CHAPTER III

MATERIALS AND METHODS

Materials

1. Samples : The 52 water samples were collected from 36 canals in Bangkok Metropolitan aera in sterile 250 ml - glass bottles. Collection were repeated twice for each sample in 4-month interval (to study seasonal variation). Collection of samples was followed as described by sites of collection (Table 5), schedule of canal monitoring (Table 6), and map of canals in Bangkok (Fig. 2). The total specimens were 156 samples. All specimens were obtained with the complement by Technical Division, Department of Drainage and Sewage, Bangkok Metropolitan Administration (BMA).

2. Containers : Each sample was collected in a sterile 250 ml glass bottle (Pyrex, Corning, NY., U.S.A.)

3. Bacterial strains : C. jejuni strains were supported by Department of Bacteriology, Armed Forces Research Institute of Medical Science (AFRIMS), Bangkok, Thailand and Department of Microbiology, Faculty of Medical Technology, Mahidol University.

4. Membrane filters : Two sizes of membrane filters were used.

4.1 The diameter of 47-mm, 0.2 um-pore Whatman membrane filters (Whatman, Maidstone, England) for concentration of water samples.

4.2 The diameter of 47-mm, 0.45 um - pore Millipore membrane filters (Millipore Corp, Bedford, MA, U.S.A.) for the isolation of Campylobacter spp. from Doyle's medium. These membrane filters were supported by Department of Bacteriology, AFRIMS, Bangkok, Thailand.

5. Media and reagents :

Media and reagents were used specifically for isolation and identification of Campylobacter spp. (see appendix).

6. Glasswares : For media preparations and intensive dilutions such as :

Beaker (Pyrex, Corning, NY., U.S.A).
Glass tubes (Pyrex, Corning, NY., U.S.A).
Cylinder (Witeg, W. Germany).
Erlenmeyer flasks (Pyrex, Corning, NY., U.S.A).
Petri dishes (Pyrex, Corning, NY., U.S.A)
Glass filter holder.

7. Equipments

Anaerobic jar (Oxoid, U.K.).
Vacuum pressure pump (Millipore Corp., Bedford,

M.A. U.S.A).

Mixer (Vortex-Genie, Scientific Industries, NY., U.S.A).

Incubators (42°C and 37°C)(Memmert, W. Germany).

Waterbath (37°C), Julabo TWB_{12} (Seelbach, W. Germany).

Analytical balance (Mettler PC440, Zurich, Switzerland).

Methods

I. Comparison of two different membrane filters for recovering C. jejuni from inoculated water.

1.1 Seeding of known C. jejuni in distilled water

The 48 h-old culture of C. jejuni on Brucella blood agar (B.B.A.) was suspended in sterile normal saline and adjusted to obtain a density of 65% transmittance at 520 nm by spectrophotometer (Beckman, Quallitech, U.S.A). The C. jejuni suspension was diluted 10-fold in 100 ml-amounts in sterile distilled water in order to yield dilutions ranging from 10^{-1} to 10^{-8}. C. jejuni remains viable for days in the water without a reduction in counts (Blaser et al. 1980). Each dilution was prepared in quadricate for evaluation of two different sizes of membrane filters.

The number of C. jejuni inoculum was determined by spread plate technique and done in duplicate on BBA. After 48 h-incubation, the colonies of C. jejuni were counted.

1.2 Evaluation of two different membrane filters

1.2.1 Evaluation of 47-mm, 0.2 um-membrane filter :

A 100 ml volume of inoculated water of each dilution were passed through membrane filters with a pore size of 0.2 um (Whatman, Maidstone, England) supported by a sterile suction flask apparatus and suctioned with pressure 1 lb/inch² (14-inch-mercury vacuum pressure pump). The membrane was changed every dilution. Each filter was placed face down on BBA plates and then removed and discarded after 30 minutes. Plates were incubated at 37°C under microaerophilic atmosphere. To achieve this atmosphere, the ambient air was removed from anaerobic jar by twice evacuating the jar to -14 inches (-37.5 cm) of mercury and then refilled it with the gas mixture of 85% N_2, 10% CO_2, and 5% O_2. The satisfactory atmosphere for incubation was also obtained when plastic bags (11.5 by 15 inches) were evacuated by suction, then reinflated twice with the gas mixture described above and sealed tightly with a rubber band. The above description was the modified technique by Kaplan (Kaplan 1980, Morris and Patton 1985). After 24-48 h

incubation, plates were read for the suspected Campylobacter colonies. This experiment was repeated again after concentration of inoculated water,each filter was not placed directly on BBA but put into 5 ml of Doyle's enrichment broth (Doyle and Roman 1982) and one membrane filter per one tube of the medium was used. Then the tubes were incubated for 24-h incubation, Doyle's media were subcultured onto BBA by spotting 10 drops of the sediment onto the sterile filter (pore size 0.45 um, Millipore Corp, Bedford, Mass) that placed on the surface of agar plate with Pasteur pipette to ensure that the drops did not extend to the edge of the filter. After 30 minutes, then the filter was removed from the plate and discarded. Plates were incubated at the same condition for 24-48 h. Read for the suspected Campylobacter colonies.

1.2.2 Evaluation of 47 mm-,0.45um-membrane filter :

The experiment was repeated in the same way as described in 1.2.1 using 47 mm-,0.45-um membrane filter (Millipore, Bedford, Mass) instead of 47 mm-, 0.2um-membrane (Whatman, Maidstone, England).

II. Methods of sample collection (Franson et al. 1985)

Each sample was collected in a sterile 250 ml-glass bottle that had been cleansed and rinsed carefully, and

given a final rinsing with distilled water then sterilized by hot air oven at 160°C for 1-2 h.

In collecting sample directly from canals, it is undesirable to take samples near the bank of canals. The sampling site would be a cross section of a stream. Take representative samples with consideration of site, method, and time of sampling. Choose a site disignated to measure estimated average pollution conditions for enough downstream to ensure complete mixing of pollutant and water. Sampling at such points does not eliminate all possible variations but will minimize any sharp fluctuation in quality. The samples were taken from the canal by holding the bottle near its base in the hand and plunging it, neck downward, below the surface then the bottle was turned until the neck points slightly upward and the mouth was directed toward the current. If there was no current, one must create the artificial current by pushing bottle forward horizontally in a direction away from the hand. The volume of sample should be sufficient to carry out all required tests, actually should not less than 100 ml (Franson et al. 1985). For this study, the water sample should not use less than 100 ml. Moreover, when the sample was collected, one must leave the ambient air space in the bottle (at least 2.5 cm) in order to facilitate shaking on examination (Franson et al. 1985).

If the sample could not be processed within 1 h after collection, it must be placed in an ice cooler for storage during transport to the laboratory. All the samples must be controlled below 10°C within 6 h of transportation. These samples were refrigerated as soon as the samples arrived in the laboratory and processed within 2 h (maximum transit 6 h, maximum processing-time 2 h). When the analysis could not study within 8 h, they must be maintained at the unfreezed-temperature below 4°C. The maximum elapse-time between collection and analysis should not exceed 30 h. Refrigeration during transportation was recommended to minimize changes in number of bacteria, particularly when ambient air temperature was over 13°C. Therefore, one should examine fresh unrefrigerated bottle samples (less than 48 h-old) within 6 h of collection and within 30 h of refrigerated samples (Franson et al. 1985).

Before examination, mixed thoroughly the samples by rapidly making about 25 complete up-and-down (or back-and-forth) movements. Optionally, one may use the mechanical shaker for 15 S. (Franson et al. 1985).

III. Bacteriological methods for isolation of Campylobacter spp from water samples (canals)

3.1 Culture for Campylobacter spp from water samples (Morris and Patton 1985, Franson et al. 1985, Steele and McDermott 1984, Doyle and Roman 1982).

A 100 ml-volume of water sample was concentrated by filtering through a sterile membrane filter with pore size of 0.2 um- (Whatman, Maidstone, England) supported by a sterile suction flask apparatus with the pressure of 1 lb/inch^2 (used 14 inch-mercury vacuum pump). Then the filter was put into 5-ml Doyle's medium tube and incubated the tube at 37°C for 18-24 h. under microaerophilic atmosphere. After incubation, Doyle's medium was subcultured onto BBA. Ten drops of sediment in the medium were spotted onto sterile filter (poresize, 0.45 um Millipore Corp, Bedford, Mass) that was placed on the surface of agar plate. Spotting with a Pasteur pipette to ensure that drops did not extent to the edge of the filter. The filter was removed and discarded after 30 minutes. Plates were incubated at the same condition for 24-48 h. Read for the suspected colonies.

3.2 Isolation for Campylobacter spp (Morris and Patton 1985).

Examine daily for the suspected Campylobacter colonies for 24-72 h, suspected colonies of Campylobacter spp. should be subcultured for the pure colonies and then were screened with three presumptive test:

a. Observation for typical motility and morphology under dark-field or phase contrast microscopy.

b. Oxidase test

c. Gram's reaction

A wet preparation of the organisms might be examined under phase or dark-field microscopy for characteristic darting motility and curve rod. Oxidase test must be positive. Colonies of Campylobacter spp. were smeared on the glass slide to examine under compound microscope by Gram's stain using 0.3% basic fuchsin as the counterstain, Campylobacter spp. are gram negative spiral rods, S-shaped bacilli, delicated seagull-wing (single or in chains) or C-shaped bacilli, with tapered end.

All isolates microscopically proved-Campylobacters were preserved in 15% glycine in brain heart infusion (BHI) broth and stored at -70°C for further biochemical tests, biotyping and serotyping of C. jejuni

3.3 Identification of Campylobacter spp.(Morris and Patton 1985, Barrett et al. 1988, Finegold and Baron 1986).

Suspicious colonies were identified by the method as described by Morris and Patton and the characteristics of biochemical tests for identification of Campylobacter are shown in Table 2 (Morris and Patton 1985, Barrett et al. 1988).

3.3.1 Preparation of inoculum for biochemical tests

All suspected isolates from stock cultures were subcultured onto BBA and incubated at 37°C for 24-48 h under microaerophilic condition (as described above). All cultures were examined for pure culture. Then the 24 h-culture was collected from the agar plate and was suspended into screw-capped bottle filled with heart infusion broth and adjusted the turbidity to match McFarland No. 1 standard ($3X10^8$ cells/ml) (Finegold and Baron 1986). The inoculum size of suspicious colonies for further biochemical tests was approximately 0.1 ml (2 drops from the Pasteur pipette) of such dilution. In case of weak or ambigous results were observed, the tests were repeated again.

3.3.2 Biochemical tests (Morris and Patton 1985, Barrett et al. 1988, Finegold and Baron 1986, Harvey 1980, Benjamin et al. 1983).

a) Oxidase test

Two or three drops of 1% tetramethyl - para - phenylenediamine dihydrochloride (BDH, Poole, England) were flooded onto a piece of Whatman filter paper No. 1. Cytochrome oxidase production was detected by placing a small amount (a loopful) of isolated colonies 1 cm. long on the saturated reagent paper. The dark-purple color developed in 5-10 seconds revealed positive reaction. Campylobacter spp. are oxidase positive.

b) Catalase test

Pour 1 ml, 3% solution of hydrogen peroxide over the 24-48 h, 37°C Campylobacter growth in heart infusion (HI) agar slant and set the slant in an inclined position. The formation of bubble (due to release of O_2) was considered a positive result. Negative results were confirmed by suspending growth in H_2O_2 on a slide then examining with disecting microscope for the presence of bubble. The test is not recommended for culture grown on blood agar because of the catalase present in the red blood cells.

c) Hippurate hydrolysis test

Hippurate hydrolysis was detected by using a modification of previously described methods (Harvey 1980, Hwang and Ederer 1975, Morris et al. 1985). For this study the rapid test described by Hwang and Ederer was used (Harvey 1980). A large loopful of 24-48-h-growth was emulsified in a thaw tube of 1% sodium hippurate and incubated in 37°C-water bath for 2 h. After incubation, 0.2 ml of ninhydrin reagent was slowly overlayed without mixing and the tube was reincubated for another 10 minutes without shaking then observed for the color development immediately. A deep purple color, crystal violet-like was considered a positive test whereas colorless or light-to medium purple color was considered negative result.

The tests are interpreted for identification and biotyping of the Campylobacter to species and subspecies level by referring to reaction in Table 2 and Table 3.

d) Susceptibility to antimicrobial agents

A standardized single disc agar diffusion method as described by Bauer et al. was used for susceptibility of Campylobacter spp. to antimicrobial agents (Bauer et al.1966). Each Campylobacter strain was inoculated by swabbing in three directions over the entire surface of the BBA with a swab saturated in the bacterial suspension (heart infusion broth) previously described. A 30-ug-nalidixic acid paper disc (NA) and a 30-ug-cephalothin paper disc were aseptically applied on the surface of inoculated agar plate. Plates were incubated at 37°C for 24-48 h under microaerophilic condition. Diameter of inhibition zone was measured and interpreted (National Committee for Clinical Laboratory Standard 1985). For NA, diameter of inhibiton zone >19 mm, 14-18 mm, <13 mm, were interpreted as susceptible, intermediate suceptible, and resistant, respectively. For CF, diameter of zone > 18 mm, 15 - 17 mm, <14 mm were interpreted as susceptible, intermediate susceptible, and resistant respectively.

e) Temperature tolerance

The ability of Campylobacter isolates to grow at 25°C and 42°C were determined on BBA plates (Morris and Patton 1985). Plates should be inoculated with a 6 mm-loop. Make a single streak of Campylobacter broth suspension across separate plates for incubation at each temperature under microaerophilic atmosphere for 3 days. Up to 4 isolates were inoculated to each plate, then examined for the presence of growth.

f) Sodium chloride (NaCl) tolerance (Benjamin et.al. 1983)

f.1) 1.5% NaCl tolerance

The ability to grow on yeast extract nutrient (YEN) agar containing 1.5% NaCl was determined by inoculating on YEN agar plate as described for temperature tolerance, then incubating at 37°C for 24-48 h under microaerophilic condition. Any growth after 3 days was considered a positive result.

f.2) 3.5% NaCl tolerance

The ability of tolerance to 3.5% NaCl was determined in Brucella albini broth medium containing 0.16% agar and 3.5% NaCl by inoculating 0.1 ml of

bacterial suspension. Any growth after 3 days was considered a positive result.

g) Glycine tolerance (Morris and Patton 1985, Barrett et al. 1988).

Growth in 1% glycine was determined in the Brucella medium of the BHI medium containing 0.16% agar and 1% glycine. Any growth after 3 days was considered a positive result.

h) Growth on MacConkey's agar

MacConkey agar slant was inoculated by dropping 1 drop of bacterial suspension to run down the slant. Any growth after 3 days was considered a positive result.

i) Hydrogen sulfide production

Two methods for examination of hydrogen sulfide production were employed as the following

i.1) Triple sugar iron (TSI) agar slant (Morris and Patton 1985, Barrett et al. 1988).

TSI agar slant was inoculated directly with needle by streaking the slant and stabbing the

butt with growth. After 37°C, for 24 h incubation, The blackening of medium was considered as a positive reaction.

i.2) FBP semisolid medium (Barrett et al. 1988, Skirrow and Benjamin 1980).

The rapid H_2S test originally described by Skirrow and Benjamin (1980) and modified by Lior (1984) (see appendix I) was performed. Suspended a large inoculum of 24-h-culture gently onto upper third of FBP semisolid medium. The tubes were incubated in 37°C water bath for 2 h. A blackening reaction around the bacterial mass represented as a positive reaction whereas a negative reaction did not show any black color.

j) Nitrate reduction test (Morris and Patton 1985).

The test for nitrate reduction was performed as described by Morris and Patton (1985). Nitrate broth medium was inoculated with bacterial suspension (0.1 ml from heart infusion broth) and inoculated at 37°C for 3 days. (Any strain that did not grow in nitrate broth medium was inoculated into a semisolid medium containing Mueller-Hinton broth, 0.3% agar, and 0.2% potassium nitrate. The semisolid medium was inoculated by being stabbed several times with 48 h-Campylobacter growth from BBA plate). Add 0.25 ml each of nitrate reagent A and

reagent B into the medium test. The development of a red reaction was a positive reaction that indicated the nitrite production of nitrate reductase from the organism. In case of no color developed, powdered zinc was added to determine whether nitrate was still present. Some organisms might generally reduce nitrite to nitrogen gas, thus if the organism did not reduce nitrate it would be reduced by the zinc to form nitrite, which could react with nitrate reagents to yield a red color. In this manner the development of the red color after the addition of zinc indicated a negative test result.

k) Tolerance to 0.04%, 2, 3, 5-triphenyl tetra zolium chloride (TTC) (Sigma, St.Louis, MO., U.S.A)

The test was done in a microtiter plate. This method was modified slightly from the method described by Nachamkin et.al. (1984). The 0.05 ml of 0.08% TTC was dropped in a microtiter plate. The bacterial suspension was diluted to a No. 0.5 McFarland standard in Brucella broth and the same amount was added into TTC in well of microtiter. So the final concentration of TTC was 0.04%. The plate was incubated at 42°C overnight under microaerophilic condition. Wells were examined for growth and production of a red formazan dye that indicated reduction of TTC.

l) DNA hydrolysis test (Smith et al. 1969, Lior 1984).

A large loopful of 24-or 48 h-37°C -culture was used for inoculating a circular area about 1 cm in diameter on a well dried methyl green deoxyribonucleic acid test agar (MG-DTA) plate. One plate could be inoculated with 4 isolates and was incubated at 37°C under microaerophilic condition for 3-5 days. An area of growth surrounded by a clear, colorless zone in the green-blue agar was considered positive for DNA hydrolysis. No change or a narrow, hazy zone around the bacterial growth was considered a negative test.

m) Aerobic growth

The ability of growth in aerobic atmosphere was determined on BBA and incubated at 37°C under aerobic condition.

n) Urease test

This test was used to detect urease production by Campylobacter spp. It was based on the ability of an organism to hydrolyze urea to ammonia and water. The urea-containing agar slant (Christensen urea agar) was inoculated heavily and incubated at 37°C and observed after incubation for 24 h. If the presence of

urease, the urea would be split into water and ammonia, resulting in an alkaline pH that turned the indicator to red or pink color.

3.4 Biotyping

Biotyping scheme of Campylobacter strains were performed as described by Lior (1984). The result obtained by hippurate hydrolysis, rapid H_2S,and DNA hydrolysis test were used for differentiating each species of C. jejuni C. coli and C. laridis into biotypes as shown in Table 4