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ISOLATION AND CHARACTERIZATION OF CAMPYLOBACTER SPECIES FROM FOOD AND WATER

A THESIS

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DECLARATION

The materials contained within this thesis is my original work and has not previously been submitted to this or any other university.

(Muhammad Asif)

This thesis by Muhammad Asif is accepted in its present form by the Department of Biological Sciences, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirements for the Degree of Master of Philosophy in Microbiology.

Internal Examiner ____________

External Examiner ____________

Chairman ____________

Dated: 17.3.91

In the Name of Allah,

the Beneficent, the Merciful.

DEDICATED TO MY PARENTS

AND BROTHER

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MUHAMMAD ASIF

LIST OF ABBREVIATIONS

^{o}C.	Degree Centigrade (Celsius)
gm	Gram
IU	International Units
L	Litre
mg	Milligram
ml	Millilitre
mm	Millimeter
pH	- log H^{+} concentration
r.p.m.	Revolutions per minute
um	Micrometer
%	Percent (percentage)

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ABSTRACT

Six hundred and seventy eight (678) food samples comprising of raw and ready-to-eat foods including mutton, beef and poultry, raw and boiled milk and water were examined for the prevalence of Campylobacter species.

Primary isolation was made by selective enrichment in Brucella broth added with Skirrow's antibiotics supplement and ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP supplement). Enriched samples were plated on selective culture medium. Microaerobic conditions were maintained under both circumstances and the temperature was kept at 42°C. Isolates were characterized morphologically, physiologically and biochemically.

Of all the samples, 39 (5.75%) were found to be positive for Campylobacter species. Only 2 of the positive isolates were identified as *Campylobacter coli*, while the rest were identified as *Campylobacter jejuni*. Isolation percentage was highest in case of raw chicken (31.25%), followed by raw mutton (4.35%), chicken tikka (3.64%), and raw milk (0.95%), respectively.

No Campylobacter could be isolated from chicken curry, raw beef, beef/mutton grills and curries. Boiled milk, chlorinated and stream waters were also free of Campylobacter.

INTRODUCTION

Being the source of energy, food is one of the most vital requirements of life. However, if the food becomes contaminated by foodborne disease causing organisms, it can also become a source of disease and even a cause of death.

Ryu (1980) has claimed that the rate of transmission of pathogens through the oral route is higher as compared to the respiratory or dermal routes.

The incidence of foodborne disease is several times greater than that is reported, probably because in case of a mild attack, the duration of discomfort is very short and is not reported to the concerned medical authorities. These illnesses are not only confined to the developing countries, but, have been also reported from several western countries including the United States (Archer and Kvenberg, 1985) and parts of Europe and North America (Sharp, 1987). In Scotland foodborne infections have been reported as early as 1956 (Sharp *et al.*, 1986).

Campylobacters are Gram negative, microaerophilic bacteria and are an important and frequent cause of human gastroenteritis (Butzler and Skirrow, 1979).

It was towards the beginning of the present century (Mc Fadyean and Stockman, 1913) that Campylobacters were first isolated. Originally, they were termed as "microaerophilic Vibrios". They were reported as causative agents of abortion in sheep. Five years later, Smith (1918) claimed the association of similar organisms with bovine abortions. A year later Smith and Taylor (1919), isolated this organism from aborted bovine fetuses and named it as *Vibrio fetus*. Eight years afterwards Smith and Orcutt (1927), isolated Vibrios from livers and spleens obtained from calves with diarrhoea.

The association of these "microaerophilic Vibrios" with human disease was first demonstrated when there was a large institutional outbreak of gastroenteritis that affected about 350 people (Levy, 1946). *Vibro fetus*, *V. jejuni* and *V. coli* were named due to their association with specific disease in animals rather than any taxonomic difference among them.

It was another eleven years after which it was suggested that human infections were caused by *Vibrio fetus*, which were termed as "related Vibrios" (King, 1957). The report presented substantive documentation indicating these "related Vibrios" (*V. jejuni*) could be a significant zoonotic cause of human enteritis. The diarrhoeal agent was difficult to recover

from fecal specimens, because of its unique growth requirements. It was also found that the agent (V. fetus) recovered from blood of patients with systematic infection would not grow at 42°C., whereas the "related Vibrios" (V. jejuni) isolated from diarrhoeal specimens grew at 42°C.

The present generic term, Campylobacter ("curved rod" in Greek), was proposed by Sebald and Veron in (1963) since the "microaerophilic Vibrios" differed significantly from Vibrio cholerae and certain other Vibrios and Vibrio-like organisms due to their biochemical and physiological properties and their DNA base pair ratios.

In 1972, at Brussels, the application of veterinary techniques to the culture of human material provided the necessary breakthrough (Dekeyser et al., 1972). C. jejuni was isolated from five percent of children with diarrhoea (Butzler et al., 1973) and later Skirrow (1977) confirmed and extended this observation.

Campylobacter jejuni is a small microaerophilic, Gram negative bacteria, belonging to family Spirillaceae, which has a characteristic spiral (S-shaped) morphology (Simbert, 1984). It constantly produces small and tightly coiled spirals having a

mean wavelength and amplitude of 1.12 and 0.48 um, respectively (Karmali *et al.*, 1981). They are extremely sensitive to the effects of low levels of hydrogen peroxide and superoxide radicals, despite the fact that catalase and superoxide dismutase are present inside the bacterial cell (Hoffman and Goodman, 1982). *Campylobacter jejuni* requires special medium and conditions for optimal growth and isolation from clinical and environmental sources. Cells from fresh cultures are curved or spiral, motile and non sporulating rods. In old cultures, cells are transformed to coccoid forms which are non-viable (Moran and Upton, 1986). The transformation is more rapid on solid media than in broth or semisolid media and is enhanced by exposure to air and room temperature (Simbert, 1978). Best growth is observed under 5% oxygen, 10% carbon dioxide, and 85% nitrogen at 42°C. Several basal media with various supplements are used for the isolation and growth of *C. jejuni* (Boltan and Coates, 1983; NG, Lai - King *et al.*, 1985; Van Landuyt *et al.*, 1987; NG, Lai-King *et al.*, 1988; Walmsley and Karmali, 1989).

Campylobacters have now been isolated from a wide range of hosts, including house flies (Rosef and Kapperud, 1983), cockroaches (Umunnabuike and Irokanulo, 1986), rats (Seguin *et al.*, 1986), crows and domestic pigeons (Ito *et al.*, 1988), cats and dogs (Latinovic and Popovic , 1987) and ducks and geese

(Pacha *et al*., 1988). It is now accepted that the animal reservoir represents a major source of infection for humans (Prescott and Munroe, 1982).

Campylobacter is now recognized as a significant human enteric pathogen. The infective dose is only 500 organisms in a glass of milk (Robinson, 1981). The route of transmission from animal resources to humans may be due to direct contact with contaminated animals or animal carcasses (Skirrow, 1977; Blaser *et al*., 1978), or more commonly, *C. jejuni* is transmitted indirectly through the infection of contaminated food or water (Knill *et al*., 1978). Person to person transmission is also possible in case of humans that are excreting large numbers of *C. jejuni*.

C. jejuni is very quickly killed by hydrochloric acid at a pH of 2.3, indicating that gastric juice is an effective barrier against infection (Blaser *et al*., 1980). However, if the pathogen manages to cross the gastric barrier, it must be able to reach and colonize the mucosal surface in order to be effective. Usually, the incubation period of the gastroenteritis ranges from 2-5 days, it may extend upto 10 days (Skirrow, 1977). The main symptoms associated with the disease are diarrhoea (94%), fever (50%), bleeding in stools (44%), abdominal pain

(31%), and Vomiting (10%) (Iwami *et al.*, 1987).

The major method by which Campylobacters cause food poisoning is through the production of potent toxins. It was Ruiz-Palacios *et al.* (1983), who described a cholera like enterotoxin produced by *C. jejuni*, which is inactivated by heat, high or low pH and has a molecular weight of about 10^4-10^5 daltons. Campylobacter, like all Gram negative organisms, also possesses lipo-polysaccharides (LPS) with endotoxic properties (Blaser and Reller, 1981).

Several reports have been published that indicate the worldwide prevalence of Campylobacter enteritis. The organism has been isolated from patients in North and South America, Europe, Africa, Asia and Australia (Stern and Kazmi, 1989).

C. jejuni or *C. coli* organisms may exist as commensals in the intestinal tracts of a wide variety of wild and domestic animals. Furthermore, recently some of the serotypes of Campylobacter that cause disease in humans have been isolated from animals (Luechtefeld *et al.*, 1980).

Most commercially raised poultry have Campylobacters in their intestinal flora. Isolations have been made from poultry

early in the growing process although some flocks apparently completely escape infection (Cruickshank et al., 1982).

During the process of slaughtering, C. jejuni spreads from the intestinal contents to the carcasses. In those flocks in which intestinal carriage was not detected, carcasses were not contaminated (Cruickshank et al., 1982).

In the United States of America, the majority of chicken carcasses sold at retail markets were contaminated with C. jejuni (Eiden and Dalton, 1980; Grant et al., 1980; Park et al., 1981).

Grant et al. (1980) reported an average of 4.4×10^6 C. jejuni per gram of feces from live chickens in a New York city (U.S.A.) poultry market. Fecal samples from laying hens in Wisconsin, U.S.A. (Doyle, 1984) and poultry ranches in California, U.S.A. (Smitherman et al., 1984) have been shown to harbour the organisms.

Other reports document the presence of C. jejuni on fresh chicken broilers (Park et al., 1981; Shanker et al., 1982; Wesley et al., 1983), wings (Kinde et al., 1983), giblets (Christopher et al., 1982; Wempe et al., 1983; Fricker, 1984),

and egg shell surfaces but not egg contents (Doyle, 1984). Turkey processing plants (Luechtefeld and Wang, 1981; Yusufu et al., 1983), eggs, poults and brooder house facilities (Acuff et al., 1982) have also been shown to harbour C. jejuni.

While C. jejuni will not grow at temperatures less than about 35°C. it does survive on raw chicken at 4°C. (Blankenship and Craven, 1982) and has been recovered from carcasses and livers (Oosterom et al., 1983; Park et al., 1983; Abram and Potter, 1984; Stern et al., 1984). Carbon dioxide may enhance survival on chicken drumsticks at 4°C. (Blankenship and Craven, 1982).

Smith and Muldoon (1974) reported incidence of C. jejuni in chicken necks (2% of 121), in chicken liver (0% of 25) and (5% of 19) from chicken carcasses.

In another study 750 chicken carcasses were screened for C. jejuni. None of the carcass was found positive for C. jejuni (Goren and de Jong, 1980).

Christopher et al. (1982), screened 60 chicken liver and 64 chicken gizzard samples, and claimed 85% samples positive in case of liver and 89% in case of gizzard.

Simmons and Gibbs (1979), found 80% of 35 chicken carcasses positive for *C. jejuni*. In case of frozen chicken carcasses Norberg (1981) claimed 22% incidence.

The first prominent outbreak of Campylobacter enteritis reported in association with undercooked chickens occurred in the Netherland among soldiers of a survival exercise (Brouwer *et al.*, 1979). Of 123 cadets given live chickens to prepare for their evening meal, 89 became ill with symptoms of enteritis within the following week.

Thirty four fecal samples yielded *C. jejuni*. The birds had been consumed in a nearly raw state, therefore, any Campylobacter contaminating the meat would likely have been consumed in a viable state and presumably, in infectious form.

Ingestion of poorly cooked chicken was thought to be an important exposure in an outbreak occurring in England after a catered banquet (Skirrow *et al.*, 1981).

The prevalence of *C. jejuni* and *C. coli* in 396 frozen and 405 fresh meat samples was examined (Stern *et al.*, 1984). About 12% of fresh meat samples were positive as compared to 2.3% of the frozen, suggesting the lethal effects of freezing on the

organisms. During a survey carried out in 185 samples (Stern *et al.*, 1985) of retail meat sold in the United States, it was revealed that *C. jejuni* or *C. coli* both were present in 3.6% of ground beef, 4% of beef flank, 8.1% of lamb stew, 4.2% of pork sausage, 5.0% of pork chop, and 29.7% of chicken samples. Hence, the types of food epidemiologically implicated as vehicles of outbreaks of Campylobacter enteritis also have been shown to convey *C. jejuni*.

Turnbull and Rose (1982) reported the isolation of *C. jejuni* from only 21 of 2015 (1%) retail beef samples (minced meat) and 0.1% of 1448 beef and swine sausage/sausage meat.

Stern (1981) screened freshly slaughtered carcasses of cattle and sheep. He claimed 2% of 58 samples in case of cattle and 24% of 59 samples in case of sheep, positive for *C. jejuni*.

Hudson and Roberts (1981) could not get any positive results from 100 freshly slaughtered beef carcasses.

Unpasteurized milk has been the most commonly implicated vehicle in foodborne outbreaks of Campylobacter enteritis (Stern and Kazmi, 1989).

The largest reported foodborne outbreak of Campylobacter enteritis was associated with consumption of raw milk, and resulted in illness among 2500 school children. In that outbreak, two strains of *C. jejuni* were recovered from ill children, but the organism was not isolated from the milk.

During 1983, a survey was carried out in Cincinnati, Ohio (U.S.A.), in which 1.5% of incidence of *C. jejuni* in milk from bulk tanks was observed (Lovett *et al.*, 1983). The method was capable of detecting less than 1 organism per ml. In a study carried out in the United States during 1986 (McManus and Lanier, 1987), raw milk samples were collected from bulk tank trucks of milk suppliers in Wisconsin, Michigan and Illinois. Only one sample out of 237 yielded *C. jejuni*.

In a study carried out at the University of Wisconsin (Doyle and Roman, 1982), *C.jejuni* was found in 1 of 108 (0.9%) milk samples obtained from the bulk tanks of nine grade A dairy farms.

Since the organism exists in cow feces, it is not surprising that it may be found in raw milk, and the degree of contamination would be expected to vary depending upon milking procedures.

Lander and Gill (1980) have shown that _C.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.jejuni_ in milk.

Raw milk was identified as a vector in an outbreak in England during 1980 (Robinson and Jones, 1981). About 75 out of 300 college students were affected. _C. jejuni_ was isolated from the milk samples, and 46 students had antibodies against it.

Christopher _et al_. (1982) screened 100 milk samples from bulk tank and were unable to recover Campylobacters from any of the hundred samples.

Outbreaks have been reported in Colorado in 1978 (Blaser _et al_., 1979), and Oregon, Kansas, Arizona, Georgia, and Minnesota in 1981 (Blaser _et al_., 1984), with a total of over 500 persons affected.

In England, outbreaks have been associated with unpasteurized and with improperly pasteurized milk. In the U.S.A., outbreaks have been reported only affecting persons who prefer to drink raw milk (Blaser _et al_., 1984).

An outbreak described at a boy scout camp was associated with cake icing, *C. jejuni* could not be isolated from any of the icing ingredients, but the icing could have been contaminated at the time of preparation by the cook (Blaser *et al.*, 1982).

Water has been implicated in several large outbreaks of Campylobacter enteritis. There have been several major outbreaks in the U.S.A. of human infection with *C. jejuni*, which resulted in an interest in the aquatic environment as vehicle of transmission of this organism. Municipal water systems (Mentzing, 1981; Palmer *et al.*, 1983) and water from mountain streams (Taylor *et al.*, 1983) have been implicated as probable sources of infection.

A number of reports describe the isolation of *C. jejuni* from various aquatic habitats (Sokal and Rohlf, 1969; Pearson *et al.*, 1977; Knill *et al.*, 1978; Bolton *et al.*, 1982), but there are only a few instances in which the organism has been isolated from the water system implicated as the source of human infection (Mentzing, 1981; Vogt *et al.*, 1982; Palmer *et al.*, 1983; Taylor *et al.*, 1983).

In October 1980, a municipal outbreak occurred in

Sweden involving an estimated 2,000 persons, approximately 20% of the community at risk (Mentzing, 1981). During this outbreak, C. jejuni was isolated from rectal swabs from 221 (84%) of 263 persons cultured. The water system was deep ground water.

Another outbreak occurred in a small community in Northern British Columbia in July 1980, where an estimated 700 persons had Campylobacter enteritis. The outbreak correlated geographically with the unchlorinated town water supply, which was gravity fed from a reservoir or pumped directly from a creek. (Health and Welfare, Canada, 1981).

In nature, C. jejuni has been isolated from stream and river water, (Knill et al., 1982), and from sea water (Knill et al., 1978).

In a study of isolations from riverine water in the Southampton area in England, Campylobacters were isolated from 50.4% of 540 water samples. (Knill et al., 1982).

In the United States, at least two major waterborne outbreaks have been reported, affecting almost 4,300 people (Blaser and Reller, 1981; Vogt et al., 1982). In these outbreaks, C. jejuni was implicated by its isolation from

individuals suffering from diarrhoea, but the organism could not be isolated from the incriminated water sources.

Fresh mushrooms (Agaricus bisporus) were obtained from grocery stores and it was possible to isolate C. jejuni from 1.5% samples (Doyle and Schoeni, 1986).

Campylobacter jejuni is now recognised as an important cause of foodborne disease throughout the world. A recent case-control study of Salmonellosis and Campylobacteriosis conducted in Seatle, Washington (Seatle-King County Department of Public Health, 1984) reported that the incidence of Campylobacteriosis was 2.5 fold greater than that of Salmonellosis.

In Pakistan Campylobacter species has been isolated from stools of patients with diarrhoea/enteritis (Siddiqui, 1987; Pakistan Medical Research Council, 1989), but, its isolation has never been attempted from foods. Therefore, to link the transmission of this causative agent of diarrhoea with various foods and waters the present study was conducted. This study shall elucidate the epidemiology of Campylobacter enteritis, involvement of different food types in transmission of Campylobacterosis and would also spotlight the problems of foods and sanitation. The results of this study would help to minimize

foodborne Campylobacterosis hazards due to the consumption of infected foods.

MATERIALS AND METHODS

COLLECTION OF SAMPLES

Six hundred seventy eight (678) samples comprising of raw and ready-to-eat meat (including mutton, beef and poultry), raw and boiled milk and water were randomly collected from food service shops of various markets, street vendors and road-side hotels of Talagang, Murree, Barakohu, Rawat, Kalarseyedan, Taxila, Fatehjang and townships of District Rawalpindi/ Islamabad. The samples were collected aseptically in sterile glass vials and were transported to the laboratory on ice. Microbiological examination of the samples was proceeded as soon as they reached the laboratory.

CHEMICALS AND CULTURE MEDIA

All chemicals and culture media used in this study were of laboratory grade and were purchased from Sigma Chemicals Co. St. Louis, Missouri, U.S.A.; E. Merck, Darmstadt, Federal Republic of Germany; Oxoid Limited, Basingstoke, Hampshire, U.K.; Difco Laboratories, Detroit, Michigan, U.S.A.; Riedel-de-Haen, Seelze, Germany; BDH Chemicals Limited, Poole, U.K.; Api, Analytab Products, Sherwood Medical, Plainview, New York, U.S.A.;

Gibco Laboratories, Life Technologies Inc., Madison, Wisconsin, U.S.A. and BBL Microbiology Systems, Becton Dickinson and Co. Cockeysville, MD 21030 U.S.A.

CULTURE MEDIA USED FOR PRIMARY ISOLATION

The following culture media were used:

Brucella broth (Difco)

Brucella Agar

To Brucella broth (Difco) 1.5 percent Bacto-Agar (Difco) was added.

FBP Supplement

FBP Supplement which contained following chemicals was incorporated in one litre of the medium, in the amounts prescribed by George et al.(1978).

Ferrous sulphate (Merck)	0.5 gm
Sodium metabisulphite (Merck)	0.5 gm
Sodium pyruvate (BDH)	0.5 gm

Skirrow's Antibiotics Supplement

This supplement was prescribed by Skirrow, (1977). To

500 ml of medium which was cooled to 50°C. one vial of antibiotics supplement (Oxoid code SR 69) containing the following antibiotics was added.

Vancomycin	5.0 mg
Trimethoprim Lactate	2.5 mg
Polymyxin B	1250.0 IU

Defibrinated Sheep Blood

Sheep blood was collected aseptically from jugular vein in a sterile flask containing glass beads. The collected blood was shaken to avoid clotting.

Blood Agar

Blood Agar Base (Oxoid)	40.0 gm
Defibrinated sheep blood	70.0 ml
Distilled water	1.0 L

To Blood Agar Base, which was cooled to 50°C. 7% defibrinated sheep blood was added.

PREPARATION OF SELECTIVE ENRICHMENT BROTH

Enrichment broth was prepared as prescribed by

Park et al. (1983). Brucella broth was prepared according to instructions provided by the manufacturer. It was sterilized by autoclaving at 121°C. for 15 minutes. The Brucella broth was later on cooled to 50°C. and filter sterlized FBP supplement was added, after this 2 vials of Skirrow's antibiotic supplement (each reconstituted in 5 ml of distilled water) filter sterilized were added. Finally 70 ml of defibrinated sheep blood was added and was shaken gently for thorough mixing.

PREPARATION OF SELECTIVE AGAR PLATES

For preparing selective agar plates, 15 gm of Bacto-Agar was added to one litre Brucella broth and the rest of the procedure was similar as described in case of enrichment broth. The medium was then poured into petri plates, which were then wrapped in brown paper to protect from light, were sterility tested and used fresh.

ISOLATION FROM MEAT

Isolations were made from chicken, mutton and beef. Raw and cooked meats were used for primary isolation. Cooked meat was in the form of kabab, tikka and curry.

The protocol as prescribed by Wesley et al. (1983) was followed. Twenty gram of sample was added to 50 ml of 0.1% peptone water in a sterile polythene bag and hand massaged. Ten

millilitre of rinsed fluid was then added to 100 ml of enrichment broth in case of raw meat while for cooked meat, the sample was blended with 100 ml of enrichment broth using a Mounilex blender at low speed (1000 r.p.m.) for 2 minutes. This mixture was transferred to 250 ml round bottomed flask with a tubing for the passage of gas and incubated at 42°C. in an anaerobic jar containing an atmosphere of 5% oxygen, 10% carbon dioxide and 85% nitrogen, which was created by using Campylobacter gas generating kit (BBL) for 48 hours. After 48 hours incubation a loopful of this enrichment broth was streak cultured on selective agar plates, and were incubated under microaerobic conditions at 42°C. for 24 hours.

ISOLATION FROM MILK

The protocol of Wesley et al. (1983) was used with slight modifications. About 40 ml of milk was added to 100 ml of enrichment broth in a round bottomed flask with a tubing for passage of gas. The enrichment broth was then incubated at 42°C. in an anaerobic jar containing an atmosphere of 5% oxygen, 10% carbon dioxide and 85% nitrogen for 48 hours. After 48 hours incubation a loopful from this broth was streaked on selective agar plates which were incubated for 24 hours at 42°C. under microaerobic conditions.

ISOLATION FROM WATER

The protocol as prescribed by Blaser and Cody (1986) was followed. Two hundred and fifty millilitre of water sample were collected in round bottomed flask, prefiltered through a sterlized millipore filter (0.6 um) and then filtered through a sterlized millipore filter (0.45 um). After filteration, filter paper was removed from the holder with the help of sterile forceps and placed face down on the selective agar plate and incubated for 12 hours in microaerobic environment. After 12 hours incubation, filter paper was removed and the growth was streaked to get the isolated colonies.

Suspected colonies were picked and streaked on Blood agar plates to get ample amount of pure culture for further studies and storage.

CHARACTERIZATION OF CAMPYLOBACTER ISOLATES

MORPHOLOGY

Isolated Campylobacter colonies were tested for cork-screw type motility and stained with Gram stain for examining typical morphology of isolates. The colonies were maintained on Blood agar.

For further characterization and identification of *C. jejuni* and *C. coli* the following tests were employed.

HIPPURATE HYDROLYSIS

Composition of the medium used

Sodium Hippurate (Sigma)	10.0 gm
Distilled water	1.0 L

Chemicals used

Ninhydrin (Sigma)	3.5 gm
Acetone (Merck)	50.0 ml
Butanol (Riedel)	50.0 ml

PROCEDURE

Hippurate hydrolysis test as described by Harvey (1980) was performed. A 1 % solution of sodium hippurate was prepared in sterile distilled water, dispensed in 2 ml amounts into screw-capped tubes, and stored at -20°C. until used. A 3.5 % solution of ninhydrin was freshly prepared in a 1:1 mixture of acetone and butanol. At the time of test, tubes were thawed and a heavy suspension of 24-48 hours growth from plate was emulsified and the suspension was incubated for 2 hours at 37°C. After incubation, 0.2 ml of the ninhydrin solution was added and mixed well, the screw cap was tightened, and the tubes were reincubated. After 10 minutes incubation, the tubes were examined

for colour development, a deep purple colour was indicative of positive test.

CATALASE TEST

A drop of 3% hydrogen peroxide (Merck) was placed on a clean microscopic slide. A visible amount of bacterial growth was added aseptically with the help of an inoculating loop, and mixed. Bubble formation was taken as positive catalase test.

OXIDASE TEST

A 1% solution of oxidase reagent was freshly prepared by dissolving tetramethyl para-phenylene diamine dihydrochloride (Sigma), (0.05 gm in 5 ml distilled water). A sterile filter paper stripe was impregnated with 2-3 drops of the reagent and heavy suspension of bacteria was smeared on filter paper. Development of a purple colour within 10-20 seconds was a positive reaction.

NITRATE REDUCTION TEST

Composition of the medium used

Heart Infusion Agar (Difco)	25.0 gm
Potassium Nitrate (Merck)	2.0 gm
Distilled water	1.0 L

Chemicals used

Nitrate reagent "1" (api)

Sulfanilic Acid	8.0 gm
5N Acetic Acid	1.0 L

Nitrate reagent "2" (api)

N,N-Dimethyl alpha-naphthylamine	6.0 ml
5N Acetic Acid	1.0 L

PROCEDURE

To 1000 ml of distilled water 25 gm of Heart Infusion agar and 2 gm of potassium nitrate were added. The mixture was cultured and incubated under microaerobic conditions at 42 ^{o}C. for 24 hours. By adding 0.25 ml of each nitrate reagent "1" and "2". Development of red colour indicated positive results.

H_2S PRODUCTION

Composition of the medium used

Kligler's Iron Agar (Gibco)

Peptone (Animal Tissue Casein Polypeptone)	20.0 gm
Lactose	10.0 gm
Dextrose	1.0 gm
Sodium Chloride	5.0 gm
Ferric Ammonium Citrate	0.2 gm

Sodium Thiosulphate	0.3 gm
Agar	12.5 gm
Phenol Red	0.025 gm
Distilled water	1.0 L

PROCEDURE

For detection of H_2S gas Kligler's iron agar (KIA) slants with butt were used. Twenty four hours old culture in Brucella broth was streaked and stab cultured in Kligler's iron agar and incubated for 48 hours under microaerobic conditions. A filter paper stripe impregnated with 10% lead acetate was hanged into the tube with the rim of the tube containing KIA. Blackening of stripe indicated H_2S production.

TEMPERATURE TOLERANCE

Composition of the medium used

Brucella broth (Difco)	28.0 gm
Bacto-Agar (Difco)	15.0 gm
Defibrinated sheep blood	100.0 ml
Distilled water	900.0 ml

PROCEDURE

Thermophilic nature of individual isolate was observed by incubating cultures at 25°C., 37°C. and 42°C. (Butzler &

Skirrow, 1979). Cultures were streaked on Blood agar plates (Brucella agar with 10% sheep blood) and incubated at 25°C., 37°C. and 42°C. for 24 hours in microaerobic environment, and growth was recorded.

SODIUM CHLORIDE TOLERANCE

Composition of the medium used

Brucella Broth (Difco)	28.0 gm
Bacto- Agar (Difco)	1.6 gm
Sodium chloride (Merck)	35.0 gm
Distilled water	1.0 L

PROCEDURE

Test culture was inoculated in brucella broth containing 0.16% Bacto-Agar and sodium chloride in concentration of 3.5%. Tubes were incubated for 24 hours at 42°C. in microaerobic atmosphere.

GLYCINE TOLERANCE

Composition of the medium used

Brucella broth (Difco)	28.0 gm
Bacto-Agar (Difco)	1.6 gm
Glycine (BDH)	10.0 gm
Distilled water	1.0 L

PROCEDURE

Freshly grown Campylobacter isolates were inoculated into Brucella broth with 0.16% Bacto-Agar and glycine. Tubes were then incubated at 42°C. for 48 hours in an atmosphere of 5% oxygen, 10% carbon dioxide and 85% nitrogen. A cloudy appearance, mostly near the surface, indicated growth of C.jejuni.

STORAGE AND MAINTENANCE OF CAMPYLOBACTER

Short term storage was made in Thioglycollate medium for routine experiments.

Composition of the medium used

Fluid Thioglycollate Medium (BBL)

Pancreatic Digest of Casein	15.0 gm
L-Cystein	0.5 gm
Dextrose (anhydrous)	5.0 gm
Yeast Extract	5.0 gm
Sodium Chloride	2.5 gm
Sodium Thioglycollate	0.5 gm
Resazurin	0.001 gm
Agar	0.75 gm
Distilled water	1.0 L

PROCEDURE

All the cultures were inoculated in Thioglycollate semisolid medium having 0.16% Bacto-Agar , in screw capped tubes and subcultured on Blood agar after every fortnight again inoculated with sterile swabs in semisolid medium for further two weeks.

LONG TERM STORAGE IN FREEZING MEDIUM

Composition of the medium used

Nutrient broth (Oxoid)	13.0 gm
Bacto-Agar (Difco)	1.2 gm
Ferrous sulphate (Merck)	0.5 gm
Sodium metabisulphite (Merck)	0.5 gm
Sodium pyruvate (BDH)	0.5 gm
Glycerol (Merck)	200.0 ml
Distilled water	800.0 ml

PROCEDURE

Long term storage was made in FBP broth (Freezing medium), which contained Nutrient broth (Oxoid) with FBP supplement and glycerol. Twenty-four hours old growth of the isolates was inoculated with a sterile swab into the freezing medium and then stored at -70°C.

RESULTS

Occurrence of Campylobacter species in foods, including raw and cooked meats, milk and water was studied. Food and milk samples were collected in sterile glass vials and water samples were collected in sterile round bottomed flasks. Sample collection was made from vendor shops, hotels and restaurants of Talagang, Murree, Barakohu, Rawat, Kalarseyedan, Taxila, Fatehjang and townships of District Rawalpindi/Islamabad. Collected samples were transported to the laboratory on ice and were processed as soon these samples reached the laboratory.

Efforts were made to isolate Campylobacter species out of the aforementioned samples using selective enrichment broth and selective agar. The isolates were confirmed in their identity on the basis of morphological, physiological and biochemical properties.

MORPHOLOGY OF THE ISOLATES

For morphological studies of the isolates, Blood agar plates were used. After 24 hours incubation in microaerobic conditions at 42°C., Blood agar plates showed grey, flat and irregular colonies, measuring 1-3 mm in diameter (Fig. 1).

Growth on Blood agar plates was Gram stained and examined under the microscope after 24 hours of initial incubation. They were Gram negative, placed scattered, S-shaped, some were spiral in shape with several undulations, 0.5 to 8 um in length and 0.2 to 0.5 um in width with tapering ends (Fig. 2).

For further confirmation, the cultures were again examined after every 24 hours till 96 hours. Transformation from S-shapped to coccoid shape was recorded (Figs. 3-5).

MOTILITY

Motility of the isolates was checked using hanging drop method. Blood agar plates were used for culturing the isolates for motility testing. All isolates showed typical cork-screw like motility.

BIOCHEMICAL CHARACTERISTICS OF THE ISOLATES

HIPPURATE HYDROLYSIS

The isolates were subjected to hippurate hydrolysis. Thirty seven out of 39 isolates showed positive reaction i.e., they hydrolysed the hippurate. The remaining 2 isolates were negative for this test.

CATALASE TEST

All the isolates showed positive catalase test by producing bubbles, with hydrogen peroxide on a test colony.

OXIDASE TEST

All the isolates were found to be positive for oxidase test as they produced a purple colouration in 10-20 seconds on a sterile filter paper stripe, impregnated with 2-3 drops of tetramethyl para-phenylene diamine dihydrochloride.

NITRATE REDUCTION TEST

All the isolates were found positive for nitrate reduction, since red colouration was observed in all the cases using Heart Infusion agar with potassium nitrate and nitrate reagents "1" and "2".

HYDROGEN SULPHIDE PRODUCTION

Hydrogen sulphide gas was produced by all the isolates. Kligler's Iron agar was employed as a growth medium, a filter paper stripe impregnated with 10% lead acetate was hanged into the tube for this test.

TEMPERATURE TOLERANCE

When the isolates were tested for temperature

tolerance, all the isolates showed growth at 42°C. and 37°C., whereas all the isolates failed to grow at 25°C.

SODIUM CHLORIDE TOLERANCE

None of the isolates could grow on a medium having 3.5% concentration of sodium chloride.

GLYCINE TOLERANCE

One percent glycine was tolerated by all the test isolates.

On the basis of aforementioned morphological, physiological and biochemical tests 37 isolates were confirmed as _Campylobacter jejuni_, because they were found to be Gram negative, motile, hippurate hydrolysing, catalase and oxidase positive, showed growth at both 42°C. and 37°C., but did not grow at 25°C., hydrogen sulphide gas was produced, did not grow in the medium containing 3.5% concentration of sodium chloride and 1% glycine was tolerated. Two isolates were identified as _Campylobacter coli_ as they showed all results similar to _Campylobacter jejuni_ except they did not hydrolyse hippurate (Table 1). Both the isolates identified as _Campylobacter coli_ were recovered from raw chicken.

INCIDENCE RATE OF CAMPYLOBACTER SPECIES

Out of 678 food samples, 39 (5.75%) were positive. Table number 2 shows that maximum number of Campylobacter species were isolated from raw chicken (31.25%), followed in decending order by raw mutton (4.35%), chicken tikka (3.64%) and raw milk (0.95%).

Not a single isolate was recovered from water, boiled milk, chicken curry, mutton (in the form of kabab, tikka and curry) and beef (raw as well as in the form of kabab, tikka and curry).

Table 1. MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF CAMPYLOBACTER ISOLATES

Characteristics	*C. jejuni*	*C. coli*
Gram Reaction	-	-
Motility	+	+
Hippurate Hydrolysis	+	-
Catalase	+	+
Oxidase	+	+
Nitrate reduction	+	+
H_2S in KIA (with 10% lead acetate stripe)	+	+
Growth at		
- 25°C	-	-
- 37°C	+	+
- 42°C	+	+
Growth in		
- 1% Glycine	+	+
- 3.5% NaCl	-	-

Table 2. OCCURRENCE OF CAMPYLOBACTER SPECIES IN FOODS, MILK AND WATER

Type of Meat/ Dish, Milk and Water	No. of samples Tested	No. of samples Positive	Percentage (%)
BEEF (125)			
- Raw	19	0	0.0
- Kabab	53	0	0.0
- Tikka	36	0	0.0
- Curry	17	0	0.0
MUTTON (117)			
- Raw	23	1	4.35
- Kabab	47	0	0.0
- Tikka	27	0	0.0
- Curry	20	0	0.0
CHICKEN (196)			
- Raw	112	35	31.25
- Tikka	55	2	3.64
- Curry	29	0	0.0
MILK (150)			
- Raw	105	1	0.95
- Boiled	45	0	0.0
WATER (90)			
- Chlorinated water	83	0	0.0
- Stream water	7	0	0.0
TOTAL	678	39	5.75

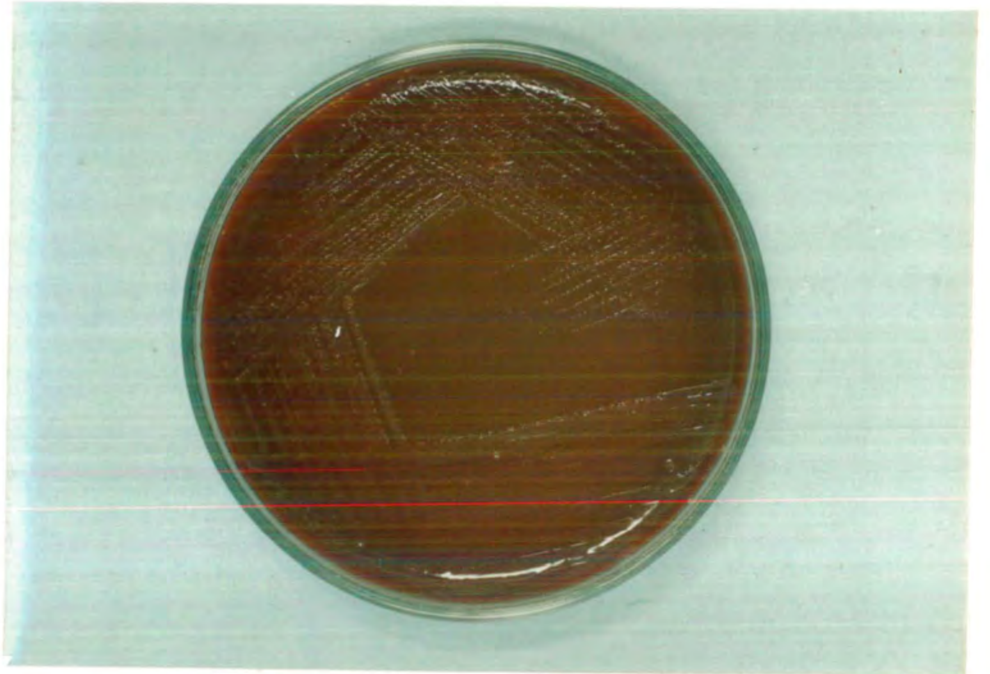

Figure 1. Typical *Campylobacter jejuni* colonies on Blood agar plate after 24 hours incubation.

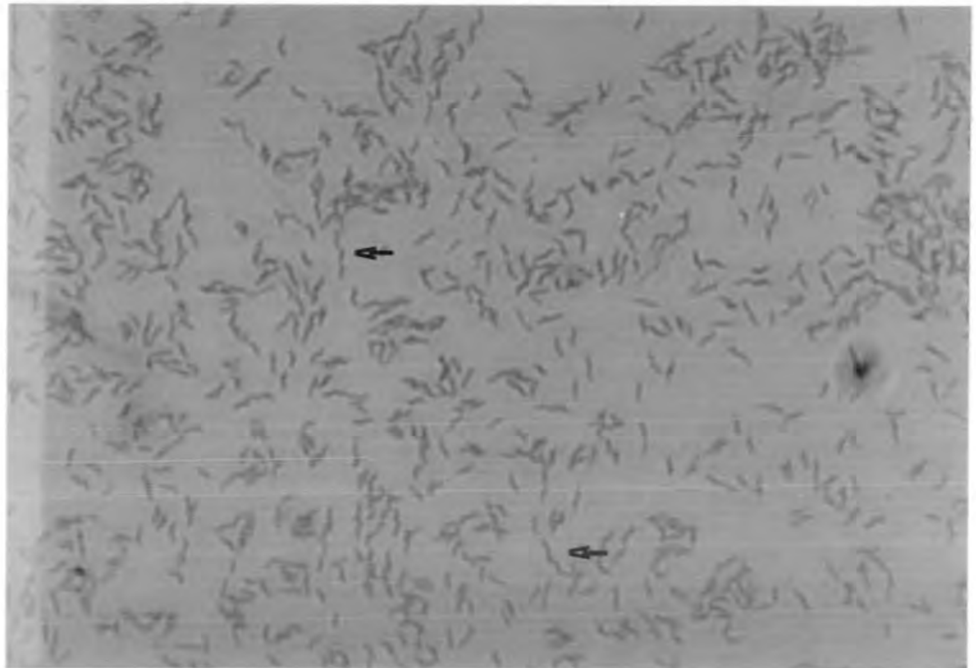

Figure 2. Photograph of Gram stained smear prepared from 24 hours old culture of _Campylobacter jejuni_ on Blood agar showing typical spiral form and arrangement of the Campylobacter species . x576.

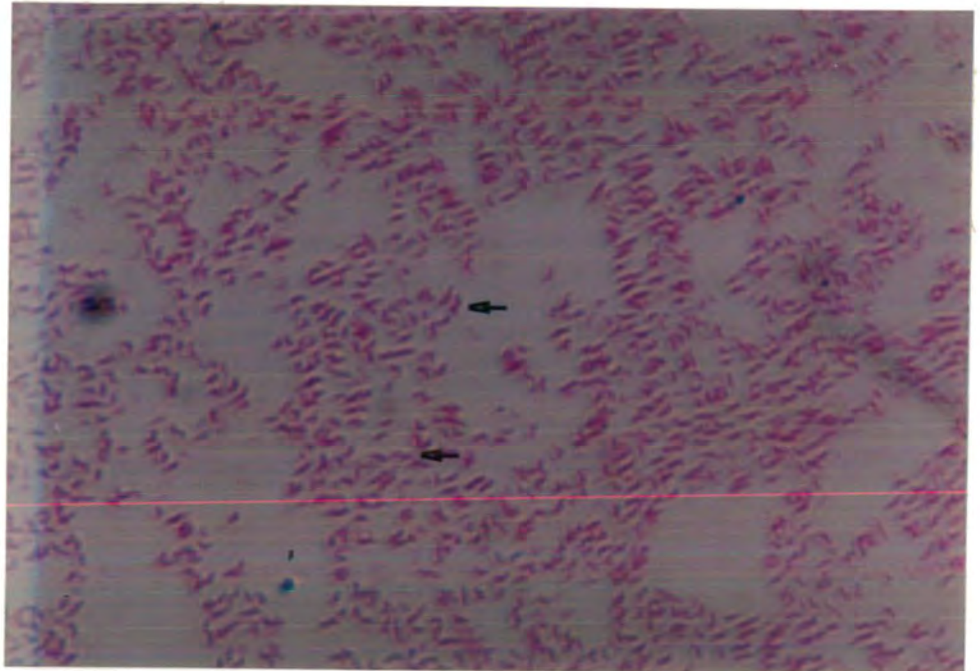

Figure 3. Photograph of Gram stained smear prepared from 48 hours old culture of Campylobacter jejuni on Blood agar showing transformation from typical spiral shape to S-shape . x576.

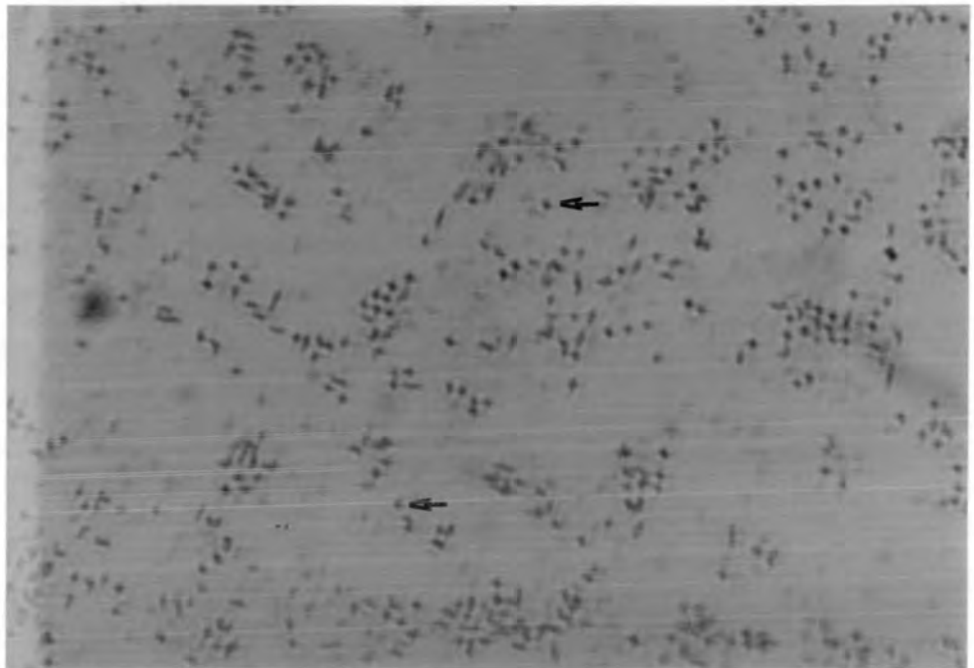

Figure 4. Photograph of Gram stained smear prepared from 72 hours old culture of Campylobacter jejuni on Blood agar showing transformation from S-shape to coccoid shape . x576.

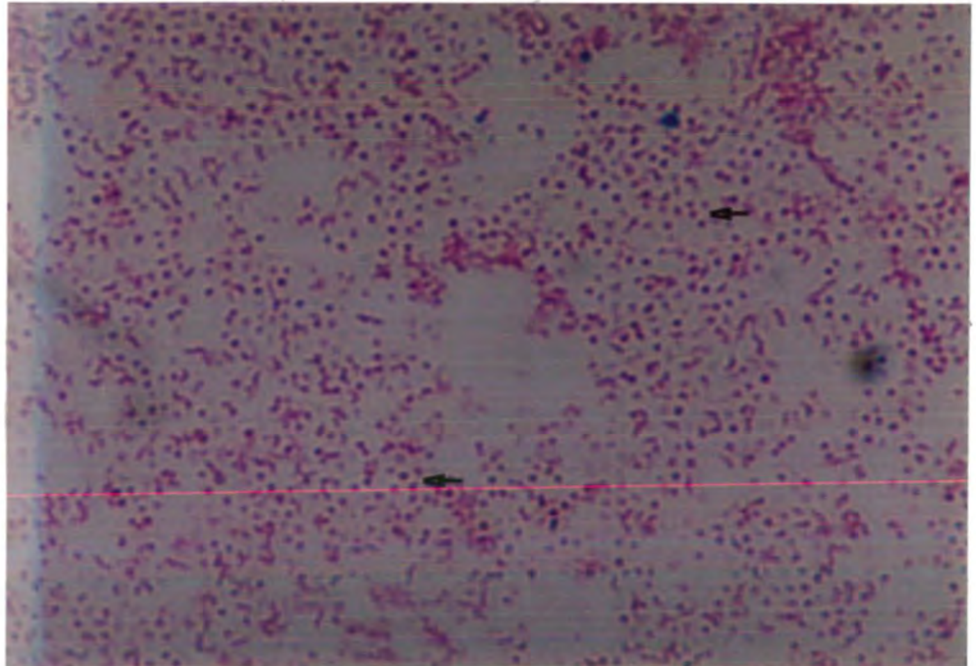

Figure 5. Photograph of Gram stained smear prepared from 96 hours old culture of Campylobacter jejuni on Blood agar showing increased transformation to coccoid form x576.

DISCUSSION

Six hundred and seventy eight (678) food samples consisting of raw and cooked meats, milk and water were examined for the presence of Campylobacter species. The isolates were characterized on the basis of their morphological, physiological and biochemical properties. Only 39 samples (5.75%) were found to be positive for Campylobacter species.

In case of poultry, upto 89% positive samples have been reported (Christopher et al., 1982). Whereas , in another study out of 750 samples no positive sample for C. jejuni could be reported (Goren and de Jong, 1980). In the present study 31.25% positivity was detected in case of raw chicken.

Though heat is sufficient to kill any C. jejuni present in raw meat, yet undercooked meat can be a source of Campylobacter contamination (Brouwer et al., 1979). Out of 55 samples of chicken tikka, 2 (3.64%) were positive for Campylobacter species, which may be due to cross contamination between raw and prepared tikkas attributable to poor food hygiene and neglect in food handling and defective method of mannual preparation of tikkas where the same person handles the raw and prepared meat.

Of 29 samples of chicken curry, none was found to be positive for Campylobacter species. The reason could be that curries do not involve intervention of human hands in their cooking and distribution, thus cross contamination is less possible.

Regarding the raw mutton samples, only 1 out of 23 (4.35%) was positive for Campylobacter. No isolation could be made from cooked mutton (kababs, tikkas and curries).

Raw and cooked dishes of beef were also free from Campylobacter species. In case of freshly slaughtered beef carcasses Hudson and Roberts (1981) were unable to find any Campylobacter positivity, while Stern (1981) was able to find 2% positivity in case of beef carcasses and 24% Campylobacter positivity in case of sheep carcasses. One of the reasons for not finding many isolates in case of mutton and beef in the present study could be the fact that Campylobacters are very sensitive to environmental conditions, and as time passes after the slaughter, they become inactivated and are not likely to be isolated from raw meat. In the present study sampling was made atleast ten hours after the actual slaughtering of the animal. Prolonged storage of meat also makes it impossible to isolate Campylobacter. Undercooked meat also behaves similarly. On the

other hand, freshly slaughtered poultry can yield Campylobacter species both under raw or undercooked conditions.

Since Campylobacter jejuni can establish itself in the bovine udder (Lander and Gill, 1980), it may thus be able to contaminate raw milk. Campylobacter species could only be isolated from one (0.95%) out of 105 samples of raw milk in the study under discussion. These findings are in consistence with the results of previous workers who could also isolate Campylobacter from raw milk but from a very few samples (Lovett et al., 1983) 1.5%, (McManus and Lanier, 1987) 1 of 237 samples, (Doyle and Roman, 1982), 0.9%. Christopher et al. (1982) were unable to isolate any Campylobacter from 100 samples of raw milk.

It has been reported (Christopher et al., 1982), that 10^6 to 10^7 cells/ml of C. jejuni do not survive heating at 60°C. for 1 minute in case of milk. In the present study, 45 samples of boiled milk were also studied, all of these were found to be negative for Campylobacter species, since the boiling was sufficient to kill any organism which might have been present in the raw milk, therefore, it is possible that well boiled milk could be always Campylobacter free until it is cross contaminated subsequent to boiling.

For the isolation of Campylobacter species from water, 90 drinking water samples were studied. None of these samples harboured Campylobacter species. Blaser and Reller, (1981) and Vogt *et al*. (1982) have also not been able to isolate Campylobacter in their studies of water samples. It has been claimed (Butterfield *et al*., 1946; Butterfield, 1948; Hoff and Geldreica, 1981; Peterson *et al*., 1983; Blaser *et al*., 1986) that presence of chlorine in water is the possible cause of failure of isolation of Campylobacter from water. In the present study most of the water samples were chlorinated and this could be the possible reason for failure to isolate Campylobacter from water in the present study. On the other hand, Blaser *et al*. (1983) were able to isolate Campylobacter from drinking water and they too were able to isolate Campylobacter from untreated or inadequately treated waters. Since our water samples were mostly chlorinated ,therefore, our findings substantiate the veiws of (Butterfield *et al*., 1946; Butterfield, 1948; Hoff and Geldreica, 1981; Peterson *et al*., 1983; Blaser *et al*., 1986) that is, Campylobacter and other enteropathogens are rapidly inactivated by chlorine used for disinfection of water.

On the basis of this study, it could be concluded that treated drinking water supplied by municipal systems is safe with regards to Campylobacter infections and so is the case with

boiled milk. Properly cooked foods do not pose any health hazard, provided no unhygienic conditions are met with the foods after cooking and during food service.

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