 200µl absolute ethanol were added and placed at -80°C for 30 minutes.

(17) The solution was centrifuged for 10 minutes and the pellet was drained.

(18) **Carefully,** the pellet was rinsed with 200µl ice cold 70% ethanol.

(19) The pellets were drained and dried at 65°C or under vacuum.

(20) The DNA was dissolved in 16µl T0.1E (10mM Tris.HCl pH8.0, 0.1mM EDTA). This was stored at -20°C for a week at most.

Sequencing reactions

(1) Annealing reaction:

DNA(denatured)	7µl
reaction buffer	2µl
primer M13 R (≥5ng/µl)	1µl

Incubate for 30 min. at 37°C, then cool to room temperature and store on ice.

(2) Dilute labelling mix 1:5 (can store at -20°C)

(3) Dilute T7 DNA polymerase 1:4 in ice cold enzyme dilution buffer, enough for inmediate use only.

(4) Put 2.5 µl of each termination mixture A, G, C and T into separate tubes.

(5) Labelling reaction:

Template/primer	10µl
0.1M DTT	1µl
Dilute label mix	1µl
[α-^{35}S] dATP	1µl
Dilute T7 polymerase	1µl

Mix thoroughly, avoiding bubbles and incubate for 2 mins. at room temperature. If more than two samples for sequencing make one mix and split for each template.

(6) Transfer 3.2 µl of the labelling reaction to each termination tube, mix and continue incubation at 37°C for 5 mins.

(7) Terminate the reaction by adding 4 µl of stop solution (gel loading buffer) or by freezing.

(8) Boil the tubes (> 90°C for 2 mins.) inmediately before loading to denature the DNA.

DNA Sequencing Solution Recipes

Template preparation

Solution I

50mM Glucose	1.0ml 1M glucose
25mM Tris.HCl pH 8.0	1.0ml 0.5M Tris.HCl pH 8.0
10mM EDTA	2.0ml 100mM EDTA
	16.0ml H_2O

Solution II

0.2M NaOH	0.5ml .4M NaOH
1% SDS	0.5ml 2% SDS

Solution III

5M Acetate	60.0ml 5M Potassium acetate
3M Potassium	11.5ml Glacial acetic acid
	28.5ml H_2O

LB broth

Tryptone	10g
Yeast Extract	5g
NaCl	10g

pH 7.5 with NaOH
Water to final volume of 1000ml

Sequencing solution recipes

Reaction buffer

200mM Tris HCl pH7.5
100mM $MgCl_2$
250mM NaCl

Labelling mix

7.5 µM dGTP
7.5 µM dCTP
7.5 µM dTTP

Enzyme dilution buffer

10mM Tris HCl pH7.5
5mMDTT
0.5mg/ml BSA

Termination mixes

ddGTP mix:	80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 8 µM ddGTP, 50 µM NaCl
ddGTA mix:	as above but 8 µM ddATP instead of ddGTP
ddGTT mix:	as above but 8 µM ddTTP instead of ddGTP
ddGTC mix:	as above but 8 µM ddCTP instead of ddGTP

Sequencing gel preparation

Vertical slab gels were prepared using treated glass plates. Before assembly the glass plates were washed with a household detergent (Fairy liquid), followed by cleaning the inside surfaces with methanol. The top plate was treated with dimethyldichlorosilane (BDH) in a fume cabinet. This was followed by cleaning with methanol. Strips of discarded x-ray film were used as spacers, after careful cleaning with detergent and methanol. The glass plates were assembled tightly with interfocus gel tape. The comb was tested to ensure it fitted the gap.

A pipette was loaded with the 1/2x mix followed by the 1 1/2x mix, allowing a few bubbles and poured between the plates (holding the gel apparatus almost vertical). The comb was inserted and covered with cling film to prevent contact with air. One or two clamps were applied and the gel was allowed 1 hour to set. To avoid air bubbles entering the wells, the gel was covered with water when removing the comb. Before loading the samples urea was removed from the wells by rinsing with buffer.

The gel was run with 1x TBE buffer in the bottom tank and 1/2x TBE buffer in the top tank at ~22mA and 48 W. Pre-running for 20 mins. followed by changing the buffers was carried out. After the gel had been run for 2 to 3 hours, the tape, spacers and clamps were removed leaving the gel on the bottom plate.

The gel was immersed in 10% methanol/10% acetic acid for at least 20 mins. to fix and then dried in a hot oven (70^{o}C). The auto radiograph was exposed overnight or longer if necessary.

Sequencing gels

sequencing gel loading buffer	98% deionised formanmide
	10mM EDTA pH8.0
	0.025% xylene cyanol FF
	0.025% bromophenol blue

Gradient sequencing gel solutions

	1/2x	2 1/2x
40% acrylamide stock	15ml	9ml
(38g acrylamide + 2g bisacrylamide)		
urea	42.0g	25.2g
10x TBE	5.0 ml	15.0 ml
sucrose	-	3.5g
bromophenol blue	-	1 grain (to colour)
total volume	100 ml	60 ml
for each gel	16 ml	9.5 ml
add		
TEMED	19µl	11µl
APS (0.1g in 2 ml)	120µl	70µl

APPENDIX IX. DNA extraction protocols useful with anaerobic bacteria.

1. Guanidinium thiocianate/sodium citrate/sarcosyl/mercaptoethanol (GITC/C/SARC/BME) method.

(1) Overnight culture pellet was resuspended in 100 µl of TES buffer and homogenised by whirlymixing.

(2) 400 µl of the GITC/C/SARC/BME mix were added wearing gloves and safety glasses. The mixture was incubated at room temperature for 5 minutes.

(3) This was followed by adding 500 µl of phenol:chloroform (3:1), mixed carefully and spun at 13,000 *g* for 5 minutes.

(4) The supernatant layer (top) was transfered to a fresh Eppendorf tube.

(5) Then the same procedures as described for other DNA extractions were followed.

Reagent recipes

TES buffer (pH 8.0)	50mM Tris-HCl 50mM EDTA 15% sucrose
GITC/C/SARK	4M guanadinium thiocyanate 25mM sodium citrate pH 7.0 0.5% (w/v) sarcosyl

Stable for three months at room temperature.

GITC/C/SARK/BME	50ml of above 360 µl (0.72%) β-2-mercaptoethanol

Stable for 1 month at room temperature.

DNA purification (cont.)

2. Extraction of bacterial genomic DNA by SDS lysis (Wren, Mullany and Lamb, 1991).

This procedure is based on : lysozyme digestion of the cell wall; lysis with SDS; disruption of protein-nucleic acid complexes with pronase and finally phenol:chloroform extraction to remove proteins.

(1) Grown the bacteria to the late log phase in 10-25 ml of the appropriate medium.

(2) Harvest the cells by centrifugation at 5000 *g* for 5 minutes.

(3) Resuspend the pellet in 2ml of 50mM Tris-HCl pH 8.0 containing 25% sucrose.

(4) Add 1 ml of freshly prepared lysozyme (10mg/ml) and incubate on ice for 30 min.

(5) Add 250 µl of 5% SDS in 50mM Tris-HCl pH 8.0, 0.75 ml of TE and 0.5 ml of pronase (20 mg/ml). Incubate at 56°C for 1 hour.

(6) Perform a phenol:chloroform extraction as detailed elsewhere.