STUDIES ON *CAMPYLOBACTER FETUS* SUBSPECIES *JEJUNI*

ISOLATED FROM CATTLE IN ZARIA AREA, NIGERIA

BY

UCHENNA E. IBEZIAKO
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DECLARATION

I hereby declare the originality of this piece of work which was carried out by me in the laboratory of the Department of Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria, under the supervision of Dr. J. O. Adekeye.

The work of other investigators is duly acknowledged and referred to in this presentation. No part of this thesis has been presented elsewhere for a degree or diploma.

Ubezlako. U.E

U. E. UBEZIAKO

Ahmadu Bello University

1ST JUNE 1985

CERTIFICATION

This thesis entitled "Studies on Campylobacter fetus subspecies jejuni isolated from Zaria Area, Nigeria" meets the regulation governing the degree of Master of Science of Ahmadu Bello University, Zaria, and is approved for it's contribution to scientific knowledge and literary presentation.

SUPERVISOR

HEAD OF DEPARTMENT

DEAN
Postgraduate School

Date: 27/ix/85

AHMADU BELLO UNIVERSITY, ZARIA.

DEDICATION

Dedicated to JOHN and LEAH, my parents

in deep appreciation for all you have done for me.

AND

OBIORA, CHIEDU and NKIRU my brother and sisters,

with all my love.

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ABSTEACT

A strong correlation has been reported between the occurrence of gastroenteritis in man following the consumption of unpasteurized raw milk and the isolation of biotypically in distinguished Campylobacter fetus subspecies jejuni from the faeces of the herd supplying the milk. To date in Nigeria, virtually no work has been done to determine the role of cattle in milk contamination by C. fetus subspecies jejuni. This work was therefore designed to determine the prevalence of the organism in cattle faeces, milk and milk products. In addition, attempts were made to determine the presence of virulence markers of the isolates, determine their susceptibility to antimicrobial agents commonly used in Nigeria and study factors that affect their survival in milk and milk products.

A total of 1,000 rectal swabs and 300 raw milk samples were collected from cattle in randomly selected livestock,farms, Fulani herds and abattoirs. Also, 140 samples of locally fermented milk (nono) and local butter (maishanu) were purchased from Zaria markets in Zaria and it's environs. All tyese were cultured for Campylobacter fetus subspecies jejuni using Butzler's selective medium.

Eleven isolates (1.1%) were obtained from the rectal swabs. No isolations were made from any of the raw milk samples and the milk products. There was no statistically significant difference in isolation rates among animals from different sources ($P>0.05$, $X^2 = 1.36$). The low carriage rate observed in cattle faeces arid the absence of the organism in the milk and milk products suggest that cattle may not be a major souce of the organism in this area.

Enterotoxin production and typical epithelial cell invasive process were not demonstrable using rabbit ligated ileal loop test, infant mouse test and guinea pig conjunctivitis test. These results suggest that the isolates obtained may not be pathogenic.

The survival profiles of the isolated *C.* fetus subsp. jejuni were determined at selected storage temperatures at 4 °C, 25 °C and 42 °C and pH of 5, 7 and 8.5. These studies reveal that the test strains survived best in milk held at pH 7.0 at all temperatures, and survival at this pH was better at 4° C than at 25 °C. The viability of the test strains under all the test conditions indicated the possible danger of transmission of infection to individuals who consume milk and low acid milk products such as fermented milk products and cheese.

The antibiotic sensitivity tests showed that almost all the 11 isolates were sensitive to chloramphenicol, gentamicin, nalidixic acid, streptomycin, neomycin, doxycycline, tetracycline, and kanamycin. Those antibiotics may be recommended as drugs of choice for the treatment of Campylobacteriosis due to C. fetus subspecies jejuni.

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CHAPTER ONE

INTRODUCTION

Campylobacter fetus subspecies jejuni is now recognized as a leading cause of acute bacterial gastroenteritis in humans (Butzler and Skirrow, 1979). It has been associated with diarrhea in adult and young cattle (Jones et al., 1931), and epizootic abortion in sheep and cattle (Bokkonheuser and Mosenthal, 1981). It is also commonly found in normal, apparently healthy animals (Doyle, 1981, Sticht-Groh, 1981), and is often present in raw foods of animal origin (Simmons and Gibbs, 1979, Grant et al., 1980, Park et al., 1981). Several types of food including pork, poultry, ground beef and most commonly raw milk have been implicated as vehicles of outbreaks of Campylobacter enteritis (Doyle and Roman, 1982).

Reports from different countries associate outbreaks of Campylobacter enteritis with the distribution and consumption of improperly pasteurized cow's milk (Blaser et al., 1979, Taylor, et al., 1979, Robinson et al., 1979, Svedhem and Kaijser, 1980). In Britain, virtually all major outbreaks of Campylobacter enteritis have been associated with the distribution of improperly pasteurized cow's milk (Robinson and Jones, 1981).

Although there is no direct association yet of outbreaks of Campylobacter infection in man with the consumption of bovine milk, nevertheless, the strong correlation between the occurrence of symptoms and consumption of unpasteurized

milk and the isolation of biotypically indistinguishable organisms in the faeces of the milking herd suggests that milk was indeed the vehicle.

The exact mode of contamination of milk by these organisms has not been fully understood, but it is believed that the milk is probably contaminated with bovine faeces (Robinson, et al., 1989). This may be a possible route since the bovine intestinal tract is a known reserviour of C. fetus subspecies jejuni (Smibert, 1978).

In addition, Lander and Gill (1980) have shown that C. fetus subspecies jejuni may establish infection and multiply within the bovine udder subsequently resulting in the production of Campylobacter contaminated mastitic milk. Hence, the bovine udder may also serve as a source of C. fetus subspecies jejuni in milk.

These reports thus implicate C. fetus subspecies jejuni as the cause of enteritis following contamination of milk from animal source. It is also well established that raw milk and milk products pose the greatest human health problems in various countries as a vehicle of transmission of a large number of food-borne infections, even in countries where milk is sold only in pasteurized form (W.H.O. 1979).

Furthermore, it has been documented that the organisms survive in milk for varying lengths of time depending on the prevailing temperature and pH. At refrigeration temperatures and at the normal milk pH, the organisms

survive for as long as two weeks whereas they survive for only 3 days at 25°C (Blaser et al., 1980). The presence and possible persistence of Campylobacter fetus subspecies jejuni in milk indicates that under appropriate conditions large numbers of the organism may survive in raw milk. These further suggest that milk could serve as a vehicle of transmission of C. fetus subspecies jejuni infections.

Since C. fetus subspecies jejuni has been implicated as agents of foodborne diseases, it's public health significance cannot be overemphasized. If it is accepted that the organism is a primary cause of enteritis in man, and milk has been identified as a vehicle of many foodborne infections, then milk may be an important source of yet another human disease - Campylobacteriosis, This underlines the potential danger inherent in the consumption of unpasteurized milk.

To date in Nigeria, virtually no work has been done in this area yet, moreso, in the Northern States where unpasteurized milk contained in various milk diets such as skim milk (nono) Butter milk (nono mesami) Yoghurt (Kindirmu) Butter (Maishanu) and Cheese (Chukui) is widely consumed.

The mechanism of pathogenicity of C. fetus subsp. jejuni is still not well understood. Several hypothesis have been proposed based on clinical observations including suggestions that the organism can produce enterotoxins andthat it is invasive (Butzler and Skirrow, 1979). Those studies that have been reported

are preliminary, although a number of potential animal models have recently been described (Field et al., 1981, Prescott et al., 1981 and Ruiz Palacois et al., 1981).

The foregoing problems have therefore led to a study with the following specific objectives:

(i) Isolation of Campylobacter fetus subsp. jejuni from cattle rectal swabs and raw milk obtained from abattoir, government and local herds and from milk products purchased from markets in and around Zaria.

(ii) Determination of the presence of virulence markers in any isolates of C. fetus subsp. jejuni by employing animals.

(iii) Determination of the susceptibility of the isolates to some commonly used antimicrobial agents.

(iv) Determination of the effects of pH and temperature on the survival of C. fetus subsp. jejuni in sterile milk.

It is hoped that the pursuit of these objectives would help in understanding the incidence of the organism in cattle, possible mode of contamination of milk and milk products, the ability of these organism to survive in such products and highlight the pathogenic potentials of the isolates.

CHAPTER TWO

L I T E R A T U R E R E V I E W

HISTORICAL BACKGROUND

The disease potential of microaerophilic vibrios (Campylobacter fetus subspecies) was first recognized in Great Britain by McFadyean and Stockman who in 1913 reported that such organisms were associated with abortion in cattle and sheep. Their findings were corroborated by Smith and Taylor (1919) who isolated a spirillum from aborted bovine fetal fluid and named this organism Vibrio fetus. It soon became clear that the organism was responsible for epizootic abortion in sheep and cattle.

Jones et al., (1931) isolated a vibrio from cattle with intestinal disorders (winter scours). To definitively prove that such organism caused dysentery in cattle the authors reproduced the disease in healthy cattle after feeding them a pure culture of vibrio that had been isolated from diseased animals. These investigators judged that the jejunum was the first site in the intestinal tract to be infected; hence, they proposed that these vibrios be designated Vibrio jejuni.

Doyle (1944) isolated a microaerophilis Vibrio in apparently pure culture from the mucosa of the colon of hogs with dysentery. After feeding this culture to 8 pigs, 6 developed diarrhea. Doyle therefore proposed that swine dysentery was caused by microaerophilic vibrios which he subsequently named Vibrio coli.

The first putative cases of human infection due to microaerophilic vibrios were reported by Levy (1946). The infection occurred in two Illinios penal institutions where 357 of 6019 inmates were inflicted with acute gastroenteritis. "Vibrio-like" organisms were observed in 31 of 73 stool specimens that were examined, however, the organisms were not fully characterized before being lost during subculture.

The first case of human infection that was definitively attributed to a microaerophilic Vibrio was reported by Vinzent et al. (1947). These investigators isolated V. fetus from two blood cultures of a pregnant woman who was ill with influenza-like symptoms that included fever and headache. After five-week of illness, she prematurely delivered a stillborn infant, and a placenta that was infected with V. fetus.

Approximately ten years later Hofstad (1956) associated Vibrios with hepatitis in poultry when he reported the isolation of a Vibrio from the liver of diseased adult chickens. Peckham (1958) substantiated the fact that aimicroaerophilic Vibrio was the causative agent of this disease by reproducing hepatitis in healthy chickens after challenging them with a Vibrio that was originally isolated from the livers of diseased birds. He then proposed that this disease be designated avian vibrionic hepatitis.

In 1957, King reported the result of a study that was designed to compare the characteristics of

microaerophilic vibrios that were obtained from different sources. She observed that two distinct groups of vibrios were present among several isolates that were obtained from blood cultures from infected humans. The characteristics of one group corresponded with the then existing description of V. fetus. The characteristics of the second group were very similar to those of V. fetus except that their optimum temperature for growth was much higher than that of other isolates. Hence King designated this group of organisms as "related vibrios". Interestingly, she noted that all of the organisms in the group of "related" vibrios were isolated from blood cultures of patients with gastroenteritis and astutely speculated that these organisms were a more common cause of gastroenteritis than was recognized at that time (King, 1957; King, 1962). Very little could be done to confirm or disprove King's hypothesis since there was no method available to isolate "related" vibrios from stool specimens of patients with gastroenteritis. However, the first major break through needed to confirm King's theory came in 1972 when Dekeyser and others developed a procedure to selectively isolate microaerophilic vibrios from stool specimens. Shortly after this technique was developed, Butzler et al (1973) used it to screen the stools of a large number of patients with diarrhea to assess the occurrence of Campylobacters in this specific population of patients. Of 800 children and 100 adults with diarrhea, C. fetus subspecies jejuni was isolated from the stools

of 5.2% and 4.0% of each population, respectively. The organism was isolated from only 1.3% of 1000 individuals who did not have diarrhea and served as control group. No other reports concerning the enteropathogenic potential of C. fetus subspecies jejuni appeared in literature until 1977 when Skirrow (1977) reported the results of a study he had conducted using a more direct method to isolate C. fetus. Using this technique Skirrow assayed faecal specimens from 803 patients with diarrhea and 194 patients without. C. fetus subspecies jejuni was isolated from 57(7.1%) of the patients with diarrhea and none was isolated from those patients who did not have diarrhea.

Not only did Skirrow's study lend credence to the earlier observations made by Butzler et al. (1973) and support the hypothesis of King (1957 and 1962), it appeared to generate a substantial amount of interest to determine the disease potential of Campylobacters in humans.

CLASSIFICATION AND TAXONOMY

Campylobacter was originally assigned to the genus Vibrio by Smith and Taylor (1919) following their first description by McFadyean and Stockman in 1913. The type species was named V. fetus because they were associated with bovine and ovine abortions. They used the morphology of the organism as their primary criteria for classification.

In 1959 and 1960 Florent studied V. fetus and divided the species into two subsepcies, V. fetus venerealis and V. fetus intestinalis. V. fetus venerealis was the cause of vibrionic abortion in cattle, it was venereally transmitted, was hydrogen sulphide negative (using lead acetate strip) and did not grow in 1% glycine or in 3.5% sodium chloride. V. fetus intestinalis caused sporadic abortion in cattle (not considered vereally transmitted), was either H_2S negative or weakly positive and did not grow in 3.5% NaCl but did grow in 1% glycine.

Bryner et al. (1962) divided V. fetus into three biotypes. Type I was H_2S positive and grew in 1% bile but not in 1% glycine or 3.5% NaCl containing medium. Subtype I 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.

This classification (of Campylobacter in the genus Vibrio) stood until Sebald and Veron (1963) studied various Vibrios and other gram-negative rods. They found that the mol % guanine and cytosine DNA content for V. fetus averaged 34.3% (33.35%) while that for classical cholera and halophilic species of Vibrio averaged 47.2% (44-50%). It then became evident that V. fetus had a significantly different DNA base pair ratio from that of the classical species of Vibrio (Doyle, 1981). Furthermore, V. fetus had phenotypic differences from

other species of Vibrio which include the inability to ferment or oxidize carbohydrate and a requirement for microaerophilic conditions for growth. Most species of Vibrio can ferment carbohydrates and are facultative anaerobes (Smibert, 1974). Hence, Sebald and Veron (1963) concluded that V. fetus should be removed from the genus Vibrio and be reclassified as Campylobacter a name compounded from the Greek Campylo ("curved") and bacter ("rod").

The genus Campylobacter is divided into two groups according to the ability of the organisms to produce catalase. Catalase-negative campylobacters (C. sputorum, C. bubulus) are not known to be pathogenic for man. The catalase-positive group consists of C. fetus and a closely related set of organisms first recognized by King (1957) and referred to as "Related Campylobacter".

Veron and Chatelain (1973) reclassified C. fetus. C. fetus subsp. fetus was the same as the V. fetus intestinalis of Florent (1960); likewise, C. fetus venerealis was the same as Florent's V. fetus subsp. venerealis. C. fetus subsp. venerealis biotype intermedius is similar to subtype I of Bryner et al. (1962). C. coli is similar to V. coli described by Doyle (1944) and C. jejuni is similar to V. jejuni of Jones et al. (1931).

Smibert (1974) classified the genus Campylobacter as follows: C. fetus subspecies fetus which is the same as V. fetus subsp. venerealis of Florent. C. fetus subsp. intestinalis, the same as Florent's V. fetus subsp.

intestinalis; C. fetus subsp. jejuni represents the C. coli and C. jejuni of Veron and Chatelain (1973) and the "related Vibrios" of King (1957).

The different nomenclatures of classification caused confusion and until the publication of approved lists of bacterial names in 1980, there were two main classification of Campylobacter: the French (Veron and Chatelain, 1973) and the American (Smibert, 1974). Almost all strains associated with acute enteritis belong to the group with thermophilic characteristics first described by King (1957) and referred to by her as "related vibrios" (Skirrow and Benjamin, 1980). In the American classification all these strains were included in a single subspecies (Campylobacter fetus subsp. jejuni) whereas in the French and currently accepted classification, they are divided into two species: C. jejuni and C. coli (Skirrow and Benjamin, 1980). These are names derived from the Vibrio jejuni of Jones et al. (1931) isolated from calves and the V. coli of Doyle (1948) isolated from pigs.

THE ORGANISM

The genus Campylobacter consists of small micro-aerophilic oxidase-positive, gram negative non-sporing bacteria with a characteristic curved S-shaped or spiral morphology (Veron and Chatelain, 1973, Smibert, 1974). They are generally from 0.2-0.4um wide to 1.5-3.5um long (Doyle, 1981). Single cells have

one curve or twist and appear vibriod. They may also appear ribbon-shaped, being comprised of a chain or single curved cells (Smibert, 1978). Occasionally spiral morphology is not obvious and in some cases spindle shaped bacilli predominate (Butzler and Skirrow, 1979).

Morphological differences between groups of Campylobacter were first observed by Florent (1959) and later confirmed by King (1962) who noted that the undulations in "related Vibrios" tended to be closer together than those in "Vibrio fetus". These observations have been extended by Karmali et al. (1981) who measured the wavelength and amplitude of representative strains and confirmed that C. jejuni, C. fetus subsp. fetus and C. fetus subsp. venerealis may be distinguished from each other on morphological grounds by direct microscopy. C. fetus also differs from "Related Campylobacters" because it tends to have blunt rather than tapering poles. Furthermore, many cells are monotrichate rather than amphitricate when stained with a flagella stain or viewed with an electron microscope (Butzler and Skirrow, 1979).

Cells of old cultures become coccoid which appear to be a degenerative form as cultures composed mainly of coccoid forms are non-viable (Smibert, 1978). It has been reported that cultures of C. fetus subsp. jejuni undergo coccal transformation much more rapidly than cultures of C. fetus subsp. fetus or

C. fetus subsp. venerealis in normal atmospheric air (Karmali et al., 1981).

The organisms are actively motile, with a characteristic cork screw-like motion whereby cells quickly move across a microscopic field twirling or rotating in a spiral-like motion. Motility and rotation of the cells are so rapid that the curvation of the cells may be overlooked. They possess a long single polar flagellum two to three times the length of the cells. Some cells may have a polar flagellum at each end of the bacterium (Smibert, 1974, Smibert, 1978).

Cultural Characteristics

Non-haemolytic colonies about 1 to 1.5mm in diameter usually appear on agar 24 -72 hours after incubation at 37°C. C. fetus colonies undergo smooth and rough variations (Bryner et al., 1962). Bryner et al. (1962) described several colony types. Most strains were smooth on primary isolation from animals. Colonial variation proceeds from smooth colonies to smooth cut glass and to rough colonies. Smooth colonies, the most frequently found, are small round slightly raised, smooth colourless, and slightly translucent. Cut glass colonies are 1mm in diameter, round raised, transluscent and granular with reflecting facets. Rough colonies are rare and similar to smooth colonies with the exception of being granular and more opaque (Smibert, 1978).

"Related Campylobacters" colonies are typically flat, glossy and effuse with a tendency to spread along the direction of streak leading to confluent growth (Butzler and Skirrow, 1979). When well spaced out, they resemble droplets of fluid that have been splattered on the agar. Smibert (1965) described two types of colonies on primary isolation. One is low flat, grey finely granular and transluscent with an irregular edge. It spreads along the direction of streak and tends to coalesce. The other is round 1-2mm in diameter, raised convex entire smooth and glistening. It has a transluscent edge with a darker dirty brownish slightly opaque centre. With continued incubation, growth thickens and most strains develop a metallic sheen not unlike that seen on cultures of Pseudomonas aerugionsa (Skirrow and Benjamin, 1980).

Physiology and Nutrition

Organisms of the Campylobacter fetus group are rather limited in their metabolic capabilities. None of these organisms is able to ferment or oxidize carbohydrates. Fatty acids are not produced as end products of fermentation although small amounts of ethanol and n-propanol may be produced in cultures (King, 1957; Smibert, 1978). Kiggens and Plastridge (1958) reported that no oxygen uptake occurred with glucose, adonitol, arabinose, mannose, raffinose, rahmnose, salicin, sorbitol, sucrose, trehalose, xylose and glycerol. Glycolytic intermediates were not utilized either but

there was oxygen uptake of cells or cell extracts with pyruvate and lactate.

Physiological studies have shown that Tricarboxylic acid (T.C.A) cycle intermediates and amino acids serve as primary energy sources for these organisms. The T.C.A. enzymes have been demonstrated and an intact TCA is operative (Hoffman, 1978). C. fetus was found to oxidize citrate, cis-aconitase, isocitrate, α-ketoglutaric acid, succinate, fumarate, malate and oxaloacetate. Of 23 amino acids tested, oxygen uptake occurred with glutamic acid, glutamine, aspartic acid, asparagine, proline and cysteine. Of eleven short and long chain fatty acids tested, only acetate was oxidized (Smibert, 1978).

Strains of C. fetus vary considerably in their minimal nutritional requirements. The vitamin niacin is required by all; other vitamins are merely stimulatory (Mehlman and Romero, 1982). Smibert (1978) recognised five groups of C. fetus based on their nutritional requirements. One subgroup grows with four amino acids glutamine and aspartic acid, proline and leucine. A second group required methionine, in addition to those listed. A third group also requires alanine and arginine and the fourth and fifth groups require 17 and 18 amino acids respectively. Casamino broth which contains casamino acid, two amino acids three vitamins and three inorganic salts have been reported to be satisfactory for growth of Campylobacter when used individually or in conjunction with protose peptone No. 3 (Mehlman and Romero, 1982).

Purines and Pyrimidines are not required for growth but Tritz and Ogg (1967) reported that the addition of DNA and RNA to a chemically defined media resulted to a shorter lag time of the culture.

Biochemical Characteristics

Campylobacter fetus does not utilize carbohydrates as a source of energy and is thus relatively inert upon routine biochemical testing (Kiggens and Plastridge, 1958). This gram-negative organism which is identified by it's typical vibriod morphology and darting motility is strongly oxidase and catalase positive. They are capable of reducing nitrates to nitrites. Glutamic acid, glutamine aspartic acid and asparagine are deaminated and ammonia is produced in culture. They grow in semi-solid medium containing 1% bile but not in medium containing 3.5% soidum chloride. They also grow in semi-solid medium containing 0.12% - 0.2% agar when incubated under aerobic conditions. Growth in semi-solid medium occurs only within the first few millimeters below the surface of the medium. They do not possess lipase activity and cannot hydrolyse gelatin, urea, casein, ribonucleic acid, DNA and esculin. Generally, they do not produce an alkaline phosphatase and aryl sulphatase. Smibert (1978) however noted that some strains of C. fetus subsp. jejuni hydrolyse casein, ribonucleic acid and DNA and also produce alkaline phosphatase and aryl sulphatase.

C. fetus organisms are indole negative and do not produce hydrogen sulphide in Triple Sugar Iron (T.S.I) slant; there is only an alkaline reaction on this medium. Hydrogen sulphide is however produced in a medium containing cysteine with lead acetate impregnated paper strip as method of detection (Smibert, 1978).

Campylobacter fetus subsp. *jejuni* has been differentiated into two biotypes based on their ability to form hydrogen sulphide in an iron-containing medium (Skirrow and Benjamin, 1980). *C. fetus* subsp. *jejuni* biotype 2 produced H_2S in iron containing medium while the biotype 1 does not.

Currently, there are only two tests which are generally used to differentiate between *C. fetus* subsp. *jejuni* and subsp. *intestinalis*: ability to grow at 42°C or at 25°C and susceptibility to nalidixic acid (Smibert, 1978). *C. fetus* subsp. *jejuni* is characterized by growth at 42°C only and sensitivity to high concentrations of nalidixic acid while most strains of *C. fetus* subspl *intestinalis* will not grow at 42°C and show no zone of inhibition surrounding the disks (Smibert, 1978).

Organisms which show hippuricase activity are able to hydrolyse hippurate to benzoic acid and glycine. Hwang and Ederer (1975) described a 2½ hour rapid method for the detection of the end products glycine by use of the indicator ninhydrin. Recently, Harvery (1980) studied the use of this rapid hippurate hydrolysis test for

distinguishing between the Campylobacter species and subspecies. In her test, none of the 16 strains of C. fetus subsp. intestinalis, 5 strains of C. fetus subsp. fetus, 5 strains of C. fecalis or one strain of C. sputorum hydrolysed hippurate. In contrast, 18 of 20 strains (90%) of C. fetus subsp. jejuni hydrolysed hippurate. C. jejuni and C. coli have been divided into four biotypes according to nalidixic acid sensitivity, hippurate hydrolysis and hydrogen sulphide production in an iron containing medium (Skirrow and Benjamin, 1980). Thermophilic Campylobacter which are sensitive to nalidixic acid but fail to hydrolyse hippurate are classified as C. coli. Evidence from DNA homology studies to this point indicates that at least two different thermophilic Campylobacter species exist and that the hippurate hydrolysis test may be used to distinguish between these two species.

Analysis of fatty acid content by gas-liquid chromatography may be used to differentiate between subspecies of Campylobacter. A recent report on the cellular fatty acid composition of C. jejuni and C. fetus showed that strains of C. fetus subsp. jejuni contain a C19 cyclopropane acid which is not present in strains of other subspecies of C. fetus (Blaser et al., 1980). The C19 cyclopropane acid was identified as lactobacillic acid (cis-11, 12 methyleneoctadecanoic acid).

That sensitivity to 1mg of triphenyltetrazolium chloride (TTC) per millilitre may be another differentiating character was first suggested by Veron and Chatelain (1973). They found that none of the 18 strains of C. fetus subsp.

intestinalis grew on Blood Agar plates containing 1ml of TTC/ml, in contrast to 9 strains of C. coli and 1 strain of C. jejuni which grew on this medium. Butzler and Skirrow (1979) later recommended decreasing the concentration of TTC from 1mg/ml to 400ug/ml for this test (measured by the strip diffusion method). The usefulness of this test for differentiating strains within the C. coli and C. jejuni group was confirmed by Skirrow and Benjamin (1980).

The ability of strains to grow in the presence of 1% glycine may be used to differentiate members of the genus Campylobacter. C. fetus subsp. jejuni and C. fetus subsp. fetus have been reported to be tolerantto 1% glycine while C. fetus subsp. venerealis is unable to grow in the presence of 1% glycine (Veron and Chatelain, 1973). Chang and Ogg (1970) have however transduced glycine tolerance from C. fetus subsp. fetus to strains of C. fetus subsp. venerealis and suggest therefore that glycine tolerance may not be a reliable differential test.

Oxygen Requirement

Campylobacter fetus requires oxygen for growth, yet it is inhibited by oxygen at normal atmospheric partial pressure. Growth occurs at oxygen concentrations below 21% and even candle jar conditions may provide too much oxygen for some strains. This organism requires micro-aerophilic conditions for growth and optimum oxygen

concentration was determined to be 6% oxygen and optimum carbon dioxide concentration was 10% (Kiggens and Plastridge, 1956).

Luetchefeld et al. (1982) reported that an atmosphere of 5% oxygen and 8% carbon dioxide was superior to an atmosphere generated in a candle jar (17% O_2 and 3% CO_2). They found that there is statistically significant interaction or synergism occurring between incubation temperature and atmospheres. That is, the effect of the incubation temperature on the growth of Campylobacter was influenced by the particular atmosphere used and vice versa. Thus if only one of the two factors is suboptimal (i.e. use of 37°C with 5% oxygen or use of candle jar with 42°C), the incubation system is only slightly less effective than when optimal conditions are used (42°C with 5% oxygen). But if both factors are suboptimal (i.e. use of 37°C with a candle jar), then their interaction produces markedly inferior incubation conditions which yield strikingly lower Campylobacter isolation rates.

The aerotolerance of C. fetus could be increased by the addition of ferrous sulphate, sodium bisulphite, sodium pyruvate and haematin to the medium (Bowdre et al., 1974). The aerotolerance of a strain of Campylobacter was reported to be greatly increased by dihydroxyphenyl ferric iron binding compounds such as norephineprine or high concentration of iron salts (Ferric or ferrous) added to Brucella agar medium (Bowdre et al., 1976).

George et al. (1978) subsequently tested 18 laboratory strains of Campylobacter fetus subspecies jejuni grown on brucella agar without blood or on FBP agar (Brucella agar with added ferrous sulphate, sodium metabisulphite and sodium pyruvate). They found that all 18 strains grew on both media when the strains were incubated in an atmosphere of 6% oxygen at 17% oxygen all 18 strains grew on FBP but only 13 strains (72%) grew on Brufella agar.

When uninoculated Brucella broth medium is exposed to light and air, high energy radicals such as superoxide and peroxides are produced as a result of photochemical reduction of the medium. These high energy radicals in the medium inhibits growth of C. fetus. In addition, the organism is highly sensitive to hydrogen peroxide (H_2O_2). As little as 0.00124% H_2O_2 in the culture broth inhibits growth. Iron and bisulphite together act non-enzymatically to destroy superoxide radicals; whereas pyruvate can destroy H_2O_2. Hence, addition of FBP to the medium reduces the H_2O_2 toxicity by a factor of 10. With F.B.P in the medium 0.0124 H_2O_2 is required to inhibit C. fetus (Smibert, 1978).

ISOLATION OF THE ORGANISM

The isolation of Campylobacters require special selective technique because of their microaerophilic nature and the many contaminating microorganisms found in specimens containing them. The first successful technique was differential filteration of faecal suspension (Dekeyser et al., 1972). The technique depends on the

ability of Campylobacters to pass through a 0.65um filter; and it's particular advantage is that it can be used with non-inhibitory medium since very few bacteria can pass through such a filter. This method however proved too tedious and time consuming for the examination of large numbers of specimens. Subsequently, isolation by direct culture of faeces was achieved on a selective media incubated at 42°C - 43°C in a microaerobic atmosphere (Skirrow, 1977).

Skirrow's medium (Skirrow, 1977), Butzler's medium (Butzler and Skirrow, 1979) and Blaser's medium (Blaser et al., 1978) are the selective media most widely used for the isolation of Campylobacters from human faeces in the United Kingdom, Europe and the U.S.A., respectively. A modification of Blaser's medium called Campy-BAP (BBL) is also available and widely used in the USA.

Selective media currently used to isolate C. fetus subsp. jejuni consists of a basal medium, blood and an antibiotic mixture. Various workers have used different basal media in their formulations. Skirrow's medium contains Blood Agar Base No. 2 (Oxoid), Butzler's medium, thioglycoliate agar; Blaser's medium, Brucella Medium Base (Oxoid), Lander and Gills selective and enrichment medium contains Veal infusion Broth (Difco) and Preston's medium. Nutrient Broth No. (Oxoid) solidified with New Zealand Agar.

The selective media vary markedly in blood contents. Skirrow's medium contains 5-7% lysed horse blood. It is

essential to use lysed horse blood in Skirrow's medium in order to ensure neutralization of trimethoprim antagonists, otherwise Proteus spp. will grow (Butzler and Skirrow, 1979). Butzler's medium contains 15% defribinated whole sheep blood, Blaser's medium 10% defribinated whole sheep blood; Lander and Gilles medium 7% lysed horse blood and Preston's medium 5% lysed horse blood. The role of blood in Campylobacter media is uncertain but it does contain enzymes such as catalase, peroxodase and superoxide dismutase which decompose toxic oxygen derivatives (Pridovich, 1974). Studies by Bolton and Coates (1983) confirm that blood is an excellent supplement for Campylobacter medium. Lysed blood and whole blood were found equally beneficial and 1% was almost as effective as 5%. Media currently in use contain blood at concentration of 5-15% which appears unnecessarily high.

Different antibiotics have been used by various workers to ensure selectivity of the different media. These antibiotics are available commercially as freeze-dried antibiotic selective supplements. Skirrow's medium contains selective supplement SR 69 (Oxoid) which consists of Vancomycin (10mg/l), Polymyxin (2500iu/l) and Trimethoprim (5mg/l). This antibiotic supplement is designed to be used at 43°C for optimum selective effect. Butzler medium contains selective supplement SR 85 (Oxoid) which contains Bacitracin (2500 units/l), Cycloheximide (50mg/l) Colistin sulphate (10,00 units/l), Cephazolin sodium

(15mg/l) and Novobiocin (5mg/l). This supplement differs from SR 69 by it's selective action at 37°C. It therefore overcomes the need to use incubators at 43°C and it selectively isolates those strains of Campylobacter species that fail to grow at 43°C such as C. fetus intestinalis. The Blaser-Wang medium contains selective supplement SR 98 which is based on the formulation of Skirrow, but with the addition of Amphotericin B (2mg/l) and Cephalothin (15mg/l). The antibiotic supplement reduces the normal enteric flora without loss of C. fetus. The inclusion of Amphotericin B inhibits the growth of Candida albicans and Cephalothin improves the selectivity of the supplement.

Isolation of C. fetus from blood or body fluids presents no great problem once adequate microaerophilic conditions are established. However, contaminating microorganisms are a problem when isolating Campylobacter species from faeces, intestines, prepuce, semen, vagina, oral cavity and foods (Smibert, 1978). Isolation of this organism from such specimens is best achieved on selective agar. Direct plating of specimens onto antibiotic containing blood agar plates is limited by the fact that only a small amount of sample can be surface plated and solid specimens must be suspended in liquid and homogenized thereby diluting the Campylobacters which may be present (Doyle and Roman, 1982).

Most of the currently accepted methods of isolation were developed to recover C. fetus subsp. jejuni from

stool specimens where the organism is present in relatively large numbers (Butzler et al., 1973; Butzler and Skirrow, 1979). Foods that contain small numbers of the organism may require enrichment in an enrichment broth prior to subculturing on selective agar. Several types of enrichments have been developed for recovering Campylobacters from foods and faeces, however those that have been proposed have limitations in that they involve an excessive amount of time and manipulations or are not effective in recovering small numbers of Campylobacters (Blaser et al., 1979; Park et al., 1981). Most recently Doyle and Roman (1982) introduced a selective, rapid, direct and more effective enrichment procedure for the recovery of C. jejuni and C. coli from foods. The methods involved incubation of food in an enrichment broth compound of Brucella broth with horse blood, sodium succinate, cysteine hydrochloride and four antibiotics for 16 to 18 hours with agitation under microaerobic condition at 42°C and with subsequent plating on Campy-BAP agar. Effectiveness of recovery as good as 0.1 cells of Campylobacter species/gram of food was reported, and isolates were recovered from hamburger and raw milk.

With all media, plates for the isolation of Campylobacter must be incubated under microaerophilic conditions. Different methods have been devised to provide this microaerophilic condition. The most common procedure include candle jar, evacuation of two-thirds of the air from an anaerobic jar and replacement with

10% carbon dioxide/85% nitrogen or hydrogen mixture, gas mixture replacement and the application of the Fortner principle (Karmali and Fleming, 1979). An iron containing supplement (Oxoid SR 84) which increased aerotolerance of Campylobacters and which may make it possible to obtain successful isolation in a candle jar (17% oxygen) has been described (George et al., 1978). Recently commercial gas generating envelope system (BBL Microbiology System and Oxoid) have been developed to produce reduced oxygen atmosphere. Chan and McKenzie (1982) have suggested the inclusion of a blood agar plate streaked with Pseudomonas aeruginosa, Clostridium perfringens and a laboratory strain of Campylobacter fetus subsp. jejuni as a control system for the atmosphere. This simple and inexpensive procedure will indicate that the proper atmosphere to culture C. fetus subsp. jejuni has been achieved in the jar as all three of these organisms grew well in the microaerophilic atmosphere at 42°C.

RESERVIORS, SOURCES AND TRANSMISSION OF Campylobacter fetus subsp. jejuni INFECTION

Despite considerable documentation of human infections with C. fetus subsp. jejuni the epidemiolgy is still not well understood. An animal source has been suspected for human infections. This suspicion stems from the fact that this organism has a wide spread reservior in a variety of domestic and wild animals, both as pathogen and as normal microbial flora (Smibert, 1978; Butzler and

Skirrow, 1979; Prescott and Munroe, 1982). Man is a relatively unimportant secondary reservior either for direct or indirect transmission. No long term carrier has been described as Campylobacter disappear from the stool within a few weeks of illness, and transmission from person to person is uncommon unless the index patient is a young child (Skirrow, 1983). Secondary cases in common-source outbreaks are usually notable for their absence.

A wide variety of animals both wild and domestic can habour C. fetus subsp. jejuni from kangaroos to camels among mammals to Blue-crowned motmots among birds (Leutchefeld et al., 1981). Wild birds constitute an extensive reservior of C. fetus subsp. jejuni (Butzler and Skirrow, 1979; Kapperud and Roself, 1983). The organism has been isolated from black birds, startlings, sparrows and pigeons (Smibert, 1969).

The animals of special importance as sources of human infection are probably poultry, cattle, sheep, pigs, and dogs. Infections can be acquired through direct contact with infected animals but this accounts for only a small minority of infections. Contact may be occupational as in farmers, veterinarians, and those engaged in meat processing (Jones and Robinson, 1981); or domestic in which case the animal concerned is almost always a newly acquired family puppy or occasionally a kitten which is itself suffering from Campylobacter enteritis (Skirrow, 1981). An association

between infected dogs and human infection has been documented. There is now clear evidence that dogs suffer from Campylobacter enteritis and that they may pass on the infection to close human contacts (Butzler and Skirrow, 1979). However, most day-to-day infections are in patients without any direct contact with animals, so indirect pathways of infection presumably operate in these cases (Skirrow, 1982). Although information is lacking, epidemiological data have provided strong evidence that animals and foods of animal origin are the main reserviors of human infections (Skirrow, 1982).

Raw bovine milk has unquestionably been incriminated as a vehicle of infection (Robinson, et al., 1979; Taylor, et al., 1979; Robinson and Jones, 1981, Skirrow, 1982). In Britain, virtually all major outbreaks of campylobacter enteritis have been associated with the distribution of raw or improperly pasteurized cow's milk (Robinson and Jones, 1981).

Eviscerated carcasses at slaughter are frequently contaminated (Stern, 1981; Bolton et al., 1982). Contaminated meat and offal also serve as vehicles of infection. Infection may result if the contaminated product itself is eaten raw or uncooked, or the handler may become self-infected through handling the raw product or other foods that are to be eaten raw or without further cooking may become cross-contaminated from the raw product (Skirrow, 1982). Red meat are in general much less frequently contaminated because

although Campylobacters are regul· ly found on abattoir carcasses (Bolton et al., 1982), contamination falls to low levels at the time meat reach retail outlets (Turnbull and Rose, 1982). Pigs however may be an important source of infection in certain European countries where salted, smoked and rare-cooked pork and offal are popular foods. High contamination rates for such products have been recorded (Stich-Groh, 1982).

Poultry constitutes by far the largest potential source of Campylobacters to humans but not necessarily the main source (Skirrow, 1982). Intestinal carriage of C. fetus subsp. jejuni is common (Blaser, 1982). Additionally, the organism can be cultured from most chicken and turkey carcasses sold at retail outlets in several countries (Simmons and Gibbs, 1979; Grant, et al., 1980; Svedhen et al., 1981, Park et al., 1981). Outbreaks of human campylobacteriosis associated with consumption of undercook chicken have been reported (Blaser, 1982). It has been found that si,nificantly more patients with campylobacter enteritis had eaten poultry particularly broiler chickens within 48 hours of the onset of illness (Kist, 1982).

The fact that poultry and cattle isolates have been reported to share characteristics of human isolates of enteropathogenic C. fetus subsp. jejuni further supports the evidence that poultry has a role to play in transmission of human campylobacteriosis.

INCIDENCE OF *Campylobacter fetus* subsp. *jejuni* IN ANIMALS AND MAN

Campylobacter fetus subsp. *jejuni* is much more commonly associated with human diseases unlike other subspecies of the Genus. This may be attributed to the fact that it occurs more widely in nature than the other subspecies (Doyle, 1981). Besides being present in infected animals, the organism is often part of the normal intestinal flora of pigs, cattle, sheep, goats, poultry and several species of wild birds (Smibert, 1969; Smibert, 1978). As these animals mature, smaller numbers of *C. fetus* subsp. *jejuni* are generally present as part of their intestinal flora.

Several studies have been conducted to determine what portion of different animal populations habour the organism as part of their intestinal flora. Among domestic livestock, varying faecal carriage rates have been reported from poultry, cattle, sheep and pigs (Butzler and Skirrow, 1979; Prescott and Munroe, 1982). Isolation rates for *C. fetus* subsp. *jejuni* from processed chicken that range from 1.7-83% and in the faeces of birds ranging from 30-100% have been reported (Leuchtefeld *et al.*, 1980; Blaser, 1982). The organism has been reported to cause vibrionic hepatitis in chickens (Truscott and Stockdole, 1966) and it has been recovered from the bile of 21% chicken with necrotizing hepatitis and 2% of normal controls. Among wild birds the organism has been isolated from blackbirds, starlings

sparrows and pigeons (Smibert, 1969). A recent study shows that migratory water-fowl commonly shed the organisms in their faeces. Thirty-five percent isolation from faecal content of these waterfowls was reported (Leuchtefeld et al., 1980). Twenty six percent of pigeons trapped in the Denver Zoological Garden (USA), premises were found to carry C. fetus subsp. jejuni. The high body temperature (42°C) of the birds have been suggested to encourage carriage of this organism whose optimum temperature for growth is 42°C (Leuchtefeld et al., 1980).

Isolation rates of 0% of 31 cattle (Stern, 1981), 2.5% of 202 cattle (Prescot and Bruin-Mosch, 1981) and 19% of 90 cattle (Svedhem and Kaijser, 1980) have been reported. In Britain, Robinson (1982) found a marked seasonal variation in faecal carriage among lactating cows. C. fetus subsp. jejuni could be isolated from 10% of each herd in the summer declining to 0% in winter and re-emerging in spring.

Pets, such as dogs (Blaser et al., 1978) and cats (Blaser et al., 1980) have been identified as sources of C. fetus subsp. jejuni. Recent surveys of dogs and cats from kennel sources indicate that significant numbers of cats and dogs can shed the organism in their faeces (Fernie and Park, 1977, Blaser et al., 1980, Fox, 1982). Patton et al. (1981) reported that the positive rate for dogs was twice as high as that for cats.

Campylobacter enteritis is as common an infection as salmonellosis and shigellosis put together. In developed countries, C. fetus subsp. jejuni is recovered from 4-7% of

patients with diarrhea and from 0-1% of normal individuals (Bokkenheuser and Mosenthal, 1981). The corresponding figures for developing areas are 8-31% and 5-17% respectively.

ASSOCIATION OF MILK WITH C. FETUS SUBSP. JEJUNI INFECTION

Campylobacter enteritis was first suspected as being attributed to the consumption of milk when Levy (1946) described an outbreak of diarrheal illness in a closed population of inmates of an Illinois (USA) prison. An epidemiological investigation implicated unpasteurized bovine milk as the source of infection. Gram stain of stool and blood cultures of affected patients demonstrated vibrio forms. However the organisms were not fully characterized before being lost during subculturing.

Blaser et al. (1978) reported that three members of a family in Colorado (USA) had gastroenteritis following the ingestion of raw milk from their cow. Stool cultures from all these three patients and cow yielded C. fetus subsp. jejuni. Also Taylor et al. (1979) reported three cases of campylobacter enteritis in California, USA, associated with consumption of commercially available certified raw milk.

In Europe, the first milk-associated outbreak of Campylobacter enteritis was recorded in Somerset during the blizzards of 1977-1978. An outbreak occurred after the consumption of milk that had been collected and distributed under extremely difficult conditions (Pether, 1978). Subsequently, Robinson et al. (1979) described an outbreak of enteritis in the north of England that was traced to consumption of unpasteurized milk. Examination of rectal swabs from the cattle concerned and milk socks yielded

strains of thermophilic Campylobacter that were indistinguishable from those strains isolated from the patients. In addition it has been shown that many people who habitually drink raw milk have campylobacter antibody in their body (Jones et al., 1981).

An outbreak of campylobacter enteritis occurred in South Kincardineshire and North Angus, Scotland during extreme weather conditions which resulted to a failure in the public electricity supply. As a direct consequence the pasteurization plant at a large dairy was out of action and the milk distributed was unpasteurized illustrating again the association of an outbreak with some sort of breakdown in the usual hygienic routine. C. fetus subsp. jejuni was isolated from stools of 143 patients with symptoms and 57 symptomless subjects all of whom had consumed the milk. The organism was also isolated from milk socks but not from any of the bulk milk samples taken daily for seven days after the outbreak.

Campylobacter fetus subsp. jejuni has been isolated only once from incriminated milk (McNaughton et al., 1982). Perhaps the difficulty is due to low concentrations of the organisms in raw milk. Even low concentrations of the organisms in raw milk represent a health hazard (Robinson et al., 1981), and are likely to survive in raw milk long enough to reach consumers (Blaser et al., 1980, Barrell, 1981; Christopher et al., 1982). Attempts by many workers (Taylor et al., 1979, Porter and Reid, 1980) to isolate C. fetus subsp. jejuni from bovine milk have been unsuccessful. However, the organism was isolated from 1 of 108 (0.9%) milk

samples obtained from the bulk tank of nine grade A dairy farms and from 50 of 78 (64%) cows producing grade A milk. Similarly Lovett et al. (1983) reported isolation of 3 C. jejuni isolates from bulk milk tanks from 195 separate farms yielding an incidence rate of 1.5%. These studies indicate that the incidence of C. fetus subsp. jejuni in farm bulk milk is at a level of 1 to 2%.

Robinson (1981) has recently shown the human infective dose of C. fetus subsp. jejuni to be very low. He infected himself and developed symptoms of Campylobacter enteritis after ingesting 180ml of milk containing 500 cells of strain of C. fetus subsp. jejuni. This study indicated that as few as 2 to 3 cells/ml of milk can infect an individual who consumes 240ml serving of contimaminated milk.

The exact mode of contamination of milk by these organisms has not been fully understood; but Robinson et al. (1979) postulated that the milk was probably contaminated with bovine faeces. This may be a possible route since cattle frequently carry C. fetus subsp. jejuni in their intestinal floral (Smibert, 1978). The likelihood of such contamination occurring is clearly greater where there has been an outbreak of scouring in the herd associated with a breakdown in dairy hygiene (Porter and Reid, 1980). Taylor et al. (1979) s suggests that contamination of milk occurred either by placental transfer, contamination by faecal flora or transmission from infected udder. Although there is no reported case of clinical mastitis due to Campylobacter species, Lander and Gill (1980) suggests that infected bovine

udder could provide a pure and abundant source of C. fetus subsp. jejuni in milk. Furthermore, these workers suggested that the sequence of events following experimental inoculation indicate that the udder could excrete quite large numbers of organisms before there were any obvious signs of disease in the cow. Even at the time of greatest excretion of bacteria the milk itself was not grossly abnormal. They postulated that; if mild cases occur, the sudden onset and fairly rapid resolution of the mastitis could go unnoticed altogether, particularly with modern highly mechanized and streamlined routines.

Pathogenicity of Campylobacter fetus subspecies jejuni in Animals and Man.

Each subspecies of Campylobacter fetus has characteristic reservoirs and is responsible for specific types of diseases. C. fetus subsp. fetus is pathogenic only in cattle in which it causes a world-wide venereal disease characterized by endometritis, infertility and occasionally abortion (Bokkenheuser and Mosenthal, 1981). The organism has never been identified as being an agent responsible for diseases in human (Smibert, 1978). C. fetus subsp. intestinalis is pathogenic to both animals and humans beings. It causes gastroenteritis and abortion in sheep and less often in cattle. In humans it is primarily responsible for systemic Campylobacteriosis. The most common clinical manifestation of this syndrome is bacteremia without localized infection (Kahler and Sheldon 1960, Guerrant et al, 1978); and

occasionally diarrhea (Bokkenheuser and Mosenthal, 1981).

C. fetus subsp. jejuni has a wide spread host range among animals and birds in which it may be present as a part of the normal intestinal flora or as a pathogen. Like C. fetus subsp. intestinalis; C. fetus subsp. jejuni is a human pathogen; however, C. fetus subsp. jejuni is much more commonly associated with human diseases than C. fetus subsp. intestinalis. Diseases produced by C. fetus subsp. jejuni include abortion in sheep (Telfer-Brunton and Heggie, 1977; hepatitis in poultry (Hofstad, 1956), dysentery in cattle (Jones et al., 1931 ; urinary tnfections in humans (Davis and Penfold, 1979) and acute gastroenteritis in humans (Butzler et al., 1973, Skirrow, 1977), monkeys (Tribe and Frank, 1930); cats (Svedhem and Norkrans, 1980) and dogs (Blaser et al., 1978 and Hastings, 1978).

C. fetus subspecies jejuni is primarily responsible for enteric campylobacteriosis and it has been reported that more than 99% of the cases of Campylobacter enteritis are due to C. fetus subsp. jejuni (Lauwers et al., 1978; Bokkenheuser et al., 1979). The symptoms associated with Campylobacter enteritis vary widely in severity. The clinical outcome of an infection is probably determined by the infecting dose, the virulence of the organism and the resistance of the host. The younger members of a species seem to be more susceptible to pathological manifestations (Bokkenheuser and Mosenthal, 1981). The major clinical manifestations are fever, abdominal pain that is often severe and diarrhea that is usually bloody (Skirrow, 1977, Blaser et al., 1979, Butzler and Skirrow, 1979;

Karmali and Fleming, 1979). Diarrhea usually occurs at the onset of abdominal pain and fever. Typically, the diarrhea is mild to moderate but may be profuse, watery and frequently in it's severest form, there is an acute gastroenteritis, with as many as twenty stools per day (Willoughby et al., 1980), mimicking ulcerative colitis or Crohn's disease (Michalak et al., 1980). Other symptoms that may be expressed include malaise, headache, musculoskeletal pain, rigor and delirium (Skirrow, 1977; Blaser et al., 1978; Karmali and Fleming, 1979). Although vomiting may occur, it is not common and is normally observed in less than 30% of the cases (Doyle, 1981). The severity of the illness is quite variable but in most cases it is brief and self-limited (Rettig, 1979; Karmali and Fleming, 1979).

From circumstantial evidence it was estimated that the incubation period of campylobacter enteritis ranges from 2 to 11 days. Other investigators suggest that a typical incubation period is 2 to 5 days (Prescott and Karmali, 1978, Blaser et al. 1978, Karmali and Fleming, 1979).

The duration of C. fetus subsp. jejuni excretion following spontaneous clinical recovery is uncertain. The faeces of patients who are not given chemotherapy usually remain positive for about two to five weeks after an attack of Campylobacter enteritis (Butzler and Skirrow, 1979). Karmali and Fleming (1979) found that 24 untreated children with acute C. fetus subsp. jejuni enteritis ceased to excrete the organism within 4 to 7 weeks. In another study, Richardson et al., (1981) showed that of 73 apparently healthy school children examined every three months,

three excreted C. fetus subsp. jejuni in their faeces for more than a year. Mild cases may excrete the organism for only a few days, and there is always the odd patient who excretes for much longer period (Butzler and Skirrow, 1979).

Mechanism of Pathogenicity of Campylobacter fetus subsp. jejuni

Little work has been done to uncover the potential pathogenic mechanisms of disease of C. fetus subsp. jejuni. This is because there is no suitable animal model. Only a few animals have been found susceptible to experimental infections but even when success has been reported by one group of workers others have failed to confirm it (Butzler and Skirrow, 1979). Monkeys (Butzler and Skirrow, 1979; Tribe and Frank, 1980); puppies and cats (Prescot and Barker, 1980); Mice (Madge, 1980) and chickens (Butzler and Skirrow, 1979; Ruiz-Palacias et al., 1981), have been tested without uniform result.

Well characterized enteric diseases result primarily from toxin production or penetration and ulceration of the gut mucosa or both even though other factors may contribute to the pathogenic process. All enteric pathogens must attach and such factors as motility, the presence of pili, the elaboration of mucinase and other factors, play a role in their ability to colonize. The relative importance of one or more of these capabilities has not been established for C. fetus subspecies jejuni (Field et al., 1981).

Several hypotheses have been proposed for the pathogenesis of this disease based on clinical observations. These include suggestions that the organism can produce exterotoxin and is

invasive (Butzler and Skirrow, 1979). The role of toxins in the pathogenecity of campylobacter enteritis has not been determined. Results have shown that culture supernatant filtrates failed to stimulate fluid accumulation in ligated ileal loop of rabbits, calves and piglets, and failed to cause morophological changes of a cholera-toxin like or cytotoxic effect in cultural chinese hamster ovary (CHO) cells to which culture supernatants in which the pathogens grew were added (Guerrant et al., 1978; Butzler and Skirrow, 1979; Manninen et al., 1982). Both of these effects are elicited by Cholera toxin and the heat labile toxin of Escherichia coli. However, a study of the pathogenesis of the colitis seen in gnotobiotic dogs infected with C. jejuni suggests that a cytotoxin of some type was involved with the production of intestinal epithelial damage (Prescott et al., 1981).

The infant mouse test is reported to be ~~...~~ reliable and reproducible for the detection of E. coli heat stable enterotoxin (Giannella, 1976). However, only 16 of 100 cultures of C. fetus subsp. jejuni were positive when tested for a heat-stable toxin in infant mice (Butzler and Skirrow, 1979). Other workers have reported that C. fetus subsp. jejuni failed to produce heat-stable enterotoxin by the infant mouse test (Guerrant et al., 1978; Manninen et al., 1982). On the basis of these findings, production of either a cholera-like heat-labile enterotoxin or a heat-stable enterotoxin analogous to those produced by V. cholerae, noncholera Vibrios and certain strains of E. coli does not seem to make a major contribution to the pathogenesis of Campylobacter enteritis.

Invasiveness by Campylobacters is considered by some authors (Butzler and Skirrow, 1979) to be a requisite virulence factor for establishing enteritis. This concept is supported by the appearance of bloody diarrhea, bacteremia, cholecystitis, ulceration of the gut in infected human patients, development of high antibody titres after infection and the recovery of the organism from peripheral blood in the acute phase of the disease. Moreover, chicken embryo cells in culture can be invaded within 24 hours after the addition of Campylobacters (Field et al., 1981). However, attempts to reproduce diarrhea in various laboratory animal species have not been successful in many instances. Recent experiments using 3-day old chickens infected with C. jejuni detected Campylobacter within epithelial cells and mononuclear cells of the lamina propria by immunofluorescence and electron microscopy. Diarrhea accompanied by weight loss was observed in 88% of the birds (Ruiz-Palacios et al., 1981). Manninen et al., (1982) however failed to induce diarrhea in 3-day old birds. All the birds appeared healthy and no gross morphological changes were noted at necropsy. Butzler and Skirrow (1979) also failed to induce diarrhea in older (8-day old) chickens although Campylobacter was observed in epithelial cells of the caecum, blood and liver and was recovered from faeces of all inoculated birds. Several investigators have attempted to reproduce the disease in non human primates. Two of three rhesus monkeys challenged with 10^6 or more organisms developed severe enteritis but not when challenged with 10^4 or 10^2 microorganisms. In a second laboratory, a group of four adult rhesus monkeys inoculated per os with 10^7 related campylobacter

from a human, failed to develop diarrhea (Butzler and Skirrow, 1979). Recently, eight young rhesus monkeys (Macaca mulatta) were infected orally with a human strain of C. jejuni (Fitzgeorge et al., 1981). The disease induced was mild with inappetence and diarrhea of short duration but prolonged, intermittent excretion of the bacteria in the faeces occurred. The underlying mechanism by which Campylobacter seemingly induced diarrhea in some animals while other animals of the same species shed the organism in the faeces and remain asymptomatic is unknown.

Results of the HeLa cell association studies have shown that the ability to associate is not a general property of C. fetus subsp. jejuni (Manninen et al., 1982). In addition, the inability of C. fetus subsp. jejuni in several studies Guerrant et al., 1978, Manninen et al., 1982) to produce keratoconjunctivitis when inoculated into the conjunctival sac of guinea pigs excludes a typical epithelial cells invasive process. These data therefore separate the pathogenesis of Campylobacteriosis from that of shigellosis and certain invasive E. coli.

The absence of enterotoxigenicity, focal epithelial cell destruction or inflamatory invasion, are reminiscent of yet another type of enteric infection such as described for salmonellosis and yersiniosis by Cater and Collins (1978). The clinical syndrome of enteric symptoms and bacteremia sometimes seen with yersiniosis and salmonellosis in human subjects is similar to the clinical syndrome of campylobacteriosis. There is now growing evidence that related campylobacters are

pathogenic mainly by virtue of a direct invasive ability.

Antimicrobial Susceptibility of Campylobacter fetus subsp. jejuni

A number of studies have been conducted on the susceptibility of Campylobacter fetus subsp. jejuni to antimicrobial agents. The available information on the antimicrobial sensitivity of C. fetus subsp. jejuni differs somewhat from different countries (Plastridge et al., 1964, Butzler et al., 1974, Chow et al., 1978, Svedhem et al., 1981, Vanhoof et al., 1982 and Walder, 1982).

Antibiogram studies have demonstrated that the organisms is most susceptible to the aminoglycosides, erythromycin, doxycycline, chloroamphenicol and the tetracyclines and resistant to polymyxin B and bacitracin (Plastridge et al., 1964, Butzler et al., 1974, Vanhoof et al., 1978). Among the aminoglycosides, gentamicin has been found to be the most active but 8.4% of the isolates in Vanhoof's series were resistant to both drugs, while lincomycin was inactive. Of the Tetracyclines, Minocycline was the most active in Vanhoof's series but 5% of the isolates were highly resistant. All isolates were reasonably sensitive to Chloramphenicol. The penicillins showed little activity; ampicillin was moderately active against most isolates, penicillin G less so and cloxacillin totally inactive.

Data on the in-vitro antimicrobial susceptibility of clinica isolates of C. fetus reported by Chow et al., (1978) were in general agreement with those reported by Butzler et al., (1974). C. fetus was relatively resistant to penicillin and all cephalosporins tested. Ampicillin, Carbenicillin and ticarcillin were considerably more active and except for one strain highly resistant to ampicillin, all were inhibited by readily

achievable serum concentrations of these antibiotics. All strains of C. fetus studied, were found to be resistant to Vancomycin. In contrast, C. fetus was exquisitely susceptible to the tetracyclines, gentamicin and amikacin. All strains of C. fetus studied were inhibited by 1.6ug/ml of tetracycline, doxycycline and minocycline, 0.8ug/ml of gentamicin and 1.6ug/ml of amikacin. All the strains tested were inhibited at concentration of clindamycin, chloramphenicol, metranidazole and kanamycin achievable in serum but were relatively resistant to lincomycin and erythromycin.

The consistent in-vitro resistance of penicillin reported is in keeping with frequent reports of therepeutic failure with this agent in the treatment of C. fetus infection (Eden, 1962). The unique susceptilility of C. fetus to tetracycline and gentamicin reported is consonant with the generally favourable response to tetracycline therapy previously reported (Mandel and Ellison, 1963). Gentamicin has also been recommended by some as the drug of choice for severe C. fetus infection (Butzler et al., 1974); however existing clinical experience with this agent is too scanty to permit adequate evaluation of it's in-vitro efficacy. Results of therapy with clindamycin and chloramphenicol have been reported to be variable (Targan et al., 1976).

The unique activity of dimetridazole, an analog of metronidazole against strains of C. fetus have been reported (Fernie et al., 1977, Chow et al., 1978). Until recently, metronidazole and other nitroimidazole derivatives were considered to be active only against obligate anaerobes

(Tally et al., 1975), probably serving as specific electron acceptors for reduced ferredoxin, thus interferring with electron transfer in the phosphoroclastic reaction essential for survival of obligate anaerobes (O'Brien and Morris, 1972; Edwards et al., 1973). Hence the demonstration of inhibition of C. fetus by metranidazole suggests that an alternative mechanism of action effective against microaerophilic bacteria doed exist.

There is only a scanty knowledge about strains isolated from animals especially domestic animals such as dogs and chickens which constitute a possible source of infections (Simmons and Gibbs, 1979; Svedhem and Kaijser, 1980). The antimicrobial susceptibility of C. fetus subsp. jejuni obtained from different patients with diarrhea and from different healthy chickens was investigated using 18 antimicrobials (Svedhem et al., 1981). The bacteria were found to be especially sensitive to carbenicillin, penicillin G, gentamicin, chloramphenicol, nalidixic acid, clindamycin, erythromycin and doxycycline. There was no significant difference between the results obtained with the human and chicken strains, a fact which further strenghtens the idea of animals as source of human infections. Similarly, Vanhoof et al. (1982) reported that in general the minimal inhibitory concentration (MIC) distribution for the animal strain were very comparable with those of the human strains.

Recently, transmissible plasmids from C. fetus subsp. jejuni have been described (Taylor et al., 1981). Different studies suggest that animals can act as a reserviour of bacteria

habouring antibiotic resistance plasmids which could be transferred to human (Marsik et al., 1975; Saida et al., 1981). Hence increased level of antibiotic resistance in campylobacters from animals may represent a human health concern.

EFFECT OF TEMPERATURE AND pH ON THE SURVIVAL OF CAMPYLOBACTER FETUS SUBSPECIES JEJUNI

It has been reported that C. fetus subspecies jejuni inoculated into sterile milk survived better at 4°C than at 25°C (Blaser et al., 1980). At 4°C, C. fetus subsp. jejuni remained viable for as long as 22 days whereas no viable organism could be detected after 3 days at 25°C. Similar observations were made by Christopher et al. (1982) who reported an approximately four $\log_{10}$ decrease in cell population in sterile skim milk at 14 days at 1°C and a 5 to 6-$\log_{10}$ decrease after 2.5 days at 20°C. The results of these studies indicate that strains of C. fetus subsp. jejuni if present in milk could survive at commercial refrigeration temperatures for at least two weeks with the length of time depending on the strains involved and level of contamination.

Berrell (1981) reported that survival profiles of different strains of C. fetus subsp. jejuni in unpasteurized milk held at 4°C or 21°C. He observed that four of the seven strains studied were recoverable after 48 hours of refrigeration temperature and that only two of seven strains were recoverable after 48 hours at 21°C. One strain was still detected after 5 days. Similarly, Doyle and Roman (1982) reported that the survival of C. fetus subsp. jejuni in unpasteurized milk held at 4°C varied with the strains. They observed that the most

tolerant strains showed a 2 $\log_{10}$ decrease in viable cells after 14 days and the most sensitive showed a 5-log decrease after 7 days. This indicates that under appropriate conditions large numbers of Campylobacter may survive in raw milk.

Inactivation of C. fetus subsp. jejuni strains in unpasteurized milk held at 4°C paralleled but was greater than inactivation of strains in sterile milk (Doyle and Roman, 1982) suggesting that the psychrophilic microflora in raw milk may have produced metabolites toxic to C. fetus subsp. jejuni. Alternatively, unlike sterile milk, raw milk contains lactoperoxidase, which in combination with hydrogen peroxide produces metabolites that are bactericidal to many gram-negative bacteria. The activity of the lactoperoxidase system may at least partially explain why the Campylobacter strains were inactivated to a greater degree in raw milk than in sterile milk. It is believed that the hydrogen peroxide produced by bacteria growing in raw milk increases the generation of lactoperoxidase-mediated metabolites toxic to Campylobacter strains.

It has been reported that the inactivation of different strains in unpasteurized milk corresponded with an increase in milk aerobic plate count (A.P.C) and a decrease in milk pH (Doyle and Roman, 1982). However no absolute correlation has been made between the rate of change in milk Aerobic Plate Count (A.P.C.) or pH and the rate of Campylobacter inactivation.

The optimum pH for growth of strains of C. fetus subsp. jejuni has been found to be in the range of 6.5-7.5

(Doyle and Roman, 1981). None of the strains studied could grow at pH 4.7 and only one strain grew at pH 4.9. This suggests that C. fetus subsp. jejuni cannot grow in high acid foods.

It is interesting to note the ability of C. fetus subsp. jejuni to survive in response to temperature and pH. At 42°C which is within the range of the optimum temperatures for growth, the organism was inactivated more rapidly in a medium adjusted to a pH that is inhibitory to growth than in a medium having a comparable pH but incubated at 25°C. For instance, at pH 4.5 a 3-$\log_{10}$ decrease of cell population occurred within 8 hours when incubated at 4°C (Doyle and Roman, 1981). Similarly, in a medium having the same pH, C. fetus subsp. jejuni was inactivated more rapidly at 25°C than at 4°C. At low pH value such as 3 to 3.5, the organism was readily inactivated at all temperatures including 4°C.

The presence and possible persistence of C. fetus subsp. jejuni in raw milk reaffirms the need for pasteurization. $D_{55°C}$ values of 1 minute or less for the inactivation of several strains of C. fetus subsp. jejuni in skim milk has been reported (Doyle and Roman, 1981). Hence the vat process of holding milk at 62.7°C for 30 minutes should be sufficient to free milk of unusually large numbers of Campylobacter strains.

CHAPTER THREE

EXPERIMENTAL STUDIES

INTRODUCTION

Campylobacter fetus subspecies *jejuni* is regarded as an important cause of diseases in humans (Skirrow, 1977; Blaser *et al.*, 1979; Pai *et al.*, 1979). In several studies it was isolated more often than *Salmonella* species or *Shigella* species from patients with diarrhea (Blaser *et al.*, 1979; Pai *et al.*, 1979). The epidemiology of *C. fetus* subsp. *jejuni* diarrhea is beginning to be elucidated. It has been apparent that there is a vast animal reservoir for this organism and that transmission occurs by the faecal-to-oral route (Smibert, 1974; Robinshn *et al.*, 1979). Food and water are the vehicles most often incriminated during outbreaks (Blaser, 1981) and the organism has been isolated frequently from foods of animal origin including chicken turkey, pork, beef, lamb and unpasteurized milk.

Despite all these, there is no reported work on these areas in Nigeria. The purpose of this study therefore, is to ascertain the role of cattle in milk contamination by determining the prevalence of the organism in cattle faeces, milk and milk products. In addition, this work aims at determining the presence of virulence markers of the isolates, their susceptibility to antimicrobial agents and factors that affect their survival in milk.

MATERIALS AND METHODS

I ISOLATION OF CAMPYLOBACTER FETUS subspecies JEJUNI

(a) Types and sources of samples

(i) One thousand rectal swabs were collected from cattle in randomly selected livestock farms, Fulani herds and abattoirs in Zaria and it's environs. Two hundred and sixty-nine of these samples were from institutional livestock farms, 355 from Fulani herds and 376 from animals slaughtered in abattoirs.

(ii) Three hundred (300) fresh milk samples were collected from cattle whose rectal swabs has been collected.

(iii) One hundred (100) samples of locally fermented milk (nono) and 40 samples of local butter (Maishanu) all purchased from markets in Zaria and it's environs.

(b) Sample collection

Rectal swabs were obtained by inserting a sterile swab soaked in sterile F.B.P* broth 5-6cm into the rectum of each animal, rotating it for approximately 15 seconds and inserting it into a sterile test-tube containing 3mls of sterile F.B.P broth. The samples were conveyed to the laboratory ice-cooled and processed within 12 hours of collection.

An average of about 15mls of raw milk were collected aseptically into sterile universal bottles from randomly selected milking cows in each herd. The locally fermented milk samples

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*Ferrous Bisulphite Pyruvate (F.B.P) broth is used for growth transport and preservation of Campylobacter. It consists of Oxoid Nutrient broth No. 2 containing 0.12% agar and Oxoid SR 84. Oxoid SR 84 consists of 0.25g/L ferrous sulphate, sodium metabisulphite and sodium pyruvate. OXOID - Basingstoke Hampshire, ENGLAND.

were obtained by purchasing 20mls of each from different sellers in different markets. The maishanu was purchased in solid form and hence the exact volumes were not known. All samples were conveyed ice-cooled to the laboratory and processed within 12 hours of collection.

(c) Cultural media

Butzler's medium was used as the primary isolation medium. It consists of Oxoid Thioglycollate agar base with 15% defribinated sheep blood and Oxoid antibiotic supplement SR 85. SR 85 consists of Bacitracin 25,000 units/L, Cycloheximide 50mg/L, Colistin sulphate 10,000 units/L, Cephazolin sodium 15mg/L and Novobiocin 5mg/l.

(d) Culturing for Campylobacter fetus subsp. jejuni

About 0.1ml of the rectal swab suspension were inoculated onto Butzler's selective medium using the swab stick. The selective agar plates were then streaked for isolation with a wire loop. A reference strain of C. fetus subsp. jejuni (852/79) kindly supplied by Dr. M. B. Skirrow, Worcester Royal Infirmary, England, was also inoculated onto two plates of each lot of selective medium to test for the ability of the selective media to support growth of Campylobacter. The plates were incubated at 42°C for 48 hours in a microaerophilic atmosphere achieved by use of commercial Gaspak enveloped (B.B.L**) in anaerobic jars without catalyst.

Culturing for the organism from milk and milk products was carried out by employing a combination of direct plating and a

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**B.B.L Microbiological Systems, Becton Dickson and Co. Cockeyville, M.D. 21030.

modified selective enrichment procedure of Doyle and Roman (1982) followed by plating on Butzler's selective medium. Approximately 5ml of each sample was inoculated into 10ml of the F.B.P broth. The samples were then incubated at 42°C for 24 hours, and finally subcultured into Butzler's selective medium. The plates were then incubated at 42°C for 48 hours under similar microaerophilic conditions. Immediately after culturing, the pH of the raw milk and locally fermented milk were determined using a pH-meter 7010 (Electronic Instruments Limited). The pH of the local butter (Maishanu) was not determined because of it's tendency to solidify.

(e) Detection of Campylobacter fetus subspecies jejuni

All plates were examined for C. fetus subspecies jejuni after 48 hours of incubation. If growth was not observed, the plates were further incubated for up to 5 days after which they were discarded as negative.

Typical smooth, flat, tan or grey, watery and sometimes spreading non-haemolytic colonies were Gram-stained (counter-stained with carbol fucshsin), examined microscopically for motility by the hanging drop method and tested for oxidase production according to standard procedure (Cowan and Steel, 1974).

Colonies consisting of Gran-negative, oxidase-positive, curved or spiral shaped rods with very rapid, darting and jerking movements, were streaked on sheep blood agar plates, incubated for 48 hours at 42°C under microaerophilic conditions for purification and further identification.

(f) Identification and Characterization of Campylobacter fetus subsp. jejuni.

Colonies from purified growth were subjected to growth and biochemical tests according to standard procedures (Smibert 1979 Cowan and Steel, 1974, Skirrow and Benjamin, 1980). The specific biochemical tests carried out included catalase test, nitrate reduction test, Hydrogen sulphide (H_2S) and acid production in Triple sugar Iron agar (T.S.I), oxidation fermentation (O/F) test, Growth at 37°C, - 42°C and 25°C, in 1% glycine and 3.5% Nacl, aerobically and anaerobically.

Isolates that exhibited the following characteristics were identified as C. fetus subsp. jejuni: Catalase positive, reduced nitrates to nitrites, did not produce acid and H_2S in TSI, did not utilize glucose by oxidation or fermentation, Grew at 37°C and 42°C but not at 25°C, grew in 1% glycine but not in 3.5% Nacl, grew slowly anaerobically and did not grow aerobically.

Biotyping of all strains identified as C. fetus subsp. jejuni was performed according to the methods of Skirrow and Benjamin (1980) based on the ability of the organism to hydrolyse hippurate, produce hydrogen sulphide (H_2S) and it's susceptibility to nalidixic acid. Hydrogen sulphide production was tested for by using non-iron containing F.B.P medium as described by Skirrow and Benjamin (1980). Hippurate hydrolysis was tested for according to the method of Harvey (1980), while susceptibility to nalidixic acid was evaluated on blood agar plates with commercial antibiotic disks (Difco) containing 30ug/disk. Bacterial showing inhibition zones of 19mm after incubation microaerophically at 37°C for 24 hours were considered susceptible

Isolates were identified as C. jejuni biotype I if they hydrolyse hippurate, did not produce H_2S in F.B.P medium but were sensitive to nalidixic acid or occasionally resistant. They were regarded as C. jejuni biotype 2 if they were sensitive to nalidixic acid, hydrolysed hippurate and produce H_2S in F.B.P broth. They were identified as C. coli if they were sensitive to nalidixic acid but failed to hydrolyse hippurate and produce H_2S in F.B.P medium.

In addition to the standard biochemical tests used for the identification of Campylobacters, the isolates were subjected to the following biochemical and growth tests often used in the identification of other enteric organisms as described by Cowan and Steel (1974): Indole test, Methyl red and Voges Proskauer tests, citrate utilization tests, lysine and arginine decarboxylase tests, fermentation of glucose, lactose, sucrose, mannitol and salicin; urease test, growth of the organisms on the following selective media: Brilliant green agar (B.G.A), Salmonella-Shigella agar (S.S.A), Thiosulphate citrate bile salt sucrose (T.C.B.S), and MacConkey agar (MaC).

II ASSAYS FOR VIRULENCE MARKERS

The eleven isolates and the reference strain (852/79) were investigated for the presence of virulence markers as follows:

(a) Ability to invade tissues:

The invasive abilities of the isolates were determined using the method described by Sereny (1955). Each isolate was grown on 5% sheep blood agar plate for 48 hours under microaerophilic conditions at 42°C. Colonies were suspended

in sterile phosphate buffered saline (P.B.S., pH 7.2). The P.B.S. suspensions of the organisms were diluted to contain approximately 3×10^8 organisms/ml by comparison with MacFarland's nephlometer standard. One ml each of these suspensions was instilled into the upper and lower conjunctival sacs of guinea-pigs. One eye each of two different adult guinea pigs were inoculated with each isolate. A positive Shigella dysenteriae suspension, and a negative P.B.S. (pH 7.2) controls were instilled into the conjunctival sacs of control guinea pigs. Both the control and the experimental animals were examined daily for up to five days for the development of redness, purulent material or corneal opacity.

(b) Enterotoxin production

(i) Infant mouse test

A modification of the infant mouse test originally described by Dean et al. (1972) was used to assay for the presence of heat stable (ST) enterotoxin in broth culture supernatant of the isolates. Broth culture supernatants were prepared for use as sources of enterotoxin by growing the isolates in broth consisting of 2% peptone (Difco), 0.3% (wt/vol) sodium chooride, 0.1% (wt/vol) magnesium chloride and 1% (vol/vol) Horse serum (Mannninen et al., 1982). The cultures were incubated at $42^{\circ}C$ for 10 hours without shaking and for a further 38 hours with slow agitation. Cultures were centrifuged, and the supernatant fluid used immediately in the infant mouse test.

Enterotoxigenic material of known heat-stable (ST) producing enteropathogenic Escherichia coli (ETEC 64283) was prepared by inoculating Trypticase soy broth (T.S.B.) (B.B.L.) containing 1% yeast extract with overnight growth of the organism. The broth culture was incubated with slow agitation for 24 hours, centrifuged and the supernatant used for the inoculation of the positive control mice.

Infant mice were obtained from pregnant adult mice supplied by the National Veterinary Research Institute (N.V.R.I) Vom animal breeding laboratories. Seventy, 3-day-old suckling mice were separated from the dams and randomly divided into 14 groups of 5 mice each. Each of 5 3-day-old mice in 12 groups was inoculated with 0.1ml supernatant preparation (which contained 1 drop/ml 1% Evans blue dye) from eleven isolates and the reference strain. One group of 5 mice inoculated with sterile broth served as negative control and another group inoculated with supernatants from broth cultures of known S.T producing E. coli served as positive control. Inoculation was done percutaneously through the abdominal wall directly into the milk filled stomach with a 30 gauge hypodermic needle. After inoculation, all the mice were left at room temperature ($25^{\circ}C$) for 4 hours and then euthanized with ether. The abdomen of each mouse was opened, and the small intestine was examined for distention and then removed with forceps. The intestines of the 5 mice in each group were weighed together, their remaining body weights were also determined. The ratio of the gut weight to the remaining body weight was then

calculated. Ratios less than 0.07 were considered negative while those in the range of 0.07-0.09 were considered questionably positive and those over 0.09 strongly positive.

(ii) Rabbit ligated ileal loop test

This was done according to the method of Guerrant et al. (1978) to assay for the presence of heat-labile (LT) enterotoxin in whole culture suspensions of the isolates. Phosphate buffered saline (P.B.S) suspensions of 48-hour blood agar culture of the test isolates estimated to contain approximately 3×10^8 organism/ml by comparison with MacFarland's nephlometer standard were assayed for LT. P.B.S suspension of 24-hour blood cultures of known LT producing enteropathogenic E. coli (ETEC 64495 and ETECV 141941) served as positive controls.

Adult rabbits were starved for 24 hours before surgery. Under general anasthesia, the small bowel of each was isolated and flushed with 0.1ml P.B.S. (pH 7.4). Starting from the ileocaecal junction, the jejunum and anterior ileum were ligated by using a single tie of surgical silk between segments into a series of intestinal loops each averaging 4 to 5cm long. An internodal loop of about 2cm was tied between two experimental loops. Twelve loops were inoculated with 1ml of a P.B.S suspension of each of the 12 test isolates using a 30 gauge hypodermic needle. Loops inoculated with 1ml sterile P.B.S served as negative control loops and loops inoculated with suspensions of enteropathogenic E. coli served as positive controls. Control loops were placed at the beginning and end of a sequence and also interspersed within the sequence. Each isolate was tested in two different rabbits. After inoculation

the loops were carefully placed back in the abdominal cavity of the rabbit and the abdomen closed up surgically. Twenty-four hours after inoculation, the animals were killed and eviscerated. The length and fluid volume of each intestinal loop was measured and the results expressed as the ratio of volume to length. Ratios of 1 or higher were considered positive.

(c) Infection of 3-day-old chickens

The method of Ruiz-Palacios (1981) was followed to investigate the ability of the isolates to infect 3-day-old chickens inducing diarrhea and weight loss. The inoculum consisted of P.B.S suspension of 48 hour blood agar cultures of the various isolates adjusted to contain approximately 3×10^8 orgainsms/ml when compared with MacFarland's nephlometer standard.

Fifty-six 3-day old chicken (N.V.R.I. Hatchery, Vom) were divided into 14 groups of 4 chickens each. Each group was orally inoculated with 0.5ml of a suspension of each of the 11 isolates and the reference strain. 4 uninoculated chickens and 4 inoculated with sterile P.B.S. served as negative controls.

The mean body weight of each group of birds was determined immediately after inoculation and just before sacrifice. The cages and chickens were inspected daily for evidence of diarrhea throughout the duration of the experiment. Two chickens per group were sacrificed after 48 hours, eviscerated and the intestines inspected for gross pathological changes. This procedure was repeated for the remaining chickens 72 hours post inoculation.

III IN-VITRO ANTIMICROBIAL SENSITIVITY TEST

(i) Antimicrobial sensitivity testing by disc diffusion method

The antimicrobial discs (DIFCO) and their concentrations used were as follows: Tetracycline (30ug), Trimethoprim (5ug), Streptomycin (10ug), Erythromycin (15ug), Neomycin (30ug), Ampicillin (10ug), Bacitracin (10ug), Kanamycin (30ug). The locally made discs included Cloxacillin (10ug), Methronidazole (25ug), Chloramphenicol (30ug), Lincomycin (2ug), Gentamicin (10ug), Doxycycline (30ug), Cephalexin (30ug), and Nalidixic acid (30ug) A modification of the disc diffusion method of Bauer et al. (1966) was used. The organisms were grown in F.B.P broth for 24 hours. Turbidity testing was determined using MacFarland's nephelometer as standard. Sterile cotton swabs on wooden applicator sticks were dipped into the standardized suspension of the isolates and streaked evenly over the entire surface of Mueller Hinton agar enriched with 5% sheep blood. The plates were allowed to dry for about 10 minutes and the discs were then placed on the surface of the plates with sterile forceps. Approximately 7-8 antimicrobial discs were placed on each 150 x 15mm plate and the plates were incubated at 37 C in a microaerophilic condition for 48 hours. The diameter of the zones of the inhibition surrounding each disc to the nearest millimeter were measured with a ruler and the results recorded as sensitive, intermediate or resistant by referring to the manufacturer's interpretative table.

(ii) Determination of Minimal Inhibitory Concentrations (M.I.C)

The M.I.Cs of some of the antimicrobials tested by the disc method for the eleven isolates and the reference strain were determined. The antibiotics used for this study were Erythromycin, Tetracycline, Chloramphenicol, Doxycycline, Methronidazole, Kanamycin, Gentamicin, Cloxacillin, Ampicillin, Cephalexin, Streptomycin and Neomycin. The agar dilution method on Mueller Hinton agar (Difco) adjusted to pH 7.2 and enriched with 5% defribrinated sheep blood was used. Two-fold serial dilutions of each antimicrobial agent was achieved by employing a modified Ericsson and Sherris's (1971) method as shown below:

Antibiotic solution		+ Sterile water	=Intermediate concentration	Final Concentration at 1:10 agar plates	
Volume	ug or I.U/ml	Volume	ug or I.U/ml	ug or I.U/ml	log concentration
6.4	2,000	3.6	1280	128	7
2	1,280 (from above)	2	640	64	6
1	1,280 " "	3	320	32	5
1	1,280 " "	7	160	16	4
2	160 " "	2	80	8	3
1	160 " "	3	40	4	2
1	160 " "	7	20	2	1
2	20 " "	2	10	1	0
1	20 " "	3	5	0.5	-1
1	20 " "	7	2.5	0.25	-2

Two mls of the appropriate intermediate concentrations were added to 18mls of the molten Mueller Hinton agar to obtain the final concentrations in the plates. The test organisms were streaked on blood agar plates, then subcultured in F.B.P broth and incubated for 48 hours. Each broth culture was adjusted to a MacFarland number 1 nephelometer standard. Inocula consisting of 0.02ml were delivered with an Epperdorf pippette onto the antimicrobial agent containing agar plates. Inoculated plates were then incubated at 37°C under microaerophilic conditions as previously described. Blood agar plates without antimicrobial agents were inoculated with the test organisms and used as controls. All results were read at 18 hours and the minimum inhibitory concentration (M.I.C.) was the least antimicrobial concentration that yielded no visible growth.

IV EFFECT OF TEMPERATURE AND pH ON THE SURVIVAL OF C. FETUS SUBSP. JEJUNI

The survival and growth rate of 2 selected isolates and the reference strain inoculated into sterile milk at temperatures of 4°C, 25°C and 42°C; and pH 5.0, 7.0 and 8.5 were investigated.

For each temperature level, three 150ml portions of the milk were sterilized by autoclaving at 121°C for 15 minutes and adjusted to pH 5.0, 7.0 and 8.5 respectively with either HCl or NaOH using pH-meter 7010 (Electronic Instruments, Limited). Each portion was then inoculated with 3ml of a 48-hour F.B.P broth culture of the test organism, mixed, and distributed aseptically in approximately 12ml amounts into 13 sterile universal bottles. All the 39 universal bottles were then kept at the appropriate test temperature either at 42°C in an incubator, 4°C in a refrigerator or at 25°C on a laboratory bench

(room temperature).

Viable count was determined at 6-hourly intervals up to 72 hours. At each sampling time, 1ml each of the milk samples was removed from different universal bottles at the appropriate pH levels and viable counts determined for each sample by a modified Miles and Misra (1938) method, using 5% sheep blood agar plates. One tenth millilitres of appropriate dilutions was inoculated onto the agar plates which were then incubated at 42°C for 48 hours under microaerophilic conditions. At each sampling time, the pH of the milk samples were determined and appropriately adjusted to the desired pH using sterile NaOH or HCl to maintain a relatively constant pH.

A similar protocol was used to investigate the effect of storage at 4°C, 25°C and 42°C on the survival of these organisms in sterile milk at it's normal pH. At 6-hourly intervals, the pH of milk was determined and recorded but not adjusted.

RESULTS

I. ISOLATION RATES AND CHARACTERISTICS OF ISOLATE

A total of eleven isolates, representing an isolation rate of 1.1% were obtained from the 1,000 cattle rectal samples examined. The isolates were non-haemolytic, gram-negative spiral S-or comma shaped rods and oxidase and catalase positive. They were non-saccharolytic but reduced nitrates to nitrites. One isolate however reduced nitrite further to nitrous oxide. All the eleven isolates were susceptible to Nalidixic acid, hydrolysed hippurate, and did not produce hydrogen sulphide in F.B.P medium. They were therefore classified as C. jejuni biotype 1.

Of the 269 rectal swabs obtained from animals on institutional farms, only one (0.37%) sample (obtained from the A.B.U University Farm), was positive for C. fetus subsp. jejuni. Seven (1.86%) isolates of the organism were obtained from the 376 samples from the abattoir and 3(0.85%) from the 355 samples from the fulani herds (Table 1).

There is no statistically significant difference in the isolation rates of C. fetus subsp. jejuni between the abattoir and fulani herds (P 0.25, X^2 = 1.36).

The organism was not isolated from any of the 300 milk samples, nor from the 100 locally fermented milk (Nono) and 40 local butter (Maishanu) samples.

Table 2 shows the reaction of the eleven isolates and the reference strain when they were subjected to other biochemical tests routinely used in the identification of other enteric pathogens. All the eleven isolates were negative in the indole, methyl red and Voges Proskauer test. All of them were able to utilize citrate. Of the eleven, only 2(numbers 11 and 65) were able to decarboxylate the amino acid lysine. None decarboxylated arginine. None of them produced H_2S on TSI slant, neither was there any positive in the urease test. None of the isolates utilized glucose, lactose, sucrose, mannitol, and salicin either fermentatively or oxidatively. On the selective media employed, none of the eleven strains grew on Brilliant green aga (B.G.A), Salmonella-

Shigella agar (S.S.A), and Thiosulphate citrate bile sucrose agar (T.C.B.S). Two of them (strain 21 and 16) showed only slight growths on the MacConkey agar.

II VIRULENCE MARKERS

(a) Ability to invade tissues

None of the eleven isolates of C. fetus subspecies jejuni and the reference strain caused purulent discharge, redness or corneal opacity (Keratoconjunctivitis) in any of the guinea pigs used for the test. Similarly, animals inoculated with sterile P.B.S did not develop keratoconjunctivitis, but guinea pigs inoculated with Sh. dysenteriae showed progressive conjunctivitis and Keratitis from 72 hours post inoculation.

(b) Enterotoxin production

(i) Infant mouse test: Table 3 shows the ratio of gut to remaining carcass (body) weight in the infant mouse test for heat stable enterotoxin. The E. coli positive control produced a mean gut-to-body-weight ratio greater than 0.090. The C. fetus subsp. jejuni strains and the sterile broth which served as the negative control, produced mean ratios of below 0.070.

(ii) Rabbit ligated ileal loop test: Table 4 and plate 1, show the enterotoxigenic capabilities of the C. fetus subsp. jejuni isolates and the control organisms when their filtrates were inoculated into the intestinal loops of rabbits. The loops inoculated with filtrates of all the C. fetus subsp. jejuni as well as the negative control loops inoculated with only P.B.S showed no fluid accumulation and each had a volume/length ratio of less than 1. The loops inoculated with the

enterotoxigenic E. coli filtrates (ETEC 64495 and ETECV 141941) showed marked distention with ratios of volume/length 1 and above.

(c) Infection of 3-day-old chicken

Of a total of 52 chickens, 8(15.4%) inoculated with isolates 16 and 65 had soft faeces and slight intestinal and caecal haemorrhage and showed slight losses in weight and both 48 and 72 hours post inoculation. The remaining 44 birds had no diarrhea and showed a mean increase in weight; they appeared healthy throughout the duration of the experiment and showed no gross pathology at necrospy. The mean weight gain of the 52 experimental chickens at 48 and 72 hours were 6.43 ± 2.32g and 7.45 ± 5.53g respectively. The control chicken had mean weight gains of 7.35g ± 2.3g at 48 hours and 8.48g ± 0.88g at 72 hours post inoculation. There was no statistically significant difference between the mean weight gains of the experimental and control chickens (P 0.5, student t-test for unpaired means).

III ANTIBIOGRAM STUDIES

(i) Antimicrobial susceptibility testing by disc diffusion method

The results of the antimicrobial sensitivity test by the disc diffusion method are shown in Table 5. All the eleven isolates tested were susceptible to gentamicin, chloramphenicol and nalidixic acid. Ten (91%) of the eleven isolates were susceptible to streptomycin, neomycin and doxycycline whereas nine (82%) of them were susceptible to tetracycline and kanamycin. Erythromycin like ampicillin was moderately active against the organisms with 72.7% and 54.5% of the isolates being sensitive to them respectively.

Table 6 shows the percentage resistance of the isolates to the antimicrobial agents used. None of the eleven isolates

were susceptible to all the antimicrobial agents tested. All the isolates were resistant to lincomycin, cloxacillin, trimethoprim and bacitracin. All except one isolate each were resistant to Cephalexin and Metronidazole.

The patterns of the antimicrobial resistance of the isolates are shown in Table 7. Nine patterns were observed, and almost all isolates exhibited different patterns of resistance from one another. Two patterns each occurred twice: Cloxacillin - Metronidazole - Lincomycin - Trimethoprim - Bacitracin - Cephalixin (18.2%) and Cloxacillin - Metronidazole - Lincomycin - Trimethoprim - Ampicillin - Bacitracin - Cephalexin (18.2%).

(ii) Minimal Inhibitory Concentration (M.I.C)

Table 8 shows the minimal inhibitory concentrations of 14 antimicrobial agents for 11 C. fetus subsp. jejuni isolates from cattle faeces. All the strains tested were sensitive to Chloramphenicol at 8ug/ml, 27.3% and 18.2% of the strains were resistant to tetracycline and Doxycycline at M.I.C. of 4ug/ml. Doxycycline was however observed to be more active than tetracycline as lower concentrations of Doxycycline were required to inhibit the isolates, and two strains resistant to tetracycline at 4ug/ml showed cross-resistance to Doxycycline (Table 6). Ampicillin was moderately active against the isolates with 6 of the 11 isolates (54.5%) inhibited by 8ug/ml. All the 11 isolates were highly resistant to Cloxacillin, Lincomycin and Methronidazole. All the isolates were resistant at M.I.C of 128ug/ml of these antimicrobial agents. Cephalexin showed

poor activity against C. fetus subsp. jejuni isolates. Only one isolate was inhibited by 32ug/ml, the others gave M.I.Cs ranging from 64 to 128ug/ml.

IV EFFECT OF TEMPERATURE AND pH ON THE SURVIVAL AND GROWTH OF THE ISOLATED C. FETUS SUBSP. JEJUNI IN MILK.

Figures 1 to 3 show the survival and growth patterns of a reference strain of C. fetus subspecies jejuni and two other isolates from cattle rectal swabs inoculated into sterile milk at different temperature and pH levels.

At 4°C, the survival of the three test organisms was observed to be best in sterile milk adjusted to pH 7.0 and better in milk at pH 8.5 than in milk at pH 5.0 over a 24 hour period (Fig. 1). A similar trend was observed at 25°C (Fig. 2). However, in general, inactivation of the organism occurred more rapidly at 25°C than at 4°C.

Similar to observations at 4°C and 25 C, at 42 C, the survival of the 3 organisms were observed to be best in milk adjusted to pH 7.0 (Fig. 3). However, in sterile milk samples adjusted to pH 8.5 and pH 5.0, survival was better at pH 5.0 than at pH 8.5 over a 24 hour period, contrary to observations at 4°C and 25°C. In sterile milk adjusted to pH 8.5 and held at 42°C, very rapid declines in numbers occurred over a 24 hour period. An approximately 6-log decrease occurred at pH 8.5 with 42°C with three test organisms over a 24 hour period.

The survival profile of the three C. fetus subspecies jejuni test strains in sterile milk at it's normal pH, and the changes in the milk pH at 4°C, 25°C and 42°C are displayed in Figures 4a and 4b. Similar patterns of survival were observed with the

three strains at each temperature. It was observed that at the three temperature levels, all the isolates at the concentrations used persisted in milk for up to three days. With the three strains, survival and growth were best at 42°C (Fig. 4). The survival and number of organism detected in milk were better at 4°C than at 25°C over a 24 hour period. At 4°C there was no significant change from the initial number of the organisms of the test strains over a 72 hour period. At 25°C, all the three strains showed varying degrees of decline in initial numbers over a 72 hour period (Fig. 4).

There were slight or no differences in the initial pH of the milk samples inoculated with the test organism. At 4°C, the pH of the milk samples remained constant over the 72 hour period. Very slight changes occurred in the milk pH at 25°C and 42°C (Fig. 4b).

TABLE 1: Isolation Rates of C. fetus subsp. jejuni from cattle from different sources.

Source	Total No. of cattle rectal swabs collected	Number positive for C. fetus subsp. jejuni	Percentage
(a) INSTITUTIONAL FARMS/HERDS			
(i) National Animal Production and Research Institute Dairy, Zaria			
(ii) Ahmadu Bello University Farm, Zaria			
(iii) Alhaji Kowa's Farm, Zaria.	269	1	0.37%
(iv) School of Agriculture Farm, Zaria			
(v) Bitrus Sawa's Farm, Zaria.			
(vi) Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.			
(b) FULANI HERDS	355	3	0.85%
(c) ZANGO ABATTOIR, ZARIA.	376	7	1.86%
TOTAL	1,000	11	1.1%

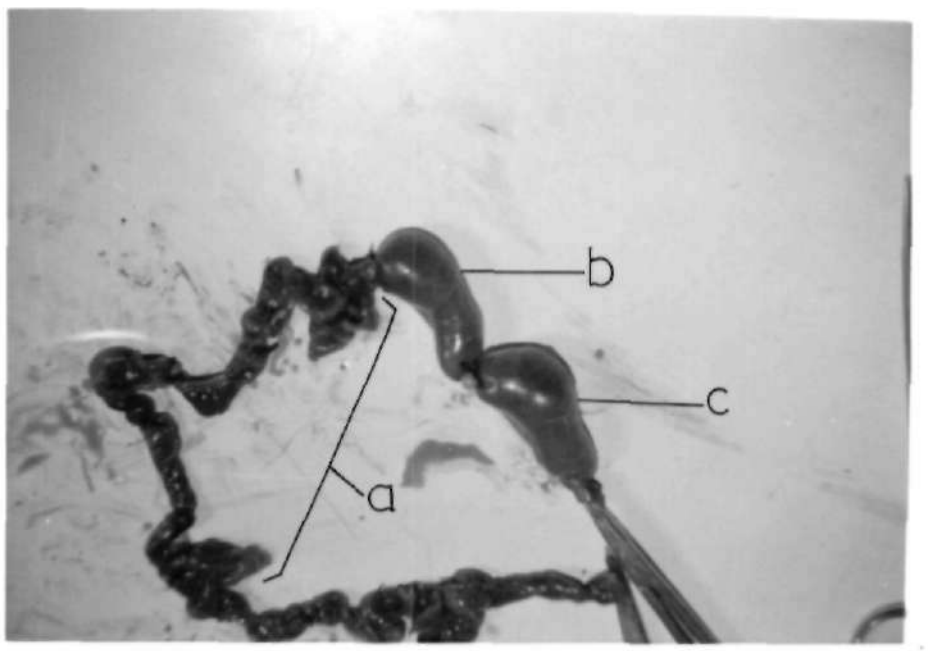
TABLE 2: BIOCHEMICAL REACTIONS OF THE ISOLATES IN TESTS NOT ROUTINELY USED FOR CAMPYLOBACTER IDENTIFICATION.

ISO-LATE	INDOLE	MR	VP	CITRATE UTILIZ-ATION	LYSINE DECARB-OXYLASE	H_2S in TSI	UREASE	BGA	SS	TCBS	MAC	GLU-COSE	LAC-TOSE	SUC-ROSE	MANNI-TOL	SALI-CIN	ARGININE DEC-ARBOXYLASE	GROWTH IN 3.5% NaCl
21	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
7	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
20	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
65	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
852/79	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-

TABLE 3: Infant Mouse test for detection of heat-stable enterotoxin from 11 C. fetus subsp. jejuni isolates.

Isolate No.	Gut Weight (G)	Body-Gut weight (C)	Ratio of Gut to remaining body weight (G/C)[a]
11	0.13	2.1	0.062
20	0.12	2.0	0.060
65	0.14	2.2	0.064
7	0.13	2.1	0.062
23	0.15	3.0	0.050
37	0.18	3.0	0.060
9 19	0.12	2.15	0.056
6 66	0.18	3.0	0.060
13	0.15	3.0	0.050
21	0.14	3.0	0.050
16	0.13	2.1	0.062
P.B.S	0.13	2.1	0.062
852/79	0.20	3.7	0.054
64283	0.17	1.8	0.094

[a] A value of 0.09 or higher was considered positive.



ENTEROTOXIGENIC ACTIVITY OF 11 C. FETUS SUBSPECIES JEJUNI USING RABBIT INTESTINAL LOOP TECHNIQUE

[a]loops inoculated with C. fetus subsp. jejuni isolates

[b]loop inoculated with strain ETEC 64495

[c]loop inoculated with ETECV 141941

TABLE 4: Enterotoxigenic activity of 11 C. fetus subsp. jejuni filtrates using rabbit intestinal ligated loop.

Isolate No.	Fluid volume in loop (ml)	Loop length (cm)	Volume/length ratio[a]
11	0.0	5.0	0.0
20	1.0	4	0.25
65	0.0	4.2	0.00
7	0.1	5.0	0.02
23	0.1	4.0	0.025
37	0.0	4.0	0.0
19	0.0	5.0	0.0
6	0.0	5.0	0.0
13	0.0	5.0	0.00
21	0.0	3.5	0.0
16	0.1	5.0	0.02
P.B.S	0.1	4.0	0.025
852/79	0.0	4.0	0.0
64495	4.5	4.0	1.125
141941	6.5	5.0	1.3

[a] A value of 1 was considered positive.

TABLE 5: Antimicrobial susceptibility of 11 C. fetus subsp. jejuni isolates to 16 antimicrobial agents by disc diffusion method.

Antimicrobial agents	Drug dose/disk (ug)	Mean zone diameter (mm)	General Interpretation	No. of susceptible isolates	Percentage susceptible (%)
Cloxacillin	1[illegible]	1.70	R	0	0.0
Methronidazole	25	5.50	R	1	9.1
Chloramphenicol	30	31.80	S	11	100.0
Lincomycin	2	3.50	R	0	0.0
Tetracycline	30	25.30	S	9	82.0
Trimethoprim	5	2.70	R	0	0.0
Gentamicin	10	26.30	S	11	100.0
Streptomycin	10	24.00	S	10	91.0
Erythromycin	15	24.20	S	9	72.7
Neomycin	30	21.00	S	10	91.0
Ampicillin	10	15.00	S	6	54.5
Nalidixic acid	30	21.60	S	11	100.0
Bacitracin	10	2.50	R	0	0.0
Doxycycline	30	21.20	S	10	91.0
Cephalexin	30	9.20	R	1	9.1
Kanamycin	30	20.00	S	9	82.0

[a]A total of 11 isolates were tested. R = Resistant; S = Susceptible

TABLE 6: Percentage resistance of 11 C. fetus subspecies jejuni strains to 16 antimicrobial agents.

Antimicrobial agents	Resistance (Percent)
Cloxacillin	11(100.0)
Methronidazole	10(90.1)
Chloramphenicol	0(0.0)
Lincomycin	11(100.0)
Tetracycline	2(18.2)
Trimethoprim	11(100.0)
Gentamicin	0(0.0)
Streptomycin	1(9.1)
Erythromycin	3(27.3)
Neomycin	1(9.1)
Ampicillin	5(45.5)
Nalidixic acid	0(0.0)
Bacitracin	11(100.0)
Doxycycline	1(9.1)
Cephalexin	10(90.1)
Kanamycin	2(18.2)

TABLE 7: Patterns of antimicrobial resistance of 11 C. fetus jejuni from cattle faeces.

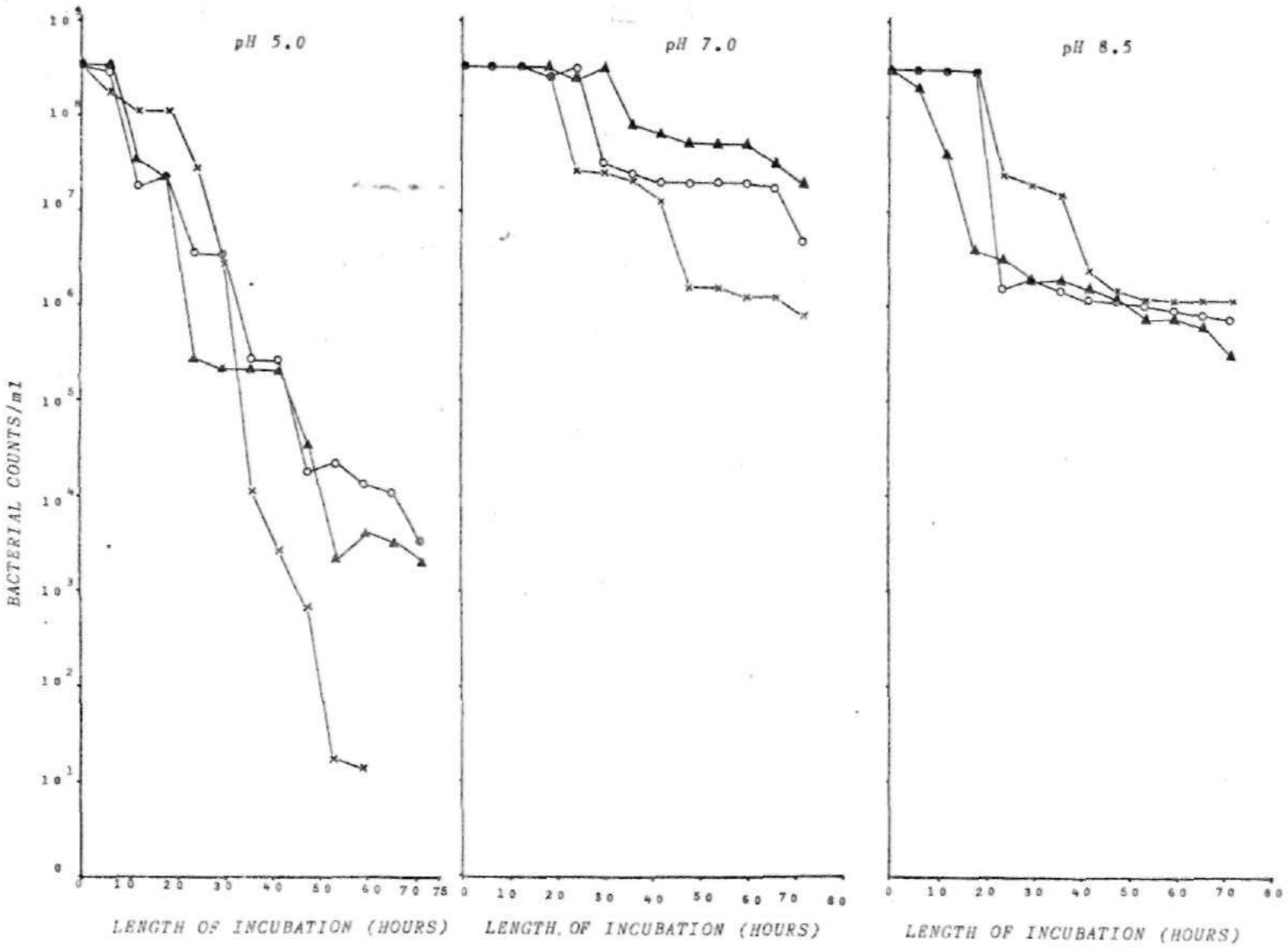
Resistance Pattern[a,b]	Number of Isolates (%)
Clox-Meth-Lin-Tri-Bac-Ceph	2(18.2%)
Clox-Meth-Lin-Tri-Bac	1(9.1%)
Clox-Lin-Tri-Neo-Bac-Ceph	1(9.1%)
Clox-Meth-Lin-Tri-Strept-Amp-Bac-Ceph-Kana	1(9.1%)
Clox-Meth-Lin-Tri-Ery-Amp-Bac-Ceph	1(9.1%)
Clox-Meth-Lin-Tri-Tetra-Ery-Bac-Ceph	1(9.1%)
Clox-Meth-Lin-Tri-Ery-Bac-Ceph	1(9.1%)
Clox-Meth-Lin-Tri-Amp-Bac-Ceph	2(18.2%)
Clox-Meth-Lin-Tri-Tetra-Amp-Bac-Doxy-Ceph-Kana	1(9.1%)

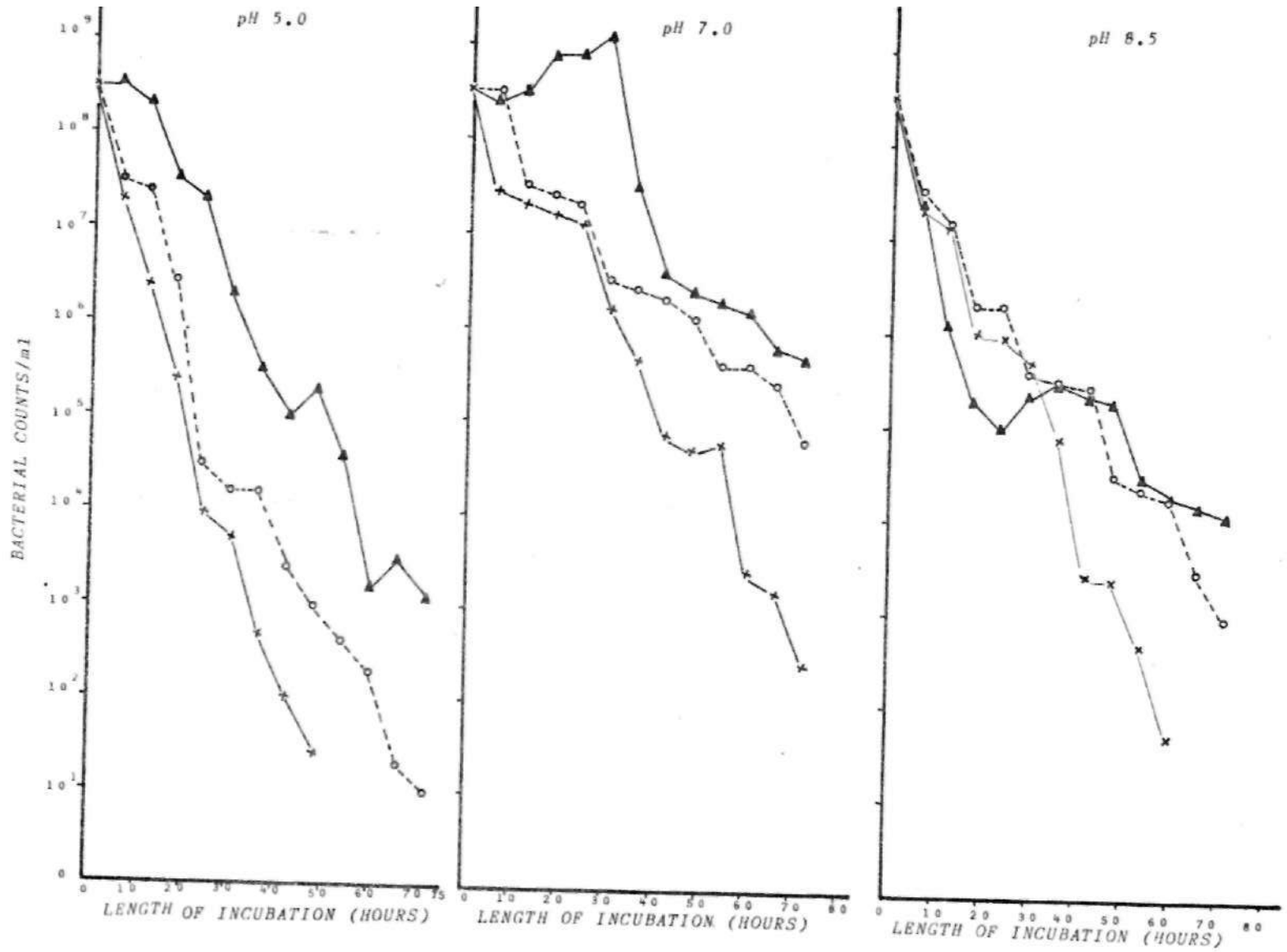
[a] Overall 9 antimicrobial resistance patterns were observed.

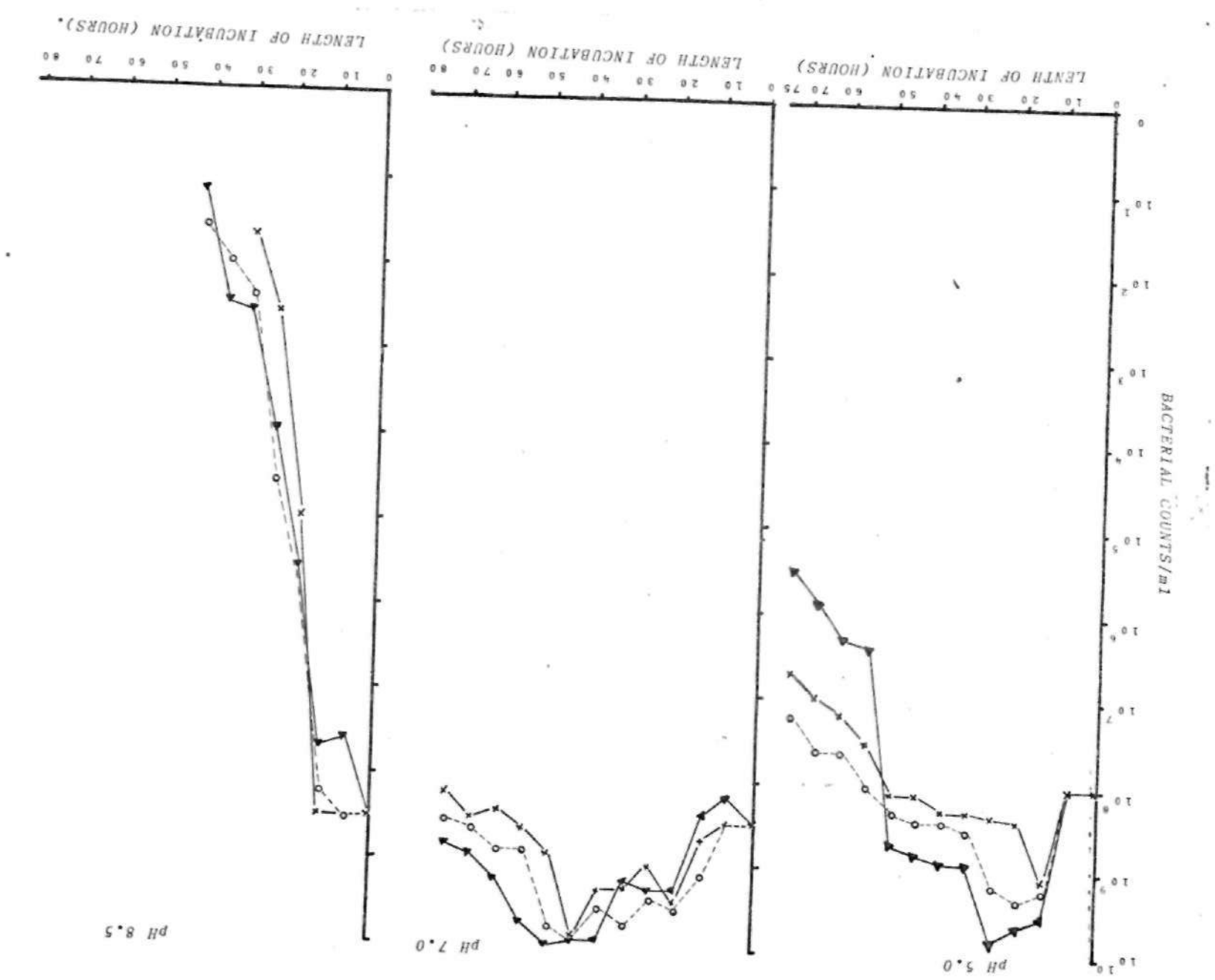
[b] Clox = Cloxacillin, Bac = Bacitracin, Tri = Trimethoprim, Meth = Methronidazole, Lin = Lincomycin, Ceph = Cephalexin, Neo = Neomycin, Strept = Streptomycin, Amp = Ampicillin, Tetra = Tetracycline, Ery = Erythromycin, Kana = Kanamycin, Doxy = Doxycycline.

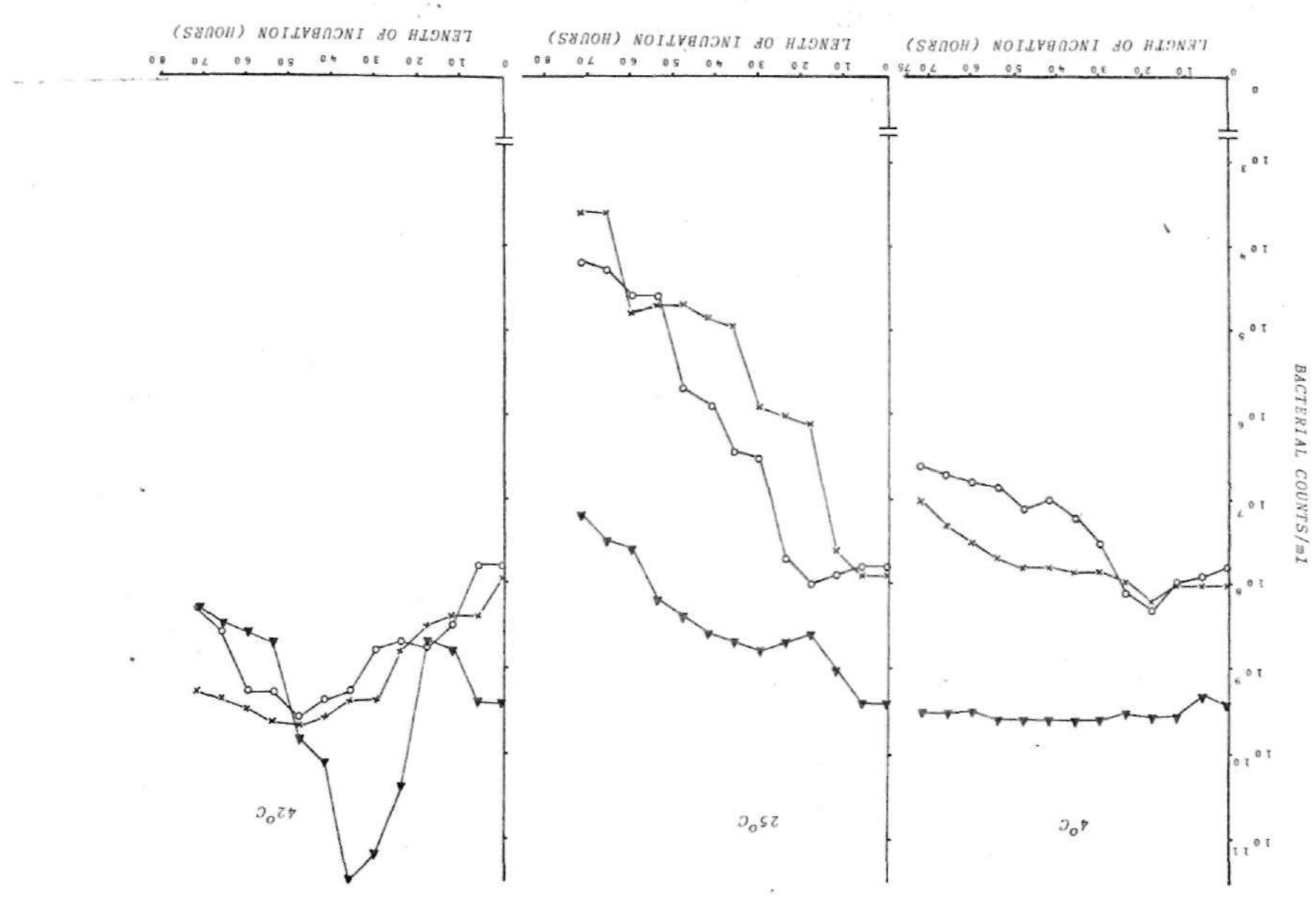
TABLE 8: MINIMAL INHIBITORY CONCENTRATIONS (M.I.C.) OF 14 ANTIMICROBIAL AGENTS FOR 11 C. FETUS SUBSP. JEJUNI ISOLATES.

	ANTIMICROBIAL AGENTS													
Isolate	ERYTHROMYCIN (ug/ml)	CHLORAMPHENICOL (ug/ml)	AMPICILLIN (ug/ml)	TETRACYCLINE (ug/ml)	CLOXACILLIN (ug/ml)	LINCOMYCIN (ug/ml)	METHRONIDAZOLE (ug/ml)	STREPTOMYCIN (ug/ml)	KANAMYCIN (ug/ml)	GENTAMICIN (ug/ml)	NEOMYCIN (ug/ml)	CEPHALEXIN (ug/ml)	DOXYCYCLINE (ug/ml)	NALIDIXIC ACID (ug/ml)
19	2	8	8	0.5	128	128	128	2	2	0.5	2	128	0.5	4
37	2	4	16	0.5	128	128	128	2	8	2	4	64	0.25	2
6	4	8	8	1	128	128	64	2	8	2	4	32	0.25	16
7	2	0.25	8	1	128	128	128	2	4	0.5	16	128	0.25	2
11	32	8	8	32	128	128	128	1	16	1	4	128	16	16
20	16	1	32	1	128	128	128	2	16	2	4	128	10.5	0.25
21	2	8	8	1	128	128	8	1	8	2	8	64	0.25	8
16	2	8	16	4	128	128	32	1	16	2	4	128	2	4
13	2	2	16	1	128	128	128	2	64	0.25	1	128	0.5	2
65	16	1	32	2	128	128	128	2	16	1	2	128	0.5	1
23	2	2	8	8	128	128	128	64	64	1	4	128	4.0	1
REFERENCE STRAIN	2	4	8	0.5	128	128	4	4	4	2	8	16	4.0	8


pH 5.0
pH 7.0
pH 8.5
BACTERIAL COUNTS/ml
LENGTH OF INCUBATION (HOURS)
LENGTH OF INCUBATION (HOURS)
LENGTH OF INCUBATION (HOURS)


pH 5.0
pH 7.0
pH 8.5
BACTERIAL COUNTS/ml
LENGTH OF INCUBATION (HOURS)
LENGTH OF INCUBATION (HOURS)
LENGTH OF INCUBATION (HOURS)


LENGTH OF INCUBATION (HOURS)
LENGTH OF INCUBATION (HOURS)
LENGTH OF INCUBATION (HOURS)
BACTERIAL COUNTS/ml
pH 5.0
pH 7.0
pH 8.5


LENGTH OF INCUBATION (HOURS)
LENGTH OF INCUBATION (HOURS)
LENGTH OF INCUBATION (HOURS)
BACTERIAL COUNTS/ml
4°C
25°C
42°C

DISCUSSION

The carriage rate (prevalence rate) of 1.1% of Campylobacter fetus subspecies jejuni by cattle in Zaria and its environment as detected in this study is low although it is in general agreement with results of Stern (1981). Prescott and Bruin-Mosch (1981) and Rosef et al. (1983) who have reported isolation rates of 0%, 2.5% and 0.8% respectively from cattle. Other workers have reported higher isolation rates ranging from 19% to 100% (Svedhem and Kaijser, 1981; Doyle and Roman, 1982; Prescott and Munroe, 1982 ; Elegbe, 1983).

The reason for the variations in the results of the various workers may be explained in part by differences in management practices by cattle owners such as the type of housing and type and quality of feeding and drinking water. These may influence the existence and spread of C. fetus subspecies jejuni in cattle. Methods for obtaining the samples and isolating the organisms may also be an important factor. Furthermore, the variation could be a true reflection of the level of infection in these environments. Results of this study show that management practices may not be an important factor because there was no statistically significant difference in the isolation rates of the organism from the three different sources studied.

Raw and improperly pasteurized milk from cows have been incriminated as the vehicle in several outbreaks of milk-borne Campylobacteriosis (Robinson and Jones, 1981; Skirrow, 1982). Many workers have tried to isolate C. fetus subsp. jejuni from milk with varying degrees of success (Taylor et al., 1979; Porter and Reid, 1980; MacNaughton et al., 1982; Lovett et al., 1983).

The result of this study in which no Campylobacter fetus subspecies jejuni was isolated from milk is in agreement with the work of Rosef et al., (1983) and Waterman and Park (1982) who obtained no isolates from milk collected from 106 and 600 dairy cattle with clinical mastitis in Norway and England, respectively. The absence of the organism in milk and milk products as observed in this study appears to be due to the low prevalence of the organism in cattle faeces which is regarded as a source of milk contamination. Failure to isolate the organism from the 300 milk samples in this study may mean that the milk was not contaminated by faecal material or it may be that cows whose milk were examined had no C. fetus subspecies jejuni in their faeces. In addition, since the technique in this study for isolating the organism from milk involved both direct plating and an enrichment procedure, it was unlikely that the organisms were present but were undetected in the raw milk and milk products examined.

The low carriage rate observed in cattle faeces and the absence of the organisms in milk and milk products suggest that cattle may not be an important source of the organism for human infection. However, since cows are intestinal carriers of C. fetus subspecies jejuni (Smibert, 1974), the faecal contamination of milk still represents a potential route leading to human infections. Although C. fetus subspecies jejuni was detected in only 11 of 1000 samples of the rectal swab examined, it's presence in the intestinal tracts of these few cattle suggest that milk handled in an insanitary manner may easily be contaminated with the organisms.

The eleven strains did not differ among themselves in most of the biochemical tests to which they were subjected. The inability of any of the strains to utilize any of the five sugars tested agrees with previous report that Campylobacter organisms have limited metabilic activities and are unable to utilize carbohydrates as sole carbon source (Smibert, 1974). Results from the study however show that routine biochemical tests used for other enteric organisms may not be necessary in the identification of Campylobacter strains.

The fact that all the isolates belong to the same biotype 1 is in agreement with work reported by Rosef et al. (1983) that majority of cattle isolates belong to biotype 1.

The results of the infant mouse test corroborated those of the ligated ileal loop test, and these show that the C. fetus subsp. jejuni strains tested neither produced a cholera-like heat-labile enterotoxin nor a heat-stable, enterotoxin analogous to those produced by Vibrio cholerae, non-cholera vibrios and certain strains of E. coli. Failure to detect toxin may mean that C. fetus subsp. jejuni does not produce enterotoxin at all or produces toxins that are not detectable by the current methods used. The results of this study are however consistent with those of Butzler and Skirrow (1979) who were unable to demonstrate heat-labile enterotoxin production in isolates by the Y1-adrenal cell test and with those of Guerrant et al. (1978), who failed to demonstrate heat-stable and heat-labile enterotoxin production by five isolates of C. fetus subsp. jejuni.

Ruiz-Palacios et al., (1981) recently described the experimental reproduction of Campylobacter diarrhea in chickens using a single human isolate. He induced diarrhea in 88% of the 3-day old chickens. The infected group failed to gain weight compared with the control group. In this study, however, a mean increase in weight was observed in the birds treated with the test organisms as well as in the controls. However, weight loss was observed in 8 of 54 birds, and although soft faeces were observed in the intestine of these birds, profuse diarrhea, mucus and perianal staining were not observed. The results of this study are in agreement with the work of Manninan et al. (1982) who also could not induce diarrhea in 3-day old chicken using human isolates of C. fetus subspecies jejuni. These differing results suggest that not all C. fetus subspecies jejuni strain have the ability to induce diarrhea, and that even with those strains that are capable of inducing diarrhea, there are differences in the severity of the diarrhea so produced. Furthermore, C. fetus subsp. jejuni isolated from human beings may be more pathogenic to chickens than those from cattle. There is also the possibility that differences between the breeds of chickens used affect induction of diarrhea.

The other results in the present study also seem unable to define the virulence factors of C. fetus subsp. jejuni because the result of guinea-pig conjunctivitis (Sereny, 1955) test also excluded a typical epithelial cell invasive process. This result however agreed with those of Guerrant et al. (1978) and Mannien et al. (1982), and separates the pathogenesis of Campylobacteriosis from that of Shigellosis and certain invasive

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E. coli. In humans however, studies suggest that C. fetus subsp. jejuni is enteropathogenic by means of invasive properties. Such evidence include the nature of the clinical symptoms the rapid development of antibody titres after infection and the recovery of the organisms from peripheral blood in the acute case of the disease (Manninen et al., 1982). It is therefore suggested that further investigation be conducted on the hypothesis that human campylobacteriosis with bacteremia may be a penetrating infection. Furthermore, more cattle isolates would have to be tested for possible invasive properties before it could be fully established that C. fetus subsp. jejuni of cattle origin is invasive or not.

The in-vitro activity of the different antimicrobial agents on the test isolates confirm other reports in literature that C. fetus subsp. jejuni isolates are susceptible to chloramphenicol, the aminoglycosides, doxycycline, tetracycline and erythromycin; and resistant to bacitracin and trimethoprim (Plastridge et al., 1964; Butzler et al., 1974; Vanhoof et al., 1978). Erythromycin resistance among our isolates was higher than the reported frequency of resistance to the same antibiotic in other parts of the world (Brunton et al., 1978; Vanhoof et al., 1978; Walderman and Forsgen, 1978; Karmali et al., 1981). This unusually high resistance to erythromycin in the present work may be relative, arising from the few (11) isolates tested.

All the isolates in this study were sensitive to chloramphenicol and were inhibited by therapeutionlly achievable serum levels of the antibiotic (M.I.C. 8ug/ml). Similar findings have been reported elsewhere (Walder, 1979; Vanhoof et al., 1980).

This antibiotic may therefore serve as a potential drug of choice in treating C. fetus subsp. jejuni infections. Its use is likely to be limited however because of the risk of serious side effect. Also, the results of this study confirmed Walder's (1979) observation that doxycycline has greater in-vitro activity against C. fetus subsp. jejuni than does tetracycline; and thus confirms its use in therapy.

The fact that all isolates in this study were highly sensitive to the aminoglycosides tested, agrees with report of earlier workers (Butzler et al., 1974; Vanhoof et al., 1978; Walder, 1979; Vanhoof et al., 1980; Karmali et al., 1981). Almost all the isolates were inhibited by gentamicin and streptomycin at 2.0ug/ml and neomycin at 4ug/ml. Hence parenteral gentamicin which has been successfully used to treat C. fetus subsp. jejuni septicemia (Butzler and Skirrow, 1979), may be useful in treating infections that may arise from bovine isolates of C. fetus subsp. jejuni in Nigeria. All strains tested were inhibited by nalidixic acid at 16ug/ml, thus confirming that our isolates were C. fetus subsp. jejuni (Veron and Chatelain, 1973).

In this study, survival of C. fetus subsp. jejuni in milk appears to be temperature-dependent. At 4°C, the initial numbers of the organism remained more or less constant over the period whereas at 25°C the numbers of each strain dropped considerably over the same period. This observation agreed with those of Blaser et al. (1980) who reported that strains of C. fetus subsp. jejuni may survive in sterile milk initially containing 10^7 cells/ml for up to 22 days at 4°C but for no more than 3 days at 25°C. Similar observations were made

by Christopher et al. (1982) who reported an approximately 4-$\log_{10}$ decrease in cells in sterile milk after 14 days at 1°C and a 5-log to 6-log decrease after 2.5 days at 20°C. From all these observations, it is obvious that Campylobacter organisms are capable of surviving for several days at refrigerated temperature (4°C) such that once contaminated, milk could serve as a source of infection for man even when refrigerated.

The increases in the initial number of each of the test isolates inoculated into sterile milk and held at 42°C can be explained in part, by the fact that the organism acclamatised and multiplied at this temperature which is regarded as optimum for growth of C. fetus subsp. jejuni (Smibert 1974). The very slight increases in the milk pH during storage at this temperature might also have contributed to a more optimum growth condition for the organism. However, since at 4°C and 25°C there was a decrease in the initial number of each organism with little or no change in the milk pH, it is possible that the suboptimal nature of the two temperatures were responsible for the decline in number. The reason for the slight increase in the pH of the milk held at 42°C is not easy to explain since C. fetus subsp. jejuni does not ferment the milk sugar, lactose. It is possible that the slight increase observed may have been as a result of the growth and multiplication of other organisms as evidenced by their presence on the growth medium.

The fact that strains of the test organism survived for 60 to 72 hours at an acid pH of 5 at 4°C in this study, would suggest that C. fetus subsp. jejuni may remain viable for days in refrigerated low acid milk products such as locally fermented

milk (nono) and local cheese (Kindrim). At room temperature (35°) the organisms were less tolerant to acid pH, so it would seem that inactivation of the organisms in low acid milk products would occur much more rapidly at room temperature than at refrigeration temperature. Increased metabolic acitivities at a higher temperature (25°C) in an unfavourable pH may have resulted in death of some cells. This may explain in part, the decline in population observed in milk kept at 25°C. It is therefore obvious that acidic milk such as local fermented milk products may serve as a vehicle for transmission of C. fetus subsp. jejuni to man with the risk being greatest with milk stored at 4°C. The possible effects of other naturally occurring microorganisms in local milk products on the survival of C. fetus subsp. jejuni can, however, not be ignored.

The survival profile observed under all test conditions suggests that the organism if present in milk in large numbers would survive for considerable lengths of time. Thus, failure to isolate these organism from the milk and milk products sampled in this study suggests the absence of the organisms in these products.

CONCLUSION

The prevalence rate of Campylobacter fetus subspecies jejuni among cattle in Zaria and it's environment is quite low. The organism was not isolated from any of the unpasteurized milk and milk products. The low carriage rate observed in cattle faeces and the absence of the organism in milk and milk products suggest that cattle may not be an important source of the organism in this area.

The mechanism of pathogenicity of the C. fetus subsp. jejuni isolates was not elucidated. The isolates did not produce enterotoxins and were not invasive by standard procedures. These results therefore separate the pathogenesis of Campylobacteriosis from those of shigellosis, certain invasive E. coli infection, enterotoxigenic E. coli infection and cholera.

A good number of C. fetus subsp. jejuni organism are capable of surviving in contaminated milk for considerable lengths of time. The long periods during which the organism remained viable and at high numbers at all temperatures used in this study emphasizes the possible danger of infection to consumers who normally consume milk and low acid milk products as skim milk.

The isolates were susceptible to a number of commonly used antibiotics. Hence antibiotics such as Gentamicin, chloramphenicol, Doxycycline, tetracycline are recommended drugs of choice for treatment of Campylobacteriosis.

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