STUDIES ON *CAMPYLOBACTER FETUS SUBSPECIES JEJUNI*

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SUMMARY

The work described in this thesis was principally directed at trying to develop a mouse model for human campylobacteriosis and also at defining the antigenic relationships between the human strains.

Human diarrhoeal and normal stool specimens were examined for Campylobacter fetus ss. jejuni by culture on plates of Campylobacter selective medium, incubated at 43°C in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2. From 290 specimens of diarrhoeal stool, 11 isolates of Campylobacter were obtained. The organism was not isolated from the stools of 49 normal people. Alkaline peptone water, pH 8.4, was examined as a possible enrichment medium but did not yield additional isolates.

For growth in liquid culture, nutrient broth supplemented with yeast extract and cystine and incubated in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2 gave the best cell yields. Bubbling the gas mixture (10% CO_2 and 90% N_2) through freshly inoculated medium gave no growth. Contrary to what has been reported with Vibrio fetus, the addition of KNO_3, $MgSO_4$, $CaCl_2$, $Na_2H_2PO_4$ to nutrient broth did not enhance the growth of C. fetus ss. jejuni.

Because of the tendency of Campylobacter colonies to spread on agar, considerable difficulty was experienced in performing colony counts on suspensions of the organism. Success was, however, achieved by using well-dried culture plates and an incubation period of less than 30 hours. Two changes in the culture medium - addition of P-nitrophenylglycerol and extra agar - which have been recommended for colony counts on Proteus strains were not satisfactory with C. fetus ss. jejuni.

Freeze-drying was found unsatisfactory for the long term (more than 1 year) preservation of C. fetus ss. jejuni. However, strains could readily be maintained by mixing the broth culture with 15% glycerol and coating glass beads which were stored at -76°C.

Mice of the HAM I/CR strain were injected with C. fetus ss. jejuni isolates of human origin by the intraperitoneal and intravenous routes, and were also given the bacteria orally. Although mice died if the dose of organisms was sufficiently large (10^{10}), viable counts on liver and spleen homogenates made at various times indicated that there was little, if any, bacterial multiplication in vivo. However, viable Campylobacter could be recovered from various organs, notably blood, liver, spleen, kidney and gastrointestinal tract. This distribution was irrespective of the route of inoculation. The most consistent recovery of the administered bacteria was from the liver and spleen; persistence in the liver being up to 21 days.

No mouse that had been given C. fetus ss. jejuni exhibited signs of diarrhoea. There was a marked age effect of susceptibility of HAM I/CR mice to the lethal effect of C. fetus ss. jejuni. Animals 7 days old were considerably more susceptible than younger (1- and 3-day old) or older (2-, 3- and 5-week old) mice. The virulence of C. fetus ss. jejuni for 7-day old mice could be increased slightly by passaging the bacteria five times through adult mice, with recovery from the spleen.

C. fetus ss. jejuni did not produce extracellular toxins, and death of the mice was due to the toxicity of the bacterial

cells themselves. Bacteria killed at 56°C had an LD_{50} of 4.1×10^9 for 7-day old mice, compared with 1.5×10^9 of the corresponding live suspension. The toxicity of bacteria killed at 56°C was not diminished by heating at 100°C for 15 min., which suggested that the toxic factor was lipopolysaccharide and that the lethal effect of live organisms was due to the content of endotoxin. This conclusion was supported by the observation that crude cell envelopes contained the toxic factor while the cytoplasm was non-toxic.

In an attempt to enhance the virulence of *C. fetus ss. jejuni* for 7-day old mice, the injected dose of bacteria was mixed with different amounts of ferric ammonium citrate. A definite enhancing effect was observed : for the LD_{50} of *C. fetus ss. jejuni* mixed with 12 μg Fe^{3+} was 4.2×10^8 (95% confidence limits $3.5 - 5.1 \times 10^8$), compared with 1.8×10^9 without added iron. Lower doses of iron were less effective. The enhancing effect was observed with 2 strains of *C. fetus ss. jejuni*. Whole-mouse homogenates made at 48 hr after injection of *Campylobacter* into 7-day old mice, showed 100-fold higher counts in animals given 12 μg Fe^{3+} with the challenge dose. This suggests that Fe^{3+} might allow the organisms to multiply to some extent if mixed with iron before injection. However, both with and without iron, the host bactericidal mechanism produced a net reduction in the viable counts, but the rate of this reduction was less in the Fe-treated animals. Ferric ammonium citrate did not increase the lethal effect of heat-killed suspensions of *C. fetus ss. jejuni* nor the toxicity of *Shigella flexneri* lipopolysaccharide for mice. This suggests that

Fe^{3+} did not increase the toxicity of LPS that might be released from Campylobacter killed in vivo.

Mucin as another possible virulence-enhancing agent was also investigated, but when added as 1% or 5% (w/v) suspension to C. fetus ss. jejuni did not affect the LD_{50} for 7-day old mice.

C. fetus ss. jejuni did not produce heat-labile, cholera-like enterotoxins detectable by the guinea pig ileal loop test, nor did it give haemagglutination with human (Group A), or with sheep, horse or rabbit red cells.

The antigenic relationships between 10 strains of C. fetus ss. jejuni were investigated with H and O antisera raised in mice and rabbits (3 strains only). Extensive cross-reactions between the various antigens and sera were observed, suggesting that C. fetus ss. jejuni from patients with diarrhoea showed one or more common antigens. The rabbit appeared to be a more suitable species for raising Campylobacter antisera than the mouse, not only because of higher titres but also because the rabbit lacks a natural agglutinin for Campylobacter which the HAM I/CR mouse possesses.

Preliminary investigation of Campylobacter whole cell proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis suggested that human strains from patients with diarrhoea were homogeneous in their protein profiles but differed from strains of animal origin.

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LIST OF ABBREVIATIONS

cAMP	-	cyclic adenosine - 3', 5' - monophosphate
CDS	-	Communicable Diseases Scotland (weekly report)
C.L.	-	confidence limits
DNA	-	deoxyribonucleic acid
g	-	gravitational force, unit of centrifugation
EDTA	-	ethylenediaminetetraacetate
G + C	-	guanine + cytosine
HA	-	haemagglutinin
Ig	-	immunoglobulin
i.p.	-	intraperitoneal
IU	-	international unit
i.v.	-	intravenous
LD_{50}	-	median lethal dose
LPS	-	lipopolysaccharide
mol. wt.	-	molecular weight
NCTC	-	National Collection of Type Cultures
No.	-	number
O.D.	-	optical density
o.u.	-	opacity unit
PAGE	-	polyacrylamide gel electrophoresis
per os	-	by mouth
PHLS	-	Public Health Laboratory Service
RBC	-	red blood cells
RNA	-	ribonucleic acid
RES	-	reticuloendothelial system
r.p.m.	-	revolution per minute

SDS	-	sodium dodecyl sulphate
ss.	-	subspecies
Tris	-	Tris- (hydroxymethyl) - methylamine
WHO	-	World Health Organisation
wk	-	week

INTRODUCTION

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I. TAXONOMY AND CLASSIFICATION

1. General remarks.

Campylobacter fetus, initially called *Vibrio fetus* was first assigned to the genus *Vibrio* by Smith & Taylor (1919). This organism was morphologically similar to the type species *V. cholerae*, but there are major differences in both the biochemical and growth characteristics and in the DNA base ratios between the true vibrios and *V. fetus* (Park, 1961). *V. fetus* neither ferments nor oxidizes carbohydrates and is microaerophilic or strictly anaerobic, but true vibrios ferment selected sugars with acid production and grow in 3% NaCl and are facultatively anaerobic. Sebald & Véron (1963) showed that the G + C content of the deoxyribonucleic acid (DNA) of *C. fetus* is different to that of *V. cholerae* and related vibrio species. They found that the G + C content of the DNA of *C. fetus* ranged from 29-35 moles %, but the value of classical vibrios is about 47 moles %. Thus, in 1963, Sebald & Véron suggested that microaerophilic vibrios should be re-classified, and a new genus, *Campylobacter* with a type species *Campylobacter fetus* be formed. The microscopic morphology of this new genus is that of a spirally curved rod, and there are many morphological physiological and anatomical features common to both the genera *Campylobacter* and *Spirillum*. *Campylobacter* was thus included in the family *Spirillaceae* (Davis & Park, 1963; Véron, 1966; Elazhary, 1968).

2. New taxonomic scheme

2.1. Morphology.

The genus Campylobacter is composed of Gram-negative, non-spore-forming, spirally curved rods, 1.5-3.5μm long by 0.2-0.4μm wide. The single cells have one curve or twist and appear vibroid (Rhoades, 1954; Ritchie, Keeler & Bryner, 1966). They may also be S-shaped or gull-shaped. Sometimes the organism may also appear ribbon-shaped, being composed of a chain of single curved cells. The cells become coccoid if incubation is prolonged. These coccoid forms are not viable (Ogg, 1962; Tritz & Ogg, 1967). Campylobacter have a characteristic corkscrew-type of motility which is achieved by a long, single polar flagellum at one end or a polar flagellum at each end of the cell. The flagellum may be 2-3 times the length of the cell.

2.2 General characteristics

According to Smibert in the Bergey Manual (1974), the genus Campylobacter is biochemically inactive. Carbohydrates are not fermented or oxidized; Kreb's cycle intermediates and amino acids are the primary energy sources of these organisms. The bacteria reduce nitrate to nitrite, but do not hydrolyze gelatin or urea. Catalase and oxidase tests are positive, but the methyl red and acetylmethylcarbinol tests are negative.

Today, the genus Campylobacter comprises 3 species : Campylobacter fetus, Campylobacter sputorum and Campylobacter faecalis. The new taxonomic scheme is shown in Table 1. Campylobacter fetus and Campylobacter faecalis are catalase

Table 1 : New taxonomic scheme of Genus Campylobacter (modified from Philip & Tilton, 1977).

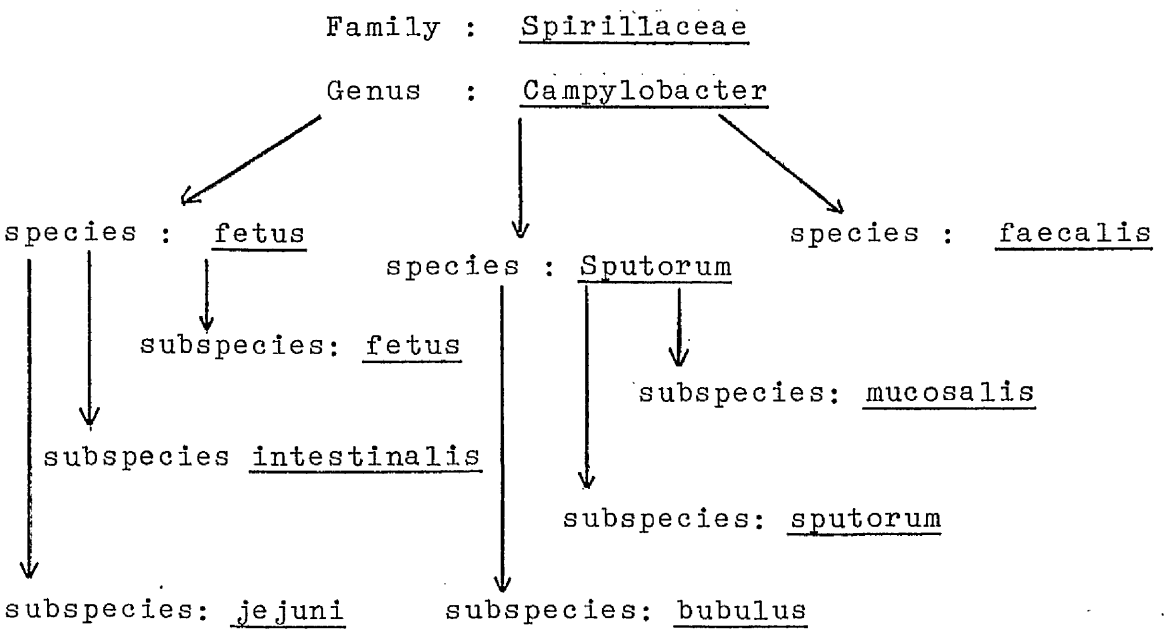


Table 2 : G + C content of Campylobacter species (From Smibert, 1974)

Species	Catalase	G + C content (moles %)
C. fetus	positive	33 - 35
C. sputorum	negative	29.5 - 30.9
C. faecalis	positive	32 - 32.8

positive, Campylobacter sputorum is catalase negative. The G + C content of Campylobacter species is summarised in Table 2.

A brief introduction to Campylobacter species, and their differentiation now follows :

a. Species : Campylobacter fetus (Vibrio fetus)

C. fetus is the most widely known species in the genus Campylobacter. It was first found to be responsible for abortion in sheep and cattle by McFadyean & Stockman (1913) in Great Britain. They described the organism, and proposed the name V. fetus ovis. Smith (1919) and Smith & Taylor (1919) in a study of infectious abortion in cattle isolated a micro-aerophilic "spirillum" from aborted calves, and suggested that this organism was identical to the "vibrio" described by McFadyean & Stockman in 1913; these workers named this organism V. fetus. In 1931, Jones, Orcutt & Little isolated a vibrio from cattle with winter scours and named it Vibrio jejuni. This was the first mention of V. jejuni in the literature. In 1944, Doyle called a Vibrio isolated from the intestine of pigs with swine dysentery V. coli.

Florent (1960) studied V. fetus isolated from cattle, and divided the species into 2 subspecies : V. fetus venerealis and V. fetus intestinalis. He reported that V. fetus venerealis was H_2S negative (lead acetate paper strip), did not grow in 1% glycine or in 3.5% NaCl. V. fetus intestinalis was either H_2S positive or weakly positive, did not grow in 3.5% NaCl but did grow in 1% glycine.

In 1962, Mohanty, Plumer & Faber divided *V. fetus* strains of bovine origin into 3 groups. Type 1 strains did not produce H_2S, and did not grow in 3.5% NaCl or 1% glycine media. Type II produced H_2S and did not grow in 3.5% NaCl but did grow in 1% glycine. Type III was H_2S positive and did not grow in 3.5% NaCl or 1% glycine. Bryner, Frank & O'Berry (1962) divided *V. fetus* strains isolated from bovine reproductive tracts or aborted foetuses into 3 biotypes. Type I was H_2S negative and grew in 1% bile but not in 1% glycine or 3.5% NaCl containing media. Subtype 1 was weakly H_2S positive and grew in 1% bile but not in 1% glycine or 3.5% NaCl. Type 2 was H_2S positive and grew in 1% bile and 1% glycine but not in 3.5% NaCl.

King (1957) first studied microaerophilic vibrios isolated from diarrhoea. She found that this organism was morphologically indistinguishable from the typical *V. fetus*, but grew better at 43°C than at 37°C. These organisms she called "related vibrios" and these are now called *C. fetus ss. jejuni.*

Véron & Chatelain (1973) reclassified *C. fetus*. *C. fetus subsp. fetus* was the same as the *V. fetus subsp. intestinalis* of Florent; likewise, *C. fetus subsp. venerealis* was the same as Florent's *V. fetus subsp. venerealis*. *C. fetus subsp. venerealis* biotype intermedium was similar to subtype 1 of Bryner *et al.*, (1962), *Campylobacter coli* was similar to *V. coli* described by Doyle (1944); except *C. coli* reduced nitrate, and *Campylobacter jejuni* was similar to *V. jejuni* of Jones *et al.*, (1931). *C. sputorum subsp. sputorum* and *C. sputorum subsp. bubulus* were *Vibrio sputorum subsp. sputorum* and *V. sputorum subsp. bubulus* of Loesche, Gibbons & Socransky (1965).

Table 3 : Comparison of various classifications of Campylobacter fetus (Smibert, 1978)

Bryner et al. (1962)	Mohanty et al. (1962)	Florent (1959)	Smibert (1974)	Véron & Chatelain (1973)	Berg et al.(1971)
Biotype 1	I	venerealis	fetus[a]	venerealis[a]	A-1
Biotype sub 1	III	venerealis	fetus[a]	venerealis[a]	A-sub-1
Biotype 2	II	intestinalis	intestinalis	fetus[a]	A-2
Biotype 2	II	intestinalis	intestinalis[a]	fetus[a]	B
			jejuni[a]	C. jejuni	C
			jejuni[a]	C. coli	

a = subspecies

In Bergey's Manual (1974), Smibert classified C. fetus in the genus Campylobacter. C. fetus subsp. fetus is the same organism as the V. fetus subsp. venerealis of Florent. C. fetus subsp. intestinalis also is the same organism named by Florent as V. fetus subsp. intestinalis. This bacterium is the cause of sporadic abortion in cattle, but it is as prevalent as the venereally transmitted disease caused by C. fetus subsp. fetus. C. fetus subsp. jejuni represents the C. coli and C. jejuni of Véron & Chatelain (1973), "related vibrios" of King (1957) and V. jejuni of Jones et al., (1931).

There is still considerable confusion and controversy about the nomenclature and classification of the species and subspecies of C. fetus within the genus. A comparison of some of the classification systems proposed for C. fetus is given in Table 3. In this review, the nomenclature of Smibert (1974) is used unless otherwise specified. The currently accepted species classification of Campylobacter from Bergey's Manual of Determinative Bacteriology is listed in Table 4, along with synonyms used by different authors in the past.

The temperature tolerance has been used as an important differential characteristic with the species C. fetus (King, 1957). Incubation at 25°C favours the growth of subspecies fetus and intestinalis and inhibits the growth of subspecies jejuni. Conversely, incubation at 43°C inhibits the growth of subspecies fetus and intestinalis while promoting the growth of subspecies jejuni. Some important differential tests for C. fetus are listed in Table 5.

Table 4 : Classification of the species *Campylobacter fetus*

Bergey's Manual	Taxonomic classification synonyms according to Véron & Chatelain (1973)	King (1957)	Florent(1959) or Jones *et al.* (1931)	Ecology	Disorders produced
1. *C. fetus ss. fetus*	*C.fetus ss. venerealis*	*V.fetus*	*V.fetus ss. venerealis* (Florent)	found in bovine semen, preputial fluid, cervical mucus; will not grow in human or animal G.I. tract	Enzootic abortion and sterility in cattle; venereal transmission, not associated with human disease.
	C.fetus ss. venerealis biotype *intermedius*				
2. *C. fetus ss. intestinalis*	*C.fetus ss. fetus*	*V.fetus*	*V.fetus ss. intestinalis* (Florent)	found in placentas and gastric content of aborted ovine and bovine foetuses, in bile and G.I. tracts, will grow in human and animal G.I. tract	Abortion in sheep; sporadic abortion in cattle; oral transmission; usual cause of systemic human diseases.
3. *C. fetus ss. jejuni*	*C.jejuni/ C.coli*	"Related vibrios"	*V.jejuni* (Jones *et al.*)	Normal G.I.flora in swine, sheep, cattle, goats, chickens, turkeys, wild birds; will grow in human and animal G.I. tracts	Abortion in sheep; enteritis in heifers, calves, avian vibrionic hepatitis; usual cause of human enteritis.

Table 5: Differential tests for Campylobacter fetus

(Smibert, 1978)

	Temperature		Nalidixic acid agar	TTC[b] agar	Brilliant green agar	8% glu-	3.5%	1%	1%	1%	H_2S production		
	25°C	43°C	40µg/ml	0.4µg/ml	1:33,000	cose	NaCl	Bile	glyc- ine	selenite reduct- ion	TSI agar	stand -ard medim	with cystine
C. fetus ss. fetus	+[a]	-	+	-	+	+	-	+	-	-	-	-	-
C. fetus ss. intestinalis	+	-	+	-	+	+	-	+	+	+	-	-	+
C. fetus ss. jejuni	-	+	-	d	-	-	-	+	+	W	-	-	+

a : + = growth, - = no growth, d = variable reaction, W = weak reaction.

b : TTC = 2,3,5-triphenyl-tetrazolium chloride.

b. Species : Campylobacter sputorum

This organism was first isolated from the oral cavity of animals by Prévot in 1940. The organism is catalase negative, and was first called V. sputorum. In 1956, Florent reported the isolation of catalase-negative vibrios from bovine reproductive organs, and he named these bacteria V. bubulus.

V. sputorum and V. bubulus were similar in microscopic morphology, both in shape and motility. Firehammer & Lovelace (1961) showed that V. bubulus isolated from semen and preputial mucus of rams and the vagina of ewes grew in 2.5% NaCl but only weakly in 3.5% NaCl. However, V. bubulus of bovine origin grew well in 3.5% NaCl.

Loesche, Gibbons & Socransky (1965) in studying V sputorum, V. bubulus and V. fetus strains, reported that the V. sputorum of Prévot was very similar to V. bubulus, but was different to V. fetus strains. V. bubulus and V. sputorum both were biochemically inactive, had no action on carbohydrates and gelatin; these strains were negative for indole, lipase, urease and acetylmethylcarbinol production; reduced nitrate to nitrite, and produced large amounts of H_2S on iron-containing medium. V. bubulus and V. sputorum grew at both 25°C and 37°C, but not at 45°C, and growth was obtained in 1% glycine. V. bubulus grew in the presence of 2.5% and 3.5% NaCl, but V. sputorum did not grow in these NaCl concentrations. Véron & Chatelain (1973) reported that V. sputorum grew in 1% bile, whereas V. bubulus did not.

In 1965, Loesche et al., recommended combining V. sputorum

and V. bubulus into one species as V. sputorum subspecies sputorum and V. sputorum subspecies bubulus. Smibert (1974) in Bergey's Manual changed the name from Vibrio to Campylobacter sputorum subspecies sputorum and C. sputorum subspecies bubulus. The main differential tests to separate the subspecies was to grow these strains in the presence of 1% bile and 3.5% NaCl.

A third subspecies, C. sputorum subspecies mucosalis has now been isolated from porcine diseases; intestinal adenomatosis, necrotic enteritis, regional ileitis and proliferative haemorrhagic enteropathy (Lawson, Rowland & Roberts, 1975; 1976; Roberts, Lawson & Rowland, 1977; Rowland & Lawson, 1974). Love, Love & Bailey (1977) compared the strains of C. sputorum subsp. mucosalis isolated from porcine intestinal adenomatosis and proliferative haemorrhagic enteropathy and found that these strains cannot be distinguished on biochemical criteria.

Biochemical characteristics of C. sputorum subsp. mucosalis generally conform to the description of C. sputorum. C. sputorum subsp. mucosalis reduces nitrate to nitrite, is strongly H_2S positive, grows in 1% glycine and 1.5% NaCl but not 2.5% NaCl. The characteristics of these 3 subspecies are shown in Table 6.

Colonies of C. sputorum subsp. mucosalis are a dirty yellow, whereas colonies of the other two subspecies are white to tan coloured.

Table 6 : Some differential characteristics of the subspecies Campylobacter sputorum (Smibert, 1978)

Subspecies	Yellow colonies	Nitrate reduction	H_2S production	1% glycine	3.5% NaCl	1% Bile
C. sputorum ss. sputorum	-	+[a]	+	+	-	+
C. sputorum ss. bubulus	-	+	+	+	+	-
C. sputorum ss. mucosalis	+	+	+	-	-	-

a : + positive test or growth - negative test or no growth

c. Species Campylobacter faecalis

Firehammer (1965) isolated a microaerophilic Vibrio from normal sheep faeces and named it V. faecalis. The organism was an actively motile, vibroid-shaped rod. These bacteria were catalase and H_2S positive, biochemically inactive, indole and urease negative, and had no action on sugars and gelatin. Nitrate was reduced to nitrite. Growth in 1% glycine and 3.5% NaCl was variable, and it was strongly H_2S positive in iron-containing medium. The organism grew at 37°C but not at 25°C. Smibert (1974) changed the name V. faecalis to C. faecalis. There are so far no subspecies.

II. CULTURAL AND ISOLATION TECHNIQUES FOR CAMPYLOBACTER SPECIES.

1. Media.

Campylobacter species are most easily grown in a semisolid medium. However, when isolating C. fetus from faeces, intestines, prepuce, semen,vagina, and oral cavity, pure cultures are seldom obtained because the accompanying bacterial species multiply more rapidly than the Campylobacter. The study of the sensitivity of C. fetus to various dyes, antibiotics and sulfa drugs by Ryff & Lee (1945), Prier (1951), Plastridge & Easterbrooks(1952) and Rolle & Mundt (1954) led to the development of selective media for the isolation of these organisms.

In 1955, Schneider & Morse incorporated ox-bile (40ml/ 1) into the medium. Kuzdas & Morse (1956) developed a selective medium which in addition to ox-bile (40ml/1), contained bacitracin (25,000 units/1), polymyxin-B sulphate (5000 units/1) and actidione (100 mg/1) in a base of Albimi brucella agar. This medium has been successful in the isolation of V. fetus from experimentally and naturally infected cattle, but contaminating bacteria e.g. Pseudomonas sp. were not inhibited, and the cultures were overgrown by Proteus if the organism was present. Florent (1956) isolated V. fetus from carrier bulls using brilliant green (1:25,000 or 1:40,000) incorporated into brucella agar; he reported that Gram-positive bacteria were completely inhibited by the dye but Pseudomonas and Proteus were occasionally still present.

Plastridge, Koths & Williams (1961) used a selective medium containing bacitracin (2IU/ml) and novobiocin (2μg/ml) to isolate V. fetus from bull semen. Their medium inhibited Streptococcus, Staphylococcus, B. subtilis and unidentified Gram-negative rods, but Pseudomonas and Proteus were not inhibited. These authors reported that the tendency for Proteus to spread appeared to be reduced, and allowed the isolation of V. fetus from bull semen.

In 1961, Plumer, Duvall & Shepler first introduced a filtration technique using millipore filters with pore sizes of 0.45μ and 0.65μ to isolate V. fetus from bull semen. The technique is based on the fact that V. fetus which has a size of about 0.2 to 0.3μ and a length of about 5μ could pass through a millipore filter and other organisms e.g. Proteus and Escherichia (diameter ranging from 0.4 to 0.6μ) are retained. These authors reported the isolation of V. fetus from 9 of 13 semen and 24 of 42 preputial samples from carrier bulls; Proteus and Pseudomonas were eliminated by this technique. Shepler, Plumer & Faber (1963) compared the selective antibiotic medium with the millipore filtration technique for the isolation of V. fetus from preputial fluid and found that neither the antibiotic medium nor the millipore filtration method had a distinct advantage. The selective medium they used contained bacitracin (15 IU/ml), polymyxin (1 IU/ml), and novobiocin (5μg/ml) in brain-heart infusion agar. Although polymyxin was the most effective antibiotic against Pseudomonas, the concentration of polymyxin which reduced the Pseudomonads also decreased the number of V. fetus colonies, and if Proteus

was present, the cultures were overgrown by this organism. Preparation of the samples for filtration required more time and equipment than simply streaking plates containing antibiotics, and fewer V. fetus colonies grew after the filtration technique. Although Pseudomonas and Proteus were eliminated using millipore filtration, they found that 8 of 56 samples were negative by millipore filter but were positive on the antibiotic medium. These samples had an average of 3 colonies per plate, and they suggested that samples containing small numbers of V. fetus were more likely to give positive cultures using the antibiotic medium.

Plastridge, Williams & Trowbridge (1964) used brucella agar containing bacitracin (2 IU/ml), novobiocin (2 μg/ml), and cyclohexamide (0.1 mg/ml) to isolate V. fetus from experimentally infected heifers. They reported that diphtheroids, alpha haemolytic streptococci were inhibited, but that coliform organisms were not inhibited, and the authors reported that these contaminants posed a serious problem in isolating V. fetus from the samples. Smibert (1965) reported the isolation of V. fetus from the faeces and intestinal contents of clinically normal sheep from 3 months to 8 years of age using both the millipore filtration technique and selective media. A membrane filter having a pore size of $0.65 \pm 0.03\mu$ was used, and the filtrate was inoculated on to the brucella agar plates containing 2 units of bacitracin/ml, and 2μg of novobiocin/ml. He isolated V. fetus from the contents of rectum, colon, caecum and ileum-jejunum of sheep.

King (1957) was the first to report the isolation of

"related vibrios" from blood of patients with diarrhoea. However, it was not until 1972 that Dekeyser *et al.*, first introduced the millipore filtration technique that isolated "related vibrios" from human faeces. The samples of faeces were first suspended in nutrient broth or 1/4 strength Ringer's solution. After homogenization, the suspension was allowed to stand for 30 minutes to 1 hr, to enable the coarse materials to settle, the supernatant fluid was then removed and centrifuged at 1,500 g for 5 minutes. The surface liquid was removed by sterile pipette, and subsequently passed through a millipore filter (mean pore size 0.65μ). The filtrate was inoculated onto thioglycollate agar medium containing 15% defibrinated ovine blood plus bacitracin (25 IU/ml), polymyxin-B sulphate (10 IU/ml), novobiocin (0.005 mg/ml) and actidione (0.05 mg/ml).

Skirrow (1977) reported, that the millipore filtration technique as a primary step in the isolation of *C. fetus ss. jejuni* from human faeces was not necessary as a selective medium containing antibiotics allowed the growth of the bacterium. The selective medium consisted of Oxoid Blood Base No. 2 plus 7% lysed horse blood containing the antibiotics vancomycin (10 mg/l), polymyxin-B sulphate (2.5 IU/ml) and trimethoprim lactate (5 mg/ml). In place of Oxoid Blood Base No. 2, Oxoid Columbia agar base could be used (Gibson, J., City Laboratory, Glasgow, Personal Communication, 1977). *Proteus* was not recovered from this selective medium, and the presence of yeasts did not affect the isolation of *Campylobacter* from the faecal specimens. If yeasts constituted a problem, amphotericin B (2 mg/l) could be incorporated in the selective medium (Wang, Blaser & Cravens, 1978).

The selective medium of Skirrow is now widely used in routine clinical laboratories for the isolation of C. fetus ss. jejuni from human faeces. The faecal suspensions are inoculated directly and spread in the normal way; but it is important that the inoculum should not be so heavy that visible faecal material can be seen on the medium. Tanner & Bullin (1977) reported that freshly voided faecal specimens should be used, and if there is any delay, the faecal specimens should be refrigerated.

2. Temperature

Microaerophilic Campylobacter grow well at 37°C, but the temperature range for growth varies between strains (King, 1957), and this characteristic has been used in classification. The strains isolated from human campylobacteriosis are normally incubated at 37°C (McDonald & Mautner, 1970; White & Walsh, 1970). The first C. fetus ss. jejuni strains isolated from human faeces followed incubation at 37°C (Dekeyser et al., 1972; Butzler et al., 1973; Cadranel et al., 1973), but Skirrow (1977) reported that Campylobacters on primary isolation from patients with diarrhoea grew better at 43°C. Dale (1977), Simmons & Gibbs (1977) confirmed this finding.

3. Atmosphere.

Campylobacter species require both oxygen and carbon dioxide, but the oxygen concentration in air at atmospheric pressure is toxic to these organisms, and the O_2 tension should be lower than that of CO_2, and the CO_2 concentrations should be greater

than that of air.

Ward (1948) incubated plates in a candle jar. Kiggins & Plastridge (1956) reported that the optimum oxygen concentration for C. fetus was 5% (v/v) and the optimum CO_2 concentration was in the range of 5-30% (v/v). Mcdonald & Mautner (1970) reported that C. fetus strains grew well in an atmosphere containing 5% CO_2; and Cooper & Slee (1971) also found that microaerophilic incubation with added CO_2 gave the most luxuriant growth of C. fetus. Morris & Park (1973) found that satisfactory growth of Campylobacter sp. was obtained by incubating the culture in an atmosphere of 30% (v/v) CO_2. This atmosphere could be achieved by reducing the pressure within the anaerobic jar by one-third and then using a CO_2 and N_2 mixture to replace the evacuated air.

Dekeyser et al., (1972) showed that colonies of C. fetus ss. jejuni isolated from patients with diarrhoea developed well after incubation in an atmosphere from which 2/3 of the air had been replaced by a mixture of 95% N_2 and 5% CO_2. Cadranel et al., (1973) isolated C. fetus ss. jejuni from stools by incubating plates in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2. Karmali & Fleming (1979b) reported that these gaseous conditions necessary to isolate C. fetus ss. jejuni from faeces could be achieved by using the Fortner principle which utilizes the ability of a rapidly growing facultative anaerobe to reduce the oxygen tension in a closed system, and this allows the subsequent growth of oxygen-sensitive organisms. They incubated the faecal inoculated plates, together with a plate of Pr. rettgeri in a polythene bag (18 by 21cm) equipped with a reversible airtight seal.

Skirrow (1977), Dale (1977) and Smibert (1978) recommended that for the isolation of C. fetus ss. jejuni from patients with diarrhoea or from infected animal tissues, the specimens should be incubated in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2, and at a temperature of 43°C.

III. CAMPYLOBACTER AS A HUMAN PATHOGEN

For years, Campylobacter was thought to cause disease only in domestic animals, but several instances of human infections had been reported (Levy, 1946). It has been increasingly recognized that Campylobacter is an important pathogen of man, and is responsible for a variety of clinical symptoms, ranging from septicaemia to abortion in women (Table 7).

The two species of C. fetus infecting man are C. fetus ss. intestinalis and C. fetus ss. jejuni. The subspecies C. fetus ss fetus has not been isolated from human infection, and this species is found associated with animal infection. C. sputorum ss. sputorum is found as part of the normal flora of the oral cavity of man (Loesche, Gibbons & Socransky, 1965).

1. Systemic campylobacteriosis in man

Systemic human C. fetus infections are mainly caused by C. fetus ss. intestinalis although C. fetus ss. jejuni can also give rise to this type of infection. Most adult patients have had one or more underlying pre-existing major medical conditions, including alcoholism, diabetes and rheumatic heart disease (Rettig, 1979) before they have contracted Campylobacter infections.

The commonest manifestation of systemic campylobacteriosis is bacteraemia (Jackson, Hinton & Allison, 1960; Hallett, Botha & Logan 1977; Robinson, 1978; Guerrant et al., 1978). C. fetus ss. intestinalis can cause bacterial endocarditis (Kilo, Hagemann & Marzi, 1965; Lawrence, Biggs & Woodward, 1967; Wozniak et al., 1978),

Table 7 : Pathological conditions associated with human *Campylobacter fetus*[a] infections

(Modified from Philip & Tilton, 1977)

Respiratory	Vascular	Nervous	Gastro-intestinal tract	Reproductive tract	Others
Pleurisy	phlebitis	Peripheral paralysis	Nausea	abortion	arthritis
Pleural effusion	Subacute bacterial endocarditis	cerebral embolus	Diarrhoea	Premature birth	reactive arthritis
Pleuropneumonia	Septicaemia	Meningitis	Abdominal cramp	-	Reiter's syndrome
Cough	-	-	Constipation	-	-
Bronchitis Empyema	Bacteraemia		Hepatomegaly with jaundice	-	-
Pulmonary embolus	-	-	-	-	-

a: subspecies : *C. fetus ss. jejuni* and *C. fetus ss. intestinalis*

meningitis (Robin et al., 1962; Gubina et al., 1976; Wright, 1979), thrombophlebitis (Kahler & Sheldon, 1960; King, 1962; Toala, McDonald & Kass, 1970; Smith, Marymont & Schweers, 1976) and septic arthritis (King & Bronsky, 1961; Kilo, Hagemann & Marzi, 1965; Kutner & Arnold, 1970). Other reported sites of infection in systemic campylobacteriosis include peritonitis (Targan, Chow & Guze, 1976), salpingitis (Brown & Sautter, 1977; Davis & Penfold, 1979) and cholecystitis (Mertens & DeSmet, 1979).

2. Campylobacter enteritis

In 1946, Levy reported an outbreak of gastroenteritis in two Illinois penal institutions; 357 of the 6019 inmates were affected. It was found that consumption of milk from a single dairy was epidemiologically associated with the illness. He isolated a "Spirillum" like bacterium from 13 of 39 blood cultures from patients with diarrhoea; the strains were lost on subculture but they appeared to be quite similar to Vibrio jejuni isolated by Jones et al., (1931) from bovine diarrhoea infections.

King (1957) called the strains isolated from blood cultures of children with bloody diarrhoea "related vibrios"; these strains were biochemically idential to V. fetus but with different optimal growth temperatures i.e. 43°C as opposed to 37°C. These strains were later called C. fetus ss. jejuni (Smibert, 1974). Wheeler & Borchers (1961) and Middelkamp & Wolf (1961) also reported cases of bloody diarrhoea in young children caused by "related vibrios". Subsequent to these

reports of infection in infants, the association of C. fetus ss. jejuni with acute enteritis in adults has been reported (Mandel & Ellison, 1963).

The first positive stool cultures of C. fetus ss. jejuni were reported by Dekeyser et al., (1972). The stool specimens were filtered through a 0.65μ millipore filter and cultured in a 5% O_2 atmosphere (p.17). Following this isolation method, Cadranel et al., (1973) and Butzler et al., (1973) reported the isolation of C. fetus ss. jejuni from the stools of patients with diarrhoea in Belgium. Skirrow (1977) using a selective medium (p.17), found that C. fetus ss. jejuni could be isolated from 7.1% of patients with diarrhoea; he corroborated Butzler's finding that these organisms are a common cause of diarrhoea.

After Skirrow reported the isolation of C. fetus ss. jejuni, there were many reports of the isolation of this bacterium from patients with diarrhoea in the U.K. (Dale, 1977; Tanner & Bullin, 1977; Pearson et al., 1977; Brunton & Heggie, 1977; Bruce, Zochowski & Ferguson, 1977; Simmons & Gibbs, 1977; Simmons, 1977; Knill, Suckling & Pearson, 1978). Enteritis caused by this organism is world wide; C. fetus ss. jejuni has been reported to have been associated with diarrhoeal conditions in Canada (Pai et al., 1979; Karmali & Fleming, 1979a, c); United States (Wang, Blaser & Cravens, 1978; Blaser & Wang, 1978; Bokkenheuser et al., 1979); Rwanda (De Mol & Bosmans, 1978); South Africa (Schewitz & Roux, 1978; Richardson & Koornhoof, 1979); Australia (Cavanagh, 1978); Spain (Brea, Molina & Baquero, 1979); Holland (Severin, 1978); Sweden (Lindquist, Kjellander & Kosunen, 1978) and Bangladesh (Greenough, W.B. International Centre for Diarrhoeal Disease Research, Decca, Bangladesh. Personal communication, 1980).

Table 8 : Reported cases of C. fetus ss. jejuni enteritis in Weekly Report Communicable Diseases of Scotland.

Year	Reported cases in CDS			Total
	Sex			
	Male	Female	Unknown	
1977[a]	73	72	3	148
1978	535	432	194	1161
1979	730	638	15	1383
1980[b]	423	372	4	799

a. Reported cases started July.

b. This survey was conducted until August.

In Scotland, the first case of C. fetus ss. jejuni enteritis was reported in July, 1977 (Communicable Diseases of Scotland, CDS). The number of reported cases increased in both 1978, 1979 and 1980 (Table 8), and it is evident from these data, C. fetus ss. jejuni is a common enteric pathogen in Scotland. In 1979, there was an outbreak of acute gastroenteritis involving C. fetus ss. jejuni in the Grampian region, Scotland (CDS, 1979), at least 200 persons were known to have been affected, and 38 positive stool cultures of C. fetus ss. jejuni were reported over a 4 week period.

All ages have been affected, Skirrow (1977) and Karmali & Fleming (1979c)reported a peak incidence in the 1 to 5 year old age group. In Scotland, the peak incidence in males was found in the 1-5 year old age group, while in females, it was among the 16-25 year olds. (Fig. 1).

Schewitz & Roux (1978); Pai et al., (1979); Jones (1979) reported that C. fetus ss. jejuni was as common as S. typhimurium and other Salmonella species in diarrhoeal faeces. In Scotland, C. fetus ss. jejuni was the second most commonly isolated enteric pathogen in 1979 (Fig. 2).

2a. Epidemiology

The source of infection in cases of Campylobacter enteritis is not known. Person-to-person transmission has been suggested (Pai et al., 1979), and members of the same family with a positive culture of C. fetus ss. jejuni have consistently been reported in Scotland (CDS, 1978, 1979). Direct contact

Fig. 1 : Reported cases of enteric campylobacteriosis in Scotland, 1977-80.

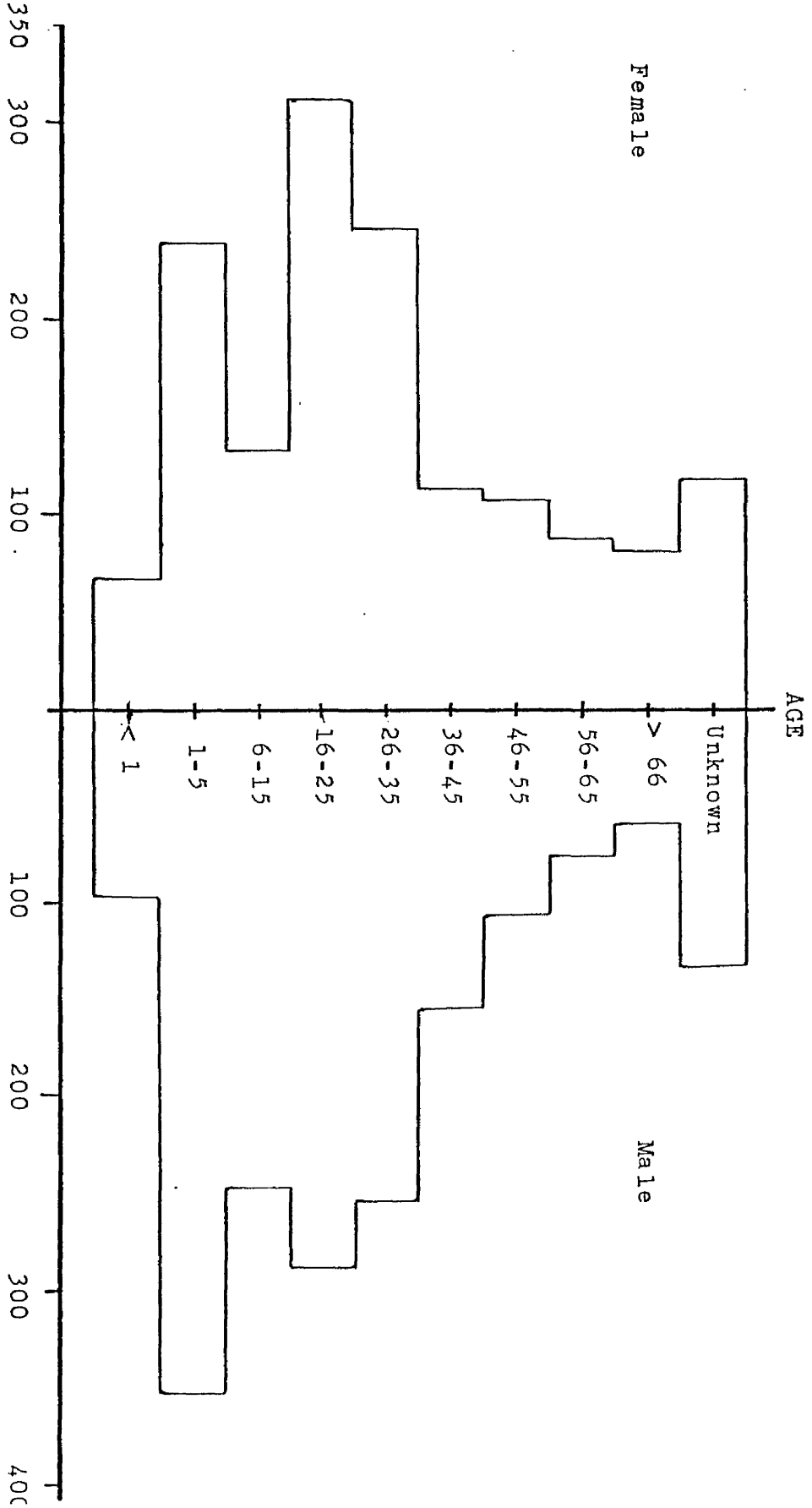
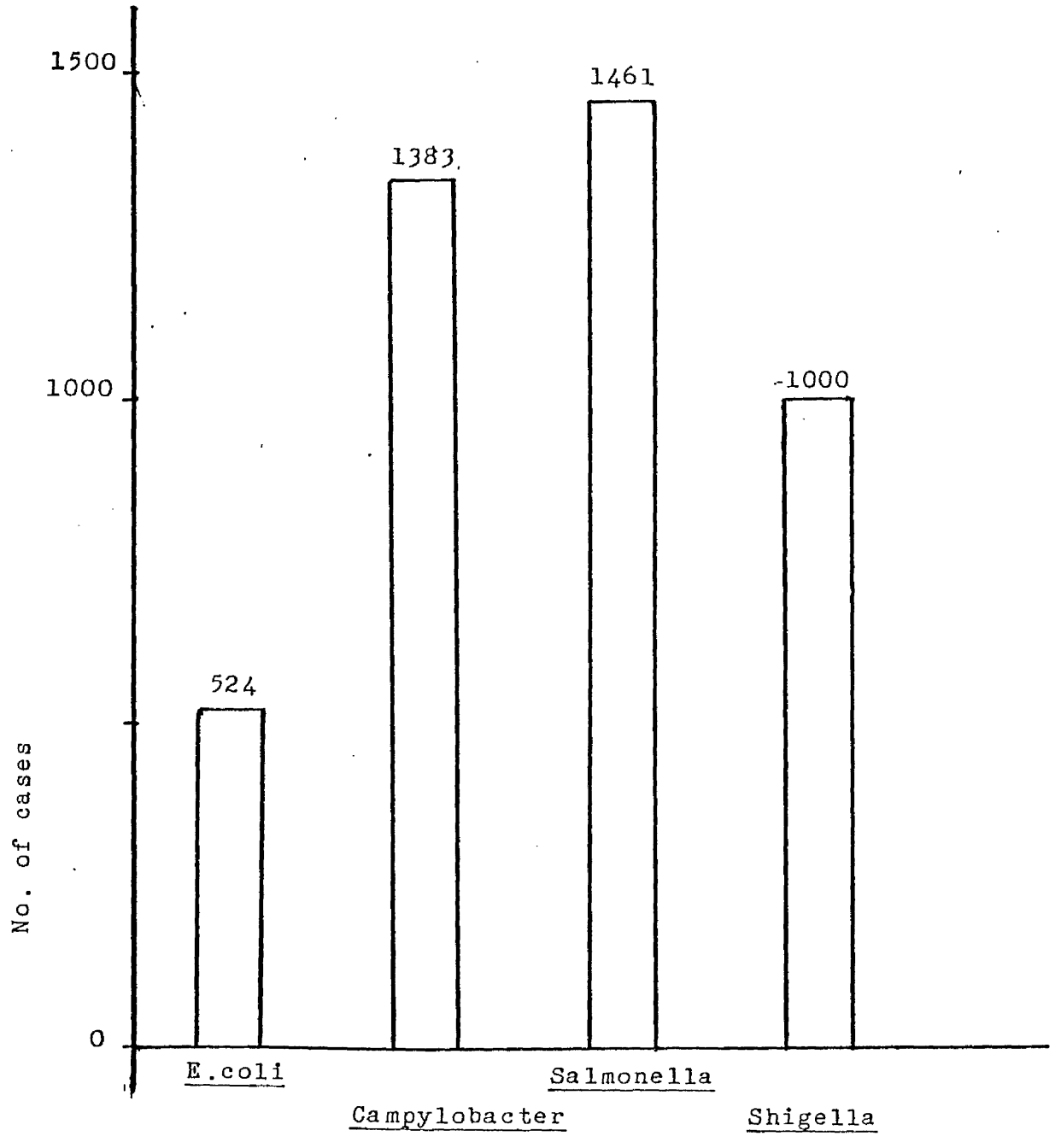


Fig. 2 : Numbers of bacterial species isolated from alimentary infection in Scotland, 1979.



with infected farm or domestic animals may be a route of infection (Skirrow, 1977; Butzler et al., 1978; Hastings, 1978). Duffell & Skirrow (1978) first documented the evidence of a link between Campylobacter abortion in ewes and human enteritis. Wheeler & Borchers (1961) reported a case of Campylobacter enteritis associated with a young dog with bloody diarrhoea; Lindquist, Kjellander & Kosunen (1978) reported the isolation of C. fetus ss. jejuni from two patients and the family dog had had bloody diarrhoea. Blaser et al., (1978) reported 5 cases of human enteric campylobacteriosis associated with infected dogs, C. fetus ss. jejuni was isolated from the faeces of both dogs and patients.

Ingestion of contaminated foods may be the route of some infections (Ribiero, 1978; Zelinger & Vargas, 1978). C. fetus ss. jejuni had been isolated from milk filters (CDS, 1978), and consumption of milk contaminated with Campylobacter has also been reported (CDS, 1978). Taylor, Weinstein & Bryner (1979) reported an outbreak of human enteric campylobacteriosis that was associated with raw milk in California. Uncooked chicken may be another means of infection (Skirrow, 1977; Brouwer et al., 1979). Simmons & Gibbs (1979) isolated Campylobacter spp. from oven-ready chickens.

2b. Clinical features of Campylobacter enteritis

The most common symptoms of Campylobacter enteritis are fever, diarrhoea and the presence of frank blood in the stools (Skirrow, 1977; Karmali & Fleming, 1979c). The diarrhoea is generally watery, profuse and foul smelling. Blood-streaked stools usually appeared after 1 to 3 days of the symptoms. Ninety-two percent of the patients with diarrhoea reported

by Karmali & Fleming (1979a) in Canada had blood or mucus in the stools, however, only 14% of 1336 patients reported in Britain in 1977 showed blood streaked stools (Communicable Disease Surveillance Centre, 1978).

Abdominal pain was an early symptom (Dale, 1977; Butzler et al., 1973), nearly all patients suffering mild or severe abdominal pain. The pain is periumbilical, cramping and may antedate other symptoms or may persist after the return of normal stools. Vomiting and dehydration are unusual, fever is variable in severity, but at least mild temperature elevation is usually present (Skirrow, 1977).

The duration of diarrhoea varies. It may last 10-14 days (Skirrow, 1977), 8-39 days (Jones, 1979) and 7 days to 21 days (Bruce, Zochowski & Ferguson, 1977).

Deaths from Campylobacter enteritis are rare. Deaths have only been reported in an 8 day-old infant (Hallett, Botha & Logan, 1977), in a five-month old baby (Evans & Dadswell, 1967) and a middle-aged farmer with cirrhosis (King, 1962).

2c. Chemotherapy

Antibiotics have been widely used in the treatment of human campylobacteriosis. All strains of C. fetus isolated from humans are highly sensitive to chloramphenicol, dihydrostreptomycin, erythromycin and streptomycin in vitro, and resistant to bacitracin (Willis & Austin, 1966; Darrell, Farrell & Mulligan, 1967), benzylpenicillin and novobiocin (Kahler & Sheldon, 1960; Collins, Blevins & Benter, 1964).

In the study of the sensitivity of 140 human strains of *C. fetus ss. jejuni* and *C. fetus* (subspecies not given) to 12 antibiotics *in vitro*, Butzler, Dekeyser & LaFontaine (1974) reported that gentamicin and erythromycin were the most active drugs against *C. fetus ss. jejuni*. Chow, Patten & Bednorz (1978) tested 11 clinical isolates of *C. fetus* (subspecies not given) against 22 antibiotics, they found that tetracyclines and clindamycin were most effective. Vanhoof *et al.*, (1978) tested 95 strains of *C. fetus ss. jejuni* from human stools against 29 antibiotics and corroborated the previous finding of high sensitivity to gentamicin, minocycline and also furazolidone *in vitro*.

C. fetus ss. jejuni strains are in general resistant to benzylpenicillin, the cephalosporins, polymyxin B, vancomycin, novobiocin and trimethoprim. Based on *in vitro* sensitivities, tetracycline, erythromycin have been suggested as potentially effective treatments (Butzler, Dekeyser & LaFontaine, 1974; Skirrow, 1977; Karmali & Fleming, 1979c).

Walder & Forsgren (1978) reported that 10% of *C. fetus ss. jejuni* strains isolated from patients with diarrhoea were resistant to erythromycin, subsequently, Vanhoof *et al.*, (1978) also reported a similar frequency of erythromycin resistance in *C. fetus ss. jejuni*. However, Brunton, Wilson & MacRae (1978) reported that among 407 *C. fetus ss. jejuni* strains isolated from patients with diarrhoea, only 2 strains (0.5%) were resistant to erythromycin.

IV. ANIMAL DISEASES CAUSED BY CAMPYLOBACTER SPECIES

The genus Campylobacter can cause infectious infertility in cattle and sheep, and is associated with swine dysentery, but the most important result of infection is abortion in cattle.

The main subspecies of C. fetus involved in infectious diseases in farm animals is C. fetus ss. fetus (Clark, 1971). C. fetus ss. fetus is associated with bovine infertility. In bulls, the organism is confined to the preputial cavity and in particular to the mucosa of the glans penis, prepuce and the distal portion of the urethra. In heifers and cows, the sites of infection are within the lumen of the vagina, cervix, uterus and oviducts. The transmission of infection occurs during coitus, or with artificial insemination of contaminated semen and movement of cows for service.

Another subspecies, C. fetus ss. intestinalis is also involved in bovine infection, it is associated with sporadic abortion and infertility in cattle, however, it is transmitted by ingestion of contaminated food and water (Park et.al.,1962). C. fetus ss. fetus can not survive or grow in the intestinal tract of cattle, whereas C. fetus ss. intestinalis can, this organism can be recovered from faeces and bile of naturally infected cattle (Bryner, O'Berry & Frank, 1964). Bryner et al., (1964) isolated C. fetus ss. intestinalis from the gallbladders of diseased cattle and sheep.

Campylobacter infection in sheep is with the subspecies intestinalis and jejuni. Pregnant ewes naturally infected with C. fetus aborted in the later stages of pregnancy

(Dennis, 1961). Bryans & Shepard (1961) reported that infected ewes developed bacteraemia, and the bacteria could cause foetal death. The infection in sheep is not transmitted venereally (Firehammer, Marsh & Tunicliff, 1956), but by ingestion of contaminated materials (Miller, Jensen & Gilroy, 1959) brought into a sheep pen on boots (Miner & Thorne, 1964); the birds found around sheep pens and barns (Waldhalm *et al.*, 1964; Dennis, 1967; Smibert, 1969).

C. fetus is associated with swine dysentery (Doyle, 1944, 1948). Peckham (1958); Hofstad, McGehee & Bennett (1958); Truscott & Morin (1964) and Hagan (1964) reported that *C. fetus* (subspecies not given) was associated with infectious hepatitis and bluecomb disease, Truscott & Stockdale (1966) found that the organism could be reisolated from bile, intestines, livers and caeca of diseased poultry. Fletcher & Plastridge (1964) stated that strains of *V. fetus* isolated from poultry grew at 42°C, and were biochemically similar to human isolates; Smibert (1974) identified these strains as *C. fetus ss. jejuni*. In 1974, Smith & Muldoon reported that *C. fetus ss. jejuni* could be reisolated from commercially processed poultry.

V. LABORATORY INFECTION OF ANIMALS WITH CAMPYLOBACTER STRAINS.

1. C. fetus from animal sources

McFadyean & Stockman (1913) and Smith (1923) showed that cattle could be experimentally aborted by intravenous inoculation of live suspensions of V. fetus (cited McEntee, Hughes & Gilman, 1954). In 1919, Smith found that V. fetus was non-pathogenic for guinea pigs, rabbits, rats and mice by the intraperitoneal route of inoculation (cited Plastridge & Williams, 1943), but Lerche (1937) found that pregnant guinea pigs were susceptible to V. fetus injection through intraperitoneal, and subcutaneous inoculations (cited Ristic & Morse, 1953). Ristic et al., (1954) found that pregnant guinea pigs could be infected experimentally by intraperitoneal injection of 1ml of V. fetus suspension. They also reported that pure cultures of V. fetus could be isolated from the aborted foetuses. In 1959, Miller, Jensen & Gilroy showed that experimental abortion in sheep could be achieved by oral administration of V. fetus cultures.

Osborne (1965) and Osborne & Smibert (1964) reported that a single intravenous injection of whole cell cultures in saline and cell free supernatant fluid of V. fetus into cattle, sheep and pigs, gave either reversible or irreversible shock and a local and generalized Shwartzman reaction. In 1965 Osborne reported that following a single 2ml intravenous injection of whole cell cultures of V. fetus (containing 10^9 cells per ml) into cattle, oedema, disseminated intravascular coagulation, stasis, embolism and thrombosis were prominent histopathologic features.

Clinical and pathological signs of swine dysentery could not be produced by oral administration of V. coli into gnotobiotic pigs (Andress, Barnum & Thomson, 1968). The bacteria were isolated from pigs with clinical signs of swine dysentery. One millilitre of a 48 hr culture grown in heart infusion broth was injected into a can of simulated sows' milk. The cans were sealed by electrocautery and incubated for 48 hr. The cans were sterilized in 2% peracetic acid for 2 hr, and then introduced into the isolator and the content fed to the pigs. In 1968, Andress, Barnum & Thomson also mentioned that by feeding gnotobiotic pigs with artificially infected, minced intestine derived from gnotobiotic pigs, no clinical signs of swine dysentery were produced and pure cultures of V. coli were reisolated from stomach, duodenum, jejunum, ileum, caecum, and colon.

In 1970, Osburn & Hoskins stated that when pregnant cows were inoculated in the jugular vein with 1ml of V. fetus cells resuspended in saline to obtain a final concentration comparable to that obtained with McFarland tube No. 3, the animals aborted in 14-15 days; and the bacteria were reisolated from the lungs, abomasum and liver of the aborted foetuses. Bryner et al. (1971) showed that when 3 biotypes of C. fetus (type 1, subtype 1 and type 2) (Bryner et al., 1962) were injected parenterally into cattle, sheep, rabbits, guinea pigs, and mice, the bacteria could be recovered from all the inoculated animals. The inocula were prepared from 3-day old cultures grown on blood agar and resuspended in broth. The inoculum for cattle intravenous administration contained 10^8 to 10^{10} viable cells per ml, and

the subcutaneous inoculum contained 10^{11} viable cells per ml. The sheep were inoculated i.v., the inoculum contained 10^{7} to 10^{11} viable cells per ml. The inoculum for other animals was as follows : 3 x 10^{9} cells per rabbit given intravenously; 2 x 10^{9} cells per guinea pig given intraperitoneally; 10^{9} cells per mouse given intraperitoneally. Bryner et al., found that no cattle died after the inoculation, and C. fetus could be reisolated from gallbladder, intestine and genital organs. One of 5 inoculated ewes died 12 hr after the injection of C. fetus Type 1, and 3 of 7 inoculated ewes died at 12, 19 and 24 hr after the inoculation of Type 2; sub type 1 was not used in sheep experiments, and the bacteria were re-isolated from blood, gallbladder and intestine. None of the rabbits died as the result of i.v. inoculation of C. fetus. Eighteen of the 46 guinea pigs inoculated died after the inoculation, and C. fetus could be reisolated from gallbladder; duodenum of dead animals, and from the animals killed on the 12^{th} day after the inoculation. No mice died after injection.

Schroeder (1920) postulated that Brucella abortus in cattle paved the way for V. fetus infection, but Ristic & Morse (1953) showed that there was no significant difference between V. fetus infection alone in pregnant guinea pigs in contrast with V. fetus infection superimposed upon Br. abortus infection. They found that by injecting V. fetus suspensions (the density approximated to tube 3 of the McFarland nephelometer) alone, and Br. abortus with V. fetus suspensions, the pregnant guinea pigs aborted at one to twelve days after the inoculation. Zaki, Krishnappa & Murthy (1979) reported the isolation of Br. abortus and C. fetus from the heart blood of aborted foetuses; however, they did

not suggest that Br. abortus predisposed to C. fetus infection.

2. C. fetus ss. jejuni from human patients with diarrhoea.

Attempts to establish Campylobacter enteritis in chickens using strains of C. fetus ss. jejuni isolated from children with diarrhoea failed (Butzler et al., 1978). Butzler et al., found that on feeding 10^6 organisms orally to chickens, no signs of enteritis developed in the infected chickens, but the bacteria could be reisolated from the stools, the liver and from the blood. Prescott & Karmali (1978) reported that human enteritis could not be established in cats and dogs by oral administration of 2.5×10^{10} organism of C. fetus ss. jejuni isolated from children with enteritis illness, but the bacteria could be isolated from the faeces of all the animals for a period of 2 to 3 days after the inoculation. In 1978, Melling (cited Butzler & Skirrow, 1979) found that gastric inoculation of four adult monkeys with a dose of 10^7 related Campylobacters (C. fetus ss. jejuni) from patients with enteritis failed to cause illness, and, in 1979, Chong Park (cited Butzler & Skirrow, 1979) reported that following rhesus monkey challenge with a dose of 10^6 or more organisms, severe enteritis developed in 2 of 3 rhesus monkeys after 13 days, but with a dose of 10^4 or 10^2 organisms, no signs of enteritis were reported. However, Steele & McDermott (1978) reported that Campylobacter enteritis could be produced in man by ingesting 10^6 pure culture of C. fetus ss. jejuni isolated from patients with diarrhoea; and Prescott & Karmali(1978) also reported that Campylobacter infection could be established in man through accidental acquisition of the organism in the laboratory.

The effect of C. fetus ss. jejuni on laboratory mice has not been studied. Reports on the use of laboratory mice, in attempts to establish Campylobacter enteritis with strains isolated from humans, could not be found in the literature.

VI. THE SEROLOGY OF CAMPYLOBACTER

1. Introduction

Little work has been done so far in studying the antigenic structure of C. fetus ss. jejuni isolated from human diarrhoeal patients. The antigenic relationships of the isolates of V. fetus (C. fetus) from human vibriosis have not been studied. At present, most of the information concerning the serology of Campylobacter has been obtained on the C. fetus isolated from animal sources.

2. Serology of animal Campylobacter strains

Animal strains are not identical serologically (King, 1957). Price, Poelma & Faber (1955) showed differences in the antigenic composition of C. fetus strains isolated from bovine and ovine sources; using heated cell suspensions (two hours boiling at 100°C) of 50 strains of bovine and ovine origin, they reported that the somatic antigens of the bovine strains were of 4 different types (Type I, II, III and IV) and one serologically unrelated ovine strain was designated as Type V.

Marsh & Firehammer (1953) investigated the serological relationships of 23 ovine and 3 bovine strains of C. fetus (antisera produced by immunizing rabbits with 0.3% formalinized bacterial cells); these authors reported that ovine strains fall into 4 types while the 3 bovine strains are of the fifth type. Using bovine and ovine strains of C. fetus and rabbits immunized with O antigens (prepared by boiling under a reflux

condenser for 2 hr), Morgan (1959) reported that the somatic antigens of C. fetus could be divided into 2 serotypes i.e. Serotype A and B. By absorbing OH sera (produced by immunizing rabbits with bacterial cells resuspended in 0.3% formol saline) with homologous O suspensions, he reported that nearly all the strains contained a common H component. Using phenol extracts of C. fetus in complement fixation tests, Mitscherlich & Liess (1958) divided C. fetus into 2 serotypes, Type 1 and 2; and C. fetus ss. bubulus represented another serotype.

In studying the biochemical and serological characteristics of 30 fresh vibrio isolates of bovine origin, Walsh & White (1968) reported that 18 strains of C. fetus type 1 (later identified as subspecies fetus) and C. fetus subtype 1 (also identified as subspecies fetus) were serologically related. Three strains of C. fetus type 2 (one representing ss. intestinalis and 2 representing ss. jejuni, based on the growth at 25°C and 42°C respectively) had greater serologic variability, and the authors found that these strains did not cross-react with each other. The other 4 strains belonged to C. sputorum ss. bubulus (V. bubulus), and 5 strains were unclassified. Their results indicated that V. bubulus strains were not cross-agglutinated by antisera of C. fetus ss. jejuni or C. fetus ss. intestinalis, and antigenically C. fetus ss. fetus represents a much more homologous group than does C. fetus ss. intestinalis or jejuni.

By combining the results of a biochemical typing system with results of a serologic typing system, Berg, Jutila & Firehammer (1971) reported that C. fetus from animal sources could be divided into 5 groups :

Serotype A - biotype 1 (glycine negative, H_2S negative)

Serotype A - biosubtype 1 (glycine negative, H_2S positive)

Serotype A - biotype 2 (glycine positive, H_2S positive)

Serotype B - (glycine positive, H_2S variable).

Serotype C - (glycine positive, H_2S positive, grew at 43°).

3. Serology of human *Campylobacter* infections

White & Walsh (1970) studied 12 human strains isolated from blood (identified as *C. fetus* ss. *intestinalis*) and divided them into two serotypes, on their heat-stable somatic antigens; results with flagellar antigens were less clear, resulting in many serotypes.

In the serology of human *Campylobacter* infections, many investigators have found agglutinating antibodies to *Campylobacter* in patients' sera (Hood & Todd, 1960; Jackson, Hinton & Allison, 1960; Wheeler & Borchers, 1961). Gubina *et al.*, (1976) reported that when the sera of patients with campylobacteriosis were tested against homologous isolated strains there was a rise in the antibody titre. Urman, Zurier & Rothfield (1977) had shown that during the patient's first attack by *C. fetus*, there was a striking increase in titre from zero to 1:28, but during the second attack the titre rose from 1:64 to 1:512. After treatment, the titre declined progressively and was negative after recovery.

Butzler (1973) using a complement fixation test found that most of the sera from patients with positive stool cultures gave titres of between 1:4 and 1:64 and that 90% of the

organisms cross-reacted with one or other of two control *C. fetus ss. jejuni* strains. Blaser *et al.*, (1978) used immunofluorescence techniques to examine acute and convalescent phase sera from 5 patients; all showed 4 fold or greater rises in titre for IgG class antibody, the highest titres being 1:512. Watson, Kerr & McFadzean (1979) found a rapid antibody response in campylobacteriosis, and reported that 77.2% of sera of 66 patients with diarrhoeal illness due to *C. fetus ss. jejuni* gave agglutination titres of 1/320 against 3 reference H antigens of *C. fetus ss. jejuni*.

There have not been any reports published in the literature regarding the serotyping of *C. fetus ss. jejuni* isolated from human patients with diarrhoea.

VII. MUCIN AND FERRIC AMMONIUM CITRATE IN EXPERIMENTAL INFECTION

1. Introduction

In experimental infections of laboratory animals with a variety of bacteria, the work has been handicapped by the fact that several million of bacteria are necessary to produce a fatal infection. When a massive dose is injected, a fatal outcome may be interpreted as due merely to the action of endotoxin liberated in the body through disintegration of the injected bacteria, or by exotoxic substances produced by bacteria. There are reports that by incorporating bacterial suspensions with virulence enhancing agents, the number of bacteria required to establish laboratory infection could be greatly reduced. Mucin and ferric ammonium citrate are two widely used virulence enhancing agents and a brief introduction is therefore given here.

2. Mucin

In 1932, Nungester, Wolf and Jourdonais reported that the fatal dose of Staph. aureus causing death in mice could be lowered from 10^6 to 10^3 bacteria with the aid of 5% mucin. Nungester & Jourdonais (1936), Nungester, Jourdonais & Wolf (1936) found that white rats were highly susceptible to the subcutaneous injection of Diplococcus pneumoniae, the pneumococci appearing in both blood and lung after the administration of a dose of 10^5 bacteria, but the symptoms of lobar pneumonia were only produced if the bacteria were simultaneously intratracheally administered

with 5% mucin. In 1939, Anderson & Oag also reported that treatment with mucin lowered the fatal dose of the R form of D. pneumoniae from more than 10^9 to 10^6 organisms in mice. In the literature, mucin preparations of different origins e.g. hog gastric mucin, salivary mucin, bovine submaxillary mucin have been shown to lower the minimum number of organisms of low pathogenicity that can cause a fatal infection in an otherwise resistant animal. Table 9 shows the different types of bacteria examined.

Rake (1935) reported that the virulence of bacteria did not increase when grown in vitro in the presence of mucin. Nungester, Jourdonais & Wolf (1936) found that Bacillus anthracis grown in a medium containing nutrient glucose (0.1%) broth and 10% gastric mucin did not appreciably increase in virulence. Nungester, Jourdonais & Wolf (1936) reported that mucin helps B. anthracis to survive in the animal body, and that, in addition, it increases the virulence of the surviving bacteria. However, Miller & Castles (1936) found that meningococci recovered from the peritoneal exudate and blood of moribund mice injected with 5% mucin at 13 hr proved no more virulent than cultures grown on artificial medium; the mortality of mice inoculated with a dose of 10^5 was the same for both cultures. Fothergill, Dingle & Chandler (1937) observed that after 10 passages through mice together with mucin, the ability of Haemophilus influenzae to kill mice in the absence of mucin was not increased.

In 1936, Miller & Castles found that 5% mucin which was most effective in promoting meningococcal infection in the mouse did not support bacterial growth in vitro. Keefer & Spink (1938)

Table 9: Enhancing effect of mucin on species of bacteria reported in the literature.

Species	References
Pseudomonas aeruginosa	Cooper, Gross & Lewis (1939)
Staphylococcus aureus	Ercoli, Lewis & Harker (1945) Tunnicliff (1940)
Neisseria gonorrhoeae	Keefer & Spink (1938) Miller & Bohnhoff (1946)
Escherichia coli	Cooper, Gross & Lewis (1939) Klinefelter (1941) Steinberg & Goldblatt (1927)
Klebsiella pneumoniae	Nungester, Jourdonais & Wolf (1936)
Salmonella typhimurium	Buttle et al., (1937)
Neisseria meningitidis	Miller (1933) Miller & Foster (1944)

also reported that mucin did not support growth of N. gonorrhoeae in vitro, but they found that gonococci survived up to 28hr in mucin, and in 1964, Sims also reported that N. gonorrhoeae remained viable for 24 hr in a mucin suspension at 37°C. Rogers (1948) reported that mucin could be a nutritive source facilitating bacterial survival in vitro, but McLeod (1941a,b) had shown that 5% mucin did not help meningococci to multiply in the peritoneal fluid in vivo.

Nungester, Jourdonais & Wolf (1936) reported that mucin did not interfere with phagocytosis, but it prevented the intracellular digestion of bacteria in the polymorphs. Keefer & Spink (1938) found that mucin lowers the bactericidal effect of fresh mouse serum on the destruction of meningococci, and Miller & Castles (1936) reported that mucin renders the barrier between the peritoneal cavity and the vascular system permeable, allowing the injected bacteria to invade the blood stream. Olitzki (1948) in his review suggested that mucin acts by forming a protective coating on bacteria, and interfering with the RES mechanism of the animal; he considered that mucin did not enhance the virulence of bacteria.

Laboratory animals e.g. rabbits, guinea pigs and mice are normally highly resistant to the genus Mycoplasma (Sheriff & Piercy, 1952). M. mycoides var. capri is the causal agent of contagious caprine pleuropneumonia or so-called oedema disease of Spartan goats (Edward, 1953); the experimental infection could be produced in goats by artificial droplet infection or subcutaneous infection, but not in mice or rabbits (Yamamoto, Adler & Cordy, 1955). But, in 1965, Smith reported that the

resistance of laboratory animals could be reduced by treatment with mucin; he produced severe pleurisy and pericarditis in rabbits by the intrathoracic inoculation of a dose of 125 x 10^6 M. mycoides var. capri suspended in 5% mucin. Smith (1967b) also reported that infection with M. mycoides var. capri could be produced in mice injected i.p. with 5% mucin; Smith found that M. mycoides var. capri was rapidly eliminated, as shown by negative blood culture, following the i.p. inoculation of a dose of 125 x 10^6 bacteria; but in the presence of 5% mucin, the organism was reisolated from the blood of all the inoculated mice 24 hr after inoculation.

M. agalactiae and M. mycoides are the other two species of Mycoplasma which cause disease in animals. M. agalactiae is the cause of contagious agalactia of sheep and goats, and M. mycoides is responsible for contagious pleuropneumonia (Turner, 1959). Infection with both species can be produced in cattle, sheep and goats by subcutaneous inoculation of the organisms alone (Castrejon-Diez, Fisher & Fisher, 1963). Smith (1967a,1967b) reported that infection with M. agalactiae and M. mycoides could be established in mice by injecting a dose of 297 x 10^6 and 207 x 10^6 organisms with 5% mucin, respectively. Mice are normally resistant to Mycoplasma.

Although the virulence-enhancing effect of mucin is well documented, I have been unable to find any papers published in the last 10 years reporting the use of mucin in laboratory infection experiments.

3. Ferric ammonium citrate

Iron compounds are essential for bacterial growth (Stephenson, 1949). Weinberg (1974) reported that Gram-negative bacteria need 0.3 to 1.8μg iron per ml and most Gram-positive bacteria need from 0.4 to 4μg iron. The concentrations of iron required for growth of a variety of common bacteria are shown in Table 10. Weinberg (1966), Bullen, Leigh & Rogers (1968) reported that the ability of a bacterium to compete successfully with the host for iron is an important factor in the establishment of many bacterial infections and is a feature of pathogenicity. Schade & Caroline (1946) first reported that iron salts could abolish the antibacterial effects of iron-binding proteins present in egg-white and serum. Pekarek et al., (1969) found that the iron concentration in sera of human volunteers decreased after exposure to an aerosol of virulent Francisella tularensis. Beresford, Neale & Brooks (1971) found that the host responds to bacterial infection by reducing the amount of iron in the blood plasma.

In experimental infection of laboratory animals, ferrous sulphate and ferric ammonium citrate have been used to enhance bacterial virulence. Jackson & Burrows (1956) reported that injection of a dose of 6.5×10^4 organisms of Pasteurella pestis with 80μg of iron supplied in the form of $FeSO_4.7H_2O$ increased the number of deaths in mice (average weight 18-29g); there were no deaths in the group of mice injected with the bacterial suspension alone, and 100% mortality (10/10) in the group of mice injected simultaneously with 80μg iron; they also reported that iron increased the virulence of non-pigmented P. pestis strains

Table 10 : Minimal quantity of iron required for complete cell growth in "demineralized" synthetic media. (Weinberg, 1974).

Species	Iron (µg/ml)
Bacillus megaterium	0.4
Bacillus subtilis	0.4
Enterobacter aerogenes	0.3
Escherichia coli	0.5
Klebsiella pneumoniae	0.5
Leptospira sp.	1.0
Mycobacterium phlei	4.0
Mycobacterium tuberculosis	1.0
Pseudomonas aeruginosa	0.3 - 1.5
Salmonella typhimurium	1.0
Staphylococcus aureus	1.6

in mice. The injection of a dose of 10^3 non-pigmented P. pestis is not lethal for mice. However, by incorporating 80 μg Fe^{3+} along with the suspension, all the 10 injected mice died.

Martin, Jandl & Finland (1963) reported that incorporation of ferric ammonium citrate with suspensions of K. pneumoniae and Ps. aeruginosa reduced the LD_{50} value in rats and mice. The dose of iron was 1000 μg per rat (average weight 190 to 210 g) and 40 μg per mouse (average weight 15g). Martin et al., reported that i.p. administration of iron with a suspension of K. pneumoniae reduced the LD_{50} value from 10^9 to 10^7 bacteria in rats, and from 10^7 to 10^6 bacteria in mice; they also reported that using the same concentration of iron with Ps. aeruginosa, the LD_{50} value in rats was reduced from 10^8 to 10^4 organisms.

In an experiment with L. monocytogenes, Sword (1966) reported that ferric ammonium citrate decreased the LD_{50} value in mice. The dose of Fe^{3+} given was 80 μg per mouse (average weight 18 to 20g). Sword found that with the incorporation of iron, the LD_{50} value was reduced from 7.2×10^3 to 1.8×10^2 bacteria. In 1965, Brubaker, Beesley & Surgalla reported that following i.p. injection of a dose of 3.8×10^5 bacteria of P. pestis with 40 μg Fe^{3+} per mouse, the LD_{50} value could be reduced to 1.4×10^1 bacteria. Consequently, the reduction of the LD_{50} value in mice with the incorporation of iron has also been reported for S. typhimurium (Chandlee & Fukui, 1965); Cl. perfringens (Rogers, 1967; Bullen & Rogers, 1969; Bullen, Cushnie & Rogers, 1967); P. septica (Bullen & Rogers, 1969 ;

Table 11 : Effect of exogenous iron on number of organisms per g of tissue during the course of experimental infection in mice. (Modified from Weinberg, 1974)

Organism	Organ	Time after inoculation (Hr)	No. of bacteria (thousand)		Reference
			No iron	iron	
L. monocytogenes (virulent strain)	Liver	24	0.1	0.1	Sword (1966)
		72	17	1,400	
		120	3	19	
L. monocytogenes (avirulent strain)	Liver	24	0.1	0.18	Sword (1966)
		72	0.3	220	
		120	0.035	220	
Cl. perfringens	Muscle	4	1,300	1,300	Bullen, Cushnie & Rogers (1967)
		8	1,000	18,000	
		12	100	60,000	
		16	13	80,000	
E. coli	Kidney	24	5.5	16	Fletcher & Goldstein (1970)
		72	30	3,000	
		120	10	10,000	

Bullen, Rogers & Lewin, 1971; Griffiths, 1971); E. coli (Bullen, Leigh & Rogers, 1968; Bornside, Bouis & Cohn, 1968; Bullen & Rogers, 1969; Griffiths, 1971); Ps. aeruginosa (Forsberg & Bullen, 1972; Miles, Khimji & Maskell, 1979) and V. cholerae (Joo, 1975).

Payne & Finkelstein (1975) reported that the addition of 62.5 μg of Fe^{3+} to inocula of the avirulent colony types of gonococci T3 and T4 increased their lethality for chick embryos after intravenous inoculation. In 11 separate experiments, Payne & Finkelstein found that the LD_{50} values for chick embryos of T3 and T4 gonococci with iron ranged from 1.2×10^5 to 1.4×10^6 bacteria, and supplementation of the inocula with 62.5 μg of iron compounds resulted in a 75-fold reduction of the mean LD_{50} to 8.1×10^3 bacteria.

The addition of 0.15, 0.5 and 2 μg of iron per ml greatly enhanced the growth of E. coli in vitro (Ratledge & Winder, 1964). Sword (1966) also reported that the addition of 10 and 100 μg of Fe^{3+} per ml increased the cell yields of L. monocytogenes in vitro. Iron compounds can also have a significant effect on the growth of bacteria in vivo (Table 11). The number of viable organisms recovered from organs, for which bacteria have an affinity, was considerably greater in animals injected with iron than in controls, and this would seem to indicate that the bacteria could multiply in vivo in the presence of Fe^{3+}.

Burrows (1962) found that iron compounds had no effect on the mobilization of polymorphs, or on their ingestive capacity, or on the formation of antibodies against P. pestis. However, Gladstone & Walton (1970) reported that the bactericidal action

of the basic protein of rabbit polymorphonuclear leukocytes was inhibited by the addition of Fe^{3+}; they found that by incubating the washed cells of staphylococci, B. anthracis, Strep. faecalis, Ps. aeruginosa and E. coli in rabbit polymorphonuclear leukocytes with ferric ammonium citrate, the bacterial action of leukocyte basic protein was abolished, and consequently, the organisms multiplied. In the case of animals receiving exogenous iron, the excess iron might favour in vivo bacterial growth (Weinberg, 1974).

OBJECT OF RESEARCH

Campylobacter fetus ss. jejuni has recently been recognized as a common cause of diarrhoea in man. The infection is worldwide, and the organism has been cultured from 5% to 30% of patients with diarrhoea. So far there has been no report of a satisfactory animal model for studying infection by C. fetus ss. jejuni, nor have the antigenic inter-relationships of strains of C. fetus ss. jejuni been investigated.

The objects of this research were :

1. To study the effect of C. fetus ss. jejuni on laboratory mice to see if infection could be established.
2. To observe the action of virulence - enhancing agents on C. fetus ss. jejuni in mice
3. To examine the antigenic relationships of strains of C. fetus ss. jejuni.

MATERIALS AND METHODS

SECTION I. MICROBIOLOGICAL MATERIALS AND METHODS

I. Bacterial strains

The strains of *C. fetus ss. jejuni* used in this study included my own isolates made from faecal specimens; isolates obtained from Mr. J. Gibson, Chief Technician, City Laboratory, Glasgow, Scotland; and Dr. M.B. Skirrow, Consultant Microbiologist, Worcester Royal Infirmary, Worcester, England. Two lyophilized strains were obtained from National Collection of Type Cultures (NCTC), Colindale, London.(Table 12).

II. Media

1. Selective medium

The selective medium consisted of Columbia agar base (Oxoid, Basingstoke, Hants) plus 6% lysed horse blood, with the following antimicrobial agents (final concentration per ml):

a. Vancomycin 10μg (Vancomycin HCl, Eli Lilly & Co. Ltd., Basingstoke, England).

b. Polymyxin B sulphate, 2.5 IU (Wellcome Reagents, Kent, England).

c. Trimethoprim lactate, 5μg (Wellcome Reagents, Kent, England).

This selective medium was used for primary isolation of *C. fetus ss. jejuni* from faecal specimens collected from patients with diarrhoea and for viable counting of *C. fetus ss. jejuni* recovered from the homogenates of mouse tissues.

Table 12 : *Campylobacter fetus ss. jejuni* cultures

Strain No.	Origin	Source
6395	Isolated in the Dept.	Patients with diarrhoea
6537	"	"
06238	"	"
06760	"	"
9428	"	"
06342	"	"
03260	"	"
6635	"	"
8346	"	"
00539	"	"
3946	"	"
2680	City Laboratory	Patients with diarrhoea
5702	"	"
4249	"	"
7972	"	"
9227	Dr. Skirrow	children with diarrhoea
5636	"	"
NCTC 5850[a]	NCTC	Animal sources
NCTC 10355	NCTC	"

a. NCTC 5850 *Campylobacter fetus* biotype B.

NCTC 10355 *Campylobacter bubulus*

2. Maintenance medium

Columbia agar base plus 6% defibrinated horse blood was a good medium for culturing Campylobacter after isolation.

3. Broth media

A wide range of broth media was tried to obtain the best medium for mass culture of C. fetus ss. jejuni. These included:

a. Nutrient broth No. 2 (Oxoid, Basingstoke, Hants)

b. Brain heart infusion broth (Oxoid, Basingstoke, Hants)

c. Thioglycollate broth (Difco Laboratories, Detroit, U.S.A.)

d. Nutrient broth No. 2 supplemented with (w/v) : 0.5% yeast extract (Difco Laboratories, Detroit, U.S.A.), 0.02% L-cystine, 0.2% L-glutamic, and 0.03% glycine (Sigma Chemical Company, St. Louis, U.S.A.). The supplements were added as a single addition.

e. Nutrient broth No. 2 supplemented with 0.5% yeast extract and 0.02% L-cystine, and with the following inorganic salts (g/l) added : 5 g $MgSO_4.7H_2O$; 0.02g $CaCl_2$; 0.75g KNO_3 and 0.25g $Na_2H_2PO_4$. (BDH Chemicals Ltd., Poole, England).

f. Nutrient broth No. 2 supplemented with 0.5% yeast extract and 0.02% L-cystine, and with the addition of 40µg, 80µg and 120µg Fe^{3+} per ml. The Fe^{3+} was supplied as ferric ammonium citrate (BDH Chemicals Ltd., Poole, England) and contained 16% Fe^{3+}.

4. Alkaline peptone water pH 8.4

Peptone water (Oxoid, Basingstoke, Hants) was adjusted to pH 8.4 using 1N NaOH. The buffered alkaline peptone water was distributed in 10ml amounts in universal bottles and autoclaved at 15 psi for 15 min.

5. Identification media

In carrying out the biochemical and tolerance tests, nutrient broth No. 2 was used as a basal medium.

5.a. Hydrogen sulphide production

Sensitive medium : To 100ml of basal broth medium. 0.02g of L-cystine (Sigma Chemical Company, St. Louis, U.S.A.) was added. The medium was dispensed in 10ml amounts in universal bottles and autoclaved at 10psi for 20 min. Lead acetate papers (Whatman, BDH) were inserted in the neck of the universal bottles.

TSI agar : Triple sugar iron agar (Difco Laboratories) was also used. The medium was dispensed in 5ml amounts in 6 x $\frac{1}{2}$" test tubes, autoclaved at 15psi for 15 min. The medium was allowed to solidify as agar slopes.

5.b. Tolerance to 1% glycine and 8% glucose

To 100ml of basal broth medium, 1% glycine (BDH Chemicals Ltd., Poole, England) or 8% glucose (BDH Chemicals Ltd., Poole, England) were added. The media were dispensed in 10ml amounts in universal bottles and autoclaved at 10psi for 20 min.

5.c. Tolerance to 3.5% sodium chloride

To 100ml of basal broth medium, 3.5% sodium chloride (Hopkins & Williams, Chadwell Heath, Essex, England) was added. The medium was dispensed in 10ml amounts in universal bottles and autoclaved at 15 psi for 15 min.

5.d. Temperature tolerance

Columbia agar base plus 6% defibrinated horse blood and nutrient broth No. 2 supplemented with 0.02% (w/v) cystine and 0.5% (w/v) yeast extract were used.

6. Sensitivity Test medium

Diagnostic sensitivity test (DST) agar (Oxoid, Basingstoke, Hants) was used. The medium was autoclaved at 15 psi for 15 min. It was cooled to 45°C, and poured as plates after the addition of 6% lysed horse blood.

III. Cultural conditions

1. Special gaseous atmosphere for the growth of C. fetus ss. jejuni

The oxygen in the air at atmospheric pressure is toxic, and the CO_2 concentration should be of a greater concentration than that of air for the growth of C. fetus ss. jejuni (Kiggins & Plastridge, 1956; Cooper & Slee, 1971). All the cultures and primary isolation plates were incubated in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2. These atmospheric conditions were achieved in a McIntosh and Fildes anaerobic jar; the catalysts were removed from the lid, 2/3 of the air was evacuated by

pumping (gauge read 500 mg Hg) and replaced with 10% CO_2/90% N_2 gas mixture (B.O.C., Special Gases, Deer Road, London).

2. Temperature

In the laboratory, the plates for primary isolation of C. fetus ss. jejuni strains from faecal specimens and the recovery of Campylobacter from 3-and 5-wk old mice were incubated at 43°C. For reasons of convenience, for all the other experiments, the plates and flasks were incubated at 37°C.

IV. Isolation of Campylobacter from faecal specimens

A 3 month study was carried out to investigate the frequency of isolation of Campylobacter strains from patients with diarrhoea and from asymptomatic people. The stool specimens were obtained from the City Laboratory, Glasgow, sent to the laboratory for investigation by general practitioners from patients with diarrhoea; in addition, routine faecal specimens from food handlers and workers in nurseries were also cultured. Stool specimens were also obtained from the Department of Bacteriology, Gartnavel General Hospital, Glasgow.

The faecal specimens were inoculated directly onto the selective medium (p 55), and spread as in the normal manner, but it was important that the inoculum should not be so heavy that visible faecal material could be seen on the medium. Faecal specimens were cultured on the same day of arrival. If there was any delay, the specimens were refrigerated.

All plates were incubated at 43°C in 5% O_2, 10% CO_2 and 85% N_2 (p 59).

V. Identification of Strains

1. Microscopic examination

Plates were examined at 24 and 48 hr. In positive specimens a fine growth should be apparent after 24 hr of incubation at 43°C. Colonies that were gray, small, round, convex or flat were Gram-stained, and studied by wet-film under phase-contrast microscopy. In Gram's method, a stronger counter stain 2% carbol fuchsin should be used, otherwise, the morphology will be indistinct. If the Gram staining confirmed the presence of small spiral or S-shaped Gram-negative bacilli, and a characteristic "darting" motility was seen under phase contrast microscopy, the colony was replated onto maintenance agar (see p. 51). Pure cultures were made from the colonies for biochemical identification.

2. Biochemical Identification

2.a. Catalase test

The production of catalase was determined as described by Cowan & Steel (1974). Organisms were grown on nutrient agar slopes at 37°C for 24 hr in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2 (see p. 59), 1ml 3% H_2O_2 was run over the slope, and the culture examined immediately and after 5 min for bubbles of gas.

2.b. Oxidase test

Oxidase activity was determined by Kovacs' method (1956).

Using 1% para-amino-dimethyl-aniline oxalate as reagent, 2-3 drops of the reagent was dropped on a piece of filter paper, and the cultures were smeared across the impregnated paper with a platinum loop. A positive reaction is indicated by the appearance of a dark purple colour on the paper within 10 sec. Ps. aeruginosa was used as a positive control.

2.c. H_2S production

Ability to produce H_2S was determined according to the method of Véron & Chatelain (1973). A loopful of bacteria was emulsified in sterile saline, 5 drops of this suspension were inoculated aseptically into a "standard medium" which was nutrient broth No. 2 and a "sensitive medium" (see p. 58). A lead acetate paper was inserted above the media after inoculation, and reading of the blackening of the lead acetate paper was done after 3 days. H_2S production on TSI agar (see p. 58) was determined by stab inoculation with the organism. A positive result was indicated by blackening of the agar after 3 days of incubation.

2.d. Tests of glycine, glucose and NaCl tolerance

Glycine tolerance was determined in nutrient broth No. 2 containing 1% glycine (Lecce, 1959 (see p. 58)). The effects of NaCl and glucose on growth were tested in nutrient broth No. 2 supplemented with 3.5% NaCl or 8% glucose (Kuzdas & Morse, 1956 (see p. 59)). Five drops of bacterial suspension were added to each test medium, and readings were made after 3 days for growth. The purity of the growth was checked by Gram's method.

2.e. Tests of temperature tolerance

Cultures were plated on 2 maintenance agar plates (see p. 57), and 5 drops of bacterial suspension were inoculated into 2 nutrient broths (see p. 57), one plate and one broth being incubated at 25°C or 43°C. The cultures were examined 3 days after incubation for growth.

3. Antibiotic Sensitivity Test

DST agar (see p. 59) was used as the sensitivity test medium. A single isolated colony was emulsified in 5ml of sterile peptone water, 1ml of the suspension was flooded over the plate, the excess fluid was removed, and a multodisk impregnated with 8 antibiotics was transferred aseptically on to the inoculated surface. The plates were incubated for 48hr at 37°C in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2 (see p. 59).

Oxoid multodisks (Oxoid Limited, England) were used. The antibiotics present and concentrations are summarized in Table 13.

VI. Maintenance of Cultures

1. Short-term preservation

The cultures were streaked onto Columbia agar slopes which were incubated at 37°C in microaerophilic conditions (see p. 59) for 48 hr, and kept at 4°C. The cultures were subcultured every 10 days.

Table 13 : Oxoid Multodisks

Antibiotic	Abbreviation	Standard concentration (µg)
Chloramphenicol	C	10
Erythromycin	E	10
Sulphafurazole	SF	100
Methicillin	CB	10
Penicillin	P	1.5 IU
Ampicillin	PN	25
Streptomycin	S	10
Tetracycline	TE	10

2. Long-term preservation

2.a. Freeze-drying :

Freeze-drying of cultures was done in a Edward's Speedivac Model 5PS centrifugal freeze dryer. The suspending fluid used was:

a. Skim milk

b. Mist. desiccans (Fry & Greaves, 1951).

The 7.5% glucose serum was made by adding 7.5g glucose to each 100ml of Wellcome horse serum No. 3 (Natural clot, unheated, no preservative). The serum was first allowed to attain room temperature before addition of the glucose, and when the glucose was dissolved, the whole was Seitz-filtered using an EKS (sterilising asbestos) pad and finally bottled in 5ml amounts.

The freeze-dried ampoules were kept at $4^{\circ}C$.

2.b. Storage of bacteria on beads at $-76^{\circ}C$

The method was described by Feltham et al., (1978). Briefly, 20-30 glass beads of diameter 3mm (Ellis & Farrier, Hanover Square, London) were placed in glass vials of 2ml capacity (Trident Ltd., London), and sterilized by autoclaving at 15 psi for 15 min. Aliquots of 10ml of 15% (v/v) glycerol in nutrient broth No. 2 (Oxoid) were prepared in universal bottles, and sterilized by autoclaving at 15 psi for 15 min.

Pure cultures were incubated in the atmosphere of 5% O_2, 10% CO_2 and 85% N_2 at $43^{\circ}C$ for 24 hr on maintenance agar (see p.57). One ml of 15% (v/v) glycerol broth was pipetted

onto the culture plate, first ensuring that there was no visible contamination of the plate, and the growth was emulsified in the broth using a sterile spreader to make a thick suspension. The suspension was pipetted with gentle shaking into each of two vials. The beads were thoroughly wetted with suspensions and the excess liquid was withdrawn from the bottom of the vial. The vial were then stored at -76°C. One vial was for routine recovery and the other was a reserve.

3. Reconstitution of cultures

The ampoules containing the freeze-dried cultures were opened aseptically, reconstituted with nutrient broth supplemented with yeast extract and cystine (see p. 57). The cultures were plated on maintenance agar (see p. 57).

For cultures stored on beads at -76°C, one bead from the vials was removed using sterile forceps. The bead was rubbed over the surface of maintenance agar with a loop. The vial was replaced immediately to prevent the contents from thawing.

All plates were incubated at 37°C for 48 hr in 5% O_2, 10% CO_2 and 85% N_2 (see p. 59).

VII. Assessment of Relative Growth Rate on Different Media

1. Cultural procedure

For a comparative study of the growth of *C. fetus ss. jejuni* on various broth media (see p. 57), strains 5636, 4249, 5702 and 06760 were used. The broth media were prepared in 90ml amounts

in 250ml dimpled conical flasks, and autoclaved at 15 psi for 15 min.

Seed cultures were grown by inoculating 250ml dimpled flasks containing 100ml of broth media with a loopful of cells from growth on maintenance agar incubated at 43°C for 24 hr. Flasks were incubated microaerophilically (see p. 59) in the 37°C hot room on a shaker (L.H. Engineering Co. Ltd., Stoke Poges, Bucks, England) at 100 r.p.m. for 24 hr. Ten ml of seed cultures were inoculated into all the broth media; the flasks were incubated as above, and 5ml of the cultures was removed aseptically for optical density (O.D.) measurement at regular intervals.

For larger scale growth, 10 250ml dimpled flasks containing 100ml amounts of broth were inoculated with 10ml of seed culture with a sterile pipette. The flasks were then incubated under microaerophilic (see p. 59) conditions in the 37°C hot room on a shaker at 100 r.p.m. The purity of each culture was checked by Gram's staining method and by inoculation onto maintenance agar (see p. 57).

2. Measurement of optical density

The optical density of the bacterial suspensions was measured at 540 nm with a Pye-Unicam, Model SP500 spectrophotometer in polystyrene cuvettes with 1cm light path.

VIII. Haemagglutination Tests

Human (Group A), horse, rabbit and sheep red cells were washed twice with KRT buffer (Appendix 1) and made up to 3% v/v in KRT. *C. fetus ss. jejuni* was grown on maintenance agar (p. 57) and incubated at 37°C in 5% O_2, 10% CO_2 and 85% N_2 for 48 hr.

The haemagglutination tests (HA) were done as described by Freer *et al.*, (1978). All materials were kept on ice and tests performed with cells taken directly from solid medium. A small amount of growth was resuspended directly in either a drop of saline or a 3% v/v suspension of washed red cells in KRT buffer on a glass slide. Reactions recorded as negative were those which showed no HA after continuous rocking for 30 min. Saline was used as a control, and *E. coli* H 10407 was used as a HA positive control.

SECTION 2 : ANIMAL INOCULATION EXPERIMENTS

I. Preparation of Inocula for Animal Inoculation

1. Harvesting of cells

Cultures were harvested by centrifugation at 9,000 r.p.m. in 250ml bottles at $4^{\circ}C$ for 15 min on an MSE 18 centrifuge. Supernatant fluid was carefully decanted and the cell pellets were resuspended in 0.85% (w/v) sterile saline.

2. Standardization of bacterial concentration by opacity units

The concentration of the bacterial suspension was estimated by comparison with the Fifth International Opacity Reference Preparation designated as having 10 international opacity units. The dilution factor used to obtain matching of cell suspension with the reference preparation was calculated and multiplied by 10 to give the concentration in opacity units of the undiluted bacterial suspension. A concentration of 1 o.u. was considered to be equivalent to approximately 10^9 organisms per ml. The opacity reference was supplied by the World Health Organisation Laboratory for Biological Standards (National Institute for Biological Standards and Controls, Holly Hill, Hampstead, London).

3. Viable counting methods for standardizing bacterial suspension for animal injection

Serial ten-fold dilutions of cultures were made in sterile saline. Duplicate 0.1ml amounts of each dilution were spread onto maintenance agar plates (p. 57). After incubation at $37^{\circ}C$ for 30 hr, plates with a countable number of colonies were

selected. The average number of colonies per dilution was multiplied by the dilution factor to obtain an estimate of the number of viable cells per millilitre of the original culture.

4. Heat-killed suspensions

Bacterial suspensions were heated in a shaking water-bath at 56°C or at 100°C for 15 or 30 min. The heat-killed suspensions were centrifuged at 9,000 r.p.m. for 15 min, the supernates were removed aseptically, and pellets were resuspended in sterile saline to equal the amount of the original volume.

5. Sonicated suspensions

Bacterial suspensions were sonicated 3 times for 30 sec each run using MSE 100 Watt Ultrasonic Disintegrator (M.S.E., London, England). The vessels were placed in ice to prevent being over-heated, and the sealing cap was used to avoid contamination during sonication. The sonicated suspensions were centrifuged at 9,000 r.p.m. at 4°C for 15 min, the supernates were removed aseptically and the pellets were resuspended in sterile saline to the initial volume.

II. Mice

1. Strain

The HAM I/CR (Charles Rivers, U.K. Ltd., Kent, England) strain was used. Litters were started by putting 1 male to a group of 10 females. The pregnant mice were removed and placed in separate cages and were checked for litters at 5 p.m. each

day. Litters born during each 24 hr period were recorded and were kept together with the lactating mother as an individual litter.

2. Age groups of mice

Mice 1-, 3-, 7-and 14-days old were used. Ten new born mice were kept together with the mother as an individual litter. The mice were separated from the lactating mother after 21 days and housed in groups of 20. Mice 3-and 5-wk old were also used. These mice were housed in a group of 20 animals per cage. In addition to these different ages of mice, mice 1-week pregnant were also used.

With mice of 1-day old, each litter was used for a single experiment. An ear punching method was used to mark mice of 3-and 7-day old, in the same litter for different inoculations (Table 14). Older animals were identified by dyeing the fur.

Table 14 : Identification Code for Mice of 3-and 7-day old

Code No.	Method of ear punching
1	Ear intact
2	Right ear, punched centre
3	Left ear, punched centre
4	Right and left ear, punched centre
5	Right ear, punched above and below
6	Left ear, punched above and below
7	Right and left ear, punched above and below

III. Injection Procedures

1. Mouth feeding

Nylon Intravenous cannula pink luer 3 FG and green luer 2 FG (Portex Limited, Hythe, Kent, England) were used for oral administration. One-half millilitre of the appropriate dilution of organisms was given. As the stomach contents have a pH of 3, some mice were given 5% $NaHCO_3$ solution to drink 18 hr prior to oral administration.

2. Intravenous injection

For 3-and 5-wk old mice, 0.2ml of the appropriate dilutions of organisms were injected via a tail vein with a 26 G 0.5 in. needle fitted to a 1ml syringe.

3. Intraperitoneal injection

For 1-and 3-day old mice, 0.05ml of the appropriate dilution of organisms was injected intraperitoneally from a 27 G needle fitted to a 0.5ml syringe. The injection was made along the dorsal midline. It was noted that back-pressure resulted in leakage of small amounts of inoculum from the site of injection. Each mouse, after the injection, was held for 2-3 min before being put back into the litter. For 7-and 14-day old mice, 0.1ml of the suspension was given using 27 G needle fitted with 1ml syringe, and with 3-and 5-wk old mice, 0.5ml of the suspension was inoculated. There was no leakage of inoculum with these mice.

All the experiments involving mice inoculation were terminated at 10 days and any surviving mice killed at that time.

IV. Assessment of Virulence

1. Death rate

The virulence of C. fetus ss. jejuni was judged from the number of mice dead after inoculation. Deaths were recorded twice daily.

2. Diarrhoea

The inoculated mice were observed for the signs of diarrhoea twice a day.

V. Passage Experiments

Strains of C. fetus ss. jejuni 4249 and 5636 were passaged through 5-wk old mice 5 times. The bacteria were grown as described in p. 66 . The cells were harvested at 24 hr; 0.2ml of the suspension containing 3×10^9 organisms was inoculated intravenously. The inoculated mice were killed at 24 hr after the injection, the spleen was removed aseptically and homogenized with a sterile glass grinder. Nutrient broth supplemented with yeast extract and cystine (p. 57), to which was added polymyxin-B sulphate (2.5 i.u/ml), trimethoprim lactate (5μg/ml) and vancomycin (10μg/ml), was used as suspending fluid. The homogenates were incubated at 43°C for 24 hr, and then plated on maintenance agar, and re-incubated at 37°C for 48 hr. The presence of Campylobacter was confirmed by Gram-staining and the characteristic "darting" motility seen under phase contrast microscopy (see p. 61). The pure culture was inoculated into nutrient broth supplemented with yeast extract and cystine, and

grown as method described in p. 66 . The cells were harvested and 0.2ml of the suspension containing 3 x 10^9 bacteria was injected intravenously into two 5-wk old mice, and simultaneously, 0.1ml of the same suspension containing 3 x 10^9 organisms was injected intraperitoneally into groups of 10 7-day old mice; the mortality of these mice was recorded daily, and this was called the 1st passage. The two inoculated 5-wk old mice were killed at 24 hr, the spleen was removed aseptically, and the procedure was repeated.

The strains of Campylobacter isolated on the 5th passage were kept at -76°C on glass beads (p. 65) and used for animal experiments.

VI. Estimation of LD_{50}

The LD_{50} value was estimated by Karber method as described by Reid (1975).

VII. Recovery of C. fetus ss. jejuni from Infected Mice

1. 3-and 5-wk old mice

Mice were anaesthetized with ether. Blood was collected by cardiac puncture; liver, spleen, stomach, small intestine, caecum and rectum were removed aseptically and placed in sterile petri-dishes. Liver and spleen were homogenized using sterile glass grinders in nutrient broth containing the following antimicrobial agents (final concentration per ml) : vancomycin 10µg, polymyxin-B sulphate, 2.5 IU and trimethoprim lactate 5µg. Blood, stomach contents, small intestine contents, caecum

contents and faecal pellets were inoculated into 10ml amounts of nutrient broth with the 3 antimicrobial agents (as above).

All the specimens were incubated at 43°C for 24 hr under microaerophilic conditions (p. 59). The specimens were then plated on maintenance agar (p. 57), and incubated at 37°C for 48 hr, when the plates were examined for the presence of Campylobacter colonies (p. 61).

For viable counting of the bacteria in both liver and spleen, serial ten-fold dilutions of homogenates were made in sterile saline. Duplicate 0.1ml amounts of each dilution were spread onto selective medium (see p. 55). After incubation at 37°C for 30 hr, plates with countable numbers of colonies were selected. The average number of colonies per dilution was multiplied by the dilution factor to obtain an estimate of the number of viable cells per gm weight of the tissues.

2. 7-day old mice

The 7-day old mice injected with suspensions were killed by decapitation at an appropriate time as required in the experiment. The body was"chopped" into 2-3 pieces in a sterile universal bottle with 10ml of sterile saline. The carcase was macerated using a Silverson mixer-emulsifier (Silverson Machines Ltd., London) fitted with a steel rotor. After the maceration of each mouse, the rotor was cleaned and sterilized as follows : the tissues in the rotor were removed, the rotor was then washed in 1/10 Hibitane, rinsed thoroughly and placed in an instrument sterilizer with boiling water for 5 min, the rotor was washed again with 70% alcohol and rinsed several times with sterile saline.

The homogenates were centrifuged for 10 min at 2,000 r.p.m. using a bench centrifuge to remove coarse particles. The number of viable Campylobacter in the supernate of emulsified whole mice was estimated by the method described by Miles, Misra & Irwin (1938). To 9ml of sterile saline, 1ml of supernate was added, and serial ten-fold dilutions were made. At each stage, the dilution was mixed well by pressing the tube against a mechanical mixer to sustain a vortex for 20 sec. Drops (0.02ml) of each dilution were put onto duplicate plates of selective medium (p. 55) dried for 12 hr at 37°C. Each plate was divided into 4 quadrants and one drop was placed in each quadrant. After the drops had been completely absorbed into the agar, the plates were incubated at 37°C for 30 hr under the gas mixture (p. 59). The first countable dilution was selected, counted and multiplied by the corresponding dilution factor to give the number of viable cells per ml in the tissue homogenates, the data were later expressed as no. of viable cells per mouse (average weight 2.5g). Where possible, two or three dilutions were counted, and the average values calculated and recorded.

VIII. Ferric Ammonium Citrate

Ferric ammonium citrate (BDH Chemicals Ltd., Poole, England) contains approximately 16% iron. The concentration of ferric ammonium citrate was prepared so as to give 3.2μg Fe^{3+} per g weight of mice; it was dissolved in 0.85% saline and sterilized by autoclaving at 15psi for 15 min. The ferric ammonium citrate solution was prepared in double strength, and these preparations were injected together with appropriate concentration of the bacterial suspensions into mice of different ages.

IX. Mucin

2.5% and 1% mucin (Bovine, submaxilliary gland, Type 1 (Sigma Chemical Company, St. Louis, U.S.A.)) were injected intraperitoneally with appropriate concentrations of live suspensions of C. fetus ss. jejuni into mice. The numbers of dead mice were counted daily after inoculation until 10 days.

X. Lipopolysaccharide

Shigella flexneri LPS (Difco Laboratories, Detroit, U.S.A.) was emulsified in 0.85% sterile saline. The doses given in 0.1ml were 400, 200, 100, 50 and 25μg per mouse. For the injection of S. flexneri LPS and 12μg Fe^{3+}, both the LPS and ferric ammonium citrate were prepared in double strength.

XI. Enterotoxin detection : Guinea-pig ileal loop test

Guinea pigs were starved of solid food and provided with a 5% glucose solution for 72 hr, as a preoperative measure. The animals were anaesthetised with ether and sedated with a dose of Veterinary Nembutal (0.6% pentabarbitone sodium; Abbott Laboratories Ltd., Queensborough, Kent) proportional to their weight. A length of small intestine above the caecum was lifted out of the peritoneal cavity, and the lumen was washed by injection of approximately 10ml of physiological saline which easily drained into the caecum.

The intestine was ligated with sterile cotton 2.0cm and 9.0cm above the caecum. A third ligature, 7.0cm above the caecum was tied loosely, resulting in a 5.0cm long test loop and a 2.0cm long interloop. To prevent leakage from the test

loops the viable C. fetus ss. jejuni culture was injected by inserting the hypodermic needle into the 2.0cm interloop and through the loop ligature into the test loop. The ligature was tightened onto the needle before injection of the culture, and finally tied off after removal of the needle. Four 5.0cm loops, separated by 2.0cm interloops were prepared in each animal.

The inoculum of C. fetus ss. jejuni was grown as described in p. 66 . The viable cell count of the culture was estimated by the method of Miles, Misra & Irwin (1938). The range of viable counts of C. fetus ss. jejuni culture at 24 hr was between 9.5 and 9.8 x 10^9 cells/ml; 0.1ml of a 24 hr culture was used for ileal loop testing. The broth culture was injected into the three loops respectively, while 0.1ml of nutrient broth supplemented with yeast extract and cystine was injected into the fourth loop.

The intestine was replaced in the peritoneal cavity and the wound was stitched with monofilament nylon (metric 2 ; Ref. No. V4022; Arnolds Veterinary Products Ltd., Ayr) and the animal was allowed to recover from the anaesthetic. The animal was killed 16 hr after the challenge and the ileal loops were examined. Measurements of the length and diameter of the loops were made using calipers (Camlab, Cambridge, type 6921). To avoid dissemination of C. fetus ss. jejuni, instead of measuring the fluid volume contained in the loop, the whole loop was cut out of the intestine, immersed in disinfectant solution, and the displaced volume measured.

SECTION 3 : BIOCHEMICAL MATERIALS AND METHODS

I. Protein Estimation

Compositions of reagents used are shown in Appendix 2. Estimates were done by the method of Herbert, Phipps & Strange (1971) with bovine albumin (Sigma Chemical Co., St. Louis, U.S.A.) as a standard.

a) 0.5ml of distilled water was dispensed into duplicate tubes as blank.

b) 0.5ml of samples was dispensed into duplicate test tubes.

c) Dilutions of the standard protein (BSA) (500, 400, 300, 200, 100 and 50 μg/ml) were prepared and 0.5ml of each duplicate was dispensed into duplicate test tubes.

d) 0.5ml of 1N NaOH was added to each of the tubes (a, b and c above). All the tubes were placed in a boiling water bath for 5 min, then removed and allowed to cool.

e) 2.5ml of reagent C was added to each of the tubes, after which the tubes were allowed to stand for 10 min.

f) 0.5ml of reagent D was added to all the tubes. Tubes were allowed to stand for 30 min for full colour development.

g) The optical density of the solution in each tube was measured at 750nm against the blanks (a).

h) A curve of the optical density values against concentrations of the standard protein was drawn and from this curve the protein concentrations in samples were estimated.

II. Extraction of Envelope Proteins

Packed Campylobacter cells were resuspended in saline at 4°C and, after freezing, were broken by three passages through an LKB X-press (LKB Instruments Ltd., South Croydon, Surrey). After thawing, the resulting suspension was diluted with saline and centrifuged at 100,000 g for 30 min. The supernatant fluid, the cytoplasmic fraction, was kept frozen until used. The opaque pellet was dispersed in distilled water using a syringe with a 23-gauge needle and the suspension recentrifuged. This washing procedure was repeated, and the deposit was again suspended in water and then centrifuged in a bench centrifuge for 10 min to remove unbroken cells and debris. The supernate was centrifuged at 100,000 g for 1 hr and the resulting envelope layer was removed, dispersed in a small volume of water and frozen until required.

III. Slab-gel Electrophoresis

The method used was based on that of Laemmli (1970) as modified by Ames (1974). Electrophoresis was done in the presence of sodium dodecyl sulphate (SDS) in a discontinuous gel and buffer system. Separating and stacking gels contained respectively 10% (w/v) and 5% (w/v) acrylamide (British Drug House Ltd., Poole, England). Both the gels and buffers contained 0.1% (w/v) SDS.

The dimensions of the slabs were approximately 8.0 x 7.0 x 0.3cm, the separating gels being 6.0 x 7.0 x 0.3cm. Details of reagents, preparation of gels, staining and destaining are

given in Appendix 2. Samples were adjusted to contain 2mg protein/ml and 0.5ml of the sample was added to 0.5ml of 0.125M Tris-HCl buffer pH 6.8 containing 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.002% (w/v) bromophenol blue. The mixture was heated at 100°C for 5 min prior to application of between 20 and 100µl to the gel.

Electrophoresis was done in a Uniscil slab gel electrophoresis unit (Universal Scientific Ltd., London) at a constant current of 15 mA/gel for approximately 3 hr. Staining and destaining was by the method of Weber and Osborn (1969). Destaining was accomplished with several changes of the destaining solution.

For molecular weight estimations, the following mixture of protein standards was run under identical conditions : bovine serum albumin (mol. wt. 67,000. Sigma Chemicals Co., St. Louis, U.S.A.), ovalbumin (mol. wt. 43,000. Sigma Chemical Co., St. Louis, U.S.A.), bovine chymotrypsinogen (mol. wt. 25,700. Miles Seravac, Maidenhead, Berkshire), trypsin (mol. wt. 23,000, Armour Pharmaceutical Co., Eastbourne, Sussex) and horse-heart cytochrome C (mol. wt. 11,700. Koch Light, Colnbrook, Buckinghamshire).

IV. Tube-gel Electrophoresis

The same method and reagents were used as for slab-gel electrophoresis ,except that the gels were prepared in clean glass tubes and run in a Shandon analytical polyacrylamide gel electrophoresis outfit (Shandon Southern Instruments Ltd., Surrey, England) at a constant current of 1mA per tube for approximately $2\frac{1}{2}$ hr.

V. Gradient-gel Electrophoresis

The same method and reagents were used as for slab-gel electrophoresis except that the concentration of acrylamide in the separating gels had a linear gradient from 5% to 25%. (Electrophoresis was done in a Uniscil slab gel electrophoresis unit as described for slab-gel electrophoresis).

SECTION 4 : IMMUNOLOGICAL MATERIALS AND METHODS

I. Bacterial Strains

Four strains isolated by myself i.e. No. 06760, 00539, 8346 and 3946; 4 isolates from the City Laboratory, strains No. 2680, 5702, 4249 and 7972, and 2 Skirrow strains 5636 and 9227 were used for antiserum production.

II. Preparation of Antigen Suspensions

Bacteria stored at -76°C on glass beads were reconstituted as in method described (p. 66). The cultures were incubated at 43°C for 24 hr; purity of cultures was checked by Gram-staining. If the cultures were pure, a loopful of organisms was inoculated into 10 250ml dimple flasks containing nutrient broth with yeast extract and cystine (p. 57). All the flasks were incubated at 37°C in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2 (p. 59) on a shaker (L.H. Engineering Co. Ltd., Stoke Poges, Bucks, England) at 100 r.p.m. for 24 hr. A portion of the cultures was removed aseptically for purity check by Gram's staining method.

For the production of heat-stable antigens (O antigens), the cells were harvested by centrifugation at 4°C at 9,000 r.p.m. for 15 min, and resuspended in 0.85% sterile saline. The bacterial suspensions were autoclaved at 2hr at 121°C. The cells collected by centrifugation were washed twice, resuspended in 0.85% sterile saline and kept frozen as stock suspensions. For production of antisera, the stock suspensions were diluted with sterile saline to optical density (O.D.) of 0.45 at 540nm

in a Pye-Unicam, Model SP 500 Spectrophotometer using polystyrene cuvettes with 1cm light path. For agglutinating antigens, the stock suspensions were diluted with sterile saline to give an optical density (O.D.) of 0.4 at 540nm.

For production of whole cell formalinized (H) antigens, the growth was formalinized to 0.3% and incubated for another 4 hr at 37°C. The cells were harvested by centrifugation, washed twice and resuspended in sterile saline as stock suspensions as above. The stock suspensions were diluted in sterile saline as for the boiled antigens and were kept frozen at -4°C until used.

III. Immunization of Mice

Five-week old HAM I/CR mice were used for antiserum production. Ten C. fetus ss. jejuni strains were used, and 40 mice were taken for each type of antigen. Two methods of immunization were employed.

Method 1. One injection of 1ml heat-killed or formalinized antigens was given intraperitoneally and the mice bled 10 days later. Mice were anaesthetized, and the blood collected by cardiac puncture. The collected blood was pooled, allowed to clot and kept in the cold room overnight. The antisera were stored at -20°C until used.

Method 2. Three injections of 0.5, 1 and 2ml were given intraperitoneally at weekly intervals, the mice were bled 10 days after the last injection.

IV. Immunization of Rabbits

Three C. fetus ss. jejuni strains 5636, 4249 and 06760 were used. The rabbits were inoculated intravenously at 3 day intervals with increasing volumes (0.5, 1.0, 2.0, 3.0 and 4ml) of the heat-killed or formalinized antigens. The rabbits were bled by cardiac puncture 10 days after the last injection. The blood was allowed to clot, and then kept at $4^{\circ}C$ overnight. The antisera were distributed in 5ml amounts and stored at $-20^{\circ}C$.

V. Agglutination Test

Agglutination titres were estimated by Dreyer's Dropping method (Edwards & Ewing, 1962). For mice, the final dilution of serum ranged from 1:25 to 1:2500 ; for rabbits, the final dilution was 1:50 to 1:10,000. The tubes were incubated at $37^{\circ}C$ for 18 hr in a water-bath. Controls included antigen plus normal rabbit serum, or mice sera; antigen plus saline, and antigens plus sera from mice inoculated with saline, nutrient broth No. 2 supplemented with yeast extract and cystine.

Complete homologous and heterologous agglutination tests were done with each antigen and antiserum. The results were quantitated from 0 (no agglutination) to 3+ (complete agglutination). The end point titre was considered to be the highest dilution of serum giving a 2+ agglutination.

RESULTS

SECTION I. CULTURAL EXPERIMENTS

1. Isolation of Campylobacter strains from faecal specimens

A total of 339 faecal specimens were studied; 290 specimens were collected from patients with diarrhoea, 49 specimens from normal people. No Campylobacters were isolated from the 49 normal samples; 11 positive isolations (3.7%) were made from the 290 diarrhoeal specimens, 5 positive (3.5%) from 142 males and 6 positive (3.9%) from 148 females.

A variety of other bacteria that are isolated from patients with diarrhoea were streaked on the antibiotic C. fetus ss. jejuni selective medium. All the plates were incubated at 43°C under microaerophilic conditions, results showed that most of these bacteria were inhibited on the plates under these conditions (Table 15).

Table 15 : Inhibitory action of the antibiotics in the Campylobacter selective medium on bacterial species found in faeces.

Bacteria tested		Result
Bacillus sp.		No growth
Escherichia coli	O 18	No growth
	O 44	Growth
Proteus sp.		No growth
Salmonella sp.		Growth
Shigella sp.		No growth
Pseudomonas sp.		No growth

2. Morphology of C. fetus ss. jejuni

On horse blood Columbia agar plates, the colonies of C. fetus ss. jejuni isolated from diarrhoeal patients were non-haemolytic. Two colony types were commonly found :

Type I. round, convex, shiny, entire edge; light grey, diameter 2mm (Plate 1)

Type II. irregular, tending to spread, raised, entire or undulate edge, diameter 2mm or more. (Plate 1).

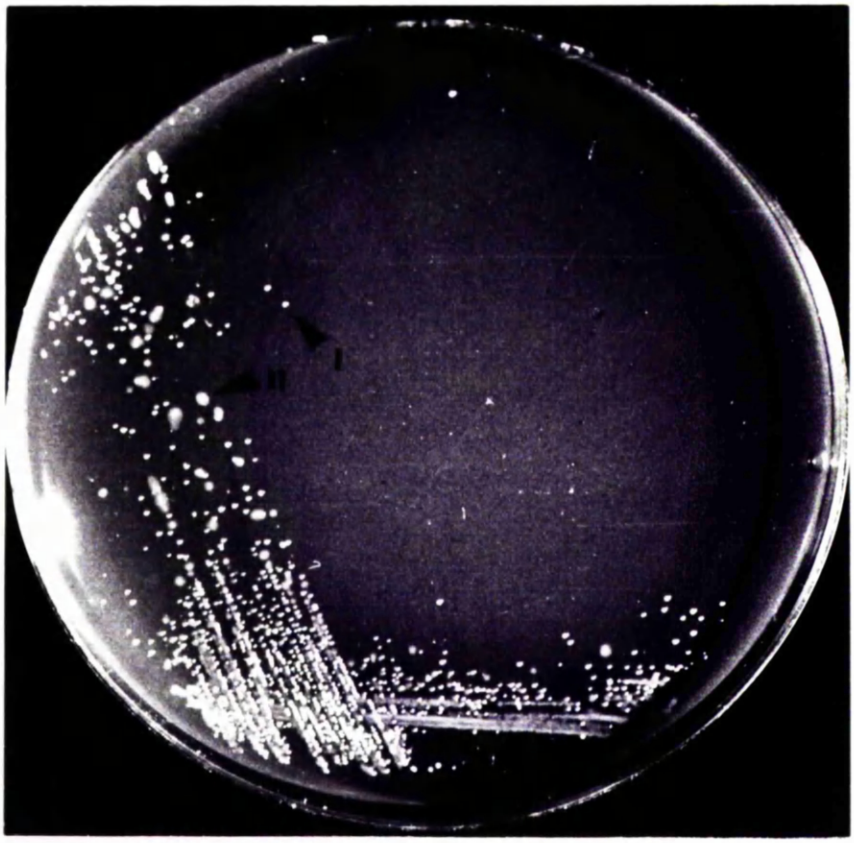
When a single isolated Type I colony was replated onto horse blood Columbia agar, and incubated under the conditions described (p. 59) at 37°C for 24 hr, both the Type I and Type II colony appeared. Similar results were obtained for the Type II colony.

A third type of colony (Plate 2) appeared after successive subculture on Columbia agar slopes over a period of 4 months. The colonies were round, raised, shiny (granular centre in large colonies), entire edge; light grey at edge of colony becoming cream towards the centre; diameter 3-5 mm.

Microscopic examination of 24 hr cultures stained by Gram's method showed that the organism was a small, Gram-negative spirally curved rod (Plate 3). After incubation for 72 hr the bacteria became coccal in shape, and at least 50% of the observed bacterial population consisted of coccal forms (Plate 4). These coccal forms did not grow when inoculated into nutrient broth No. 2 or streaked onto maintenance agar i.e. they were non-viable. The organism was not capsulated.

Plate 1 : Two colony types of *C. fetus ss. jejuni* isolated from patients with diarrhoea after incubation at 37°C for 24 hr.

Plate 2 : Colony type of *C. fetus ss. jejuni* after successive subculture for 4 months.


I
II

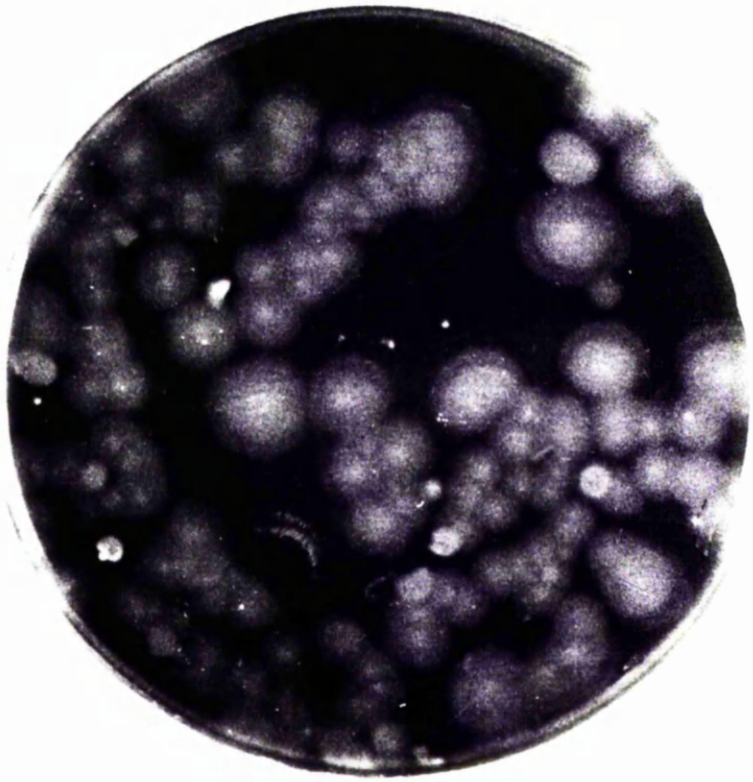


Plate 3 : Morphological form of C. fetus ss. jejuni as seen on Gram's stain smear - sea-gull forms. (1000 x 5)

Plate 4 : Morphological form of C. fetus ss. jejuni as on Gram's stain smear - coccal forms. (1000 x 5)

All C. fetus ss. jejuni strains were actively motile with a characteristic "darting" motion when examined by wet film preparations.

3. Biochemical characteristics of strains

All 15 strains (the 11 isolated by myself and the 4 isolated at the City Laboratory) were catalase and oxidase positive. Table 16 summarizes the results of the biochemical tests. The results show that the strains of C. fetus ss. jejuni produced H_2S in the sensitive medium (p. 58), grew on TSI agar but produced no H_2S; tolerated 1% glycine but not 3.5% NaCl and 8% glucose respectively. All strains grew at 43°C but not at 25°C.

4. Antibiotic sensitivity/resistance patterns of C. fetus ss. jejuni strains

Six strains of C. fetus ss. jejuni 4249, 5702, 06760, 8346 and 3946 were tested against 8 common antibiotics, using Oxoid multodisks (Table 13), and Table 17 shows that C. fetus ss. jejuni strains were resistant to both methicillin and penicillin, but sensitive to the other 6 antimicrobial agents tested.

5. Maintenance of cultures

5a. Short-term preservation

All cultures were kept on Columbia agar slopes and stored at 4°C. They were subcultured every 10 days. The cultures died if they were not subcultured.

Table 16 : Biochemical reactions of C. fetus ss. jejuni

Isolate No.	Catalase	Oxidase	H_2S production[1]		1%[2] glycine	3.5%[2] NaCl	8%[2] glucose	Temperature tolerance	
			sensitive medium	TSI				25°C	43°C
6395	+	+	+	-	+	-	-	-[3]	+
6537	+	+	+	-	+	-	-	-	+
06238	+	+	+	-	+	-	-	-	+
06760	+	+	+	-	+	-	-	-	+
9428	+	+	+	-	+	-	-	-	+
06342	+	+	+	-	+	-	-	-	+
03260	+	+	+	-	+	-	-	-	+
6635	+	+	+	-	+	-	-	-	+
8346	+	+	+	-	+	-	-	-	+
00539	+	+	+	-	+	-	-	-	+
3946	+	+	+	-	+	-	-	-	+
2680	+	+	+	-	+	-	-	-	+
5702	+	+	+	-	+	-	-	-	+
4249	+	+	+	-	+	-	-	-	+
7972	+	+	+	-	+	-	-	-	+

1. H_2S production : sensitive medium :
   Oxoid nutrient broth No. 2 + 0.02% cystine.
   TSI : Difco Triple Sugar Iron
   + : H_2S production
   - : No H_2S production

2. Nutrient broth No. 2 (Oxoid) as basal medium. 3. + : Growth - : No growth.

Table 17 : Antibiograms of C. fetus ss. jejuni

Antimicrobial agents	antibiotics (µg) per disk	Campylobacter fetus ss. jejuni 4249	5702	06760	8346	00539	3946
Chloramphenicol	10	+[a]	+	+	+	+	+
Erythromycin	10	+	+	+	+	+	+
Sulphafurazole	100	+	+	+	+	+	+
Methicillin	10	-[b]	-	-	-	-	-
Penicillin	1.5 IU	-	-	-	-	-	-
Ampicillin	25	+	+	+	+	+	+
Streptomycin	10	+	+	+	+	+	+
Tetracycline	10	+	+	+	+	+	+

a : + sensitive, no growth

b : - resistant, growth

5b. Long-term preservation

Freeze-drying

The efficacy of the suspending fluids was determined by the recovery of the bacterium from the freeze-dried stage. Comparison was made of the viability of cultures freeze-dried in 7.5% glucose serum (Mist.desiccans)and in skim-milk. It was found that skim-milk was a better suspending fluid than Mist.desiccans. The cultures freeze-dried with 7.5% glucose serum failed to grow 3 months later, the cultures in the skim-milk suspending fluid grew successfully. However, all the cultures freeze-dried in skim-milk failed to grow 1 year later.

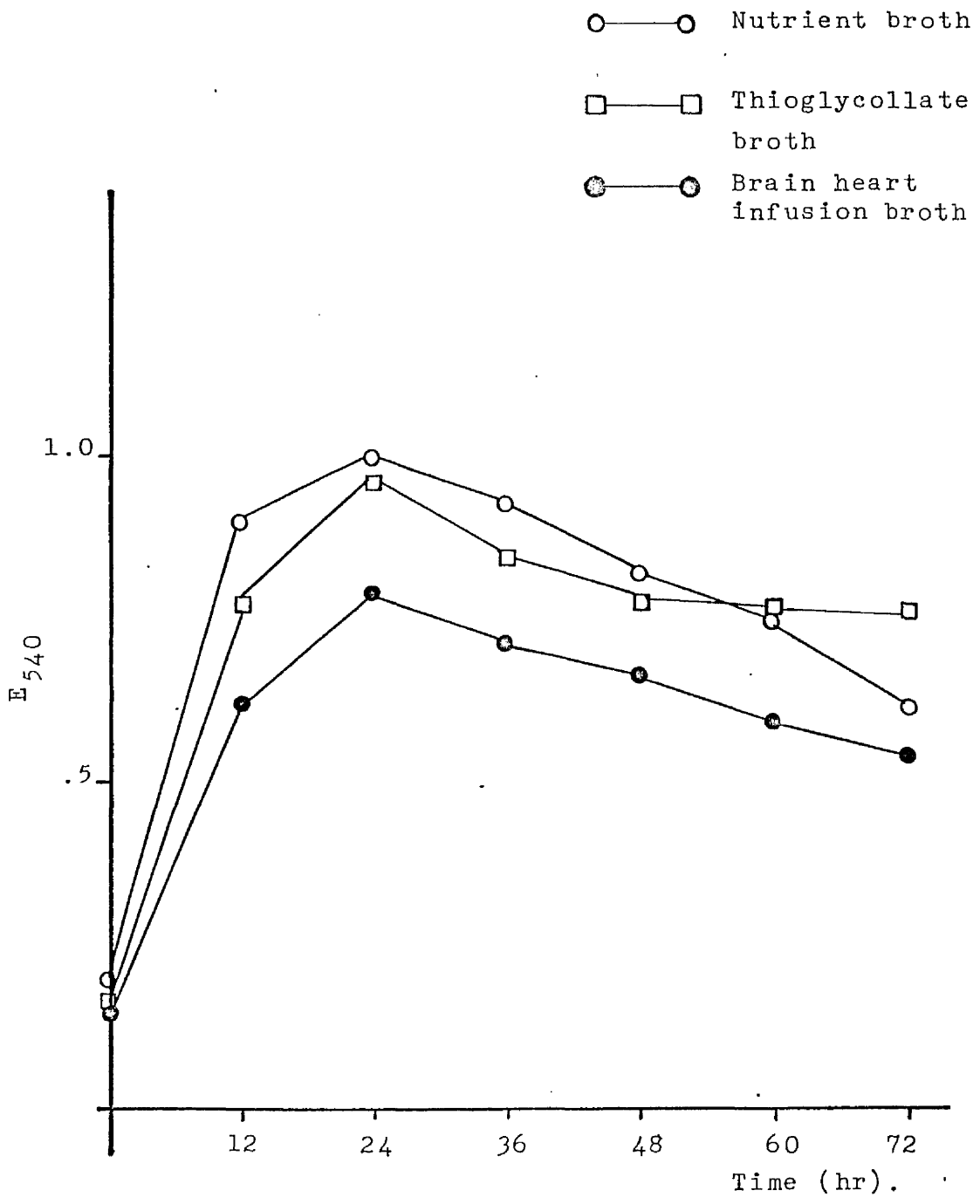
Storage at -76^{o}C on glass beads. This method was described on p. 65 . It is a safe, reliable and simple procedure for long term preservation of C. fetus ss. jejuni. All the strains could be successfully recovered 2 years later.

6. Nutrition of C. fetus ss. jejuni

6a. Growth of C. fetus ss. jejuni in different broth media.

C. fetus ss. jejuni grew in Oxoid nutrient broth No. 2. thioglycollate broth and brain heart infusion broth. Fig. 3 shows the O.D. (optical density) of strain 4249 grown in these 3 media. Both nutrient broth and thioglycollate broth gave higher cell yields than brain heart infusion. The maximum O.D. was reached in 24 hr, the O.D. for the organisms grown in nutrient broth and thioglycollate broth was 1.0 and 0.96 respectively, but it was only 0.71 in brain heart infusion broth. Similar results were obtained with strain 5636.

Fig. 3 : Growth of C. fetus ss. jejuni strain 4249 in different broth media.



6b. The cell-yields of C. fetus ss. jejuni in nutrient broth supplemented with yeast extract, amino acids and inorganic salts.

Nutrient broth was used because high cell yields were obtained when C. fetus ss. jejuni was grown in this broth medium (Fig. 3). When the amino acids cystine (0.02g/100ml), L-glutamic (200mg/100ml) and glycine (0.03g/100ml) were individually added to nutrient broth, only cystine was found to give slightly enhanced growth (Table 18). The data also show that nutrient broth supplemented with yeast extract supported good growth of the Campylobacter strains. However, with both cystine and yeast extract in the medium, the cell yields were only slightly greater. The O.D. of C. fetus ss. jejuni 4249 grown in nutrient broth with and without supplements recorded at 0, 12, 24, 36, 48 and 72 hr is shown in Fig. 4. The cell yields reached a maximum at 24 hr; the maximum O.D. recorded for the organism grown in nutrient broth supplemented with yeast extract and cystine was 1.51, but it was only 0.95 for the organisms grown in nutrient broth without supplementation. Similar results were obtained for C. fetus ss. jejuni strains 5636, 5702 and 06760. The maximum O.D. at 24 hr is recorded in Table 18. The addition of inorganic salts e.g. $MgSO_4$, $CaCl_2$, KNO_3 and $Na_2H_2PO_4$ did not affect the growth (Table 18).

6c. Effect of pH on the growth of C. fetus ss. jejuni

The pH of the nutrient broth supplemented with yeast extract and cystine was 6.9; the broth was buffered to pH 3, pH 5 and pH 8.4 to study the effect on the growth of C. fetus ss. jejuni.

Table 18 : The growth of strains of C. fetus ss. jejuni in nutrient broth with various supplements[a]

Medium[b]	C. fetus ss. jejuni			
	5636	4249	5702	06760
Nutrient broth (NB)	0.95	1.06	1.02	1.0
NB + cystine	0.97	1.05	1.04	1.0
NB + L-glutamic	0.90	0.98	1.01	0.96
NB + glycine	0.95	1.02	0.96	0.98
NB + yeast extract	1.34	1.45	1.55	1.56
NB + inorganic salts	0.98	1.05	1.00	0.98
NB + cystine + yeast extract	1.38	1.54	1.64	1.58
NB + cystine + yeast extract + inorganic salts	1.36	1.52	1.60	1.60

a : results expressed as optical density at E540, recorded at 24 hr.

b : cystine = 0.02g/100ml

L-glutamic = 0.2g/100ml

glycine = 0.03g/100ml

yeast extract = 0.5g/100ml

inorganic salts : (g/l)

5g	$MgSO_4.7H_2O$
0.02g	$CaCl_2$
0.75g	KNO_3
0.25g	$Na_2H_2PO_4$

Fig. 4 : Growth of C. fetus ss. jejuni in supplemented nutrient broth.

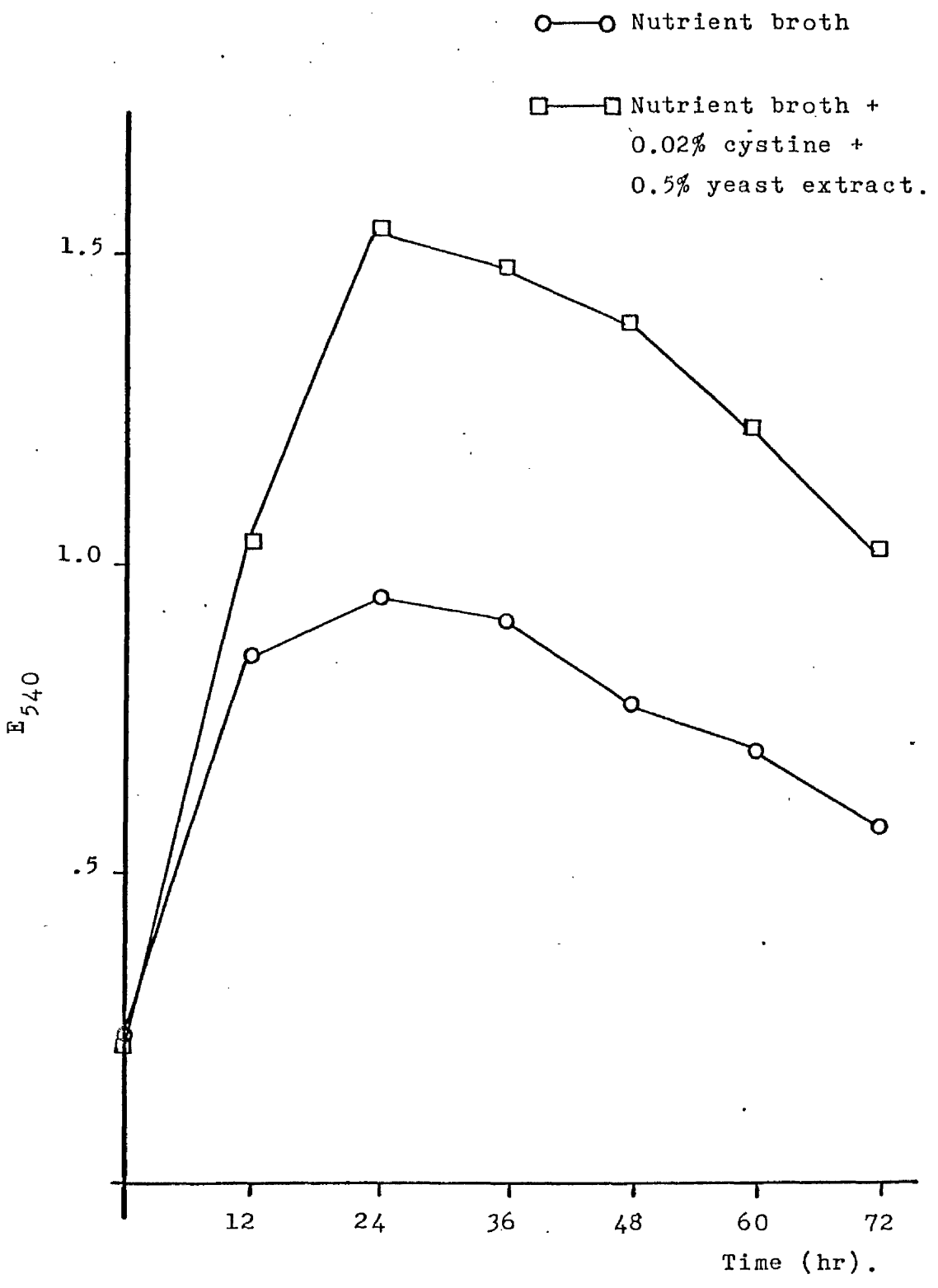
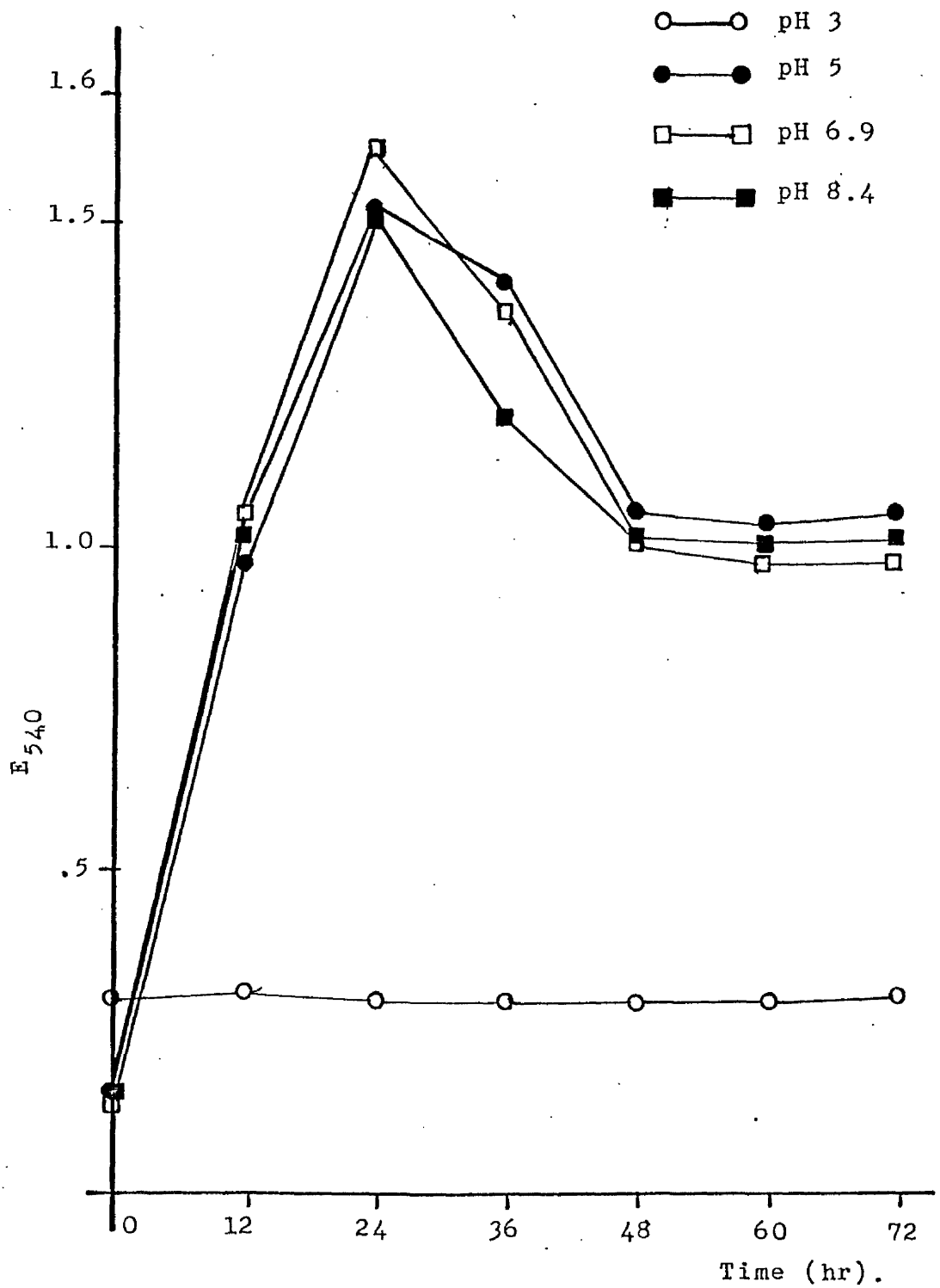


Fig. 5 : Effect of pH on the growth of C. fetus ss. jejuni 4249.



No growth was obtained when C. fetus ss. jejuni was inoculated into broth of pH 3. When bacteria were grown in broth of pH 5, pH 6.9 and pH 8.4, the cell-yields were consistent. They reached an average maximum O.D. 1.54 in 24 hr, and decreased to an average O.D. 1.3 after 3 days (Fig 5). The final pH of all the broths was between 7.2 and 7.6. C. fetus ss. jejuni 5636, 06760 and 5702 gave similar results.

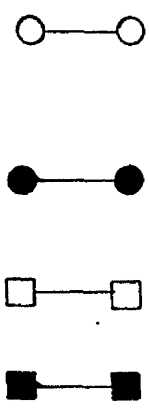
6d. Effect of ferric ammonium citrate on in vitro growth of C. fetus ss. jejuni.

Fig. 6a shows the growth of C. fetus ss. jejuni in nutrient broth supplemented with yeast extract, cystine, and 40, 80 and 120 μg Fe^{3+} per ml; the Fe^{3+} was supplied in the form of ferric ammonium citrate which contained 16% Fe^{3+}. The growth was recorded turbidimetrically by a Unicam SP 500 Spectrophotometer at E_{540}, and the data show that the addition of excess Fe^{3+} into nutrient broth did neither stimulate nor repress growth. The results obtained by viable counts showed the growth curves at 12, 24, 48, and 72 hr were similar to that of the O.D. measurement (Fig. 6b).

7. Haemagglutinating activity of C. fetus ss. jejuni.

Four C. fetus ss. jejuni strains No. : 5636, 4249, 06760 and 2680 were used in this study. The HA test procedure is described in p. 68 . The results showed that C. fetus ss. jejuni strains did not haemagglutinate with the red cells of human (Group A), sheep, horse and rabbit (Table 19).

Fig. 6a : The effect of various concentrations of Fe^{3+} on *in vitro* growth curves of *C. fetus ss. jejuni* - OD measurement



NYC (Nutrient broth + 0.02% cystine + 0.5% yeast extract)

NYC + 40 μg Fe^{3+}/ml

NYC + 80 μg Fe^{3+} /ml

NYC + 120 μg Fe^{3+}/ml

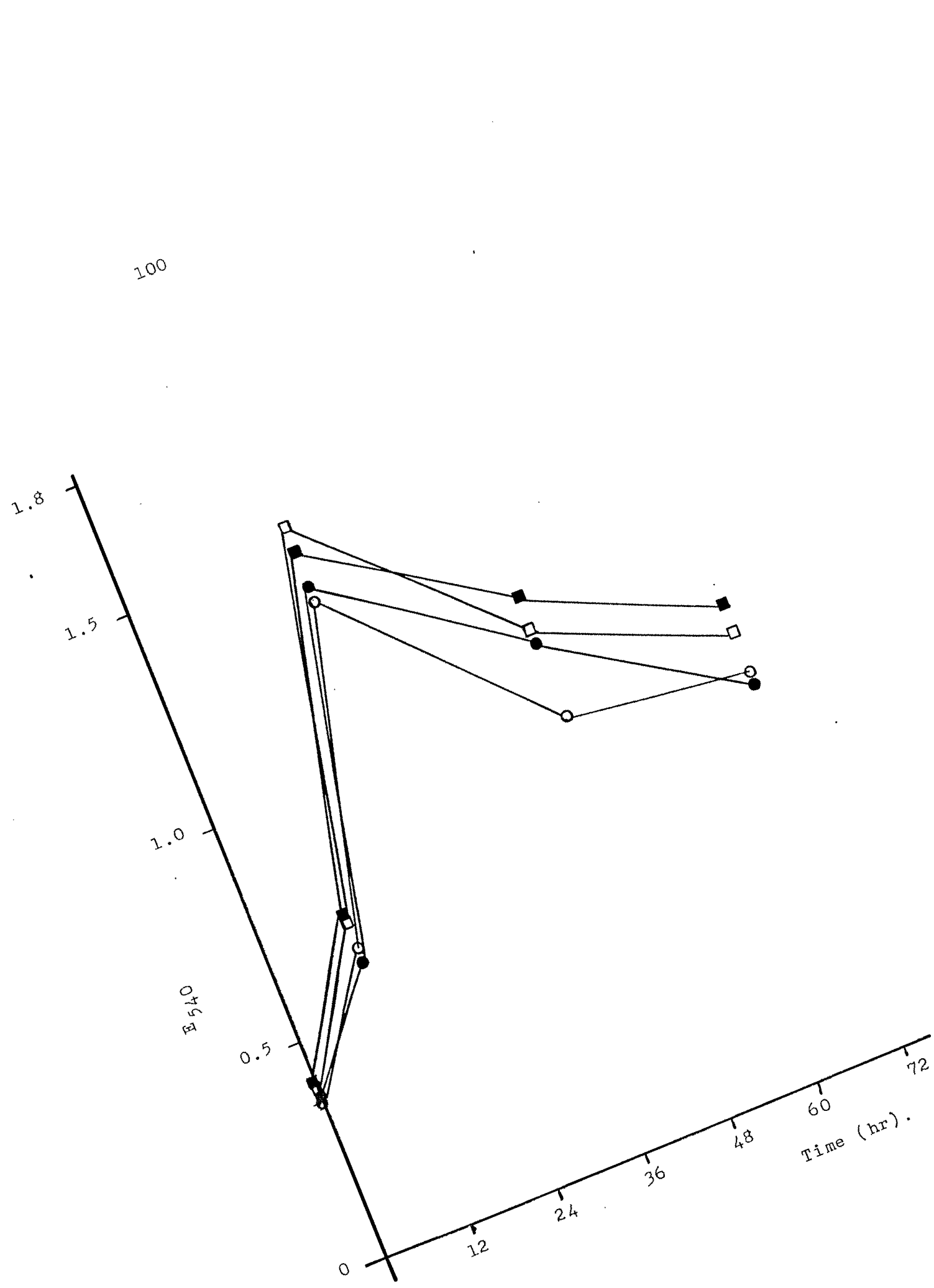
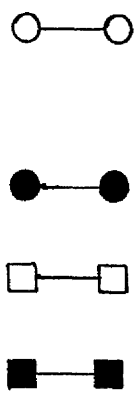

100
1.8
1.5
1.0
0.5
0
E540
12
24
36
48
60
72
Time (hr).

Fig. 6b : Effect of various concentrations of Fe^{3+} on *in vitro* growth curves of *C. fetus ss. jejuni* - viable counts.



○—○ NYC (Nutrient broth + 0.02% cystine + 0.5% yeast extract)

●—● NYC + 40 µg Fe^{3+}/ml

□—□ NYC + 80 µg Fe^{3+}/ml

■—■ NYC + 120 µg Fe^{3+}/ml

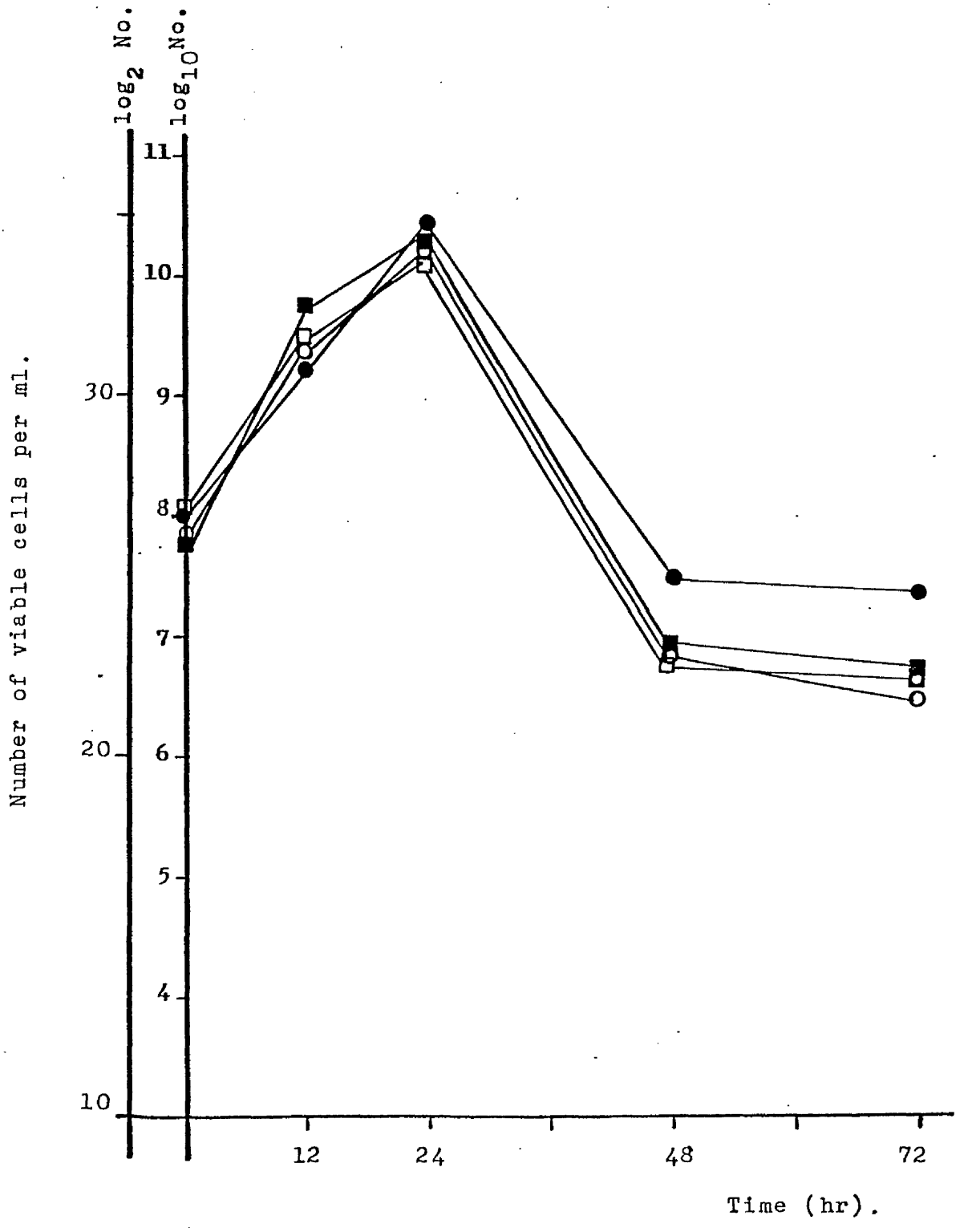

log2 No.
log10 No.
11
10
9
8
7
6
5
4
30
20
10
12
24
48
72
Time (hr).
Number of viable cells per ml.

Table 19 : Haemagglutinating activity of *C. fetus ss. jejuni* strains

Type of red cells	*C. fetus ss. jejuni* strain 5636	4249	06760	2680	Control *E. coli* H10407
Human (Group A)	-[b]	-	-	-	+[a]
Horse	-	-	-	-	N[c]
Rabbit	-	-	-	-	+
Sheep	-	-	-	-	+

a + = HA positive

b - = HA negative

c N = Not done

8. Estimation of viable *C. fetus ss. jejuni* in standardizing bacterial suspensions for animal injection

The concentration of bacterial suspensions used for animal experiments was standardized by comparison with the International Opacity Reference Preparation as described on p. 69 . The number of viable *C. fetus ss. jejuni* cells present in a 10 Opacity Units (o.u.) suspension was estimated by the method described (p.69). Table 20 summarizes the number of viable organisms obtained in 3 separate experiments. The data show that 1 o.u.

Table 20 : Number of viable C. fetus ss. jejuni cells in serial ten-fold dilutions of a 10 o.u. suspension

Opacity Units (o.u.)	No. of viable cells per ml		
	1st experiment	2nd experiment	3rd experiment
10	4.5×10^9	5.8×10^9	4.9×10^9
1	4.8×10^8	4.7×10^8	5.7×10^8
0.1	4.7×10^7	4.9×10^7	5.7×10^7

Table 21 : Number of viable Campylobacter in the dilutions used for injecting animals

Standardized bacterial suspension (o.u.)	No. of viable cells per ml	
	C.fetus ss. jejuni 5636	C.fetus ss. jejuni 4249
20	9.9×10^9	9.9×10^9
10	4.9×10^9	4.9×10^9
5	2.5×10^9	2.4×10^9
2.5	1.3×10^9	1.2×10^9
1.25	6.2×10^8	6.2×10^8

of C. fetus ss. jejuni suspensions contains 5 x 10^8 bacteria per ml.

In the animal experiments, the number of viable C. fetus ss. jejuni was estimated by the method suggested on p.69 . The results showed that a 20 o.u. suspension of C. fetus ss. jejuni strains 5636 and 4249 contained 1 x 10^{10} per ml bacteria respectively. If the doses used in animal inoculations were diluted from 1/2 - 1/16, the inocula should contain the number of viable C. fetus ss. jejuni shown in Table 21.

C. fetus ss. jejuni is actively motile. As the organisms did not grow on over-dried plates, all the plates were pre-incubated at 37°C for 12 hr before inoculation. Two types of colonies were present on the plate after incubation at 37°C for 30 hr (p. 87). Prolonged incubation resulted in spreading growth of Type II colonies and made the counting impossible.

P-nitrophenylglycerol (PNPG), a recommended antiswarming agent for Proteus (Senior, 1977), at the concentration of 0.5mM, 1mM, and 2mM did not stop the spreading of a Type II colony if the incubation at 37°C was prolonged. PNPG was found to inhibit the growth of C. fetus ss. jejuni at a concentration of 2.5mM.

When the agar concentration was increased from 1% to 3%, Type II colonies tended to spread on prolonged incubation i.e. 48 hr; and at a 6% agar concentration, C. fetus ss. jejuni grew very poorly.

The results of these experiments showed that colonies of C. fetus ss. jejuni could be counted, provided that the plates were incubated for 30 hr at 37°C.

SECTION II. ANIMAL INFECTION EXPERIMENTS

I. Injection of 5-wk old mice with live *C. fetus ss. jejuni* cultures

Mice 5-wk old, strain HAM I/CR were used in an attempt to produce human *C. fetus ss. jejuni* infection. Mice were chosen for the experiment because they are cheap, and readily available in the Departmental Animal House.

1. Effect of live *C. fetus ss. jejuni* cultures

Eight human strains of *C. fetus ss. jejuni* were used in this study. The suspensions were given i.p., i.v. and *per os*. The mice received 0.2ml of the suspensions i.v., and 0.5ml i.p. and *per os*. The number of cells contained in each amount injected was the same. These were standardized to 6 o.u. per mouse (p. 69) which contained 3×10^9 bacteria (1 o.u. contained 5×10^8 bacteria (Table 20)). Groups of 10 mice were used for each route of inoculation, a total of 240 being used. Table 22 shows that no deaths occurred after the inoculation, and the inoculated mice did not develop signs of diarrhoea or other illness.

Three strains, *C. fetus ss. jejuni* 5636, 4249 and 06760 were inoculated into groups of 15 mice which had been allowed to drink 5% $NaHCO_3$ solution 18 hr prior to the experiment. The suspensions were given *per os*, and 0.5ml containing 3.0×10^9 bacteria were given to each mouse. It was found that these groups of mice did not die or develop any illness. Thus $NaHCO_3$ had no apparent effect on the oral administration, and therefore was not used

Table 22 : Inoculation of 8 *C. fetus* ss. *jejuni*[a] strains into 5-wk old mice

C. fetus ss. *jejuni*	No. of deaths/No. of mice injected[b]		
No.	i.p.[c]	i.v.	*per os*
5636	0/10	0/10	0/10
9227	0/10	0/10	0/10
4249	0/10	0/10	0/10
5702	0/10	0/10	0/10
2680	0/10	0/10	0/10
00539	0/10	0/10	0/10
06760	0/10	0/10	0/10
8346	0/10	0/10	0/10

a : Suspensions given via each route of inoculation contained 3×10^9 organisms.

b : Recorded over a period of 10 days

c : Routes of inoculation :

i.p. : intraperitoneal
i.v. : intravenous
Per os : by mouth

in the later experiments.

As 3×10^9 live C. fetus ss. jejuni had no effect on the mice, the effect of increasing the number of organisms injected into mice was examined. Three strains of C. fetus ss. jejuni 5636, 4249 and 06760 were used. In the suspensions inoculated into each mouse the cell numbers were increased from 6 o.u. (3×10^9 organisms) to 20 o.u. (1×10^{10} organisms). These were given via i.p., i.v. and oral routes : 0.2ml of the suspensions were given i.v.; 0.5ml were given both i.p. and per os. Each injecting dose contained the same number of organisms. All the mice (10 mice for each strain) injected i.v. with 1×10^{10} organisms died within 2 hr. No deaths were recorded in the groups of mice with suspensions injected i.p. and given orally, and again, the mice did not develop diarrhoeal illness after 10 days.

2. Recovery of C. fetus ss. jejuni from 5-wk old mice injected with live cultures

Mice experimentally injected with C. fetus ss. jejuni were examined for the presence of these bacteria. The 7 sites selected for culture were blood, spleen, liver, stomach contents, small intestine contents, caecum contents, and faecal pellets in the rectum. Doses of C. fetus ss. jejuni containing 3.0×10^9 bacteria were given i.p., i.v. and per os to 160 5-wk old mice. A mouse from each route of inoculation was killed at 24, 48hr, 7, 14 and 21 days after the injection. Blood was collected by cardiac puncture, then the spleen, liver, stomach content, small intestine contents, caecum contents and faecal pellets were

Table 23 : Recovery of *C. fetus ss. jejuni* from 5-wk old mice after infection

a : I.P. : intraperitoneal

I.V. : intravenous

Per os : by mouth

b : ● *Campylobacter* was reisolated

○ No *Campylobacter* reisolated

⊗ Not done

Sites of Recovery	Routes[a] of Infection	Campylobacter fetus ss. jejuni												Control mice Uninoculated
		5636			5702			06760			00539			
		24 hr[b]	48 hr	7 Day	24 hr	48 hr	7 Day	24 hr	48 hr	7 Day	24 hr	48 hr	7 Day	
Blood	I.P.	●	○	○	○	○	○	○	○	○	○	○	○	○
	I.V.	○	○	○	○	○	○	○	○	○	○	○	○	
	Per os	●	○	○	○	○	○	○	○	○	⊗	⊗	⊗	
Liver	I.P.	○	●	○	●	○	●	●	○	○	●	●	●	○
	I.V.	●	●	○	●	●	●	●	●	○	●	●	○	
	Per os	●	○	○	●	●	○	●	○	○	⊗	⊗	⊗	
Spleen	I.P.	●	○	○	●	○	○	●	○	○	●	○	○	○
	I.V.	●	○	○	●	○	●	●	○	○	●	●	○	
	Per os	●	○	○	○	●	○	○	○	○	⊗	⊗	⊗	
Stomach Contents	I.P.	●	○	○	○	○	●	○	●	○	○	●	○	○
	I.V.	○	○	○	●	○	○	●	○	○	○	●	○	
	Per os	●	○	○	○	●	●	○	○	○	⊗	⊗	⊗	
Small Intestine Contents	I.P.	●	○	○	●	●	○	○	○	○	●	○	○	○
	I.V.	○	●	○	●	○	○	●	○	○	●	●	○	
	Per os	●	○	○	●	○	○	○	○	○	⊗	⊗	⊗	
Caecum Contents	I.P.	●	○	○	○	○	○	○	○	○	○	○	○	○
	I.V.	○	○	○	○	○	○	○	○	○	○	○	○	
	Per os	●	●	○	○	○	○	○	○	○	⊗	⊗	⊗	
Rectum faecal pellets	I.P.	●	○	○	○	○	○	○	●	○	○	○	○	○
	I.V.	○	○	○	●	○	○	●	○	○	●	○	○	
	Per os	●	○	○	●	○	○	○	○	○	⊗	⊗	⊗	

aseptically removed and cultured for C. fetus ss. jejuni (p. 66).

The plates were examined for characteristic colonies of C. fetus ss. jejuni (p.61). With groups of mice inoculated with strains 5636, 5702, 06760 and 00539, the culture procedure was carried out over a period of 7 days, and with groups of mice inoculated with strains 4249, 9227, 2680 and 8346, the culture procedure was the same except that a period of 21 days was used.

Ten control uninoculated mice were similarly cultured.

Table 23 shows the sites of isolation of C. fetus ss. jejuni from mice inoculated with strains 5636, 5702, 06760 and 00539. The organisms could be reisolated from blood, spleen, liver, stomach contents, small intestine contents, caecum contents and faecal pellets irrespective of the inoculation routes used. There was little difference in the recovery of these 4 strains from the 7 sites during the first 48 hr. In mice examined on the 7th day, C. fetus ss. jejuni 5702 was found to persist in liver, spleen, and G.I. tract but with strain 00539, the recovery of organisms was made only from the liver. The mice inoculated with strains 5636 and 06760 did not yield Campylobacter from any of the 7 sites sampled on the 7th day.

C. fetus ss. jejuni strains were obtained in pure cultures from the spleen, blood and sometimes the liver. In the peritoneal cavity of mice inoculated intraperitoneally, pure cultures of Campylobacter were obtained at 24 and 48 hr post-injection; however, the organisms were not reisolated on the 7th day.

Table 24 shows the results of examining mice during the

Table 24 : Recovery of C. fetus ss. jejuni from 5-wk old mice after infection

a : I.P. : intraperitoneal

I.V. : intravenous

Per os : by mouth

b : ● Campylobacter was reisolated

○ No Campylobacter reisolated

Sites of Recovery	Routes[a] of Infection	Campylobacter fetus ss. jejuni																			
		4249					9227					2680					8346				
		24[b] hr	48 hr	7 Day	14 Day	21 Day	24 hr	48 hr	7 Day	14 Day	21 Day	24 hr	48 hr	7 Day	14 Day	21 Day	24 hr	48 hr	7 Day	14 Day	21 Day
Blood	I.P.	●	●	○	○	○	●	○	○	○	○	○	○	○	○	○	●	○	○	○	○
	I.V.	●	●	●	○	○	●	○	○	○	○	○	○	○	○	○	●	●	○	○	○
	Per os	●	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○
Liver	I.P.	●	●	●	○	○	●	●	○	○	○	●	●	●	○	○	●	●	●	○	○
	I.V.	●	●	●	●	●	●	●	○	○	○	●	●	●	○	○	●	●	●	○	○
	Per os	●	●	○	○	○	●	●	○	○	○	●	●	○	○	○	○	○	○	○	○
Spleen	I.P.	●	●	○	○	○	●	●	○	○	○	●	○	○	○	○	●	○	○	○	○
	I.V.	●	●	○	○	○	●	●	○	○	○	●	○	○	○	○	●	●	○	○	○
	Per os	○	●	○	○	○	●	○	○	○	○	●	●	○	○	○	○	○	○	○	○
Stomach Contents	I.P.	●	●	●	○	○	○	○	○	○	○	○	○	●	○	○	○	●	○	○	○
	I.V.	●	●	●	○	○	●	●	○	○	○	●	○	●	○	○	○	○	●	○	○
	Per os	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
S.I. Contents	I.P.	○	●	●	○	○	●	●	○	○	○	●	○	○	○	○	●	●	○	○	○
	I.V.	●	●	●	●	○	○	●	○	○	○	○	●	○	○	○	●	●	○	○	○
	Per os	●	○	○	○	○	●	●	○	○	○	○	●	○	○	○	○	○	○	○	○
Caecum Contents	I.P.	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	I.V.	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○
	Per os	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Rectum faecal pellets	I.P.	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○
	I.V.	●	●	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○
	Per os	○	●	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○

21 days after injection of C. fetus ss. jejuni strains 4249, 9227, 2680 and 8346. The data show that the recovery of C. fetus ss. jejuni from these 7 sites at 24 and 48 hr was similar and agreed with the results obtained with the 4 strains previously used. The persistence of the former 4 strains was found to vary on the 7th day after the inoculations. All strains disappeared from the sites examined on the 14th and 21st day with the exception of strain 4249. This strain was reisolated from liver, small intestine contents and caecum contents on the 14th day, and from the liver on the 21st day after the injection.

Table 25 summarizes the results of reisolating the 8 strains of C. fetus ss. jejuni over a period of 21 days. The data show that the highest reisolation of organisms was at 24 hr. Campylobacter was present in 50.9% of the sites and the reisolatior rate decreased with time. On the 21st day, only one of 84 samples (1.1%) cultured showed the presence of Campylobacter.

In studying the sites of recovery of 8 strains of C. fetus ss. jejuni after different routes of inoculation in Table 23 and Table 24, the recovery rate was highest after the i.v. route. Figure 7 differentiates the organs in which Campylobacter was present after i.p., i.v. and oral routes of inoculation. The liver and spleen were the organs from which the bacteria could be regularly isolated, and the i.v. route of inoculation was the best route for subsequent recovery of the organisms.

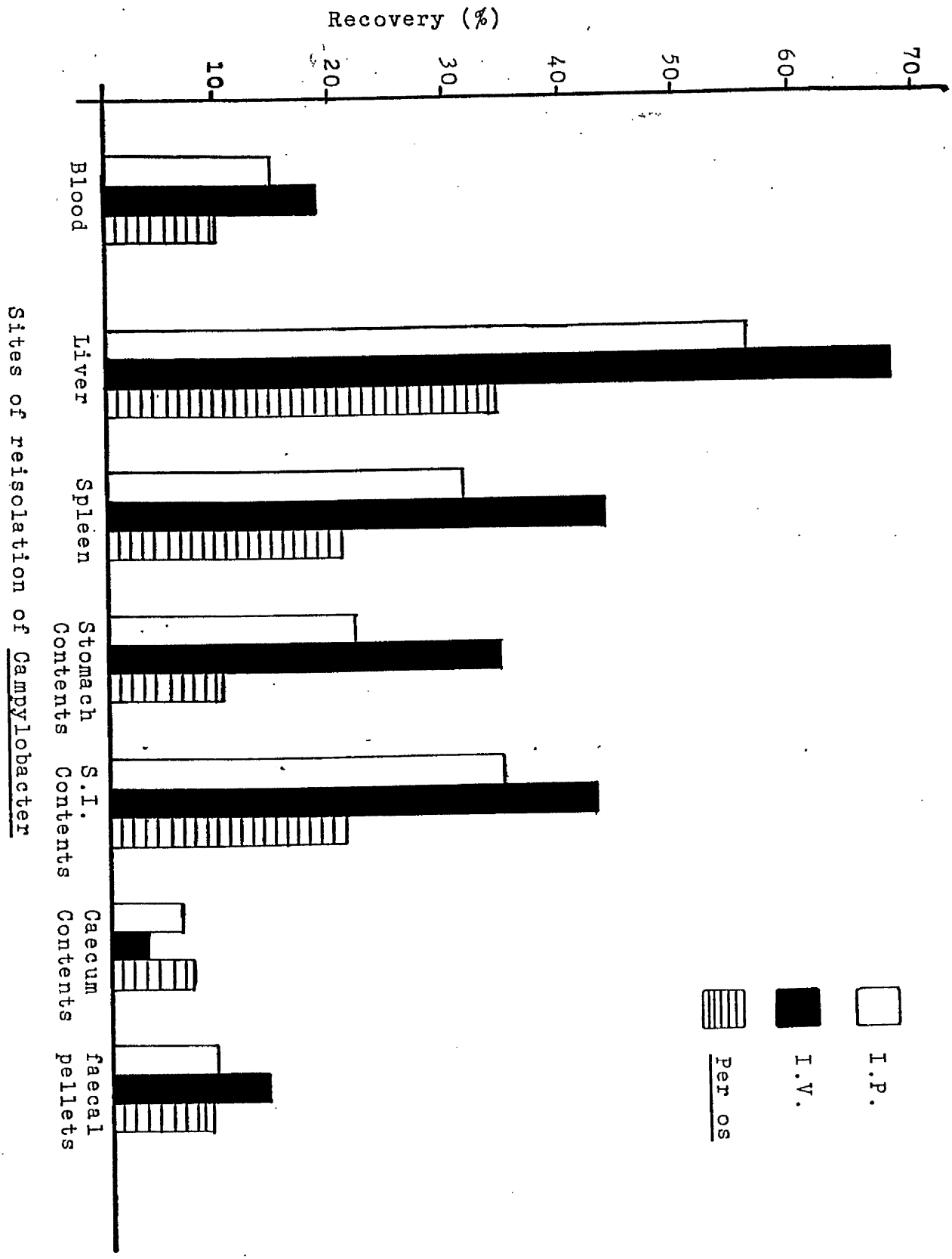
Table 25 : Reisolation of C. fetus ss. jejuni from mice after injection

C. fetus ss. jejuni	Post-injection time (Day)				
Strain	1	2	7	14	21
5636	15/21[a]	4/21	0/21	N	N
5702	11/21	5/21	5/21	N	N
06760	8/21	3/21	0/21	N	N
00539	7/14	6/14	1/14	N	N
4249	13/21	14/21	7/21	3/21	1/21
9227	12/21	11/21	0/21	0/21	0/21
2680	8/21	6/21	4/21	0/21	0/21
8346	8/21	9/21	3/21	0/21	0/21
Total (%)	82/161 (50.9%)	58/161 (36%)	20/161 (12.4%)	3/84 (3.5%)	1/84 (1.1%)

a : No. of positive cultures / No. of samples studied

N : Not done

Fig. 7 : Recovery of Campylobacter from mice infected via different routes.



Sites of reisolation of Campylobacter

3. The occurrence of C. fetus ss. jejuni in mice during the 24hr period following i.v. injection

As it was possible to isolate C. fetus ss. jejuni from the organs of 5-wk old mice inoculated by various routes and kept for 7 and 21 days, I decided to examine those sites every 2 hr during a 24 hr period to observe the clearance of Campylobacter from blood after i.v. injection.

A dose of 0.2ml containing 3.0×10^9 bacteria of the 3 strains, C. fetus ss. jejuni 4249, 5636 and 06760 was inoculated intravenously into a group of 60 5-wk old mice i.e. 20 mice for each strain. The injected mice were killed at 30 min, 1 hr, and then at 2 hr intervals after injection up to 24 hr. The procedure for the recovery of the bacteria was the same as described in Expt. I.2.

Figure 8 shows the 7 sites from which the 3 strains were recovered after inoculation. Campylobacter was reisolated from the liver, spleen and kidney after 30 min, and the organisms were regularly reisolated from these 3 sites over 24 hr.

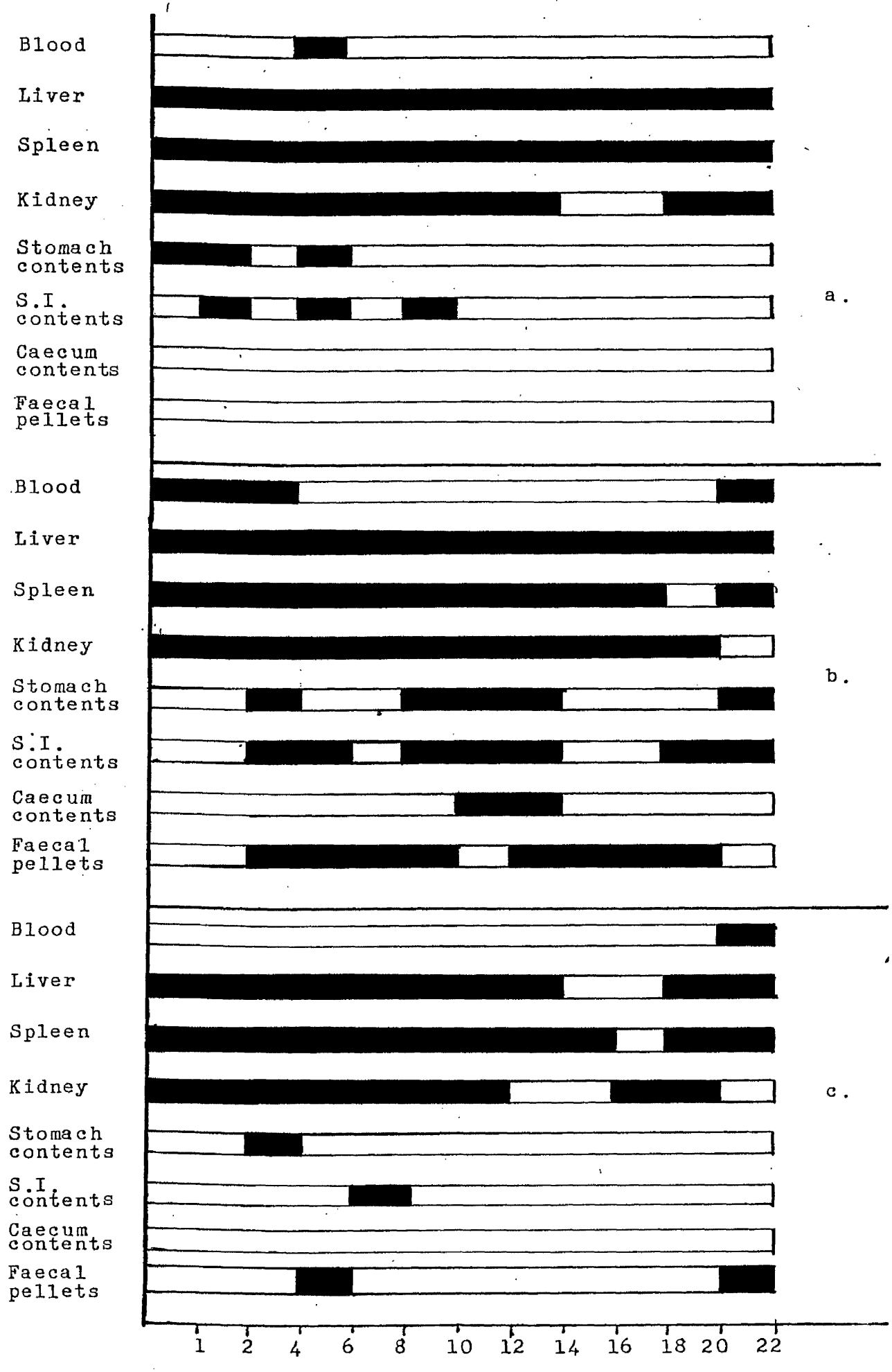
C. fetus ss. jejuni strain 4249 was recovered from the blood 30 min to 6 hr post-injection, and was reisolated again at 22 hr. However, with strains 5636, 06760, the organism was reisolated from blood at 6 hr and 22 hr only. The reisolation of C. fetus ss. jejuni from the gastrointestinal tract was very irregular; strain 4249 was found more frequently than strains 5636 and 06760.

Fig. 8 : Recovery of *C. fetus ss. jejuni* from intravenously-injected mice over a 24 hr period.

a : *C. fetus ss. jejuni* strain 5636
b : " " 4249
c : " " 06760

■ *Campylobacter* was reisolated

□ No *Campylobacter* reisolated


Blood
Liver
Spleen
Kidney
Stomach contents
S.I. contents
Caecum contents
Faecal pellets
a.
Blood
Liver
Spleen
Kidney
Stomach contents
S.I. contents
Caecum contents
Faecal pellets
b.
Blood
Liver
Spleen
Kidney
Stomach contents
S.I. contents
Caecum contents
Faecal pellets
c.
1 2 4 6 8 10 12 14 16 18 20 22
post-injection time (hr).

4. Viable counts of C. fetus ss. jejuni in the homogenates of liver and spleen following i.v. injection

Experiment I.2 had shown that both liver and spleen were the sites from which C. fetus ss. jejuni could be readily recovered after injection, irrespective of the route of inoculation. The aim of this experiment was to investigate the number of viable Campylobacter which could be reisolated from the liver and spleen of 5-wk old mice after i.v. injection. The dose given per mouse contained 3×10^9 bacteria, 2 of the injected mice were killed immediately after injection, and then at intervals of 12, 24, 48, 72 hr and 7 days. The viable counting procedure is described in p. 74 .

The number of viable Campylobacter recovered from the homogenates of spleen and liver at 0, 12, 24, 48, 72 hr and 7 days are recorded in Fig. 9 and 10. The data show that the number of viable Campylobacter decreased rapidly after the injection which suggests that there was no multiplication in either liver or spleen.

II. Injection of C. fetus ss. jejuni cultures into mice of different ages

Inoculation of 5-wk old mice with live C. fetus ss. jejuni did not produce demonstrable illness. Although the injected bacteria could be reisolated from various organs, particularly liver and spleen, there was no sign of multiplication of Campylobacter in vivo. The purpose of this experiment was to investigate if HAM I/CR mice 1-, 7-day and 3-wk old would be susceptible to C. fetus ss. jejuni.

Fig. 9 : Recovery of C. fetus ss. jejuni from the homogenates of spleen.

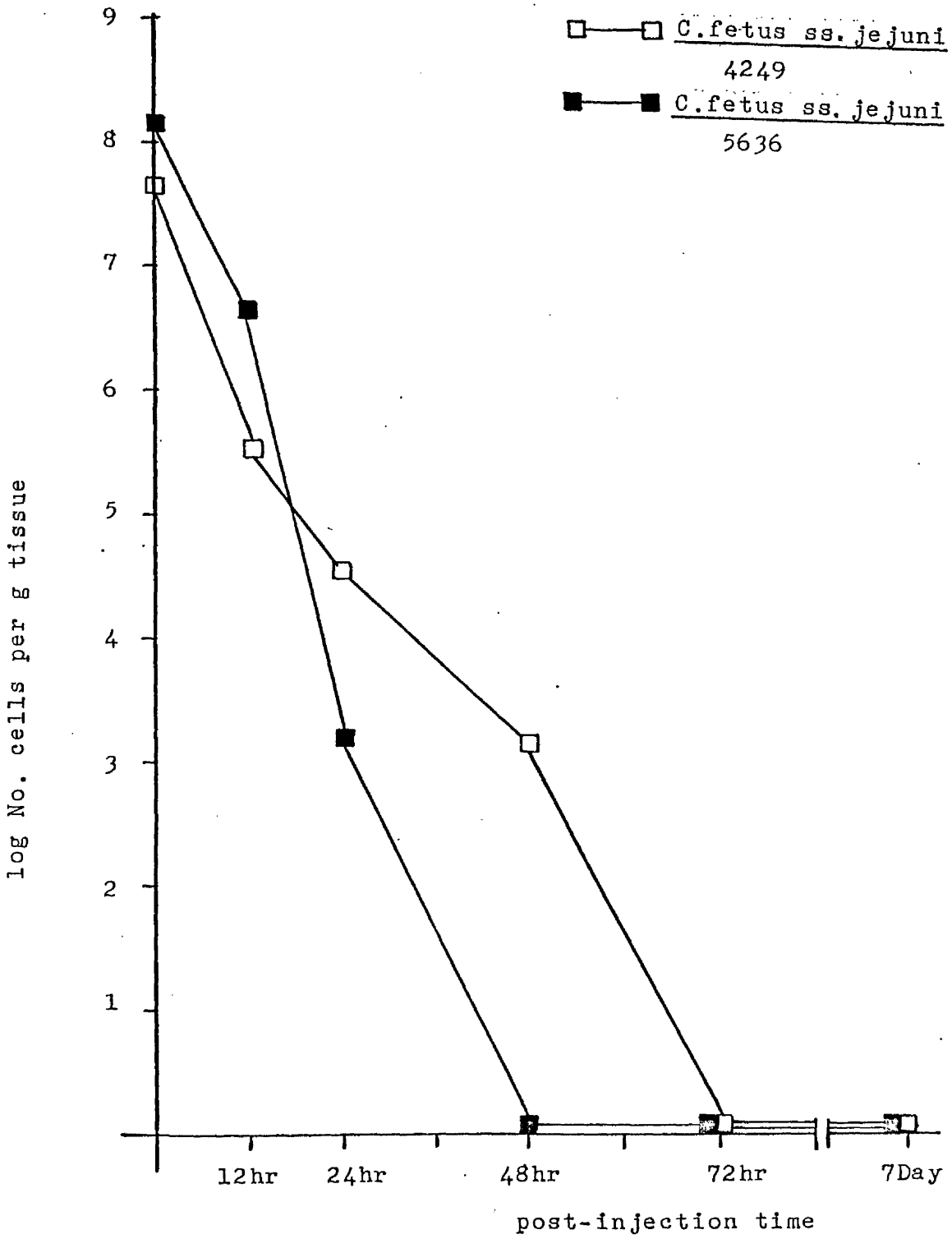
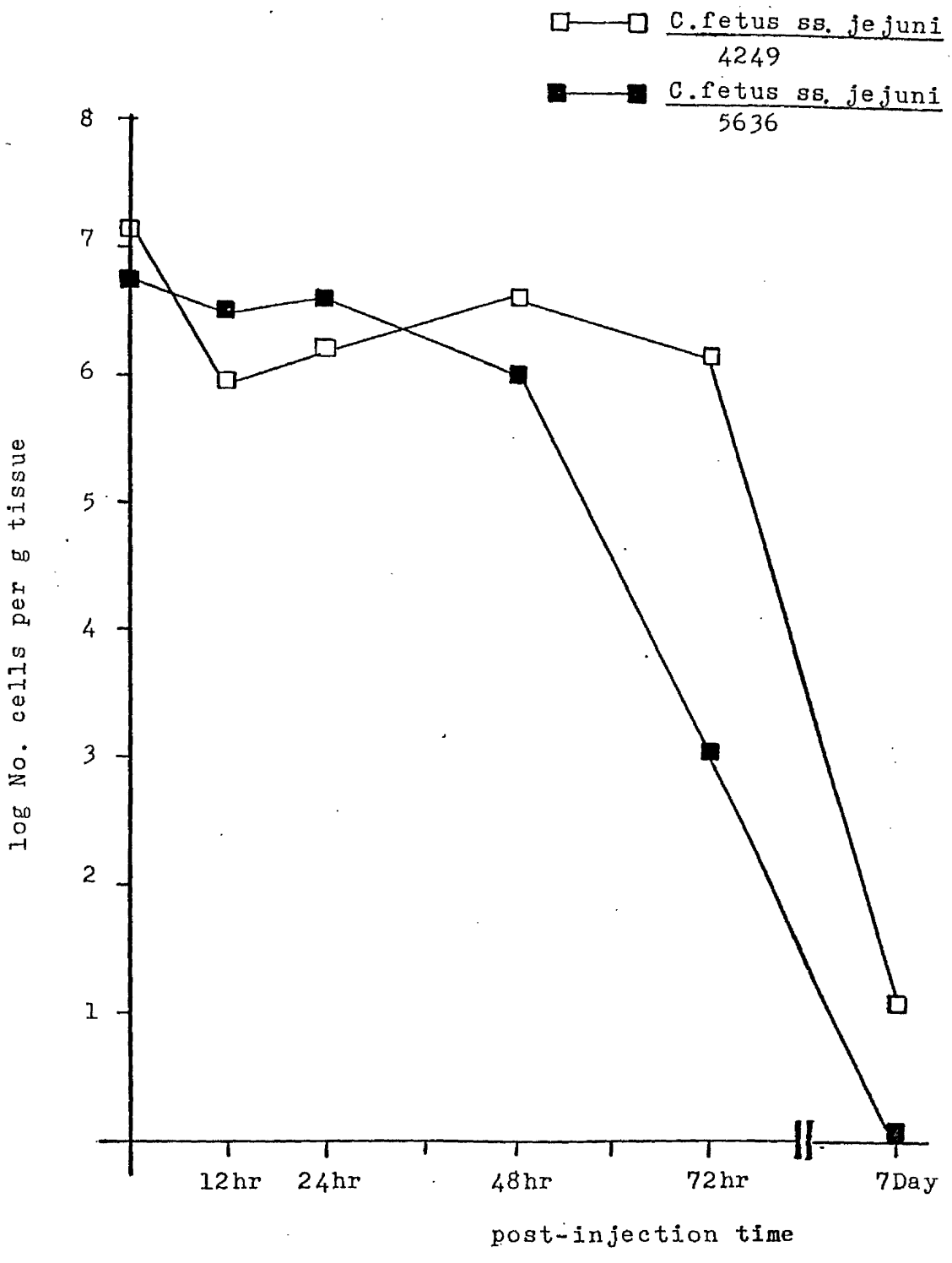


Fig. 10 : Recovery of C. fetus ss. jejuni from the homogenates of liver.



1. 3-week old mice

The 8 strains of C. fetus ss. jejuni which had been used for the 5-wk old mice experiments were used to inoculate mice 3-week old. The suspensions were freshly prepared, these were given i.p., i.v., and per os; the dosage was the same as for the 5-wk old mice, each mouse being given a dose of 3×10^9 bacteria. Table 26 summarizes the results of these experiments. No deaths and no signs of illness were observed over a period of 10 days.

The recovery sites of the 3 strains of C. fetus ss. jejuni 5636, 4249 and 06760 from the 7 sites of inoculated 3-wk old mice (Table 27) showed no significant difference from those of the 5-wk old mice. The bacteria could be recovered from the liver, spleen, blood and gastrointestinal tracts via i.p., i.v. and oral routes of inoculation. Strain C. fetus ss. jejuni 4249 persisted in the liver at the 14th day, and all the organisms disappeared from the 7 sites cultured at the 21st day.

2. 7-day old mice

Three strains of C. fetus ss. jejuni 5636, 06760 and 4249 were used to inoculate 60 7-day old mice i.e. 20 mice for each strain; 0.1ml of the suspension being given intraperitoneally, and each injecting dose contained 3.0×10^9 bacteria. Table 28 summarizes the results of this experiment. The mice which died did so within 72 hr without obvious signs of disease. Macroscopic examination of the dead mice revealed no abnormality in the organs, and pure cultures of Campylobacter were reisolated from the peritoneal cavity of the dead mice. The surviving mice grew normally and were observed over a period of 10 days.

Table 26 : Effect of C. fetus ss. jejuni in 3-wk old mice after different routes of inoculation

C. fetus ss. jejuni	No. of deaths/No. of mice injected[a]		
strain	i.p.[b]	i.v.	Per os
5636	0/10	0/10	0/10
9227	0/10	0/10	0/10
4249	0/10	0/10	0/10
5702	0/10	0/10	0/10
2680	0/10	0/10	0/10
00539	0/10	0/10	0/10
06760	0/10	0/10	0/10
8346	0/10	0/10	0/10

a : Recorded over a period of 10 days

b : Route of inoculations :

i.p. : intraperitoneal

i.v. : intravenous

Per os : by mouth

Table 27 : Recovery of C. fetus ss. jejuni from 3-wk old mice after infection

a : I.P. : intraperitoneal

I.V. : intravenous

Per os : by mouth

b : ● Campylobacter was reisolated

○ No Campylobacter was reisolated

Sites of Recovery	Routes[a] of Infection	Campylobacter fetus ss. jejuni														
		5636					4249					06760				
		24[b] hr	48 hr	7 Day	14 Day	21 Day	24 hr	48 hr	7 Day	14 Day	21 Day	24 hr	48 hr	7 Day	14 Day	21 Day
Blood	I.P.	○	○	○	○	○	●	○	○	○	○	●	○	○	○	○
	I.V.	●	○	○	○	○	●	●	○	○	○	●	○	○	○	○
	Per os	●	○	○	○	○	●	○	○	○	○	○	○	○	○	○
Liver	I.P.	●	●	○	○	○	●	●	○	○	○	●	●	○	○	○
	I.V.	●	●	●	○	○	●	●	●	●	○	●	●	○	○	○
	Per os	●	●	○	○	○	●	○	○	○	○	●	○	○	○	○
Spleen	I.P.	●	○	○	○	○	●	●	○	○	○	●	●	○	○	○
	I.V.	●	●	○	○	○	●	●	○	○	○	●	●	○	○	○
	Per os	●	○	○	○	○	●	○	○	○	○	●	○	○	○	○
Stomach Contents	I.P.	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	I.V.	○	●	○	○	○	○	●	○	○	○	○	○	○	○	○
	Per os	○	○	○	○	○	●	○	○	○	○	●	○	○	○	○
S.I. Contents	I.P.	●	○	○	○	○	●	●	○	○	○	●	○	○	○	○
	I.V.	●	●	○	○	○	○	●	●	○	○	●	○	○	○	○
	Per os	●	●	○	○	○	●	●	○	○	○	●	●	○	○	○
Caecum Contents	I.P.	○	○	○	○	○	○	○	○	○	○	●	○	○	○	○
	I.V.	○	○	○	○	○	○	●	○	○	○	●	○	○	○	○
	Per os	●	○	○	○	○	●	○	○	○	○	●	●	○	○	○
Rectum faecal pellets	I.P.	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	I.V.	○	●	○	○	○	●	○	○	○	○	○	●	○	○	○
	Per os	●	○	○	○	○	●	○	○	○	○	○	●	○	○	○

3. 1-day old mice

Table 29 shows the deaths recorded in groups of 20 1-day old mice during the 10 days following i.p. injection with the 3 strains of C. fetus ss. jejuni used in experiment II.2.; 0.05ml of the suspension was given, and the injecting dose for each mouse contained 3 x 10^9 bacteria. The data show that the mortality rate was very low in comparison to that of 7-day old mice. No mice in the group injected with strain 4249 died; 3 and 2 of the 20 injected mice died as the result of the injection of C. fetus ss. jejuni strain 5636 and 06760.

All the mice died 72 hr after the injection without showing signs of disease. Pure cultures of Campylobacter were recovered from the peritoneal cavity. The surviving mice grew normally.

The results of the foregoing experiment is summarized in Table 30. The data indicate that mice 7-day old would appear to be most susceptible to the injection of live C. fetus ss. jejuni.

4. Pregnant mice

Mice 1-week pregnant were injected with strains of C. fetus ss. jejuni 5636 and 4249. Four animals were injected i.v. with 0.2ml containing 3.0 x 10^9 bacteria. Two uninoculated mice were kept as controls. The inoculated mice showed no signs of visible upset or diarrhoea, but all the babies were still-born. Cultures of C. fetus ss. jejuni were recovered from the homogenates of the dead baby mice. The 2 uninoculated pregnant mice had normal living litters.

Table 28 : Effect of live C. fetus ss. jejuni cultures[a] on 7-day old mice

No. of deaths / No. of mice injected[b]		
C. fetus ss. jejuni		
5636	4249	06760
6/20	4/20	7/20

a : 0.1ml of the suspensions containing 3 x 10^9 bacteria were given i.p.

b : Recorded over a period of 10 days.

Table 29 : Effect of live C. fetus ss. jejuni cultures[a] on 1-day old mice

No. of deaths / No. of mice injected[b]		
C. fetus ss. jejuni		
5636	4249	06760
3/20	0/20	2/20

a : 0.05ml containing 3 x 10^9 bacteria were given i.p.

b : Recorded over a period of 10 days

Table 30: Effect of *C. fetus ss. jejuni* on mice of different ages

C.fetus ss. jejuni strains	No. of deaths/No. of mice injected age of mice [a]			
	1-Day	7-Day	3-week	5-week
5636	3/20	6/20	0/10	0/10
4249	0/20	4/20	0/10	0/10
06760	2/20	7/20	0/10	0/10
Total	5/60	17/60	0/30	0/30

a : each mouse received a dose of 3×10^9 bacteria intraperitoneally.

III. Passage Experiments

1. Effect of successively passaged strains of C. fetus ss. jejuni on 7-day old mice

The virulence of bacteria can be restored by successive passage through susceptible animals. As mice 7 days old appeared to be most susceptible to C. fetus ss. jejuni, the effect on these mice of C. fetus ss. jejuni successively passaged through 5-wk old mice was examined.

C. fetus ss. jejuni strains 5636 and 4249 were passaged 5 times. The procedures are described in p. 73. Table 31 shows that the successively passaged cultures generally increased the number of deaths in 7-day old mice. The bacterial suspensions contained 3.0×10^{10} organisms per ml, 0.1ml of these suspensions were given i.p. per mouse. The inoculated mice died within 72 hr without showing signs of diarrhoea. Pure cultures of Campylobacter were recovered from the peritoneal cavity of the dead mice.

The 5th passaged strains were stored at $-76^{\circ}C$ on glass beads, and used for further experiments.

2. Virulence of passaged C. fetus ss. jejuni for mice of different ages

Preliminary experiments (Table 30) showed that 7-day old mice were more susceptible to C. fetus ss. jejuni than 1-day, 3-and 5-wk old mice. In order to establish more precisely at what age mice became resistant to a single inoculation dose of C. fetus ss. jejuni, a further study of age-related susceptibility was carried out

Table 31 : Inoculation of 7-day old mice with successively passaged strains of C. fetus ss. jejuni 5636 and 4249.

No. of passage	No. of deaths/No. of mice injected[a]	
	C.fetus ss. jejuni 5636	C. fetus ss. jejuni 4249
1	3/10	2/10
2	5/10	5/10
3	6/10	6/10
4	10/10	5/10
5	8/10	8/10

a : The results were recorded over a period of 10 days. 0.1ml containing 3×10^9 bacteria were given i.p.

Table 32 : Susceptibility of mice of different ages to 5th passaged *C. fetus ss. jejuni* 5636.

Dose per[a] mouse	No. of deaths/No. of mice injected mice aged[c] 1-Day	3-Day	7-Day	14-Day	3-Wk	5-Wk
1×10^{10}	ND[b]	10/10	10/10	10/10	2/10	0/10
5×10^{9}	2/10	5/10	10/10	10/10	0/10	0/10
2.5×10^{9}	0/10	2/10	8/10	7/10	0/10	0/10
1.3×10^{9}	0/10	0/10	4/10	1/10	0/10	0/10
Total mice died (%)	2/30 (6.6%)	17/40 (42.5%)	32/40 (80%)	28/40 (70%)	2/40 (5%)	0/40 (0)

a : The amounts given were 0.5ml for 3-and 5-wk old mice, 0.1ml for 3-, 7-and 14-day old mice, and 0.05ml for 1-day old mice. All these amounts contained the same number of viable cells as shown.

b : ND : Not done

c : The results were recorded over a period of 10 days.

with C. fetus ss. jejuni strain 5636 which had been passaged 5 times. Mice 1-day old were injected with 0.05ml, while mice 3-, 7- 14-days 3-and 5-wk old received 0.1ml. The suspensions were inoculated intraperitoneally, and the mice were given a dose of 1×10^{10}, 5×10^{9}, 2.5×10^{9} and 1.3×10^{9} viable Campylobacter. The results are summarized in Table 32 which shows that 7-day old mice were most susceptible to C. fetus ss. jejuni; 32 of the 40 inoculated mice (80%) died in this group. The percentage mortality in both the 3-and 14-day age-groups was relatively high; 28 of the 40 inoculated mice (70%) died in the 14-day age-group and 17 of the 40 inoculated mice (40.5%) died in the 3-day old age-group. However, the percentage mortality was low in the other age-groups, only 2 of the 30 (6.6%)1-day old mice and 2 of the 40 (5%) 3-wk old mice died. No 5-wk old mice died after these injections. Death occurred within 48 hr, and there were no signs of diarrhoeal illness. The surviving mice were all killed 10 days after the experiments.

IV. 7-Day Old Mice for Toxicity/Infectivity Studies

1. Inoculation routes

With 7-day old mice, i.v. and per os routes of inoculation were not feasible because of the small size of the animals, so i.p. and intra-rectal routes of inoculation were used. It was found that inoculation of 0.1ml (3.0×10^{9} organisms) of live C.fetus ss. jejuni via the intra-rectal route had no effect on 7-day old mice i.e. no mouse died or showed signs of diarrhoeal illness.

Intraperitoneal injection of 0.1ml sterile physiological saline and nutrient broth had no effect on 7-day old mice. The weight-gain of the mice injected with saline and nutrient broth and the uninoculated mice was the same.

2. Examination of various culture preparations in 7-day old mice

Early experiments showed that 7-day old mice were most susceptible to C. fetus ss. jejuni (Table 32) and passaging the organism via mice would appear to increase the mouse virulence of C. fetus ss. jejuni (Table 31). The 5th passaged strains of C. fetus ss. jejuni 4249 and 5636 reconstituted from the cultures stored at -76°C on glass beads were used in an attempt to investigate the mechanisms causing the death in 7-day old mice.

The results obtained by inoculating 24 hr broth cultures, bacterial pellets resuspended in saline and supernate fluids into 7-day old mice are summarized in Table 33. The data show that death was directly associated with the bacterial cells. No mice in the group injected with the supernatant fluids died. This would appear to indicate that C. fetus ss. jejuni did not produce under these conditions extracellular toxin which were lethal to 7-day old mice. The 24 hr broth culture contained 9.7×10^9 cells per ml; no deaths were found in the group of mice injected with 0.1ml of these whole broth cultures. It would appear that the number of living bacteria injected into the mice was smaller than that required to cause lethal effect in 7-day old mice.

Table 33 : Effect of various culture preparations of passaged *C. fetus ss. jejuni* in 7-day old mice

No. of deaths / No. of mice injected[1]					
C. fetus ss. jejuni 5636			*C. fetus ss. jejuni* 4249		
Whole[2] broth culture (9.7×10^8 cells)	Supernatant[3] fluid	Pellet[4] (3×10^9 cells)	Whole broth culture (9.7×10^8 cells)	Supernatant fluid	Pellet (3×10^9 cells)
0/10	0/10	6/10	0/10	0/10	7/10

1. Recorded over a period of 10 days.
2. The 24hr broth cultures contained 9.7×10^9 bacteria per ml. 0.1ml of the broth culture was given i.p.
3. 0.1ml of the culture supernates was given i.p.
4. Pellets were resuspended in saline and standardized as containing 3×10^{10} bacteria per ml. 0.1ml containing 3×10^9 bacteria was given i.p.

3. Virulence of passaged *C. fetus ss. jejuni* strains for 7-day old mice

C. fetus ss. jejuni did not produce extracellular toxins lethal to 7-day old mice. Death following i.p. injection of live *Campylobacter* was directly associated with the numbers of bacterial cells (Table 33). This experiment aimed at determining the median lethal dose of *C. fetus ss. jejuni* for this age-group of mice.

Table 34 shows the results obtained when groups of 20 7-day old mice were inoculated intraperitoneally with different doses of live suspensions of passaged *C. fetus ss. jejuni* 5636. The bacterial suspension was standardized as described on p. 69 . The undiluted suspension contained 1×10^{11} bacteria per ml; from this, serial doubling dilutions of 1/2 to 1/16 were made; 0.1ml of the diluted suspensions was given intraperitoneally, and the dose per mouse was 1×10^{10}, 5×10^{9}, 2.5×10^{9}, 1.3×10^{9} and 6.2×10^{8} viable cells respectively.

The data indicate that when concentrations of 1×10^{10} or 5×10^{9} bacteria were given per mouse, all of them died. The mortality decreased as the concentration of cells in the suspension was reduced, and there were no deaths in the group of mice inoculated with a dose of 6.2×10^{8} bacteria. Most of the mice died 48 hr after the injection, but with the groups of mice inoculated with doses of 1×10^{10} and 5×10^{9} bacteria, the deaths were recorded within 24 hr, and 1 mouse in the group injected with a dose of 2.5×10^{9} died at 72 hr. There were no deaths before 12 hr and after the 4th day from the time of the

Table 34 : Virulence of passaged C. fetus ss. jejuni 5636 for 7-day old mice

Dose per[a] mouse in 0.1ml.	No. of mice dead: Post-injection time 24 hr	48 hr	72 hr	4th Day	7th Day	10th Day	No. of deaths/ No. of mice injected.
$1x10^{10}$	20						20/20
$5x10^{9}$	20						20/20
$2.5x10^{9}$	9	3	1	0	0	0	13/20
$1.3x10^{9}$	4	2	0	0	0	0	6/20
$6.2x10^{8}$	0	0	0	0	0	0	0/20

LD_{50}^{b} : 1.8×10^{9} (95% C.L. : $1.4 - 2.1 \times 10^{9}$)

a : Undiluted suspension contained 1×10^{11} viable bacteria per ml. The suspension was diluted in a 2-fold series. 0.1ml of the suspension was given i.p., and the data show the no. of viable bacteria given per mouse.

b : 95% C.L. = The 95 per cent confidence limits

injection. The LD_{50} value was 1.81×10^9 bacteria with 95% confidence limits (C.L.) of $1.4 - 2.1 \times 10^9$ bacteria.

Comparable results were obtained in repeated experiments using the same undiluted suspension which contained 1×10^{11} bacteria per ml, but was diluted from 1/10 to 1/100 (Table 35). The LD_{50} value was 2.2×10^9 bacteria with 95% C.L. of $1.6 - 3.1 \times 10^9$ bacteria.

In a similar experiment but with passaged strain 4249, the results obtained were comparable to those of passaged strain 5636. Serial doubling dilutions of 1/2 to 1/16 were made from the undiluted bacterial suspension which contained 1×10^{11} bacteria per ml; 0.1ml of the diluted suspensions which contained 1×10^{10}, 5×10^9, 2.5×10^9, 1.3×10^9 and 6.2×10^8 viable cells was given intraperitoneally to 100 7-day old mice (Table 36). There was 100% mortality in the group of mice inoculated with the two highest doses and the mortality decreased as the dose of cells was reduced. The percentage mortality for the groups injected with 2.5×10^9 and 1.3×10^9 viable Campylobacter was 45 (9/20) and 15 (3/20) respectively. There were no deaths in the group inoculated with 6.2×10^8 bacteria. Most of the mice died within 24 hr, with the exception of 1 mouse in the group inoculated with a dose of 5×10^9 bacteria which died at 48 hr, and 1 mouse from the group inoculated with 2.5×10^9 bacteria which died at 72 hr. The LD_{50} value of passaged C. fetus ss. jejuni 4249 was 2.30×10^9 with 95% confidence limits of $1.90 - 2.80 \times 10^9$ bacteria.

None of the mice that died following the injection of passaged C. fetus ss. jejuni 5636 and 4249 exhibited signs of

Table 35 : Virulence of passaged C. fetus ss. jejuni 5636 for 7-day old mice

Dose per mouse in 0.1ml[a]	No. of mice dead: Post-injection time 24hr	48hr	72hr	4 Day	7 Day	10 Day	No. of deaths/ No. of mice injected.
$1x10^{10}$	27						27/27
$1x10^{9}$	3	1	0	0	0	0	4/27
$1x10^{8}$	0	0	0	0	0	0	0/27
LD_{50}^{b} : 2.2×10^{9} (95% C.L. : $1.6 - 3.1 \times 10^{9}$)							

a : Undiluted suspension contained 1×10^{11} viable bacteria per ml. The suspension was diluted with a factor of 10. 0.1ml of the suspension was given i.p., the data show the dose inoculated per mouse.

b : 95% C.L. : The 95 per cent confidence limits.

Table 36 : Virulence of passaged C. fetus ss. jejuni 4249 for 7-day old mice

Dose per[a] mouse in 0.1ml	No. of mice dead — Post-injection time						No. of deaths/ No. of mice injected
	24hr	48hr	72hr	4 Day	7 Day	10 Day	
1×10^{10}	20						20/20
5×10^{9}	19	1					20/20
2.5×10^{9}	8	0	1	0	0	0	9/20
1.2×10^{9}	3	0	0	0	0	0	3/20
6.2×10^{8}	0	0	0	0	0	0	0/20

LD_{50} : 2.30×10^{9} (95% C.L. $1.9 - 2.8 \times 10^{9}$)

a : The undiluted suspension contained 1×10^{11} viable bacteria per ml and 0.1ml was given i.p.

Table 37 : Effect of heating at 56°C and sonication on *C. fetus* ss. *jejuni*

No. of viable cells per ml[a]		
Untreated suspension	heating at 56°C for 30 min	sonication (3x30 sec)
1×10^{10}	< 10	4.9×10^{5}

a : The number of viable *Campylobacter* was estimated by colony plate counts.

Table 38 : Response of 7-day old mice to heat-killed and sonicated suspensions

Dose per[a] mouse in 0.1ml	No. of deaths / No. of mice injected				
	Control live suspension	Sonication (3 x 30sec)	Suspensions heat-killed at		
			56°C 15 min	56°C 30 min	100°C 30min
1×10^{10}	17/17	11/17	15/17	14/17	14/17

a : The suspension was given i.p.

diarrhoea. Macroscopic examination of dead mice showed no abnormality; pure cultures of Campylobacter were recovered from the peritoneal cavity.

4. Effect of heat-killed and sonicated suspensions of C. fetus ss. jejuni 5636 on 7-day old mice

C. fetus ss. jejuni was killed by heating at 56°C in a shaking water bath for 15 min. In another sample of bacteria subjected to sonication, there was about a 50% mortality (Table 37). The lethality of these treated suspensions for 7-day old mice was studied in this experiment.

Bacterial suspensions containing 1 x 10^{10} viable Campylobacter per ml were sonicated for 3 x 30 sec, or heat-killed at 56°C for 15 min and 30 min or heat-killed at 100°C for 30 min. Then, 0.1ml of these suspensions were given i.p. to 5 groups of 17 7-day old mice, and the results of injecting live, sonicated and heat-killed (both at 56°C and 100°C) suspensions are summarized in Table 38. The data show that all the mice injected with a dose of 1 x 10^{10} Campylobacter died, the result corresponding to the early experiments (Table 34). However, with the group of mice injected with the suspension heat-killed at 56°C for 15 min, the percentage mortality was 88.2% (15/17). For the suspensions heat-killed at 56°C or 100°C for 30 min, the percentage mortality for both was 82.3% (14/17). Previous experiments had shown that about 50% of bacteria could survive after sonication, and it was found that only 64.7% (11/17) of the mice died after the inoculation of a dose of the sonicated suspension. The deaths

occurred within 24 hr, with the exception that 2 mice inoculated with sonicated suspension died 48 hr after the injection. No mice developed signs of diarrhoeal illness.

The heat-killed (56° or 100°C for 30 min) suspensions of passaged C. fetus ss. jejuni were centrifuged at 9,000 r.p.m. for 15 min. When the heat-killed suspensions and both the supernatant fluids and pellets of these suspensions were inoculated i.p. into 7-day old mice (Table 39), the number of deaths produced by heat-killed suspensions and the pellets resuspended in saline was the same. There were no deaths in the group of mice inoculated with the supernatant fluids of the heat-killed suspensions.

It would seem from the results of these experiments that although C. fetus ss. jejuni was completely killed at 56°C for 15 min, the injection of a dose of 1×10^{10} heat-killed bacteria remained lethal to the 7-day old mice. This would indicate that some factor, perhaps the LPS of Campylobacter may be responsible for death.

5. Comparative studies of the effect of heat-killed and live suspensions of C. fetus ss. jejuni on 7-day old mice

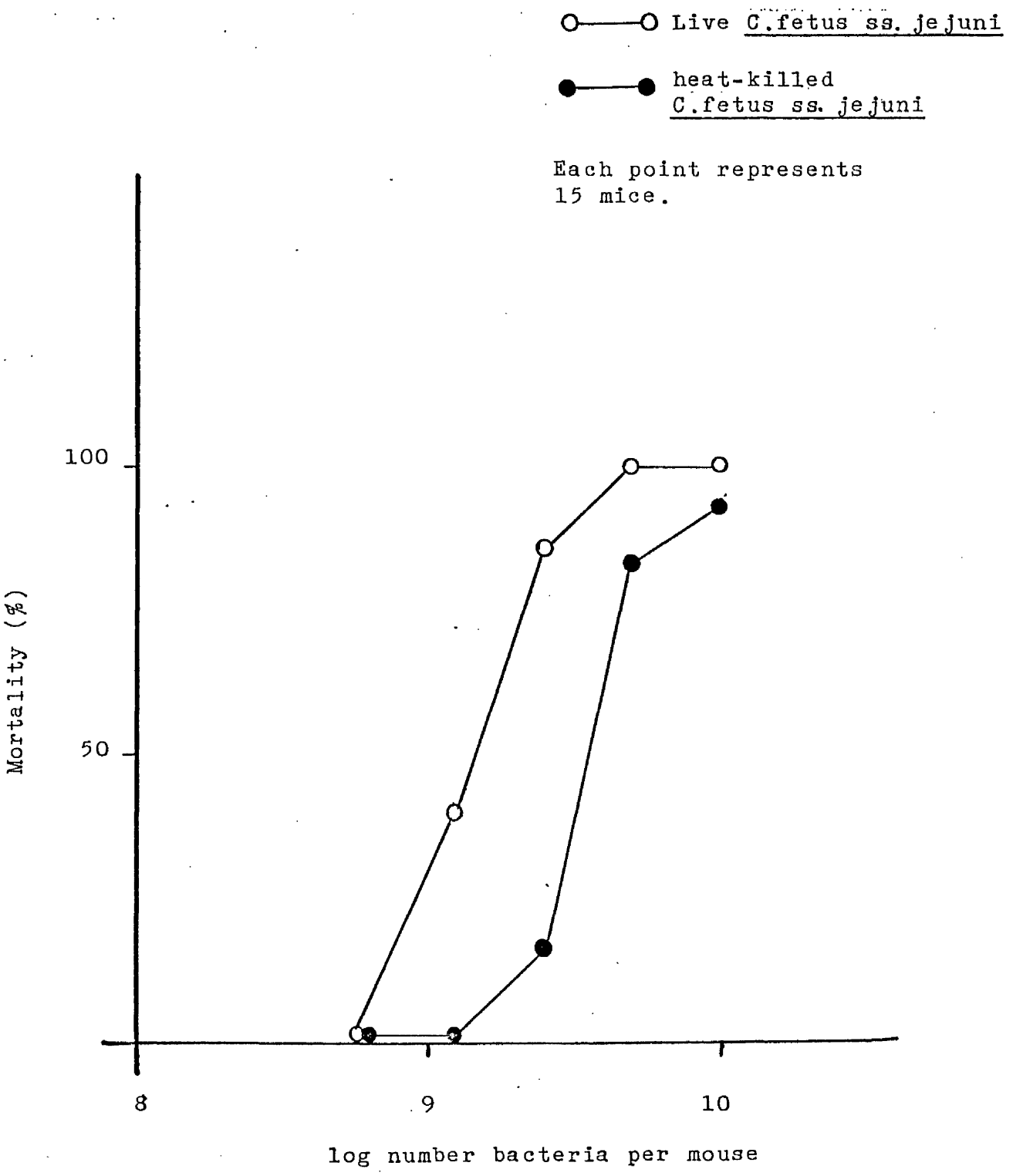
Early experiments had shown that 7-day old mice died after the injection of a dose of 1×10^{10} live and heat-killed suspensions of C. fetus ss. jejuni. In comparing the effect of different concentration of cells of both heat-killed and live suspensions in 7-day old mice, a portion of the undiluted suspension which contained 1×10^{11} bacteria per ml was heat-

Table 39 : Inoculation of 7-day old mice with heat-killed suspensions of *C. fetus ss. jejuni*

Dose per[1] mouse in 0.1 ml	No. of deaths / No. of mice injected[2]					
	Heat-killed at 56°C for 30 min			Heat-killed at 100°C for 30 min		
	Suspension	Centrifugation, 9,000 r.p.m., 15 min		Suspension	Centrifugation, 9,000 r.p.m., 15 min	
		Supernatant fluid	Pellets[3]		Supernatant fluid	Pellets
1×10^{10}	10/10	0/10	10/10	9/10	0/10	9/10

1. Suspensions contained 1×10^{11} bacteria per ml. 0.1ml of the suspensions were given i.p.
2. Recorded over a period of 10 days
3. Bacterial pellets collected by centrifugation were resuspended in sterile saline to initial volume (10ml)

Fig. 11 : Effect of live and heat-killed *C. fetus ss. jejuni* on 7-day old mice.



killed at 56°C for 15 min, and both live and heat-killed suspensions were diluted by a factor of 2; 0.1ml of these suspensions were given i.p. to groups of 15 7-day old mice. Fig. 11 shows that all the mice died after receiving a dose of 1×10^{10}, and 5×10^{9} viable Campylobacter, and the mortality decreased as the concentration of cells was reduced. In the group of mice inoculated with heat-killed suspensions, 14 of the 15 (93%) mice injected with a dose of 1×10^{10} bacteria died, and the mortality also decreased as the concentration of cells was reduced. The LD_{50} value for the live suspensions was found to be 1.5×10^{9} (95% C.L. was $1.2 - 1.8 \times 10^{9}$), and the LD_{50} value of the heat-killed suspension was 4.1×10^{9} (95% C.L. was $3.3 - 5.1 \times 10^{9}$). The mice injected with doses of live and heat-killed suspensions of 1×10^{10} and 5×10^{9} died within 24 hr; the deaths in other groups of mice were reported 72 hr after the injection.

V. Ferric ammonium citrate as an enhancing agent for virulence of C. fetus ss. jejuni in mice

As recorded in the literature, incorporation of ferric ammonium citrate with the inocula reduced the LD_{50} for a variety of bacteria. The effect of using this agent along with Campylobacter for mouse inoculation was examined. Two aspects were considered, a) would this mixture increase the lethality of C. fetus ss. jejuni for 3-and 5-wk old mice and b) would the LD_{50} for 7-day old mice be decreased?

1. The effect of injecting ferric ammonium citrate with live C. fetus ss. jejuni into 3-and 5-wk old mice

Injecting a dose of 3×10^9 live C. fetus ss. jejuni had no effect on both 3-and 5-wk old mice, irrespective of the route of inoculation, although the bacteria could be reisolated from the blood, liver, spleen and gastrointestinal tract after inoculation (Table 23 and Table 24).

The incorporation of 80 μg Fe^{3+} with a dose of 3×10^9 viable bacteria (2 passaged strains of C. fetus ss. jejuni), given i.p., i.v. and orally to 5-wk old mice (average wt 25g) had no apparent effect i.e. no mouse died or developed signs of diarrhoea over a period of 10 days.

Similarly, inoculation of 3-wk old mice (average wt 15g) concomitantly with a dose of 3×10^9 bacteria and 48 μg Fe^{3+} i.p., i.v. and per os also produced no demonstrable effect, and no mouse died as a result of these injections.

2. Effect of injecting ferric ammonium citrate with live C. fetus ss. jejuni into 7-day old mice

The LD_{50} values of passaged C. fetus ss. jejuni strains 5636 and 4249 were 1.8×10^9 and 2.4×10^9 bacteria respectively (Table 34 and Table 36). The results of repeated experiments with and without the incorporation of Fe^{3+} in live bacterial suspensions of passaged C. fetus ss. jejuni 5636 in 7-day old mice are summarized in Table 40. The undiluted suspensions contained 5×10^{10} bacteria per ml. The concentrations of bacterial suspensions and ferric ammonium citrate were prepared in double

strength. This preparation would give a final dose of 2.5×10^9, 1.3×10^9, 6.2×10^8 and 3.1×10^8 bacteria with a concentration of 4, 8, 12 μg Fe^{3+} per mouse when the bacterial suspensions were injected concomitantly with the ferric ammonium citrate solution. The injection of a dose of 4, 8, 12 μg Fe^{3+} alone had no apparent effect on 7-day old mice (Table 41).

Table 40 shows that the incorporation of Fe^{3+} in live bacterial suspensions reduced the LD_{50} values. This effect showed a correlation and parallelism with the concentration of Fe^{3+}; this parallelism being more pronounced as the Fe^{3+} concentration increased to 12 μg. The LD_{50} value of live bacterial suspensions without Fe^{3+} was 1.8×10^9 bacteria but by incorporating 4 μg Fe^{3+}, the LD_{50} value was reduced to 1.2×10^9, and with 8 and 12 μg Fe^{3+}, the LD_{50} values were 7.4×10^8 and 4.2×10^8 bacteria respectively.

Table 42 shows the results of the similar experiment with undiluted suspensions which contained 1×10^{11} bacteria per ml, and diluted in a 5-fold series. It is clear that when doses of 5×10^9, 1×10^9, 2×10^8 and 4×10^7 bacteria were injected with 8 and 12 μg Fe^{3+} per mouse, the LD_{50} values were reduced. The LD_{50} of live bacterial suspension without Fe^{3+} was 1.6×10^9, but with the incorporation of 8 and 12 μg Fe^{3+}, the LD_{50} values were reduced to 7.2×10^8 and 2.3×10^8 respectively.

The reduction of LD_{50} value with the incorporation of Fe^{3+} was also observed with another passaged strain of C. fetus ss. jejuni strain 4249. The LD_{50} of live bacterial suspension was 2.4×10^9, and with the incorporation of 4, 8 and 12 μg Fe^{3+}, the

Table 40 : Effect of ferric ammonium citrate on passaged *C. fetus ss. jejuni* 5636 on 7-day old mice[a]

Dose per[c] mouse in 0.1 ml	No. of deaths / No. of mice injected[b]			
	Suspension without Fe^{3+} i.p.	Fe^{3+} µg, intraperitoneal, 0.1ml		
		4	8	12
2.5×10^9	15/20	20/20	20/20	20/20
1.3×10^9	6/20	10/20	14/20	20/20
6.2×10^8	0/20	2/20	9/20	16/20
3.1×10^8	0/20	0/20	2/20	5/20
LD_{50} value (95% C.L.)	1.8×10^9 ($1.4\text{-}2.1 \times 10^9$)	1.2×10^9 ($0.9\text{-}1.4 \times 10^9$)	7.4×10^8 ($6.5\text{-}8.4 \times 10^8$)	4.2×10^8 ($3.5\text{-}5.1 \times 10^8$)

a : Average weight - 2.5g

b : Recorded over a period of 10 days

c : The undiluted suspension contained 5×10^{10} bacteria per ml. It was diluted by a factor of 2 and the data show the number of bacteria inoculated per mouse.

Table 41 : Toxicity of ferric ammonium citrate for 7-day old mice[a]

No. of deaths / No. of mice injected[b]					
control mice 0.1ml, saline i.p.	Fe^{3+}, μg, i.p., 0.1 ml per mouse				
	4	8	12	20	100
0/20	0/20	0/20	0/20	0/20	12/20[c]

a : Average weight - 2.5g

b : The results were recorded over a period of 3 days

c : The mice died within 12 hr after the injection

Table 42 : Effect of ferric ammonium citrate on passaged *C. fetus ss. jejuni* 5636 in 7-day old mice[a]

Dose per mouse in 0.1ml[c]	No. of deaths / No. of mice injected[b]		
	Suspension without Fe^{3+}, i.p.	Fe^{3+} µg, i.p., 0.1ml	
		8	12
5×10^9	10/10	10/10	10/10
1×10^9	2/10	6/10	10/10
2×10^8	0/10	1/10	4/10
4×10^7	0/10	0/10	0/10
LD_{50} value (95% C.L.)	1.6×10^9 ($1.5\text{-}1.7 \times 10^9$)	7.2×10^8 ($6.4\text{-}8.1 \times 10^8$)	2.3×10^8 ($1.4 - 4 \times 10^8$)

a : Average weight - 2.5g

b : The results were recorded over a period of 10 days

c : The undiluted suspension contained 1×10^{11} bacteria per ml. The suspension was diluted in a 5-fold series.

LD_{50} values were reduced to 1.7×10^9, 1.2×10^9 and 8.6×10^8 respectively. (Table 43).

Using a higher concentration of Fe^{3+} i.e. 20 μg per mouse, did not however lead to a marked lowering of the LD_{50} value of the passaged strain of C. fetus ss. jejuni 5636 for 7-day old mice (Table 44). The LD_{50} value of the suspension incorporated with 12 μg Fe^{3+} was 5.4×10^8, but with the incorporation of 20 μg Fe^{3+}, the LD_{50} value was reduced only to 5.0×10^8. This would seem to indicate an optimal amount of Fe^{3+} was required to produce the enhancing effect in vivo.

3. Effect of ferric ammonium citrate with heat-killed suspensions on 7 day-old mice

The injection of live C. fetus ss. jejuni with ferric ammonium citrate increased the number of deaths in 7-day old mice (Table 40). In this experiment the effect of incorporating ferric ammonium citrate with heat-killed C. fetus ss. jejuni was investigated. Table 45 shows the results obtained when the heat-killed suspensions were inoculated into 7-day old mice with the incorporation of 12 μg Fe^{3+}. The dose given per mouse for passaged strains of C. fetus ss. jejuni 5636 and 4249 was 2.5×10^9 bacteria. The bacterial suspensions were heat-killed at 56°C for 30 min. The data show that the incorporation of ferric ammonium citrate in heat-killed suspensions did not increase the mortality in 7-day old mice. With the group of mice inoculated with a dose of 2.5×10^9 live C. fetus ss. jejuni 5636, the percentage mortality was 30 (6/20), and the mortality increased to 100% when a dose of

Table 43 : Effect of ferric ammonium citrate on passaged *C. fetus ss. jejuni* 4249 in 7-day old mice[a]

Dose per[c] mouse in 0.1 ml	No. of deaths/ No. of mice injected[b]			
	Suspension without Fe^{3+}, i.p.	Fe^{3+} µg, i.p., 0.1ml		
		4	8	12
5×10^9	10/10	10/10	10/10	10/10
2.5×10^9	4/10	7/10	10/10	10/10
1.2×10^9	1/10	3/10	4/10	7/10
6.2×10^9	0/10	0/10	1/10	3/10
LD_{50} value (95% C.L.)	2.4×10^9 ($1.8\text{-}2.9 \times 10^9$)	1.7×10^9 ($1.3\text{-}2.4 \times 10^9$)	1.2×10^9 ($1.2\text{-}1.3 \times 10^9$)	8.6×10^8 ($6.5\text{-}11.7 \times 10^8$)

a : Average weight - 2.5g

b : The results were recorded over a period of 10 days

c : The undiluted suspension contained 1×10^{10} bacteria per ml. The suspension was diluted by a factor of 2.

Table 44 : Influence of various concentrations of ferric ammonium citrate on *C. fetus ss. jejuni* 5636 on mice[a]

Dose per[c] mouse in 0.1 ml	No. of deaths/No. of mice injected[b]			
	Suspension without Fe^{3+}, i.p.	Fe^{3+} µg, i.p., 0.1ml		
		8	12	20
2.5×10^9	7/10	10/10	10/10	10/10
1.3×10^9	2/10	6/10	10/10	10/10
6.2×10^8	0/10	1/10	7/10	6/10
3.1×10^8	0/10	0/10	2/10	2/10
LD_{50} value (95% C.L.)	1.8×10^9 ($1.4\text{-}2.1 \times 10^9$)	8.4×10^8 ($7.3\text{-}9.1 \times 10^8$)	5.4×10^8 ($4.5\text{-}5.9 \times 10^8$)	5.0×10^8 ($4.6\text{-}6 \times 10^8$)

a : Average weight - 2.5g

b : Recorded over a period of 10 days

c : The undiluted suspension contained 5×10^{10} bacteria per ml. It was diluted by a factor of 2, and the data show the number of bacteria inoculated per mouse.

Table 45 : Effect of Fe^{3+} with live and heat-killed suspensions of C. fetus ss. jejuni on 7-day old mice[a]

passaged C.fetus ss. jejuni strains	Dose per mouse in 0.1 ml, i.p.	No. of deaths/No. of mice injected[b]			
		without 12µg Fe^{3+}		with 12µg Fe^{3+}	
		live suspensions	heat-killed suspensions[c]	live suspensions	heat-killed suspensions
5636	2.5×10^9	6/20	0/20	20/20	0/20
4249	2.5×10^9	4/20	0/20	16/20	0/20

a : Average weight - 2.5g

b : Recorded over a period of 10 days

c : The suspensions were heat-killed at 56°C for 30 min.

live 2.5 x 10^9 bacteria was given along with 12 μg Fe^{3+}. However, there were no deaths after the injection of a dose of heat-killed 2.5 x 10^9 bacteria or with the incorporation of 12 μg Fe^{3+}. Similar results were obtained for passaged C. fetus ss. jejuni 4249.

4. Injection of S. flexneri lipopolysaccharide with or without ferric ammonium citrate into 7-day old mice

The addition of ferric ammonium citrate to live C. fetus ss. jejuni reduced the LD_{50}; however, Fe^{3+} did not increase the number of deaths when injected with heat-killed suspensions (Table 45). The aim of this experiment was to study the possibility that the incorporation of ferric ammonium citrate could reduce the LD_{50} value of the lipopolysaccharide of S. flexneri in 7-day old mice. S. flexneri lipopolysaccharide was used instead of Campylobacter lipopolysaccharide because the latter was unobtainable.

Sixty 7-day old mice in groups of 5 were inoculated i.p. with doses of 400, 200, 100, 50 and 25 μg of S. flexneri lipopolysaccharide alone and also with 12 μg Fe^{3+} (Table 46). All the mice in the groups inoculated with 400 and 200 μg LPS died; 4 and 2 of the two groups of 5 mice injected respectively with 100 and 50 μg LPS died. There was no death in the group of mice injected with 25 μg of S. flexneri LPS. The data also show that the incorporation of 12 μg Fe^{3+} with LPS did not significantly increase the number of deaths.

Table 46 : Effect of S. flexneri lipopolysaccharide with ferric ammonium citrate on 7-day old mice

Dose of LPS per mouse in 0.1 ml, i.p. (μg)	No. of deaths / No. of mice injected[a]	
	LPS alone	LPS + 12 μg Fe^{3+}
400	5/5	5/5
200	5/5	5/5
100	4/5	5/5
50	2/5	3/5
25	0/5	0/5
LD_{50} value (95% C.L.)	61.5 μg (39.7-95.4μg)	46.6 μg (33.2-65.5μg)

a : All deaths were recorded 24 hr after the injection.

5. Susceptibility of 7-day old mice injected with a non-lethal dose of *S. flexneri* LPS to *C. fetus ss. jejuni*

Preliminary experiments had shown that ferric ammonium citrate increased the virulence of *C. fetus ss. jejuni* for 7-day old mice, but did not increase the toxicity of *S. flexneri* LPS. The aim of this experiment was to investigate whether the susceptibility of 7-day old mice to *C. fetus ss. jejuni* would be affected by an injection of *S. flexneri* LPS.

The 7-day old mice were injected with a non-lethal dose of 25 μg of *S. flexneri* LPS 4 hr before the experiment. The doses given per mouse were 1.3×10^9, 6.2×10^8, 3.1×10^8, 1.6×10^8 and 7.8×10^7 bacteria respectively. Table 47 shows that mice injected with LPS were more susceptible to *C. fetus ss. jejuni*. With the untreated mice i.e. mice not pre-injected with LPS, 2 of the 5 mice injected with a dose of 1.3×10^9 bacteria died, and there were no deaths in the groups of mice injected with doses of 6.21×10^8 bacteria or lower concentrations of cells. By incorporating 12 μg Fe^{3+} with these dosages, the mortality increased, but there were deaths in the groups of mice injected concomitantly with 1.6×10^8 and 7.8×10^7 bacteria.

The injection of the same dosages of *C. fetus ss. jejuni* in the groups of mice which had been injected with 25 μg of LPS 4 hr before the experiment, gave an increase in mortality. All the mice injected with 1.3×10^9 and 6.2×10^8 bacteria died, and the mortality decreased as the concentration of cells was reduced; there were 3 deaths in the groups of mice injected with 1.6×10^8 and 7.8×10^7 bacteria, as compared to that of the non-treated mice in which the same injection had no apparent effect. By

Table 47 : Effect of C. fetus ss. jejuni 5636 on 7-day old mice pretreated with a non-lethal dose of S. flexneri LPS

Dose per mouse in 0.1ml, i.p.	Suspensions alone	Suspensions + 12μg Fe^{3+}	25μg LPS, 4hr prior[1] to the injection	
			Suspensions alone	Suspensions + 12 μg Fe^{3+}
1.3×10^9	2/5[2]	5/5	5/5	5/5
6.2×10^8	0/5	3/5	5/5	5/5
3.1×10^8	0/5	2/5	4/5	5/5
1.6×10^8	0/5	0/5	3/5	5/5
7.8×10^7	0/5	0/5	3/5	4/5

1. Mice were injected with a dose of 25μg S. flexneri LPS. There was no death after the injection of 25μg LPS.
2. No. of deaths/No. of mice injected.

incorporating the same suspensions with 12 μg Fe^{3+}, all the mice injected with 1.6×10^8 or higher concentration of cells died, and 4 of the 5 injected mice succumbed in the group of mice receiving 7.8×10^7 bacteria.

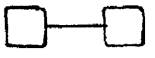
All the mice died 48 hr after the injection, and there was no death recorded before 18 hr.

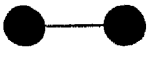
VI. Recovery of viable C. fetus ss. jejuni from 7-day old mice after intraperitoneal injection with or without ferric ammonium citrate

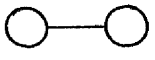
1. The recovery of C. fetus ss. jejuni from whole mouse homogenate without ferric ammonium citrate

Forty 7-day old mice were inoculated i.p. with doses of 2.5×10^9, 1.3×10^9 and 6.2×10^8 viable C. fetus ss. jejuni. Two of the inoculated mice from each group were killed immediately and at 12, 18, 24 and 48 hr after the inoculation. On each occasion the whole mouse was homogenized, and the homogenates were used for viable counts. Fig 12 shows the number of viable C. fetus ss. jejuni recovered from the homogenates of whole mice at 0, 12, 18, 24 and 48 hr.; each point represents 2 mice. The data show that C. fetus ss. jejuni did not multiply in the peritoneal cavity, and there was a direct eliminating process in the animal and the number of bacteria fell over a period of 48 hr.

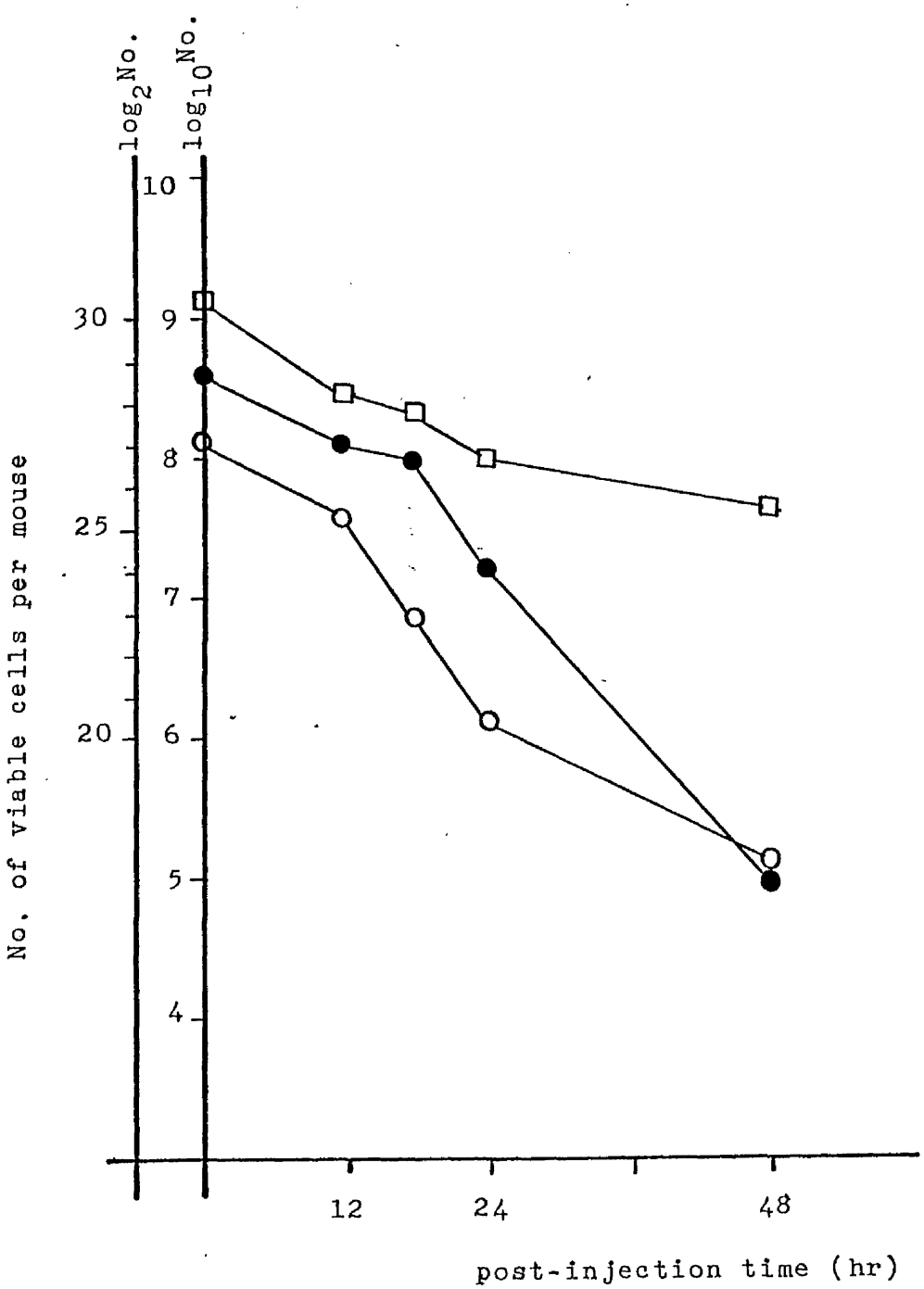
Fig. 12 : The number of C. fetus ss. jejuni recovered from the whole mouse homogenate after intraperitoneal injection without ferric ammonium citrate.


2.5 x 10^9 bacteria per mouse


1.3 x 10^9 bacteria per mouse


6.2 x 10^8 bacteria per mouse

Each point represents 2 mice.

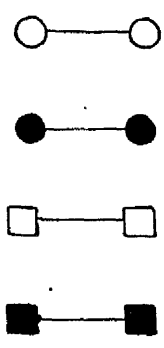

log2No.
log10No.
10
9
8
7
6
5
4
30
25
20
No. of viable cells per mouse
12
24
48
post-injection time (hr)

2. The effect of ferric ammonium citrate on the recovery of *C. fetus ss. jejuni* from whole mouse homogenates

Previous experiments had shown that ferric ammonium citrate would appear to enhance the virulence of *C. fetus ss. jejuni* for 7-day old mice by reducing the LD_{50} value (Table 40 and 43), and ferric ammonium citrate had no effect on the *in vitro* growth of *C. fetus ss. jejuni*. The aim of this experiment was to investigate the effect of ferric ammonium citrate on the number of *Campylobacter* reisolated from the inoculated mice.

Sixty 7-day old mice were injected intraperitoneally with 0.1ml of the bacterial suspension containing 6.2×10^8 bacteria along with 4, 8 or 12 μg Fe^{3+}. Two mice from each group were killed immediately following the injection, and then at 12, 18, 24 and 48 hr respectively. Fig 13 shows the viable bacterial counts obtained in groups of 7-day old mice inoculated with 6.2×10^8 bacteria with varying concentrations of Fe^{3+}; each point represents 2 mice. The results show that the incorporation of Fe^{3+} in the bacterial suspensions affects the number of cells reisolated. The number of viable *Campylobacter* was found to be consistently greater in the groups of mice given the higher concentrations of Fe^{3+}. The number of *Campylobacter* recovered at 48 hr from the homogenates of mice injected with 12 μg Fe^{3+} was 2 log units higher than that of *Campylobacter* reisolated from the homogenates of mice injected with suspensions alone. The number was also repeatedly higher than the number of viable *Campylobacter* reisolated from the whole mice homogenates inoculated with 4 and 8 μg Fe^{3+}; with the exception at 24 hr

Fig. 13 : Recovery of C. fetus ss. jejuni from whole 7-day old mice homogenates with various concentrations of Fe^{3+}.



○——○ 6.2×10^8 bacteria per mouse

●——● $6.2 \times 10^8 + 4\mu g\ Fe^{3+}$ per mouse

□——□ $6.2 \times 10^8 + 8\mu g\ Fe^{3+}$ per mouse

■——■ $6.2 \times 10^8 + 12\mu g\ Fe^{3+}$ per mouse

Each point represents 2 mice.

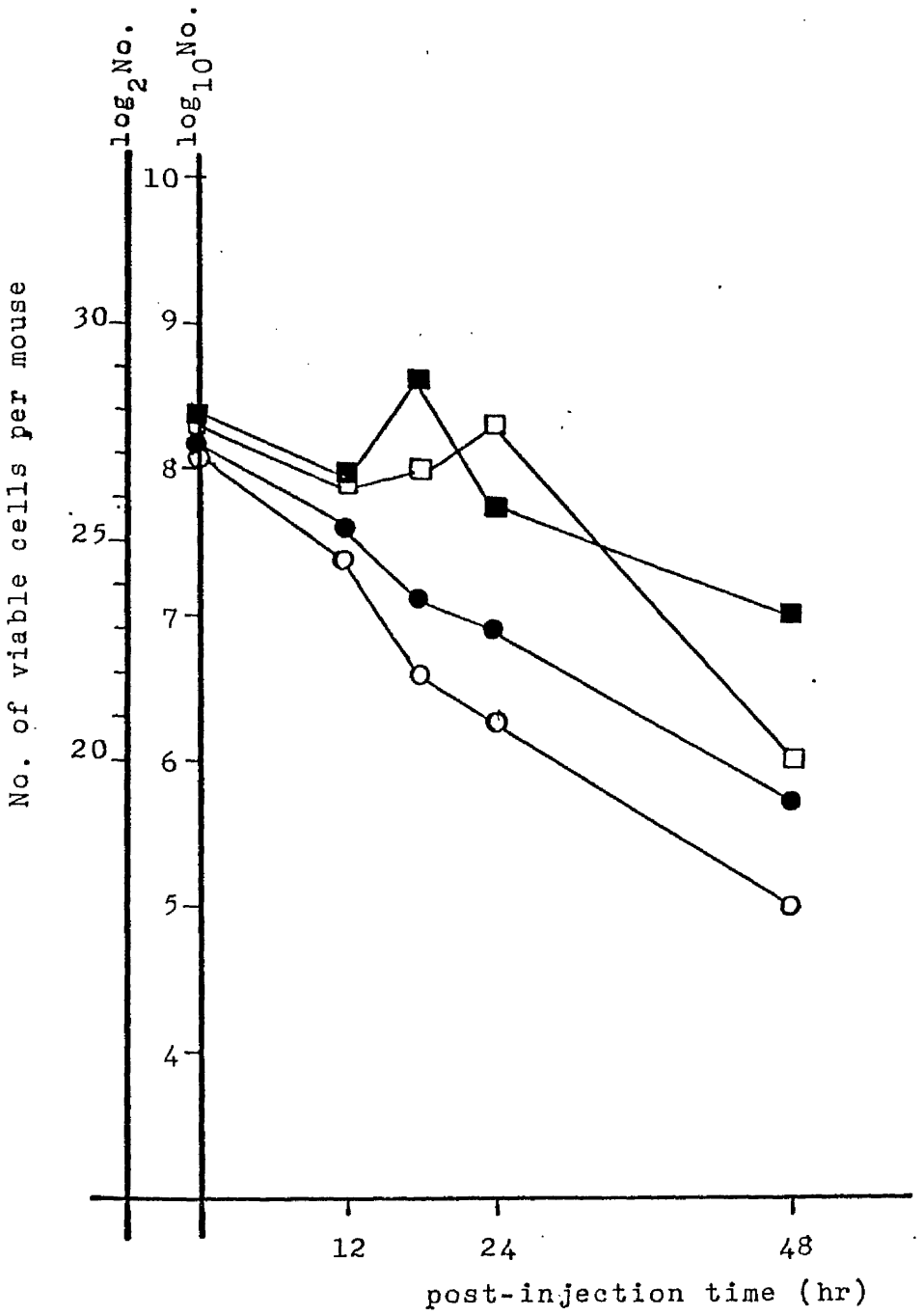
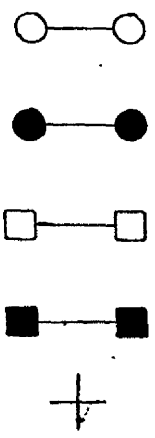

log2No.
log10No.
10
9
8
7
6
5
4
30
25
20
No. of viable cells per mouse
12
24
48
post-injection time (hr)

Fig. 14 : The number of C. fetus ss. jejuni reisolated from whole mice homogenates after i.p. injection with various concentrations of Fe^{3+}



1.3×10^9 bacteria per mouse

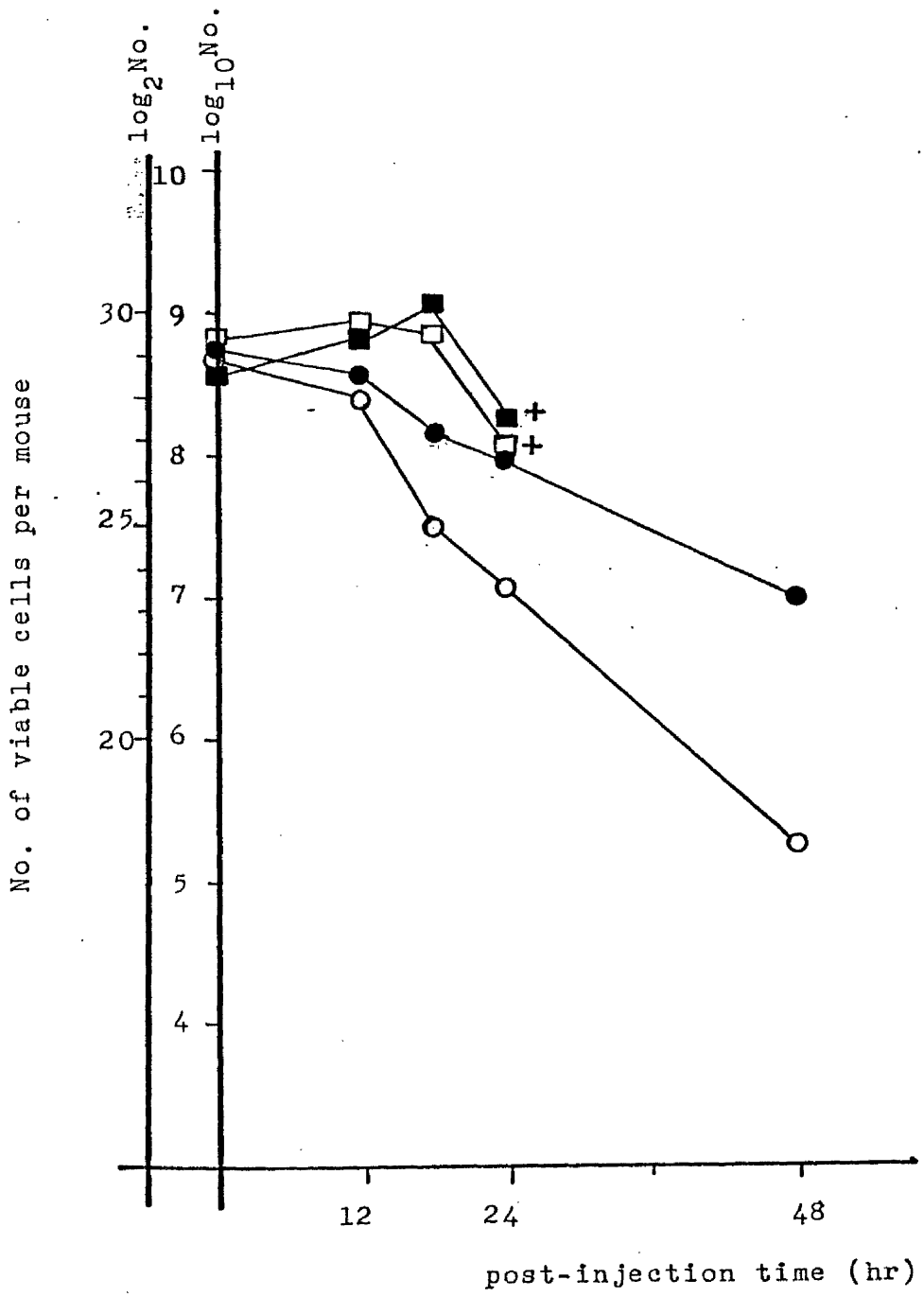
$1.3 \times 10^9 + 4\mu g\ Fe^{3+}$ per mouse

$1.3 \times 10^9 + 8\mu g\ Fe^{3+}$ per mouse

$1.3 \times 10^9 + 12\mu g\ Fe^{3+}$ per mouse

All the mice died

Each point represents 2 mice.


log2No.
log10No.
10
9
8
7
6
5
4
30
25
20
+
+
12
24
48
post-injection time (hr)
No. of viable cells per mouse

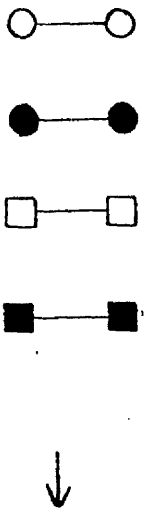
that the number of viable cells from the homogenates of mice injected with 8 μg Fe^{3+} was higher. The viable count data of the homogenates of mice injected with suspensions alone showed a clear fall in cell numbers which probably indicates an eliminating process over a period of 48 hr. Although the viable numbers of Campylobacter isolated from the homogenates of mice injected with 4 μg Fe^{3+} also showed a decrease, the number was higher than that of the homogenates of mice without Fe^{3+}. The elimination of Campylobacter in mice inoculated concomitantly with 8 and 12 μg Fe^{3+} was considerably delayed for 24 hr, and the number of viable bacteria declined abruptly at 48hr.

Fig. 14 shows the results of a similar experiment in which 60 7-day old mice were given a dose of 1.3×10^9 bacteria incorporating 4, 8 or 12 μg Fe^{3+}. The data show that the number of viable Campylobacter recovered from the homogenates of mice inoculated together with Fe^{3+} was higher than those without Fe^{3+}. The groups of mice inoculated with 1.3×10^9 bacteria with 8 or 12 μg Fe^{3+} died after 24 hr. Both groups of mice injected with 1.3×10^9 bacteria and 1.3×10^9 bacteria with 4 μg Fe^{3+} showed a clear direct eliminating process. But the elimination of bacteria in the presence of Fe^{3+} was considerably delayed; and the number of viable Campylobacter reisolated from the homogenates of whole mouse with 4 μg Fe^{3+} was higher than that of the number of bacteria reisolated from the whole mice homogenates without Fe^{3+}. There was 1 log unit difference in the number of viable Campylobacter at 24 hr, and 2 log units at 48 hr respectively.

3. Effect of excess ferric ammonium citrate on the recovery of C. fetus ss. jejuni from the whole mice homogenates.

C. fetus ss. jejuni was eliminated in 7-day old mice following the intraperitoneal injection with or without the incorporation of ferric ammonium citrate (Fig. 13 and 14). However, elimination was considerably delayed, and the number of viable bacteria was consistently higher in the presence of Fe^{3+}. By giving excess 12 μg Fe^{3+} at 18 hr to groups of mice which did not receive Fe^{3+} with the bacterial suspension in the first injection, and to the group of mice which received 12 μg Fe^{3+} with bacterial suspension initially, it was found that Fe^{3+} had a significant effect on the recovery of bacteria from the animal (Fig. 15). The number of viable Campylobacter cells recovered from the homogenates of mice not injected with Fe^{3+} initially at 12, 18, 24 and 48 hr showed a clear eliminating process i.e. fall in numbers, but by giving 12 μg Fe^{3+} intraperitoneally at 18 hr, the eliminating process was averted, there was a 1 log unit difference in the number of viable cells recovered at 24 hr, and 3 log units difference at 48 hr. With the group of mice injected with the suspension concomitantly with 12 μg Fe^{3+} initially, and then given excess 12 μg Fe^{3+} at 18 hr intraperitoneally, the delay in the elimination of bacteria could be maintained. The number of viable cells was 2 log and 3 log units higher at 18 and 48 hr than the number of viable Campylobacter reisolated from the group of mice injected without Fe^{3+}; the number of viable cells was also 1 log unit higher at 48 hr than the number obtained from the homogenates of whole mouse injected with 12 μg Fe^{3+} initially but not receiving excess 12 μg^{3+} at 18 hr.

Fig. 15 : Effect of excess ferric ammonium citrate on the recovery of C. fetus ss. jejuni after i.p. injection



○—○ 6.2×10^8 bacteria alone

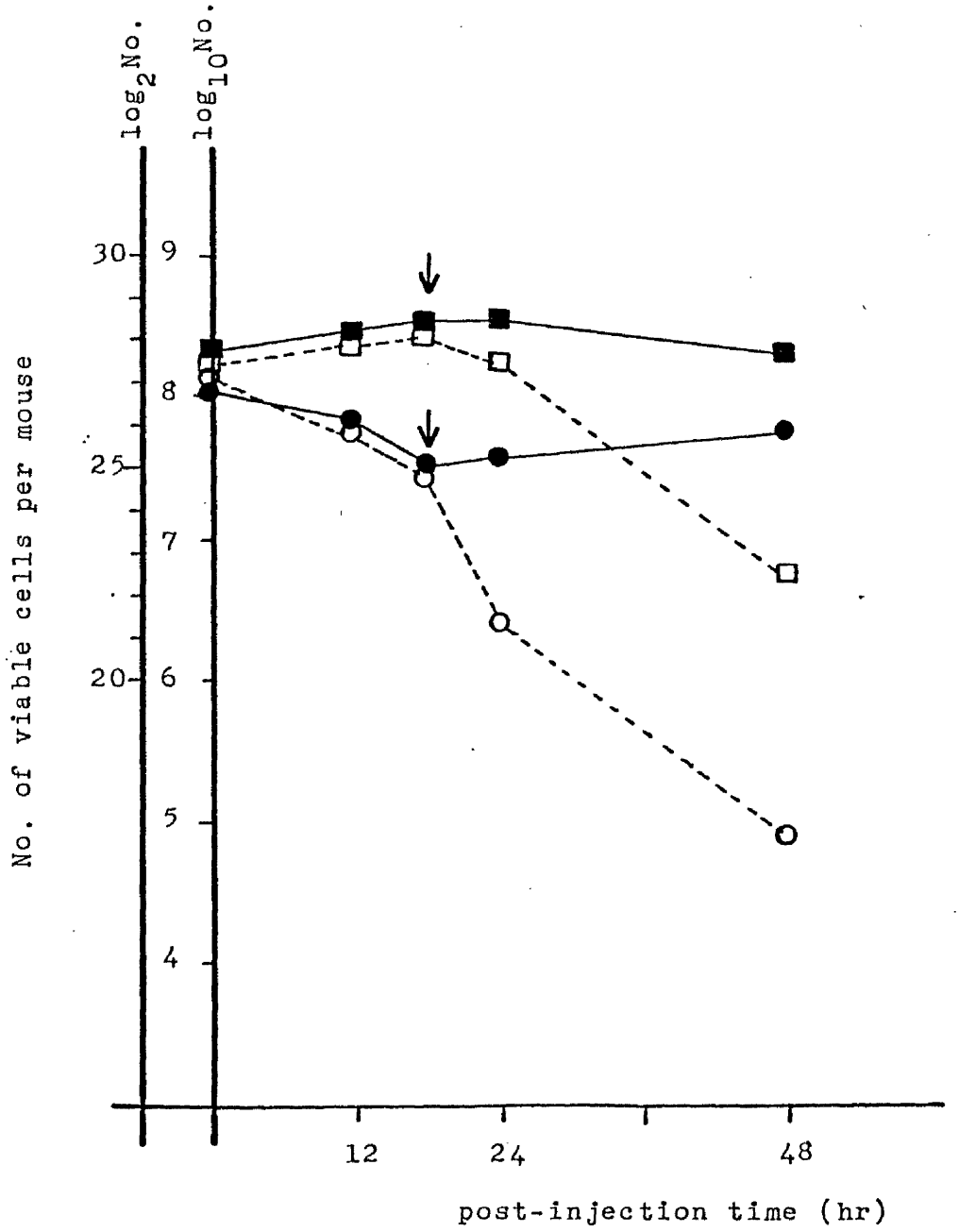
●—● $6.2 \times 10^8 + 12\mu g\ Fe^{3+}$ at 18 hr

□—□ $6.2 \times 10^8 + 12\mu g\ Fe^{3+}$

■—■ $6.2 \times 10^8 + 12\mu g\ Fe^{3+}$ + Excess $12\mu g\ Fe^{3+}$ at 18 hr

↓ Excess $12\mu g\ Fe^{3+}$ given i.p. at 18 hr.

Each point represents 2 mice.


log2No.
log10No.
30
9
8
25
7
20
6
5
4
No. of viable cells per mouse
12
24
48
post-injection time (hr)

The effect of ferric ammonium citrate on C. fetus ss. jejuni in vivo was again demonstrated by the data shown in Fig. 20. Each point represents 3 mice. Thirty 7-day old mice were inoculated intraperitoneally with a dose of 6.2×10^8 bacteria without Fe^{3+}, at 18 hr after the injection 12 μg Fe^{3+} was given intraperitoneally to 15 mice which had initially been inoculated with 6.2×10^8 bacteria. The data show that with the injection of Fe^{3+}, the number of viable bacteria recovered was 1 log unit different at 24 hr, and 2 log units different at 48 and 72 hr respectively.

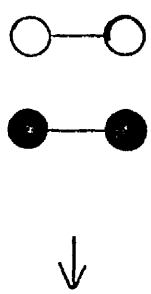
VII. Effect of injecting mucin with live C. fetus ss. jejuni on 7-day old mice

The LD_{50} value of passaged C. fetus ss. jejuni on 7-day old mice was 1.8×10^9 bacteria; by adding to this 12 μg Fe^{3+}, the LD_{50} value was reduced to 2.3×10^8. The effect of mucin, another virulence enhancing agent, was also examined. The doses given per mouse were the same as Experiment V.2. The results show (Table 48) that the incorporation of 1 and 2.5% mucin with the suspensions of C. fetus ss. jejuni 5636 did not reduce the LD_{50} value. A similar result was obtained using passaged strain C. fetus ss. jejuni 4249 (Table 49).

VIII. Guinea-pig ileal loop tests

Guinea-pig ileal loop tests are used to detect the enterotoxin produced by a variety of enteric bacteria (Sack, 1976). Examination of C. fetus ss. jejuni broth cultures for an enterotoxin-like effect on intestinal secretion or for the production

Fig. 16 : Recovery of C. fetus ss. jejuni from 7-day day old mice with and without ferric ammonium citrate.



6.2×10^8 bacteria per mouse

6.2×10^8 + 12μg Fe^{3+} 18 hr after the injection

12μg Fe^{3+} given i.p. at 18 hr

Each point represents 3 mice

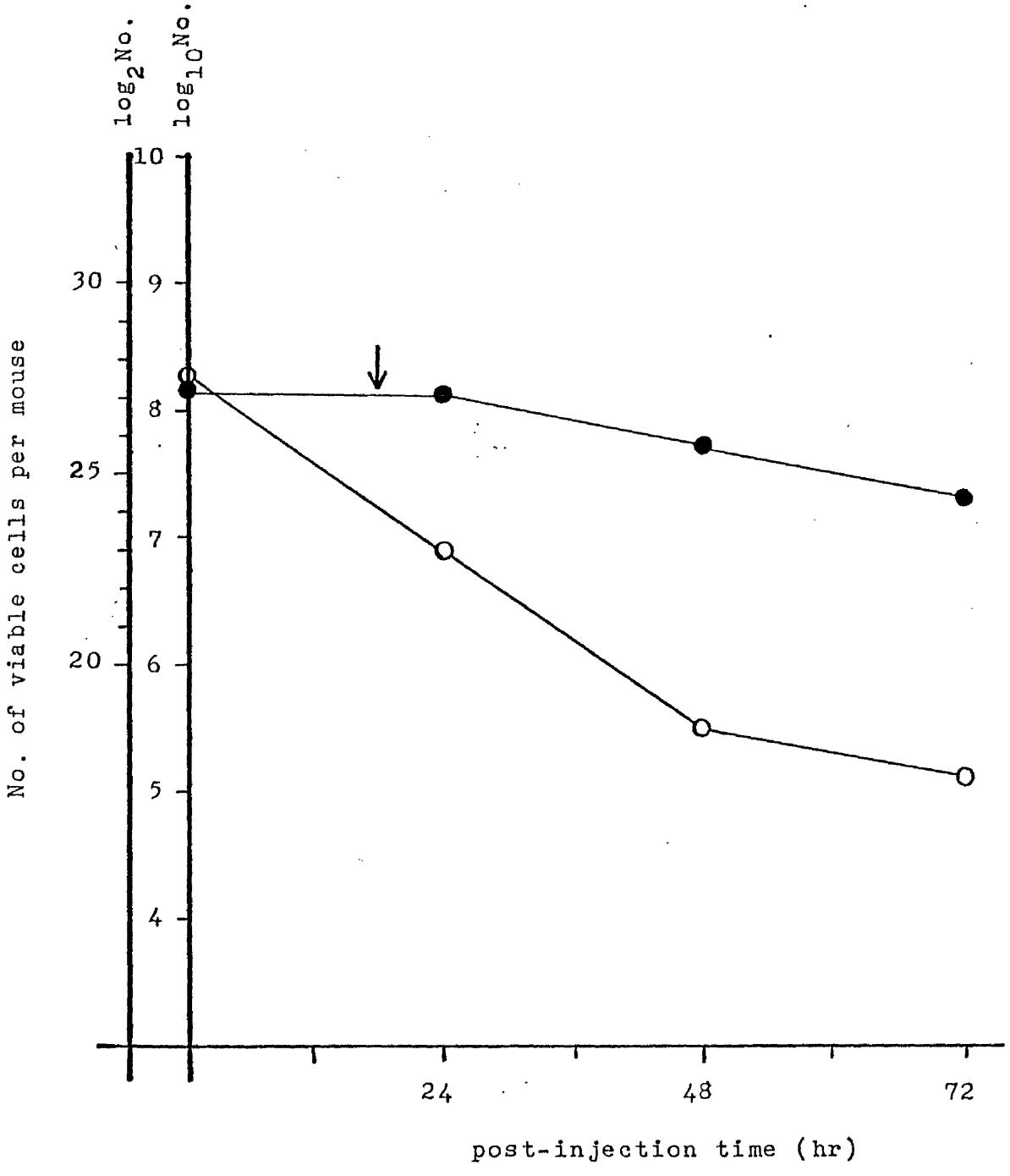

log2No.
log10No.
10
9
8
7
6
5
4
30
25
20
No. of viable cells per mouse
24
48
72
post-injection time (hr)

Table 48 : Effect of mucin on passaged C. fetus ss. jejuni 5636 on 7-day old mice

Dose given per mouse in 0.1ml	Suspensions without mucin, i.p.	Mucin, %, i.p., 0.1ml per mouse[a]	
		1%	2.5%
2.5×10^9	7/10	9/10	10/10
1.3×10^9	4/10	5/10	5/10
6.2×10^8	0/10	0/10	0/10
LD_{50} value (95% C.L.)	1.8×10^9 ($1.4\text{-}2.1 \times 10^9$)	1.3×10^9 ($0.9\text{-}1.6 \times 10^9$)	1.3×10^9 ($0.9\text{-}1.6 \times 10^9$)

a : Mucin was given intraperitoneally mixed with the bacterial suspension.

Table 49 : Effect of mucin on passaged C. fetus ss. jejuni 4249 on 7-day old mice

Dose given per mouse in 0.1 ml	Suspensions without mucin, i.p.	Mucin, %, i.p., 0.1 ml per mouse[a]	
		1%	2.5%
5×10^9	10/10	10/10	10/10
2.5×10^9	4/10	6/10	7/10
1.2×10^9	0/10	0/10	1/10
6.2×10^8	0/10	0/10	0/10
LD_{50} value (95% C.L.)	2.3×10^9 ($1.9\text{-}2.8 \times 10^9$)	2.2×10^9 ($1.9\text{-}2.9 \times 10^9$)	2×10^9 ($1.6\text{-}2.6 \times 10^9$)

a : Mucin was given intraperitoneally mixed with the bacterial suspensions.

Table 50 : Fluid accumulation and length of guinea-pig ileal loops inoculated with live *C. fetus ss. jejuni*.

Test loop No.	*C. fetus ss. jejuni* cultures 0.1ml		Broth (Control) 0.1ml	
	fluid accumulation (ml)	loop length (cm)	fluid accumulation (ml)	loop length (cm)
1	5	9.8	5	10.2
2	4	6.3	5	8.5
3	2	9	2.5	6

of a cholera-like enterotoxin in guinea-pig ileal loop failed to reveal enterotoxic activity. There was no fluid accumulation in the loop 18 hr after the inoculation of whole broth cultures, and there was no significant difference between the lengths of the test loops inoculated with C. fetus ss. jejuni and the controls (Table 50). No test loops were inoculated with crude cholera toxin, hence, no direct comparison could be made between the effect of the crude toxin and Campylobacter cells.

SECTION 3. COMPARISON OF CAMPYLOBACTER CELL PROTEINS BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

C. fetus ss. jejuni has been isolated from normal people (Lauwers, DeBoeck & Butzler, 1978) as well as from patients with diarrhoea. It has not been possible to differentiate these isolates by the usual methods because members of this subspecies are biochemically inactive. However, Campylobacter strains isolated from various animal sources have been distinguished by their electrophoretic profiles (Morris & Park, 1973). The aim of the present experiment was a comparison, by SDS-polyacrylamide gel electrophoresis, of the cell proteins of C. fetus ss. jejuni strains from patients with diarrhoea with those of animal strains.

Ten strains of C. fetus ss. jejuni (4 isolated by myself, strains No. 06760, 00539, 8346 and 3946; 4 isolated from the City Laboratory, strains No. 2680, 5702, 4249 and 7972; and 2 Skirrow strains 5636 and 9227) and 2 Campylobacter species of animal origin, C. fetus biotype B (NCTC 5850) and C. bubulus (NCTC 10355), were used. All the cultures were grown in nutrient

Plate 5 : Electrophoretic patterns of whole cell proteins of *C. fetus ss. jejuni*, *C. bubulus* and *C. fetus* biotype B.

k	:	standard proteins	
a	:	*C. fetus ss. jejuni*	5636
b	:	"	06760
c	:	"	00539
d	:	"	2680
e	:	"	9227
f	:	"	3946
g	:	"	5702
h	:	"	4249
i	:	*C. fetus* biotype B	NCTC 5850
j	:	*C. bubulus*	NCTC 10355

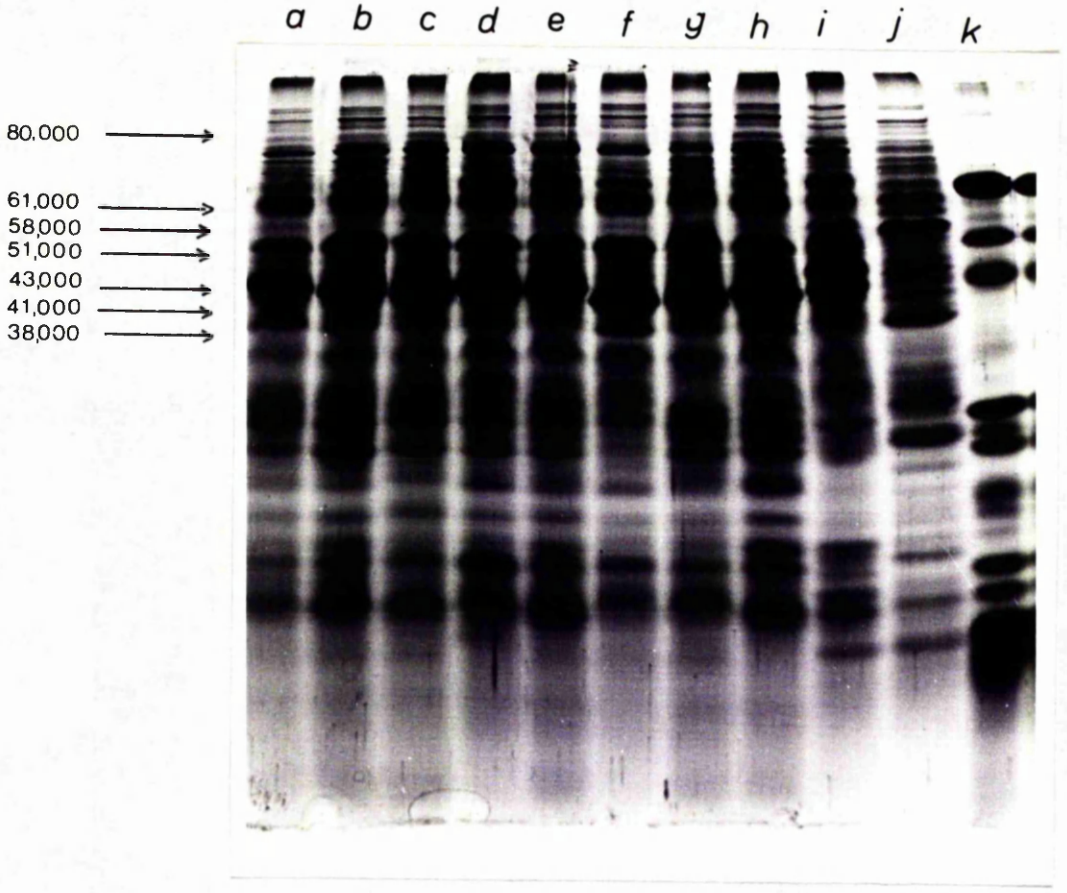

a b c d e f g h i j k
80,000
61,000
58,000
51,000
43,000
41,000
38,000

Plate 6 : Electrophoretic patterns of envelope proteins of *C. fetus ss. jejuni* strains isolated from patients with diarrhoea.

a, l	:	standard proteins	
b	:	*C. fetus ss. jejuni*	5636
c	:	"	06760
d	:	"	00539
e	:	"	2680
f	:	"	9227
g	:	"	3946
h	:	"	5702
i	:	"	4249
j	:	"	8346
k	:	"	7972

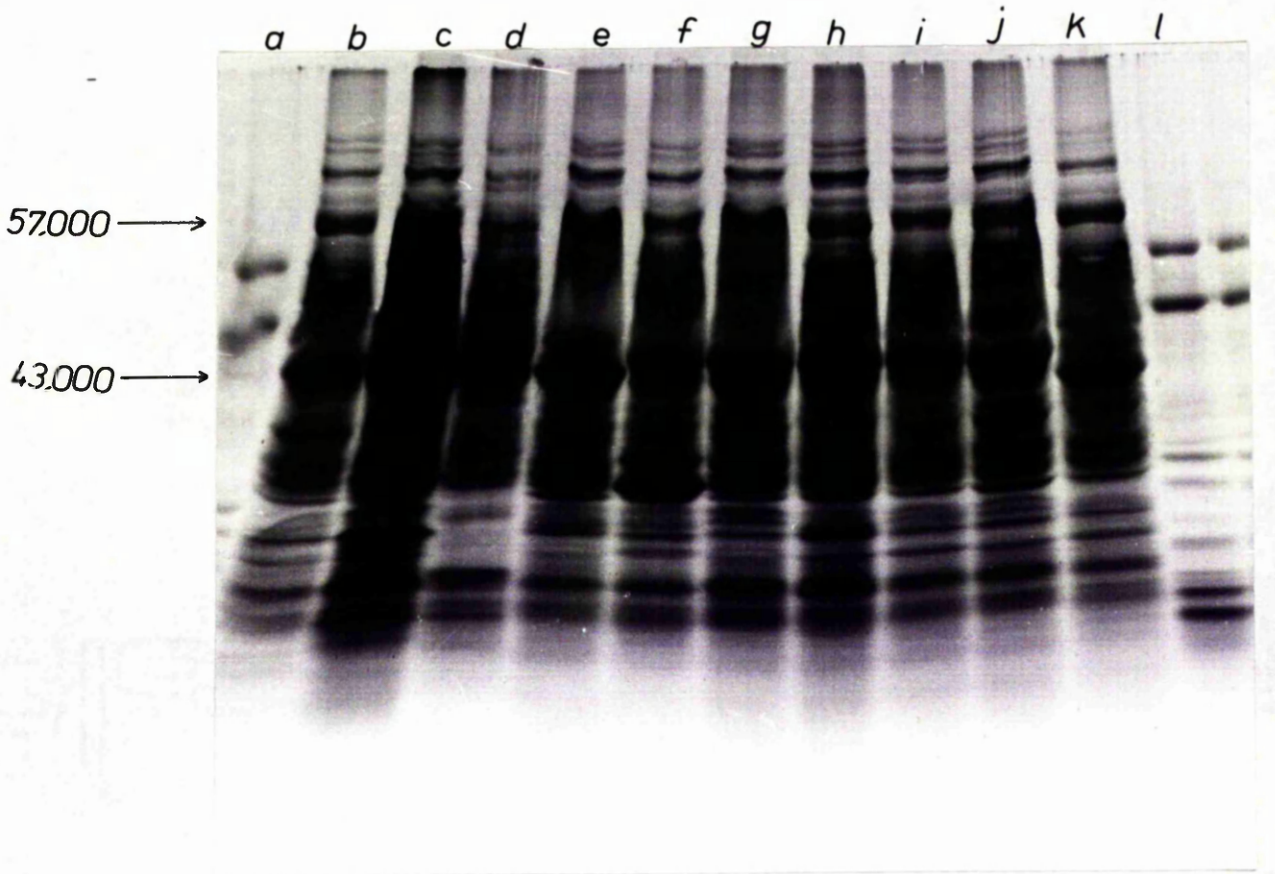
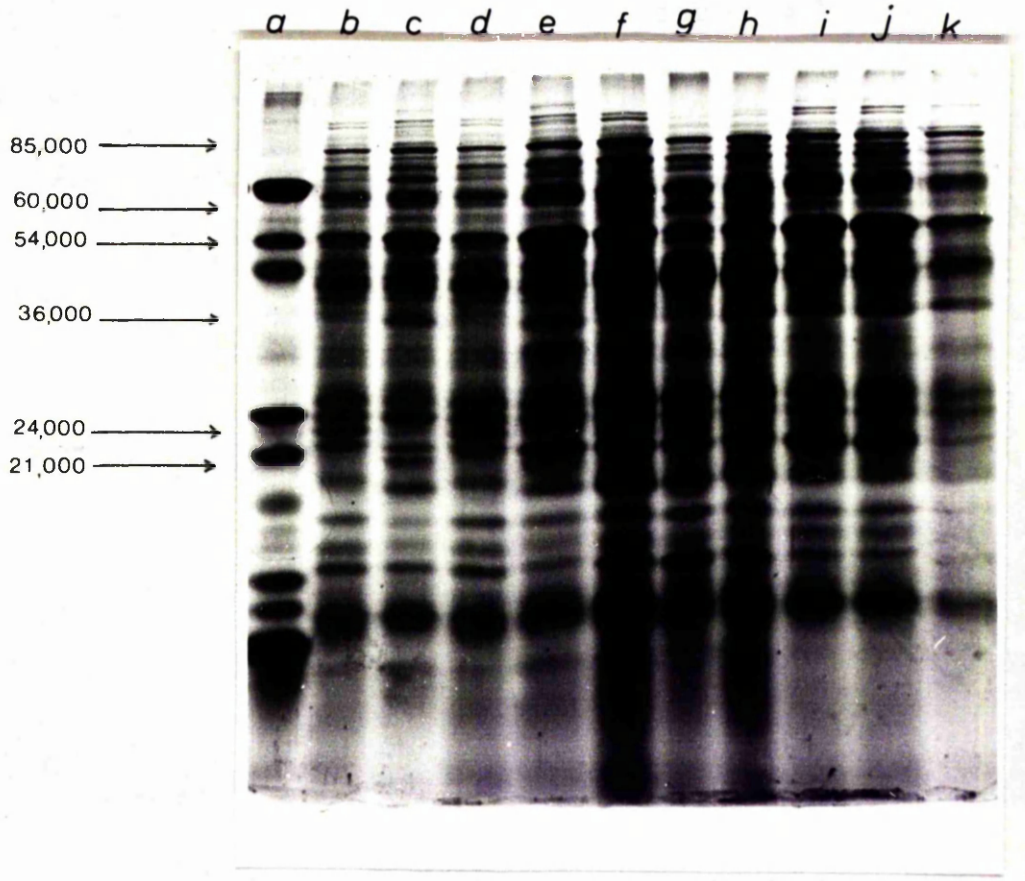

a
b
c
d
e
f
g
h
i
j
k
l
57.000
43.000

Plate 7 : Electrophoretic profiles of cytoplasmic proteins of *C. fetus ss. jejuni* isolates.

a	:	standard proteins	
b	:	*C. fetus ss. jejuni*	5636
c	:	"	06760
d	:	"	00539
e	:	"	2680
f	:	"	9227
g	:	"	3946
h	:	"	5702
i	:	"	4249
j	:	"	8346
k	:	"	7972


a b c d e f g h i j k
85,000
60,000
54,000
36,000
24,000
21,000

broth supplemented with yeast extract and cystine.

Plate 5 (p.169) shows the protein profiles obtained from whole cells of 8 strains of C. fetus ss. jejuni, and the 2 animal strains. All 8 human strains showed a degree of similarity, but the two animal strains differed from each other and from the human strains. Strain C. fetus biotype B (profile J) was deficient in a band of mol. wt. 80,000, but contained a prominent band of mol. wt. 61,000 which was absent in the others. Both animal strains contained a component of mol. wt. 58,000 which was not prominent in the human strains. All the 8 human strains, together with C. fetus biotype B contained a major polypeptide component of mol. wt. 51,000. In addition, some differences in the profiles of the 8 human strains were evident; strains No. 5636 (profile a), 06760 (profile b), 2680 (profile d), 9227 (profile e), and 4249 (profile h) contained another major polypeptide component at 43,000, whereas strains 00539 (profile c), 3946 (profile f) and 5702 (profile g) had another major band at 41,000; strain 3946 also had a prominent band at 38,000, which was also present in strain C. fetus biotype B.

Envelope fractions were prepared from the 10 strains of C. fetus ss. jejuni and were subjected to electrophoresis as above. However, no satisfactory protein profiles of the envelope samples could be obtained by tube or slab-gel electrophoresis. The polypeptide bands in the molecular weight range of 43,000 - 57,000 were consistently distorted. Gradient-gel electrophoresis, as expected, gave better resolution of the low molecular weight polypeptide bands (Plate 6) but did not eliminate this distortion. No meaningful comparison of envelope profiles could therefore be made.

The protein profiles of the cytoplasmic fractions of the 10 human isolates were generally similar (Plate 7). However, a few minor qualitative and quantitative differences were noted : three strains, 5702, 4249 and 8346 (profiles h, i, j respectively) contained a prominent band of mol. wt. approximately 85,000; strain 3946 (profile g) was the only strain showing a prominent band at 60,000. The major polypeptide component in all strains, except strains No. 5636 (profile b), 00539 (profile d) and 3946 (profile g), was a major band of mol. wt. 54,000. Strains 5636 and 00539 differed from all other strains in lacking a band at 36,000, but possessing a band at 24,000. Strain 06760 (profile c) was the only strain containing a prominent band of mol. wt. 21,000.

SECTION 4 : STUDIES ON THE ANTIGENIC STRUCTURE OF C. FETUS SS. JEJUNI

I. Mouse anti-C. fetus ss. jejuni sera

1. Effect of temperature on the agglutination titre

O and H sera from three strains - C. fetus ss. jejuni 5636, 4249 and 06760, were used. The agglutination titres were estimated by Dreyer's Dropping technique. After mixing, one set of tubes was left at 37°C for 18 hr, and the second set was left at 56°C for 2 hr followed by overnight incubation at 4°C. The results are shown in Table 51 and Table 52. The higher agglutination titres were obtained from the set incubated at 37°C for 18 hr, this temperature of incubation was thus chosen for routine use.

Table 51 : Effect of temperature of incubation on the agglutination titres- heated antigens

O antisera (mouse)	O antigens	Temperature : Reciprocal titres[a]	
		56°C for 2 hr overnight	37°C for 18 hr
5636	5636	50	250
4249	4249	25	500
06760	06760	50	500

a : Titres represent the highest serum dilution giving a 2++ reaction

Table 52 : Effect of temperature of incubation on the agglutination titres- formalinized antigens

H antisera (mouse)	H antigens	Temperature : Reciprocal titres[a]	
		56°C for 2 hr overnight	37°C for 18 hr
5636	5636	0	500
4249	4249	25	500
06760	06760	0	250

a : Titres represent the highest serum dilution giving a 2++ reaction

2. Comparing the methods for producing mouse anti-*C. fetus ss. jejuni* sera.

Two methods of immunization were described in p. 84 . Analysis of antisera obtained by these methods (Table 53 and Table 54) revealed that 3 injections of increasing amounts at weekly intervals consistently gave higher agglutination titres than single injection of 1 ml. Method 2 was chosen for producing antisera.

3. Analysis of the O antigen

The results of cross-agglutination tests using 10 O sera and 10 O antigens are given in Table 55. The results showed that all strains cross-agglutinated with each other, and occasionally with the reduction of titres, usually a one tube difference.

All O antigens of *C. fetus ss. jejuni* could agglutinate with the sera collected from normal uninoculated mice to a titre of 25 or 50.

4. Analysis of H sera

Results of cross-agglutination tests using 10 H sera and 10 H antigens showed that all strains shared a common agglutinin (Table 56). The antisera of homologous and heterologous strains cross-agglutinated, and an apparent serologic relationship was found among the strains isolated from patients with diarrhoea.

Table 53 : Titres obtained using two different methods of immunization - heated antigens

O antisera (mouse)	O antigens	Method[a] : Reciprocal titres[b]	
		Method 1	Method 2
5636	5636	125	250
4249	4249	50	250
06760	06760	125	500

a : Method 1 : one injection of 1ml, mice bled 10 days after the injection.

Method 2 : three injections of increasing amounts i.e. 0.5, 1, and 2ml, injections were given weekly intervals, mice bled 10 days after immunization.

b : Titres represent the highest serum dilution giving a 2++ reaction.

Table 54 : Titres obtained using two different methods of immunization - formalinized antigens

H antisera (mouse)	H antigens	Method[a] : Reciprocal titres[b]	
		Method 1	Method 2
5636	5636	125	500
4249	4249	250	500
06760	06760	125	250

Table 55 : Cross-agglutination titres of isolates of C. fetus ss. jejuni isolated from patients with diarrhoea - heated antigens

O antigens	Mouse antisera : Reciprocal agglutination titres[a]										
	5636	5702	4249	3946	8346	2680	00539	06760	9227	7972	Control sera[b]
5636	250	125	250	125	250	250	250	125	125	250	50
5702	125	250	250	125	125	250	250	125	125	125	25
4249	250	250	500	250	250	125	125	125	250	125	50
3946	125	125	125	250	125	125	125	250	125	125	50
8346	125	125	250	125	250	250	250	125	125	250	50
2680	250	125	500	250	125	250	250	125	125	250	25
00539	125	125	125	125	125	250	500	250	250	125	50
06760	125	250	250	125	250	125	500	250	125	125	25
9227	125	125	125	250	125	125	125	125	500	250	50
7972	250	125	250	125	125	250	250	250	250	250	50

a : Titres given represent the highest serum dilution giving a 2++ reaction

b : Sera collected from 10 uninoculated mice.

Table 56 : Cross-agglutination titres of isolates of C. fetus ss. jejuni isolated from patients with diarrhoea - formalinized antigens

H antigens	Mouse antisera : Reciprocal agglutination titres[a]										
	5636	5702	4249	3946	8346	2680	00539	06760	9227	7972	Control sera[b]
5636	500	250	250	500	500	250	125	500	125	250	25
5702	250	250	125	250	250	250	250	250	250	250	50
4249	500	125	250	250	250	125	250	250	125	250	50
3946	500	250	125	500	250	250	125	250	125	125	50
8346	250	125	250	500	500	250	125	500	125	125	50
2680	250	250	250	125	250	250	250	250	250	250	50
00539	250	250	125	250	500	125	250	250	125	250	25
06760	125	125	250	250	500	250	125	500	125	250	25
9227	125	125	250	250	250	250	125	250	500	125	50
7972	250	250	125	250	500	250	125	500	250	250	25

a : Titres given are reciprocal and represent the highest serum dilution giving a 2++ reaction

b : Pooled sera of 10 uninoculated mice.

II. Rabbit anti-*C. fetus ss. jejuni* sera

Rabbits were also used to raise anti-*C. fetus ss. jejuni* sera against 3 strains of *C. fetus ss. jejuni* viz. 5636, 4249 and 06760.

The results of cross-agglutination of these 3 O and H antisera against 10 O and H antigens are summarized in Table 57 and Table 58. Higher agglutination titres were obtained using rabbit antisera; there was no natural antibody in uninoculated rabbits which would agglutinate with the O and H antigens. All strains cross-agglutinated with each other, and this agreed with the analysis of O and H mouse anti-*C. fetus ss. jejuni* sera that strains of *C. fetus ss. jejuni* isolated from patients with diarrhoea showed a common antigen.

The absorption of sera was not done because of shortage of time.

Table 57 : Cross-agglutination titres of isolates of *C. fetus* ss. *jejuni* isolated from patients with diarrhoea - heated antigens

O antigens	Rabbit antisera : Reciprocal Titres[a]			
	5636	06760	4249	Control[b]
5636	5,000	1,000	2,000	0
06760	2,000	2,000	1,000	0
4249	1,000	1,000	5,000	0
3946	2,000	500	2,000	0
5702	2,000	2,000	1,000	0
8346	5,000	1,000	2,000	0
00539	2,000	1,000	1,000	0
2680	2,000	500	1,000	0
9227	1,000	500	500	0
7972	2,000	1,000	2,000	0

a : Titres represent the highest serum dilution giving a 2++ reaction

b : Control uninoculated rabbit sera

Table 58 : Cross-agglutination titres of isolates of *C. fetus* ss. *jejuni* isolated from patients with diarrhoea - formalinized antigens

H antigens	Rabbit antisera : Reciprocal titres[a]			
	5636	06760	4349	Control[b]
5636	500	1,000	2,000	0
06760	500	1,000	1,000	0
4249	500	500	2,000	0
3946	250	500	1,000	0
5702	250	1,000	500	0
8346	500	1,000	500	0
00539	500	1,000	1,000	0
2680	250	500	1,000	0
9227	250	500	500	0
7972	500	1,000	1,000	0

a : Titres represent the highest serum dilution giving a 2++ reaction

b : Sera from control uninoculated rabbit

DISCUSSION

I. Isolation of C. fetus ss jejuni from faecal specimens

The Millipore filtration technique suggested by Dekeyser et al., (1972) was not routinely used in this investigation for the isolation of Campylobacter because this technique required more time and equipment; instead, a selective medium which contained polymyxin-B sulphate (2.5 IU/ml), trimethoprim lactate (5 mg/l) and vancomycin (10 mg/l) was used; growth of the common enteric bacteria i.e. Proteus, Pseudomonas and Shigella was inhibited on this media. Yeasts are the commonest organism capable of growth on this selective medium, but amphotericin B (2 mg/l) was not incorporated because yeasts did not interfere with the isolation of Campylobacter.

Faecal specimens should be plated on the same day as received from the patients; if this is not possible, these should be refrigerated. Those specimens positive for Campylobacter when fresh failed to yield the same result if they were left at room temperature for 48 hr; however, when the specimens were stored at 4°C, they all remained positive. Tanner & Bullin (1977) reported that alkaline peptone water (pH 8.4) was a satisfactory enrichment medium for the isolation of Campylobacter; they reported that two samples of faeces which were negative on direct plating yielded Campylobacter after enrichment in alkaline peptone water and subsequent plating on selective medium. Using this alkaline peptone water, there was no increased incidence in the isolation of Campylobacter. Specimens yielding no Campylobacter on direct plating remained negative after enrichment in alkaline peptone water, incubated at 43°C for 24 hr and then plated onto selective medium.

II. Incidence of Campylobacter enteritis

Enteritis caused by C. fetus ss. jejuni has been reported in many parts of the world. The organism has been cultured from between 5% and 6% of patients with diarrhoea in Belgium (Lauwers, DeBoeck & Butzler, 1978). Skirrow (1977) examined stools from about 800 patients with diarrhoea, and isolated C. fetus ss. jejuni from 7.1% of these cases. Severin (1978) in Holland, examined the stools of 584 patients with diarrhoea, and isolated C. fetus ss. jejuni from 11% of the specimens, but, Bokkenheuser et al., (1979) in South Africa cultured C. fetus ss. jejuni from 30% of infants with diarrhoea.

In this study, C. fetus ss. jejuni was isolated from the faeces of 11 (3.9%) from a total of 290 specimens of patients with diarrhoeal illness, but was absent in cultures from healthy people. Although Lauwers, DeBoeck & Butzler (1978) in Brussels reported the isolation of this organism in 1.2% of asymptomatic normal people, these findings have not been reproduced in several series (Brunton & Heggie, 1977; Skirrow, 1977; Hayek & Cruikshank, 1977; Bruce, Zochowski & Ferguson, 1977; Karmali & Fleming, 1979a) nor in my investigation.

III. Identification of C. fetus ss. jejuni

C. fetus ss. jejuni and C. fetus ss. intestinalis are the two subspecies isolated from human campylobacteriosis. Both subspecies are biochemically inactive, but could be differentiated by the temperature tolerance test. Incubation at 43°C favours the growth of C. fetus ss. jejuni, but not subspecies intestinalis.

C. fetus ss. intestinalis grows at 25°C, but not subspecies jejuni. Other major biochemical characteristics of the subspecies intestinalis and jejuni are summarized in Table 5.

All the 11 strains I isolated and the 4 strains obtained from the City Laboratory were identified as subspecies jejuni. No C. fetus ss. intestinalis was isolated from faecal samples. All samples were plated on the selective medium and incubated at 43°C and 25°C respectively, only plates incubated at 43°C yielded Campylobacter. This result confirms early reports by various authors (King, 1957; Dale, 1977; Rettig, 1979) that C. fetus ss. jejuni can be isolated from human faeces resulting from diarrhoeal illnesses, whereas C. fetus ss. intestinalis is mainly isolated from the blood of patients with systemic campylobacteriosis e.g. bacterial endocarditis, meningitis, and thrombophlebitis.

IV. Maintenance of cultures

Subculturing C. fetus ss. jejuni every 10 days is a cumbersome task. Skirrow (Personal Communication, 1978) mentioned that the freeze-drying technique is not reliable for long term preservation of C. fetus ss. jejuni. In search of a satisfactory method for long term maintenance of cultures, a freeze-drying method using 2 different suspending fluids (skim milk and Mist. desiccans), and a simple method of storage of bacteria on glass beads at -76°C described by Feltham et al., (1978) were tested.

In freeze-drying, the type of reconstituting medium used had no effect on the recovery of Campylobacter i.e. nutrient

broth supplemented with yeast extract and cystine, or this nutrient broth with 10% Horse serum No. 3 added. Freeze-dried ampoules which yielded no growth of Campylobacter with the nutrient broth supplemented with yeast extract and cystine, also remained negative with the other reconstitution broth. The cultures freeze-dried in skim milk had a longer period of viability i.e. one year than the cultures in Mist. desiccans, which only survived for three months. For long term preservation of C. fetus ss. jejuni, the method described by Feltham et al., should be used, as after 2 years, the bacteria are still viable.

V. Concentration of oxygen for the growth of C. fetus ss. jejuni

C. fetus ss. jejuni requires both O_2 and CO_2, however, this bacterium did not grow in the normal atmospheric levels of oxygen. There are reports suggesting that C. fetus ss. jejuni grew well in an atmosphere of 10% CO_2, 5% O_2 and 85% N_2 (Skirrow, 1977; Dale, 1977; Smibert, 1978), but the mechanism of oxygen toxicity in this organism is not understood.

Fridovich (1974) stated that the superoxide dismutase (SOD) in eukaryotic and prokaryotic organisms gives an important protection against oxygen toxicity. Tally et al., (1977) found a correlation between the degree of aerotolerance of anaerobic bacteria and their superoxide dismutase content. This enzyme catalyses the dismutation of O_2^- to H_2O and O_2; superoxide dismutase prevents O_2^- or other even more reactive groups like the hydroxyl radical and singlet oxygen from exerting their detrimental effects on vital cell components (Koppenol & Butler, 1977).

Ware & Colley (1978) reported the presence of superoxide dismutase activity in C. fetus (subspecies not given); they found that most C. fetus strains had at least two bands of SOD activity by gel electrophoresis. The enzyme was also demonstrated by Niekus et al., (1977; 1978) in C. sputorum ss. bubulus; these authors reported the cell-free extracts of C. sputorum ss. bubulus contained superoxide dismutase, and the enzyme activity was localized in the cytoplasm.

There have been no papers published reporting the presence of SOD activity in C. fetus ss. jejuni, and the reason for C. fetus ss. jejuni being able to grow in an atmosphere of 10% CO_2, 5% O_2 and 85% N_2 is not yet understood; but it is possible that C. fetus ss. jejuni does possess this enzyme which may act in this way.

Bowdre et al., (1976) reported that the aerotolerance of C. fetus ss. jejuni was enhanced by incorporating dihydroxyphenyl ferric iron binding components, such as norepinephrine or high concentrations of iron salts (ferric or ferrous) in brucella agar. George et al., (1978) also reported that brucella agar supplemented with 0.2% $FeSO_4.7H_2O$, 0.025% sodium metabisulfite and 0.05% sodium pyruvate supported the growth of 89% of the 64 strains of C. fetus in 21% O_2. The reason for the increase of aerotolerance using these inorganic salts is not clear, however, Smibert (1978) suggested that iron and bisulfite act together non-enzymatically to destroy superoxide radicals and hydrogen peroxide which are harmful to the vital cell components.

VI. Growth of *C. fetus ss. jejuni* *in vitro*

Mass culture of *C. fetus ss. jejuni* presents a problem in obtaining adequate cell-yields. In this laboratory attempts were made to grow *C. fetus ss. jejuni* in 2 l flasks by bubbling the special gas mixture (10% CO_2/90% N_2) through the supplemented nutrient broth (p. 57) for 48 hr. The flasks were incubated at 37°C but the bacteria did not grow.

With the OD measurement of strains of *C. fetus ss. jejuni* grown in nutrient broth, brain heart infusion and thioglycollate broth, maximum cell yields were reached in 24 hr. The organisms grew better in nutrient broth (Fig. 3) and with the addition of yeast extract; growth was greatly enhanced (Table 18). This agreed with Smibert (1963) who reported that the addition of yeast extract enhanced the cell-yields of *C. fetus* isolated from bovine sources and Batlin & Wilson (1950) and Zemjanis & Hoyt (1960) reported that the addition of cystine, glutamate, lactate and glycine greatly enhanced the cell-yields. However, L-glutamic and glycine did not increase the cell-yields of *C. fetus ss. jejuni*; cystine had little effect on the growth *in vitro*. Manclark & Pickett (1960) found that the addition of potassium, magnesium, nitrate and iron stimulated the *in vitro* growth of *C. fetus*, but in this work inorganic salts e.g. $MgSO_4$, $CaCl_2$, $Na_2H_2PO_4$, KNO_3 and ferric ammonium citrate had no growth stimulatory effect on *C. fetus ss. jejuni*.

C. fetus ss. jejuni could grow in the broth at pH values between 5 and 8.4. The OD measurements of the strains grown in broth media with the pH adjusted to 3, 5, 6.9 and 8.4, showed that highest cell-yields were obtained by growing the organisms

in broth of pH 6.9. C. fetus ss. jejuni did not grow in the broth medium of pH 3. The organisms did not survive after inoculation into this broth.

It appeared from these experiments that nutrient broth with yeast extract and cystine at pH 6.9 was a satisfactory medium for the growth of C. fetus ss. jejuni. All the flasks were incubated microaerophilically on a shaker (p. 66) since Dennis & Jones (1959), and Roberstad & Morrison (1957) reported that shake cultures in flasks gave much better cell yields than stationary cultures.

VII. The effect of the successive passage through mice on C. fetus ss. jejuni

The virulence of P. septica and E. coli can be maintained by repeated passage through susceptible animals (Bullen, Leigh & Rogers, 1968). There have been no reports on the loss of virulence of C. fetus ss. jejuni after storage. However, successive passage of C. fetus ss. jejuni through 5-wk old mice increased the virulence of this bacterium for 7-day old mice ; the mortality being increased from 20% to 80% after 5 successive passages (Table 31). In the literature, there have been reports that enterotoxin plasmids of enterotoxic E. coli may be lost during storage (Smith & Halls, 1968; Formal et al., 1971; Goldschmidt & DuPont, 1976). Echeverria et al., (1976) and French (1976) found that human enteropathogenic strains of E. coli which had previously given positive results with the rabbit-ileal loop test were negative when subsequently retested, and suggested that loss of virulence may be due to the loss of the plasmid. Sack (1976) found that the lost enteropathogenic property of

E. coli could be restored by successive passage through susceptible animals. It may be that by successive passage of C. fetus ss. jejuni through mice, the pathogenicity of this bacterium can be increased in a manner similar to that of E. coli.

VIII. Effect of live C. fetus ss. jejuni cultures on 3- and 5-wk old mice

C. fetus ss. jejuni could not survive in broth at pH 3 (Fig. 5). The pH of mouse stomach contents is about 3, therefore oral administration of live C. fetus ss. jejuni, without first reducing the acidity of the stomach contents, may kill the organisms immediately. A solution of $NaHCO_3$ could reduce the acidity of the stomach (Levine et al., 1978), therefore 5-wk old mice were given 5% $NaHCO_3$ solution to drink 18 hr prior to the oral administration of C. fetus ss. jejuni suspensions. It was found that by giving 3×10^9 live bacteria orally to these groups of mice and to groups of mice without prior dosage of $NaHCO_3$ solution, there was no difference between the 2 groups i.e. the pre-treated mice did not show any signs of diarrhoeal illness.

McEntee, Hughes & Gilman (1954) and Bryner et al., (1971) reported that following intravenous injection of C. fetus (10^8 to 10^{10} viable cells per ml) into cattle and sheep, the bacteria could be reisolated from the gall-bladder, intestine and genital organs. Plastridge, Williams & Petrie (1947) reported that after the intraperitoneal injection of C. fetus (from cattle) into guinea pigs and rats, the bacteria could be reisolated from the blood, liver, spleen and gastrointestinal tract. Butzler et al., (1978) using

Campylobacter isolated from children with diarrhoea, found that after oral administration of 10^6 organisms to chickens, the bacteria could be isolated from the blood, spleen and liver. Oral administration of 3×10^9 of live C. fetus ss. jejuni to 3-and 5-wk old mice could cause bacteraemia; the injected bacteria were reisolated from liver, spleen and gastrointestinal tract. This result showed that although C. fetus ss. jejuni could not survive at pH 3 in vitro, the administration of a dose of 3×10^9 bacteria per os resulted in the bacteria reaching the gastrointestinal tract. This may be due to the presence of food particles in the stomach which protect the bacteria against the acid environment.

In the groups of 3-and 5-wk old mice inoculated i.p. and i.v. with a dose of 3×10^9 bacteria; C. fetus ss. jejuni could also be reisolated from blood, liver, spleen and gastrointestinal tract (Table 23 and 24). The injected bacteria could be reisolated from blood, liver, spleen and kidney 30 min after intravenous injection ; the bacteraemia occurred intermittently, and the bacteria persisted in the liver over a period of 21 days. Viable count of the homogenates of liver and spleen at 12, 24, 48hr and 7 days after intravenous injection showed that C. fetus ss. jejuni did not multiply in vivo even though liver and spleen are the two sites where Campylobacter could readily be reisolated, irrespective of the routes of inoculation (Fig. 9 and 10).

There are reports that C. fetus ss. jejuni infection can not be established in chickens (Butzler et al., 1978), cats and dogs (Prescott & Karmali, 1978) or monkeys (Butzler & Skirrow, 1979). But Steele & McDermott (1978) reported that Campylobacter enteritis

could be reproduced in man by ingestion of a pure culture of C. fetus ss. jejuni. McDermott ingested a dose of 10^6 Campylobacter recently isolated from a patient with diarrhoea; three days later he developed vague abdominal discomfort, the organisms appearing in the stools on the fourth day, and on the fifth day, diarrhoea with cramping abdominal pain developed; the diarrhoea was not severe but the abdominal pain persisted for three days. An accidentally acquired infection of C. fetus ss. jejuni was recorded by Prescott & Karmali (1978), they reported that one of them developed moderate diarrhoea, flatus and mild central abdominal pain on the sixth day after an experiment attempting to transmit Campylobacter enteritis to dogs and cats. Blood and mucus were present in the stools, and the bacteria were recovered from the diarrhoeal faeces. These two incidents indicate that man may develop symptoms of enteritis after ingestion of Campylobacter.

In the experimental inoculations with 3-and 5-wk old mice, no signs of diarrhoeal illness could be produced with the administration of a dose of 3×10^9 bacteria by the i.p., i.v. and oral routes. Although the organism could still be recovered from the apparently normal mice, this finding seems to indicate that HAM I/CR mice are not susceptible laboratory animals for establishing a Campylobacter infection.

Inoculation of a dose of 3×10^9 live C. fetus ss. jejuni i.v. to pregnant mice may cause abortion. This suggests that this bacterium can cause foetal death; the mechanism of this effect is not known. However, in 1960, Rieder & Thomas reported that Gram-negative endotoxin causes haemorrhage and congestion

of the placenta; Parant & Chedid (1964) suggested that this could trigger foetal death, and perhaps this finding with C. fetus ss. jejuni could be explained on these grounds.

IX. Effect of C. fetus ss. jejuni on 7-day old mice

Mice at 7 days of age were found to be susceptible to C. fetus ss. jejuni (Table 32). There was no detectable toxicity in culture supernatant fluids and the lethal effect was associated with the bacterial cells (Table 33). Seven-day old mice injected i.p. with live C. fetus ss. jejuni died within 72 hr; and although pure cultures of the injected bacteria could be reisolated from the peritoneal cavity of the dead mice, there was no signs of diarrhoeal illness when these mice were injected with various concentrations of living cells; as higher dosages i.e. 1×10^{10} bacteria were given, all the inoculated mice died, and as a converse, the mortality decreased as the concentration of cells was reduced (Table 34 and 36). From this evidence, it would appear that it is the total number of living cells that causes death in mice, and the organism does not multiply in this host. If multiplication did occur, a dose of 6.2×10^{8} bacteria would eventually give the same mortality rate as that of higher doses. This did not happen.

Seven-day old mice died after the inoculation of a dose of 1×10^{10} heat-killed bacteria. After a live suspension of 1×10^{10} bacteria was sonicated 3 x 30 sec (Table 37), 50% of the bacteria could survive, the injection of this sonicated suspension caused death in 7-day old mice (Table 38) whereas a dose of 6.21×10^{8} live bacteria had no apparent effect on 7-day

old mice. There were no deaths in the groups of mice injected with the supernates of these heat-killed and sonicated suspensions. These results suggest that death in 7-day old mice was due to endotoxin, i.e. the lipopolysaccharide. This was illustrated by viable count of the homogenates of whole 7-day old mice inoculated with doses of 2.5×10^9, 1.3×10^9 and 6.2×10^8 bacteria. The bacterial numbers of the viable count decreased at 12, 18, 24 and 48 hr suggesting that C. fetus ss. jejuni did not multiply or multiplied at a rate slower than the bactericidal activity in the animals (Fig. 12). The injected bacteria were probably rapidly eliminated by macrophage phagocytic activity, as suggested by Medearis & Kenny (1968) for E. coli, and death could be due to the release of the LPS of the injected bacteria in the animals.

The LD_{50} of the heat-killed suspension was higher than that of the live suspension which suggested that a toxic factor which could be destroyed at 56°C for 30 min (Fig. 11) might be present.

X. Age-related susceptibility

It is well established that mice exhibit an age-related susceptibility to many experimental viral infections (Sigel, 1952; Sawicki, 1961; Marrenikova & Kaptsova, 1965; Hirsch, Zisman & Allison, 1970; Reinarz, Broome & Sagik, 1971; and Hirsch et al., 1972). McKay (1975) first reported that mice exhibit an age-related susceptibility to experimental staphylococcal infection when challenged by the subcutaneous route. It has been shown that neonatal mice lack immunocompetence

because they lack antigen-recognising cells in the form of functional macrophages (Argyris, 1968; Hirsch et al., 1972), and that functional development of the reticuloendothelial system (RES) is related to the macrophage population (Reade & Casley-Smith, 1965).

In the preliminary studies, the i.p. injection of 1-, 7-day, 3-and 5-wk old mice with a dose of 3 x 10^9 live C. fetus ss. jejuni showed that mice at 7 days of age were most susceptible to C. fetus ss. jejuni (Table 30). Using passaged C. fetus ss. jejuni, the mice of 1-, 3-, 7-, 14-days, 3-and 5-wk were inoculated i.p. with doses of 1 x 10^{10}, 5 x 10^9, 2.5 x 10^9 and 1.3 x 10^9 bacteria, the highest mortality rate was again recorded in the 7-day old mice group (Table 32). Dubos (1954) mentioned that the young mouse possesses a smaller reserve of dietary materials than does the adult, and he showed that this protein deficiency greatly increased bacterial infection in the juveniles. The immunological differences between neonatal and adult macrophages do not appear to be related to differences in phagocytic activity, since it has been shown in vivo that phagocytes from new born and weanling mice are equally able to clear carbon particles from the blood (Hackbarth, Reinarz & Sagik, 1973). However, Benacerraf et al., (1954) had reported that phagocytic activity of the RES in mice, increased as the liver and spleen weight increased. This might explain the finding that older mice e.g. 3-and 5-wk are resistant, and 7-day old mice are most susceptible to C. fetus ss. jejuni. The fact that 1-and 3-day old mice are less susceptible may be due to the presence of protective antibodies from the mother, as it has been shown that there are natural antibodies in mice which

could agglutinate the O and H antigens of C. fetus ss. jejuni.

XI. Experimental studies on the pathogenic mechanisms of C. fetus ss. jejuni

The major enteric pathogens can be classified into two groups: On the one hand are those that cause disease by elaboration of a toxin, V. cholerae being a prime example; on the other hand are those that cause disease by invasion of tissue such as salmonellae and shigellae, Fig 17 shows the pathogenic mechanisms for both types.

The pathogenic mechanism of C. fetus ss. jejuni is not known. Guerrant et al., (1978) found that C. fetus ss. jejuni isolated from cases of human campylobacteriosis did not produce heat-labile cholera-like enterotoxins detectable by Chinese hamster ovary cells (Guerrant et al., 1974), and heat-stable E. coli-like toxins detectable by the suckling mouse method (Dean et al., 1972). Stavric et al., (1979) reported that a cell-free filtrate of C. fetus ss. jejuni produced a morphological response and increased cyclic-AMP levels in Y-1 adrenal cells, but had no apparent effect on rabbit ileal loops or suckling mouse intestine. However, Butzler & Skirrow (1979) reported that sixty of 100 strains of C. fetus ss. jejuni showed heat-stable enterotoxin activity using the suckling mouse method, but did not show heat-labile enterotoxin activity on Y-1 adrenal cells. In this laboratory, Dr. D.E.S. Stewart-Tull and I observed that following the injection of 0.1ml of a 24 hr culture of C. fetus ss. jejuni into guinea pig ileal loops, there was no fluid accumulation after 18 hr. These results indicated that C. fetus ss.

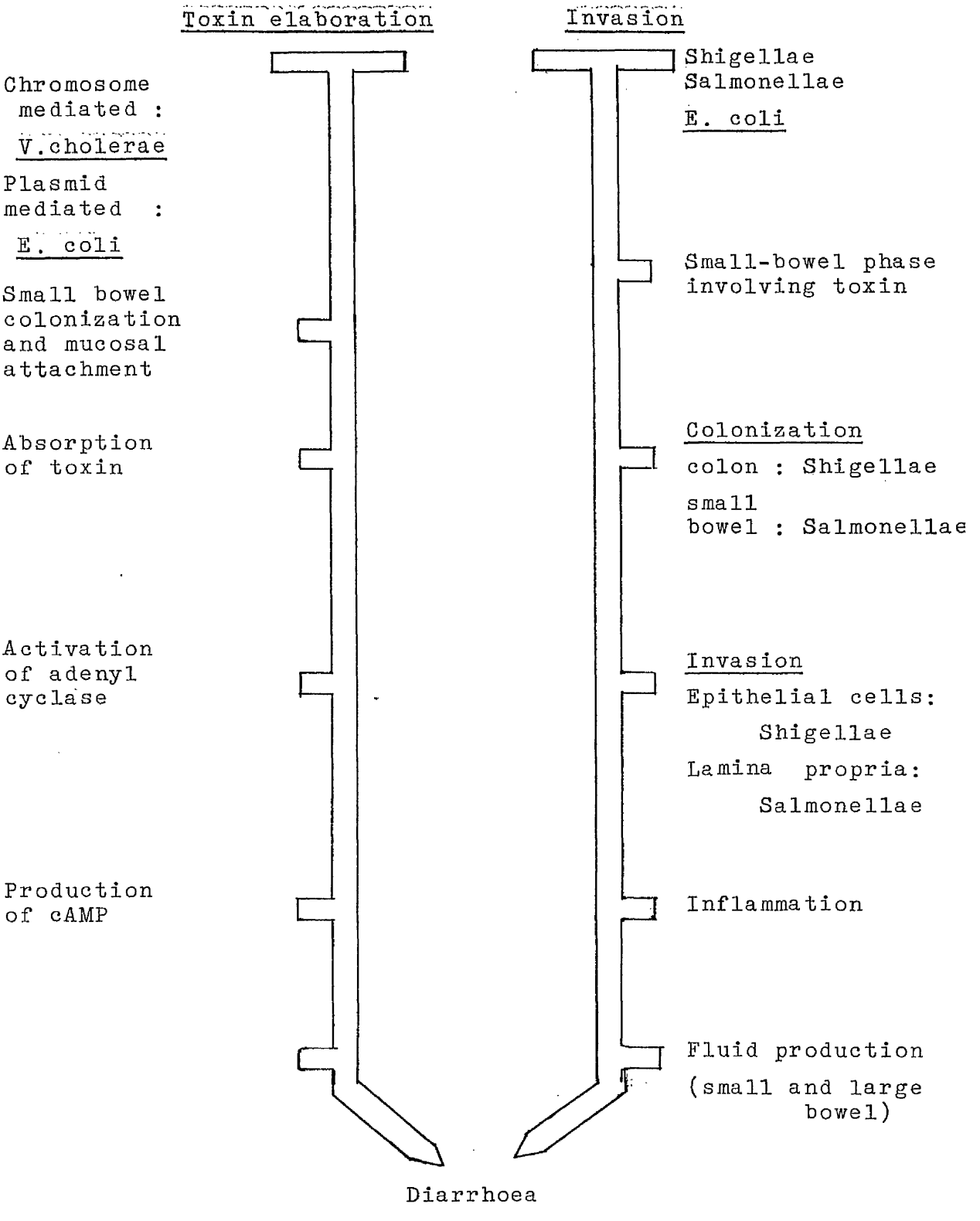


Fig.17 : Pathogenic mechanisms of acute bacterial diarrhoea. (Modified from Sack, 1976)

jejuni did not produce heat-labile V. cholerae-like entero-toxin detectable at this level.

Butzler et al., (1978) reported that after oral administration of 10^6 organisms to chickens, C. fetus ss. jejuni could be reisolated from liver, spleen, and blood. Following oral administration of 3 x 10^9 organisms to 3-and 5-wk old mice, C. fetus ss. jejuni could be reisolated from blood, liver and spleen. This suggests that C. fetus ss. jejuni may invade the blood stream from the intestine, which may explain the origin of the bacteraemia in human campylobacteriosis (King, 1957, Guerrant et al., 1978). In 1979, Butzler & Skirrow reported that C. fetus ss. jejuni could invade chick embryo cells, and the caecal wall of 8-day old chicks after the inoculation of a dose of 10^8 organisms. Butzler & Skirrow suggested that the pathogenic mechanism of C. fetus ss. jejuni which results in diarrhoea, is that of a direct invasive action. However, there is not sufficient evidence to suggest that Campylobacter is entero-invasive because Guerrant et al., (1978) reported that loopfulls of C. fetus ss. jejuni cultures inoculated onto the upper and lower conjunctival sacs of guinea pigs failed to cause conjunctivitis (Sereny Test). Clearly, more investigation has to be carried out to elucidate the pathogenic mechanism of C. fetus ss. jejuni, which causes gastroenteritis in man.

Many enteric bacteria e.g. E. coli and V. cholerae produce fimbriae which enable them to attach to the mucosa and thereby resist dispersal by the flow of the gut contents. The adhesive action of fimbriae is further suggested by their role in haemagglutination (Smith & Linggood, 1971). In 1973, Morris & Park

reported that catalase-positive Campylobacter sp. isolated from the genital or intestinal tract of cattle, sheep and pigs all agglutinated red blood cells of man, guinea pigs and sheep. However, the strains of C. fetus ss. jejuni studied here did not haemagglutinate red blood cells of man (Group A), horse, rabbit and sheep. Rhoades (1954) and Pead (1979) had reported that Campylobacter did not possess fimbriae.

XII. Virulence enhancing effect of ferric ammonium citrate in experimental infection

Iron is essential for the growth of most, if not all, bacteria (Stephenson, 1949). The total quantity of iron in animal fluids, such as milk and plasma is more than sufficient for microbial growth, but iron in these fluids is unavailable to many bacteria because of the iron-binding proteins transferrin and lactoferrin (Aisen & Leibman, 1968). These proteins are single-chain glycoproteins, having molecular weights of about 76,000 and are capable of binding 2 Fe^{3+} ions (Palmour & Sutton, 1971). In man, all the inorganic iron (20 to 50 μM) in circulating plasma is bound to transferrin, although only about 30% of the available iron-sites are occupied (Bothwell & Finch, 1962). Aasa et al., (1963) found that free Fe^{3+} is present at a concentration of about 10^{-18}M, a level far too low to support normal bacterial growth.

In this investigation, the injection of 4, 8 and 12 μg Fe^{3+} per mouse had no demonstrable toxic effect, but as the concentration of Fe^{3+} increased to 100 μg, there was a 60% mortality at 12 hr (Table 41). This result agreed with early reports that

Fe^{3+} at sufficiently high concentrations is toxic for this species (Martin, Jandl & Finland, 1963; Joo, 1975).

Campylobacter infection could not be established in strain HAM I/CR mice. The administration of 3-and 5-wk old mice with a dose of 3×10^9 bacteria by the i.p., i.v. and oral routes did not kill the mice or produce a diarrhoeal illness. Bullen, Leigh & Rogers (1968) reported that iron compounds can greatly enhance the virulence of E. coli for guinea-pigs. The incorporation of 40 μg and 80 μg Fe^{3+} with 3×10^9 live C. fetus ss. jejuni; however, did not increase the virulence of this bacterium for 3-and 5-wk old mice, and again, the mice did not die nor was diarrhoea found - irrespective of the inoculation routes.

Brubaker, Beesley & Surgalla (1965), Sword (1966), Rogers (1967), Bullen & Rogers (1969) reported that the incorporation of ferric ammonium citrate with live bacterial suspensions reduced the LD_{50} value. The LD_{50} value of passaged C. fetus ss. jejuni strain 5636 for 7-day old mice was 1.8×10^9; the LD_{50} values were reduced to 1.2×10^9, 7.4×10^8 and 4.2×10^8 respectively when 4, 8 and 12 μg Fe^{3+} was incorporated. Comparable reduction of the LD_{50} value was also obtained with passaged C. fetus ss. jejuni strain 4249. The mechanism by which iron promotes the pathogenicity of bacteria in animals may be difficult to assess because of the complexity of the situation in vivo. However, many reports have suggested that iron appears to enhance virulence by interfering with two non-specific defense mechanisms. a) Injected bacteria are deprived of iron essential for in vivo growth because iron is tightly bound to proteins

like transferrin and lactoferrin (Bullen, Rogers & Griffiths, 1974) therefore injected excess iron provides sufficient iron for bacterial growth. b) The bactericidal action of the cationic lysosomal proteins of neutrophils is neutralized by excess iron (Gladstone, 1973; Bullen & Wallis, 1977).

Rogers (1967) found that transferrin, together with the β_2 and γ-globins, is an essential component of the bacteriostatic system in horse-serum, which has a powerful inhibitory effect on Cl. welchii (Cl. perfringens) Type A *in vitro*, and this inhibitory effect on bacterial growth is abolished by adding ferric ions (final concentration : 10 μg/ml ferric ammonium sulphate) to saturate transferrin. Bullen, Cushnie & Rogers (1967) reported that passive immunization of guinea-pigs with 100-200 units of Cl. welchii Type A antitoxin protected the animal against a dose of 5×10^4 to 1.3×10^5 Cl. welchii, but intravenous injection of 5 mg iron/kg immediately before infection resulted in the deaths of all fifteen animals passively immunized with antitoxin. These authors reported that the growth of Cl. welchii in the infected muscle of immunized control guinea-pigs and in passively immunized guinea-pigs injected with 5 mg iron/kg was very similar; the growth occurred rapidly for 4 hr, and continued without interruption, and just before death at 18 hr, the viable count had risen to 10^7 - 10^8 bacteria per g. wt. of infected muscle of guinea-pigs. In the case of passively immunized guinea-pigs, growth also took place during 4 hr, but ceased abruptly after 4 hr, and at 18 hr the viable count decreased to 10^3 per g. wt. of guinea-pigs. In a similar experiment, Bullen & Rogers (1969) found that

passive immunization of mice with P. septica horse anti-serum protected the animal against intraperitoneal injection of a dose of 2.9×10^6 P. septica. However, the protective effect of P. septica antiserum in mice was abolished when ferric ammonium citrate (5 mg Fe^{3+}/kg live weight, the average wt. of the mice 25 - 28 g) was injected intraperitoneally. These results are good evidence indicating that iron compounds can abolish or interfere with both the bacteriostatic effect of serum in vitro and passive immunity in vivo and strongly suggest that the bacteriostatic effect of serum proteins may play a decisive role in resistance to infection.

There was a continuous loss of viable Campylobacter in the homogenates from mice not receiving Fe^{3+}; but, in the presence of 4, 8 and 12 μg Fe^{3+}, the number of viable Campylobacter at 48hr reisolated was 5, 10 and 100 fold greater than with the injection of a dose of about 10^9 Campylobacter alone. These findings agreed with the reports about the effect of Fe^{3+} on the recovery of L. monocytogenes (Sword, 1966), Cl. perfringens (Bullen, Cushnie & Rogers, 1967) and E. coli (Fletcher & Goldstein, 1970) from liver, muscle and kidney. These results suggest that in the presence of Fe^{3+}, the process of bacterial elimination is delayed.

The delay in the process of elimination could be maintained by further injection of excess Fe^{3+} (Fig. 15). The number of viable Campylobacter recovered at 48 hr from the homogenates of mice a) receiving no iron and b) with 12 μg Fe^{3+} initially, were 6.7×10^4 and 6.8×10^6 respectively. By injecting an excess dose of 12 μg Fe^{3+} at 18 hr into mice which received a

dose of 12 μg Fe^{3+} at the first injection, the number of viable Campylobacter reisolated increased to 2.3×10^8. Similarly, by inoculating a dose of 12 μg Fe^{3+} at 18 hr into a group of mice injected with the suspension alone, the number of viable Campylobacter at 48 hr was 5.5×10^7 as compared to that of 6.7×10^4 bacteria reisolated from the homogenates of mice receiving no iron. The effect of Fe^{3+} on delaying the elimination of bacteria was confirmed by a further experiment (Fig. 16) in which the viable counts of Campylobacter from the homogenates of mice were examined not only at 48 hr but at 72 hr.

The incorporation of 12 μg Fe^{3+} with a dose of 2.5×10^9 heat-killed Campylobacter did not increase the mortality for 7-day old mice (Table 45); this result was in agreement with Bullen, Leigh & Rogers' (1968) report that Fe^{3+} did not increase the virulence of heat-killed suspensions of E. coli for guinea-pigs. Ferric ammonium citrate did not increase the toxicity of S. flexneri LPS, the mice injected with a dose of 25 μg or 25 μg with 12 μg Fe^{3+} did not die (Table 46). These results suggested that Fe^{3+} did not increase the toxic activity of lipopolysaccharide, and the death in 7-day old mice with living cells in the presence of Fe^{3+} was due to the increase of Campylobacter cells. The delay in the process of elimination in the presence of Fe^{3+} may represent a balance between multiplication and elimination. This demonstrates that the availability of iron allows the multiplication of the bacteria to equal or overtake the host's bactericidal activity, and when the critical concentration of C. fetus ss. jejuni endotoxin is reached in 7-day old mouse tissue, death occurs.

Human and guinea-pig heterophil leukocytes contain the iron-binding protein - lactoferrin, in the cytoplasmic granules which are discharged into phagocytic vacuoles upon the onset of phagocytosis (Hirsch & Cohn, 1960; Cohn & Hirsch, 1960; Zucker-Franklin & Hirsch, 1964; Masson, Heremans & Schonne, 1969; Baggiolini et al., 1970); the lactoferrin possesses bacteriostatic properties (Masson et al., 1966; Oram & Reiter, 1968). Van Snick, Masson & Heremans (1974) found that the lactoferrin in polymorphs is present largely in the iron-free form, and lactoferrin extracted from leukocytes was only about 8% saturated with iron. Bullen & Wallis (1977) mentioned that when iron is presented in the form of a ferritin-antibody complex, the metal was readily phagocytosed and appeared in the phagosomes, and they found that when polymorphs were exposed to a ferritin-antibody complex, the viable bacterial count of Ps. aeruginosa fell slowly for 5 hr but thereafter increased rapidly and was 346 times greater than the control at 8 hr. These authors thus suggest that the unsaturated or only partly saturated iron binding capacity of the proteins in polymorphs may be essential for antibacterial function.

Since the method by which the animal body disposes of bacteria is predominantly by intracellular destruction in polymorphs, and the basic proteins e.g. lactoferrin are secreted into the phagocytic vacuole, these basic proteins probably play an important part in the destruction of bacteria. It is likely that Fe^{3+} enhances the virulence of bacteria by combining with these basic proteins inside the cells, and following injection of C. fetus ss. jejuni with Fe^{3+} iron, the reduction in the LD_{50}

value and the greater number of viable Campylobacter recovered from the homogenates may be due to these phenomena.

XIII. Influence of mucin on C. fetus ss. jejuni in mice

Mucin is known to enhance the virulence of a variety of bacteria (p. 45). However, the incorporation of 1% and 5% mucin did not reduce the LD_{50} value of C. fetus ss. jejuni in 7-day old mice. This may be due to no increase in the amount of LPS in the animal tissues, as Keefer & Spink (1938) reported that mucin could not provide a nutrient source in vivo. This result would also suggest that mucin acts in a different manner from that of ferric ammonium citrate in vivo.

XIV. Effect of S. flexneri LPS on the susceptibility of 7-day old mice to C. fetus ss. jejuni

Biozzi, Benacerraf & Halpern (1953); Benacerraf & Sebestyen (1957) reported that 12 hr after the administration of endotoxin to mice or rabbits, there was a depression of the phagocytic activity of the reticuloendothelial system also shown by Howard Rowley & Wardlaw in 1958. Benacerraf et al., (1954) had reported that depression of phagocytic activity of the RES affects the uptake of carbon particles by phagocytes, and Heller (1953) suggested that LPS inhibits the cellular response of the RES thus impairing the defense mechanism of the animals against invading bacteria. Mice injected with a non-lethal dose of S. flexneri LPS 4 hr beforehand were more susceptible to Campylobacter. The injection of doses of 6.2×10^8, 3.1×10^8 or lower concentration of cells had no apparent effect on 7-day

old mice, however, as the same dosages were given to groups of 7-day old mice inoculated with S. flexneri LPS 4 hr before the experiment, deaths did occur (Table 47). This finding agreed with Benacerraf et al's suggestion concerning the phagocytic repression by LPS. It would be very interesting to see if Campylobacter could multiply in the LPS-treated mice, thus resulting in a larger accumulation of LPS in animal tissues which would lead to death.

XV. Classification of C. fetus ss. jejuni strains by SDS-polyacrylamide gel electrophoresis of cell proteins

Polyacrylamide gel electrophoresis has proved to be a useful aid in the classification of certain groups of organisms, e.g. in differentiating species of Mycoplasma (Razin & Rottem, 1967; Rottem & Razin, 1967), Mycobacteria (Haas, Davidson & Sacks, 1971), Bacteroides (Strom et al., 1976; Poxton & Brown, 1979) and Xanthomonas (El-Sharkaway & Huisingh, 1971); in comparing strains of group-A streptococci (Larsen, Webb & Moody, 1969) and E. coli (Schnaitman, 1970 a, b); for identifying rumen bacteria (Wojciechowicz, Ziolecki & Tomerska, 1973) and L-forms of Staphylococcus and Proteus (King, Theodore & Cole, 1969).

Morris & Park (1973) examined 40 Campylobacter strains isolated from cattle, sheep, pigs and fowls, and reported that the electrophoretic patterns of the acid - plus phenol - soluble proteins allowed the isolates to be placed in three groups which correlated with their habitat. Group 1 contained all the Campylobacter from the bovine genital tract; Group II all the strains isolated from cattle faeces and all the strains

associated with sporadic abortions in cattle and sheep; Group III all the Campylobacter from healthy pigs and from pigs with swine dysentery.

The present survey of electrophoretic profiles of Campylobacter strains suggests that differentiation of human and animal strains is possible. The two animal strains had characteristic whole-cell protein profiles which were quite distinct from each other and from those of the human strains. The human strains showed a marked overall similarity but minor differences were evident; as were minor differences in the profiles of their cytoplasmic proteins. In view of these findings, it would be interesting to examine a larger number of human and animal isolates. This would show whether it was possible to assign the isolates to a particular category, each of which had a characteristic electrophoretic profile. Such a classification scheme could then be compared with schemes based on other characteristics.

The distortion seen in the envelope profiles is not understood. It is possible that by altering the electrophoretic conditions, this distortion could be eliminated and the envelopes could be compared. Comparison of whole cells or cytoplasmic fractions is difficult because of their numerous protein constituents which result in complex polypeptide profiles. The use of extraction techniques e.g. for outer membrane proteins (Valtonen et al., 1975) would be worth investigating, since simpler profiles could perhaps be obtained.

SDS-polyacrylamide gel electrophoresis has also been used to demonstrate that certain membrane components of bacteria are associated with virulence (Valtonen et al., 1977; Parton &

Wardlaw, 1974). Changes in envelope protein profiles have also been seen when cells are grown under different conditions or at different stages of growth (Robinson & Tempest, 1973; Ames, 1974). An investigation to determine if such changes occur in Campylobacter species would be worthwhile. For example, normal Campylobacter strains could be compared with mouse-passaged strains; cells in the spiral form could be compared with those in the coccal form.

XVI. The antigenic relationships of C. fetus ss. jejuni strains isolated from patients with diarrhoea

Many investigators have found agglutinating antibodies to Campylobacter in sera of patients with Campylobacter infections. (Hood & Todd, 1960; Jackson, Hinton & Allison 1960 ; White, 1967; Cooper & Slee, 1971; Butzler, 1973; Gubina et al., 1976; Urman, Zurier & Rothfield, 1977; Watson, Kerr & McFadzean, 1979). However most serological studies have been carried out with ovine and bovine strains (McCoy et al., 1975, McCoy, Wiltberger & Winter, 1976; Berg, Jutila & Firehammer, 1971), and very few studies have been done on C. fetus ss. intestinalis from human systemic campylobacteriosis and C. fetus ss. jejuni isolated from patients with diarrhoea.

In this study, strains of C. fetus ss. jejuni isolated from patients with diarrhoea were used. The preparation of C. fetus ss. jejuni suspensions for agglutination tests posed a problem because the suspensions were autoagglutinable; similar problems have been encountered (Skirrow, 1978, personal communication). Although there have been suggestions that this

autoagglutination could be reduced by using only young log-phase growth, which can be conveniently harvested from the culture grown on plates, the cultures are normally very sticky, and a satisfactory suspension could not be obtained. Butzler & Skirrow (1979) suggested that a serological reaction other than the agglutination test must be used. However, CFT (complement fixation test) and fluorescent antibody tests (Blaser et al., 1978) and indirect haemagglutination tests (Bokkenheuser, 1972) have been found unsatisfactory.

In the present investigation the cultures for antisera production and agglutination tests were grown in broth medium (p. 57). Autoagglutination did not occur with the formalinized antigens (H), but autoagglutination occurred in the heated suspensions (O) which were autoclaved for 20 min at 121°C (Skirrow, personal communication, 1977) , the density of the suspensions being equivalent to that of the H antigens. When the heated-suspensions were autoclaved for 2 hr at 121°C, the conditions used by Kauffmann (1947) for the determination of the O antigens of E. coli, autoagglutination was eliminated. This seems to indicate that a cell-surface component stable at 121°C for 20 min was responsible for the autoagglutination and that it could be removed by extending the 121°C treatment to 2 hr.

Ten human strains were used for producing antisera in mice. Analysis of the O and H antisera of the homologous strains showed that mice gave low agglutination titres, and all the antisera agglutinated with the heterologous antigens tested. There were antibodies in the uninoculated mice which agglutinated with the O and H antigens of C. fetus ss. jejuni. These natural antibodies

against Campylobacter may explain why mice are not suitable for establishing infections with this organism.

Three strains of C. fetus ss. jejuni were used to produce antisera in rabbits. The analysis of O and H antisera showed that reasonably high agglutination titres could be obtained, and these 3 antisera, both O and H, could be agglutinated by the heterologous strains tested. This suggests that C. fetus ss. jejuni strains isolated from patients with diarrhoea shared common antigens.

Because of shortage of time, I was unable to carry out antisera absorption tests, but obviously this is an area worthy of further work, and possibly a serotyping scheme could be developed.

PERSPECTIVES AND FURTHER RESEARCH ON C. FETUS SS. JEJUNI

There are now numerous reports of the isolation of C. fetus ss. jejuni from man, but the majority of the reports are concerned with the clinical aspects of Campylobacter infections. There are only a few papers attempting to deal with the pathogenic mechanisms of C. fetus ss. jejuni (Butzler et al., 1978; Guerrant et al., 1978, Stavric et al., 1979) and the information is contradictory. There have been attempts to transmit Campylobacter enteritis to cats and dogs (Prescott & Karmali, 1978), chickens (Butzler et al., 1978) and monkeys (Butzler & Skirrow, 1979), but until the present study, laboratory mice do not appear to have been used. Although Campylobacter enteritis could not be established in the HAM I/CR strain of mice through i.p., i.v. and oral routes, 7-day old mice were the most susceptible age group to C. fetus ss. jejuni.

The epidemiology of Campylobacter infection is not yet known; chickens, milk and domestic animals have been incriminated as the sources of infection. The natural habitat of C. fetus ss. jejuni is not known, but it is likely that improvements in food hygiene would help to reduce infection. Death caused by C. fetus ss. jejuni is very rare, but the nuisance to the infected person is considerable, and there may be time lost from work.

For future research on C. fetus ss. jejuni, the following aspects should be investigated :

1. Formulation of media which will give improved growth of this bacterium. This would contribute to the development of techniques for the mass production of Campylobacter cells which is essential for successful LPS extraction and envelope analysis.

2. To study the pathogenic mechanisms of C. fetus ss. jejuni ; the use of other strains of mice, guinea-pigs, rats and rabbits in an attempt to establish Campylobacter infection. The role of a virulence enhancing agent e.g. ferric ammonium citrate, and the repression of phagocytes by LPS on animals should be investigated.

3. This bacterium is biochemically inactive, SDS-polyacrylamide gel electrophoresis could be useful in differentiating the strains of C. fetus ss. jejuni isolated from patients with diarrhoea also to distinguish free-living Campylobacter strains isolated from human and animal infections.

4. There would be considerable value in developing a serotyping scheme for this bacterium, and the understanding of the antigenic relationships between human and animal strains, because this would be important in the epidemiology of Campylobacter infection.

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APPENDICES

Appendix 1

KRT Buffer (Krebs - Ringer Solution)

NaCl	7.5g
KCl	383mg
$MgSO_4.7H_2O$	318mg
$CaCl_2$	305mg

Dissolve in 1 litre of distilled water, and buffer with 0.01M Tris hydroxymethyl aminomethane-hydrochloric acid at pH 7.4.

Appendix 2

Slab and tube-gel electrophoresis

Stock solutions

1. Acrylamide solution

   acrylamide (B.D.H., Poole, England) 30g

   NN'-methylenebisacrylamide (B.D.H., Poole, England) 0.8g

   Add distilled water to 100ml. Filter and store in brown bottle at 4°C.

2. Tris/HCl buffer.pH 8.8

   1M Tris (hydroxymethyl-methylamine) 50ml

   1N HCl 8.1ml

   Add distilled water to 100ml

3. Tris/HCl buffer.pH 6.8

   1M Tris (hydroxymethyl-methylamine) 50ml

   1N HCl 45ml

   Add distilled water to 100ml

4. Tris/glycine buffer.pH 8.3 (10x concentrated)

   glycine 144.13g

   Tris 30.28g

   Add distilled water to 1000ml

5. Ammonium persulphate

   0.8% (w/v) in distilled water

6. Sodium dodecyl sulphate (SDS)

   20% (w/v) in distilled water

7. Bromophenol blue (B.D.H. Ltd., Poole, England)

   0.1% (w/v) in distilled water

8. Solubilizing buffer

Tris/HCl (6.8)	2.0ml
SDS	3.2ml
β-mercaptoethanol	1.6ml
glycerol	3.2ml
Bromophenol blue	0.32ml
distilled water	5.68ml

Preparation of gel plates

The gel plates are prepared with glass plates (80mm x 80mm), separated with spacers and sealed at three sides with adhesive tapes. Seal corners by dipping in molten paraffin wax.

Preparation of separating gel solution

acrylamide solution	14.7ml
Tris/HCl (8.8)	15.0ml
20% SDS	0.2ml
N',N',N',N'tetramethyl-ethylenediamine (TEMED)	10μl
ammonium persulphate	4.0ml
distilled water	6.1ml

Mix thoroughly and pipette approximately 14ml into the gel plates standing vertically in a rack. Carefully overlay with 5% (v/v) ethanol without disturbing the surface of the gel. This removes the meniscus from the gel and leaves a perfectly flat surface. Allow to polymerise and prepare stacking gel solution.

Preparation of stacking gel solution

acrylamide solution	1.7ml
Tris/HCl (6.8)	1.25ml
20% SDS	0.05ml
TEMED	2.5µl
ammonium persulphate	1.0ml
distilled water	6.0ml

Mix thoroughly but without aeration. Pour off overlay solution from separating gel. Replace the plates in the rack and fill to within 3-4mm of the top with stacking gel solution. Suspend the sample "combs" in the solution and fill any remaining space with gel solution. Allow to polymerise then remove the "combs" carefully under running buffer (Tris/glycine buffer, pH 8.8, diluted 1 to 10).

Electrophoresis conditions

Dilute the Tris/glycine stock buffer 1 in 10 and add SDS to give a final concentration of 0.1% (w/v). Fill the lower electrode tank with approximately 2.5L of the diluted buffer.

Remove the tape from the base of the gel plates and insert into the upper electrode tank using a liberal amount of grease around the gaskets to prevent leakage. Fill the vessel with running buffer and then layer 25-50µl of samples into the sample wells.

Connect electrode unit to power supply and run at a constant current of 15mA/gel for approximately $2\frac{1}{2}$h. When the tracking dye reaches the end of the gel, switch the power off. Carefully remove the gels from the plates and

immerce in fix-staining solution for 90 min. Destain by soaking in several changes of destaining solution.

Fixing and staining solution

Coomassie brilliant blue R250	1.25g
50% (v/v) methanol	454ml
glacial acetic acid	46ml
methanol	50ml
glacial acetic acid	75ml
distilled water	875ml