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Standard Procedures for Bacteriological Analysis

Procédures pour analyses bactériologiques

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STANDARD PROCEDURES FOR BACTERIOLOGICAL ANALYSIS

PROCÉDURES POUR ANALYSES BACTÉRIOLOGIQUES

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STANDARD PROCEDURES FOR BACTERIOLOGICAL ANALYSIS | **PROCÉDURES POUR ANALYSES BACTÉRIOLOGIQUES**

No.	Date
1	15/05/98

Bulletin

TO: All Holders of the Standard Procedures for Bacteriological Analysis Manual

SUBJECT: MODIFICATIONS TO THE PROCEDURES

Attached are modifications to various Chapters and Appendices of the Bacteriological Analysis manual. The changes have been incorporated in this bulletin format in order to provide the updates to manual holders as quickly as possible and also due to the uncertainty as to whether the manual will continue to be used as a bacteriological procedures reference by the Agency.

The changes to the respective sections have been incorporated on separate pages so the pertinent page(s) may be inserted with the relevant chapter/appendix.

If required, these modifications will be incorporated in the manual at a later date.

Cam Prince
Director
Fish, Seafood and Production Division

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FOREWORD

This is the third edition of the Bacteriological Procedural Manual and supersedes the second edition published in April 1972.

The Bacteriological Procedural Manual was developed to provide a consolidated reference of the procedures and methods used in the Fish Inspection Laboratories of the Department of Fisheries and Oceans for the bacteriological analysis of fish and fish products. The procedures for this manual have been selected from a number of sources and appropriate references are given. No claim is made that these procedures are superior to others that may be available.

The manual is published in loose-leaf format to permit flexibility in the revision of procedures and in the addition of new ones as they are published. As new procedures become available, they will automatically be forwarded to holders of this manual. Comment and suggestions for improvement are welcome.

Requests for copies should be directed to:

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Fisheries and Oceans | Pêches et Océans

Date 31/03/88

STANDARD PROCEDURES FOR BACTERIOLOGICAL ANALYSIS

PROCÉDURES POUR ANALYSES BACTÉRIOLOGIQUES

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CHAPTER 1

THE COLLECTION AND STORAGE OF SAMPLES FOR ANALYSIS

1.1 Introduction

It is of the utmost importance that all samples accurately reflect bacteriological conditions at the time that sampling is done. All sampling must be carried out aseptically in order that no question as to the source of the bacteria present on a sample can arise. Samples must be processed as soon after collecting as is practicable. In the interim they must be held under conditions that will preserve the original bacterial flora as completely as possible, permitting neither die-off nor multiplication. Chilling the sample and holding it at the temperature of melting ice is usually the only feasible way in which samples can be stored without significantly changing the bacteriological picture. In some cases, samples must be frozen but it should be recognized that this may diminish bacterial numbers in the sample. Protracted frozen storage may further reduce the viability of bacteria in the samples. Do not freeze samples destined for Vibrio parahaemolyticus analysis.

1.1.1 Definitions

Lot: A quantity of food produced and handled under uniform conditions at a fixed location.

Sample: A representative portion of a lot.

Sample Unit: One of a number of individual containers that make up a sample.

Analytical Unit: The amount of product withdrawn from the sample unit for analysis. Unless otherwise specified, it will be 100g.

1.2 Sampling Plans

An attribute sampling plan or a variable sampling plan may be used to estimate compliance of product lots & water sources to established microbiological specifications.

Attribute plans are based on the presence or absence of a specific characteristic. They can be further subdivided into either two or three class plans. For example, Salmonella guidelines are a two class plan whereas a three class plan introduces degrees of acceptability; for example, Escherichia coli guidelines show the product can be acceptable, marginally acceptable or unacceptable.

Definitions of defective sample units, sample size and acceptance numbers are detailed in the latest Departmental microbiological guidelines.

Variable sampling plans are based on the measurement of some continuous data and use of these plans requires knowledge of frequency and distribution. These plans are used in the evaluation of shellfish growing waters and the performance of depuration plants (International Commission on Microbiological Specifications for Foods-ICMSF).

1.2.1 Sample Size

Sampling should be representative of the lot. Five sample units per lot will be drawn randomly for analysis unless otherwise specified.

1.3 Laboratory Apparatus
(Some specifications are outlined in Appendix B)

Sterile screw-cap or ground glass stoppered bottles, or autoclavable, nontoxic plastic bottles, with or without added thiosulfate.
Felt pen.
Glass-marking crayon.

Sterile, wide mouth screw-cap jars - glass or autoclavable, nontoxic plastic.
Long forceps, stainless steel, or of noncorrodible, nontoxic material.
Sterile plastic bags.
Depth water sampler.
Insulated containers.
Ice or dry ice.

1.4 Media and Reagents
(Some specifications are outlined in Appendices C and D)

Denatured Ethyl Alcohol 95% for flaming instruments, or 70% for general disinfection. Alternative disinfectants are acceptable if they leave no toxic residues that may be transmitted to the sample.
Sodium Thiosulfate, 10% solution

1.5 Procedure

1.5.1 Water Samples

Collect the samples of water in clean, sterile bottles of suitable size. One hundred to two hundred mL ground-glass stoppered or screw-cap bottles will hold sufficient water for a routine analysis. Screw-cap closures must not contain toxic or bacteriostatic substances. Autoclavable, nontoxic plastic screw-cap bottles for sampling are also acceptable. The tops and necks of sample bottles must be enclosed within a protective covering prior to sterilization. The protective covering may be aluminum foil, rubberized cloth, heavy impermeable paper or bottle cover caps.

Sample bottles to be used for collecting chlorinated water shall contain 0.1mL of a 10% solution of sodium thiosulfate prepared with distilled water. This is sufficient for a 100mL water sample. For larger bottles, add a proportionately larger amount of the thiosulfate solution. Add the thiosulfate solution to the bottles before they are sterilized. Use sufficient thiosulfate solution to neutralize all chlorine present (5 parts thiosulfate will neutralize 1 part chlorine).

In order to obtain a representative sample from a tap, open the tap fully and allow to run for 2 or 3 minutes, or a sufficient time to permit clearing of the service line. Remove the stopper or cap of the sample bottle and hold by the protective covering. After the sample is drawn, replace the stopper or cap in such a way that the protective covering remains in place.

When a still body of water is to be sampled, remove the cap from the bottle as already outlined, hold the bottle near the base and plunge neck downward below the surface to a depth of about 30cm. Then tilt it with the neck pointed slightly upward and during filling, push the bottle horizontally forward in a direction away from the hand to avoid contamination.

If any current exists, direct the mouth of the bottle against the current.

When applicable, use a depth sampler. The more popular devices utilize the sample bottle and are designed so that, upon reaching the desired depth, the stopper may be raised to fill the bottle. Such devices are useful to depths of 10 to 20 metres. Beyond that depth, hydrostatic pressure makes it impossible to remove the stopper. When such samplers

are not suitable, the capillary tube water sampler in general use for oceanographic work may be used.

The number of water samples to be taken from any one source may be left to the discretion of the laboratory concerned, but keep in mind that sampling must be sufficient to detect contamination under all conditions that might influence the quality of the water. These would include current, tides, wind action, precipitation, landwash, temperature and salinity gradients.

Whenever possible, start the bacteriological examination of water samples immediately after collection. When this is not feasible, sample bottles must be held at temperatures below 5°C until analysed. The holding time should not exceed 6h for impure waters and for all sea water samples, and should not exceed 24h in any case. Should this time limit be exceeded, record actual time between sampling and analysis.

During sampling, allow for sufficient head space in the bottles to permit adequate mixing of the sample by shaking.

Analysis of water samples can be initiated in the field. Samples should be inoculated into screw cap fermentation tubes of Lauryl Tryptose Broth (LTB) or lactose broth. The screw cap tubes are required to prevent sample spills during transportation to the laboratory where they will be incubated at 35°C. The portable membrane filtration kit, discussed in Chapter 3, can also be used for on-site analysis. Samples should be analysed in the laboratory within 72h of sampling.

1.5.2 Fishery Products

Take samples at the end of the processing line, i.e. the point beyond which no further handling of the product takes place. Take samples as packaged by the processor or in new polyethylene bags. Samples may be transferred to the bags by the operators who normally handle the fish at the end of the line. Frozen samples may consist of factory produced packages, or of portions removed aseptically from such packages, and must be kept frozen. Fresh samples must be adequately refrigerated until analysed.

Analysis of unfrozen fillets should take place within 24h of sampling, otherwise report the time of sampling and the time of analysis. Reports must state whether or not the samples analysed have been frozen.

Inspections shall consist of 5 end-of-line samples spaced so as to be representative of the production of the plant for that particular run. When special sampling of a plant is being done, the point at which samples are to be taken and the numbers of samples to be taken will be left to the discretion of the laboratory. In principle, however, reported data should be based on a sampling schedule comparable in scope to that used in reporting results for end-of-line samples.

Store frozen samples at the laboratory at a temperature not higher than -20°C. The samples may be defrosted at room temperature for a period of 3h or overnight at 5°C to simplify sample preparation.

1.5.3 Raw Shellfish (Molluscs)

Samples of shellstock and of shucked unfrozen shellfish should be examined within 24 hours after collection. When analysis is unavoidably delayed beyond this point, actual time elapsed between collection and analysis must be reported.

Heavy plastic bags (6 mil gauge) are suitable for shellstock. Keep shellstock samples in refrigerated storage but avoid freezing. Do not permit shellstock to come into direct contact with ice.

In general, take 12-18 shellfish in order to obtain a representative sample and to allow for the selection of 10 sound animals suitable for shucking. For most species this sample size will yield approximately 200g of meats and shell liquor.

A sterile, wide-mouth jar of suitable capacity with water-tight closure is an acceptable container for samples of shucked shellfish taken in shucking houses. Transfer the shellfish to the sample jar with sterile forceps or spoon. Samples of the final product may be taken in the packing cans or containers. Consumer packages are acceptable for examination. Refrigerate samples of shucked shellfish immediately after collection by packing in crushed ice and keep them in ice until examined. The shellfish must not come into direct contact with ice.

1.5.4 Breading and Batter

Transport dry ingredients in sterile wide-mouth screw-cap jars or in polyethylene bags. These need not be refrigerated.

Transport batter in sterile jars and keep at 5°C or lower until analysed.

1.5.5 Brines and Dips

Collect and handle samples in this category as outlined in Section 1.5.1.

1.5.6 Canned Fish

Randomly select samples from lot(s) following appropriate directives for sample size. Transport the sample(s) to the laboratory at ambient temperature. Take special precautions when transporting cans that are obviously swollen or under pressure. Place swollen cans in a plastic bag and transport inside a box or a cooler. These cans should be examined immediately on arrival at the laboratory. DO NOT INCUBATE SWOLLEN CANS.

Except for swollen cans, each sample unit must be judged by the laboratory as to whether or not preliminary incubation would be desirable. When cans are suspected of being non-sterile due to some apparent defect or because of loss of vacuum, such cans may also be opened without prior incubation.

CHAPTER 2: PROCEDURES FOR THE DETERMINATION OF THE AEROBIC PLATE COUNT

Title

Change,

"PROCEDURES FOR THE PREPARATION OF AEROBIC PLATE COUNT"

to read:

"PROCEDURES FOR THE PREPARATION OF *SAMPLES AND THE DETERMINATION* OF AEROBIC PLATE COUNT".

2.4.6 **Pooled Samples**

Delete section 2.4.6.

2.5.1 **Counting Protocol**

Change, paragraph 5, sentence 2:

"If no colonies are found, record as less than 0.5 x the dilution factor. (e.g., ≤ 500/g)."

to read:

"If no colonies are found, record as less than the dilution factor. (e.g., *< 1000/g*)."

CHAPTER 2

PROCEDURES FOR THE DETERMINATION OF THE AEROBIC PLATE COUNT

2.1 Introduction

The Aerobic Plate Count, commonly known as the Standard Plate Count (SPC), is applicable in both water and food analysis and estimates the number of viable organisms in a sample. Although no single set of cultural conditions will permit all organisms present in a sample to grow, strict adherence to the prescribed procedure will produce results that will permit comparison between samples.

The sample is, if necessary, homogenized and diluted. An aliquot of each chosen dilution is pipetted into a petri dish and a liquefied, tempered agar medium is added and mixed with the inoculum. The nutrient agar medium is selected to promote the best achievable growth of the widest spectrum of bacterial species. It is usual to prepare duplicate plates of each dilution to enhance precision. The plates are incubated under specified conditions of time and temperature to produce visible colonies, which can then be counted.

Some selectivity will be exercised by the temperature at which the inoculated plates are incubated. When psychrophiles make up a large proportion of the bacteria being sought, as is the case with raw fish, the incubation temperature should fall within the range of 20-25°C. When organisms of public health significance are being determined, 35-37°C should be used. Where thermophiles are concerned, plates may be incubated at 45°C or even 55°C. In such cases, special selective media are often required and the plates must be protected against dehydration during incubation.

In many cases, such as in the analysis of a prepared food product at the end of a processing line, the SPC is less a measure of the quality of the food than an assessment of plant practices. The SPC is also an index of the probable keeping quality of the product sampled.

2.2 Laboratory Apparatus

(Some specifications are outlined in Appendix B)

Forceps - stainless steel
Tongs - stainless steel
Scissors (with serrated blades)

Knife
Balance - electronic, automatic tare, sensitivity 0.5g
Blender - equipped with auto-transformer for stepless speed control or other suitable homogenizing equipment
Blender jars - 1000mL capacity - stainless steel or glass
- 4000mL capacity - stainless steel
pressure fitted or screw top covers
Bottles for preparing decimal dilutions
Large bottles of diluent (approx. 300mL - 1000mL)
Pipettes (must be tip-calibrated)
- 25mL delivery with wide tip opening
- 10 or 11mL delivery, including some with wide tip opening
- 5mL graduated delivery including some with wide tip opening
- 2.2mL delivery
- 1.1mL delivery

Petri dishes - Borosilicate glass or disposable plastic, 100 X 15mm size
Incubators (25°C and 35°C)
Quebec type colony counter, darkfield model
Electric laboratory timer
Glass-marking pencil
Hand-held counter or touch counter to tally colony count
Water bath - for tempering melted agar (45°C ± 1°C.)

2.3 Media and Reagents

(Some specifications and descriptions are outlined in Appendices C and D)

Peptone water (0.1% peptone) or phosphate buffered water for dilutions
Plating medium - Standard Plate Count Agar
Alcohol, for sterilizing instruments
Sterilizing solution for working surfaces - iodophore or phenolic derivative

2.4 Procedure

2.4.1 Water

Wipe the counter surface with a suitable disinfectant.

Set out appropriately labelled, sterile petri dishes.

Prepare control plates to check on the sterility of the diluent, the glassware, and the agar medium; the possibility of air contamination will also be assessed by the use of these control plates.

Shake the sample bottle vigorously 25 times within 10 seconds or use a mechanical shaker. Prepare a decimal dilution by pipetting 10mL of sample into a labelled 90mL dilution blank (or 11mL into 99mL). Prepare further decimal dilutions if deemed necessary. Shake each dilution bottle in the prescribed manner before withdrawing aliquots.

Into duplicate sets of petri dishes pipette 1mL aliquots from each prepared dilution beginning with the highest dilution. Lift the cover of the culture dish only enough to permit the introduction of the pipette containing the inoculum or the mouth of the bottle with the melted agar.

Add approximately 12-15mL of tempered agar to each inoculated plate. Flame the lips of test tubes, flasks or bottles containing the liquefied medium before pouring into the plates. Melt only such quantities of agar medium as will be used within 3h. Do not remelt unused agar.

Mix the inoculated medium carefully and thoroughly. Allow the medium to solidify, then invert the plates and place in the incubator. Stacks of dishes must be separated so that air circulation is not unduly impeded.

Incubate at 35°C $\pm$ 0.5°C for 48h $\pm$ 2h or at 20°C to 25°C $\pm$ 0.5°C for 72h $\pm$ 3h depending on what organisms are under investigation.

Report temperature and time of incubation.

2.4.2 Fishery Products

To weigh the sample into a sterile, tared blender jar, hold with sterile forceps and cut with sterile scissors or knife. The sample size should be approximately 100g weighed to a tolerance of $\pm$ 0.5g. Add sufficient sterile diluent to give a dilution of 1 part sample to 3 parts diluent (1 in 4 or 1:4). If the sample available is much less than 100g, an initial dilution of 1:10 may be made directly in the blender jar by adding 9 parts diluent to 1 part sample.

Sterilize instruments used in handling the samples by wiping with alcohol, and then flaming. After each use, rinse off adhering organic

matter before again wiping the instruments with alcohol and reflame. Take all reasonable aseptic precautions in the manipulation of the samples.

Blend the diluted sample in the jar for 2 minutes. As soon as practicable after the blending, add 40g of the 1:4 dilution gravimetrically to a 60mL dilution blank. It is preferable that a wide-mouth pipette of 11mL or 25mL capacity be used in making these transfers. If transfer is delayed, particulate matter will settle out. Should this occur, rehomogenize the sample by reblending for 5-10 seconds before making transfer. In order to minimize changes in bacterial numbers, do not prepare more samples than can be diluted and plated in 20 minutes.

Shake the resulting 1:10 dilution 25 times in the prescribed manner, and immediately add 10g of this dilution to a 90mL dilution blank which has been tared on a balance. Make further decimal dilutions volumetrically and plate in the manner outlined in section 2.4.1. Vigorously shake each dilution bottle immediately before making transfers to re-establish homogeneity.

Incubate inverted plates at 25°C for 72h $\pm$ 3h.

2.4.3 Raw Shellfish (Molluscs)

When unfrozen shellstock is used, include only live animals in the sample. Discard gapers. Unless otherwise directed, select 10 shellfish to constitute a sample. (See Section 1.5.3).

It is advisable to wear heavy gauge rubber gloves to protect the hands. Scrub hands (gloves) thoroughly with soap and water, rinse with potable water and rinse again with 70% alcohol. Alternatively, protective gloves may be dipped into an iodophore solution and rinsed with water of potable quality.

Scrape off all extraneous growth and loose material from the shell and scrub the shellfish with a sterile stiff brush under running water of potable quality, paying particular attention to the crevices at the juncture of the shells. Place the cleaned shellfish in clean containers or on clean towels and allow to drain.

Prior to shucking shellstock, re-disinfect hands (or gloves) as described previously. Open the shellstock as directed in "Laboratory Procedures for the Examination of Sea Water and Shellfish" (latest

edition) and collect shell liquor and meats in a sterile blending jar or other suitable sterile container.

The quantity of shellstock used and the initial preparation of samples should be:

1. Shuck 10 shellfish into a tared sterile blender jar, weigh the meats and shell liquor and add an equal weight of diluent (phosphate buffer or 0.1% peptone water). Blend for 90 seconds and dilute to 1:10 by promptly adding 20g of the homogenate to 80mL of diluent;

2. When the shucked quantity from 10 specimens greatly exceeds 200g, and when the consistency of the sample permits, grind undiluted for 30 seconds, then transfer 200g of this preliminary grind to a second sterile blender jar, add an equal weight of diluent and proceed as outlined above;

3. When the consistency of a 1:2 dilution would result in a mixture too thick for effective blending, use 100g of shucked meats and add 300mL of the diluent. Blend for 2 minutes and transfer 40g of the ground material to 60mL of diluent;

4. When 10 shellfish yield a quantity of shucked material much less than 200g, make a 1:10 dilution directly in the blender jar by adding 90mL diluent for every 10g of sample. Blend for 90 seconds;

5. When specimens are very large, and only a part of the animal is used for food, use only the edible portion for analysis (e.g. the foot of the surf clam or the siphon of the geoduck); 100-200g of sample is then blended as outlined in paragraph 1 above.

Prompt transfers will ensure that the blended sample does not separate out in the blender jar. Wide mouth 25mL capacity pipettes are convenient for making these transfers.

Each of the above procedures results in a 1:10 dilution suitable for analysis. Make further decimal dilutions as required and plate as outlined in Section 2.4.1

Incubate inverted plates at 35°C $\pm$ 0.5°C for 48h $\pm$ 3h

2.4.4 Breading and Batter

Make an initial 1:10 dilution in the blender jar. In all other respects, carry out plating as described under section 2.4.2. Incubate inverted plates at 35°C ± 0.5°C for 48h ± 3h or at 25°C ± 0.5°C for 72h ± 3h, depending on the type of organism under investigation.

2.4.5 Brines and Dips

Process these samples as outlined under section 2.4.1. Greater dilution than for water samples may be necessary.

2.4.6 Pooled Samples

In analysing product having a satisfactory record it is permissible to pool sample units for analysis. By pooling the samples the end result will, in effect, be an arithmetic rather than a geometric mean of the Standard Plate Counts.

a) Tare a sterile 4L jar on a large capacity balance, and add approximately 100g from each of the sample units to the jar. Add sterile diluent in the proportion of 3 parts diluent to 1 part fish flesh.

   Blend the diluted sample at "Medium" speed for 2 minutes;or

b) When pooled plate counts - but individual faecal coliform analyses - are to be conducted, the following method is recommended:

   Pool the 5 sample units by pipetting 10g from each of the 1:10 dilutions into a sterile empty dilution bottle. Shake the resulting 1:10 pooled dilution 25 times in the prescribed manner, and immediately add 10g of this dilution to a 90mL tared dilution blank. Make further dilutions and plate in a manner outlined in section 2.4.1.

2.5 Counting Colonies and Recording Results

After the required incubation period, examine plates and if possible select those with 30-300 colonies for counting. Use a hand tally, or other mechanical means of recording the count, and a Quebec type colony counter equipped with a guide plate ruled in square centimetres.

If it is not feasible to count plates at the end of the incubation period, the plates may be stored at refrigeration temperature (1°- 4°C) for up to 24 hours before counting.

2.5.1 Counting Protocol

Use the following protocol to count the colonies, calculate the standard plate count and record the results.

Count the colonies on spreader-free plates having 30 to 300 colonies. Average the counts of the duplicate plates and multiply by the dilution factor.

If only one of two plates at a given dilution contains 30 to 300 colonies, count both plates unless there are spreader colonies. Calculate the average and multiply by the dilution factor.

If plates from consecutive decimal dilutions yield 30 to 300 colonies, calculate the counts of each dilution and then average the results. However, should the higher result be more than twice the lower, report the lower result only.

If plates of all dilutions show fewer than 30 colonies each, record the actual number of colonies counted in the lowest dilution and report the computed result as Estimated Standard Plate Count (ESPC)/g. If no colonies are found, record as less than 0.5 x the dilution factor. (e.g. < 500/g).

If plates of all dilutions show more than 300 colonies each, use such plates as have a count nearest 300. Divide each selected plate into radial sections (i.e. one-half, one-quarter, one-eighth) and count the colonies in one such section. Multiply the total in each case by the appropriate factor to obtain an estimate of the total number of colonies on the plate. Average the estimates of the two plates and multiply by the dilution factor. Report the computed result as ESPC/g.

If the number of colonies appreciably exceeds 300, and the colonies are reasonably evenly distributed, count the colonies in 13 one-centimetre squares (using the ruled guide in the Quebec Colony Counter). Select 7 consecutive squares horizontally across the plate and 6 consecutive squares at right angles. The sum of the counts on the 13 cm^2 multiplied by 5 yields the estimated count per plate (a glass petri dish has an approximate area of 65 cm^2).

When there are more than 10 colonies per cm^2 count the colonies in 5 representative squares. Multiply the total number by 13 and by the dilution factor to obtain the ESPC/g. (Note: If a petri dish other than the standard size glass plate is used, the area of the dish must be calculated in order to obtain the proper factor).

If spreader colonies are encountered and do not cover more than half the plate, count the colonies on the acceptable half and multiply by two. Reject plates more than half covered by spreaders and report as "spr".

Record the results of these counts as "Standard Plate Count" (SPC) or "Estimated Standard Plate Count" (ESPC) per g (per mL) at 35°C (25°C). Avoid fictitious accuracy by recording only the two left-hand digits, rounding off numbers if necessary (e.g. report 142 as 140, and 145 as 150). A two-digit count is recorded as such.

CHAPTER 3: PROCEDURES FOR THE ENUMERATION OF COLIFORMS, FAECAL COLIFORMS AND ESCHERICHIA COLI

3.6.1 Water

Change, paragraph 1, sentence 3:

"Inoculate five 10 mL aliquots of the sample into test tubes containing 20 mL of 1.5 strength, or 10 mL of double strength, or 5 mL of triple strength Lauryl Tryptose Broth (LTB)."

To read:

"Inoculate five 10 mL aliquots of the sample into test tubes containing 20 mL of 1.5 strength, or 10 mL of double strength Lauryl Tryptose Broth (LTB)."

Change, in paragraph 2, sentence 2:

"Under ordinary circumstances, inoculate three 10 mL portions (into 1.5, double, or triple strength LTB as already indicated), three 1 mL portions, and three 0.1 mL portions into 10 mL of LTB (single strength)."

To read:

"Under ordinary circumstances, inoculate three 10 mL portions (into 1.5 or double strength LTB as already indicated) three 1 mL portions, and three 0.1 mL portions into 10 mL of LTB (single strength)."

3.6.2 Fishery Products

Change, in paragraph 1, sentences 4 and 5:

"For samples which are expected, through past experience and plant history, to be of good quality, the 5:1:1 or 3:3:3 multi-tube series may be used. When greater precision is required, or for products in which the quality is suspect or unknown, use the 5:5:5 series."

To read:

"For fisheries products, the 5:5:5 multi-tube series is prescribed."

Delete, paragraph 3, sentence 2

"If necessary" from sentence.

3.6.3 Raw Shellfish (Molluscs)

Change, in paragraph 2, sentences 3 and 4:

"However, since the standard for molluscan shellfish is based on faecal coliforms, classification beyond EC gas positive is not required. Report as Faecal Coliform MPN/100 g."

To read:

"Standards for molluscan shellfish may be based on either faecal coliforms or *E. coli*. For faecal coliform analysis, classification beyond EC gas positive is not required on a routine basis. Report as Faecal Coliform MPN/100 g or *E. coli* MPN/100 g depending on standard being assessed."

3.6.4 Breading and Batter

Delete, "for faecal coliforms" from sentence.

CHAPTER 3

PROCEDURES FOR THE ENUMERATION OF COLIFORMS, FAECAL COLIFORMS, AND ESCHERICHIA COLI

3.1 The Coliform Group

The coliform group is made up of aerobic and facultatively anaerobic, gram-negative, nonspore-forming, cytochrome oxidase negative, rod-shaped bacteria capable of fermenting lactose with gas formation in 48h at 35°C. This group has historically been used to demonstrate insanitation because it is frequently associated with the intestinal contents of warm-blooded vertebrates. For this reason, coliform bacteria are primarily used as a measure of faecal contamination, and thus can potentially indicate the presence of enteric pathogens. This indirect method provides an effective means of detecting conditions that may signify risk to public health.

It is applied to the analysis of potable water and ice.

3.2 The Faecal Coliform Group

Since the coliform group contains some members that are demonstrably of non-faecal origin, a test has been devised that more accurately detects bacteria directly associated with faecal contamination. The faecal coliform group comprises those members of the coliform group that are able, in a defined medium, to ferment lactose with gas formation in 24h at 44.5°C. Faecal coliforms are believed to be more directly associated with faeces from warm-blooded vertebrates than are other members of the coliform group.

Faecal coliform analysis is applied to the examination of bivalve molluscan shellfish and to seawater from shellfish-growing areas.

The terms "coliform" and "faecal coliform" have no taxonomic validity. Thus a faecal coliform count is meaningful only when expressed in terms of the test procedure, i.e. medium, time and temperature of incubation. The principal member of the faecal coliform group is Escherichia coli, which is almost invariably found in human faeces; other faecal coliforms, such as Klebsiella and Citrobacter are less commonly present. These may also proliferate in an environment outside the intestine, as for instance, Klebsiella pneumoniae does in pulpmill effluent. This characteristic reduces their effectiveness as an indicator of faecal pollution.

Escherichia coli is less likely than other members of the coliform group to multiply in an environment outside the intestine. Thus it can, with considerable certainty, be accepted as an indicator of faecal pollution. It is valuable also in that it is closely related to the pathogenic species Salmonella, and will react to environmental stresses in a manner similar to Salmonella. Escherichia coli analysis is applied to most fishery products.

3.3 Methods

3.3.1 Most Probable Number (MPN)

The enumeration of coliforms, faecal coliforms and Escherichia coli is usually arrived at by the Most Probable Number (MPN) method (see Appendix E, and refer also to "Standard Methods for the Examination of Water and Waste-water"). The tables in Appendix E include only some of the possible combinations of positive tubes; those not included are inherently unlikely to occur with any degree of frequency. If they occur in more than 1% of tests, it is an indication of faulty technique or that assumptions underlying the MPN estimate are not being fulfilled. ("International Standards for Drinking Water", 2nd edition). The most common inoculation series are as follows, in ascending degree of precision:

1. Three 10mL portions, three 1mL portions, three 0.1mL portions, total 33.3mL.

2. Five 10mL portions, total 50mL.

3. Five 10mL portions, one 1mL portion, one 0.1mL portion, total 51.1mL.

4. Five 10mL portions, five 1mL portions, five 0.1mL portions, total 55.5mL.

For brevity, these four categories will be coded in this section as 3:3:3, 5:-:-, 5:1:1, and 5:5:5 respectively.

It must be kept in mind that the precision of the MPN procedure is not of a high degree, and caution should be used in interpreting, in terms of sanitary significance, the results of tests unless they are based on an adequate series of analyses of the same source.

3.3.2 Modified A-1 Method

This method is specifically applicable to the analysis of shellfish growing waters and may be used in place of the procedures for the enumeration of faecal coliforms and E. coli previously mentioned.

3.3.3 Membrane Filter Technique

The membrane filter technique is a useful rapid test for drinking water or raw fresh water supplies. Initially it is desirable to conduct parallel tests with the multiple-tube fermentation technique to demonstrate applicability. It also serves as a rapid check on water supplies that have been chlorinated and thus are unlikely to contain coliforms. It has the additional advantage of permitting much larger volumes of water to be analysed than can readily be processed by the multi-tube method. Furthermore, results are obtained sooner than by the multi-tube method.

The membrane filter technique does not readily lend itself to the evaluation of other than fluid samples and even in these the amount of suspended matter will markedly affect the performance of the method. This material can clog the filter or cover it with a film that may interfere with typical colony formation.

3.4 Laboratory Apparatus
(Some specifications are outlined in Appendix B)

Data Sheets should be designed such that presumptive coliform, confirmed coliform, faecal coliform, EMB agar results and IMViC results or those of a comparable test for any one sample may be recorded together on the same sheet.

Homogenization and Dilution: Same apparatus as listed in Chapter 2.

Incubator (35°C ± 0.5°C)
Waterbath - circulating, (44.5°C ± 0.2°C)
Test tubes - 150 x 16mm and 150 x 25mm or similar dimensions
Durham tube - 50 x 6mm and 70 x 12mm
Test tube racks - stainless steel or autoclavable plastic
Standard inoculating loop - platinum or platinum-iridium (5mm diameter) or sterile wooden applicator sticks
Inoculating needle - platinum or platinum-iridium
Certified thermometer calibrated in 0.1°C

3.5 Media and Reagents

(Some specifications are outlined in Appendices C and D)

Lauryl Tryptose Broth (LTB)
Brilliant Green Lactose Bile 2% (BGLB)
EC Medium (EC)
Levine's EMB Agar (EMB)
Simmons Citrate Agar
Brain Heart Infusion Agar (BHI Agar)
Brain Heart Infusion Broth (BHI Broth)
MR-VP Medium
Tryptone 1%
Distilled Water
Lactose Broth (LB)
Kovac's Reagent
alpha Naphthol solution
40% KOH solution
Methyl Red solution
Commercial Rapid Identification Systems
A-1 Medium Modified

NOTE: All tubes of fermentation media, i.e. Lauryl Tryptose Broth, Lactose Broth, EC Medium and Brilliant Green Lactose Bile must contain an inverted Durham tube to trap gas if produced.

3.6 Most Probable Number Method

3.6.1 Water

When analysing a chlorinated water supply, or one known to be of a uniformly high standard, the 5:-:- or 5:1:1 inoculation series may be used. Shake the water sample in the prescribed manner. Inoculate five 10mL aliquots of the sample into test tubes containing 20mL of 1.5 strength, or 10mL of double strength, or 5mL of triple strength Lauryl Tryptose Broth (LTB).

For work in the field, the 3:3:3 series may be used in order to conserve media and glassware. Under ordinary circumstances, inoculate three 10mL portions (into 1.5, double, or triple strength LTB as already indicated), three 1mL portions, and three 0.1mL portions into 10 mL of LTB (single strength).

When water samples are suspected of being heavily polluted, decimal dilutions of the sample may be made. In this way the equivalent of 0.01mL or even 0.001mL of the original sample may be planted. It is desirable, whenever the multi-tube procedure is employed, to plant dilutions which will avoid the reporting of indeterminately high results, such as "more than 2400 per 100mL".

The most precise of the MPN series is the 5:5:5 series. Use this schedule unless there is good cause for selecting one of the less precise series already discussed.

Use tip-calibrated pipettes (serological or delivery), 10mL capacity for large volume transfers, and 1mL (or graduated 5mL) for 1mL transfers. Do not use pipettes to transfer less than 10% of their total volume.

Agitate all inoculated tubes well to ensure adequate mixing of the inoculum and the medium, before the tubes are placed in the incubator.

Incubate the LTB tubes at 35°C $\pm$ 0.5°C for 24h and examine for gas formation. Active effervescence in the medium on agitation or any amount of gas in the Durham tube is a positive result. Reincubate negative tubes for a further 24h and examine again. Discard tubes negative at that time. Report positive results as "Presumptive Coliforms MPN/100mL".

To confirm presumptive results, transfer one 5mm loopful of agitated presumptive broth, (or use a sterile wood applicator stick) from each gas-positive tube to a test tube containing 5-7mL Brilliant Green Lactose Bile Broth (BGLB). Incubate the BGLB tubes at 35°C $\pm$ 0.5°C and examine for gas formation at 24h and 48h. Report gas-positive tubes as "Confirmed Coliforms MPN/100mL".

If it is necessary to determine the faecal coliform count, transfer one loopful from each 24h gas-positive LTB tube to a fermentation tube containing 5-7mL of EC medium. Because 48h gas-positive LTB tubes only very rarely produce gas in EC medium, it is unnecessary to transfer these in routine analyses; they may be considered to be EC negative. Preferably, the EC tubes are preheated and immediately after inoculation are transferred to a circulating water bath for incubation at 44.5°C $\pm$ 0.2°C. The water level in the bath must be at least 1cm above the level of the medium in the tubes. Incubate EC tubes for 24h and report gas-positive results as "Faecal Coliforms MPN/100mL". Discard tubes which are negative after 24h.

When EC gas-positive tubes are to be classified further, streak a loopful on Levine's EMB Agar and incubate the inverted plates at 35°C ± 0.5°C for 18-24h. Record discrete colonies, with or without sheen, showing nucleated centres as typical E. coli.

Other coliform organisms, e.g. Klebsiella, Enterobacter, or Citrobacter, may form mucoid or otherwise atypical colonies. Colourless colonies are not coliforms and need not be considered further.

For further classification of cultures use either the traditional biochemical tests, IMViC or any of the accepted commercial rapid identification systems.

3.6.1.1 IMViC

Transfer selected well-isolated colonies from EMB plates to Brain Heart Infusion Broth and to Brain Heart Infusion Agar Slants and incubate these at 35°C. After visible growth has occurred (4h or overnight), inoculate the following from the BHI Broth:

a) by needle, a Simmons Citrate Agar Slant and a fermentation tube of Lactose Broth, in that order; and

b) by loop, a fermentation tube of EC Medium, and one tube of 1% Tryptone.

Just prior to inoculation, transfer 0.5mL amounts of sterile MR-VP medium aseptically to sterile 13x100mm tubes. Prepare only enough tubes for immediate use.

Using a loop, transfer enough growth from each BHI Agar slant to two 13x100mm tubes containing 0.5mL MR-VP Medium to make an evenly distributed turbid suspension.

Incubate the EC tube at 44.5°C for 24h and record gas formation.

Incubate the Lactose Broth, Simmons Citrate Agar, 1% Tryptone, and MR-VP tubes at 35°C for 24h.

Record acid and gas in Lactose Broth at 24h. If negative, incubate for a further 24h and check again.

Perform the indole test on the 1% Tryptone tube at 24h by adding 3 drops of Kovac's reagent so that it forms a layer on the surface of the medium. A red colour developing in the reagent layer in 1min is a positive result.

Perform the Methyl Red test by adding 1 drop of Methyl Red indicator to an MR-VP tube incubated for 24h. A red colour in the medium indicates a positive reaction. Yellow is negative.

Perform the Voges-Proskauer test on the other tube (24h incubation) of MR-VP Medium. Add, in the following order, the reagents specified, mixing the contents well after each addition:

a) 2 drops saturated Creatine solution
b) 3 drops alpha naphthol reagent
c) 2 drops 40% KOH solution

A pink to bright red colour developing within 30 min is a positive reaction.

Read the Simmons Citrate Agar Slant at 24h, and if negative, again at 48h. Evidence of growth and a blue colour developing in the medium constitutes a positive reaction. (See Appendix I for summary of IMViC reactions).

3.6.1.2 Commercial Rapid Identification Systems

Commercial rapid identification systems can be used as an alternative to the traditional IMViC system to identify Enterobacteriaceae. Laboratories must establish familiarity with the system prior to using it to identify unknown members of this family. Known strains of E. coli and other Enterobacteriaceae should be run periodically.

3.6.2 Fishery Products

In general, the 1:10 dilution will be the lowest dilution used in coliform analyses. When larger coliform numbers are anticipated, make further decimal dilutions. Avoid indeterminate results. For samples which are expected, through past experience and plant history, to be of good quality, the 5:1:1 or 3:3:3 multi-tube series may be used. When greater precision is required, or for products in which the quality is suspect or unknown, use the 5:5:5 series. Other than the use of an original 1:10 dilution the coliform tests for fishery products will conform with the method outlined in section 3.6.1. Do not use pipettes to transfer less than 10% of their total volume.

Transfer all 24h gas-positive tubes of the presumptive test to EC medium for the faecal coliform determination. 48h gas-positive LTB need not be transferred in routine analyses. Confirmed coliform counts, using BGLB, are generally not required in the analysis of fishery products.

Since EC gas-positives in this category may include a significant proportion of coliforms other than E. coli, process all EC gas-positive tubes through EMB agar. If necessary use IMViC to establish their identity. A rapid identification system may also be used for this purpose.

3.6.3 Raw Shellfish (Molluscs)

For molluscan shellfish, the 5:5:5 MPN series is prescribed. Transfer five 10mL and five 1mL aliquots of the 1:10 dilution, and five 1mL aliquots of the $1:10^2$ dilution. Do not transfer 0.1mL aliquots of shellfish homogenates.

Preparation of 1:10 dilution of all molluscan shellfish has been previously described in section 2.4.3. Continue as outlined in section 3.6.2. However, since the standard for molluscan shellfish is based on faecal coliforms, classification beyond EC gas positive is not required. Report as Faecal Coliform MPN/100g.

3.6.4 Breading and Batter

Analyse for faecal coliforms as outlined in section 3.6.2, keeping in mind that EC gas-positive results may include a high proportion of coliforms other than E. coli.

3.6.5 Brines and Dips

Proceed as prescribed in section 3.6.2. Since the presence of salt in the brines may suppress gas formation by coliforms present in the sample, it is possible that positive results may be obtained in higher dilutions even when the largest inocula appear to be non-gas forming. Subculturing a loopful from such non-gas producing inocula to fresh LTB may be successful in demonstrating the presence of gas forming coliforms.

3.7 Modified A-1 Method

3.7.1 Sea Water

The most precise of the MPN series is the 5:5:5 series. Use this schedule unless there is good cause for selecting one of the less precise series. Avoid the reporting of indeterminately high results.

Shake the water sample and prepare a decimal dilution as previously described.

Plant five 10mL aliquots of the sample in fermentation tubes containing 20mL of 1.5 strength or 10mL of double strength A-1 Medium. Although salt inhibition is not anticipated as a problem, the likelihood will be less with the use of the larger volume of medium. Plant five 1mL aliquots of the sample in fermentation tubes containing 10mL of single strength A-1 Medium. Plant five 1mL aliquots of the decimal dilution of the sample into fermentation tubes containing 10mL of single strength A-1 Medium.

Agitate inoculated tubes well to ensure adequate mixing of the inoculum and the medium, before the tubes are placed in the incubator.

Incubate the racks of inoculated tubes in an air incubator, or in a water bath, at 35°C $\pm$ 0.5°C for 3h.

Then transfer the racks of tubes to a water bath at 44.5°C $\pm$ 0.2°C for 21h $\pm$ 2h. Maintain the water level in the bath above the level of the inoculated medium in the tubes. This will ensure adequate temperature control.

Presence of gas in the inverted vial or active effervescence when the tube is slightly agitated constitutes a positive result. Discard negative tubes. Report results as "Faecal Coliforms MPN/100 mL".

For further characterization of faecal coliforms, follow instructions in Section 3.6.1.

3.8 Membrane Filter Technique

3.8.1 Laboratory and Field Apparatus and Materials
(Some specifications are outlined in Appendix B)

Culture dishes, 60mm X 15mm, 50mm X 9mm, plastic

Filtration units, glass or stainless steel
*Portable membrane filter field kit for field use; temperature set at 35°C ± 0.5°C

Filter membranes, presterilized, individually wrapped, grid-marked, 47mm diameter, pore size 0.45um
Absorbent pads, presterilized (for use with nutrient medium in culture dishes)
Forceps, flat, smooth tipped
Sterile pipettes, 10mL and 1mL
Sterile sample bottles, polypropylene or glass
Sterile dilution blanks, 99mL and 90mL (0.1% peptone)
Sterile 0.1% peptone in larger bottles (for rinsing filters after filtration)
Vacuum pump, with gauge; or
Vacuum/pressure pump, hand-operated (for field work)
Portable butane or alcohol burner
Hand tally for counting
Fluorescent lamp (portable, for field use)
Magnifying glass (field)
Dissecting microscope (magnification 5-10X)
Graduated cylinder, autoclavable plastic, 250mL capacity.

*NOTE: Includes a portable incubator with its components such as a stainless steel measuring cup, stainless steel filtration apparatus, syringe pump, stainless steel forceps with smooth tips to permit handling of filters without damage. This incubator may be connected to 115 volt AC, 230 volt AC, 6 volt DC, 12 volt DC, and 24 volt DC using the adaptors provided.

IMPORTANT! Turn the selector switch to the proper indicated voltage. The temperature is set to 35°C ± 0.5°C.

3.8.2 Media and Reagents

(Some specifications are outlined in Appendices C and D)

m-ENDO Agar LES
m-ENDO Agar MF
m-FC Agar
1% Rosolic Acid in 0.2N Sodium hydroxide
Methanol for sterilization purposes
Lauryl Tryptose Broth
95% Ethanol

The medium of choice is m-Endo Agar LES. This medium can be prepared in the laboratory and used in the field. After filtration, membranes may be placed directly on the surface of the agar medium with or without the suggested pre-enrichment in Lauryl Tryptose Broth. The prepared medium should be stored in the dark at 4°C and used within seven days. Uninoculated controls should also be used.

3.8.3 Procedure

The amount of water to be sampled is governed by the expected bacterial density. Ideally, the quantity filtered will result in 20 to 80 coliform colonies, and not more than 200 colonies of all types, on the membrane.

Suggested sample quantities:

a) Treated waters - duplicate 100mL to 500mL volumes.

b) Well water - three aliquot volumes with 1mL, 10mL and 100mL portions of each aliquot.

c) Polluted water - as (b) above but reduced volumes in accordance with the degree of pollution.

When less than 20mL is to be filtered, dilute with sterile diluent to minimum volume of 30mL just before filtration.

Pipette 4mL sterile melted m-ENDO Agar LES into a sterile plastic culture dish. Allow agar to solidify, then place a sterile absorbent pad inside the cover of the culture dish. Saturate the pad with sterile Lauryl Tryptose Broth (1.8mL to 2.2mL).

Using sterile forceps, place a sterile filter membrane over the porous plate or stainless steel screen of the filter holding unit, grid side up. Attach the funnel unit. Pour the water sample into the funnel and filter under partial vacuum. Rinse funnel walls with 30mL of sterile diluent. Remove the funnel and transfer the membrane, grid side up, aseptically with sterile forceps to the surface of the absorbent pad containing the Lauryl Tryptose Broth. The membrane must be carefully "rolled" onto the surface to avoid trapping air bubbles between membrane and medium.

Resterilize funnel prior to analysing additional samples. Incubate the culture dishes at 35°C $\pm$ 0.5°C for 3h. Then, with sterile forceps,

transfer the membrane from the absorbent pad in the cover of the dish to the m-ENDO Agar LES surface. Exercise care that no air is trapped between the membrane and the agar surface. Incubate the culture dish at 35°C ± 0.5°C for 18h to 24h.

For counting, remove the cover from the culture dish. Make counts under adequate magnification (5-10X) using oblique lighting to demonstrate a metallic sheen formation on the colonies.

Report as "Coliforms/100mL", taking into account the actual volume of sample filtered.

3.8.4 Faecal Coliforms

Use m-FC Agar with added rosolic acid. However, if background non-target organisms are not a problem, the rosolic acid may be omitted. Transfer the inoculated membrane to the agar surface and incubate the culture dish, enclosed in a plastic bag to exclude moisture, submerged in a circulating water bath at 44.5°C ± 0.2°C for 24h ± 2h.

Count all colonies showing a degree of blue colour. Report results as "Faecal Coliforms/100mL", taking into account the volume of sample filtered.

3.8.5 Membrane Filter Analyses in the Field

Use the portable stainless steel filter holder. To sterilize, remove the stainless steel receptacle from the funnel base assembly. Pour methanol onto the asbestos wick around the base and ignite. After 30s, place the inverted receptacle over the funnel and leave in place for 15min. Sterilization takes place due to the formaldehyde formed when air is excluded from the ignited methanol. Remove the receptacle and set it upright and place the filter holder base on it. Before use, rinse the funnel with sterile diluent to remove possible toxic residues.

Using sterile forceps, place a sterile membrane filter on the stainless steel screen of the filter holder base. Lock the funnel in place.

Attach the manually operated vacuum pump to the filter holder assembly by the plastic tubing provided. Add the sample to the funnel and filter the sample, using the pump to produce the necessary vacuum.

Process the inoculated membrane filter as outlined previously.

3.8.6 Confirmation of Colonies

Biochemical confirmation of the enumerated colonies should be periodically carried out to establish the reliability of the technique. The minimum cultural and biochemical confirmation should include inoculation into LTB or BGLB Media.

CHAPTER 4: PROCEDURE FOR THE ENUMERATION OF COAGULASE-POSITIVE STAPHYLOCOCCI

4.1 Introduction

Delete, paragraph 5 in its entirety.

"Although a plating technique using a selective agar medium is most commonly employed, an MPN multiple-tube enrichment/confirmatory technique may be used when the estimated total number of the staphylococci is small. It may also be desirable at times to use a combination of media for the enumeration of staphylococci from certain products. The medium used should have the potential to recover stresses/injured staphylococci without occurrence of crowding by non-target organisms."

4.2.1 Laboratory Apparatus

Delete, "Incubator, humidified, (45 °C + 0,5 °C) optional"

4.2.2 Media and Reagents

Delete,

"Several agar media may be used for primary plating for analysis of food samples; these include:"
"Tellurite Polymyxin Egg Yolk Agar (TPEY)"
"Staphylococcus 110 medium with added egg yolk (S110Y)"
"Staphylococcus 110 medium with added egg yolk and azide (S110YZ)"
"Nutrient Agar, Trypticase Soy Agar; or"
"Brain Heart Infusion Agar"
"Note: * Sterile egg yolk suspension with or without tellurite is available commercially.
** The added azide (0.05 g/L of medium) will inhibit spreaders frequently encountered in samples with cereal content."

4.2.3.1 Presumptive *S. aureus*

Change, in paragraph 1, sentence 1:

"Prepare plates of one of the staphylococcus media listed in 4.2.2 (minimum of 20 mL per plate)."

to read:

"Prepare plates of *Baird-Parker agar* (minimum of 20 mL per plate)."

Delete, in paragraph 3, sentences 4 and 5:

"When using S110Y or S110YZ, plates may be incubated at 45 °C + 0.5 °C for 24h. A humidified incubator atmosphere is essential during 45 °C incubation."

4.2.3.2 Counting Colonies and Recording Results

Change:

"Typical S. aureus colonies are described below:

a) S110Y and S110YZ:

- 2mm white to yellow colonies with an opaque white precipitation zone.

b) BP:

- circular, smooth, convex colonies, 2-3mm diameter that:

1) are grey to black in colour, with or without an off-white margin.

2) may or may not produce a zone of opaque white precipitation.

3) may or may not produce an outer cleared zone.

Note: When first isolated from frozen material, staphylococci may produce rough colonies.

c) TPEY:

- black colonies, 1-2mm diameter, with a white opaque precipitation zone around the colony, and a cleared zone (halo) around the colony. Not every strain will show all of the above characteristics."

to read:

"Typical colonies on BP are circular, smooth, convex colonies, 2-3 mm in diameter that:

1) are grey to black in colour, with or without an off-white margin;
2) may or may not produce a zone of opaque white precipitate; and
3) may or may not produce an outer cleared zone.

Note: When first isolated from frozen material, staphylococci may produce rough colonies."

4.3 The Most Probable Number Method

Delete, entire section.

CHAPTER 4

PROCEDURE FOR THE ENUMERATION OF COAGULASE-POSITIVE STAPHYLOCOCCI

4.1 Introduction

The presence of small numbers of enterotoxigenic staphylococci on a product is not uncommon. Only when the product is contaminated with large numbers of these organisms and is handled in such a way that the staphylococci are provided with an opportunity to multiply and produce toxin in the food, does their presence become a serious matter.

The ability of certain staphylococci to produce enterotoxins, with few exceptions, is limited to those strains that are coagulase-positive and may produce a heat-stable nuclease (DNAse).

The normal flora found on raw fish and shellfish make refrigeration of such foods essential. Enterotoxigenic staphylococci which may be present on the food cannot grow at such reduced temperatures. If the temperature of the food should rise to a point where staphylococcal reproduction could take place, the other bacterial flora, which greatly outnumber the staphylococci, would render the food inedible through spoilage long before the staphylococci could increase to dangerous levels; indeed they might inhibit the growth of staphylococci very effectively through competition. For this reason, water and raw fish or raw seafood products need not be analysed routinely for the presence of staphylococci.

Only when procedures used in preparing a food product eliminate or greatly reduce the original bacterial flora on the product is there a danger that subsequently introduced staphylococci might increase to a point where they could render the food toxic. Such foodstuffs, which include precooked foods and breaded and battered products, may remain at room temperature or warmer for some time after they have been prepared. This extended period, during which relatively high temperatures would favor staphylococcal growth, may be necessitated by manufacturing methods, such as the peeling of shrimp or the manual packaging of fish sticks. During this handling, and at any subsequent period before the product is chilled or frozen, staphylococci may be introduced into the product and may then multiply sufficiently to render the food toxic.

Although a plating technique using a selective agar medium is most commonly employed, an MPN multiple-tube enrichment/confirmatory

technique may be used when the estimated total number of the staphylococci is small. It may also be desirable at times to use a combination of media for the enumeration of staphylococci from certain products. The medium used should have the potential to recover stressed/injured staphylococci without occurrence of crowding by non-target organisms.

4.2 Plate Count Procedure

4.2.1 Laboratory Apparatus
(Some specifications are outlined in Appendix B)

Apparatus required for homogenization and dilution as outlined in 2.2

Culture tubes, sterile, 12 x 75mm or similar dimensions
Glass spreader bar
Incubator (35°C ± 0.5°C)
Incubator, humidified, (45°C ± 0.5°C) optional
Petri dishes (plastic or glass)
Rotating petri dish table (optional)
Thermometer (0 - 100°C) (1° division)
Water bath (35-37°C ± 0.5°C)

4.2.2 Media and Reagents
(Some specifications are outlined in Appendices C and D)

Several agar media may be used for primary plating for analysis of food samples; these include:
Baird-Parker agar with added egg-yolk tellurite enrichment (BP)*
Tellurite Polymyxin Egg Yolk Agar (TPEY)
Staphylococcus 110 medium with added egg yolk (S110Y)*
Staphylococcus 110 medium with added egg yolk and azide (S110YZ)**

Brain Heart Infusion Broth
Toluidine Blue DNA Agar
Nutrient Agar, Trypticase Soy Agar; or
Brain Heart Infusion Agar
Coagulase Plasma EDTA

NOTE: * Sterile egg yolk suspension with or without tellurite is available commercially

** The added azide (0.05g/L of medium) will inhibit spreaders frequently encountered in samples with cereal content.

4.2.3 Procedure

4.2.3.1 Presumptive S. aureus

Prepare plates of one of the staphylococcus media listed in 4.2.2 (minimum of 20mL per plate). Air dry the plates sufficiently to permit absorption of the 0.5mL inoculum which is subsequently pipetted into each plate. This drying can be accomplished by placing the plates, inverted, with the covers slightly off to one side, in an air-circulating 35°C incubator or in a horizontal laminar flow hood for 1-2 hours.

With a pipette, inoculate and distribute 1mL of inoculum over two plates, approximately 0.5mL per plate. The 1:10 or 1:100 dilution as prepared in section 2.4 should also be used. Spread the inoculum evenly over the agar surface with the tip of the pipette, or with a sterile glass spreader bar. Do not spread the inoculum to the agar edge, since this may cause confluent growth at the interface between the agar and the petri dish.

Leave the inoculated plates in an upright position until the inoculum has been absorbed by the medium (approximately 10 minutes on properly dried plates). If the inoculum is not readily absorbed, the plates may be placed in an upright position in a 35°C incubator for 1-2h, or until the liquid on the surface of the plates disappears. Invert the plates and incubate at 35°C $\pm$ 0.5°C for 48h $\pm$ 2h. When using S110Y or S110YZ, plates may be incubated at 45°C $\pm$ 0.5°C for 24h. A humidified incubator atmosphere is essential during 45°C incubation.

4.2.3.2 Counting Colonies and Recording Results

Typical S. aureus colonies are described below:

a) S110Y and S110YZ:
   - 2mm white to yellow colonies with an opaque white precipitation zone.

b) BP:
   - circular, smooth, convex colonies, 2-3mm diameter that:

   1) are grey to black in colour, with or without an off-white margin.

   2) may or may not produce a zone of opaque white precipitate.

3) may or may not produce an outer cleared zone.

NOTE: When first isolated from frozen material, staphylococci may produce rough colonies.

c) TPEY:
- black colonies, 1-2mm diameter, with a white opaque precipitation zone around and under the colony, and a cleared zone (halo) around the colony. Not every strain will show all of the above characteristics.

Where possible, select plates containing 20-200 presumptive coagulase-positive colonies per plate. Combine the counts for both plates of the same dilution. If several types of colonies indicative of S. aureus are observed, record counts for each type separately. Calculate the count, using the dilution factor, and report as "Presumptive Coagulase-Positive Staphylococci/g".

If the plates contain fewer than 20 presumptive colonies, record as number counted x dilution factor. Counts on crowded plates may be estimated. Record as "Estimated S. aureus/g". If any sample remains, the analysis should be repeated using higher dilutions so that accurate counts can be made.

When no presumptive colonies are found on plates inoculated with the 1:10 dilution, report results as "less than 10/g".

4.2.4 The Coagulase Test

Select a representative number of each type of colony and inoculate into Brain Heart Infusion Broth. Incubate overnight at 35°C, then transfer 0.2mL (3-4 drops) of each BHI culture to sterile 12-13mm tubes containing 0.5mL Coagulase Plasma EDTA. Mix thoroughly but avoid foam formation.

Incubate the plasma tubes at 35°C and examine at half hour intervals over a 6h period for clot formation. Do not agitate the tubes during examination. If firm clotting has not taken place after 6h, continue incubation overnight and re-examine tubes for clot formation.

Only a firm or large organized clot is considered as a positive coagulase reaction. (Figure 1)

Cultures yielding weak coagulase reactions must test positive for the most able nuclease before being included as S. aureus. Test known positive and negative cultures simultaneously with suspect colonies.

Report as "Confirmed coagulase-positive staphylococci per gram" taking into account the number of positive colonies of each type and dilution used. It is necessary to record positive results in such a way as to indicate how many cultures were tested and what proportion were positive.

4.2.5 The Thermostable Nuclease Test (Ancillary Test)

Pipette melted Toluidine Blue DNA Agar medium into a flat bottom petri plate or onto a microscope slide to a thickness of 1.5-2.0mm.

With a pasteur pipette, cut wells 2mm in diameter, approximately 12mm apart, in the solidified agar layer and remove agar plugs by aspiration.

In separate sterile screw-capped tubes, heat 0.5mL aliquots of broth cultures used for the coagulase test in a boiling water bath for 15min, then cool rapidly under tap water.

Fill duplicate wells with paired heated and unheated broth cultures and incubate at 35°C for up to 4h.

A pink halo surrounding the perimeter of both wells within 4h is indicative of thermostable nuclease activity. A pink halo around the unheated control only is a negative result.

4.3 The Most Probable Number Method

4.3.1 Laboratory Apparatus
(Some specifications are outlined in Appendix B)

Water bath (35°C)
Other apparatus from section 4.2.1

4.3.2 Media and Reagents
(Some specifications are outlined in Appendices C and D)

Baird-Parker Agar with added egg yolk tellurite enrichment (BP)
Sodium Chloride c.p.
Tellurite Polymyxin Egg Yolk Agar (TPEY)
Trypticase Soy Broth with 10% NaCl.

4.3.3 Procedure

Prepare decimal dilutions of the food sample as outlined in section 3.6.2.

Pipette 1mL of the food homogenate into each of 5 tubes of Trypticase Soy Broth with 10% Salt for each dilution to be tested.

Incubate the inoculated tubes at 35°C for 24h.

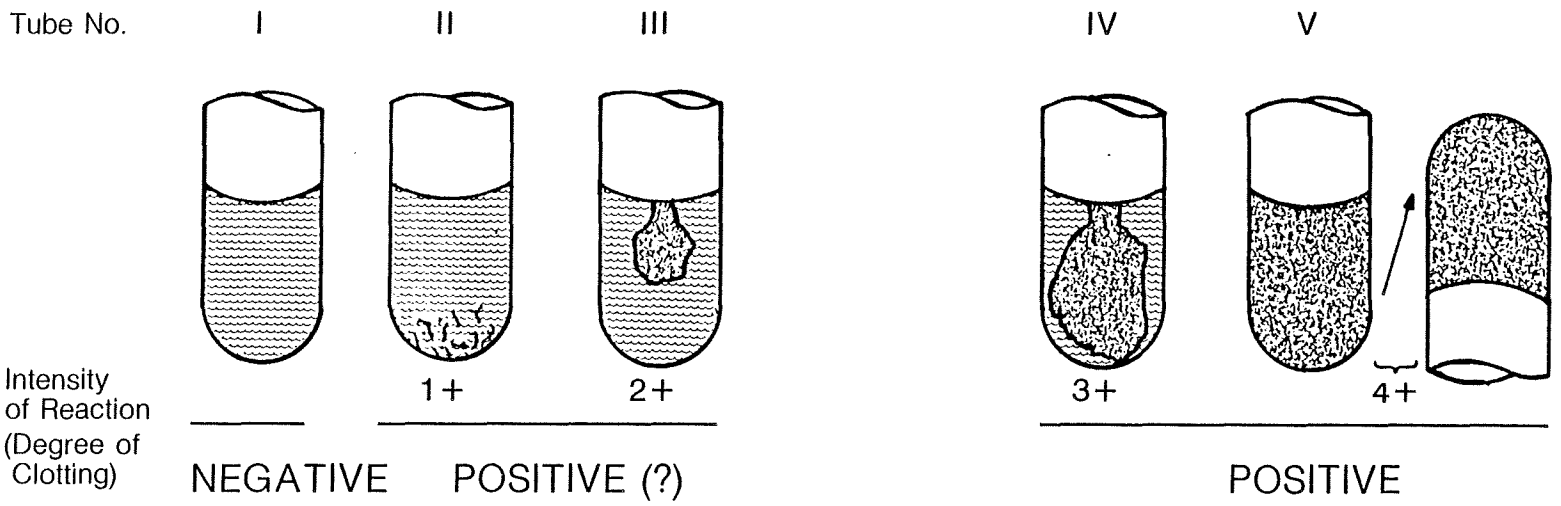
Streak a loopful of material from each incubated tube onto a plate of BP or TPEY agar so as to obtain isolated colonies.

Incubate the plates at 35°C for 48h. Select typical colonies for coagulase testing as in Section 4.2.4.

All tubes from which coagulase-positive colonies have been isolated are considered to be positive. Calculate and report "Coagulase-positive Staphylococci MPN/100g" or "per gram" using the MPN tables in Appendix E.

FIGURE 1

COAGULASE TEST REACTIONS



CHAPTER 5: PROCEDURE FOR THE DETERMINATION OF SALMONELLAE

5.3 Media and Reagents

Change,
"II. Selective Enrichment media:
Tetrathionate Broth with added Brilliant Green
Selenite Cystine Broth (optional)"

to read:
"II. Selective Enrichment media:
Tetrathionate Broth with added Brilliant Green
Selenite Cystine Broth"

5.4.1 Sample size

Change,
"A sample consists of one to five units of 50 g each which may be analyzed individually or may be pooled for analyses. The exception is fishmeal where a sample consists of 100 g."

to read:
"A sample consists of one to five units of *25 g* each which may be analyzed individually or may be pooled for analyses."

5.4.2 Pre-Enrichment

Change, in paragraph 1 :
"The pre-enrichment is combined with the sample in a 4 to 1 ratio (200 mL are required per 50 g)."

to read:
"The pre-enrichment *medium* is combined with the sample in a *9 to 1 ratio (25 g in 225 mL enrichment broth or if a pool of five sub-samples then 125 g in 1125 mL).*"

5.4.3 Selective enrichment

Change,
"After pre-enrichment is completed swirl containers gently, then using a mechanical pipetting device add 1 mL of the sample to the screw-cap tube(s) containing 10 mL of selective enrichment medium. After careful mixing, incubate the inoculated tube(s)."

Tetrathionate Broth containing Brilliant Green
(1) 43 °C for 18-24h
(2) 35 °C for 18-24h (optional)

Selenite Cystine
35 °C for 18-24h"

to read:
"After pre-enrichment is completed swirl container gently, then using a mechanical pipetting device add 1 mL of the *mixture to 10 mL of tetrathionate broth and add 1 mL of mixture to 10 mL of Selenite Cystine broth.* After careful mixing, incubate the inoculated tubes as follows:

Tetrathionate Broth containing Brilliant Green
(1) 43 °C for 18-24h
(2) 35 °C for 18-24h (optional)

Selenite Cystine
35 °C for 18-24h"

CHAPTER 5

PROCEDURE FOR THE DETERMINATION OF SALMONELLAE

5.1 Introduction

The general procedure for isolating enteric pathogens of the Salmonella group from foods is outlined below:

I. Non-Selective Enrichment or Pre-Enrichment

This step favours the recovery of stressed or injured microorganisms. This state may have been caused by heat, freezing, desiccation, preservatives, high osmotic pressure or drastic temperature change. Generally, a sample is suspended in a nutrient medium (Lactose Broth, Lauryl Tryptose Broth) followed by homogenization and incubation.

II. Selective Enrichment

After incubation in the pre-enrichment medium, transfers are made into selective enrichment medium which retards or inhibits the growth of competing microorganisms and favours the growth of Salmonellae. This step involves the addition of an aliquot from the pre-enrichment medium to the selective enrichment medium (Tetrathionate Broth containing Brilliant Green) followed by incubation.

III. Selective Plating

Selectively enriched cultures are streaked onto selective agars to permit the isolation of presumptive Salmonellae. The media employed are Bismuth Sulfite and Brilliant Green Sulfa Agar (both media are necessary), along with supplementary media if desired. Such supplementary media may include XLD Agar and/or Hektoen Enteric Agar.

IV. Purification

Purification of suspect colonies by streaking on a differential medium, e.g. MacConkey Agar.

V. Biochemical Screening

VI. Serological Confirmation

5.2 Laboratory Apparatus
(Some specifications are outlined in Appendix B)

Blender or other suitable homogenizing apparatus (large blender may be necessary for pooled analyses), with variable speed control
Jars - Screw-cap, wide-mouth 1L, 2L, 4L capacity, polypropylene, autoclavable
Pipettes: 10mL graduated in 0.1mL
1mL graduated in 0.01mL
Petri dishes, 100 X 15mm, borosilicate glass or plastic
Inoculating loops, 3mm or 5mm diameter, platinum-iridium
Inoculating needle, platinum-iridium
Balance, top loading, 1600g capacity, sensitivity $\pm$ 0.5g
Incubator 35°C $\pm$ 0.5°C
Water bath 43°C $\pm$ 0.2°C
Mechanical pipetting device

5.3 Media and Reagents
(Some specifications are outlined in Appendices C and D)

I. Non-selective enrichment media:
Lactose Broth
Lauryl Tryptose Broth
Nutrient Broth

II. Selective enrichment media:
Tetrathionate Broth with added Brilliant Green
Selenite Cystine Broth (optional)

III. Selective agar media:
BG Sulfa Agar
Bismuth Sulfite Agar
Hektoen Enteric Agar (optional)
XLD Agar (optional)

IV. Purification Media:
MacConkey Agar

V. Basic Confirmation Media:
Triple Sugar Iron Agar (TSI)
Lysine Iron Agar (LIA)
Cytochrome Oxidase Test Strips
Nutrient Agar (NA)

VI. Additional Confirmation Media:
Commercial Rapid Identification Systems
or the following:
Dulcitol Fermentation Broth
ONPG Differentiation Discs
Malonate Broth, Modified
Simmons Citrate Agar
Tryptone 1%
Urea Broth or Agar

Other Ingredients and Reagents:

Iodine solution (for Tetrathionate Broth)
Brilliant Green: 0.1% aqueous solution (for Tetrathionate Broth)
NaOH, 1N
pH paper, 5.0 to 7.0 range
NaCl, 0.85% aq. soln., sterile

Commercial Salmonella Antisera and QC Antigens

5.4 Procedure

5.4.1 Sample Size

A sample consists of one to five units of 50g each which may be analysed individually or may be pooled for analysis. The exception is fishmeal where a sample consists of 100g.

5.4.2 Pre-Enrichment

The pre-enrichment medium is combined with the sample in a 4 to 1 ratio (200mL are required per 50g sample).

Weigh each sample unit into a sterile blender jar. Add about half the pre-warmed (35°C), sterile pre-enrichment medium to permit proper blending. By restricting the amount of medium added before homogenizing, foaming will be minimized.

Blend for 2min (fishmeal samples do not require blending). Add the remainder of the medium from the polypropylene jar to the homogenate and swirl carefully to suspend the blended material. Return the entire contents of the blender jar to the polypropylene jar.

Test the mixture with pH paper strip, and if the pH is 6.4 or lower, adjust to pH 6.8 by adding 1N NaOH. Replace the screw-cap loosely and incubate the jar at 35°C for 24h.

5.4.3 Selective Enrichment

After pre-enrichment is completed, swirl container gently, then using a mechanical pipetting device, add 1mL of the sample to the screw-cap tube(s) containing 10mL of selective enrichment medium. After careful mixing, incubate the inoculated tube(s).

Tetrathionate Broth containing Brilliant Green

(1) 43°C for 18-24h
(2) 35°C for 18-24h (optional)

Selenite Cystine
35°C for 18-24hrs.

5.4.4 Selective Plating

Streak one loopful from each of the inoculated enrichment media on to a plate of each of the following selective or differential media:

a) BG Sulfa Agar (BGS)
b) Bismuth Sulfite Agar (BiS)
c) XLD Agar (optional)
d) Hektoen Enteric Agar (optional)

Incubate the inoculated plates for 22-24 h, and the BiS plates for 24 and 48h, all at 35°C.

5.4.5 Isolation and Purification

Examine plates for presumptive Salmonella colonies (Appendix F). Pick at least 1 of each type to a TSI Agar slant and to a LIA Agar slant. Streak the slant and stab the butt of the TSI tube, then, without returning the needle to the colony, streak the slant and stab the butt of the LIA tube 2 or 3 times. Concurrently streak a MacConkey Agar Plate to ensure culture purity and to permit the selection of smooth colonies for serological analysis. Incubate at 35°C for 24h and check the MacConkey plate and read both slants. LIA slants with doubtful reactions at 24h must be re-incubated for further 24h.

When crowded plates are encountered, pick with a needle from an area with characteristic medium reaction and restreak on a second plate of the same medium. Incubate the plate as in sec. 5.4.4. and examine for suspect colonies. If such colonies are found on the second plate, proceed as in paragraph above.

If rough and smooth suspect Salmonella colonies are observed on the purity plate, pick a smooth colony to fresh TSI Agar before proceeding with the serology. If only rough colonies are seen on the purity plate, pick one to a Nutrient Agar slant. Incubate at 35°C for 24h and use for serology testing.

Salmonella cultures on TSI show alkaline (red) slants and acid (yellow) butts, with or without H_2S production (blackening of the agar) and may produce gas (splitting the agar). Salmonella cultures on LIA show alkaline (purple) reaction throughout the medium, with or without H_2S production (blackening). TSI cultures showing yellow slants should not be excluded from further testing if the corresponding LIA slant gives a typical Salmonella reaction (refer to Appendices G and H).

5.4.6 Screening

Screen presumptive positive cultures from TSI and LIA slants as follows:

a) serological screening - Somatic Polyvalent O Antiserum

b) biochemical screening - commercial Rapid Identification System or traditional media method (Appendix J)

5.4.6.1 Serological Screening

If serological testing is not performed within 48 hours, streak suspect isolates from the biochemical media onto Nutrient Agar slants. Incubate at 35°C ± 0.5°C for 24h ± 2h. Subsequently these slants may be stored at 2°C - 8°C. To avoid false-positive results such as spontaneous auto-agglutination from slant cultures older than 48h, re-culture on fresh nutrient agar slants prior to serological testing.

Using Polyvalent O antisera (A to I and Vi) perform the agglutination test as follows:

a) Mark off with a wax pencil paired sections about 2x2cm each on the inside of a glass petri dish, or glass serological slides, and label + and -.

b) Place a drop of sterile physiological saline in each section.

c) Remove sufficient culture material from a TSI slant or nutrient agar slant, with a sterile loop or wooden applicator stick, and emulsify the culture in each of the two drops of saline. Add one drop of Poly O (A to I) Antiserum to the emulsion marked +. Mix the emulsion with the antiserum. Tilt the petri dish back and forth for 1 minute.

d) Examine under magnification and good illumination against a dark background to detect cell clumping. Consider any degree of agglutination as a positive reaction (Figure 2). Use QC Antigens to evaluate the activity of the Poly O antisera; if none is available use a known Salmonella culture as a positive control.

e) Poly O Antiserum (A to I and Vi) will not agglutinate all Salmonella serotypes. Should Poly O (A to I and Vi) fail to provide positive agglutination, continue with Poly O groups D and E and, on rare occasions, F and G. Should the laboratory not carry Poly O Antiserum D, E, F and G, it is possible to obtain reasonable positive identification by relying on typical colony characteristics on selective agars (Bismuth Sulphite Agar and Brilliant Green Sulpha Agar plus typical results on TSI and LIA agar slants). It is recommended that such unusual serotypes be confirmed by applying a Rapid Identification System before taking product action. It is, however, of utmost importance that the isolate under investigation be identified as a pure culture.

f) The results are invalid if the negative control shows clumping (auto-agglutination). If the results of serological screening are inconclusive proceed to Biochemical Screening.

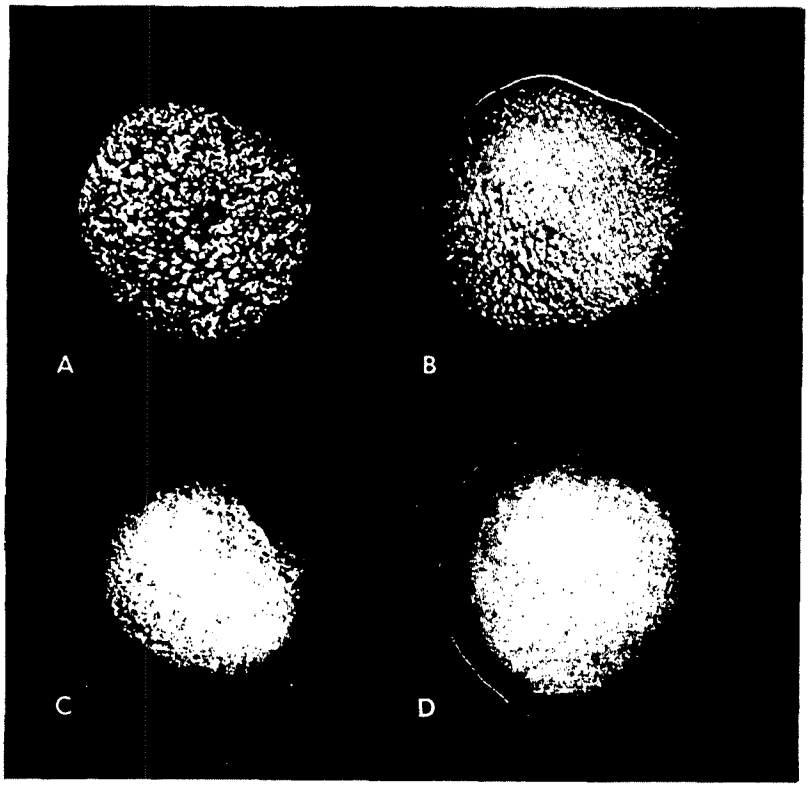
5.4.6.2 Biochemical Screening

Use a commercial rapid identification system to confirm identification of Poly O positive cultures or use the traditional methodology outlined in Appendix J.

Submit the confirmed Salmonella isolates to a serotyping center for complete identification.

Figure 2

AGGLUTINATION TEST REACTIONS



A - Strongly positive

B - Positive

C - Weakly positive

D - Negative

CHAPTER 6: PROCEDURE FOR THE EXAMINATION OF CANNED PRODUCTS FOR STERILITY

Title

Change,

"PROCEDURE FOR THE EXAMINATION OF CANNED PRODUCTS FOR STERILITY"

to read:

"PROCEDURE FOR THE EXAMINATION OF CANNED PRODUCTS FOR *COMMERCIAL* STERILITY"

6.2 **Laboratory Apparatus**

Change,

"Test tubes, 16x150 mm, screw-cap"

to read:

"Test tubes, 16x150 mm or *20x150 mm*, screw-cap"

Change,

"Rubber bulbs for pipetting"

to read:

"Rubber bulbs for pipetting *or mechanical pipetting device*"

Add: "pH-meter
Microscope, 100X oil-immersion objective (recommended a phase-contrast microscope, 100X oil-immersion phase-contrast objective)
Slides and coverslips
Surgical absorbent cotton
Cotton for pipette plugging"

6.3 **Media and Reagents**

Add, the following sentence at the end of paragraph 1:

"Select at least two (2) of the following media for cultivating anaerobic, microaerophilic and aerobic micro-organisms and for detecting the presence of viable micro-organisms in canned products."

Change:

"Cooked Meat Medium (optional)"

to read:

"Cooked Meat Medium"

Delete, from the list:

"Liver Veal Agar (optional)"
"Surgical absorbent cotton"
"Cotton for pipette plugging"

Add, to the list:

Immersion oil
Crystal violet, Methylene blue or Gram stain
Xylene

6.4 **Procedure**

Replace, the first paragraph with:

"On arrival at the laboratory, strip label from cans and examine the cans for external defects. Pre-incubate only the normal cans, which have been sampled less than 30 days after the conditioning process (heat-process). Incubate these cans at 35 °C for 7 days before opening. Examine daily and remove any can showing evidence of swelling or pressure. Do not incubate cans suspected of being non-sterile such as swollen or under-pressure cans, cans with apparent external defects, with imperfect seams, with serious corrosion, etc. These cans should be examined immediately on arrival at the laboratory.

Before opening the can for examination, scrub the cans with soap (or detergent sanitizer solution) and water, using a stiff brush. Rinse with potable water and wipe dry. If available, use a vertical laminar flow cabinet. Wipe counter top with detergent sanitizer solution immediately before placing washed and dried cans on it."

Change, paragraph 2, sentence 1:

"Wipe the top of each can to be opened with a cotton pledget soaked in 95% ethyl alcohol."

to read:

"Wipe the *non-coded end* of each can to be opened with a cotton pledget soaked in 95% ethyl alcohol."

Change, in paragraph 2, sentence 4:

"Place the can upright on the counter and immediately cover the flamed top with a sterile petri dish half, to reduce the possibility of recontamination."

to read:

"Place the can upright on the counter and immediately cover the flamed *non-coded end of the can* with a sterile petri dish half, to reduce the possibility of recontamination."

Change, paragraph 6, sentence 1 and 2:

"To transfer material from the can to the duplicate tubes of thioglycollate medium, remove the petri dish cover, and introduce a sterile cotton-plugged, long-tipped, transfer pipette with attached rubber bulb into the opening in the can. Transfer approximately 0.5 to 1 mL of fluid from the can into duplicate tubes of medium of choice."

to read:

"To transfer material from *each* can to the duplicate tubes of *each selected medium*, remove the petri dish cover, and introduce a sterile cotton-plugged, long-tipped, transfer pipette with attached rubber bulb *(or mechanical pipetting device)* into the opening in the can. Transfer approximately 0.5 to 1 mL of fluid from *each* can into duplicate tubes of *each* medium of choice."

Change, paragraph 7, sentence 9:

"Introduce the material into duplicate tubes of the medium, disturbing the medium as little as possible during transfer."

to read:

"Introduce the material into duplicate tubes of *each medium chosen*, disturbing the medium as little as possible during transfer."

Change, paragraph 10, sentence 4:

"If growth is doubtful, but not definitively negative, transfer material from the suspected tube by Pasteur pipette to a fresh tube of thioglycollate and an additional medium and reincubate for one week."

to read:

"If growth is doubtful, but not definitively negative, transfer material from the suspected tube by Pasteur pipette to fresh tubes of *chosen media* and reincubate for one week."

6.5 **Interpretation of Results**

Delete, number 3, sentence 2:

"Other factors will be taken into account in determining the fate of the lot."

CHAPTER 6

PROCEDURE FOR THE EXAMINATION OF CANNED PRODUCTS FOR STERILITY

6.1 Introduction

This type of examination is to be carried out on processed canned products in which the main or only means of preservation is heat. The application of heat must be sufficient to destroy all organisms of public health significance and organisms that might grow in the food and cause it to spoil under normal storage conditions - commercial sterility.

When nonsterility is detected without supporting evidence (microscopic examination and organoleptic abnormality) one must consider the possibility of adventitious contamination in the laboratory. Proper investigation must rule out this cause before the can is declared to be nonsterile.

Canned products which depend on factors other than heat for preservation are not necessarily commercially sterile. The presence of salt or another chemical, or a relatively low pH may be enough to act as a bacteriostat. If such products are examined for viable bacteria, the method of analysis will of necessity change conditions by removing or reducing the concentration of inhibitory preservatives, thus permitting the bacteria to grow. Such findings of nonsterility would not be an indication that the food concerned presents a health hazard, or that it is bacteriologically unacceptable.

Heat processed canned foods present two possible problems: either insufficient heat may have been applied to destroy all organisms capable of multiplication under the conditions present in the canned foodstuff, or the can contents may have been subsequently contaminated due to some can defect. This defect may be microscopic and may have been resealed after the contamination has taken place.

Viable organisms in the can may be of a type which will not grow under normal storage conditions. The presence of such organisms, as for example obligate thermophilic anaerobes, need not necessarily make the product unacceptable.

6.2 Laboratory Apparatus
(Some specifications are outlined in Appendix B)

Spike, with long-tapered point, stainless steel, 150-200mm in length
Hammer, one-piece, steel
Test tubes, 16 X 150mm, screw-cap
Test tube racks
Pipettes, disposable glass transfer, long tip, 225mm length, cotton-plugged, sterile
Rubber bulbs, for pipetting
Spatula, narrow blade, stainless steel
Petri dishes, sterile
Bunsen burner
Can opener - can be flame sterilized
Incubator (35°C $\pm$ 0.5°C)

NOTE: Other more sophisticated equipment is recommended if conditions are critical, or if many cans are routinely examined for sterility, e.g. sterile chamber, vertical laminar flow hood.

6.3 Media and Reagents
(Some specifications are outlined in Appendices C and D)

The laboratory, in routine large scale sterility testing, must choose among many media available for the recovery of viable organisms possibly present in the sample being tested. The recovery of attenuated organisms, and particularly spore outgrowth, will be strongly influenced by the medium into which the food sample is inoculated. When a particular food preparation, or a particular lot under suspicion, gives a doubtful reaction in the medium routinely used, it is the responsibility of the laboratory to investigate other media which may prove more satisfactory.

Fluid Thioglycollate Medium
PE-2 Medium
Cooked Meat Medium (optional)
Liver Veal Agar (optional)
Surgical absorbent cotton
Cotton for pipette plugging
95% alcohol for flaming
Iodophore solution (150ppm iodine)
2% iodine in 70% alcohol.

6.4 Procedure

Refer to section 1.5.6 for instructions on sampling and transport of cans for examination. Pre-incubate the cans at 35°C for 14 days before opening. Examine daily and remove any can showing evidence of swelling or pressure. After the incubation period, remove the cans from the incubator and scrub the cans with soap and water using a stiff brush. Rinse with potable water and wipe dry.

Wipe the top of each can to be opened with a cotton pledget soaked in 95% ethyl alcohol. Then grasp the can in the hand and invert over the flame of a bunsen burner, distributing the heat over the top with a circular motion. Do not play the burner down upon the top of the can because this will result in a concentration of heat at the top, causing scorching of the contents and possibly leading to spurting when the opening is made. Place the can upright on the counter and immediately cover the flamed top with a sterile petri dish half, to reduce the possibility of recontamination.

DO NOT FLAME A SWOLLEN CAN! Sanitize the swollen can end using 2% iodine in 70% alcohol or equivalent and wipe off with a sterile towel. Make the perforation through a sterile pledget or pledget of cotton saturated with alcohol or iodophore. This will serve to absorb and sterilize any material that may spurt from the opened can. Use a pre-sterilized spike.

Remove the petri dish cover from the top of the can and drive the sterilized tip of the spike into the can to a depth of about 10mm, using a hammer. Immediately replace the petri dish cover over the can top. The puncture is usually made towards one side of the can lid, so that the fluid portion of the contents is more easily reached.

When a large number of cans are to be opened, it may be necessary to use several spikes in rotation. After each use, wipe adhering material from the spike with a sterile paper towel. Then immerse the spike in an iodophore solution for a minimum of two minutes. Before re-use, rinse the spike in sterile distilled water, then dip into 95% alcohol again and flame.

To transfer material from the can to the duplicate tubes of thioglycollate medium, remove the petri dish cover, and introduce a sterile cotton-plugged, long-tipped, transfer pipette with attached rubber bulb into the opening in the can. Transfer approximately 0.5 to 1mL of fluid from the can into duplicate tubes of medium of choice. The depth of the medium in the test tube should not be less than 70mm.

Deposit the inoculum in the bottom of the tube with as little disturbance of the medium as possible in order to minimize oxidation of the tube contents. If there is no liquid component capable of being transferred by pipette, the can top must be removed, or a larger opening made. Use a can opener of a design that can be easily cleaned and which allows sterilization. When a large number of cans are to be opened it is desirable to use several can openers in rotation. After each use, wipe adhering material from the instrument with a sterile paper towel, then immerse the tool in an iodophore solution for a minimum of 2 minutes. Before re-use, rinse the tool in sterile distilled water, then dip into the 95% alcohol and flame. To transfer material from the opened can, use a sterile narrow spatula, and remove approximately 0.5 to 1g of material. A separate sterile spatula must be used for each can. Introduce the material into duplicate tubes of the medium, disturbing the medium as little as possible during transfer. It is important to recognize the greater danger of chance contamination during this method of inoculation. Every precaution must be taken to ensure that surfaces in the working area have been sterilized by wiping with a suitable bactericidal agent, and that the area is free from drafts. If large numbers of cans are examined for sterility, the use of a sterile inoculating chamber is recommended.

Direct microscopic examination of the material must be made at the time that the original tube transfers are made. Prepare a direct smear from the contents of each can onto a microscope slide. Dry, fix and stain with methylene blue, crystal violet or gram stain. If product is oily, add a drop of xylene to warm fixed film, rinse and stain. If product washes off slide during preparation, examine contents as a wet mount or hanging drop.

It is not easy to differentiate microscopically between viable and non-viable organisms in the can contents. Phase-contrast microscope equipment is recommended for this type of examination, especially if a large number of examinations are to be made. A 40X phase contrast objective may be used for more detailed examination. Examine can contents for odor, colour, texture and pH and the can lining for blackening, detinning and pitting. DO NOT TASTE THE PRODUCT.

Incubate the inoculated tubes for 1 week at the temperature at which the cans were held - generally 35°C - and then examine for evidence of bacterial growth. Reincubate tubes showing no evidence of growth for a further week and examine again. Inspect, microscopically, all tubes in which growth appears to have taken place, as outlined previously. If growth is doubtful, but not definitely negative, transfer material from

the suspected tube by pasteur pipette to a fresh tube of thioglycollate and an additional medium and reincubate for one week. Since this transfer introduces very little extraneous matter to the new tube, subsequent bacterial growth is easily seen. This evidence must again be verified by microscopic observation. The reduction of the indicator in the surface layer of the fluid thioglycollate medium is frequently a useful sign of bacterial activity.

6.5 Interpretation of Results

1) Only double-tube nonsterility is to be considered.

2) The presence of mesophilic anaerobic spore-formers is a criterion for rejection (considered to be evidence of under-processing).

3) A mixed bacterial flora indicates post-process contamination. Other factors will also be taken into account in determining the fate of the lot.

4) The presence of strict aerobes is not by itself a reason for rejection.

5) In a fat-containing product, otherwise organoleptically normal, the presence of anaerobic or microaerophilic bacteria of the following morphology - loosely clumped micrococci, diphtheroids, or pleomorphic rods - is considered to be evidence of fat-protection, and will not be used as grounds for rejection. In such instances, single tube nonsterility is not infrequent.

Rejection of lots should be instituted only in cases where one or more of the following conditions are satisfied:

1) There is an organoleptic quality change

2) Change in pH from what is considered normal

3) Detection of defects in can integrity

4) Can Swells

5) Detection of anaerobic mesophilic spore-formers.

CHAPTER 7: PROCEDURE FOR THE ENUMERATION OF HALOPHILIC BACTERIA

Delete in its entirety

CHAPTER 8: PROCEDURE FOR THE ENUMERATION OF VIBRIO PARAHAEMOLITYCUS

Delete in its entirety

CHAPTER 9

BACTERIAL ASSESSMENT OF FISH AND FISH PROCESSING PLANTS

9.1 Sampling Procedure for the Bacteriological Assessment of Fish Processing Plants

9.1.1 Introduction

The bacteriological survey should be made, if possible, when the plant has a sufficiently large enough supply of fish available so as to ensure that all samples taken during the survey can reasonably be assumed to be part of the same lot of fish. Such a survey is to be carried out if the bacteriological quality of the final product is unsatisfactory. Its purpose is to determine the point or points at which the product is contaminated and to assess the effect of remedial measures as these are introduced.

Although the number of samples taken at each sampling point depends on the resources at the disposal of the examining laboratory, it may be accepted as a general principle that a sufficient number of samples should be assessed to minimize the effect of any one aberrant sample that might unduly influence results. To assess plant cleanup procedure, examine the plant before commencement of operations. This should be done before the first fish is handled. Using this inspection procedure, areas where increased cleaning effort is necessary can be pointed out prior to the survey.

As a general principle, it is necessary that the responsible laboratory personnel have firsthand knowledge of all plant operations likely to affect fish quality, so that proper interpretation of findings in the laboratory may be made and effective remedial procedures recommended.

9.1.2 Laboratory Apparatus

Swabs, commercial, sterile
Sterile sample bags

9.1.3 Media and Reagents

Dependent on the organisms being sought. (Refer to previous sections)

9.1.4 Procedure

In order to establish the efficiency of the fish washing operation, take swabs from the skin surface of a sufficient number of unwashed fish and again of an equal number of washed fish. Process these samples as outlined in section 9.2.4.

Establish other sampling points immediately following each successive step in the fish handling operation, as for example in a fish filleting plant:

1) after cutting
2) after skinning
3) after candling
4) after trimming
5) after dipping
6) after weighing
7) after packaging
8) immediately prior to freezing

Sample the product at each point and carry out the required bacteriological examination of each sample using procedures outlined in the appropriate section of this manual.

Sample all water sources at the principal plant outlets and test for coliform bacteria.

In the case of chlorinated supplies determine chlorine residuals at various plant outlets.

After the problem areas have been defined and plant action taken, another survey should be made to determine the effect of the remedial measures.

9.2 Procedure for Determination of Surface Bacterial Counts of Fish and Fish Processing Plants (Swab Technique)

9.2.1 Introduction

The purpose of swabbing is to determine bacterial numbers on various surfaces, particularly when other methods of taking samples are difficult or impossible to apply.

Swabbing is most reliable when the surface being examined is flat, smooth and impermeable, such as the top of a stainless steel table. When porous or rough surfaces or those having irregular contours are examined, the precision of the method is diminished.

A further possible source of imprecision is the difficulty of ensuring that all of the bacteria removed with the swab are brought into homogeneous suspension during subsequent treatment of the swab. Swabbing the surface of a fish heavily coated with slime will produce more erratic and less reliable results than swabbing a wet, clean, stainless steel tray.

It is important that these inherent sources of imprecision in the method be kept in mind when interpreting results based on this method of analysis.

9.2.2 Laboratory Apparatus

Sterile swabs, commercial
Test tubes, 150 X 18mm
Other apparatus as required

9.2.3 Media and Reagents

Dependent on the organisms being sought. (Refer to previous chapters)

9.2.4 Procedure

The standard area for swabbing is $25cm^2$ in a square 5cm X 5cm. A wire swab guide or steel template of the correct size, which is readily sterilized by wiping with alcohol and flaming, may be used to outline the area.

When a dry area is to be swabbed, dip the swab into the sterile diluent before use.

Establish swabbing points on the equipment used in each successive step of the operation.

Place the swab immediately into a bottle containing 100mL of the diluent. Break off and discard that portion of the stick that has been touched by the hand.

If the swab in its diluting medium is to be shipped, hold it under the same conditions as water samples being transported.

Shake the bottle to disintegrate the swabs satisfactorily.

For SPC determination, pipette duplicate 1mL amounts from the bottle containing the swab, or a decimal dilution (if required), into sterile petri dishes. Incubate at 25°C for 72h. (Chapter 2)

For coliform determination by the MF technique, filter an appropriate amount from the bottle containing the swab, or a decimal dilution, onto an MF filter. (Chapter 3)

For coliform determination by the MPN technique, use the diluent containing the swab plus two further decimal dilutions for inoculating a 5:5:5 series. (Chapter 3)

For staphylococcus determination by the plating technique, plate out 0.5mL from the bottle containing the swab, in duplicate, on an appropriate medium. Premoistened swabs may also be applied directly to the surface of prepoured staphylococcus plating media. (Chapter 4)

Calculate the bacterial numbers in the original bottle containing the swab. This represents the number of bacteria from the swabbed area.

Report as number of bacteria/cm^2.

WASHING AND STERILIZATION

1. Clean all glassware thoroughly with a suitable detergent and hot water, rinse with hot water to remove all traces of residual washing compound, and finally, rinse with distilled water. If mechanical glassware washers are used, influent plumbing should be of stainless steel or other nontoxic material. Copper piping should not be used to distribute distilled water. Plumbing for the rinse system shall be of stainless steel or other nontoxic material.

2. Glassware, except when in metal containers, shall be sterilized for not less than 60min at a temperature of 170°C. Glassware in metal containers should be heated to 170°C for not less than 2h. Sample bottles not made of plastic may be sterilized similarly, or in an autoclave at 121°C for 15min.

3. Autoclave all media, except sugar broths or other media with specific sterilization requirements at 121°C for 15min. When the pressure reaches zero, remove the medium from the autoclave and cool it quickly. To permit uniform heating and rapid cooling, pack materials loosely and in small containers. The maximum elapsed time for exposure of sugar broths to any heat (from the time of closing the loaded autoclave to unloading) is 45min. Preheating the autoclave before loading can reduce total needed heating time to within the 45min limit. Alternatively, filter-sterilize the heat-labile sugar solution and add it to the basal medium after this has been autoclaved.

4. Sterilize in the autoclave at 121°C for 30min all inoculated petri dishes, flasks or culture tubes before washing. This will eliminate the possibility of disseminating pathogenic bacteria that may be present. Also, before washing, disinfect all glassware and utensils that may have come in contact with any cultured organisms.

SPECIFICATIONS FOR APPARATUS AND EQUIPMENT

It is essential for accurate and satisfactory laboratory work that good equipment in proper working order be provided. The laboratory equipment listed must be available, and all items should meet the minimum requirements given. Some additional items of equipment not listed may also be required and they too should meet similar standards of quality.

AUTOCLAVE: This must be of adequate size to prevent crowding of the interior. It should provide uniform temperatures within the chamber up to and including the sterilization temperature of 121°C. It must be equipped with an accurate thermometer, properly located to register minimum temperatures within the sterilizing chamber, pressure gauge, and properly adjusted safety valves. It should be capable of reaching the desired temperature within 30 minutes. Portable autoclaves may be substituted as an alternative in situations where a standard autoclave cannot be used. In emergencies, a pressure-cooker may be substituted for an autoclave provided that it is equipped with an efficient pressure gauge and with a thermometer, the bulb of which is 2.5cm above the water level.

BALANCE: Balances shall provide a sensitivity of at least 0.1g at a load of 150g. An analytical balance having a sensitivity of 1mg under a load of 10g shall be used for weighing small quantities (less than 2g) of materials. For rapid routine weighing of larger units, such as blender jars, a single pan, 3-4kg capacity, electronic balance, with at least 0.5g sensitivity and an automatic taring device is desirable.

BLENDING DEVICE: A blender that has several operating speeds or one with a rheostat should be used. For routine work, 1L capacity blender jars, preferably stainless steel, are required. For large, pooled, or refractory samples, a 4L blender jar is recommended. Other suitable homogenizing equipment is acceptable.

CAN OPENER: The design should be such that it may be easily cleaned and sterilized. Special, commercially available, bacteriological can openers are preferable.

COLONY COUNTER: Standard apparatus providing a magnification of 1.5x, such as a Quebec type colony counter, dark field model preferred.

DISTILLING APPARATUS: For preparation of media and reagents, only distilled water should be used. The still must be of a capacity to meet the water demand required for routine laboratory analyses.

FILTRATION UNITS: The filter holding assembly consists of a seamless funnel fastened to a base bearing a porous plate for support of the filter membrane. The design should ensure that the membrane filter be held securely on the porous plate without mechanical damage and allow all fluid to pass through the membrane during filtration. Must be autoclavable.

GLASSWARE: Bottles and tubes should be made of resistant glass, preferably borosilicate, or of autoclavable, nontoxic plastic. Caps, equipped with liners that do not produce toxic or bacteriostatic compounds on sterilization, should be used for covers.

Tip calibrated (serological), borosilicate glass pipettes should be used. The error of calibration should not exceed 2.5%. Use only pipettes with unbroken tips and having graduations distinctly marked. Pipettes with large tip openings may have to be used for transfer of initial fish homogenates.

HOT-AIR STERILIZING OVEN: Should be of sufficient size to prevent internal crowding and constructed to give uniform and adequate sterilizing temperatures. It should be able to reach and maintain 170°C $\pm$ 10°C for periods of up to 2h. It should be equipped with suitable thermometers capable of registering accurately in the range of 160°C to 180°C.

INCUBATORS: Incubators must maintain a uniform and constant temperature within $\pm$ 0.5°C of the desired setting at all times. An accurate thermometer, with bulb continuously immersed in liquid, should be maintained within the incubator and daily readings of the temperatures should be recorded. It is desirable when laboratory temperatures vary excessively that the incubators be kept in special rooms which may be maintained at a few degrees below the recommended incubator temperature.

INOCULATING EQUIPMENT: Needles and wire loops shall be made of 22 to 24 gauge Nickel-chromium or platinum-iridium alloy. Single service disposable hardwood applicator sticks are also satisfactory. These must be long enough to reach the bottom of the culture tube with at least 3cm extending out of the tube for manipulation.

MICROSCOPE: A microscope with an oil immersion objective lens (95-100X) and 10X ocular is recommended. A phase contrast microscope is recommended when doing sterility testing of canned fish products.

PETRI DISHES: Borosilicate glass or sterile plastic petri dishes (15x100mm) may be used. The bottom should be free from bubbles and scratches and should be flat, so that the medium will be of uniform thickness throughout the plate.

pH METER: For the determination of pH values of media. Should be accurate to at least 0.1 pH units and be fitted with a temperature compensator.

THERMOMETERS: Both partial and total immersion mercury thermometers of various temperature ranges should be available. Calibration should be checked at least once per year. Where a record of temperature is desired, automatic temperature recording instruments may be used. These should also be calibrated at least once a year.

WATER BATHS: Must maintain a uniform and constant temperature at all times ($\pm$0.5°C for most water baths; 44.5°C $\pm$ 0.2°C for faecal coliforms test). To obtain the temperature required and to maintain it throughout the test, a water bath with a circulating system should be used. It should also be equipped with a gabled cover to reduce water and heat loss. It must be possible to immerse tubes in water to 1cm above the level of the medium.

APPENDIX C: MEDIA AND OTHER BACTERIOLOGICAL PREPARATION INDEX

Delete, from the list (and delete description from body of text):

N°.

6. Bismuth Sulfite Salt Broth
10. BTB Teepol Agar
11. Carbohydrate Media using Purple Broth Base
14. Diluents
    a) NaCl 20%
    c) Peptone (1%) + NaCl (3%)
18. Glucose Salt Teepol Broth
20. Hugh Leifson Glucose Medium
24. Liver Veal Agar
25. Lockhead's Skim Milk Agar, Modified
32. Nutrient Gelatin
37. Staphylococcus 110 Medium with Added Egg Yolk
38. Staphylococcus 110 Medium with Added Egg Yolk and Added Azide
40. Thiosulfate-Citrate-Bile-Sucrose (TCBS) Agar
41. Tellurite Polymyxin Egg Yolk (TPEY) Agar
45. Trypticase Broth
46. Trypticase Soy Broth with 10% NaCl
50. Vibrio maintenance Medium

MEDIA AND OTHER BACTERIOLOGICAL PREPARATIONS INDEX

34. Plate Count Agar
35. Salmonella Polyvalent (Poly 0) Antisera and Quality Control Antigens
36. Selenite Cystine Broth

39. Simmons Citrate Agar

42. Tetrathionate Broth Base with Brilliant Green
43. Toluidine Blue DNA Agar
44. Triple Sugar Iron (TSI) Agar

47. Tryptone 1%
48. Urea Broth and Agar
49. Vaspar

51. XLD Agar

Introduction

All preparations are listed in alphabetical order.

Media commonly available in dehydrated form are listed by name only. In many cases, several manufacturers market similar products which may vary in name and performance. If unsatisfactory results are obtained with one medium, another package of the media with a different control number or that of another manufacturer should be investigated. It is important that a record of all control numbers be maintained.

Where specific media are required, the section of the manual in which the medium is called for is indicated.

Instructions and Comments

1. A-1 Medium Modified (Chapter 3)

Although A-1 Medium has been offered commercially, it can be prepared in the laboratory. Store the prepared medium in a dark place at room temperature and use within 7 days of preparation. Store dehydrated ingredients under conditions that will prevent absorption of moisture.

	INGREDIENTS
Lactose	5g
Tryptone	20g
NaCl	5g
Salicin	0.5g
Triton X-100	1mL
Distilled Water	1L

Suspend the dry ingredients in the distilled water. Heat with stirring to dissolve the ingredients. Using a pipette, add the Triton X-100. Adjust the pH to 6.9 $\pm$ 0.1, if necessary. Dispense the completed medium in tubes containing inverted fermentation vials. Autoclave at 121°C (15 lbs pressure) for 10 minutes. Formation of a flocculent precipitate, particularly in double strength medium, does not impair performance.

2. Agar

Solidifying media.

3. Baird-Parker Agar Base with Added Egg Yolk Tellurite Enrichment (Chapter 4).

   For the enumeration of coagulase-positive staphylococci.

4. BG Sulfa Agar (Chapter 5)

   A selective medium for the isolation of Salmonellae

5. Bismuth Sulfite Agar (Chapter 5)

   A selective medium for the isolation of Salmonellae. This medium should be prepared, and the plates poured, 24h in advance of their intended use, since the freshly prepared medium is excessively toxic to Salmonellae. It must also be remembered that extended storage of the plates gradually reduces their selectivity.

6. Bismuth Sulfite Salt Broth (Chapter 8)

   For the enrichment of Vibrio parahaemolyticus

   Formula for basal medium

Peptone	10g
Sodium chloride	25g
Potassium chloride	0.7g
Magnesium chloride hexahydrate	5g

   Directions for completed medium: dissolve above ingredients in 950mL of distilled water and adjust pH to 9.1 by addition of 10% aqueous solution of Na_2CO_3. Sterilize by autoclaving at 121°C for 15 minutes, cool to room temperature, and add aseptically 100mL of bismuth sulfite solution and 1mL of 95% ethyl alcohol. Mix well and dispense in 10mL portions into sterile culture tubes. Do not reheat.

   Bismuth sulfite solution: dissolve (a) 20g of sodium sulfite in 100mL of boiling water, (b) 0.1g of ammonium bismuth citrate in 100mL of boiling water, (c) 20g of mannitol in 100mL of boiling water. Mix solutions (a) and (b), boil the mixture for 1 minute, and add solution (c). The final mixture is white in color and turbid; mix well before using. The reagent remains usable for 1 month if stored in the refrigerator.

7. Brain Heart Infusion Broth

For subculturing bacteria, particularly staphylococci in preparation for the coagulase test. Also used in the preparation of egg yolk emulsion.

8. Brain Heart Infusion Agar

For subculturing bacteria

9. Brilliant Green Lactose Bile Broth 2% (Chapter 3)

For confirming presumptive coliform results.

10. BTB Teepol Agar (Chapter 8)

A selective agar for the isolation of Vibrio parahaemolyticus.

11. Carbohydrate Media using Purple Broth Base

Add the desired carbohydrate at a concentration of 0.5 - 1.0%, dispense in 5mL amounts to fermentation tubes.
Autoclave at 118°C (12 lbs) for 12-15 minutes. DO NOT OVEREXPOSE TO HEAT.

The following carbohydrates are most commonly required:
Lactose - in conjunction with the IMViC test (Chapter 3)
Sucrose and Mannitol - to differentiate Vibrio species (Chapter 8)
Dulcitol - to differentiate Salmonella arizonae from other Salmonellae (Chapter 5).

If desired, a filter-sterilized solution of the carbohydrate can be added aseptically to the pre-sterilized base.

12. Coagulase Plasma EDTA (Chapter 4)

For the detection of coagulase production by staphylococci.

13. Cooked Meat Medium (Chapter 6)

For the anaerobic cultivation of bacteria. This medium must be tubed for use, commonly in 16 x 150mm screw-cap culture tubes. Using a calibrated scoop, add 1.25g of the desiccated medium to each tube, then add 10mL of distilled water. Larger tubes may be used, with a corresponding adjustment of the ingredients, but it is essential that the reconstituted liquid be sufficiently deep to assure anaerobic conditions (at least 50mm above the granules).

14. Diluents

a) 20% NaCl solution (Chapter 7)
Used in the determination of halophilic bacteria.

b) Peptone 0.1%
This is the standard diluent used throughout the manual.

c) Peptone (1%) + Salt (3%) (Chapter 8)
Used in the determination of Vibrio parahaemolyticus

Peptone	10g
NaCl	30g
Distilled H_2O	1L

Add ingredients to 1L of distilled water, adjust pH to 7.2, distribute in screw-cap bottles and autoclave at 121°C for 15min.

d) Phosphate Buffer

(1) Stock Phosphate Buffer Solution:

Monobasic potassium phosphate, KH_2PO_4	34.0g
Distilled water	500mL
1N NaOH solution added to adjust to pH 7.2 (usually about 175mL)	
Distilled water to make	1000mL

(2) Final Phosphate Buffered Dilution Water:

Stock phosphate buffer solution	1.25mL
Distilled water to make	1000mL

Final reaction after sterilization: pH 7.0 $\pm$ 0.1

e) Saline 0.85% NaCl

For use in serotyping Salmonellae
Dissolve 8.5g sodium chloride in 1000mL distilled water.

15. EC Medium (Chapter 3)

For the detection of faecal coliforms. Dispense medium in 5-7mL volumes in fermentation tubes.

16. Egg Yolk Suspension (Chapter 4)

Commercially available sterile egg yolk preparations with or without added tellurite are used in staphylococcus plating media as directed.

17. Fluid Thioglycollate Medium (Chapter 6)

For determination of mesophilic anaerobic spore-formers in canned products. Use screw-capped tubes. The depth of the medium in the tube must not be less than 70mm to ensure anaerobic conditions within the medium. After storage of the prepared medium, reheat no more than once in boiling water or flowing steam to drive off absorbed oxygen.

18. Glucose Salt Teepol Broth (Chapter 8)

For the enrichment of Vibrio parahaemolyticus

Beef Extract	3g
Peptone	10g
NaCl	30g
Glucose	5g
Methyl violet	0.002g
Teepol	4mL
Distilled Water	1000mL

Dissolve ingredients in distilled water and adjust pH to 7.4. Dispense single strength medium in 16x150mm tubes and 1.5 strength medium in 25x150mm tubes. Autoclave at 121°C (15 lbs) for 15 min.

19. Hektoen Enteric Agar (Chapter 5)

A differential medium for the isolation of Salmonella

20. Hugh Leifson Glucose Medium (Modified) (Chapter 8)

Used in the differentiation of Vibrio species.
Prepare as per manufacturer's instructions and modified by the addition of 2.5g NaCl per 100mL medium to bring the final concentration to 3%.

21. Lactose Broth

For pre-enrichment in the isolation of Salmonella. Also used as an alternative to Lauryl Tryptose Broth for the analysis of coliforms in water.

22. Lauryl Tryptose Broth (Chapters 3 and 5)

Used in the presumptive test for coliforms and also as a diluent of food samples for pre-incubation in the isolation of Salmonella.

23. Levine's EMB Agar (Chapter 3)

Used in the differentiation of faecal coliforms.

24. Liver Veal Agar (Chapter 6)

For the cultivation of anaerobic bacteria.

25. Lockhead's Skim Milk Agar, Modified (Chapter 7)

For the enumeration of halophilic bacteria.
Prepare medium in two parts:

Part 1.	NaCl	250g
	$MgSO_4 \cdot 7H_2O$	29g
	$Ca(NO_3)_2$	2.5g
	KCl	2.0g
	Agar	20g
	Distilled Water	700mL

Combine the above ingredients and dissolve by heating to 100°C stirring constantly. Adjust pH to 7.4. Cool to 60°C before combining with Part 2.

Part 2.	Skim milk powder	50g
	Blood Serum (dehydrated)	10g
	Yeast extract	1.0g
	Distilled Water	300mL

Dissolve ingredients. Heat to 40°C and combine with Part 1 with gentle mixing. Pour plates, air dry them, and store them in refrigerator in closed polyethylene bags.

26. Lysine Iron Agar

For the differentiation of Enterobacteriaceae. Dispense in tubes and prepare slants with a generous butt.

27. MacConkey Agar (Chapter 5)

For the purification of suspected colonies from primary selective media in the analysis for Salmonellae.

28. Malonate Broth (Modified) (Chapter 5)

Used in the differentiation of Enterobacteriaceae

29. Membrane Filter Media (Chapter 3)

a) m-Endo Agar LES
For the enumeration of coliforms in water by the membrane filter method.

b) m-FC Agar
For the enumeration of faecal coliforms in water by the membrane filter method.

c) m-Endo Agar MF
Used as an alternative to m-Endo Agar LES for the enumeration of coliforms in water by the membrane filter method. Prepare by adding 1.2 - 1.5% agar to the m-Endo Broth MF before boiling.

30. MR-VP Medium Broth

Used in the Methyl Red and Voges-Proskauer tests for differentiation of faecal coliforms and Vibrio species. Tube in 0.5mL volumes. For use in Chapter 8 add sufficient NaCl to bring the final concentration to 3%.

31. Nutrient Broth and Agar

32. Nutrient Gelatin (Chapter 8)

Used in the differentiation of Vibrio species.
Prepared as per manufacturer's instructions and modified by the addition of NaCl to bring the final concentration to 3%.

33. PE-2 Medium Modified

Preparation:

Yeast Extract	3.0g
Peptone	20.0g
Bromcresol Purple (2% w/v in 95% ethanol)	2.0mL
Distilled Water	1.0L

Dissolve ingredients in distilled water. Dispense in 10mL amounts in 16 x 150mm screw-cap tubes. Add 5 untreated Alaska Seed Peas (W.H. Perron Seed Company, 515 La Belle Blvd., Laval, P.Q.). Allow to stand for 1 hour. Autoclave 15 minutes at 121°C. Store tightly capped in the dark. If not used within 5 days, deaerate by boiling tubes in a waterbath for 10 minutes prior to use. Do not store beyond 4 weeks at room temperature in the dark.

Method: Intended for use in sterility testing, this medium is inoculated in duplicate and incubated for 14 days. Not all organisms will produce a colour change in the medium. Check for turbidity and examine microscopically.

34. Plate Count Agar (Chapter 2)

Used in all standard plate count procedures.

35. Salmonella Polyvalent O (Poly O) Antisera and Quality Control (QC) Antigens (Chapter 5)

Rehydrate a vial of Salmonella Antiserum with 3mL sterile 0.85% NaCl solution and rotate gently to dissolve completely. Store both desiccated and reconstituted antisera at 2-8°C. Do not expose the rehydrated serum to room temperature unnecessarily.

Store QC Antigens at 2-8°C. Bring to room temperature prior to use, but return promptly to the refrigerator after use.

36. Selenite Cystine Broth (Chapter 5)

For selective enrichment in the isolation of Salmonellae.

37. Staphylococcus 110 Medium with Added Egg Yolk (Chapter 4)

For the enumeration of coagulase-positive staphylococci.
Prepare Basal Medium as per manufacturer's instructions.
Egg Yolk Suspension: Immerse a fresh egg (shell on) in 70% alcohol for 2h, rinse with sterile distilled water, separate the yolk aseptically and pass it through four layers of sterile surgical gauze or cheese cloth into 100mL sterile Brain Heart Infusion in an erlenmeyer flask. Swirl gently to mix contents and dispense into sterile large screw-cap tubes in 40mL volumes. The suspension may be refrigerated until needed. Immediately before use, resuspend any precipitate in the preparation. Add the egg yolk suspension (10% by volume) with a sterile pipette to the melted, tempered agar medium, mix gently and pour plates in about 15-20mL amounts. Allow to harden and then air dry in a 45°C incubator or a horizontal laminar flow hood.

38. Staphylococcus 110 Medium with Added Egg Yolk and Added Azide (Chapter 4)

For the enumeration of coagulase-positive staphylococci. Sodium azide inhibits spreaders which may be encountered when breaded foods or other similar products are analysed.
Prepared as for "Staphylococcus 110 Medium with Added Egg Yolk" adding 0.05g sodium azide per liter of medium.

39. Simmons' Citrate Agar

Used in the differentiation of Enterobacteriaceae and Vibrio by the IMViC tests. Glassware used in the preparation of this medium must be scrupulously clean to eliminate all possible carry-over of contaminating carbon sources. Tube in 5mL volumes and slant. For use in Chapter 8, add sufficient NaCl to bring the final concentration to 3%.

40. Thiosulfate-Citrate-Bile-Sucrose (TCBS) Agar (Chapter 8)

A selective agar for the isolation of Vibrio parahaemolyticus

41. Tellurite Polymyxin Egg Yolk Agar (TPEY) (Chapter 4)

For the enumeration of coagulase-positive staphylococci.

42. Tetrathionate Broth Base with Brilliant Green (Chapter 5)

For the selective enrichment of Salmonellae.
Prepare 500mL single strength Tetrathionate Broth Base. Allow to cool and add 1.0mL 1:1000 Brilliant Green solution per 100mL of the medium. With constant agitation to ensure uniform suspension of the precipitate, dispense aseptically in 10mL volumes into sterile 25x150mm screw-cap tubes. Cap tightly and store in the dark at room temperature.

Complete Tetrathionate Medium

To 10mL tubes of single strength TETBG Basal Medium add 0.2mL KI/I solution just prior to use. Mix the medium by gentle agitation prior to and following inoculation.

Iodine Potassium Iodide Solution

Iodine	6g
Potassium Iodide	5g
Distilled Water	20mL

Dissolve the Potassium Iodide in the water with stirring. Add the Iodine crystals and stir until dissolved. Store in a tightly capped bottle. AVOID SKIN CONTACT!

43. Toluidine Blue DNA Agar (Chapter 4)

Deoxyribonucleic Acid (DNA)	0.3g
Calcium Chloride (anhydrous)	1.1mg
Sodium Chloride	10g
Toluidine Blue	83mg
Agar	10g
*Tris (hydroxymethyl) aminomethane	6.1g

*Dissolve the Tris Buffer in 1.0L of distilled water and adjust to pH 9.0 with 0.2N HCl. To this solution add the remaining ingredients except the Toluidine Blue.
Heat to dissolve the agar.
Dispense the complete medium in smaller volumes (3.0mL) in sterile screw-cap tubes. Store at room temperature in the dark.

44. Triple Sugar Iron (TSI) Agar

For the differentiation of Enterobacteriaceae and Vibrio species. Tube and slant so as to leave a generous butt. For use in Chapter 8, add sufficient NaCl to bring the final concentration to 3%.

45. Trypticase Broth (Chapter 8)

Used in the differentiation of Vibrio species.

Trypticase or tryptone	10g
Yeast extract	2g
Distilled water	1000mL

Add 0, 30, 70, 100g of NaCl per litre to the above medium to make, respectively, 0, 3, 7 and 10% NaCl trypticase broth for salt tolerance tests. Dissolve ingredients in salt water and adjust pH to 7.5. Dispense in screw-cap tubes in 5mL volumes and autoclave at 121°C (15 lbs) for 15 minutes.

46. Trypticase Soy Broth with 10% NaCl (Chapter 4)

For the enumeration of coagulase-positive staphylococci by the most probable number method. Add sufficient NaCl to bring the final concentration to 10%.

47. Tryptone 1%

Prepare in a 1% solution and use in detecting indole production in the IMViC tests.
For use in Chapter 8, add sufficient NaCl to bring the final concentration to 3%.

48. Urea Broth and Agar

Used to differentiate Proteus from other members of the family Enterobacteriaceae.

49. Vaspar

Used as a sealing agent in tubed media to exclude atmospheric oxygen

White petroleum jelly ..... 1 part
Paraffin ..... 1 part

Heat the two together until melted. Mix well. Store in screw capped flasks or large test tubes.

50. Vibrio Maintenance Medium (Chapter 8)

For the maintenance of Vibrio cultures.

NaCl	24.0g
KCl	0.7g
$MgCL_2$	5.3g
$MgSO_4.7H_2O$	7.0g
Yeast extract	3.0g
Proteose peptone	10.0g
Agar	15.0g
Distilled water	1000mL

Heat to dissolve and dispense in screw-cap tubes. Autoclave at 121°C (15 lbs) for 15 minutes. Allow to set in slanted position.

51. XLD Agar (Chapter 5)

A differential medium for the isolation of Salmonellae.

Appendix D: Chemicals, reagents, indicators and other materials

Delete, the following:

Phenylenediamine solution

Add, the following:

Crystal Violet	To stain a direct smear from the contents of a can
Methylene Blue	To stain a direct smear from the contents of a can
Xylene	To help in the staining of oily product

Change, "Naphthol Solution"

To read: "alpha-naphthol"

CHEMICALS, REAGENTS, INDICATORS AND OTHER MATERIALS

NAME	USE AND DESCRIPTION
Absorbent Cotton	Surgical grade. For applying disinfectant or alcohol.
Alcohol	a) 95% Ethanol, for the preparation of reagents. b) 95% Denatured, for flaming and disinfection. c) 70% Denatured, for disinfection. NOTE: Isopropanol or Methanol may be used for (b) and (c) above
Aluminum Foil	As wrapping for sterilization and as tube caps.
Applicators	Wood
Brilliant Green - 1:1000 aqueous solution	For use in preparation of Media to inhibit growth of non-target organisms.
Creatin Solution	For performing the Voges-Proskauer Test. Dissolve 1g in 75mL distilled water. Store in refrigerator.
Hydrochloric Acid-1N	To adjust the pH of media. HCl (37%) c.p. .....10 mL Distilled water sufficient to make 100 mL Preserve in screw-capped polypropylene dropping bottle or rubber stoppered glass bottle.
Hydrogen Peroxide 10%	For testing catalase activity of cultures H_2O_2 30% c.p. .....33 mL Distilled water sufficient to make 100 mL
Indole Test Papers	Strips of filter paper are impregnated with a solution of: - p-dimethylaminobenzaldehyde ....5g - methanol .....50mL - o-phosphoric acid .....10mL Drop onto the filter paper. Do not immerse. Dry at 70°C as quickly as possible.

Iodine Reagent	Starch indicator used in the starch hydrolysis test. Prepare Lugol's Iodine Solution (Gram's modification) as follows: - Iodine ..... 1g - Potassium Iodide ..... 2g - Distilled water 300ml
Iodine Solution (Iodine and KI)	For Tetrathionate Broth. - Iodine ..... 6g - Potassium Iodide ..... 5g - Distilled water 20mL
Iodophore	General sanitizing agent, dilution is usually 100-150ppm iodine.
Kovac's Reagent	- p-dimethylaminobenzaldehyde.... 5g - Amyl or butyl alcohol ..... 75mL - Hydrochloric acid (37%) c.p.... 25mL Dissolve the first ingredient in the alcohol, then add the acid.
Methyl Red Indicator Solution	- Methyl red .....0.1g - 95% ethanol .....250mL - Distilled water .....250mL Used in the performance of the Methyl Red test. Add 1 drop of the indicator to a 1 day old culture of the test organism in 0.5mL of MR-VP medium. A red colour reaction is positive, yellow is negative.
Naphthol Solution	a) Used in the IMViC analysis - 5% Naphthol 5g Ethyl Alcohol (95%) 100mL b) Used in cytochrome oxidase test - 1% Naphthol 1g Ethyl Alcohol (95%) 100mL
ONPG Differentiation Disks	Used in the rapid differentiation of late lactose-fermenting Enterobacteriaceae (ONPG: ortho- Nitrophenyl Beta -D- galactopyranoside)

Non-absorbent Cotton	For pipette plugging.
Phenolic Derivatives, e.g., Lysol, Lyseptol, Cycol	Used as general disinfectant. 3% solution for routine disinfection 10% solution for jars to hold contaminated glassware.
Phenylenediamine solution	Used in Vibrio determinations NN-dimethyl -p- phenylenediamine dihydrochloride ... 1g Distilled H_2O - 1000mL
Potassium Hydroxide 40% Solution	Used in the IMViC analysis - KOH c.p. .....40g - Distilled water sufficient to make 100mL
Potassium Hydroxide N/2	- KOH c.p. ..... 28.05g - Distilled water to make 1000mL
Potassium Phosphate (Monobasic) KH_2PO_4	Used in the preparation of phosphate buffer.
Potassium Tellurite	Prepare a 1% solution of the salt and sterilize by passing through a membrane filter. Store the solution in sterile screw-cap tubes in the refrigerator. Used in the preparation of selective bacteriological media.
Quaternary Ammonium Compounds	Used as a sanitizing agent or germicide. A small amount added to the water in serological baths prevent bacterial growth and the development of a surface scum.
Rosolic Acid 1%	Used in the preparation of m-FC Agar. Dissolve 1g in 100mL 0.2N NaOH. This solution is stable for 2 weeks at 2-8°C
Sodium Hydroxide 0.2N	- NaOH .....8g - Distilled water to make 1000mL
Sodium Hydroxide 1N	For pH adjustment of media. - NaOH c.p. .....4.0g - Distilled water .....100mL Preserve in a screw-cap polypropylene dropping bottle or rubber-stoppered glass bottle.

Sodium Thiosulfate - 10% solution	- Sodium Thiosulfate ..... 10g - Distilled water to make 100mL a) Used for the neutralisation of chlorine in water samples b) As a neutralizer for chlorine and iodine sanitizers
Swabs	For sanitary surveys Wrap wooden applicator sticks with a thin layer of absorbent cotton to make a swab 5cm long. Sterilize in bundles in large tubes or wrapped in aluminum foil. NOTE: suitable prepared swabs are available from medical supply houses.
Triton X-100	Ingredient of A-1 medium.

APPENDIX E

ESTIMATION OF BACTERIAL DENSITY
Most Probable Number Tables

Comb'n of pos. Tubes	MPN Index* 3.3.3 Series	95% Conf. Limits		MPN Index* 5.5.5 Series	95% Conf. Limits	
0-0-0	<3			<2		
0-0-1	-			2	<0.5	7
0-1-0	3	<0.5	13	2	<0.5	7
0-2-0	-			4	<0.5	11
1-0-0	4	<0.5	20	2	<0.5	7
1-0-1	7	1	21	4	<0.5	11
1-1-0	7	1	23	4	<0.5	11
1-1-1	-			6	<0.5	15
1-2-0	11	3	36	6	<0.5	15
2-0-0	9	1	36	5	<0.5	13
2-0-1	14	3	37	7	1	17
2-1-0	15	3	44	7	1	17
2-1-1	20	7	89	9	2	21
2-2-0	21	4	47	9	2	21
2-3-0	-			12	3	28
3-0-0	23	4	120	8	1	19
3-0-1	39	7	130	11	2	25
3-1-0	43	7	210	11	2	25
3-1-1	75	14	230	14	4	34
3-2-0	93	15	380	14	4	34
3-2-1	150	30	440	17	5	46
3-2-2	210	35	470	-		
3-3-0	240	36	1300	-		
3-3-1	460	71	2400	-		
3-3-2	1100	150	4800	-		
3-3-3	≥2400			-		
4-0-0	-			13	3	31
4-0-1	-			17	5	46
4-1-0	-			17	5	46
4-1-1	-			21	7	63
4-1-2	-			26	9	78
4-2-0	-			22	7	67
4-2-1	-			26	9	78
4-3-0	-			27	9	80
4-3-1	-			33	11	93
4-4-0	-			34	12	93
5-0-0	-			23	7	70
5-0-1	-			31	11	89
5-0-2	-			43	15	110
5-1-0	-			33	11	93
5-1-1	-			46	16	120
5-1-2	-			63	21	150
5-2-0	-			49	17	130
5-2-1	-			70	23	170

Comb'n of pos. Tubes	MPN Index* 5.5.5 Series	95% Conf. Limits	
5-2-2	94	28	220
5-3-0	79	25	190
5-3-1	110	31	250
5-3-2	140	37	340
5-3-3	180	44	500
5-4-0	130	35	300
5-4-1	170	43	490
5-4-2	220	57	700
5-4-3	280	90	850
5-4-4	350	120	1000
5-5-0	240	68	750
5-5-1	350	120	1000
5-5-2	540	180	1400
5-5-3	920	300	3200
5-5-4	1600	640	5800
5-5-5	≥2400		

Comb'n of pos. Tubes	MPN Index* 5.1.1 Series	95% Conf. Limits	
0-0-0	≤2		
0-1-0	2	0.05	13
1-0-0	2.2	0.05	13
1-1-0	4.4	0.52	14
2-0-0	5	0.54	19
2-1-0	7.6	1.5	19
3-0-0	8.8	1.6	29
3-1-0	12	3.1	30
4-0-0	15	3.3	46
4-0-1	20	5.9	48
4-1-0	21	6.0	53
5-0-0	38	6.4	330
5-0-1	96	12	370
5-1-0	240	12	3700
5-1-1	>240	-	-

Comb'n of pos. Tubes	MPN Index* 5:0:0 Series	95% Conf. Limits	
0-0-0	≤2.2		
1-0-0	2.2	0.1	12.6
2-0-0	5.1	0.5	19.2
3-0-0	9.2	1.6	29.4
4-0-0	16.0	3.3	52.9
5-0-0	>16.0	-	-

* MPN Index per 100 mL using 10 mL, 1 mL and 0.1 mL inocula.

COLONY CHARACTERISTICS OF ENTEROBACTERIACEAE ON SELECTIVE AND DIFFERENTIAL MEDIA

MEDIA	COLONY CHARACTERISTICS
	NOTE: All reactions described are those most typically encountered. Each laboratory must learn through experience how to interpret atypical results.
Bismuth Sulfite Agar	Plates may be read after 24h at 35°C. Incubate negative plates for a further 24h before discarding. Salmonella - colonies appear brown, grey, to black, sometimes with metallic sheen. Medium surrounding colony usually brown, darkening to black on further incubation. Some strains produce green colonies with little or no darkening of surrounding medium.
BG Sulfa Agar	Incubate plates 18-24h at 35°C. Salmonella - pink colonies surrounded by brick-red medium. Lactose/sucrose fermenters - almost completely inhibited, occasionally yellow-green colonies surrounded by yellow-green zone. Proteus spp - some strains produce red colonies.
Hektoen Enteric Agar	Incubate plates 18-24h at 35°C. Shigella, Providencia - green colonies, moist, raised. Salmonella, Hafnia, Proteus - blue-green to blue colonies with or without black centres. Pseudomonas - green or brownish colonies, flat, irregular. Coliforms - salmon to orange-coloured colonies.

MEDIA	COLONY CHARACTERISTICS
Levine's EMB Agar	Incubate plates 24h at 35°C. E. coli - small (2-3mm), discrete, nucleated, with or without metallic sheen. Klebsiella and E. aerogenes - 4-6mm colonies, raised, mucoid, tendency to confluent growth, often colourless, metallic sheen rare.
MacConkey Agar	Incubate plates 24h at 35°C. Lactose fermenters - intense violet-red colonies with peripheral precipitated bile salts in medium. Salmonella - colourless colonies, no precipitated zone. May form clear zones when found in proximity to lactose fermenters. Lactose fermenters are rarely encountered unless isolated from Bismuth Sulfite Agar.
XLD Agar	Incubate plates 18-24h at 35°C. Salmonella, including S. arizonae - red colonies, black centres. Occasional strains may not have black centres. Shigella, Providencia - red colonies. Some Pseudomonas, Proteus rettgeri - red colonies. Escherichia, Citrobacter, Klebsiella, Enterobacter, Proteus - yellow colonies.

THE REACTIONS OF ENTEROBACTERIACEAE IN TRIPLE SUGAR IRON (TSI) AGAR MEDIUM

GENERA AND SPECIES	SLANT	BUTT	GAS	H_2S
Escherichia	A(K)	A	+(-)	-
Shigella	K	A	-	-
S. typhi	K	A	-	+(-)
Other Salmonellae	K	A	+(-)	+++(-)
S. arizonae	K(A)	A	+	+++
Citrobacter	K(A)	A	+	+++
Edwardsiella	K	A	+	+++
Klebsiella	A	A	++	-
Enterobacter	A	A	++	-
Hafnia	K	A	+	-
Serratia	K or A	A	-	-
Proteus vulgaris	A(K)	A	+	+++
P. mirabilis	K(A)	A	+	+++
P. morganii	K	A	+(-)	-
P. rettgeri	K	A	-	-
Providencia	K	A	+ or -	-

K - alkaline A - acid

Symbols enclosed in parentheses indicate occasional reactions.

Alk. slant - lactose and sucrose not fermented

Acid slant - lactose and sucrose fermented

Alk. butt - dextrose not fermented

Acid butt - dextrose fermented

THE REACTIONS OF ENTEROBACTERIACEAE IN LYSINE IRON AGAR (LIA)

GENERA AND SPECIES	SLANT	BUTT	GAS	H_2S
Escherichia	K	K or N	- or (+)	-
Shigella	K	A	-	-
Salmonellae	K	K or N	-	+(-)
S. typhi	K	K	-	+ or -
S. paratyphi-A	K	A	+ or -	- or +
S. arizonae	K	K or N	-	+(-)
Citrobacter	K	A	- or +	+ or -
Edwardsiella	K	K	- or +	+
Klebsiella	K or N	K or N	+ or -	-
Enterobacter				
E. cloacae	K or N	A	+ or -	-
E. aerogenes	K	K or N	+(-)	-
Hafnia	K	K or N	- or +	-
Serratia	K or N	K or N	-	-
Proteus				
P. vulgaris	R	A	-	-(+)
P. mirabilis	R	A	-	-(+)
P. morganii	K or R	A	-	-
P. rettgeri	R	A	-	-
Providencia	R	A	-	-

An alkaline or neutral reaction in the butt of this medium indicates decarboxylation.

K = alkaline
N = neutral
A = acid
R = red (oxidative deamination)

INTERPRETATION OF IMViC REACTIONS

ORGANISM	INDOLE	METHYL RED	VOGES-PROSKAUER	CITRATE
Escherichia coli				
Variety I	+	+	-	-
Variety II	-	+	-	-
Citrobacter freundii	-	+	-	+
Citrobacter diversus	+	+	-	+
Klebsiella - Enterobacter group	+ or -	-	+	+

Details of Reactions

1. Indole - Use 24h incubation of 1% tryptone culture. Run 0.5mL of Kovac's Reagent down side of tube to form a layer on the surface of the medium. Do not agitate.

   Wine red colour .....positive
   Unchanged (yellowish-green) .....negative

2. Methyl Red - Use 24h incubation of 0.5mL MR-VP medium. Add 1 drop of methyl red solution and shake.

   Red colour .....positive
   Yellow colour .....negative

3. Voges-Proskauer (Acetyl methyl carbinol) - Use 24h incubation of 0.5mL MR-VP medium. Add, in sequence, 2 drops creatine solution, 3 drops alpha-naphthol (5%), 2 drops 40% KOH. Agitate vigorously between additions, so that tube contents are strongly oxygenated. Re-incubate at 35°C for not longer than 30 min.

   Red colour .....positive
   Yellow colour .....negative

4. Citrate - Inoculate Simmons Citrate Agar slant by needle with a single surface stroke. Read results after 24 and 48h incubation at 35°C.

   Blue colour in medium and evidence of surface growth .....positive
   Green (unchanged) .....negative

CONVENTIONAL MEDIA METHODOLOGY FOR THE BIOCHEMICAL IDENTIFICATION OF SALMONELLA

For satisfactory biochemical identification, culture purity must be assured. Therefore, pick an isolated colony from the MacConkey Agar purification plate (Chapter 5) and inoculate a Nutrient Agar slant. Incubate at 35°C for 18-24h.

Perform a Cytochrome Oxidase test. Remove a portion of the bacterial growth from the slant with a platinum loop or needle (wire containing iron may cause false-positive results) and rub the inoculum on a strip of impregnated filter paper (commercially available). A blue colour developing within 2 min. where the culture material touched the filter paper constitutes a positive result. Salmonella is oxidase-negative. Oxidase-positive cultures require no further testing and can be discarded. At least 6 tests can be performed consecutively on each strip. Handle used strips carefully since they contain live cultures. Discard such strips into a jar containing germicide.

Screen oxidase-negative cultures as follows:

a) Urea: Using a heavy inoculum from the Nutrient Agar slant, inoculate a tube of Urea Broth or a Urea Agar Slant. Incubate at 35°C for 24h. Salmonella is urea-negative. Urease-positive cultures (red reaction) may be discarded.

b) Tryptone 1%: Inoculate and incubate at 35°C for 24h. Perform the test for indole. Salmonella is indole-negative.

c) ONPG Differentiation Disks:
(o-Nitrophenyl-B-D-Galactopyranoside)
Dispense 0.2mL of sterile 0.85% saline into a sterile 75mm x 10mm culture tube. Disperse a loopful of the culture from the TSI slant in the saline, add an ONPG disc, cover the tube with a square of sterile aluminum foil, and incubate at 35°C. Examine at 30 min. intervals for up to 4h. A yellow colour is positive. S. arizonae is ONPG positive.

d) Dulcitol Fermentation Broth: Inoculate lightly with a needle and incubate at 35°C. Examine at 24 and 48h. Most Salmonellae form acid and gas in this medium. S. arizonae does not.

e) Malonate Broth: Inoculate lightly with a needle and incubate at 35°C. Examine at 24h and 48h. A blue colour is positive. S. Arizonae is malonate positive.

f) Simmons' Citrate Agar: Inoculate lightly with a needle and incubate at 35°C for up to 7 days. A blue colour is positive. Most Salmonellae are citrate-positive. Delayed or negative reactions are uncommon.

TABLE I

ONPG TEST REACTIONS OF ENTERIC BACTERIA

Organism	ONPG Reaction
S. arizonae	+
Citrobacter	+
Salmonella	-
Escherichia	+
Shigella	+ or -
Enterobacter	+
Klebsiella	+
Hafnia	+
Serratia	+
Alcaligenes	-
Proteus	-
Providencia	-
Pseudomonas	-

\+ positive (yellow)
\- negative (no colour change)

TABLE II

SOME INDICATIVE BIOCHEMICAL TESTS FOR SALMONELLAE DIFFERENTIATION

	Salmonella			S. arizona	Citrobacter
	Subgenus I (Typical)	Subgenus II	Subgenus IV	(Subgenus III)	
Dextrose	AG	AG	AG	AG	AG
Dulcitol	d	+	-	-	d
Lactose	-	-	-	+ or x	d
Sucrose	-	-	-	-	d
Salicin	-	-	+	-	d
Indole	-	-	-	-	-
MR	+	+	+	+	+
VP	-	-	-	-	-
Citrate	+	+	+	+	+
H_2S(TSI)	+	+	+	+	+ or -
Urea	-	-	-	-	d(weak)
Malonate	-	+	+	+	-
Lysine	+	+	+	+	-
ONPG	-	-/x	-	+	- or +
Gelatin	-	+	+	+	-

A - Acid
G - Gas
+ - positive
- - negative
d - different biochemical types
x - delayed and irregularly positive

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