Manual of Bacteriological Examination of Drinking Water

English Edition

Abdurazzaq Suliman Al-Tomi
Microbiology Researcher

Biotechnology Research Centre
Microbiology Department

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PREFACE

Water microbiology, by its very nature, is a dynamic science. It is constantly evolving as more information is added to the continuum of knowledge and microbiological techniques are rapidly being modified and refined. In this edition of "*Manual of Bacteriological Examination of Drinking Water*", I have endeavored to provide a blend of traditional methodologies with the more contemporary procedures, in an attempt to meet the pedagogical needs of all water microbiologists.

The structure of the manual includes specific explanations and detailed directions precede each examination, with the hope that it will enable water microbiologists more readily to comprehend the concepts and purposes of each examination.

A. S. Al-Tomi*
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* ***Tomei_abdu@yahoo.com***

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INTRODUCTION

The world health organization has estimated that up to 80% of all sickness and disease in the world is caused by inadequate sanitation, polluted water, or unavailability of water. Approximately three out of five persons in developing countries do not have access to safe drinking water, and only about one in four has any kind of sanitary facility.

Against such a background of human suffering, the benefits to be derived from improved sanitation and water supplies are immense. To secure such benefits, however, resources must be made available and communities, health workers, and health authorities must work together to bring about improvements.

Supplies of drinking water contaminated with sewage or other excreted matter from man and animals may cause diseases like typhoid fever, cholera, campylobacteriosis, amoebiasis, helminthiasis and other diseases as shown in Table 1. In the interests of public health, supplies should be tested regularly to confirm their freedom from such contamination. It is impracticable to attempt to detect directly the presence of all the different kinds of water-borne pathogens, any of which may be present only intermittently. Instead, reliance is placed on testing the supply for faecal indicator bacteria. These are the common intestinal commensal bacteria, *Escherichia coli, Streptococcus faecalis* and *Clostridium perfringens* (*C. welchi*), which are universally present in, and excreted in large numbers by man and animals. In themselves, they are not dangerous (with the exception of certain types of pathogenic *E. coli*), but their presence indicates that faecal matter has entered the water supply, that the faecal bacteria have not been killed or removed by purification processes and that the supply is therefore liable to contamination with dangerous intestinal pathogens.

It is necessary not only to attempt to detect the presence of the indicator bacteria, but also to enumerate them, for the greater their number, the greater the danger of infection from the supply. The presence of very small numbers of the bacteria in unchlorinated water is usually disregarded, being assumed to have originated from animals and not from the more dangerous excretions of man. Separate counts are made of presumptive coliform bacteria and confirmed *E. coli* bacteria. If the results of these tests are difficult to interpret, counts are also made of faecal streptococci and *C. perfringens.*

Table 1. Diseases that may be associated with contaminated drinking water.

Organism	Disease Caused
Bacteria	
Escherichia coli (some types)	Gastroenteritis
Leptospira spp.	Leptospirosis
Salmonella typhi	Typhoid fever
Salmonella spp.	Salmonellosis
Shigella spp.	Shigellosis (bacillary dysentery)
Vibrio cholerae	Cholera
Protozoa	
Balantidium coli	Balantidiasis
Cryptosporidium parvum	Cryptosporidiosis
Entamoeba histolytica	Amebiasis (amoebic dysentery)
Giardia lamblia	Giardiasis
Helminths	
Ascaris lumbricoides	Ascariasis
Taenia solium	Taeniasis
Trichuris trichiura	Trichuriasis
Viruses	
Enteroviruses (72 types) e.g., polio echo and coxsackie viruses)	Gastroenteritis, hear anomalies, meningitis
Hepatitis A virus	Infectious hepatitis
Norwalk agent	Gastroenteritis
Rotavirus	Gastroenteritis

Good quality water:

Good quality water is odourless, colourless, tasteless, and free from faecal pollution and chemicals in harmful amounts.

Collection of water samples:

Glass bottles used for water sampling should have a capacity of at least 200 ml. They should be fitted with ground glass stoppers or screw caps. The stopper or cap and neck of the bottle should be protected from contamination by a suitable cover either of paper or thin aluminum foil. Silicon rubber liners, that will withstand repeated sterilization by autoclaving, should be used inside screw caps. After being sterilized the bottle should not be opened before the sample is collected.

1. For collection, use heat-sterilized bottles containing a sufficient volume of sodium thiosulphate to neutralize the bactericidal effect of any chlorine or chloramine in the water. Each bottle of ٢00ml capacity should contain 0.1 - 0.2ml of a fresh 30 g/L (3%w/v) aqueous solution of sodium thiosulphate.

Note: *Sodium thiosulphate at concentration of approx. 18mg/L has no significant effect on the coliform or E. coli content of a water sample. It should neutralize up to 5mg/L of residual chlorine.*

2. When collecting the sample, exercise extreme care to avoid contaminating it with bacteria from the environment. Flame the mouths of taps and hydrants and allow water to run to waste for 3-5 min before running it into the bottle. When sampling from streams or lakes, open the bottle at a depth of about 20-30cm with it is mouth facing the current and ensure that water entering the bottle has not been in contact with the hand. Sample wells with weighted bottles. Collect at least 100ml in each bottle.

3. Stopper the bottle, label it with full details, and deliver it to the laboratory as quickly as possible, at least within 6 hours, keeping it in cool container and protected from light. The changes that may occur in the bacterial content of water on storage can be reduced to a minimum by ensuring that samples are not exposed to light and are kept cool, preferably between 4 and 8^{0}C, but not frozen. Examination should begin as soon as possible after sampling and certainly within 24h of sampling. If neither condition can be met, the sample should not be analysed.

I- Collecting a sample from a tap:

1- Remove any external fittings from the tap, such as an anti-splash nozzle or rubber tube. Clean carefully the outside nozzle of the tap, especially any grease which has collected (see Figure 1).

2- Turn the tap on full, and allow the water to run to waste for 3-5 minutes. This allows time for the nozzle of the tap to be flushed and any stagnant water in the service pipe to be discharged.

3- Sterilize the tap using the flame of a blowlamp or gas torch, or by igniting a piece of cotton wool soaked in alcohol and holding it with a pair of tongs close to the nozzle until the whole tap is unbearably hot to the touch.

4- Allow the tap to cool by running the water to waste for a few seconds.

5- Fill the sample bottle from a gentle flow of water, and replace the cap of the bottle.

6- Using a water-proof marker or grease pencil, number the bottle with the sample code number.

Note: *Leaking taps may cause contamination of the sample from sources outside the water pipe and therefore leak should be reported when sampling. A bacteriological sample should not be taken until the leak is repaired.*

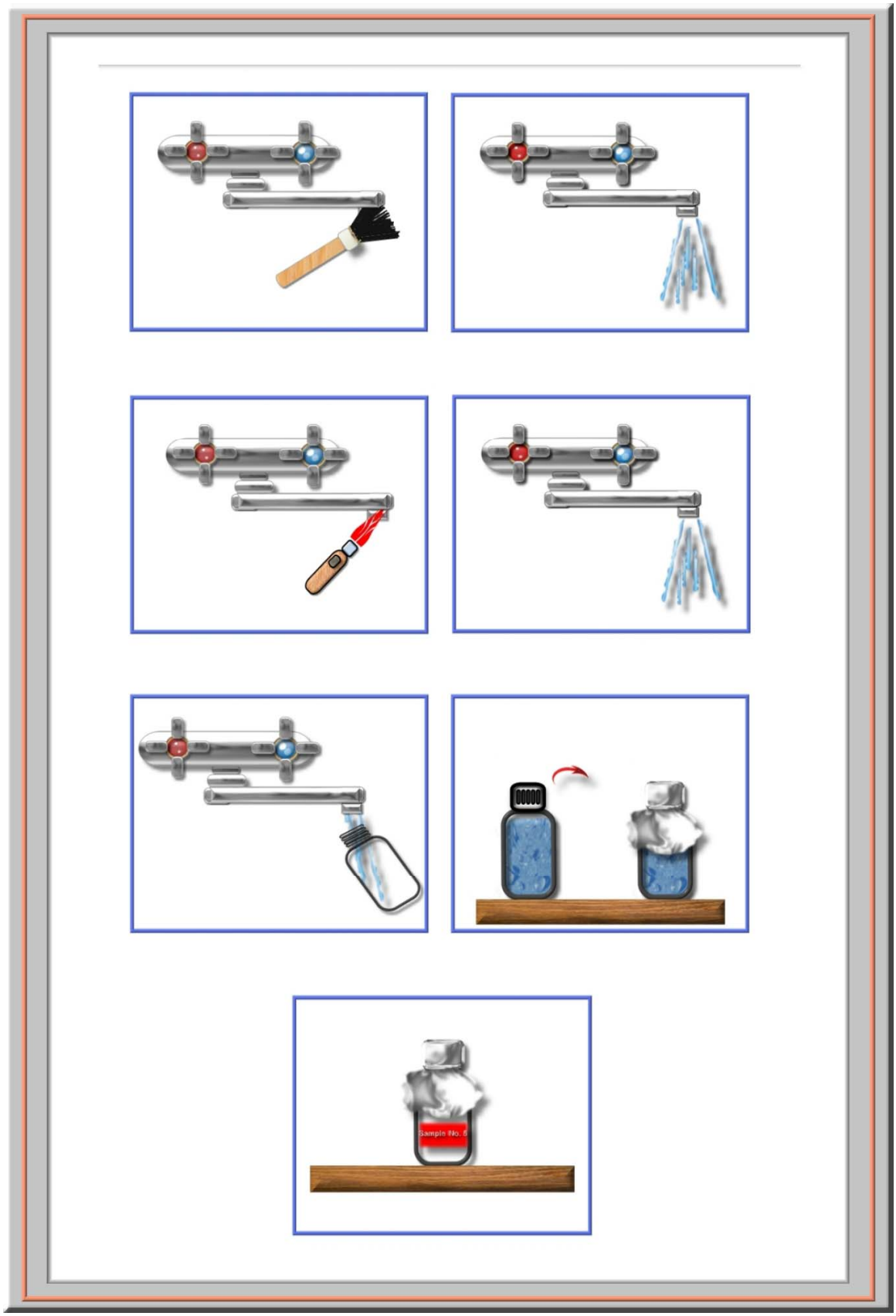

Figure No.1

II- Collecting a sample from a river, stream, or other surface water:

1- Aseptically remove the cap and cover of the sterile sample bottle, and face the mouth of the bottle upstream.

Note: *To avoid entering the water, the bottle should be clamped to the end of a stick. One way of doing this is to fix the bottle neck in a resort stand clamp and mount this on a stick.*

2- Plunge the neck downwards about 20-30cm below the water surface (Figure 2), and then tilt the neck slightly upwards to let it fill completely before carefully replacing the cap and cover. Where there is no current, push the bottle forward horizontally until it is filled.

3- Label the bottle with the sample code number.

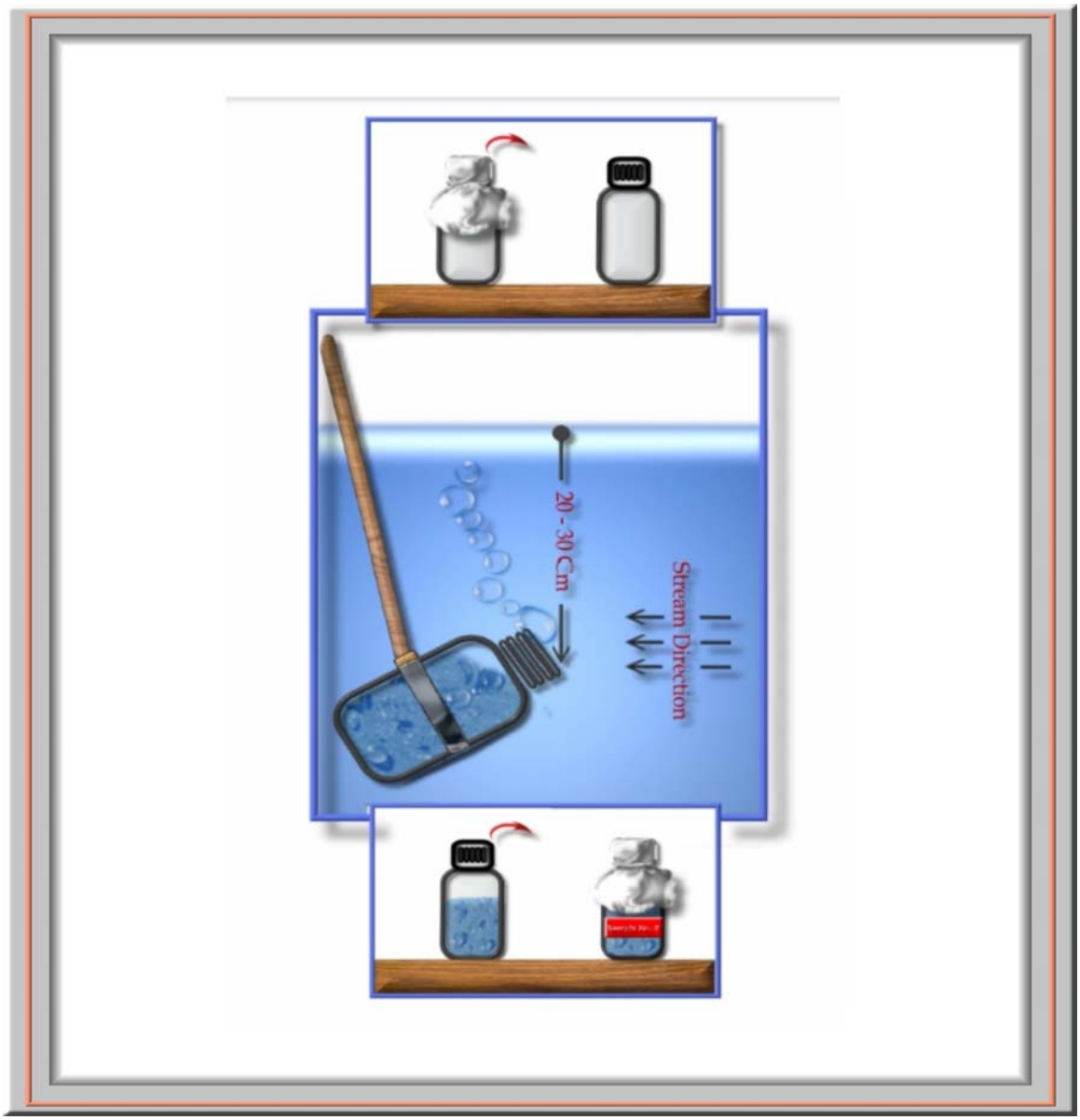

Figure No.2

III- Collecting a sample from a street hydrant:

Collect a sample from a suitable tap supplying water direct from the main. Where this is not possible, obtain a sample from a street hydrant by the following method:

1- Pour hypochlorite solution into the duckfoot or hydrant bowl. If the hydrant box is full of water it must be baled out until 1 inch below the top of duck-foot or bowl and enough water must be displaced to allow room for the hypochlorite solution.

2- Immediately screw on the standpost with the bibcock shut.

3- Open the hydrant valve and bibcock until water runs, then shut the bibcock.

4- Allow at least one minute to elapse.

5- Open the bibcock fully and allow water to run for at least 2 minutes.

Caution: *Avoid being splashed by hypochlorite charged water*.

6- Make a residual chlorine test to make sure all the hypochlorite charged water has been flushed to waste.

7- Screw down the bibcock to a small stream, and aseptically collect the samples.

8- Number each bottle with the sample code number.

IV- Collecting a sample from an open well:

If the well is one from which water can be raised only by means of a bucket or can, use a weighted bottle to collect the sample as follows:

1- Tie a sterile sample bottle on to a weighted length of rope or strong string. Use a stone or piece of metal weighing about 500 grams as the weight, and attach the bottle just above the weight (Figures 3 & 4).

2- Aseptically remove the cap from the bottle, and lower the bottle into the well to a depth of about 1 meter from water surface.

3- When no more air bubbles rise to the surface, raise the bottle out of the well and carefully replace the cap.

4- Label the bottle with the sample code number.

Figure No.3

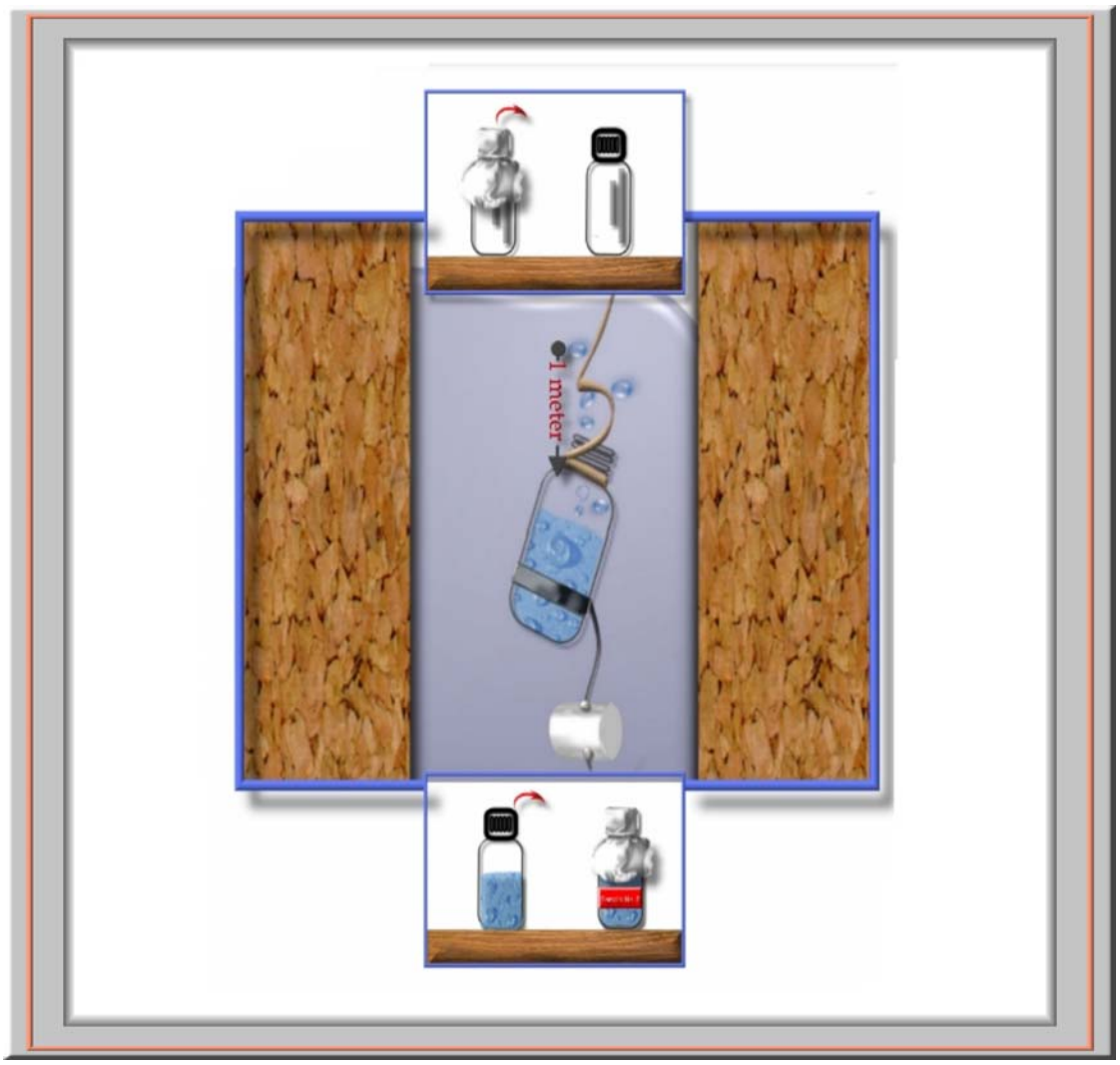

Figure No.4

V- Collecting a sample from a tube-well:

1- Continuously operate the handpump for 5 minutes.

2- Heat the mouth of the pump, preferably by means of a blowlamp or gas torch, and pump several gallons of water to waste.

3- Aseptically collect a sample of water by allowing the water from the pump to flow directly into the sterile bottle. Carefully replace the bottle cap and cover.

4- Label the bottle with the sample code number.

Information to be supplied with the samples:

- Code number of the sample.

- Reasons for examination, for example whether a routine sample or Otherwise.
- Source from where the water has been collected, for example whether from a well, spring, lake, reservoir, or piped supply.

  Mention also the exact place from where the water was taken. If the sample was collected from a house-tap, mention whether the water was drawn from a cistern or direct from the main.
- Whether the water has been filtered, chlorinated, or treated in some other way.
- If the water is from a well, give details of it is depth, whether covered or uncovered, and whether recently constructed or altered.
- If the sample is spring water, describe the stratum from which it issued and whether the sample was taken directly from spring or from a collection chamber.
- If the water is from river or stream, mention the depth at which the sample was collected, whether from the side or the middle of the stream, whether the water level was above or below average, and whether there had been heavy rainfall or flooding.
- If the water is from a lake or reservoir, give the exact position and the depth at which it was collected.
- Temperature of the source of the sample.
- Mention any possible sources of pollution in the area and their approximate distance from the sampling point.
- Date and time when the sample was taken and dispatched.

Sampling frequencies and procedure:

The frequency of sampling will be determined by the resources available. The more frequently the water is examined, the more likely is that chance contamination will be detected. Two main points should be noted. Firstly, the chance of detecting pollution which occurs periodically, rather than randomly, is increased if samples are taken at different times of the day and on different days of the week. Secondly, frequent examination by a simple method is more valuable than frequent examination by a complex test or series of tests. Sampling frequencies for raw water sources will depend on their overall quality, their size, the likelihood of contamination, and the season of the year. They should be established by local control agencies and are often specified in national regulations and guidelines. The results, together with information from the sanitary inspection of the gathering grounds, will often indicate whether increased vigilance is needed.

Sampling frequencies for treated water leaving the waterworks depend on the quality of the water source and the type of treatment. Minimum frequencies are one sample every 2 weeks for waterworks with a ground water source, and one sample every week for waterworks with a surface water source.

The frequency of sampling should be greater where a large number of people are supplied because of the greater number of people at risk. Advice on the design of sampling programmes and on the frequency of sampling is given in ISO standards and in many national regulations. The minimum number of samples to be taken each month for water in the distribution system is given for different population sizes as shown in Table 2.

Table 2. Minimum sampling frequencies for drinking water in the distribution system.

Population served	Samples to be taken monthly
Less than 5000	1 sample
5000-100 000	1 sample / 5000 population
More than 100 000	1 sample / 10 000 population, plus 10 additional samples.

Samples should be taken at random intervals in each month from fixed points, such as pumping stations and tanks, from random locations throughout the distribution system and from taps connected directly to the mains in houses and large multioccupancy buildings, where there is a greater risk of contamination through cross-connections and back-siphonage. The frequency of sampling should be increased at times of epidemics, flooding, and emergency operations, or following interruption of supply or repair work. With systems serving small communities, periodic sanitary surveys are likely to yield more information than infrequent sampling.

No general recommendation can be made for unpiped supplies and untreated water because the quality and likelihood of contamination will vary seasonally and with local conditions. The frequency should be established by the local control agency and reflect local conditions, including the results of sanitary surveys.

When samples of disinfected water are taken, the concentration of residual disinfectant at the sampling point and the pH should be determined at the time of collection.

The frequency of sampling water in distribution pipes, unchlorinated water supplies before distribution, and chlorinated water before distribution is as follows:

Water in distribution pipes:

It is inevitable that water quality deteriorates in distribution, mainly as a result of corrosion in pipes allowing soil contamination to enter. The larger the population served, the longer is the distribution system and therefore the greater the risk of contamination.

At least one sample per 5000 population per month should be examined and every effort should be made to establish a random routine sampling procedure.

Unchlorinated water supplies before distribution:

The world health organization has suggested that the maximum interval between successive samples for bacteriological analysis should be as shown below.

Population served	**Maximum interval**
Less than 20 000	One month
20 000 - 50 000	Two weeks
50 000 - 100 000	four days

Chlorinated water supplies before distribution:

Large supplies, serving populations of greater than 100 000 should be subjected to daily sampling of bacteriological analysis in conjunction with continuous chlorine residual recording.

Small rural supplies, serving populations, often significantly less than 10 000, where even sampling for bacteriological analysis at weekly intervals may be impractical. In such cases it is vital that the free and combined residual chlorine in water leaving treatment plants should be checked at least once daily.

Choice of technique:

When starting routine bacteriological control of water quality, the following factors will need to be considered when deciding whether to use the Most Probable Number (MPN) method or the membrane filtration technique as shown below:

FACTORS	Most Probable Number	Membrane Filtration*
Cost	**Large quantities of culture media and glassware are required, relatively large capacity autoclave.**	**Smaller amounts of media and glassware are used, but costly membrane filters are required.**
Accuracy	**Suffers from large sampling error.**	**Significantly more accurate.**
Adaptability, speed, simplicity.	**Requiring up to 48 hours to obtain presumptive *E. coli* count and bio-chemical confirmation may then be required. Requires less specialized equipment and for those with little experience in practical bacteriology, it is simpler to do.**	**Using membranes, the conditions of incubation can be varied to encourage the growth of slow growing organisms; membranes can also be transferred to another temperature or even to another medium to improve growth and recovery of bacteria. An *E. coli* colony may be obtained in 12-18h and does not depend on the use of probability tables**

**If water testing can be performed in the lab the membrane filtration technique is recommended.*

Interpretation of results:

E. coli is the most numerous coliform in human and animal intestine and is derived almost exclusively from these sources. It does not survive long in water. It is therefore the best indicator of recent human or animal faecal pollution. It is presence in water indicates a potentially dangerous pollution, high counts a heavy or recent pollution, and low counts a slight or more remote one.

Other coliform bacilli, e.g. klebsiellae and citrobacters, are much less abundant in faeces than *E. coli* and enter water mainly from soil and vegetation, where they grow as saprophytes. They also survive longer in water than *E. coli*. Their presence in water may therefore indicate either

contamination from soil and vegetation or contamination with faecal material at a time remote enough to have allowed the *E. coli* bacteria to die out. The presence of any coliform bacilli in chlorinated water indicates either a failure of the chlorination process or a contamination after chlorination, and the fault should at once be investigated and corrected.

Faecal streptococci are less abundant in faeces than *E. coli* and are often more abundant in animal than human faeces. Specification of isolates may help to trace the source of pollution, for *Streptococcus bovis* is usually derived from cattle and sheep, *S. equines* from horses and *S. avium* from birds; *S. faecium* and *S. durans* are derived from both man and animals, and *S. faecalis* more often from man. Faecal streptococci survive longer in water than *E. coli* and are more resistant to chlorination than the coliforms. Their presence with coliforms, despite the absence of *E. coli*, confirms faecal pollution of the water.

Clostridium perfringens is present in faeces in even smaller number than the streptococci and is the least sensitive indicator of faecal pollution. But it is spores may survive chlorination and persist in water much longer than the other indicator bacteria, so that it is finding in the absence of the latter implies an intermittent or remote pollution, or the result of chlorination in killing the vegetative bacteria, and thus any bacterial pathogens in recently polluted water. In these circumstances the spores themselves do not constitute a hazard to health when the water is used for drinking, but the finding of *C. perfringens* as well as coliform bacilli is suggestive of faecal pollution even in the absence of *E. coli*.

The plate count is useful mainly for purposes other than assessing water safety, but a sudden increase in the count at 37^{0}C may be an early sign of pollution and should be investigated.

Bacteriological standards of water quality:

1. Water entering a distribution system:

The final treatment of water, usually by chlorination, before it enters the system of pipes distributing it to the public is an effective disinfection. If correctly done, this treatment ensures that no viable coliform bacteria remain in the water. The supply here can be regarded as satisfactory only if coliform bacteria are undetectable in all tested samples of 100ml. Any deviation from this standard, however small, should be reported at once, so that the treatment process can be investigated for faults.

2. Water drawn from the distribution system:

For various reasons, pure water entering the distribution system may deteriorate in quality before it reaches the consumer's tap, e.g. as a result of

unsatisfactory pumping repairs, use of contaminated materials in construction, and access of coliforms through air valves, hydrants, pumps and leaks in pipes where they are under negative pressure. Thus, although ideally all samples drawn from the distribution system should be free from coliforms, a minimal contamination within the following limits may be tolerated.

E. coli should not be detected in any 100ml sample. No more than three coliform bacilli should be found in any 100ml sample. Coliform bacilli should not be detected in any two consecutive samples of 100ml from the same or closely related sampling point. Coliform bacilli should not be found in more than 5% of routine samples from the system among at least 50 samples examined at regular intervals throughout the year.

When any coliforms are found, the disinfection process should be checked and the water re-sampled from the same and related sites to confirm the original positive finding and locate the possible source of contamination. Even when satisfactory results are obtained on re-testing, the frequency of routine sampling should be increased for a while. The results of the tests should be reviewed annually to obtain an indication of the overall quality of the supply, which may be graded as shown in Table 3, if results from at least 50 samplings are available.

Table 3. Grades of the quality of drinking water supplies determined by the result of periodic *Escherichia coli* and coliform count.

Quality of supply	Results from routine samples		Tolerance
	Coliform count/ 100ml	*E. coli* count/100ml	
1- Excellent	**0**	**0**	**In all samples.**
2- satisfactory	**1-3**	**0**	**Provided that coliform organisms do not occur in consecutive samples or in more than 5% of samples.**
3- Intermediate	**4-9**	**0**	
4- Unsatisfactory	**10 coliforms or more, *E .coli* or any coliform organisms present in consecutive samples. or presence of any coliform organisms in more than 5% of routine samples.**		**In any sample.**

3. Water from unpiped rural supplies:

In the absence of a piped supply, the source of water, e.g. a lake or well, should be protected from obvious sources of excremental pollution.

The quality of the water may be considered satisfactory if the coliform count is less than 10/100ml sample. Water in which this is exceeded, or in which more than minimal numbers of *E. coli* are found, should be condemned for drinking.

Most untreated water supplies contain faecal bacteria but in the case of protected ground water, for example springs, sealed wells, and tubewells, it should be possible to achieve very low levels of contamination. The following guidelines are therefore suggested as shown below in Table 4.

Table 4. Suggested bacteriological criteria for drinking water from unchlorinated rural hand pumps and other sources.

Mean count* 44^0**C, 100 ml** ***E . coli* count**	**Category**	**Comments**
0	**A**	**Excellent**
1 – 10	**B**	**Acceptable: But make regular sanitary checks on equipment**
10 – 50	**C**	**Unacceptable: Look for and correct structural faults and poor maintenance of pump and plinth. Then disinfect equipment and source.**
More than 50	**D**	**Grossly polluted: Look for alternative source, or carryout necessary repairs, and disinfect well.**

**** Guidelines can only be applied when routine survey data are available, e.g. 5-10 consecutive weekly samples.***

Detection and counting of indicator organisms:

As the number of indicator bacteria in the water may be small, large volumes of the water have to be cultured. Two methods are available for this purpose, the multiple tube method, and membrane filtration method. Membrane filtration has advantages over multiple tube test in requiring less labour and materials and in giving results earlier, so that any corrective action required to render the supply safe may be taken sooner. The multiple tube method has the advantages that it can show gas formation by the bacteria and is suitable for the examination of turbid waters containing small numbers of the indicator bacteria, e.g. waters containing numerous saprophytic bacteria that might suppress growth of the coliforms.

Summary: If water testing can be performed in a microbiology laboratory, the membrane filtration technique is recommended for it is accuracy and speed of result.

MULTIPLE TUBE TEST
The Most Probable Number technique (MPN)

Measured volumes of water and the dilutions of water are added to a series of tubes or bottles containing a liquid indicator growth medium. The media receiving one or more of the indicator bacteria show growth and a characteristic colour change which is absent in those receiving an inoculum of water without indicator bacteria. From the number and distribution of positive and negative reactions, the most probable number (MPN) of indicator organisms in the sample may be estimated by reference to statistical tables (McCardy tables).

The indicator medium most used has been MacConkey broth containing bromocresol purple to indicate by it is colour change to yellow the formation of acid from the lactose in the broth. An inverted Durham tube is placed in each bottle or tube of the medium. Bacteria capable of growth and the production of acid and gas in MacConkey broth are assumed to be coliform bacilli, i.e. "presumptive coliforms".

An alternative selective indicator medium is lauryl tryptose broth in which fermentation of lactose is judged by gas formation and the absence of a pH indicator dye allows indole production to be observed by the addition of indole reagent after growth. The medium most strongly recommended is the minerals modified glutamate medium containing lactose and bromocresol purple. In comparative trials it has given more isolations of *E. coli* than either MacConkey broth or lauryl tryptose broth.

A. Presumptive Coliform Count:

1. Place 10ml volumes of indicator broth at "MacConkey broth" double – strength concentration and two sets of 5ml volumes of single – strength into suitable sized tubes containing inverted Durham tube. Cap and sterilize. After sterilization check that the Durham tubes are free from air bubbles.

2. Invert the bottle containing the sample of water rapidly several times to mix and distribute any deposit. Aseptically discard a little of the water, replace the cap and shake the bottle up and down 25 times.

3. Aseptically pipette five 10ml volume, five 1.0ml volume and five 0.1ml volumes of the water sample into tubes containing corresponding one set of five tubes containing 10ml double strength medium, two sets of five tubes containing 5ml single strength medium.

4. Incubate the seeded media aerobically at 37^{0}C.

5. After 24h and 48h of incubation, inspect the media and note the number of cultures of each volume of water that show the production of acid (colour change) and gas (a bubble large enough to fill the concavity at the top of the Durham tube). These acid and gas producing cultures are considered "presumptive positive" growths of coliform bacilli, e.g. *Escherichia*, *Klebsiella* or *Citrobacter* species. Cultures not showing production of both acid and gas at 48h are considered negative.

6. By reference to tables of most probable numbers in respect of the combination of positive and negative results observed, read off the most probable number (MPN) of presumptive coliform bacilli to be present in 100ml of the sampled water.

B. Confirmed *Escherichia coli* Count (Eijkman test):

Some spore-bearing bacteria give false-positive reactions in the presumptive coliform test. Their presence is most likely to be misleading in the examination of chlorinated drinking water, for the spores are more resistant to chlorination than coliform bacilli. It is necessary, therefore, to confirm the presence of true coliform bacilli in each tube showing a presumptive positive reaction and to determine whether these coliform bacilli are *E. coli*. The Eijkman test is used for this purpose, as it gives valid results with inocula of mixed bacteria from the cultures grown in the presumptive coliform test and does not require the preliminary isolation of the bacteria in pure culture.

The test is done by incubating subcultures from the positive presumptive tests at 44^{0}C and 37^{0}C in a lactose-containing medium inhibitory to spore-forming bacteria e.g. lauryl tryptose broth or brilliant-green lactose bile broth, and other subcultures at 44^{0}C in tryptone water.

The presence of coliform bacilli is confirmed by the production of gas from lactose at 37°C. That of *E. coli* is confirmed by the production at 44°C of gas from lactose and indole from tryptophan. Two atypical types of *E. coli*, known as irregular types II and VI, are unable to form indole at 44°C. The formation of indole at 44°C without the formation of gas from lactose at 44°C, even if acid is formed, are the reactions of bacteria other than *E. coli* and are dismissed as negative.

1. Prepare tubes containing 5-10ml of either lauryl tryptose (lactose) broth or brilliant green lactose bile broth and an inverted Durham tube, and other tubes containing 5-10ml tryptone water. Sterilize and check that afterwards the Durham tubes are free from gas bubbles.
2. Before inoculation, incubate the lactose media in thermostatically controlled waterbaths at 44 $\pm$ 0.5°C, and at 37°C, and the tryptone waters at 44 $\pm$ 0.5°C.

3. When the media have reached the incubation temperature, inoculate a loopful or drop of each presumptive positive culture into a tube of the lactose medium at 37°C, another tube of the lactose medium at 44°C and a tube of tryptone water at 44°C. Immediately re-incubate the tubes in the waterbath at their correct temperature.

With each batch of tests, include and treat similarly control cultures known to give the appropriate positive (*E. coli*) and negative (*Klebsiella aerogenes*) results.

4. Remove the tubes incubated at 44°C after 24h. Add a few drops of indole reagent to the tryptone water cultures, also if desired to the lauryl tryptose cultures. After a few moments check that the reactions of the control organisms are correct. Then examine each culture for gas production and indole formation and record the results.

5. Remove the tubes incubated at 37°C after 48h, examine them for gas production, and record the result.

6. Refer to the tables of most probable number. From the combination of positive and negative results for gas production at 37°C, read off the MPN of coliform bacilli per 100ml of water. This value is known as the confirmed coliform count. From the combination of positive and negative results for gas and indole production at 44°C, read off the MPN of *E. coli* per 100ml of water. This latter value is known as the confirmed *E. coli* count (Figure 5).

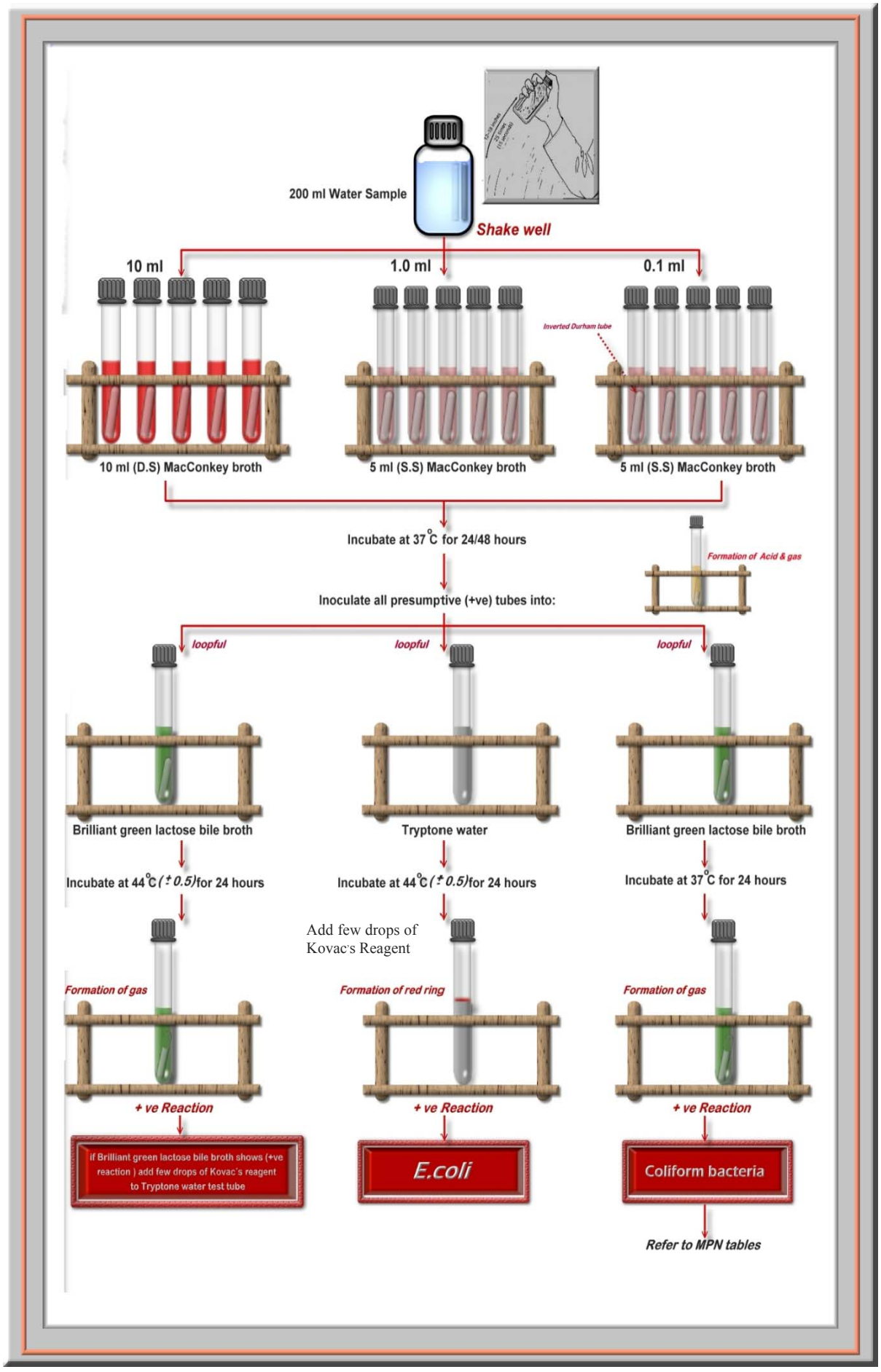
200 ml Water Sample
Shake well
10 ml
1.0 ml
0.1 ml
Inverted Durham tube
10 ml (D.S) MacConkey broth
5 ml (S.S) MacConkey broth
5 ml (S.S) MacConkey broth
Incubate at 37°C for 24/48 hours
Formation of Acid & gas
Inoculate all presumptive (+ve) tubes into:
loopful
loopful
loopful
Brilliant green lactose bile broth
Tryptone water
Brilliant green lactose bile broth
Incubate at 44°C (± 0.5) for 24 hours
Incubate at 44°C (± 0.5) for 24 hours
Incubate at 37°C for 24 hours
Add few drops of Kovac's Reagent
Formation of gas
Formation of red ring
Formation of gas
+ ve Reaction
+ ve Reaction
+ ve Reaction
if Brilliant green lactose bile broth shows (+ve reaction) add few drops of Kovac's reagent to Tryptone water test tube
E.coli
Coliform bacteria
Refer to MPN tables

Figure No.5

Calculation of Most Probable Numbers (MPNs):

Table 5 shows the most probable numbers of coliforms per100ml/g of test material corresponding to the number of gas-positive tubes in the coliform test.

Table 5 has been adapted from a conversion table prepared for the analysis of drinking waters where 10, 1.0, and 0.1ml of the water under test are used as test portions. When other sized portions of the test material are placed in the tubes, MPN values obtained from Table 6 must be multiplied by an appropriate number, to correct for the actual amount of test material in the tubes, and also to obtain the MPN per ml/g as is usually done for foods, rather than per 100ml/g, for which the values are given in the table. The volume of diluent added to the tubes (and which accompanies the test material) is ignored when calculating the MPN.

Example:

The following inoculated tubes give a positive reading:

1. Five tubes with 10ml of 1:10 dilution of test material- All 5 are positive.
2. Five tubes with 1ml of 1:10 dilution of test material- 1 is positive.
3. Five tubes with 1ml of 1:100 dilution of test material- None are positive.

The quantities in each of the five tubes of the three dilution series represent 1, 0.1, and 0.01g (ml), respectively of the test material. According to Table 5, a reading of (5 – 1 – 0) gives a value of 33 when 10, 1 and 0.1g (ml) respectively are used. However, since only 1/10 of these amounts were actually used in the analysis, the value of 33 obtained from Table 5 must be multiplied by giving 33 X 10 = 330 organisms per 100 ml/g of test material. If the results need to be expressed per ml/g, the MPN value is 330 ÷ 100 = 3.3. When higher dilutions are used, the same procedure is followed, but the multiplier (dilution factor) is enlarged to relate the amount of test material actually present to the values given for 10, 1.0, and 0.1ml/g in Table 5.

Dilution factor = reciprocal of the dilution of the analytical unit.

For calculating the MPN, use the dilution factor of the middle set of the three dilutions selected.

To determine which consecutive dilutions to use, refer to the combinations shown below:

1- If only three dilutions are made, use the results for those 3 dilutions to Compute the MPN. Examples ***a*** and ***b***, Table 6.
2- If more than 3 dilutions are employed, use the results of only 3 consecutive dilutions. Select the highest dilution (last dilution, i.e. dilution

with the smallest quantity or product), in which all 5 tubes are positive and 2 subsequent higher dilutions. Examples ***c*** and ***d***, Table 6.

3- If more than 3 dilutions are made, but none of the dilutions tested have all 5 tubes positive, use the first 3 dilutions. Example ***a***, Table 6.

4- If a positive tube occurs in the dilution higher than the 3 chosen to rule, the number of such positive tubes should be added to those of the next lower dilution. Example f, Table 6.

5- If the tubes of a dilution series are positive, choose the 3 highest dilutions of the series and indicate by a "greater than" symbol (>) that the MPN is greater than the one calculated. Example ***g***, Table 6.

Refer to Table 6 and look up the value which corresponds to the number of positive tubes obtained.

MPN/100ml = No. of microorganisms (Table 6) X dilution factor of middle set of tubes.

Table No. 5: Most Probable Number of Bacteria Per 100 ml or g of Test Material Using 5 Tubes With 10,1 and 0.1 ml or g of Test Material

Pos. 10,1,0.1	MPN	Pos. 10,1,0.1	MPN	Pos. 10,1,0.1	MPN	Pos. 10,1,0.1	MPN	Pos. 10,1,0.1	MPN	Pos. 10,1,0.1	MPN
0.0.0	‹1.8	1.0.0	2	2.0.0	4.5	3.0.0	7.8	4.0.0	13	5.0.0	23
0.0.1	1.8	1.0.1	4	2.0.1	6.8	3.0.1	11	4.0.1	17	5.0.1	31
0.0.2	3.6	1.0.2	6	2.0.2	9.1	3.0.2	13	4.0.2	21	5.0.2	43
0.0.3	5.4	1.0.3	8	2.0.3	12	3.0.3	16	4.0.3	25	5.0.3	58
0.0.4	7.2	1.0.4	10	2.0.4	14	3.0.4	20	4.0.4	30	5.0.4	76
0.0.5	9	1.0.5	12	2.0.5	16	3.0.5	23	4.0.5	36	5.0.5	95
0.1.0	1.8	1.1.0	4	2.1.0	6.8	3.1.0	11	4.1.0	17	5.1.0	33
0.1.1	3.6	1.1.1	6.1	2.1.1	9.2	3.1.1	14	4.1.1	21	5.1.1	46
0.1.2	5.5	1.1.2	8.1	2.1.2	12	3.1.2	17	4.1.2	26	5.1.2	64
0.1.3	7.3	1.1.3	10	2.1.3	14	3.1.3	20	4.1.3	31	5.1.3	84
0.1.4	9.1	1.1.4	12	2.1.4	17	3.1.4	23	4.1.4	36	5.1.4	110
0.1.5	11	1.1.5	14	2.1.5	19	3.1.5	27	4.1.5	42	5.1.5	130
0.2.0	3.7	1.2.0	6.1	2.2.0	9.3	3.2.0	14	4.2.0	22	5.2.0	49
0.2.1	5.5	1.2.1	8.2	2.2.1	12	3.2.1	17	4.2.1	26	5.2.1	70
0.2.2	7.4	1.2.2	10	2.2.2	14	3.2.2	20	4.2.2	32	5.2.2	95
0.2.3	9.2	1.2.3	12	2.2.3	17	3.2.3	24	4.2.3	38	5.2.3	120
0.2.4	11	1.2.4	15	2.2.4	19	3.2.4	27	4.2.4	44	5.2.4	150
0.2.5	13	1.2.5	17	2.2.5	22	3.2.5	31	4.2.5	50	5.2.5	180
0.3.0	5.6	1.3.0	8.3	2.3.0	12	3.3.0	17	4.3.0	27	5.3.0	79
0.3.1	7.4	1.3.1	10	2.3.1	14	3.3.1	21	4.3.1	33	5.3.1	110
0.3.2	9.3	1.3.2	13	2.3.2	17	3.3.2	24	4.3.2	39	5.3.2	140
0.3.3	11	1.3.3	15	2.3.3	20	3.3.3	28	4.3.3	45	5.3.3	180
0.3.4	13	1.3.4	17	2.3.4	22	3.3.4	31	4.3.4	52	5.3.4	210
0.3.5	15	1.3.5	19	2.3.5	25	3.3.5	35	4.3.5	59	5.3.5	250
0.4.0	7.5	1.4.0	11	2.4.0	15	3.4.0	21	4.4.0	34	5.4.0	130
0.4.1	9.4	1.4.1	13	2.4.1	17	3.4.1	24	4.4.1	40	5.4.1	170
0.4.2	11	1.4.2	15	2.4.2	20	3.4.2	28	4.4.2	47	5.4.2	220
0.4.3	13	1.4.3	17	2.4.3	23	3.4.3	32	4.4.3	54	5.4.3	280
0.4.4	15	1.4.4	19	2.4.4	25	3.4.4	36	4.4.4	62	5.4.4	350
0.4.5	17	1.4.5	22	2.4.5	28	3.4.5	40	4.4.5	69	5.4.5	440
0.5.0	9.4	1.5.0	13	2.5.0	17	3.5.0	25	4.5.0	41	5.5.0	240
0.5.1	11	1.5.1	15	2.5.1	20	3.5.1	29	4.5.1	48	5.5.1	350
0.5.2	13	1.5.2	17	2.5.2	17	3.5.2	32	4.5.2	56	5.5.2	540
0.5.3	15	1.5.3	19	2.5.3	26	3.5.3	37	4.5.3	64	5.5.3	920
0.5.4	17	1.5.4	22	2.5.4	29	3.5.4	41	4.5.4	72	5.5.4	1600
0.5.5	19	1.5.5	24	2.5.5	32	3.5.5	45	4.5.5	81	5.5.5	›1600

Pos. = Number of Positive Tubes with each of 3 Volumes Used(10,1,0.1).

Table 6: Dilutions to be Used and Calculations of MPN per g or ml of Test Material.

	Dilutions*					Combination to be Used	MPN from Table	Dil. Factor of Middle dil.	MPN per g or ml
	Undiluted		1/100	1/100	1/1000				
	Amount of Original Material g (ml)								
	10	1	0.1	0.01	0.001				
a	5/5**	5/5	2/5	-	-	5.5.2	540	1	5.4
b		5/5	5/5	2/5	-	5.5.2	540	10	54
c		5/5	5/5	2/5	2/5	5.2.2	95	100	95
d		5/5	5/5	2/5	0/5	5.2.0	49	100	49
e		2/5	2/5	1/5	0/5	2.2.1	12	10	1.2
f		5/5	2/5	1/5	1/5	5.2.2	95	10	9.5
g		5/5	5/5	5/5	5/5	5.5.5	›1600	100	›1600

* ***Dilutions To Be Used are Undiluted***

** ***No. of Positive Tubes/No. of Tubes Inoculated***

Count of faecal streptococci:

If there is difficulty in interpreting the results of the presumptive coliform and confirmed *E. coli* tests, as when presumptive coliforms are present but *E. coli* is absent, a demonstration of the presence of faecal streptococci will confirm the faecal origin of the coliform bacilli. The term faecal streptococci refer only to those streptococcal species of Lancefield's group D which normally occur in human and animal faeces. These enterococci can grow in the media used in the presumptive coliform test, where they ferment the lactose with the production of acid but not gas.

1. Incubate tubes containing 5ml sterile glucose azide broth in a water bath thermostatically controlled at 44-45^{0}C. It should be noted that sodium azide is highly toxic and forms explosive compounds with metals. Exercise extreme care in preparing and discarding the medium.

2. When the tubes have warmed to the incubation temperature, seed them with heavy inocula from all the tubes in the presumptive coliform test that showed the formation of either acid and gas, or acid only. Immediately reincubate at 44-45^{0}C.

3. After incubation for 48h, inspect the cultures for acid production shown by a yellow colour change in the bromocresol purple in the medium. Those producing acid contain faecal streptococci.

4. Confirm the presence of faecal streptococci by subculturing each positive glucose azide culture onto a plate of bile aesculin azide agar incubated at 44-45^{0}C.

5. Examine the plates after a few hours of incubation for a brown-black colouration around the inoculum, which is evidence of hydrolysis of the aesculin and confirms the presence of faecal streptococci.

6. Refer to the tables of most probable numbers and from the proportion of tubes in the presumptive coliform test from which a confirmed streptococcal result was obtained, determine the most probable number of faecal streptococci in 100ml of the water sample as shown in Figure 6.

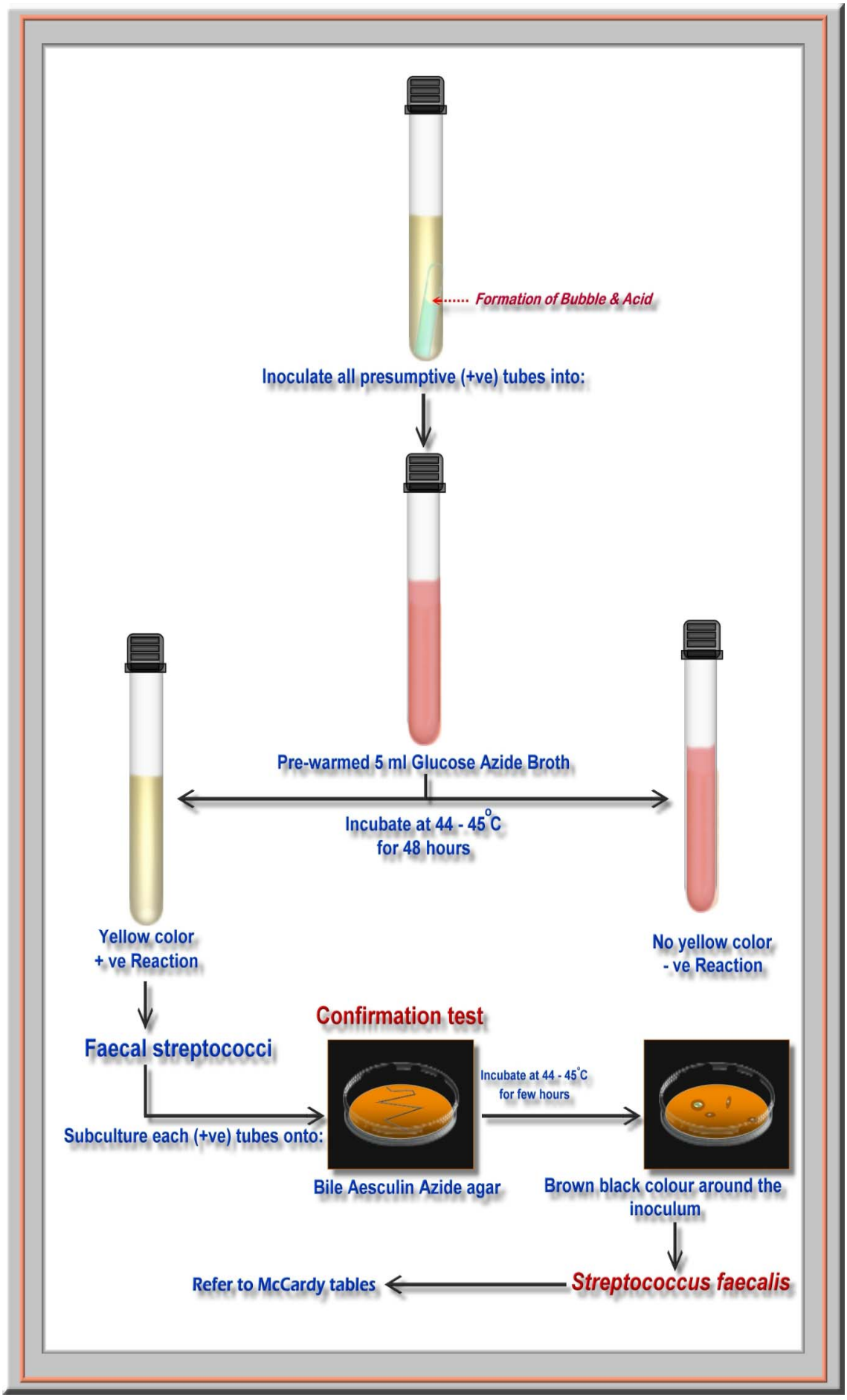
Formation of Bubble & Acid
Inoculate all presumptive (+ve) tubes into:
Pre-warmed 5 ml Glucose Azide Broth
Incubate at 44 - 45°C
for 48 hours
Yellow color
+ ve Reaction
No yellow color
- ve Reaction
Confirmation test
Faecal streptococci
Subculture each (+ve) tubes onto:
Bile Aesculin Azide agar
Incubate at 44 - 45°C
for few hours
Brown black colour around the
inoculum
Streptococcus faecalis
Refer to McCardy tables

Figure No.6

Count of *Clostridium perfringens*:

C. perfringens is a normal inhabitant of the intestine. Although present in a smaller numbers than *E. coli*, its spores can survive in water for long periods and persist when all other faecal bacteria have died. An examination for their presence in water is valuable in demonstrating remote or intermittent pollution, and in confirming the presence of faecal pollution when only coliforms other than *E. coli* are cultured from the water.

A count is made of the spores of sulphate reducing clostridia by first heating the water sample to kill all vegetative bacteria and then doing a multiple tube test by culturing it in differential reinforced clostridial medium (DRCM). This medium contains cysteine to permit the growth of anaerobic clostridia and a combination of sodium sulphate and ferric citrate to reveal any growth of sulphate reducing bacteria by the production of a black precipitate of ferrous sulphide.

1. Kill vegetative bacteria by heating the water sample in a waterbath for 10min at 75^{0}C. Control the heating by having beside the sample in the waterbath an equal volume of tap water and a thermometer in the same kind of container as the sample. Start timing the heating period when the thermometer in the tap water reaches 75^{0}C.

2. Aseptically add 50 ml of heated sample to a vessel containing 50 ml double strength of DRCM, five 10ml volumes of the sample to vessels containing 10ml double strength DRCM, and five 1ml volumes to vessels containing 25ml single strength DRCM. If the water may be highly polluted, 1ml volumes of a 1 in 10 and a 1 in 100 dilution of the sample should be cultured in other vessels containing 25ml single strength DRCM. The culture vessels should be stout screw-capped glass bottle of azide just sufficient to hold the water and the medium. If necessary, aseptically add single strength DRCM to the bottle until the level reaches the foot of the neck of the bottle; this arrangement minimizes the content of residual air.

3. Screw the caps on the bottles tightly and place the bottles in plastic bags to retain their contents should they explode during incubation. Incubate them for 48h at 37^{0}C.

4. After incubation, examine the bottles for blackening of the medium, a reaction that can be given by species of *Clostridium*. Refer to the probability tables and read from the combination of positive and negative results the most probable number of spores of sulphate reducing clostridia in 100ml of water sample.

5. Examine for the presence of *C. perfringens* in the positive, sulphate reducing cultures by subculturing a loopful from each such culture into a

separate tube of litmus milk medium that has been freshly steamed and cooled and to which a piece of iron wire or nail heated to redness has just been added. The litmus milk should be at least 70 mm deep. Incubate in air for up to 48h at 37^{0}C.

6. After 48h incubation, observe the tubes for production of "stormy clot" reaction, in which, through fermentation of the lactose, the milk is acidified and coagulated and clot is disrupted by bubbles of gas and often blown to the top of the tube. This reaction indicates that the inoculated cultures contained *C. perfringens*.

7. Refer to the probability tables and read from the combination of positive "stormy clot" results in litmus milk and negative results in both litmus milk and DRCM the most probable number of *C. perfringens* spores in 100ml of water sample as shown in Figure 7.

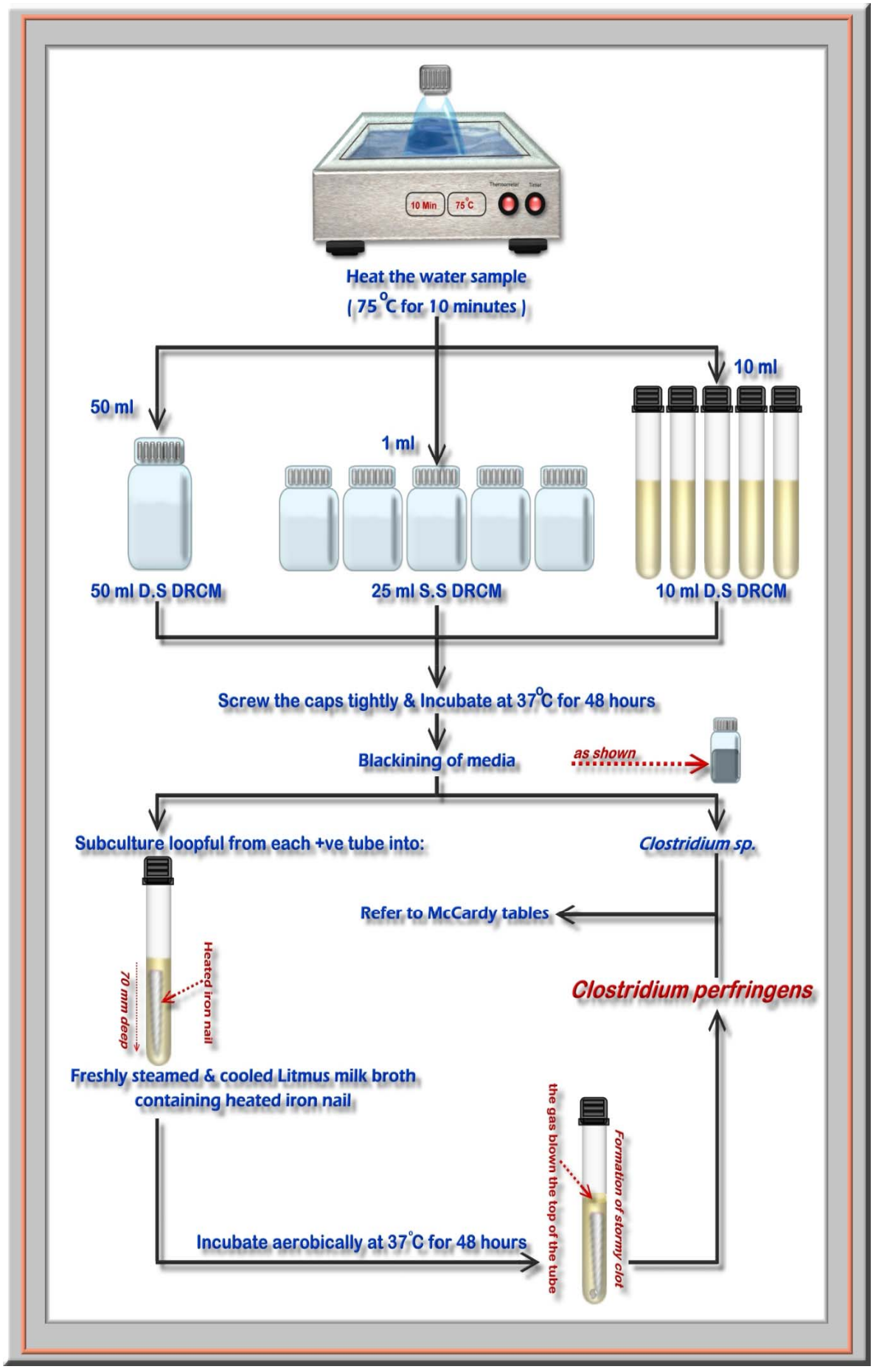
10 Min
75°C
Heat the water sample
(75°C for 10 minutes)
50 ml
1 ml
10 ml
50 ml D.S DRCM
25 ml S.S DRCM
10 ml D.S DRCM
Screw the caps tightly & Incubate at 37°C for 48 hours
Blackining of media
as shown
Subculture loopful from each +ve tube into:
Clostridium sp.
Refer to McCardy tables
Clostridium perfringens
70 mm deep
Heated iron nail
Freshly steamed & cooled Litmus milk broth
containing heated iron nail
the gas blown the top of the tube
Formation of stormy clot
Incubate aerobically at 37°C for 48 hours

Figure No.7

MEMBRANE FILTRATION TESTS

All of the gridded membranes are made from ccellulose nitrate, a material that assures excellent retention and optimum colony growth. The various colours allow the one to be chosen which gives the best contrast to the colonies that are to be counted.

The membrane filter of the appropriate pore size is placed in a filter holder, and the sample is filtered. In the process microorganisms in the test sample are retained on the filter surface by the screening action of the membrane filter. Growth inhibitors can be removed by flushing the holder with sterile water following filtration. Afterwards, the membrane filter is placed on a culture medium and incubated. Nutrients and metabolites are exchanged through the pore system of the membrane filter. Colonies, which have developed on the membrane filter surface during incubation, are counted and related to the sample volume as shown in Figure 8.

The advantages:

- Compared with the direct method, considerably larger sample volumes can be tested. This concentration effect increases the accuracy of microbial detection.
- The membrane filter with colony growth can be filed as a permanent record of the test.
- The visible colonies can be related directly to the sample volume. They give quantitative results.

Note: *To use* ***the Portable Membrane Filtration Unit*** *(See Page 39-41).*

Filtration procedure

1. The filtration apparatus consists of a base supporting a porous disk under a graduated funnel. Sterilize the apparatus and two extra funnels by autoclaving. Then connect the base to a vacuum source.

2. Procure from a commercial source filter membranes of high quality, control grade, 47mm in diameter and 0.45μm of pore size.

3. Remove the funnel from the apparatus and with flamed and cooled flat-ended forceps grasp the edge of a sterile membrane and place it, grid side up, over the porous disk. Replace the sterile funnel securely on the filter base.

4. While the vacuum is still turned off, pour or pipette the requisite volume of the water sample into the funnel. The volume should be chosen so that the colonies to be counted on the membrane will number between 10 and 100. For chlorinated waters, filter a 100ml volume. For unknown waters, filter a range of different volumes smaller than 10ml,

but add at least 20ml sterile water to the filter before addition of the sample to ensure dispersion of the bacteria over the membrane.

When filtering different volumes of the same sample, the funnel can be re-used without boiling between uses provided that the smallest volumes are filtered first. When filtering different samples, always sterilize the funnel in boiling water and allow it to cool between its uses for each sample. Always filter pure and chlorinated samples before samples known to be polluted.

5. Filter the sample slowly through the membrane by applying a vacuum of about 500 mm of mercury. Stop the evacuation as soon as the sample has been filtered so that as little contaminated air as possible is drawn through the membrane.

6. Remove the funnel and aseptically transfer the membrane, keeping its upper side upwards, onto a sterile paper pad saturated with selective indicator broth or onto a plate of selective indicator agar medium. Ensure that no air bubbles are trapped between the membrane and the medium.

The absorbent paper pads should be free from toxic substances, approximately 1mm thick and of at least as great a diameter as the membrane. They can be sterilized in foil-wrapped bundles by autoclaving for 20min at 121^{0}C. Place the pads in separate sterile Petri dishes before soaking with the special membrane medium. Pour off any excess medium from the saturated pad either before or after the membrane is in position so as to prevent the erroneous formation of confluent growth on the membrane.

7. Incubate the plates holding the membranes under appropriate conditions. Those with absorbent pads must be held in polythene bags or airtight tins containing wads of cotton-wool moistened with water to prevent the pads drying out.

8. After incubation, immediately count the characteristic colonies in a good light and with the aid of a magnifying glass. Express the result as the number of indicator bacteria per 100ml of water sample.

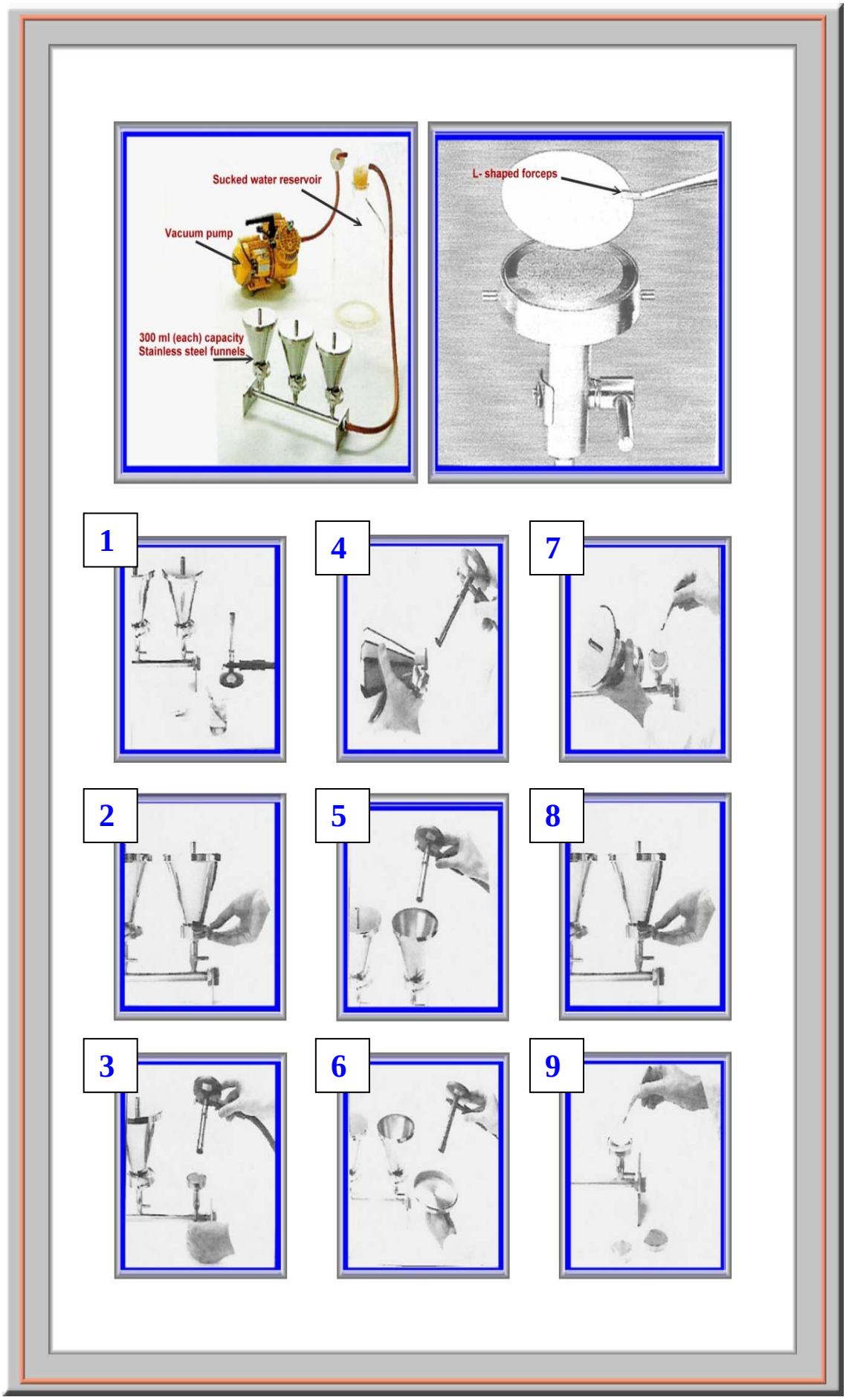
Sucked water reservoir
Vacuum pump
300 ml (each) capacity
Stainless steel funnels
L- shaped forceps
1
4
7
2
5
8
3
6
9

Figure No.8

Presumptive coliform count by filtration:

For these test the membrane are cultured on pads soaked with membrane lauryl sulphate broth, or equivalent medium (as shown in Table 7), which is inhibitory to many non-coliform bacteria and on which, because it contains lactose and phenol red, the coliforms grow as yellow-coloured colonies.

1. Filter the water sample as directed above.

2. Incubate the membranes on pads soaked with membrane lauryl sulphate broth first for 4h at 30^{0}C and then for 14 h at 37^{0}C .

3. Immediately on removal from the incubator, count all the yellow colonies irrespective of size and, with reference to the volume of water filtered, calculate the number of presumptive coliform bacteria per 100ml of sample.

Confirmed coliform and *E. coli* counts by filtration:

As some non-coliform bacteria that produce acid but not gas from lactose can form yellow colonies in the presumptive coliform test, the number of yellow colonies that are coliforms must be confirmed by the demonstration of gas formation at 37^{0}C and the number that are *E. coli* by the demonstration that they can form acid and gas from lactose at 44^{0}C and indole from tryptophan at 44^{0}C.

1. Subculture every yellow colony or a sufficient representative number of them, from the membranes cultured for the presumptive coliform count, each into two tubes of lactose peptone water with phenol red containing an inverted, medium filled Durham tube, and a tube of tryptone water.

2. Incubate one of the tubes of lactose peptone water at 37^{0}C and the other at 44^{0}C; after about 6h, subculture growth from the lactose peptone water incubated at 37^{0}C onto a plate of nutrient agar; incubate this plate at 37^{0}C and re-incubate the tube at 37^{0}C.

3. After 24h incubation; (a) do an oxidase test on colonies on the nutrient agar plate; (b) add a few drops of Kovac's (indole) reagent to the tryptone water culture and look for the development of a pink colour denoting indole formation; (c) inspect the lactose peptone water cultures for the formation of acid (yellow colour change) and gas, re-incubating those that are negative for re-examination after a further 24h at 37^{0}C.

Yellow colonies on membranes are confirmed as being coliform bacteria if they are oxidase negative and form acid and gas in lactose

peptone water incubated at 37^{0}C. The oxidase test is required to exclude those *Aeromonas* species that form acid and gas from lactose but are oxidase positive.

Yellow colonies on membranes are confirmed as being *E. coli* if they are oxidase negative, form acid, and gas in lactose peptone water incubated at 44^{0}C and form indole in tryptone water incubated at 44^{0}C.

4. From the results, calculate the confirmed coliform count and confirmed *E. coli* count per 100ml water sample.

Table7. Different types of membrane filtration media.

Type of Medium	Purposes	Description
Endo	**Selective medium for detecting *E. coli* & coliform bacteria.**	***E. coli* and coliform develop sharply contoured, dark red colonies with a dark red point on the underside of the membrane filter.**
Tergitol TTC	**Detection of coliform bacteria & *E. coli.***	**Coliform bacteria form red colonies. *E. coli* & *Enterobacter aerogenes* colonies are yellow to orange with a yellow zone. The medium prevents the *Proteus* colonies from swarming.**
Teepol	**Detection of *E. coli* & faecal coliform bacteria.**	**They form 1-2mm diameter yellow colonies surrounded by a yellow zone.**
M-FC	**Detection of *E. coli* & coliform bacteria.**	***E. coli* & coliform bacteria develop blue colonies with diameter of 1-2mm.**
ECD	**Selective culture medium for detecting and identifying *E. coli.***	**Colonies with light blue fluorescence in U.V light indicate *E. coli.***
MacConkey	**Isolation and differentiation of enterobacteria.**	***E. coli* forms large red or reddish colonies. Coliform bacteria form large pink coloured, sometimes slimy colonies, lactose (–ve) enterobacteria form colourless colonies.**
Azide	**Detection of enterococci**	**Enterococci forms red to reddish brown colonies with smooth peripheries.**
Bismuth-sulfite	**Selective culture medium for detecting salmonellae in water**	**Most *Salmonella* spp. form light coloured colonies with brown to black centres, surrounded by a black zone with a metallic sheen. Some *Salmonella* spp. develop uniformly dark brown to black colonies.**
Cetrimide	**Detection and determination of the CFU count of *Pseudomonas aeruginosa***	***Ps. aeruginosa* forms blue colonies with 1-2mm diameter and blue zones. Occasionally, the colonies can also be bluish-green, yellowish green or colourless. Other *Pseudomonas* sp. develops whitish colonies.**

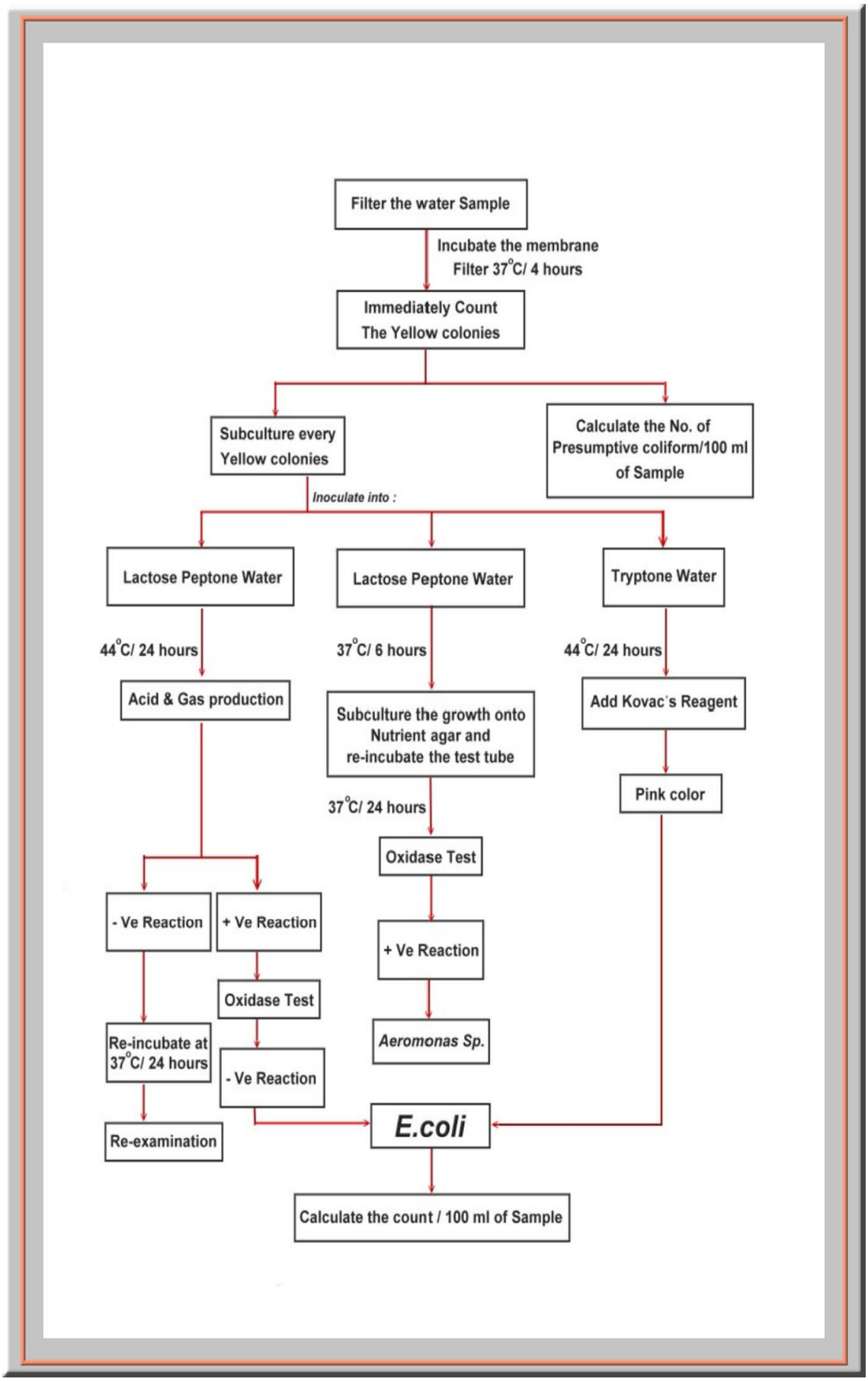
Filter the water Sample
Incubate the membrane Filter 37°C/ 4 hours
Immediately Count The Yellow colonies
Subculture every Yellow colonies
Calculate the No. of Presumptive coliform/100 ml of Sample
Inoculate into :
Lactose Peptone Water
Lactose Peptone Water
Tryptone Water
44°C/ 24 hours
37°C/ 6 hours
44°C/ 24 hours
Acid & Gas production
Subculture the growth onto Nutrient agar and re-incubate the test tube
Add Kovac's Reagent
Pink color
37°C/ 24 hours
Oxidase Test
- Ve Reaction
+ Ve Reaction
+ Ve Reaction
Oxidase Test
Re-incubate at 37°C/ 24 hours
Aeromonas Sp.
- Ve Reaction
Re-examination
E.coli
Calculate the count / 100 ml of Sample

Figure No.9

Faecal streptococci count by filtration:-

1. Filter the water sample as detailed above.

2. Place the membrane on surface-dried plate of membrane enterococcus agar and incubate for 48h at 37^{0}C.

3. Count all red, maroon, and pale pink colonies as being presumptive faecal streptococci.

4. Subculture these presumptive colonies onto slopes of bile aesculin azide agar and incubate for a few hours in a waterbath at 44-45^{0}C. Inspect the slopes for the development of a brown-black colour, which confirms the presence of faecal streptococci.

5. Calculate the number of confirmed faecal streptococci per 100ml of water sample.

Clostridium perfringens count by filtration:-

1. Kill vegetative bacteria by heating the water sample at 75^{0}C as described for the MPN test and, after cooling, filter an appropriate volume through a membrane.

2. Place the membrane face upwards either (a) on a surface-dried plate of membrane clostridial agar or, (b) on the base of a small Petri dish (ensuring no air bubbles are trapped) over which 18ml molten membrane clostridial agar cooled to 50^{0}C is carefully poured and allowed to set.

3. Incubate the plates in an anaerobic cabinet or jar at 37^{0}C.

4. After 24h and 48h of incubation, count all black colonies on the plates as being sulphate-reducing clostridia.

5. Subculture each black colony into a tube of freshly steamed and cooled litmus milk medium containing an iron nail as described for the MPN test. Incubate for 48h at 37^{0}C.

6. Count the colonies that produce a "stormy clot" as *C. perfringens* and calculate the count of confirmed *C. perfringens* per 100ml of water sample.

Portable Membrane Filtration Unit

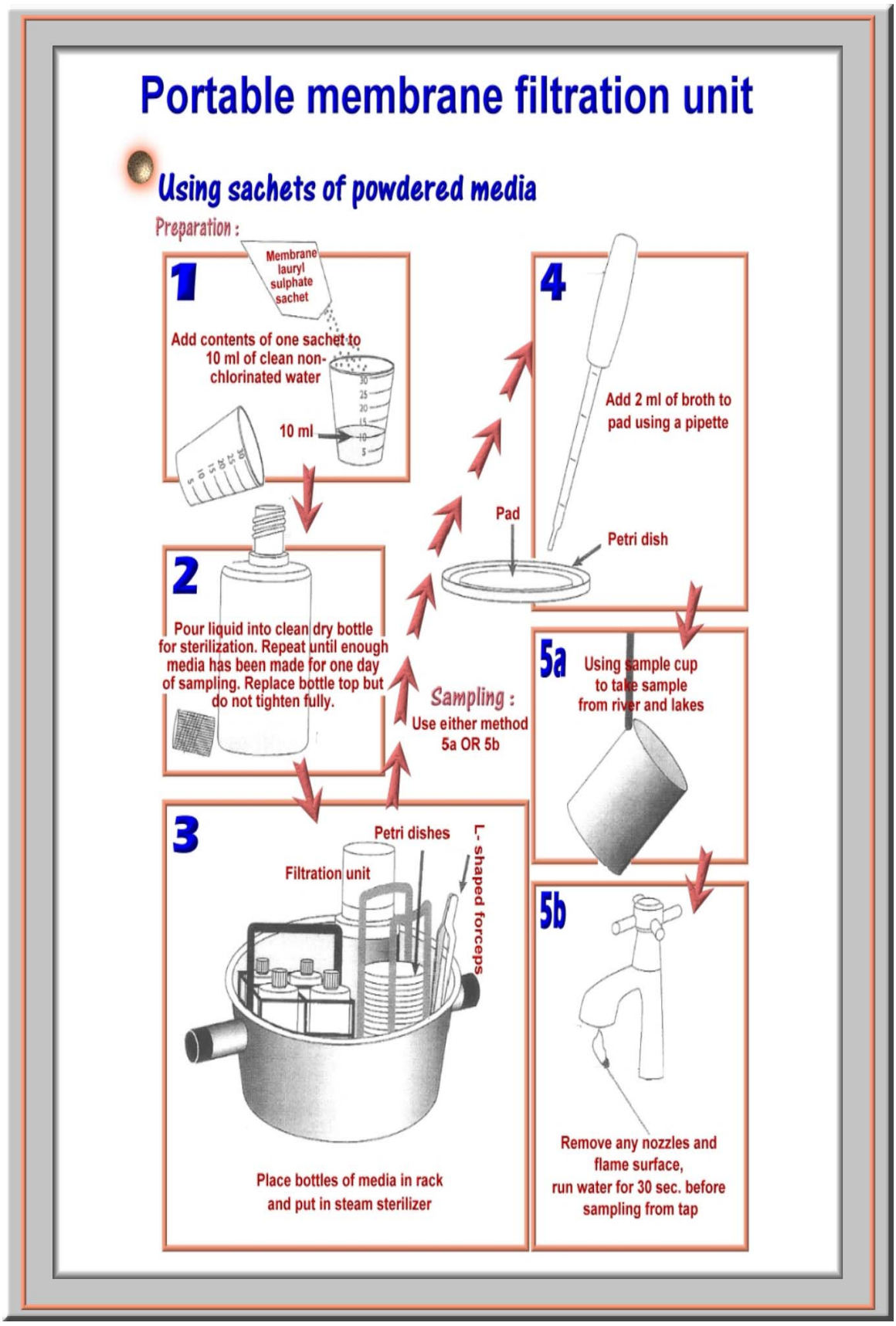

Figure.10

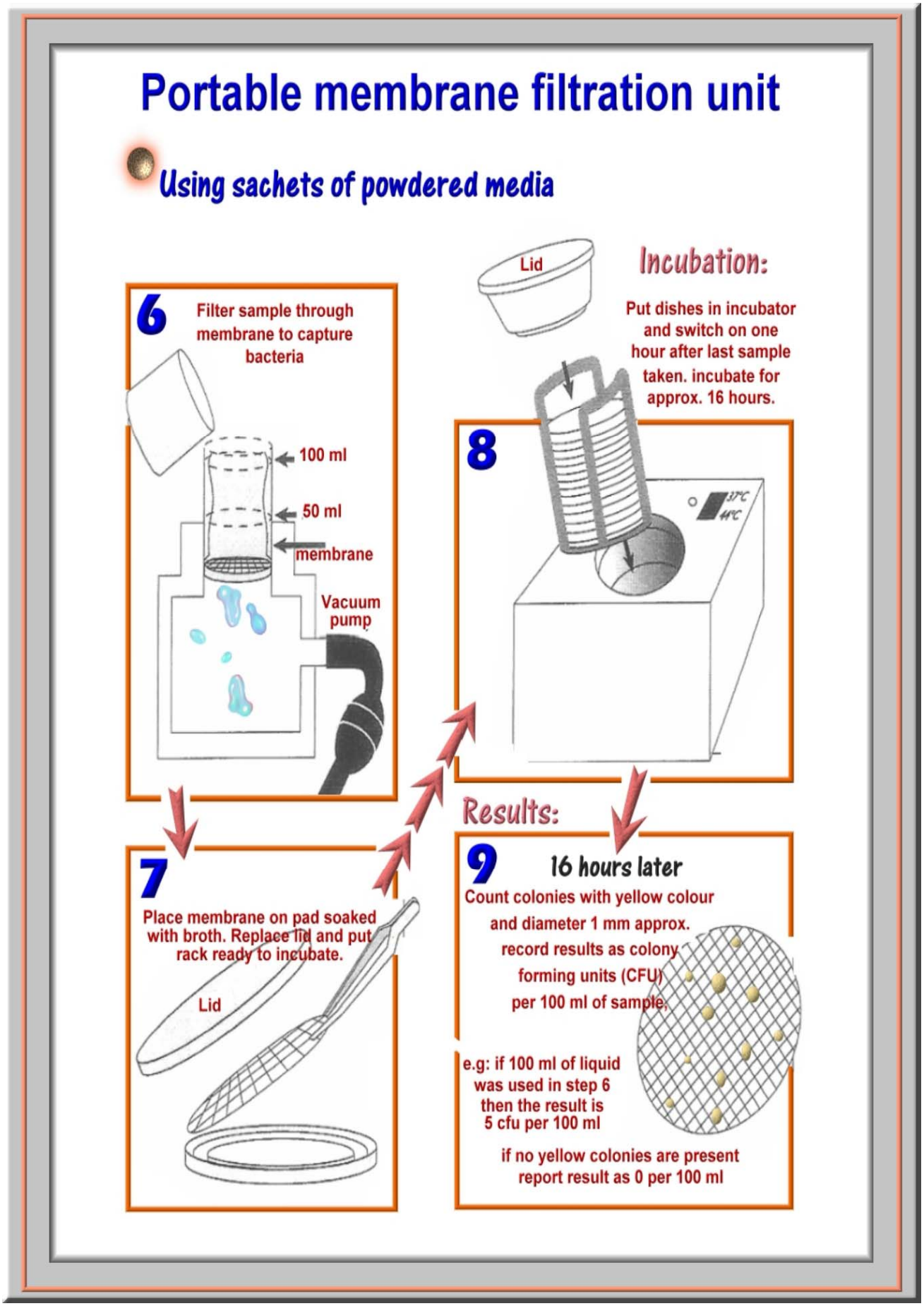
Portable membrane filtration unit
Using sachets of powdered media
6
Filter sample through membrane to capture bacteria
100 ml
50 ml
membrane
Vacuum pump
7
Place membrane on pad soaked with broth. Replace lid and put rack ready to incubate.
Lid
Lid
Incubation:
Put dishes in incubator and switch on one hour after last sample taken. incubate for approx. 16 hours.
8
37°C
44°C
Results:
9
16 hours later
Count colonies with yellow colour and diameter 1 mm approx. record results as colony forming units (CFU) per 100 ml of sample.
e.g: if 100 ml of liquid was used in step 6 then the result is 5 cfu per 100 ml
if no yellow colonies are present report result as 0 per 100 ml

Figure. 11

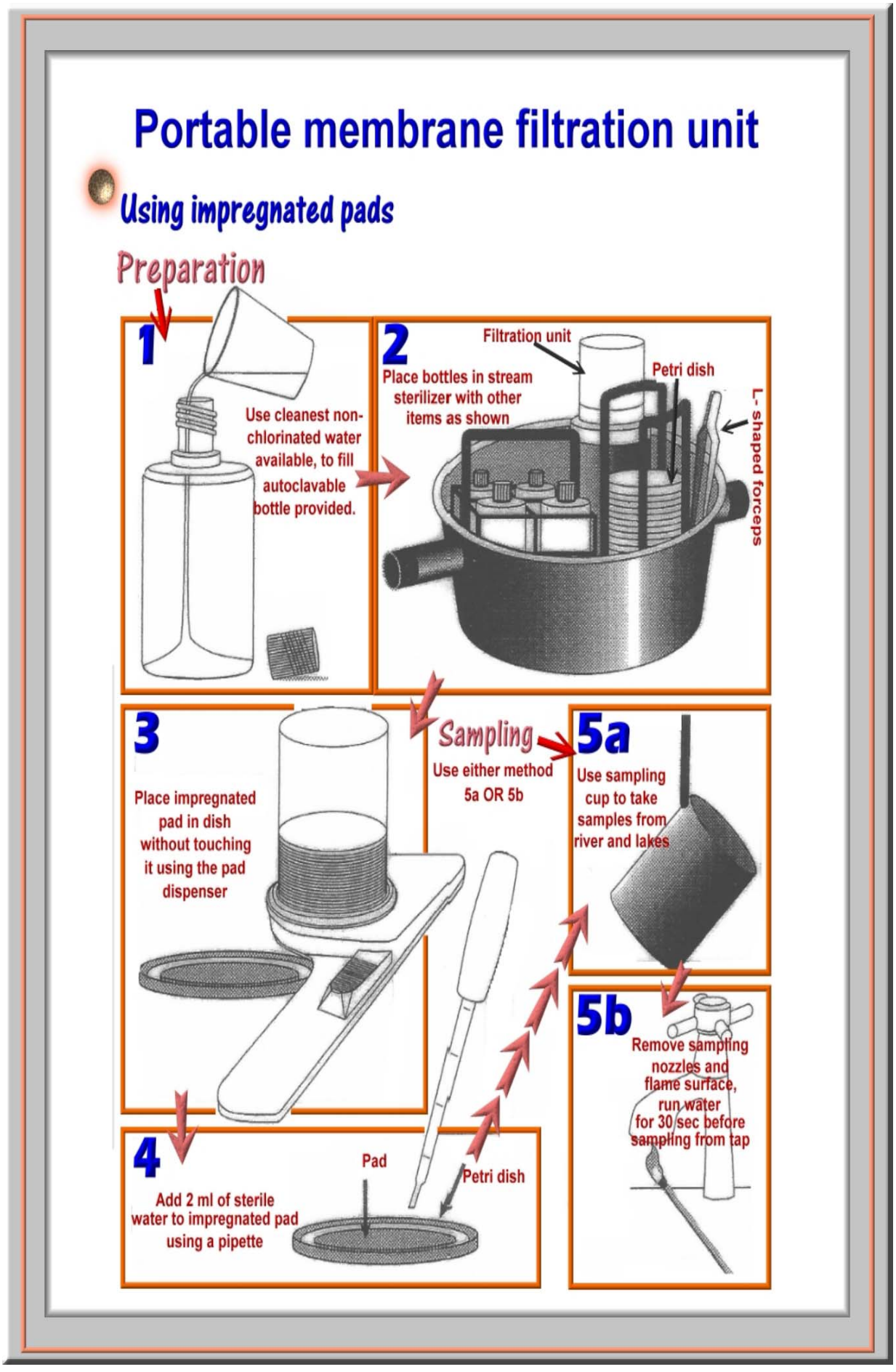
Portable membrane filtration unit
Using impregnated pads
Preparation
1
Use cleanest non-chlorinated water available, to fill autoclavable bottle provided.
2
Filtration unit
Petri dish
Place bottles in stream sterilizer with other items as shown
L- shaped forceps
3
Place impregnated pad in dish without touching it using the pad dispenser
Sampling
Use either method 5a OR 5b
5a
Use sampling cup to take samples from river and lakes
5b
Remove sampling nozzles and flame surface, run water for 30 sec before sampling from tap
4
Add 2 ml of sterile water to impregnated pad using a pipette
Pad
Petri dish

Figure. 12

Counting, Computing, and Reporting Results:

Optimal colony density per filter is 20 to 200. Report all colonies counted as colony-forming units (CFU)/ml. Include in the report the method used, the incubation temperature and time, and the medium. For example, 98 CFU/l, ml, 35°C, 24h, m-TGE broth.

* 1 to 2, or fewer colonies per square:

Count all of the colonies on the filter, and divide the results by the volume of original sample used. For example**,** if there are 122 colonies on the filter, and the volume of original sample used was 10ml, compute results as follows:

$$\frac{\text{122 colonies}}{\text{10 ml sample}} = 12.2 \text{ CFU/ml}$$

* 3 to 10 colonies per square:

Count all colonies in 10 representative squares and divide by 10 to obtain an average number of colonies per square. Multiply this number by 100 and divide by the volume of original sample used. For example**,** if you calculate an average of 8 colonies per square, and the volume of original sample used was 0,1ml, compute results as follows:

$$\frac{\text{8 colonies/square X 100}}{\text{0.1ml sample}} = 8000 \text{ CFU/ml}$$

* 10 to 20 colonies per square:

Count all colonies in 5 representative squares and divide by 5 to obtain an average number of colonies per square. Multiply this number by 100 and divide by the volume of original sample used. For example, if there are averages of 17 colonies per square, and the volume of original sample used was 0.1ml, compute results as follows:

$$\frac{\text{17 colonies/square X 100}}{\text{0.1ml sample}} = 17{,}000 \text{ CFU/ml}$$

* More than 20 colonies per square:

If there are more than 20 colonies per square, record the count as >2000 divided by the volume of original sample used. For example, if the

original volume of sample used was 0.01 ml, result would be >2000/0.01 or >200,000 CFU/ml.

Note: *Report average counts as estimated CFU/ml. Make estimated counts only when there are discrete, separated colonies without spreaders.*

Heterotrophic Plate Count

Microorganisms will normally grow in water and on surfaces in contact with water as biofilms. Growth following drinking water treatment is normally referred to as "regrowth" Growth is typically reflected in higher HPC values measured in water samples. Elevated HPC levels occur especially in stagnant parts of piped distribution systems, in domestic plumbing, in bottled water and in plumbed-in devices, such as softeners, carbon filters, and vending machines.

The principal determinants of regrowth are temperature, availability of nutrients and lack of residual disinfectant. Nutrients may derive from the water body and/or materials in contact with water.

Use of HPC in water management:

HPC testing has a long history of use in water microbiology. At the end of the 19th century, HPC tests were employed as indicators of the proper functioning of process (and of sand filtration in particular) and thereby as indirect indicators of water safety. Use as a safety indicator declined with the adoption of specific faecal indicator bacteria during the 20th century.

HPC measurements nevertheless continue to figure in water regulations or guidelines in many countries. HPC measurements are used:

- to indicate the effectiveness of water treatment processes, thus as an indirect indication of pathogen removal;
- as a measure of number of regrowth organisms that may or may not have a sanitary significance; and
- as a measure of possible interference with coliform measurements in lactose-based culture methods. This application is of declining value, as lactose-based culture media are being replaced by alternative methods that are lactose-free.

The plate count expresses the number of all colony-forming bacteria in 1 ml water. It is of limited value by itself, but as a supplementary test it provides information about the amount and type of organic matter in the water which may be useful in indicating the efficiency of the processes used for water treatment or the suitability of the water for large-scale production of food and drink.

Separate plate cultures are incubated at 20-22^{0}C and at 37^{0}C., that at 20-22^{0}C grows mainly the natural saprophytes of soil and water, whilst that at 37^{0}C grows mainly parasitic bacteria derived from human and

animal excretions. The true number of viable bacteria in the water will be in excess of the counts on these plates, for many of the microorganisms occur in clumps and many fail to grow under the cultural conditions used.

A. Pour plate technique

It is the procedure usually employed. Molten agar, cooled to 45^{0}C, is poured into Petri dish containing specified amount of diluted sample. Following addition of the molted agar, the cover is replaced, and the plate is gently rotated in a circular motion to achieve uniform distribution of microorganisms. This procedure is repeated for all dilutions to be plated. Dilutions should be plated in duplicate for greater accuracy, incubated overnight, and counted on a Quebec colony counter either by hand or by an electronically modified version of this instrument.

Plates suitable for counting must contain not fewer than 30 or more than 300 colonies. The total count of suspension is obtained by multiplying the number of cells per plate by the dilution factor, which is the reciprocal of the dilution.

Advantages:

1. Only viable cells counted.

2. It allows isolation of discrete colonies that can be subcultured into pure culture, which may then be easily identified.

Disadvantages:

1. Overnight incubation is necessary before colonies develop on the agar surface.

2. It is necessary to use more glassware in this procedure.

3. The need for greater manipulation may result in erroneous counts due to error in dilution or plating.

The procedure:

1. Melt sterile Plate count agar, cool it to, and maintain it at 50^{0}C until ready to pour plates.

2. Shake the bottle of water sample as described for the MPN test. Then aseptically prepare a series of 10-fold dilutions of the water by successively transferring 1ml volumes through a series of bottles holding 9ml sterile Peptone water as diluent. According to the count expected, one or two or, rarely, three dilutions will be required.

3. Starting with the highest (most dilute) dilution of the sample and working towards the undiluted sample, aseptically pipette exactly 1ml of the dilution or sample into each of four sterile Petri dishes.

4. To each plate add 15ml molten, cool (~50^{0}C) Plate Count Agar and immediately, and for a brief period, gently rotate the plate clockwise and then anticlockwise to mix the water with the medium. Then leave the agar to set. The interval between preparing the dilutions and mixing them with agar should not exceed 15min.

5. Incubate two of the four plates of each dilution for 3 days at 20-22^{0}C and the other two for 24h $\pm$ 3h at 37^{0}C.

6. Immediately after these periods of incubation, count the colonies on each plate. If there has to be any delay, meantime refrigerate the plates at 4^{0}C, but count them at least within 24h. For counting, use an illuminated colony counting apparatus fitted with a magnifying lens and an automatic count recorder. Select for counting the dilution plates that bear between 30 and 300 colonies. Multiply the mean count by the dilution to obtain the plate counts, expressed as the number of colonies per ml water sample after 3 days at 20-22^{0}C or 24h at 37^{0}C.

If there are less than 30 colonies on the plate from the undiluted sample, report the count as approximate only. If the plates from the highest dilution contain more than 300 colonies either try to count them and report the result as approximate or express the result as more than X colonies per ml. Calculating X as dilution factor multiplied by 300. (Figures 13 and 14).

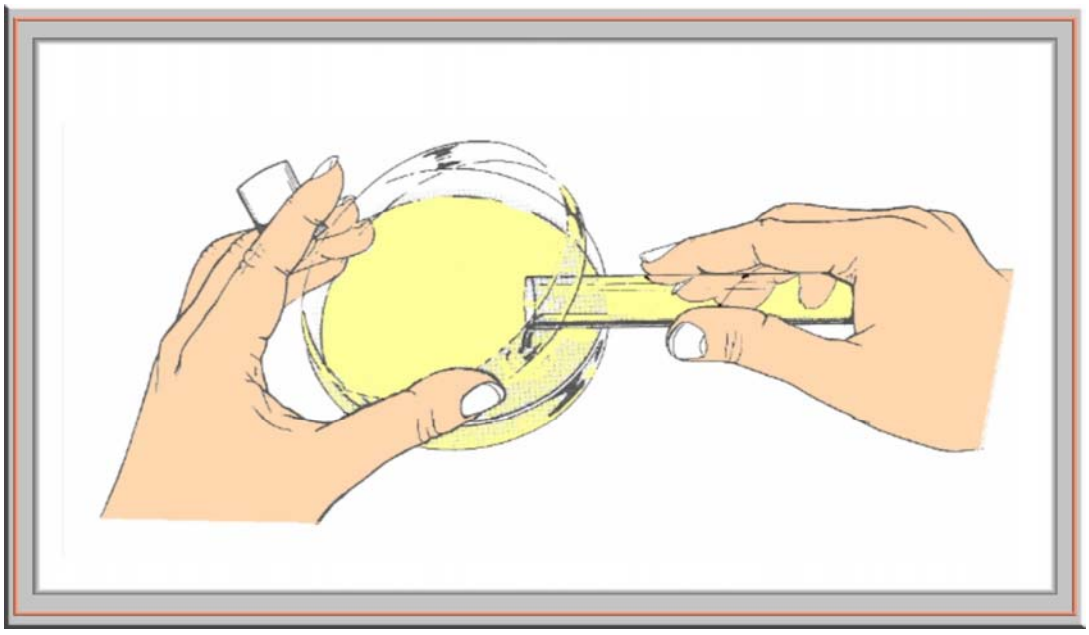

Figure. 13

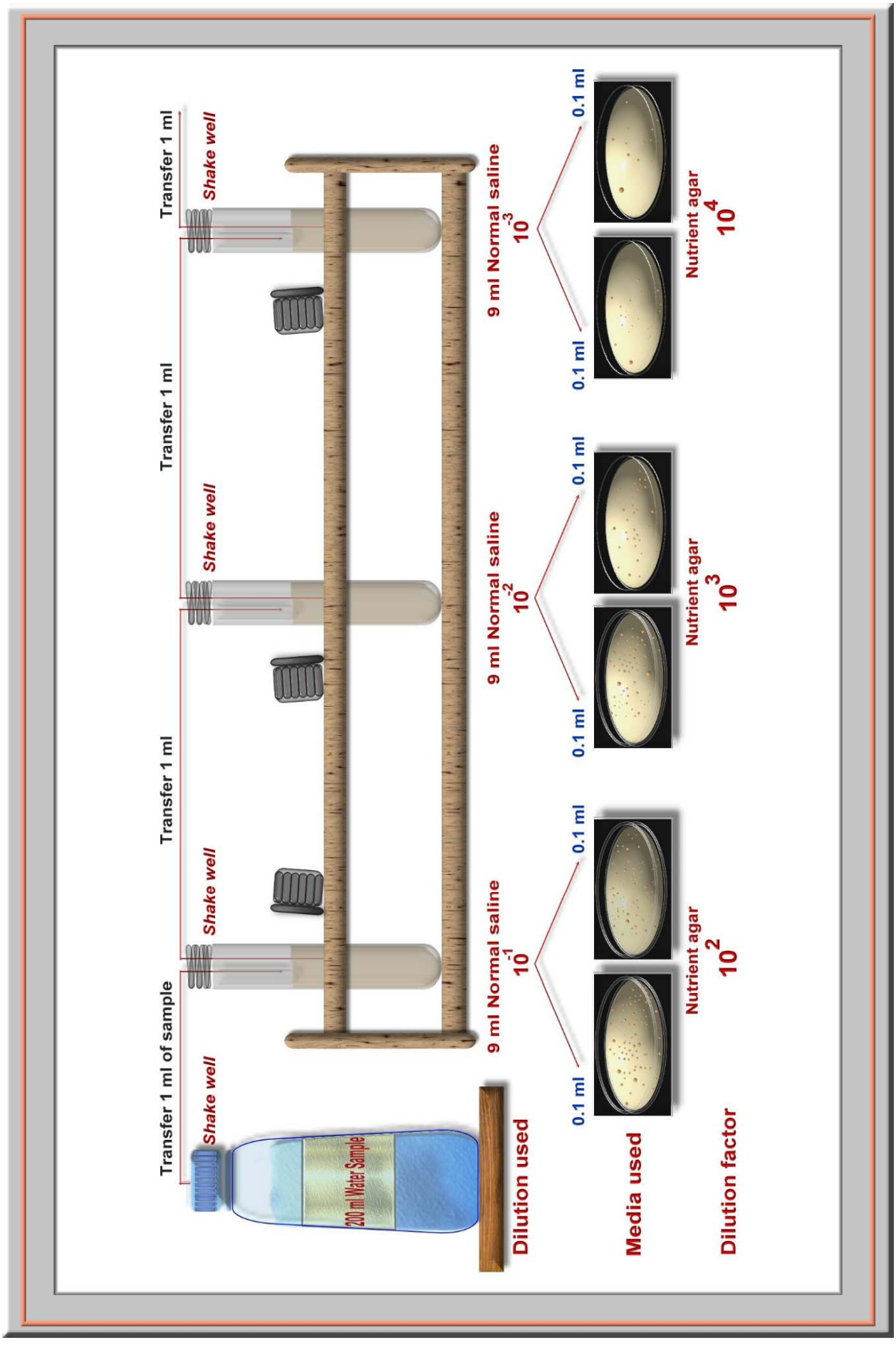
Transfer 1 ml of sample
Shake well
200 ml Water Sample
Transfer 1 ml
Shake well
9 ml Normal saline
10^{-1}
Transfer 1 ml
Shake well
9 ml Normal saline
10^{-2}
Transfer 1 ml
Shake well
9 ml Normal saline
10^{-3}
0.1 ml
0.1 ml
0.1 ml
0.1 ml
0.1 ml
0.1 ml
Nutrient agar
Nutrient agar
Nutrient agar
10^{2}
10^{3}
10^{4}
Dilution used
Media used
Dilution factor

Figure. 14

Interpreting and reporting results:

Count all colonies on selected plates promptly after incubation. If count must be delayed temporarily, store plates at 5-10^{0}C for no more than 24h, but avoid routine delays. Report all counts as colony-forming units (CFU)/ml. Include in the report the method used, the incubation temperature and time, and the medium. For example, 75CFU/ml, pour plate method, 35^{0}C/48h, plate count agar.

Generally, results are obtained by averaging the number of colonies on all plates from the same undiluted or diluted sample volume, and multiplying by a dilution (described below). In this case, results should be rounded to two significant digits to avoid creating false precision. For three-digit results raise the middle digit if the last digit is 5 or greater. Retain the middle digit if the last digit is 4 or smaller. The last digit will be zero. For example, 143 would become 140, 255 would become 260. Two digit numbers require no rounding.

Be familiar with the following terms before counting and reporting results:

* Average number of Colonies/Plate:

The average number of colonies per plate is derived by dividing the total number of colonies on all plates that were inoculated with the same sample volume or dilution volume, and dividing that sum by the number of plates used. For example, if two plates were each inoculated with 1.0 ml of diluted sample, and there were 89 colonies on one plate and 103 on the other, then the average number of colonies/plate would be:

$$\frac{89 \text{ colonies} + 103 \text{ colonies}}{2 \text{ plates}} = 96 \text{ colonies}$$

* Colony-Forming Units (CFU)/ml:

This is the unit used for reporting bacterial density. To derive the number of CFU/ml, multiply the average number of colonies/plate by the dilution factor of the incubated sample.

Note: *In some instances where a large number of colonies are observed, the average of colonies/plate is obtained by adding colonies counted only in a specified number of squares on each plate.*

* Dilution Factor:

The dilution factor is the reciprocal of the volume of original, undiluted sample plated, and is used to standardize the results according to the sample volume. For example, if 1.0ml of original sample was used, the dilution factor is 1.0. If 0.1ml of original sample was used, the dilution factor is 10.0. The dilution factor for 1ml of diluted sample (0.01ml of original sample) is 100, and the dilution factor for 0.1ml of diluted sample (0.001ml of original sample) is 1000.

* Representative Colony Distribution

When counting colonies in a specified number of squares (as seen through the colony counter), count those squares that appear to have an average number of colonies. Avoid counting squares that have many less or many more colonies than most of the other squares on the plate.

* Spreaders:

Are colonies of bacteria which grow in such a way that they appear to be (spread) across the plate as shown in Figure 15.

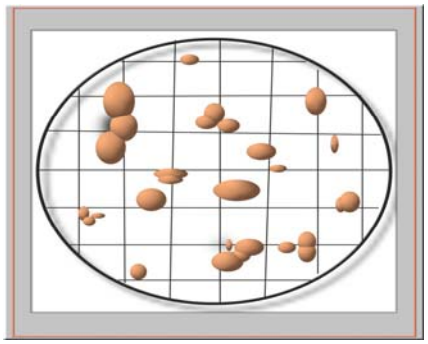

Figure.15

It is preferable when counting and recording results, to consider plates having between 30 and 300 colonies. However, this is not always the case, so when counting and recording colonies, choose the situation that best describes your results.

If spreader are encountered on the plates selected, count colonies on representative portions only when the colonies are well distributed in spreader-free areas, and the area covered by the spreaders does not exceed one-half of the plate area.

When spreading colonies must be counted, count each of the following types as one colony:

1. A chain of colonies that appears to be caused by disintegration of bacterial clumps as agar and sample were mixed.
2. A spreaders that develops as a film of growth between the agar and the bottom of the Petri dish.
3. A colony that forms in a film of water at the edge or over the agar surface.

Count as individual colonies the similar-appearing colonies growing in close proximity but not touching, provided that the distance between them is at least equal to the diameter of the smallest colony. Also count as individual colonies those colonies which are touching, but are different in appearance, such as morphology or colour.

To obtain results, multiply the **Average Number of Colonies/Plate** by the **Dilution Factor**, report counts as CFU/ml.

If plates have excessive spreader growth, report as (spreaders). When plates are uncountable because of missed dilution, accidental dropping, or contamination, or the control plates indicate that the medium or other material or lab ware was contaminated, report as "laboratory accident".

* No Colonies:

If plates from all dilutions of any sample have no colonies, repot the count as less than one (<1) times the dilution factor for the largest volume of original sample used. For example, if no colonies develop using 0.1 ml of original sample, report the count as less than 10 (<10) estimated CFU/ml.

Less Than 30 Colonies/Plate:

Ordinarily, no more than 1.0ml of sample is plated. Therefore, when the total number of colonies developing from 1.0ml is less than 30, record the number of colonies as CFU/ml.

* 30 to 300 Colonies/Plate:

Compute bacterial count per ml by multiplying the **Average Number of Colonies/Plate** by the **Dilution Factor**. Report counts as CFU/ml. For example, 0.1ml of undiluted sample was used to inoculate two plates. After incubation, the plates had colony counts of 115 and 145. The CFU/ml value is computed as follows:

$$\frac{115 \text{ colonies} + 145 \text{ colonies}}{2 \text{ plates}} \times 10 \text{ (dilution factor)} = 1300 \text{ CFU}$$

* Greater Than 300 Colonies/Plate:

If no plates have 30 to 300 colonies, and one or more plates have more than 300 colonies, use the plates having a count closest to 300 colonies. Compute the count by multiplying the **Average Number of Colonies/Plate** by the **Dilution Factor**, and report as estimated CFU/ml.

* More Than 300 Colonies/Plate:

If there are greater than 300 colonies/plate, ***do not*** report the results as "too numerous to count (TNTC)". Instead, follow these guidelines for reporting:

* Less Than 10 Colonies/cm2:

If there are fewer than 10 colonies per cm^2 (one "square" as seen through the colony counter), then count colonies in 13 squares having **Representative Colony Distribution**. If possible, select seven consecutive squares horizontally across the plate, and six consecutive squares vertically, being careful not to count a square more than once (Figure 16).

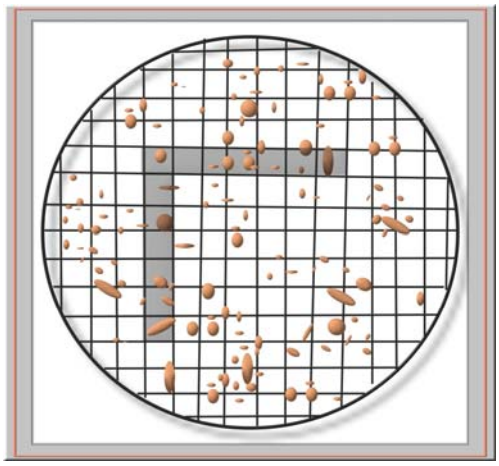

Figure.16

Add the number of colonies in each square. Multiply this sum by 4.38 when the plate area is $57cm^2$ (disposable plastic plates). Multiply this sum by 5 when the plate's area is $65cm^2$ (glass plates). To determine colony-forming units (CFU)/ml, compute the **Average Number of Colonies/Plate** and multiply the result by the dilution factor. Report as estimated CFU/ml.

* More Than 10 Colonies/cm2:

When there are more than 10 colonies per cm^2 (one "square" as seen through the colony counter), count colonies in four squares having

Representative Colony Distribution. Add the number of colonies in these four squares, and divide the sum by 4, to get the **Average Number of Colonies/Square**. Multiply this number by 57 when the plate area is 57cm^2 (disposable plastic plates). Multiply this number by 56 when the plate area is 65cm^2 (glass plates). To determine CFU/ml, compute the average number of colonies/plate and multiply the result by 1000 (see "**Note**" below). Report as estimated CFU/ml.

Note: *One thousand is the dilution factor for 0.1ml of diluted sample. This is the sample volume that should be used when bacterial counts are this high.*

Avoiding Errors:

Avoid inaccuracies in counting due to damaged or dirty optics that impair vision, or due to failure to recognize colonies. Be careful not to contaminate plates due to improper handling. Laboratory workers, who cannot duplicate their own counts on the same plate within 5% and counts of other analysts within 10%, should discover the cause and correct such disagreements.

B. Spread Plate Technique

A previously diluted mixture of micro-organisms is used, during inoculation; the cells are spread over the surface of a solid agar medium with a sterile, L-shaped bent rod while the Petri dish is spun on a turntable as shown in figure 17.

The procedure:

1. Place the bent glass rod into the beaker and add a sufficient amount of 95% ethyl alcohol to cover the lower, bent portion.

2. With a sterile pipette, place 1ml of fluid to be tested in the center of the appropriately labeled Nutrient agar plate that has been placed on the turntable and replace the cover.

3. Remove the glass rod from the beaker and pass it through the Bunsen burner flame. With the bent portion of the rod pointing downward, allow the alcohol to burn off the rod completely and cool the rod for 10 to 15 seconds.

4. Remove the Petri dish cover and spin the turn table.

5. While the turntable is spinning, lightly touch the sterile bent rod to the surface of the agar and move it back and forth. This will spread the culture over the agar surface.

6. When the turntable comes to a stop, replace the cover. Immerse the rob in alcohol and reflame.

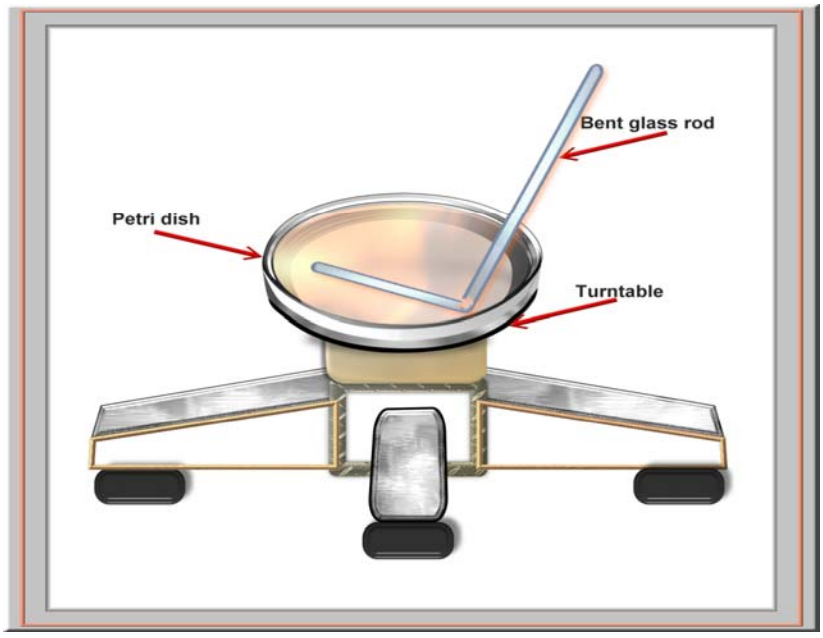

Figure.17

Culture Media

1-Bile aesculin azide agar:

- Peptone	10g
- Meat extract	10g
- Ox-bile purified and dehydrated	10g
- Sodium chloride	5g
- Aesculin	1g
- Sodium azide	0.15g
- Ferric ammonium citrate, green scales	0.5g
- Agar	10g
- Distilled water	1litre

Dissolve the ingredients in the water by heating at 100^{0}C. Adjust the pH to 7.0 and sterilize at 115^{0}C for 10 min. pour into plates and store at 4^{0}C in sealed containers to prevent drying.

2- Brilliant green lactose broth:

- Peptone	10g
- Lactose	10g
- Ox bile, purified and dehydrated	20g
- Brilliant green, 0.1 %(*w/v*) in water	13ml
- Distilled water	1litre

Dissolve the peptone in 500ml distilled water. Dissolve the Ox bile in 200ml distilled water; the pH should be between 7.0 and 7.5. Add the bile solution to the peptone solution, make up with distilled water to about 975ml, add the lactose, and adjust the pH to 7.4. Add the brilliant green and make up the volume to 1 litre. Distribute 5ml volumes in tubes containing inverted Durham tubes and autoclave at 115^{0}C for 10min.

3- Differential reinforced clostridial medium (DRCM):

Make up double strength medium as follows and dilute with an equal volume of distilled water to prepare single strength medium.

1. Basal medium:-

- Peptone	20g
- Meat extract	20g

- Sodium acetate, hydrated	10g
- Yeast extract	3g
- Soluble starch	2g
- Glucose	2g
- L (-) Cysteine hydrochloride	1g
- Distilled water	1litre

Prepare the basal double strength by adding the peptone, meat extract, sodium acetate, and yeast extract to 800ml of the water. Dissolve the starch in the remaining 200ml, first make it into slurry with a little water, then boiling the rest of the water and stirring it into the cold slurry. Add the glucose and cysteine, dissolve, and adjust to pH 7.1-7.2.

Distribute 10ml and 50ml volumes of double strength basal medium into, respectively, 28ml and 125ml screw capped bottles and 25ml volumes of the single strength basal medium into 28ml screw capped bottles. Autoclave at 121^{o}C for 15min.

Sodium sulphate and ferric ammonium citrate solutions:

Prepare solutions of sodium sulphate (anhydrous) 4% and ferric ammonium citrate (green scales) 7% in distilled water, heating the latter to dissolve. Sterilize by filtration. The solution may be stored at 4^{o}C for up to 14 days.

2. Final medium:

On the day of use, mix equal volumes of sodium sulphate and ferric ammonium citrate solutions. Freshly steam and cool the basal media to exclude dissolved oxygen. Aseptically add 0.4ml and 2.0ml, respectively, of the sulphate-iron mixture to each 10ml and 50ml volume of the double strength basal medium and 0.5ml to each 25ml volume of the single strength medium.

4- Glucose azide broth:

-Peptone	20g
-Sodium chloride	10g
- Di-potassium hydrogen phosphate	10g
- Potassium dihydrogen phosphate	4g
- Glucose	10g
- Yeast extract	6g
- Sodium azide	0.5g

- Bromocresol purple, 1.6 %(w/v) in ethanol 4ml
- Distilled water 1litre

Prepare double strength medium as follows and dilute with an equal volume of distilled water to prepare single strength medium.

Dissolve the ingredients in the water and adjust to pH6.6-6.8. distribute in tubes or bottles and autoclave at 115^{0}C for 10min. Note: Sodium azide is highly toxic if ingested or inhaled. Solutions containing it should not be discharged through metal pipe work.

5-Lactose peptone water with phenol:

- Peptone 10g
- Sodium chloride 5g
- Lactose 10g
- Phenol red, 0.4 %(w/v) in water 2.5ml
- Distilled water 1litre

Dissolve ingredients in the water and adjust to pH7.5. Add the phenol red and distribute in 5ml volumes in tubes with inverted Durham tubes. Autoclave at 110^{0}C for 10min. alternatively, steam at 100^{0}C for 10min on three successive days. Test for sterility by incubating at 37^{0}C for 24h.

6- Lauryl sulphate broth: (Lauryl tryptose broth)

- Tryptose 40g
- Lactose 10g
- Sodium chloride 10g
- Di-potassium hydrogen phosphate 5.5g
- Potassium dihydrogen phosphate 5.5g
- Sodium lauryl sulphate, pure(BDH 44244) 0.2g
- Distilled water 1litre

Add the tryptose, sodium chloride, lactose, and phosphates to the water and warm to dissolve. Add the sodium lauryl sulphate, mixing gently to avoid frothing. Adjust to pH6.8. Prepare single strength medium by diluting the double strength medium with an equal volume of distilled water. Distribute the double strength medium in 10ml and 50ml volumes and the single strength medium in 5ml volumes in tubes or bottles each containing an inverted Durham tube. Autoclave at 115^{0}C for 10min.

7- Litmus milk broth:-

Lactose fermenting bacteria in litmus milk form acid and cause it become pink, large amount of acid will precipitate the casein as a clot and if gas is formed during coagulation the clot will be disrupted by it (stormy clot). Proteolytic bacteria may decompose milk proteins to a transparent solution of soluble products. In litmus milk this shows as a change to a clear dark purple solution, usually taking several days and preceded by the formation of soft, easily disintegrated clot.

- ***Skimmed milk:-***

Steam fresh milk for 20min and allow it to stand for 24h in order that the cream may separate. Siphon the milk off from the cream.

- ***Litmus solution:-***

- Litmus granules	80g
- Ethanol, 40% aqueous	300ml
- Hydrochloric acid, HCl 1mol/l	q.s.

Grind up the granules, add to a flask containing 150ml aqueous ethanol and boil for 1min. decant and combine the two solutions, making the volume up to 300ml with 40% aqueous ethanol. Add hydrochloric acid drop by drop, shaking continuously until the solution becomes purple. To test for the correct reaction, boil a tube of a tap water and another of distilled water and add a drop of the solution to each. The tap water should be blue and the distilled water mauve.

- ***Preparation of complete medium:-***

Distribute in 5ml amounts in tubes or screw-capped bottles. Steam for 20min on three successive days, in bulk (e.g. 250ml) and add the litmus immediately before distribution.

8- MacConkey broth:-

- Peptone	20g
- Lactose	10g
- Bile salts	5g
- Sodium chloride	5g
- Neutral red	0.075g
- Distilled water	1litre

Sterilize at 121^{0}C for 15min, adjust the pH to 7.4 +/- 0.2.

9- Nutrient agar:-

- Lab-Lemco' powder 1g
- Yeast extract 2g
- Peptone 5g
- Sodium chloride 5g
- Agar 15g
- Distilled water 1litre

Sterilize at 121^{0}C for 15min adjust the pH to 7.4 +/- 0.2.

10- Tryptone water:-

- Tryptone 10g
- Sodium chloride 5g
- Distilled water 1litre

Sterilize at 121^{0}C for 15min, adjust the pH to 7.5 $^{+}$/. 0.2.

Reagents

1-Kovac's Reagent:

- *p*- Dimethylaminobenzaldehyde 2g
- Isoamyl alcohol 30ml
- Hydrochloric acid, conc. 10ml

Dissolve the dimethylaminobenzaldehyde in isoamyl alcohol, add conc. HCl, and mix well. Then transfer to a clear dark bottle, store it at 2-8^{0}C, renew monthly.

2- Oxidase reagent:-

Prepare fresh before use:

- Tetramethyl-*p*-phenylenediamine dihydrochloride 0.1g
- Distilled water 10ml

The reagent is not stable. It is therefore best prepared immediately before use. Some commercially available oxidase reagents are more stable and can be stored for several days at 2-8^{0}C.

Materials

The Most Probable Numbers Technique:

- Pipette: 1ml, 0.1ml, 5ml, and 10ml.
- Durham tubes.
- Screw capped test tubes.
- 200ml Capacity autoclavable container.
- Water bath (44 – 45 ^{0}C).
- Incubator (37 ^{0}C).
- Test tube rack.
- Rubber bulb.
- Inoculating loop.
- Bunsen burner.

Reagents:

- Kovac's reagent.

Culture media:

- MacConkey broth.
- Brilliant green lactose bile broth.
- Tryptone water.

Counting faecal *streptococcus*:

- Screw capped test tubes.
- Pipettes 5ml.
- Petri dishes.
- Inoculating loop.
- Incubator (44^{0}C).

Culture media:

- Glucose azide broth.
- Bile Aesculin azide agar.

Counting *Cl. Perfringens*:

- 50ml Flask capacity.

- Screw capped test tubes.
- Pipettes:- 1ml, 10ml and 25ml.
- Incubator.
- Iron nail.
- Incubator (37^{0}C).
- Water bath (75^{0}C).

Culture media:

- Differential reinforced clostridial medium (DRCM).
- Freshly steamed & cooled Litmus milk broth

Confirmation & counting of *E. coli* by Membrane Filtration Technique:

- Membrane filter (0.45Um).
- Complete membrane filtration unit.
- Petri dishes.
- Test tubes.
- Inoculating loop.
- Incubator (37^{0}C and 44^{0}C).

Reagents:

- Kovac's reagent.
- Oxidase reagent.

Culture media:

- Tryptone water.
- Lactose peptone water.
- Nutrient agar.
- MacConkey agar.

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- Bridosn EY. 1998. The Oxoid Manual (8th edn). England.
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- Collee JG., Duguid JP, Fraser AG. And Marmion BP. 1989 Practical Medical Microbiology (13th edn). Churchill Livingstone, Edinburgh.
- Cheesbrough M. 1984. Medical Laboratory Manual for Tropical Countries (1st edn). Tropical Laboratory Technology, England.
- Polyscience Publications. 1993. Microbiological Examination of Mineral Water.
- World Health Organization. 1996. Guidelines for Drinking Water Quality. WHO, Geneva.

Recommended web sites:

- www.cdc.gov
- www.eleint.co.uk
- www.epa.gov
- www.fda.gov
- www.hach.com
- www.hc-sc.gc.ca/food-aliment
- www.iso.com
- www.oxoid.co.uk
- www.sartorius.com
- www.who.int

Notes

Diarrhoea

- *1.8 million people die every year from diarrhoeal diseases (including cholera): 90% are children under 5, mostly in developing countries.*
- *88% of diarrhoeal diseases is attributed to unsafe drinking supply, inadequate sanitation and hygiene.*
- *Improved water supply reduces diarrhoea morbidity by 21%.*
- *Improved sanitation reduces diarrhoea morbidity by 37.5%.*
- *The simple act of washing hands at critical times can reduce the number of diarrhoeal cases up to 35%.*
- *Additional improvement of drinking water quality, such as point of use disinfection. Would lead to a reduction of diarrhoea episodes of 45%.*