UNITED NATIONS ENVIRONMENT PROGRAMME OCTOBER 1995

Determination of Camphylobacter in sea water and sewage

Reference Methods For Marine Pollution Studies No. 68

Prepared in co-operation with

WHO

UNEP 1995

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REGIONAL SEAS

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PREFACE

The Regional Seas Programme was initiated by UNEP in 1974. Since then the Governing Council of UNEP has repeatedly endorsed a regional approach to the control of marine pollution and the management of marine and coastal resources, and has requested the development of regional action plans. The Regional Seas Programme at present includes 12 regions and has over 140 coastal states participating in it (1), (2).

One of the basic components of the action plans sponsored by UNEP in the framework of the Regional Seas Programme is the assessment of the state of the marine environment and of its resources, and of the sources and trends of the pollution, and the impact of pollution on human health, marine ecosystems and amenities. In order to assist those participating in this activity, and to ensure that the data obtained through this assessment can be compared on a world-wide basis and thus contribute to the Global Environment Monitoring System (GEMS) of UNEP, a set of Reference Methods and Guidelines for marine pollution studies is being developed as part of a programme of comprehensive technical support which includes the provision of expert advice, reference methods and materials, training and data quality assurance (3). The methods are recommended to be adopted by Governments participating in the Regional Seas Programme.

The methods and guidelines are prepared by, or in cooperation with, the relevant specialized bodies of the United Nations system as well as other organizations, and are tested by a number of experts competent in the field relevant to the methods described.

In the description of the methods and guidelines the style used by the International Organization for Standardization (IS0) is followed as closely as possible.

The methods and guidelines, as published in UNEP's series of Reference Methods for Marine Pollution Studies, are not considered as final. They are planned to be periodically revised taking into account the development of our understanding of the problems, of analytical instrumentation and the actual need of the users. In order to facilitate these revisions, the users are invited to convey their comments and suggestions to:

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GREECE

which is responsible for the development and preparation of microbiological and other health-related Reference Methods.

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(1) UNEP: Achievements and planned development of the UNEP's Regional Seas Programme and comparable programmes sponsored by other bodies. UNEP Regional Seas Reports and Studies No. 1, UNEP, 1982.

(2) P. HULM: A strategy for the Seas. The Regional Seas Programme: Past and Future, UNEP 1983.

(3) UNEP/IAEA/IOC: Reference Methods and Materials: A Programme for comprehensive support for regional and global marine pollution assessments. UNEP, 1990.

This first substantive issue of Reference Methods for Marine Pollution Studies No. 68 was prepared by the World Health Organization on the basis of a review of the Method during expert meetings and comments from individual scientisits who tested the Method. The assistance of all those who contributed to this revised issue of the Reference Method is gratefully acknowledged.

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1. INTRODUCTION

The overall objectives of the Long-term Programme of Pollution Monitoring and Research in the Mediterranean Sea (MED POL Phase II), which constitutes the environmental assessment component of the Mediterranean Action Plan, adopted by the governments of the region in 1975, include the assessment, on a continuing basis, of the state of pollution of the Mediterranean Sea, the identification of the sources, pathways and effects of pollutants entering into it, and the establishment of temporal trends in pollution levels.

In order to assist laboratories participating in this activity and to ensure the necessary degree of intercomparison of data, a set of reference methods and guidelines has been developed by the Regional Seas Programme of UNEP in cooperation with the relevant specialized agencies of the United Nations system, as well as other organizations. In these reference methods and guidelines, the style used by the International Organization for Standardization (ISO) is followed as closely as possible.

In common with other microbiological methods and guidelines regarding effluents, coastal recreational waters and shellfish areas, the original version of this document was prepared by the World Health Organization within the framework of the MED POL Programme and issued by UNEP's Regional Seas Programme as part of its Reference Methods for Marine Pollution Studies series.

Actual preparation of methods and guidelines falling within the responsibility of WHO is entrusted to competent microbiologists with experience of the relevant characteristics of the Mediterranean marine environment. Following its preparation in draft form, each method is reviewed by international experts in the field, either individually or (normally) during consultation meetings. Following their issue in substantive form, methods and guidelines are distributed to Mediterranean laboratories and are periodically updated and/or revised on the basis of experience.

2. SCOPE AND FIELD OF APPLICATION

The method described is suitable for the determination of *Campylobacter* sp. presence in coastal bathing waters of temperate and tropical seas. It is designed to be used in sanitary surveillance of coastal waters and effluents.

This method employs a membrane filter procedure, which allows concentration of the bacteria prior to incubation, and is a great advantage in estimating low numbers of bacteria.

Thermophilic campylobacters have received considerable attention in recent years as a major cause of bacterial enteritis in man (Jones *et al.*, 1984; Fricker, 1987; Skirrow, 1987). *Campylobacter jejuni* can be found worldwide, and in developed countries is now recognized as one of the commonest causes of bacterial diarrhoea (Skirrow, 1987). To a lesser extent, enteritis is also associated with *C. coli*. Enteritis is produced also by the Nalidixic Acid Resistant Thermophilic *Campylobacter* (NARTC) formally described as a new species with the proposed name of *C. laridis* (Benjamin *et al.*, 1983), and by a fourth thermophilic species named *C. upsaliensis* (catalase negative or weakly reacting) (Sandstedt and Ursing, 1986).

Fresh water is potentially an important reservoir of thermophilic campylobacters and is an established vehicle for the transmission of these organisms to man and domestic animals (Hoff and Geldreich, 1982; Bolton *et al.*, 1987). *Campylobacter* sp. have a low infective dose.

No obvious relationship has been observed in freshwater between total and faecal coliforms concentrations and the occurrence of *Campylobacter* sp. (Carter *et al.*, 1987; Taylor *et al.*, 1983; Bolton *et al.*, 1987; Martikainen *et al.*, 1990). Consequently, faecal bacterial indicators may not be good predictors for the presence of *Campylobacter* sp. in seawater.

3. DEFINITION

Campylobacters are slender, spirally curved rods, 0.2 to 0.5 µm wide and 0.5 to 5 µm long. The rods may have one or more spirals and can be as long as 8 µm. They also appear S shaped and gull-winged when two cells form short chains. They are non spore-formers and Gram-negative. Cells in old cultures may form spherical or coccoid bodies. They are motile with a characteristic corkscrew-like motion by means of a single polar flagellum at one or both ends of the cell. Campylobacters are microaerophilic, with a respiratory type of metabolism, and require an oxygen concentration of between 3 and 14% and a carbon dioxide concentration of between 3 and 5%. They are chemo-organotrophs, and they don't ferment or oxidize carbohydrates. They don't hydrolyze gelatin or urea. They are methyl red and Voges Proskauer negative. They have no lipase activity and they are oxidase-positive (Smibert, 1984).

A number of characteristic properties (based on biochemical tests, resistance patterns, and growth temperatures) of *Campylobacter* species is given in Table 1 (Griffiths and Park, 1990).

4. PRINCIPLES

Methods for isolating campylobacters are not standardized and therefore must be considered research procedures, subject to future modifications.

In environmental studies microbiologists face the problem of detecting a few campylobacters within a large and diverse bacterial flora (Stelzer *et al.*, 1991). Additional difficulties emerge from the necessity to recover environmentally stressed campylobacters (Stelzer *et al.*, 1991). Therefore, extremely sensitive isolation methods are required. Sub-lethally damaged cells are able to effect repair at 37 °C, and pre-enrichment at this temperature is found to increase the isolation of *Campylobacter jejuni* from water significantly (Humphrey, 1989). However, the pre-enrichment is problematic in non-selective media without antibiotics due to over-growth by other organisms (Stelzer *et al.*, 1991). From this point of view, a pre-enrichment is recommended in selective broth at 37 °C for 4 h (Humphrey, 1989).

Table 1. Conventional tests for distinction between *Campylobacter* spp. associated with enteritis (Griffiths and Park, 1990).

Campylobacter	Oxidase	Catalase	Nitrate	1% glycine	Hippurate hydrolysis	Indoxyl acetate	Growth at			Susceptibility	
							25°C, 37°C, 42°C			Nalidixic acid	Cephalotin
jejuni	+	+	+	+	+	+	-	+	+	S	R
coli	+	+	+	+	-	+	-	+	+	S	R
laridis	+	+	+	+	-	-	-	+	+	R	R
upsaliensis	+	-/±	+	-	-	U	-	+	+	S	S
cinaedi	+	+	+	+	-	-/±	-	+	-	S	I
fennelliae	+	+	-	+	-	+	-	+	-	S	S
hyointestinalis	+	+	+	+	-	-	+	+	+	R	S
cryaerophila	+	+	+	-	-	+	+	+	-	V	R
fetus subsp. *fetus*	+	+	+	+	-	-	+	+	-	R	S

Note: R = resistant; S = sensitive; I = intermediate zones; V = variable; U = not reported.

A volume of 100 ml for seawater samples near sewage discharge points and a volume of 1000 ml for seawater in less polluted bathing areas are filtered through 0.45 µm pore size membrane filters. After filtration the membranes are removed and introduced in flasks with 100 ml of Preston *Campylobacter* selective enrichment broth (PB) (Bolton and Robertson, 1982) or *Campylobacter* blood-free selective enrichment broth (CBFSB) (Bolton *et al.*, 1984). The flasks are placed in an anaerobic jar (the catalyst must be removed from the anaerobic jar) in an atmosphere of 5% O_2 and 10% CO_2, and pre-incubated at 37 °C for 4 hours (to recover damage cells) before transfer to 42 °C for 44 hours. From each enrichment flask a loop is streaked on plates (two per flask) of Preston *Campylobacter* selective agar (PA) or *Campylobacter* blood-free selective agar (CBFSA). Other media are available in the medical field, such as Skirrow's and Butzler's media. The plates are placed in an anaerobic jar, containing 5% O_2 and 10% CO_2, and incubated at 42 °C for 48 hours.

Confirmation of the growth of campylobacters is made from colony morphology and microscopic appearance (campylobacters are characteristically gram negative and curved or spiral in shape). Cultures of campylobacters are purified by subculturing on blood agar base (without blood) added with 0.025% each of ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP) (to increase the aerotolerance of campylobacters) (George *et al.*, 1978, Hoffman *et al.*, 1979) and tested for catalase, nalidixic acid sensitivity (30 µm disk) and hippurate hydrolyse. These tests are minimal to distinguish *C. jejuni* or *C. coli* from other *Campylobacter* species.

Residual chlorine, if present, is neutralized by adding thiosulphate to the sampling bottle before sterilization.

5. APPARATUS AND GLASSWARE

5.1 Sample bottles of dark coloured borosilicate glass for surface seawater or effluent, of 200-300 ml capacity, wide-mouthed and with ground-glass stoppers, or plastic containers of similar capacity with well fitting stoppers.

5.2 Sample rod of non-corrosive material with a clamp to hold the sampling bottle (Figure 1).

5.3 Subsurface sampler of the type shown in Figure 2, or similar, complete with plastic rope and weight.

5.4 Thermoisolated plastic boxes with cooling pads or similar cooling units (camping equipment) for storage of samples.

5.5 Thermometer, 0 to 50 °C, precision ± 1 °C, preferably of unbreakable plastic, to be used for checking temperature in plastic boxes.

5.6 Filtration apparatus for 4.7 cm diameter membrane filters consisting of at least three filter funnels for simultaneous filtration, made of borosilicate glass or other non-toxic sterilizable material (excluding metal filter holders and funnels), complete with electric or water vacuum pump.

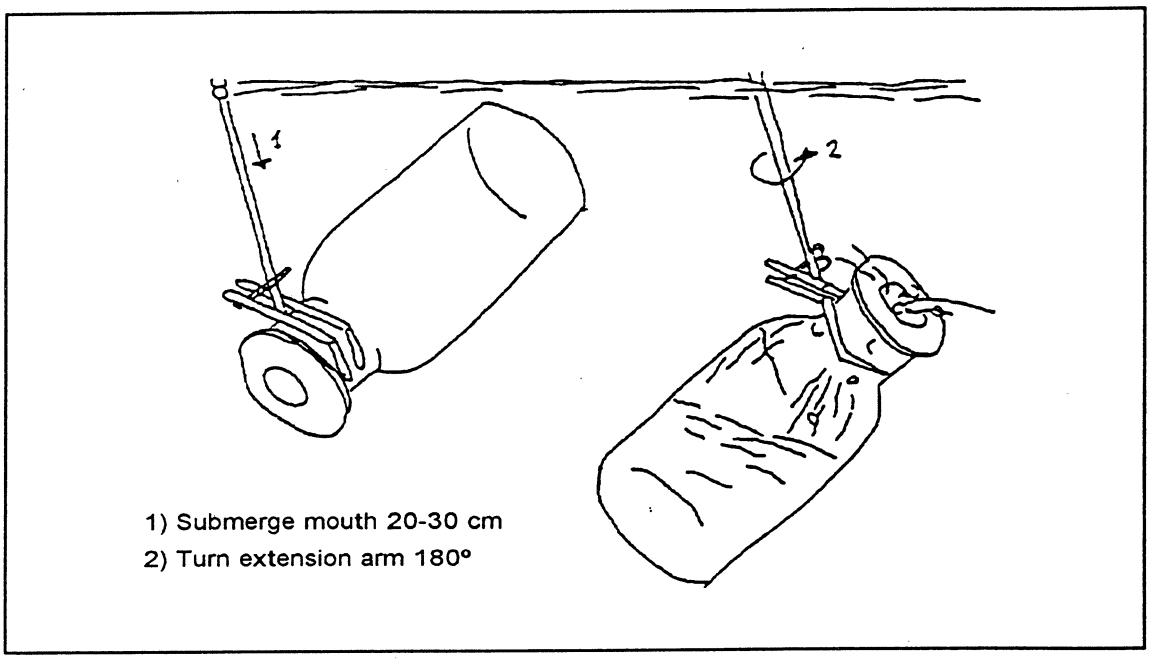

Figure 1. Subsurface sampling with extension arm.

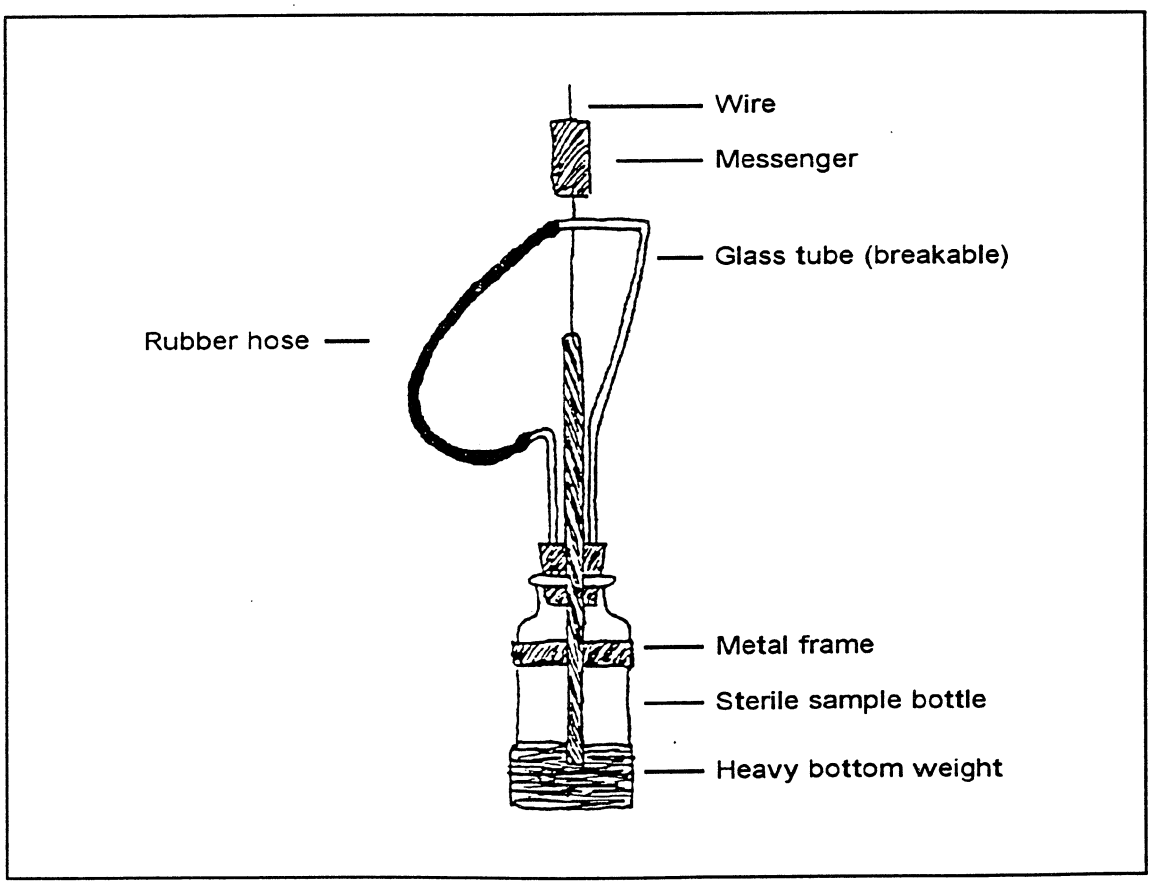

Figure 2. Sampler for sterile subsurface sampling.

5.7 Water bath thermostatically controlled at 37 ± 1 °C and 42.0 ± 0.2 °C, or alternatively air incubator thermostatically controlled 37 ± 1 °C and 42.0 ± 0.2 °C, preferably with a water jacket.

5.8 Stereomicroscope with a magnification 10-15x, or dark field colony counter, with a magnification 2-3x.

5.9 Autoclave, with a maximum pressure of 2 atm, electric or gas.

5.10 Drying oven for sterilization up to 170 °C.

5.11 pH meter, precision ± 0.1 pH units.

5.12 Stainless steel forceps.

5.13 Analytical balance, precision ± 1 mg.

5.14 Refrigerator thermostatically controlled at 4 ± 2 °C.

5.15 Vibrator (Vortex type) for mixing liquids in culture tubes.

5.16 Petri dishes of borosilicate glass, 9 cm diameter, complete with stainless steel containers for sterilization, or disposable pre-sterilized plastic petri dishes.

5.17 Erlenmeyer flasks of borosilicate glass for media preparation, of 250 ml, 500 ml and 1 litre.

5.18 Borosilicate glass bacteriological culture tubes.

5.19 Pipettes of borosilicate glass with total volume (blow-out) of 1, 5 and 10 ml capacity, with stainless steel containers for sterilization.

5.20 Graduated borosilicate glass cylinders of 100, 500 and 1000 ml capacity with glass beakers for cover.

5.21 Bacteriological loops of 3 mm diameter.

5.22 Heavy wrapping paper.

5.23 Aluminium foil (household quality).

5.24 Membrane filters, 0.45 µm pore size, 4.7 cm diameter.

Note: The 0.45 µm pore size membrane filters should be certified by the manufacturer to be free from substances which may hinder the growth and development of bacteria. Maximum recoveries are obtained using membranes composed of mixed esters of cellulose.

5.25 Filtration apparatus for preparing sterile solutions (Seitz filter or similar).

5.26 Anaerobic jar of metal or polycarbonate, without catalyst.

5.27 Brightfield microscope, with objectives 10x, 20x, 40x, and 100x.

5.28 Glass slides and cover glass.

5.29 Small screw-capped vials.

5.30 Filter paper.

6. CULTURE MEDIA, CHEMICALS AND STOCK CULTURE

Note: The composition of the media is based on one litre solutions or similar units. Before preparation of media and solutions, the actual needs have to be established and amounts must be chosen accordingly. Most of the following media are commercially available.

6.1 Preston Broth (PB)

6.1.1 Broth base

Beef extract	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Distilled water	950.0 ml

Preparation: Dissolve the ingredients in the distilled water. Sterilize the medium at 121 °C for 15 min. The pH should be 7.5 ± 0.2. After sterilization, cool the medium at 50 °C in a water bath and add aseptically 50 ml of sheep blood and 4 ml of antibiotics solution (6.1.2). To increase the aerotolerance of campylobacters add 4 ml of FBP solution (6.1.3) to cooled PB medium. Distribute 100 ml portions in flasks.

6.1.2 Antibiotics solution

Polymixin B	5 000.0 IU
Rifampicin	10.0 mg
Trimethoprim lactate	10.0 mg
Cycloheximide	100.0 mg
Acetone or distilled water	4.0 ml

Preparation: Dissolve the antibiotics in 4 ml of an equal volume mixture (1:1) of acetone and sterile distilled water.

6.1.3 FBP solution

Ferrous sulphate	0.25 g
Sodium metabisulphite	0.25 g
Sodium pyruvate	0.25 g
Distilled water	4.0 ml

Preparation: Dissolve the ingredients in 4 ml of sterile distilled water.

6.2 *Campylobacter* Blood-Free Selective Broth (CBFSB)

Beef extract	10.0 g
Peptone	10.0 g
NaCl	5.0 g
Bacteriological charcoal	4.0 g
Casein hydrolysate	3.0 g
Sodium deoxycholate	1.0 g
Sulphate ferrous	0.25 g
Sodium pyruvate	0.25 g
Distilled water	1.0 litre

Preparation: Let the ingredients soak in distilled water for 10 min. Mix to suspend undissolved material and autoclave at 121 °C for 15 min. Cool to 50 °C and aseptically add 4 ml of antibiotic solution (6.2.1). Distribute 100 ml portions in flasks.

6.2.1 Antibiotic solution

Cefoperazone	32.0 mg
Distilled water	4.0 ml

Preparation: Dissolve the antibiotic in 4 ml of sterile distilled water.

6.3 Preston Agar (PA)

6.3.1 Agar base

Beef extract	10.0 g
Peptone	10.0 g
Sodium chlorite	5.0 g
Agar	15.0 g
Distilled water	950.0 ml

Preparation: Dissolve the ingredients in the distilled water. Sterilize the medium at 121 °C for 15 min. The pH should be 7.5 ± 0.2. After sterilization, cool the medium at 50 °C in a water bath and add aseptically 50 ml of sheep blood and 4 ml of antibiotics solution (6.3.2). To increase the aerotolerance of campylobacters add 4 ml of FBP solution (6.3.3) to cooled PA medium. The pH of the medium should be 7.5 ± 0.2. Mix well and pour into petri dishes.

Note: If exposed to sunlight, PA becomes toxic for campylobacters. The culture media plates should not be overcooked dried. Prior drying of the surface of the medium should be kept to a minimum.

6.3.2 Antibiotics solution

Polymixin B	5,000.0 IU
Rifampicin	10.0 mg
Trimethoprim lactate	10.0 mg

Cycloheximide	100.0 mg
Acetone or distilled water	4.0 ml

Preparation: Dissolve the antibiotics in 4 ml of an equal volume mixture (1:1) of acetone and sterile distilled water.

6.3.3 FBP solution

Ferrous sulphate	0.25 g
Sodium metabisulphite	0.25 g
Sodium pyruvate	0.25 g
Distilled water	4.0 ml

Preparation: Dissolve the ingredients in 4 ml of sterile distilled water.

6.4 *Campylobacter* Blood-Free Selective Agar (CBFSA)

Beef extract	10.0 g
Peptone	10.0 g
NaCl	5.0 g
Bacteriological charcoal	4.0 g
Casein hydrolysate	3.0 g
Sodium deoxycholate	1.0 g
Sulphate ferrous	0.25 g
Sodium pyruvate	0.25 g
Agar	15.0 g
Distilled water	1.0 litre

Preparation: Let the ingredients soak in the distilled water for 10 min. Dissolve the ingredients by gentle boiling and autoclave at 121 °C for 15 min. Cool to 50 °C in a water bath and add aseptically 4 ml of antibiotic solution (6.4.1). Mix well and pour into petri dishes.

6.4.1 Antibiotic solution

Cefoperazone	32.0 mg
Distilled water	4.0 ml

Preparation: Dissolve the antibiotic in 4 ml of sterile distilled water.

6.5 Blood Agar Base

Beef extract	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients by gentle boiling. Autoclave at 121 °C for 15 min. The pH of the medium should be 7.5. To increase the aerotolerance of

campylobacters add 4 ml of FBP solution (6.5.1) to the cooled medium. Mix well and pour into petri dishes.

6.5.1 FBP solution

Ferrous sulphate	0.25 g
Sodium metabisulphite	0.25 g
Sodium pyruvate	0.25 g

Preparation: Dissolve the ingredients in 4 ml of sterile distilled water.

6.6 Hippurate Broth

Sodium hippurate	1.0 g
Distilled water	100.0 ml

Preparation: Dissolve the ingredient in the distilled water and dispense 0.4 ml portions in small screw-capped vials. Store at - 10 °C and discard after 6 months.

6.7 Ninhydrin Reagent

Ninhydrin	3.5 g
Acetone-butanol mixture 1:1	100.0 ml

Preparation: Dissolve the ingredient in the acetone-butanol mixture.

6.8 Nalidixic Acid (30 µg) Disk

6.9 Hydrogen Peroxide Reagent

Hydrogen peroxide 30%	10.0 ml
Distilled water	90.0 ml

The reagent may be stored in a brown bottle for up to 6 months.

Note: Shaking the reagent before use will help to expel any dissolved oxygen. False positive reactions may occur if the hydrogen peroxide reagent contains dissolved oxygen.

6.10 Oxidase Reagent

Tetramethyl-p-phenylenediamine	0.1 g
Distilled water	10.0 ml

Preparation: Dissolve the ingredient in the distilled water. The reagent is not stable and therefore should be prepared fresh immediately before use.

6.11 Immersion Oil

6.12 Gas Generation Sachets that Produce 5% O_2 and 10% CO_2

6.13 Aqueous Basic Fuchsin

Basic fuchsin	1.0 g
Distilled water	100.0 ml

Preparation: Dissolve 1.0 g basic fuchsin in 100 ml of distilled water, and filter the solution to remove any undissolved crystals.

6.14 Crystal Violet Gram Stain

Crystal violet	20.0 g
Ammonium oxalate	9.0 g
Ethanol or methanol, absolute	95.0 ml
Distilled water to	1.0 litre

Preparation: Weigh the crystal violet on a piece of clean paper (preweighed). Transfer to a clean brown bottle. Add the absolute ethanol or methanol and mix until the dye is completely dissolved. Weigh the ammonium oxalate and dissolve in about 200 ml of distilled water. Add to the stain. Make up to 1 liter with distilled water, and mix well. The stain is stable for several months at room temperature.

Note: Ammonium oxalate is a toxic chemical, and therefore it must be handled with care.

6.15 Lugol Iodine Gram Stain

Potassium iodide	20.0 g
Iodine	10.0 g
Distilled water to	1.0 litre

Preparation: Weigh the potassium iodide, and transfer to a clean brown bottle premarked to hold 1 litre. Add about a quarter of the volume of water, and mix until the potassium iodide is completely dissolved. Weigh the iodine, and add to the potassium iodide solution. Mix until the iodine is dissolved. Make up to the 1 litre mark with distilled water, and mix well. Label the bottle, and mark it toxic. Store the solution in a dark place at room temperature. Renew the solution every one or two weeks (depending on storage conditions) or when its colour fades.

Note: Iodine is dangerous to health if inhaled or allowed to come in contact with the eyes, and therefore it must be handled with care in a well ventilated room.

6.16 Decolorizer Gram Stain

Acetone	500.0 ml
Ethanol or methanol, absolute	475.0 ml
Distilled water	25.0 ml

Preparation: Mix the distilled water with the absolute ethanol (ethyl alcohol) or methanol (methyl alcohol). Transfer the solution to a 1 litre clean bottle. Measure the acetone, and add immediately to the alcohol solution. Mix well. Label the bottle, and mark it "HIGHLY FLAMMABLE". Store in a safe place at room temperature. The reagent is stable indefinitely.

6.17 Safranin Gram Stain

Safranin	
(2.5 g/100 ml of 95% ethyl alcohol)	10.0 ml
Distilled water	90.0 ml

Preparation: Dissolve 2.5 g of safranin dye in 100 ml of 95% ethyl alcohol. Add 10 ml of this solution into 90 ml of distilled water.

6.18 Phosphate Buffer (pH = 7.2)

K_2HPO_4	3.0 g
KH_2PO_4	1.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients in the distilled water.

6.18.1 P-buffer for filtration

Preparation: Autoclave the buffer at 121 °C for 15 minutes.

6.19 Thiosulfate Solution

Preparation: Prepare a 10% (10 g/100 ml) sodium thiosulfate solution in distilled water and sterilize by filtering through a sterile membrane filter (5.25 or 8.4). The sterilization can be omitted if the solution is prepared every 2-3 weeks and kept in the refrigerator.

6.20 Distilled Water

Use only water distilled in all-glass or all-quartz distillation apparatus or reverse osmosis (RO) water. De-ionized water is also acceptable if produced in apparatus not releasing toxic substances.

Note: Commercially available distilled water is often produced in copper and zinc apparatus and is highly toxic for bacteria. Before using such water its toxicity should be checked with a stock culture of *Campylobacter* (6.23).

6.21 Detergents for Cleaning Glassware and Apparatus

Use only detergents recommended by the supplier for bacteriological use. If such a detergent is not available, check normal household detergents with a biotest using a stock culture of *Campylobacter* (6.23).

Note: Never use toxic chromic-sulphuric acid mixture for cleaning glassware.

6.22 95% Ethanol for Analysis

6.23 Stock Culture

Stock culture of a *Campylobacter* strain from a type collection.

7. SAMPLING

Details of a sampling plan are provided in Part I of these guidelines.

7.1 Sampling of Surface Water

Attach a clean sterile bottle to a clean sampling rod (5.2). Immediately before submerging the sample bottle, remove the ground glass stopper from the bottle without touching the stopper cone. Immerse the bottle from the bow of the boat or from the windward side while the boat is moving forward slowly. Push the bottle with the sampling rod 25 cm under the water surface with the mouth of the bottle downwards, in order to avoid contamination by surface film, then turn the sample bottle upwards and take the sample (Figure 1). The sterile sample bottle may also be filled directly by hand (Figure 3).

Retrieve the bottle and discard some water, if necessary, so that some air space remains in the closed bottle. This space is needed for homogenizing the water sample at the receiving laboratory. Replace the glass stopper and store the samples in the clean thermoisolated box with cooling pads at about 4 °C, avoiding exposure to more than + 10 °C. Separate bottles from each other with clean wrapping paper to avoid breakage. Check the temperature with a thermometer every 3 hours. Report irregularities in the test report. Label sample bottles indicating the sampling station, the time of sampling, and other factors relevant to the interpretation of the results.

7.2 Sampling of Subsurface Water

Lower the sterile subsurface sampler (5.3) after attaching it to a clean plastic rope, without letting the weight disturb the bottom sediments (Figure 2). Release the messenger and after one minute retrieve the sampler and store it in a thermoisolated box. Proceed as for sampling of surface water (7.1).

7.3 Sampling of Sewage Effluents

Sampling of sewage is relatively difficult because raw wastewater varies both in composition and in flow. Sampling should be performed at points where there is a good mixture of the material to be sampled, unaffected by earlier deposits. Composite samples should be obtained when the aim is to measure average quality over a period not exceeding 24 hours. Sampling should be performed as described in 7.1 above. If the bottle is filled directly by hand, long rubber gloves, covering the hand and fore-arm should be worn.

The water sample represents the test solution.

Note: It is known that the die-away rate of bacteria at ambient temperature in the presence of light is very high. Therefore, all efforts should be made to collect only the number of samples than can be filtered and incubated the same day. If this is not possible, the samples should be stored at + 4 °C and analyzed not later than 24 hours after sampling.

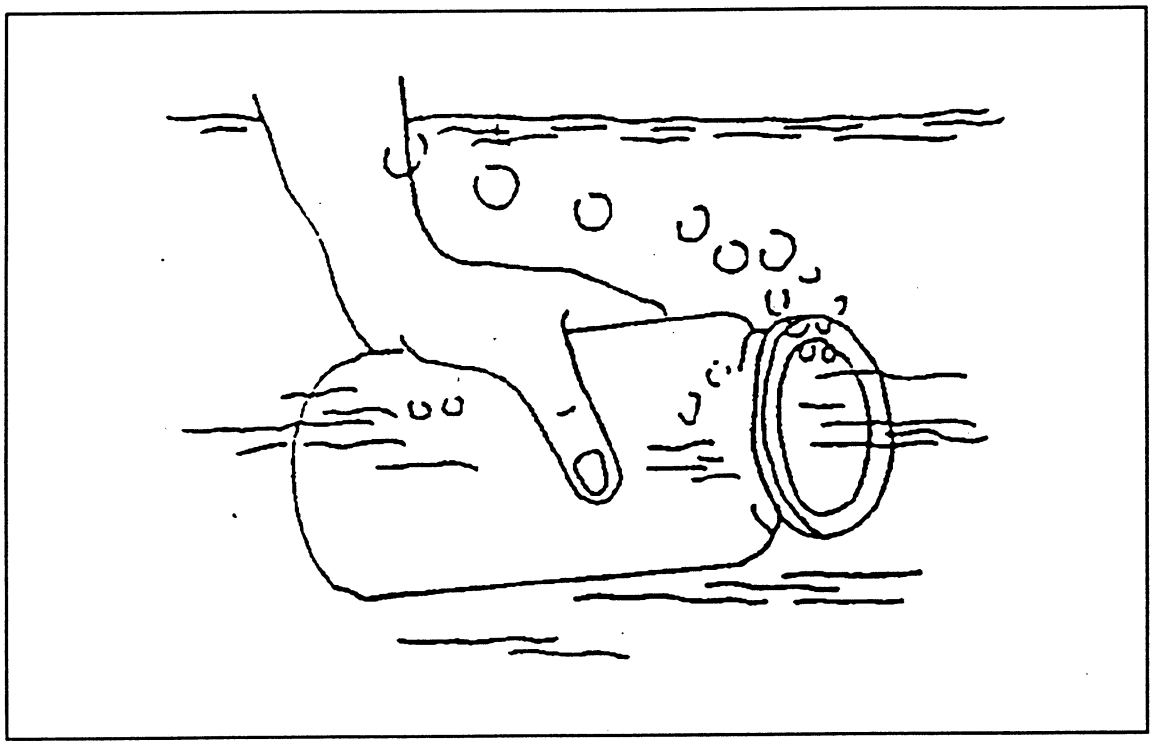

Figure 3. Subsurface sampling by hand.

8. TEST PROCEDURE

8.1 Washing of Glassware and Equipment

All glassware and apparatus should be washed with non-toxic detergent first, rinsed thoroughly with hot tap water and then rinsed at least three with distilled water.

8.2 Sterilization of Glassware and Equipment

8.2.1 Surface sample bottles

Wash glass bottles as described under 8.1. Dry and sterilize them in a drying oven at 160 °C for 2 hours, or at 170 °C for 1 hour. Before sterilization, place a small piece of filter paper in the neck of each bottle to prevent the ground glass stopper from sticking after cooling. After cooling to ambient temperature in the drying oven, remove this filter paper with sterilized forceps and fit the ground glass stopper securely into the neck of each bottle. Put the bottles into detergent-cleaned thermoisolated boxes. Separate the bottles from each other with clean wrapping paper to avoid breakage.

Wash plastic bottles as described under 8.1, then treat them with 95% alcohol. After adding an appropriate volume of alcohol to each bottle, shake them thoroughly, keeping its stopper in place. After 2-3 alcohol washings, the bottles must be well drained and kept in an oven at 40-50 °C temperature until complete evaporation of the alcohol. Stoppers are also placed on a sterile petri dish to dry.

Note: If residual chlorine is suspected in the water sample, add 0.1 ml of a 10% thiosulfate solution (6.19) for each 100 ml sample to the contents of the sample bottle before sterilization. This amount is sufficient to neutralize about 15 mg of residual chlorine per litre.

8.2.2 Subsurface sampler (5.3)

Wash the subsurface sampler as described under (8.1), rinse with tap and distilled water. Enclose each sampler in heavy wrapping paper or aluminium foil and sterilize them in an autoclave at 121 °C for 15 minutes.

8.2.3 Petri dishes and pipettes

Clean dishes and pipettes, complete with a cotton plug in the mouthpiece, are put into suitable stainless steel containers and sterilized in a drying oven at 160 °C for 2 hours, or at 170 °C for 1 hour.

Note: Disposable pre-sterilized plastic petri dishes may be more economical to use than re-usable glass petri dishes.

8.2.4 Filter funnels of filtration apparatus (5.6)

Loosen the filter-holding assembly slightly and wrap the whole filter funnel in heavy wrapping paper or aluminium foil. Sterilize in an autoclave at 121 °C for 15 minutes, or in a drying oven at 160 °C for 2 hours, or at 170 °C for 1 hour.

8.2.5 Membrane filters

Remove the paper separator (if present) and place 10 to 12 clean membrane filters into petri dishes. Autoclave them at 121 °C for 15 minutes. Once sterilization is completed, let the steam escape rapidly in order to minimize the accumulation of condensate on the membrane filters.

Note: Sterilized membrane filters are commercially available.

8.2.6 Forceps

Sterilize forceps by dipping them into 95% ethanol and flaming them. Let them cool before taking the membrane filters.

8.3 Selection of Sample Size

If previous experience for selecting the sample size is not available, filter 1 litre of clean seawater or 100 ml of contaminated waters. Smaller volumes have to be filtered for contaminated waters and sewage effluents.

8.4 Filtration Procedure (selective enrichment technique)

Each water sample must be vigorously shaken to insure a uniform dispersion of bacteria in each sample portion tested.

Begin filtration with the smaller portion of a water sample to avoid contamination from higher bacterial concentrations. Use a sterilized filtration funnel for each water sample. Place the sterilized membrane filters with flamed sterilized forceps over the porous plate of the filtration apparatus. Carefully place the matching funnel unit over the receptacle and lock it in place. Add into the funnel about 20 ml of P-buffer solution (6.18.1). Add into the funnel the volume of sample to be filtered. Filter with a partial vacuum. Wash the funnel walls with approximately 20 ml of P-buffer solution (6.18.1). Filter with a partial vacuum. Wash the funnel walls two more times with 20 ml of buffer solution each time (6.18.1). Unlock and remove the funnel, immediately remove the membrane filter with flamed sterilized forceps, by rolling it, and transfer the membrane filter to a flask containing 50 to 100 ml of selective enrichment broth (5.1 and/or 5.2). Before filtering the next sample portion in the same manner, pass 20 ml of P-buffer solution (6.18.1) through the assembled filtration unit.

In the case of sewage effluents, as the sample would be sometimes turbid due to numerous suspended particles, a blending step is generally necessary.

8.5 Incubation

The flasks containing the membrane filters are placed in an anaerobic jar containing 5% O_2, 85% N_2 and 10% CO_2, and are preincubated at 37 °C for 4 hours before transfer to 42 °C for 44 hours. As a sterility check, incubate also one blank without a membrane filter, i.e. a flask containing only PB (5.1) or CBFSB (5.2). As a control, incubate also one stock culture of a *Campylobacter* strain from a type collection (6.23), using a flask containing PB (5.1) or CBFSB (5.2).

8.6 Selective Isolation

From each enrichment flask streak a loop on two petri dishes containing PA (5.3) or CBFSA (5.4). To inoculate a plate, spread the inoculum over a small area near the edge of the plate. This is area 1. Apply the loop lightly to the medium to avoid digging into it. Flame the loop and cool it for 5 seconds. Make five or six streaks from area 1 into area 2. Stay near the edge as shown in Figure 4. Flame the loop again and allow it to cool. Make six or seven streaks from area 2 into area 3. Flame the loop again and make as many streaks from area 3 to area 4 as possible.

Note: The objective of the petri dishes inoculation technique is to provide single colonies for identification and determination on whether a culture is pure or mixed, i.e. consisting of a single type of organisms or several different types of organisms.

8.7 Incubation

The inoculated petri dishes are incubated immediately at 42 °C for 48 hours in an anaerobic jar, using gas generation sachets to achieve a microaerophilic

atmosphere. As a sterility check, incubate also a blank, i.e. a petri dish containing PA (5.3) or CBFSA (5.4), but without inoculum.

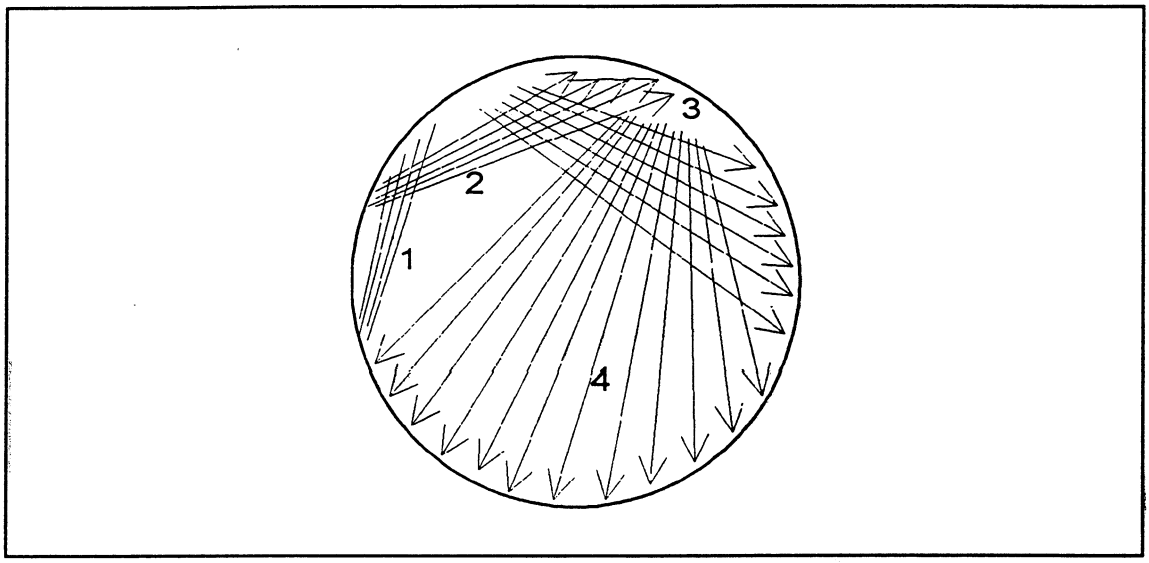

Figure 4. Streak plate pattern.

8.8 Interpretation

Campylobacter colonies are non-hemolytic on PA (5.3). They spread along the direction of the streak and tend to swarm and coalesce. The colonies are 1 to 2 mm in diameter.

The morphology of campylobacters colonies can be used as a guideline for their identification to species level on CBFSA (5.4). *C. jejuni* strains produce grey, moist flat spreading colonies after 48 hours incubation at 42 °C. Some strains may have a green hue or a dry appearance, with or without a metallic sheen. *C. coli* strains tend to be creamy-grey in color, moist, slightly raised and often produce discrete colonies. *C. laridis* strains vary in their morphology: some produce an appearance like *C. jejuni* and *C. coli* strains, while others produce grey, discrete colonies.

Confirmatory tests (basic fuchsin or Gram stain) should be performed on *Campylobacter* colonies isolated on the selective medium, PA (5.3) or CBFSA (5.4). They can be identified by the scheme outlined in Figure 5.

Note: *Campylobacter* species are rapidly killed when exposed to air.

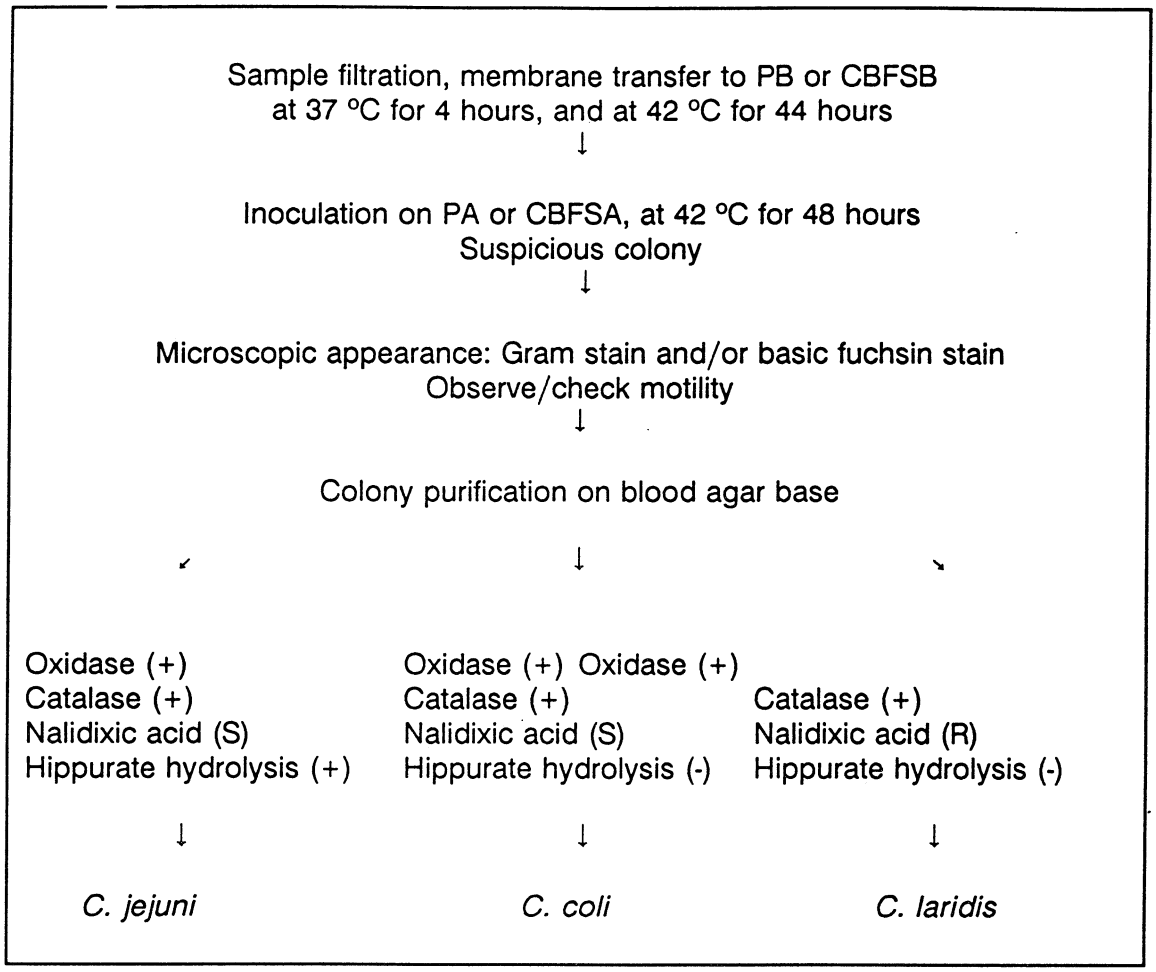

Figure 5. Schematic diagram for isolation and identification of campylobacters.

8.9 Confirmatory Tests

8.9.1 Basic fuchsin stain

Smears are spread covering an area of about 15-20 mm diameter on a slide. Spread *Campylobacter* colonies thinly using a sterile wire loop. The flame-sterilized loop must be allowed to cool before use. Colonies must be emulsified in sterile phosphate buffer (5.18) before spread thinly. After smears are prepared, the slides should be left in a safe place to air-dry, protected from insects and dust.

Smears are fixed by heating to prevent being washed from slides during staining. Allow the smear to air-dry completely. Rapidly pass the slide, smear uppermost, three times through the flame of a Bunsen burner. Allow the smear to cool before staining.

Note: After passing the slide three times through the flame, it should be possible to lay the slide on the back of the hand without feeling uncomfortably hot. Otherwise, too much heat has been used.

The fixed smears are flooded with basic fuchsin (6.13) for 10-20 seconds and then thoroughly rinsed with tap water.

Examine the smear microscopically, first using the 40X objective to check the staining and observe bacterial distribution, and then using the oil immersion objective (100X) to examine bacterial morphology. The light condenser should be fully open when using the oil immersion lens. Only smears displaying spirally curved rods are considered positive.

Note: Cells in old cultures may form spherical or coccoid bodies.

8.9.2 Gram stain

Prepare the campylobacters smears as described in 8.9.1. Cover the smear with crystal violet (6.14). Let stand for 20 seconds. Briefly wash off the stain, using a wash bottle of distilled water. Drain off excess water. Cover the smear with Gram's iodine solution (6.15) and let stand for one minute. Pour off the Gram's iodine and flood the smear with decolorizer Gram stain (6.16) for 10 to 20 seconds.

Note: The decoloration step is critical. Thick smears will require more time than thin ones. Decolorization has occurred when the solvent flowing from the slide has no colour.

Stop the action of the alcohol by rinsing the slide for a 5-10 seconds using a wash bottle. Cover the smear with safranin (6.17) for 5 minutes. Wash gently for 5-10 seconds and let dry at room temperature.

Examine the smear microscopically, first using the 40X objective to check the staining and observe bacterial distribution, and then using the oil immersion objective (100X) to examine bacterial morphology. The light condenser should be fully open when using the oil immersion lens. Gram negative bacteria are pink and Gram positive bacteria are purple. Campylobacters are Gram negative.

8.10 Species Identification

8.10.1 Oxidase test

Place a piece of filter paper in a clean petri dish and add 2-3 drops of freshly prepared oxidase reagent (6.10). A colony of the test organism, purified on nutrient agar, is then smeared onto the filter paper. If the organism is oxidase-producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour within 10 seconds.

Note: Ignore any blue-purple colour that develops after 10 seconds.

8.10.2 Catalase test

Transfer a *Campylobacter* colony isolated from a blood-free medium (6.5) (as red cells possess catalase activity) to a test tube with 2-3 ml of the hydrogen peroxide solution (6.9). Active bubbling indicates catalase production. Absence of bubbles

indicates lack of catalase production. Campylobacters are catalase positive, except *C. upsaliensis* that is catalase negative or weakly reacting.

Note: Colonies should be transferred using sterile wooden sticks or glass rods. Nichrome wire loop should not be used because it may result in a false positive reaction.

8.10.3 Hippurate hydrolysis

Inoculate sodium hippurate (6.6) with enough number of 18 to 24 hours old *Campylobacter* colonies to obtain a milky suspension. Incubate the suspension at 35 °C for 2 hours. Add 0.2 ml of ninhydrin reagent (6.7). Mix well and incubate for another 10 to 15 minutes. A Deep purple color indicates hippurate hydrolysis (*C. jejuni*). No color change indicates a negative test (*C. coli*).

Note: Using a light inoculum or cells from an old culture may give a gray to very slight purple color; this should be interpreted as a negative test.

8.10.4 Nalidixic acid sensitivity (30 µg disk)

A light suspension of *Campylobacter* colonies is streaked with a cotton swab over the surface of sheep blood agar (blood agar (6.5) supplemented with sheep blood), and then a nalidixic acid (30 µg) disk is applied onto it. The plate is incubated at 42 °C for 1-2 days in an anaerobic jar, using gas generation sachets to achieve a microaerophilic atmosphere.

Strains showing inhibition zones of 20 mm indicate sensitivity to nalidixic acid. *C. coli* and *C. jejuni* are sensitive to nalidixic acid (30 µg), while *C. laridis* is resistant.

9. EXPRESSION OF RESULTS

9.1 Express the results in terms of *Campylobacter* presence or absence per 100 ml of sample (contaminated waters), 1 litre sample (clean seawater), or any other sample volume analyzed.

9.2 Record in the test report (Table 2, item 11) the anomalies observed during the test procedure, such as lack of growth of *Campylobacter* strain controls on enrichment and plating media, lack of false-positive results from control media not inoculated, and temperature deviations from those prescribed for sample storage and incubation.

10. TEST REPORT

Table 2. *Campylobacter* spp. in seawater and sewage samples.

1. Sampling area
   country: _____
   area: _____

2. Sampling point code number: _____
   (station) longitude: _____
   latitude: _____

3. Time of sampling hour: ___.___ day: _____ month: _____ year: _____

4. Sampling and environmental conditions
   Sampling depth: _____ Container number: _____
   Temperature at sampling depth: _____
   Salinity at sampling depth: _____ Duration of storage: _____
   (other factors which may influence the results should be reported under 11)

5. Time of filtration hour: ____ . ____ day: ____ / ____ / ____
6. Start of incubation hour: ____ . ____ day: ____ / ____ / ____
7. End of incubation hour: ____ . ____ day: ____ / ____ / ____

8. Confirmatory tests carried out:
   Basic fuchsin stain: positive: _____
   negative: _____
   Gram stain: positive: _____
   negative: _____
   Species identification: _____

9. Qualitative results:
   Absence of *Campylobacter* spp.: _____
   Presence of *Campylobacter* spp.: _____
   in 100 ml of sample: _____
   in 1 000 ml of sample: _____
   in _____ ml of sample

11. Anomalies observed in the test procedure:
   _____
   _____
   _____
   _____

12. Full address of the institution which carried out the analysis:
   _____
   _____
   _____
   _____

13. Name(s) and signature(s) of the person(s) who carried out the analysis:
   _____
   _____
   _____
   Date: _____

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