LABORATORY MANUAL

ON

"BACTERIA OF PUBLIC HEALTH SIGNIFICANCE"

ICAR-CENTRAL INSTITUTE OF FISHERIES TECHNOLOGY

Willingdon Island, Matsyapuri P.O.,

Cochin – 682029 Kerala (INDIA)

LABORATORY MANUAL

ON

Bacteria of Public Health Significance
for M.Sc. students of Sacred Heart College, Cochin

(11 April - 18 April, 2023)

COURSE DIRECTORS

Dr. George Ninan

Dr. Femeena Hassan

COURSE CO-ORDINATORS

Dr. Pankaj Kishore

Dr. Devananda Uchoi

Dr. Ranjit K Nadella

Dr. Niladri S. Chatterjee

ICAR- CENTRAL INSTITUTE OF FISHERIES TECHNOLOGY

Willingdon Island, Matsyapuri P.O., Cochin – 682029

Ph: 091-0484-2412300, Fax: 091-484-2668212, E-mail: cift@ciftmail.org, directorcift@gmail.com

www.cift.res.in

2023

Published by: Director, ICAR-CIFT, Cochin- 682029

Compiled and edited by:

Dr. Devananda Uchoi

Dr. Pankaj Kishore

Dr. Ranjit Kumar Nadella

Dr. Niladri S. Chatterjee

Dr. Femeena Hassan

For citing the Training manual:

Uchoi, D., Nadella, R.K., Kishore, P., Chatterjee, N., Femeena, H. and Ninan, G. (eds.) (2023) Laboratory Manual on Bacteria of Public Health Significance. Central Institute of Fisheries Technology, Cochin, India. pp 101.

For citing the chapter

Pankaj Kishore, Devananda Uchoi and Ranjit Kumar Nadella. Determination of Total Plate Count. In: Uchoi, D., Nadella, R.K., Kishore, P., Chatterjee, N., Femeena, H. and Ninan, G. (eds.) (2023) Laboratory Manual on Bacteria of Public Health Significance. Central Institute of Fisheries Technology, Cochin, India. p. 1-6.

Design and Cover page: Dr. Devananda Uchoi

© 2023 ICAR- Central Institute of Fisheries Technology, Cochin

CONTENTS

Chapter No.	Topic	Page No.
1	Determination of Total Plate Count - *Pankaj Kishore Devananda Uchoi and Ranjit Kumar Nadella*	1 - 6
2	Most Probable Number (MPN) technique - *Devananda Uchoi, Pankaj Kishore and Ranjit Kumar Nadella*	7 - 15
3	Isolation and Identification of faecal coliforms - *Femeena Hassan, Devananda Uchoi, Pankaj Kishore and Ranjit Kumar Nadella*	16 - 19
4	Isolation and identification of *Vibrio* spp. - *Pankaj Kishore, Ranjit Kumar Nadella and Devananda Uchoi*	20 - 24
5	Conventional Identification techniques - *Devananda Uchoi, Pankaj Kishore and Ranjit Kumar Nadella*	25 - 28
6	Isolation and Identification of *Salmonella* spp. - *Ranjit Kumar Nadella, Devananda Uchoi and Pankaj Kishore*	29 - 36
7	Isolation and Identification of *Escherichia coli* - *Femeena Hassan, Pankaj Kishore, Ranjit Kumar Nadella and Devananda Uchoi*	37 - 42
8	Isolation and Identification of *Listeria monocytogenes* - *Pankaj Kishore, Ranjit Kumar Nadella and Devananda Uchoi*	43 - 47
9	Isolation and Identification of *Bacillus sp. and Clostridium sp.* - *Ranjit Kumar Nadella, Pankaj Kishore and Devananda Uchoi*	48 - 53
10	Isolation and Identification of *Yersinia enterocolitica* - *Pankaj Kishore, Ranjit Kumar Nadella and Devananda Uchoi,*	54 - 58
11	Isolation and Identification of *Shigella* spp. - *Ranjit Kumar Nadella, Pankaj Kishore and Devananda Uchoi*	59 - 62
12	Isolation and Identification of *Staphylococcus aureus* - *Devananda Uchoi, Ranjit Kumar Nadella and Pankaj Kishore*	63 - 66
13	Molecular detection Techniques for Identification of Bacterial Pathogens - *Pankaj Kishore Ranjit Kumar Nadella and Devananda Uchoi*	67 - 70

14	Spectral Fingerprinting of Pathogenic Bacteria by Fourier Transform Infrared Spectroscopy (FTIR) and Chemometric Models for Rapid Identification - *Niladri Sekhar Chatterjee, Toms C. Joseph, Minimol V.A.*	71 - 81
15	Automated Microbial Detection Techniques in Food Microbiology - *Ranjit Kumar Nadella, Pankaj Kishore and Devananda Uchoi*	82 - 93
16	Glossary	94 - 101

CHAPTER 1

Determination of Total Plate Count

Pankaj Kishore, Devananda Uchoi and Ranjit Kumar Nadella

Email: pkishore2007@gmail.com

Introduction:

Total Plate Count (TPC) is the enumeration of aerobic, mesophillic organisms that grow in aerobic conditions under moderate temperatures of 20-45°C or TPC provides an estimate of the total number of aerobic microorganisms in foods including fish and fishery products. This method is used to determine the level of aerobic bacteria, yeast, molds and fungi. This count includes all pathogens and non-pathogens and is used to determine the hygienic status and quality of food. TPC is also called as Aerobic plate count (APC) or total viable count (TVC). This method is useful in determining spoilage/ deterioration of the perishable commodities like seafoods. This is an indicator of the sanitary conditions under which the food has been processed and/or produced and also of the level of Good Manufacturing Practices (GMPs) adopted during processing. It is enumerated by serial dilution of food homogenate and plating on general purpose media such as Plate count agar (PCA) or other non-selective agar medium and further incubation as per the required time temperature of the method used. All the colonies developing on the medium are counted and bacterial load is expressed as colony forming units (CFU) per gram of sample.

Equipment and materials

1. Phosphate buffer or Physiological saline (0.85%) or 0.1% Peptone water
2. Biosafety cabinet/Laminar air flow
3. Petri dishes, glass or plastic (at least 15 × 90 mm)
4. Micropipettes of 1, 5, and 10 ml
5. Dilution bottles borosilicate-resistant glass, with rubber stoppers or plastic screw caps
6. Pipet and petri dish containers, adequate for protection
7. Circulating water bath, for tempering agar, thermostatically controlled
8. Incubator
9. Colony counter, dark-field, Quebec, or equivalent, with suitable light source
10. Plate count agar
11. Refrigerator, to cool and maintain samples at 0-5°C, if needed
12. Freezer, to maintain frozen samples from -15 to -20°C

Procedure

About 25 g of fish muscle is weighed aseptically and homogenised with 225 ml physiological saline in a homogeniser / blender. Serial decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4} and others as appropriate depending on the type of sample are prepared with 9 ml physiological saline. 0.1 ml of inoculum from each of the serial dilution is poured onto Plate Count Agar (PCA) plates and spread using sterile glass spreaders for spread plate technique. But, in the case of pour plate technique, 1 ml of inoculum is placed into the petriplates and the sterile cooled (50°C) media is poured over it and mixed by gently swirling in clockwise and anticlockwise direction. The plates are incubated at 37 $\pm 1^{0}$C for 48 h. All the colonies developed on the agar plates are then counted and counts per gram of sample calculated. It is advisable to choose dilutions which give colonies between 30 – 300 ranges. Plates having crowded or spreading colonies which cannot be counted shall be discarded.

Calculation

Pour Plate Technique

Aerobic Plate Count (cfu/g) = (Number of colonies x Dilution) / Weight of sample

Spread Plate Technique

Aerobic Plate Count (CFU / g of sample) = (Number of colonies x Dilution x 10) / Weight of sample

Phosphate Buffer Solution

Stock solution

Potassium dihydrogen orthophosphate (KH_2PO_4) - 34.0 g

Distilled water - 1000.0 ml

Working solution

Dilute 1.25 ml of stock buffer solution to 1000 ml with distilled water. Adjust the pH to 7.2 before use. Sterilize at 121°C for 15 minutes.

Media composition

Nutrient Agar (NA)

Peptone	-	5.0 g
NaCl	-	5.0 g
Beef Extract	-	1.5 g
Yeast Extract	-	1.5 g
Agar	-	15.0 g
Distilled water	-	1000 ml
pH	-	7.4 ± 0.8

Sterilize at 121°C for 15 minutes.

Plate Count Agar (PCA) or Standard Methods Agar

Tryptone	-	5.0 g
Yeast Extract	-	2.5 g
Dextrose	-	1.0 g
Agar	-	15.0 g
Distilled water	-	1000 ml
pH	-	7.0 ± 0.2

Sterilize at 121°C for 15 minutes.

Soyabean Casein Digest Agar (Trypticase Soy Agar, TSA)

Tryptone	-	17.0 g
Soya peptone	-	3.0 g
NaCl	-	5.0 g
Dipotassium phosphate	-	2.5g
Agar	-	15.0 g
Distilled water	-	1000 ml
pH	-	7.3 ± 0.2

Sterilize at 121ºC for 15 minutes.

Rules for enumerating counts

- The suitable colony counting range is **25-250**/30-300. 15-150 for yeast and molds.
- Always choose replicate plates containing colonies.
- count all colony forming units (cfu) including those of pinpoint size.
- Record the volume and dilution used for plating, and average number of colonies counted.

  Example:

Dilutions	10^{-1}	10^{-2}	10^{-3}
Colonies counted	TNTC	182	45
	TNTC	165	39

TNTC- Too Numerous to count

Since only 10^{-2} dilution shows colonies between 30 and 300, only this pair of plates is considered and average counts determined.

Average number of colonies x dilution factor

CFU/ml = ---

Volume of sample plated

Average number of colonies in 10^{-2} = 80 + 70 ÷ 2 = 75

Volume of sample plated: 1 ml

CPU/ml = $(75 \times 10^2) = 7.5 \times 10^3$

Result reported as = 7.5×10^3 CFU/ML

2. If there are 2 consequetive dilutions showing between 30-300 colonies then the count in each of the dilutions computed. If the count of one dilution is not more than double the other, then the average of both dilutions taken and results reported.

   Example:

   Counts in 10^{-2} dilution : 280 and 290 colonies. CFU/ML = 2.85×10^4

   10^{-3} dilution: 40 and 44 colonies. CFU/ML = 4.30×10^4

   Since 4.30 is not more than double of 2.85, the average is considered

   $(2.85 + 4.30) \div 2 = 3.47$)

   Result is report as : 3.57×10^4 CFU/ML

3. If there are 2 consequetive dilutions showing between 30-300 colonies, and if the count of one dilution is more than double than the other then the lower value is reported.

   Example:

   Counts in 10^{-2} dilution: 280 and 290 colonies. CFU/ML = 2.85×10^4

   10^{-3} dilution: 80 and 70 colonies. CFU/ML = 7.50×10^4

   Since 7.50 is more than double of 2.85, the lower value is considered.

   Result is reported as : 2.85×10^4 CFU /ML

4. If there are no colonies in any of the dilutions, then the counts is reported as less than one times the lowest dilution plated.

   Example:

   10^{-1} dilution: No colonies

   10^{-2} dilution: No colonies

   Results reported as: **Estimated count** $< 1\times10^1$ CFU/ML

   or Est. $< 1\times10^1$ CFU/ML

5. If all the dilutions show more than 300 colonies, then the dilution where the colonies are countable is considered. Then all the colonies in that dilution counted and the results computed as **Estimated count.**

   Example:

   10^{-1} dilution : Too numerous colonies to count

10^{-2} dilution: Too numerous to count

10^{-3} dilution : 450 and 460 colonies (Average: 455 colonie)

Results reported as : **Estimated count** 4.55×10^5 CFU/ML

6. If there are too numerous or too many colonies making the counting difficult, then results can be expressed by following one of the following ways.

   Count all the colonies in 13 square centimeters using a colony counter and multiply the counts by 5. This number should be multiplied by the dilution factor used for plating to report as estimated count.

   OR

   Count all colonies in in 5 square centimeters using a colony counter and multiply by 13. This number should be multiplied by the dilution factor used to report the Estimated count.

   OR

   If the number of colonies is more than 100 per sq. cm, then count all colonies in one sq.cm and multiply it by 64. This number should be multiplied by the dilution factor to report the Estimated count.

   (*Multiplying by 5 or 13 depends on the area of the petridish used. The standard petridish of diameter 9 cm has an area of approximately 65 sq. cm)*

References

- IS 5402 : 2012 (Indian Standard MICROBIOLOGY OF FOOD AND ANIMAL FEEDING STUFFS — HORIZONTAL METHOD FOR THE ENUMERATION OF MICRO-ORGANISMS — COLONY-COUNT TECHNIQUE AT 30°C)/ISO 4833 : 2003 (‘MICROBIOLOGY OF FOOD AND ANIMAL FEEDING STUFFS — HORIZONTAL METHOD FOR THE ENUMERATION OF MICROORGANISMS — COLONY-COUNT TECHNIQUE AT 30 °C’)
- ISO 6887-3 : 2003 Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products
- Refer: http://ecoursesonline.iasri.res.in/mod/page/view.php?id=88202

CHAPTER 2

Most Probable Number (MPN) Technique

Devananda Uchoi, Pankaj Kishore and Ranjit Kumar Nadella

Email: uchoidev514@gmail.com

Introduction

Most Probable Number (MPN) is a method used to estimate the concentration of viable microorganisms in a sample by means of replicate liquid broth growth in ten-fold dilutions. It is commonly used in estimating microbial populations in soils, waters, agricultural products and is particularly useful with samples that contain particulate material that interferes with plate count enumeration methods. Most probable number method is used when there is a chance of occurrence of low bacteria no. in the sample. MPN is only a statistical approximation of the test bacteria in the given sample and not the actual no. It is called dilution extinction technique. In this technique liquid medium is used for the enumeration of bacteria.

Principle

Food or Water sample to be tested is diluted serially and inoculated in lactose broth. Coliforms if present in sample utilize the lactose present in the medium to produce acid and gas. The presence of acid is indicated by colour change of the medium and the presence of gas is detected as gas bubbles collected in the inverted Durham's tube present in the medium. The number of total coliforms is determined by counting the number of tubes giving positive reaction (i.e. both colour change and gas production) and comparing the pattern of positive results (the number of tubes showing growth at each dilution) with standard statistical tables.

Importance of MPN Method

MPN is most commonly applied for quality testing of water i.e., to ensure whether the water is safe or not in terms of bacteria present in it. The presence of very few faecal coliform bacteria would indicate that a water probably contains no disease- causing organisms. While the presence of large numbers of faecal coliform bacteria would indicate a very high probability that the water could contain disease - producing organisms making the water unsafe for consumption.

Equipment and materials

- Covered water bath, with circulating system to maintain temperature of 44.5 ± 0.2°C. The temperature for water baths for the shellfish program is 44.5°C ± 0.2°C. Water level should be above the medium in immersed tubes.
- Immersion-type thermometer, 1-55°C, about 55 cm long, with 0.1°C subdivisions, certified by National Institute of Standards and Technology (NIST), or equivalent Incubator, 35 ± 0.5°C.
- Balance with capacity of >2 kg and sensitivity of 0.1 g
- Blender and blender jar
- Sterile graduated pipets, 1.0 and 10.0 mL
- Sterile utensils for sample handling
- Dilution bottles made of borosilicate glass, with polyethylene screw caps equipped with Teflon liners.
- Quebec colony counter, or equivalent, with magnifying lens
- Longwave UV light [~365 nm], not to exceed 6 W.
- pH meter

Media and Reagents

- Brilliant green lactose bile (BGLB) broth, 2%
- Lauryl tryptose (LST) broth
- Lactose Broth
- EC broth
- Levine's eosin-methylene blue (L-EMB) agar
- Tryptone (tryptophane) broth
- MR-VP broth
- Koser's citrate broth
- Plate count agar (PCA) (standard methods)
- Butterfield's phosphate-buffered water or equivalent diluent
- Kovacs' reagent
- Voges-Proskauer (VP) reagents
- Gram stain reagents
- Methyl red indicator
- Violet red bile agar (VRBA)

- VRBA-MUG agar
- EC-MUG medium
- Lauryl tryptose MUG (LST-MUG) broth
- Peptone Diluent, 0.5%

Three steps in MPN test

1. Presumptive test.
2. Confirmatory test.
3. Completed test

1. **Presumptive test**

It is a screening test to sample water for the presence of *coliforms* organisms. If the presumptive test is negative, no further testing is performed, and the water source is considered microbiologically safe. If, however, any tube in the series shows acid and gas, the water is considered unsafe and the confirmed test is performed on the tube displaying a positive reaction.

The method of presumptive test varies for treated and untreated water.

***Requirements*:**

- Medium: Lactose broth or Mac Conkey Broth or Lauryl tryptose (lactose) broth
- Glassware: Test tubes, Durham tube
- Others: Sterile pipettes

***Preparation of the Medium*:**

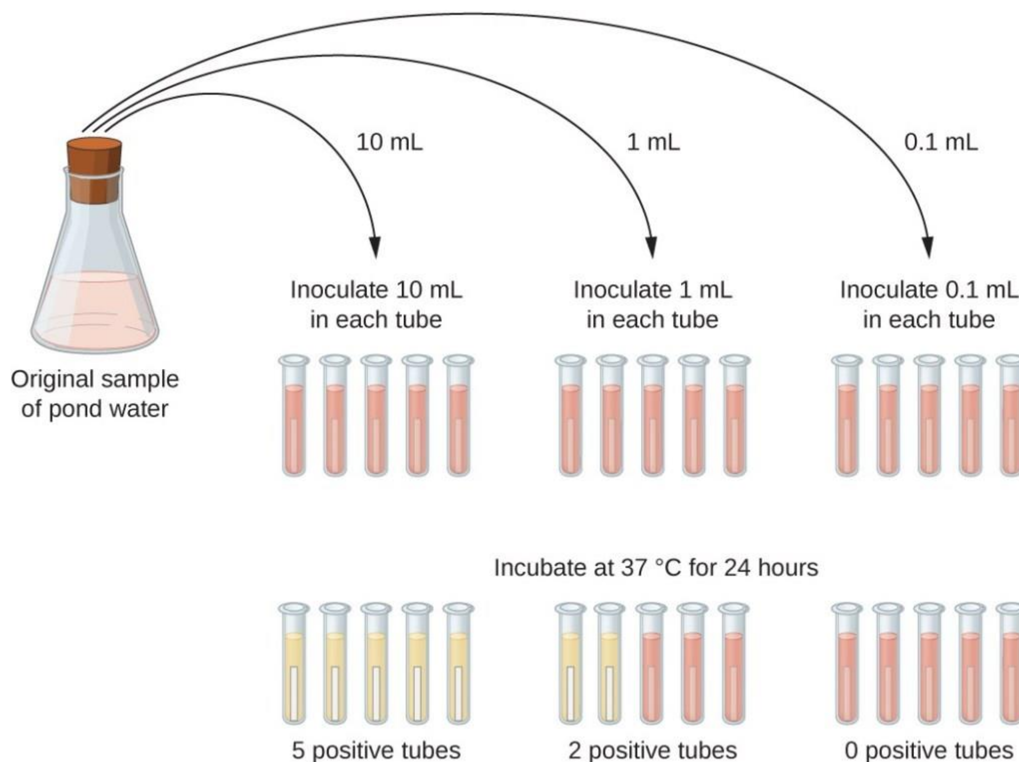
Prepare medium (either mac Conkey broth or Lactose broth) in single and double strength concentration.

<u>For untreated or polluted water:</u>

Dispense the double strength medium in 10 tubes (10 ml in each tube) and single strength medium in 5 tubes (10 ml in each tube) and add a Durham's tube in inverted position.

<u>For treated water:</u>

Dispense the double strength medium in 5 tubes (10ml in each tube) and 50 ml single strength medium in 1 bottle and add a Durham's tube in inverted position. Examine the tubes to make sure that the inner vial is full of liquid with no air bubbles. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Take 5 tubes of double strength and 10 tubes of single strength for each water sample to be tested.



Using a sterile pipette add 10 ml of water to 5 tubes containing 10 ml double strength medium. Similarly add 1 ml of water to 5 tubes containing 10 ml double strength medium and 0.1 ml water to remaining 5 tubes containing 10 ml double strength medium. Incubate all the tubes at 37°C for 24 hrs. If no tubes appear positive re-incubate up to 48 hrs. Compare the number of tubes giving positive reaction to a standard chart and record the number of bacteria present in it. *For example: a water sample tested shows a result of 3–2–1 (3 × 10 ml positive, 2 × 1 ml positive, 1 × 0.1 ml positive) gives an MPN value of 17, i.e., the water sample contains an estimated 17 coliforms per 100 ml*.

MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when five 10-ml, five 1-ml and five 0.1 ml test portions are used)

No. of tubes giving a positive reaction :			MPN (per 100 ml)	95% confidence limits	
5 of 10 ml	5 of 1 ml	5 of 0.1 ml		Lower	Upper
0	0	0	<2	<1	7
0	1	0	2	<1	7
0	2	0	4	<1	11
1	0	0	2	<1	7
1	0	1	4	<1	11
1	1	0	4	<1	11
1	1	1	6	<1	15
2	0	0	5	<1	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	9	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34
3	2	1	17	5	46

For treated water

Take 1 tube of single strength (50ml) and 5 tubes of double strength (10ml) for each water sample to be tested. Using a sterile pipette add 50 ml of water to the tubes containing 50 ml single strength medium. Similarly add 10 ml of water to 5 tubes containing 10 ml double strength medium Incubate the tubes at 37°C for 24 hrs. If no tubes appear positive re-incubate up to 48 hrs. Compare the number of tubes giving positive reaction to a standard chart and record the number of bacteria present in it.

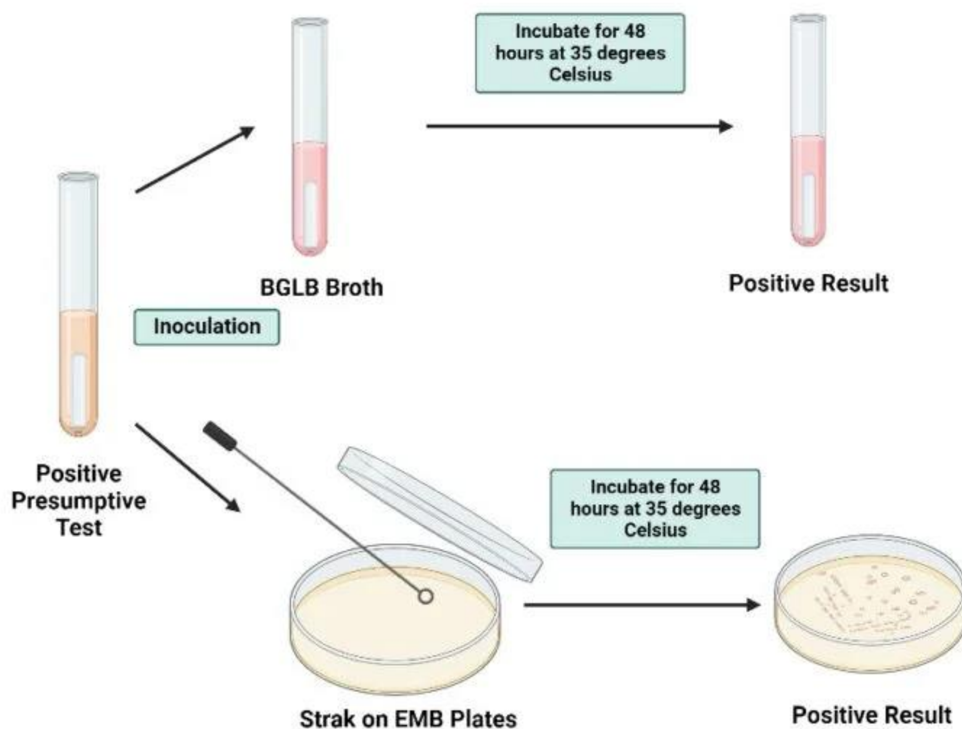
For example: a water sample tested shows a result of 1-4 (1 × 50 ml positive, 4 × 10 ml positive) gives an MPN value of 16, i.e., the water sample contains an estimated 16 coliforms per 100 ml.

MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when one 50-ml and five 10-ml test portions are used)

No. of tubes giving a positive reaction		MPN (per 100 ml)	95% confidence limits	
1 of 50 ml	5 of 10 ml		Lower	Upper
0	0	<1	—	—
0	1	1	<1	4
0	2	2	<1	6
0	3	4	<1	11
0	4	5	1	13
0	5	7	2	17
1	0	2	<1	6
1	1	3	<1	9
1	2	6	1	15
1	3	9	2	21
1	4	16	4	40
1	5	>18	—	—

Confirmed test

Some microorganisms other than coliforms also produce acid and gas from lactose fermentation. In order to confirm the presence of coliform, confirmatory test is done. From each of the fermentation tubes with positive results transfer one loopful of medium to 5 ml lactose-broth or brilliant green lactose fermentation tube, to an agar slant and 5 ml tryptone water. Incubate the inoculated lactose-broth fermentation tubes at 37°C and inspect gas formation after 24 ± 2 hours. If no gas production is seen, further incubate up to maximum of 48 ±3 hours to check gas production. The agar slants should be incubated at 37°C for 24± 2 hours and Gram-stained preparations made from the slants should be examined microscopically. The formation of gas in lactose broth and the demonstration of Gram negative, non-spore-forming bacilli in the corresponding agar indicates the presence of a member of the coliform group in the sample examined. The absence of gas formation in lactose broth or the failure to demonstrate Gram-negative, non-spore-forming bacilli in the corresponding agar slant constitutes a negative test (absence of coliforms in the tested sample).



Completed test

Since some of the positive results from the confirmatory test may be false, it is desirable to do completed tests. For this inoculum from each positive tube of the confirmatory test is streaked on a plate of EMB or Endo agar. In this process, a loopful of sample from each positive BGLB tubes is streaked onto selective medium like Eosin Methylene Blue agar or Endo's medium. One plate each is incubated at 37°C and another at 44.5± 0.2°C for 24 hours. High temperature incubation (44.5 ±0.2) is for detection of thermotolerant *E. coli.* Following incubation, all plates are examined for presence of typical colonies. Coliforms produce colonies with greenish metallic sheen which differentiates it from non-coliform colonies (show no sheen). Presence of typical colonies on high temperature (44.5 ±0.2) indicate presence of thermotolerant *E. coli.*

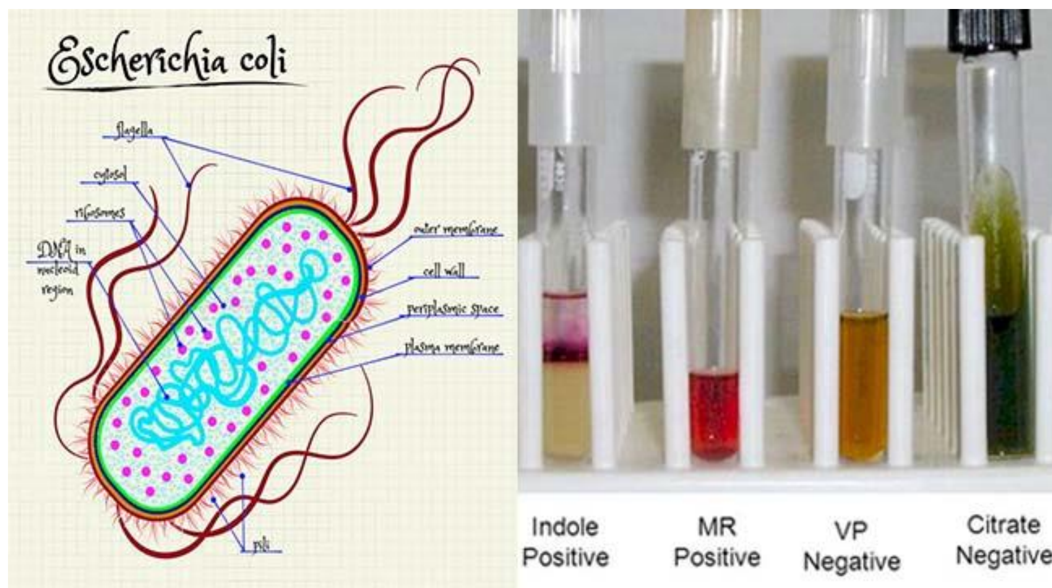
Biochemical test

- Indole test (tryptone broth)- Kovert's reagent pink colour ring (24-48 h)
- Methyl red test (glucose peptone phosphate broth)- red colour due to acid production
- Voges Proskaur test (GPP)-40% KOH and α napthol, (48 h) port wine colour (2-6 h)-few grains of creatine phosphate enhance the reaction.

- Citrate test (Simmon's citrate media) –bromothymole blue (blue) >pH 8.4

Positive result for *E. coli*

- Indole test: positive
- MR: positive
- VP: negative
- Citrate: negative



Advantages of MPN Techniques

- Interpretation of the results requires minimal experience and training as results can be got by simply observing for the presence of gas or no gas.
- Water samples with high turbidity can be analyzed, since there is no apparent deleterious effect.
- MPN technique is the effective method for analyzing samples such as muds, sludges, sediments etc.
- Uses comparatively less expensive materials
- Larger volume of sample
- Enrichment with liquid
- Possible to make selective qualitative method into quantitative method

Disadvantages of MPN Techniques

- MPN procedure takes very long time for the confirmed test result.
- In MPN the results are probability calculations and cannot be accurate.
- MPN requires more glass wares and media.
- False positive results are of common occurrence.
- Sterilization may need a larger autoclave
- Not suitable for field tests.

CHAPTER 3

Isolation and Identification of Faecal Coliforms

Femeena Hassan, Devananda Uchoi, Pankaj Kishore and Ranjit Kumar Nadella

Email: drfemeenahassan@gmail.com

The term "coliform" was coined to describe this group of enteric bacteria. Coliform is not a taxonomic classification but rather a working definition used to describe a group of Gram-negative, facultative anaerobic rod-shaped bacteria that ferments lactose to produce acid and gas within 48 h at 35°C. Although coliforms were easy to detect, their association with fecal contamination was questionable because some coliforms are found naturally in environmental samples. This led to the introduction of the fecal coliforms as an indicator of contamination. Fecal coliform, first defined based on the works of Eijkman is a subset of total coliforms that grows and ferments lactose at elevated incubation temperature, hence also referred to as thermotolerant coliforms. Fecal coliform analyses are done at 45.5°C for food testing, except for water, shellfish and shellfish harvest water analyses, which use 44.5°C. The fecal coliform group consists mostly of *E. coli* but some other enterics such as *Klebsiella* can also ferment lactose at these temperatures and therefore, be considered as fecal coliforms.

Currently, all 3 groups are used as indicators but in different applications. Detection of coliforms is used as an indicator of sanitary quality of water or as a general indicator of sanitary condition in the food-processing environment. Fecal coliforms remain the standard indicator of choice for shellfish and shellfish harvest waters; and *E. coli* is used to indicate recent fecal contamination or unsanitary processing. Almost all the methods used to detect *E. coli*, total coliforms or fecal coliforms are enumeration methods that are based on lactose fermentation. The Most Probable Number (MPN) method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases. In the assay, serial dilutions of a sample are inoculated into broth media. Analysts score the number of gas positive (fermentation of lactose) tubes, from which the other 2 phases of the assay are performed, and then uses the combinations of positive results to consult a statistical table, to estimate the number of organisms present. Typically only the first 2 phases are performed in coliform and fecal coliform analysis, while all 3 phases are done for *E. coli*. The 3-tube MPN test is used for testing most foods. Analysis of seawater using a multiple dilution series should not use less than 3 tubes per dilution (5 tubes are recommended); in certain instances a single dilution series using no less than 12 tubes may also be acceptable. Likewise, analysis of bivalve molluscan shellfish should be performed using a multiple dilution MPN series whereby no fewer than 5- tubes per dilution should be used. There is also a 10-tube MPN method that is used to test bottled water or samples that are not expected to be highly contaminated.

MPN - Presumptive test for coliforms, fecal coliforms and *E. coli*

Weigh 50 g of food into sterile high-speed blender bag. Frozen samples can be softened by storing for <18 h at 2-5°c, but do not thaw. Add 450 mL of phosphate-buffered saline and blend for 2 min. If <50 g of sample are available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. Prepare decimal dilutions with sterile phosphate diluent or equivalent. Number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions by vortex mix for 7 s. Using at least 3 consecutive dilutions, inoculate 1 mL aliquots from each dilution into 3 LST tubes for a 3 tube MPN analysis (other analysis may require the use of 5 tubes for each dilution). Lactose Broth may also be used. For better accuracy, use a 1 mL or 5 mL pipet for inoculation. Hold pipet at angle so that its lower edge rests against the tube. Not more than 15 min should elapse from time the sample is blended until all dilutions are inoculated in appropriate media. Incubate LST tubes at 35°C± 0.5°C . Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 3 h. Perform confirmed test on all presumptive positive (gas) tubes.

MPN - Confirmed test for coliforms

From each gassing LST or lactose broth tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. (a sterile wooden applicator stick may also be used for these transfers). Incubate BGLB tubes at 35°C ± 0.5°C and examine for gas production at 48 ± 3 h. Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

MPN - Confirmed test for fecal coliforms and *E. coli*

From each gassing LST or Lactose broth tube from the Presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). Incubate EC tubes 24 ± 2 h at 44.5°C and examine for gas production. If negative, reincubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN. The EC broth MPN method may be used for seawater and shellfish since it conforms to recommended procedures.

MPN - Completed test for *E. coli*.

To perform the completed test for *E. coli*, gently agitate each gassing EC tube, remove a loopful of broth and streak for isolation on a L-EMB agar plate and incubate for 18-24 h at 35°C ± 0.5°C. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic

sheen. Transfer up to 5 suspicious colonies from each L-EMB plate to PCA slants, incubate them for 18-24 h at 35°C ± 0.5°C and use for further testing.

NOTE: Identification of any 1 of the 5 colonies as *E. coli* is sufficient to regard that EC tube as positive; hence, not all 5 isolates may need to be tested.

Perform Gram stain. All cultures appearing as Gram-negative, short rods should be tested for the IMViC reactions below and also re-inoculated back into LST to confirm gas production.

Indole production. Inoculate tube of tryptone broth and incubate 24 ± 2 h at 35°C ± 0.5°C . Test for indole by adding 0.2-0.3 mL of Kovacs' reagent. Appearance of distinct red color in upper layer is positive test.

Voges-Proskauer (VP)-reactive compounds. Inoculate tube of MR-VP broth and incubate 48 ± 2 h at 35°C± 0.5°C . Transfer 1 mL to 13 × 100 mm tube. Add 0.6 mL α-naphthol solution and 0.2 mL 40% KOH, and shake. Add a few crystals of creatine. Shake and let stand 2 h. Test is positive if eosin pink color develops.

Methyl red-reactive compounds. After VP test, incubate MR-VP tube additional 48 ± 2 h at 35°C± 0.5°C . Add 5 drops of methyl red solution to each tube. Distinct red color is positive test. Yellow is negative reaction.

Citrate. Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate for 96 h at 35°C ± 0.5°C . Development of distinct turbidity is positive reaction.

Gas from lactose. Inoculate a tube of LST and incubate 48 ± 2 h at 35°C ± 0.5°C . Gas production (displacement of medium from inner vial) or effervescence after gentle agitation is positive reaction.

Interpretation: All cultures that (a) ferment lactose with gas production within 48 h at 35°C, (b) appear as Gram-negative nonsporeforming rods and (c) give IMViC patterns of ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*. Calculate MPN (see Appendix 2) of *E. coli* based on proportion of EC tubes in 3 successive dilutions that contain *E. coli*.

NOTE: Alternatively, instead of performing the IMViC test, use API20E or the automated VITEK biochemical assay to identify the organism as *E. coli*. Use growth from the PCA slants and perform these assays as described by the manufacturer.

Solid medium method - Coliforms

Prepare violet red bile agar (VRBA) according to manufacturer's instructions. Cool to 48°C before use. Prepare, homogenize, and decimally dilute sample as above so that isolated colonies will be obtained when plated. Transfer two 1 mL aliquots of each dilution to petri dishes, and use either

of the following two pour plating methods, depending on whether injured or stressed cells are suspected to be present. Pour 10 mL VRBA tempered to 48°C into plates, swirl plates to mix, and let solidify. To prevent surface growth and spreading of colonies, overlay with 5 mL VRBA, and let solidify. If resuscitation is necessary, pour a basal layer of 8-10 mL of tryptic soy agar tempered to 48°C. Swirl plates to mix, and incubate at room temperature for 2 ± 0.5 h. Then overlay with 8-10 mL of melted, cooled VRBA and let solidify. Invert solidified plates and incubate 18-24 h at 35°C. Incubate dairy products at 32°C. Examine plates under magnifying lens and with illumination. Count purple-red colonies that are 0.5 mm or larger in diameter and surrounded by zone of precipitated bile acids. Plates should have 25-250 colonies. To confirm that the colonies are coliforms, pick at least 10 representative colonies and transfer each to a tube of BGLB broth. Incubate tubes at 35°C. Examine at 24 and 48 h for gas production.

NOTE: If gas-positive BGLB tube shows a pellicle, perform Gram stain to ensure that gas production was not due to Gram-positive, lactose-fermenting bacilli.

Determine the number of coliforms per gram by multiplying the number of suspect colonies by percent confirmed in BGLB by dilution factor.

Alternatively, *E. coli* colonies can be distinguished among the coliform colonies on VRBA by adding 100 µg of 4-methyl-umbelliferyl-β-D-glucuronide (MUG) per mL in the VRBA overlay. After incubation, observe for bluish fluorescence around colonies under longwave UV light.

CHAPTER 4

Isolation and Identification of *Vibrio* spp.

Pankaj Kishore, Ranjit Kumar Nadella and Devananda Uchoi

Email: pkishore2007@gmail.com

Fish and shellfish are consumed in various forms across the globe and its contribution is significant. Bacteria of genus Vibrio may contaminate fishery products at different point of handling/processing and hence this can pose risk to human population. Vibrios are abundant in the aquatic environment. Most of them require 2 to 3% NaCl or a seawater base for optimal

growth. Vibrios are associated with live seafood as they form part of the indigenous microflora of the marine environment. Foodborne infections with Vibrio spp. are common in Asia. Vibrio spp. are Gram-negative, facultatively anaerobic motile curved rods with a single polar flagellum. Among the members of the genus, 12 species have so far been reported to be pathogenic to humans, where eight of these may be associated with foodborne infections of the gastrointestinal tract. V. mimicus is a recognized pathogen with similar characteristics to V. cholerae, except an ability to ferment sucrose. Other species within the genus, such as V. alginolyticus, V. fluvialis, V. furnissii, V. metschnikovii, and V. hollisae are occasional human pathogens. Vibrio species account for a significant proportion of human infections from the consumption of raw or undercooked shellfish. Three main Vibrio species, V. cholerae, V. parahaemolyticus and V. vulnificus, are potentially pathogenic to humans. Most of these foodborne infections are caused by *V. parahaemolyticus* and *V. cholerae*, and to a lesser extent by *V. vulnificus*. These species are responsible for a dramatic increase of seafood-borne infections worldwide.

V. cholerae

V. cholerae is a major concern among the genus vibrios, because of its ability to cause cholera and infect human population through food and other environmental sources. *V. cholerae* can be divided into serogroups on the basis of the O antigen. Of the more than 200 serogroups that exist, only O1 and O139 are associated with the epidemiological features and clinical syndrome of cholera. However, organisms of *V. cholerae* serogroups other than O1 and O139 (non-O1 non-O139 serogroups) have been associated with sporadic cases of foodborne outbreaks of gastroenteritis, but have not spread in epidemic form. The most important virulence factor associated with *V. cholerae* O1 and O139 serogroups is the cholera toxin. Non-O1 non-O139 serogroups are generally nontoxigenic.

Cholera remains a public health threat globally causing hundreds of thousand cases every year. However, this threat is much reduced in places with safe water supply and good standards of hygiene and sanitation. Cholera is an acute intestinal infection. Its incubation period ranges from a few hours to five days, usually two to three days. Although asymptomatic infection is more common, clinical illness may be exhibited. Symptoms include a sudden onset of profuse painless watery diarrhoea that can quickly lead to rapid dehydration, acidosis, circulatory collapse, hypoglycaemia in children, renal failure and death if treatment is not promptly given. Nausea and vomiting also occurs early in the course of illness. Cholera is transmitted through ingestion of food or water contaminated with the bacterium, especially via faeces or vomitus of infected

persons, directly or indirectly. Human volunteer feeding studies utilising healthy individuals have demonstrated that the infective dose is approximately one million organisms. However, conditions which decrease acidity in the stomach such as antacid consumption markedly lowers the infective dose. *V. cholerae* is a mesophilic organism that grows in the temperature range of 10 to 43°C, with optimum growth at 37°C. The pH optimum for growth is 7.6 although it can grow in the pH range of 5.0 to 9.6. *V. cholerae* can grow in the salt range of 0.1 to 4.0% NaCl, while optimum is 0.5% NaCl.

V. parahaemolyticus

V. parahaemolyticus was first identified as a foodborne pathogen in Japan. In early 1970s, *V. parahaemolyticus* was recognised as a cause of diarrhoeal disease worldwide, although most common in Asia and the United States. The illness caused by *V. parahaemolyticus* food poisoning is a gastroenteritis characterised by watery diarrhoea and abdominal cramps in most cases, with nausea, vomiting, fever and headache. The incubation period is usually between 12 and 24 hours and the disease usually resolves in three days. The infection is typically acquired through consumption of contaminated seafood. These could be raw or inadequately cooked, or that have been cross-contaminated by improper handling. Poor temperature control of storage favours bacterial proliferation. *V. parahaemolyticus* is a slightly halophilic bacterium. The optimum growth NaCl concentrations range from 2 to 4% and poor growth is exhibited in media below 0.5% NaCl. The bacterium is inactivated rapidly in distilled water and growth at levels of 10% NaCl is inhibited. The organism grows at a temperature range between 5 and 43°C, with optimum growth at 37°C. The optimum pH range for growth is 7.8 to 8.6, although it can grow in the pH range of 4.8 to 11.

V. vulnificus

V. vulnificus is an opportunistic pathogen that can cause wound infections and primary septicaemia. This bacterium has less often been described as a cause of gastroenteritis, and its role as a primary cause of gastrointestinal disease remains to be determined. Wound infections occur in connection with puncture wounds after handling of raw seafood or trauma and exposure to saline environments that harbour the organism. *V. vulnificus* is very similar to *V. parahaemolyticus* in cultural characteristics and sensitivity to processing procedures. It differs principally in salt requirement and tolerance, growing in media containing between 0.1 and 5%

NaCl. Same as *V. parahaemolyticus*, the organism grows optimally at 37ºC although it can grow at a temperature range between 08-43ºC. The pH range for growth of *V. vulnificus* is 05- 10, with an optimum at 7.8.

Procedures

***V. cholerae*:**

A. Enrichment and plating

- Weigh 25 g of sample and Add 225 ml APW to jar. Thoroughly mix the sample or blend 2 min at high speed.
- Incubate APW at 35 ±2°C for 6 to 8 h. Re-incubate for 18 to 21 h at 42 ±0.2°C.
- An enumeration technique by most probable number (MPN) may also be performed if desired.
- Prepare may also be included.
- Transfer a 3-mm loopful from the surface pellicle of APW culture to the surface of a dried TCBS plate (and mCPC or CC), and streak to isolated colonies.
- Incubate TCBS overnight (18 to 24 h) at 35°±2°C. Incubate mCPC and CC overnight at 39-40°C.
- Typical colonies of *V. cholerae* on TCBS agar are large (2 to 3 mm), smooth, yellow and slightly flattened with opaque centers and translucent peripheries.
- Typical colonies of *V. cholerae* on mCPC or CC agar are small, smooth, opaque, and green to purple in color, with a purple background on extended incubation.

V. parahaemolyticus

- Weigh 50 g of seafood sample into a blender and Add 450 ml APW and blend for 1 min at 8,000 RPM.
- Incubate APW overnight at 35 ±2°C.
- Streak a 3-mm loopful from the top 1 cm of APW tubes containing the three highest dilutions of sample showing growth onto TCBS (and mCPC or CC agars for *V. vulnificus* isolation)
- Incubate TCBS plates at 35 ±2°C (and mCPC or CC plates at 35-37°C overnight.
- *V. parahaemolyticus* appear as round, opaque, green or bluish colonies, 2 to 3 mm in diameter on TCBS agar. Interfering, competitive *V. alginolyticus* colonies are, large, opaque, and yellow. Most strains of *V. parahaemolyticus* will not grow

on mCPC or CC agar. If growth occurs, colonies will be green-purple in color due to lack of cellobiose fermentation.

Table 1. Biochemical characteristics of human pathogenic Vibrionaceae commonly encountered in seafood

	V. alginolyticus	*V. cholerae*	*V. parahaemolyticus*	*V. vulnificus*
TCBS agar	Y	Y	G	G
mCPC agar	NG	P	NG	Y
CC agar	NG	P	NG	Y
Oxidase	+	+	+	+
Arginine dihydrolase	−	−	−	−
Ornithine decarboxylase	+	+	+	+
Lysine decarboxylase	+	+	+	+
Growth in (w/v):				
0% NaCl	−	+	−	−
3% NaCl	+	+	+	+
6% NaCl	+	−	+	+
10% NaCl	+	−	−	−
Voges-Proskauer	+	V	−	−
Urease	−	−	V	−
Sensitivity to - 10 µg O/129	R	S	R	S
Sucrose	+	+	−	−
D-Cellobiose	−	−	V	+
Lactose	−	−	−	+

Arabinose	−	−	+	−
D-Mannose	+	+	+	+
D-Mannitol	+	+	+	V
ONPG	−	+	−	+

Note:

TCBS, thiosulfate-citrate-bile salts-sucrose;

mCPC, modified cellobiose-polymyxin B-colistin;

Y = yellow NG = no or poor growth S = susceptible nd = not done

G = green V = variable among strains R = resistant P = purple, V = variable

KK = Slant alkaline / Butt alkaline KA = Slant alkaline /Butt acidic, Ka = Slant alkaline/ Butt slightly acidic.

Refer:

1. BAM
2. https://pubmed.ncbi.nlm.nih.gov/28956623/
3. https://www.cfs.gov.hk/english/programme/programme_rafs/programme_rafs_fm_01_02_vss.html

CHAPTER 5

Conventional Identification Techniques

Devananda Uchoi, Pankaj Kishore and Ranjit Kumar Nadella

Email: uchoidev514@gmail.com

Conventional detection of pathogenic bacteria is mainly based on cultivation procedures, which use enrichment broths followed by the isolation of colonies on selective media, biochemical identification and confirmation of pathogenicity. This culture method is selective for the search of one type of pathogen at a time.

Conventional techniques for identification of bacterial cultures includes:

1. Gram's staining and microscopy
2. Motility
3. Test for catalase

4. Penicillin sensitivity
5. H&L glucose O/F reaction
6. Cytochrome oxidase test
7. Fermentation of glucose
8. Pigmentation
9. Growth at zero NaCl level

a).Catalase test

Place a speck of young culture on a clean glass slide and flood with 2 drops of 3% H_2O_2 (H2O2 bottle is usually stored in a refrigerator). Bring the temperature of H_2O_2 to room temperature before the test). Evolution of gas from the culture indicates positive test for catalase. Note: Catalase is an enzyme, which decomposes H2O2.

b).Cytochrome oxidase test

Smear a little of the young culture on the test paper (already impregnated with Kovac's cytochrome oxidase reagent). Development of a blue colour in a few seconds indicates a positive test.

c).Penicillin sensitivity

Prepare pre-set antibiotic agar plates and dry the surface at 56°C for 45 min. Cool to room temperature. Divide each plate into 4 quarters by drawing lines on the bottom so that we can use one plate for 4 cultures. A little of the culture is smeared over about 4 cm2 area in each quarter. A filter paper disk impregnated with penicillin (each disk contains 2.5 IU penicillin) is placed on the surface of each smear. Plates are incubated without inverting for 18-24 h. Examine for clear zones of inhibition around the discs. Cultures showing clear zones of inhibition are sensitive to 2.5 IU penicillin.

d).H&L glucose O/F reaction test.(Hugh &Leifson's oxidative verses fermentative reaction test using glucose as substrate)

Using a platinum wire (needle), a little of the culture is stab-inoculated into the H&L glucose O/F medium, in such a way that at least 2 cm long column of the medium at the bottom of the tube remains uninoculated. Incubate for 18-24 h and observe the changes. A colour change into yellow indicates acid production from glucose. A deepening of red colour of the medium indicates an increase in pH to alkaline level.

Growth of bacteria along the line of inoculation and an yellow colour throughout the medium indicate fermentative reaction (Fermentative with acid but no gas; FANG). If gas bubbles are also seen trapped in medium, reaction is fermentative with gas production (Fermentative with acid and gas; FAG).

If yellow colour appears only at the top part of the medium, the reaction is oxidative. Sometimes, a deep pink colour develops near the top surface, indicating change of pH to alkaline side (Alkaline top). In both these cases, the reaction is nonfermentative (NF).

e). Fermentation of glucose (Durham tube method)

Inoculate the culture into glucose fermentation broth and incubate. Note acid and gas production after 24-48 h. A deep pink colour shows acid production. Gas bubble in the Durham's tube shows fermentation of glucose with gas production.

f).Pigmentation

Note the colour of the bacterial culture on TGA slants after 72-96 h. Some bacterial cultures are pigmented yellow, red, pink, violet, brown or red.

g).Growth at zero NaCl level

Inoculate the culture to peptone medium without NaCl (P1No) medium and incubate. Note growth after 48 h. Turbidity indicates bacterial growth.

Gram's staining. Gram's staining, originally devised by Christian Gram (1884) is a differential staining. By this method of staining, bacteria are divided to two groups: Gram Positive and Gram Negative.

h) Gram's Staining

Staining solutions used are:

i) Gram's Crystal violet

ii) Gram's iodine

iii) Safranine

iv)Ethyl alcohol for destaining

a) Preparation of smear

Only young cultures shall be used for staining. Usually 16-24 h old cultures are considered young. Take a dust free, dirt free and oil free microscopic glass slide. A speck of young culture is emulsified with a drop of sterile water in the middle of the slide and spread uniformly. Dry in the air. Fix by passing the slide 3-4 times through the blue flame of bunsen burner (Care not to char the smear).

b) Staining

i) Place the slide on a staining bridge.

ii) Flood the smear with Gram's crystal violet for 1 min.

iii) Wash with water.

iv) Flood with Gram's iodine for 1 min.

v) Wash with water.

vi) Destain with dropwise addition of alcohol until washings are free from violet colour(Note: collect alcohol washings in a bottle for recovery of alcohol).

vii) Wash with water.

viii) Counterstain with Safraninefor 1 min.

ix) Dry in air.

c) Microscopy

Observe the slides under the microscope using oil immersion objective (95X or 100X). Cells stained violet, bluish violet or bluish purple are Gram +ve. Cells stained red are Gram -ve. Note the shape, size and arrangement of cells. Also examine whether the culture is pure or mixed. In case the cells are G+ve rods, examine whether there is spore formation (Note: All G+ve rods are not spore formers).

i) Motility of bacteria

Some bacteria exhibit the property of movement in a liquid phase, with the help of their flagella. This movement is referred as motility. Motility is observed under a microscope using high power objective (40X, 45X or 50X). Two methods are usually used to observe bacterial motility.

1) Hanging drop method

A small drop of DW is placed on the middle of a cover slip, a speck of young culture from agar slant is emulsified with it. A cavity slide is taken, the margin of the cavity is smeared with a little paraffin jelly. The slide is inverted on the cover slip in such a way, that the cover slip gets

attached to the slide and on turning upside down, the culture drop hangs into the cavity. Observe under the microscope.

2) Plain slide method

Make an emulsion of the culture in a drop of DW on an ordinary microscopic slide, place a cover slip on it and observe under microscope.

An actual movement of the cells (individually) indicates motility. Depending on the intensity and speed of movement, motility is referred as weakly motile, moderately motile and actively motile.

After conventional identification of bacterial cultures upto genus level, species level idenfication can be carried out by employing DNA based PCR assay.

CHAPTER 6

Isolation and Identification of *Salmonella* spp.

Ranjit Kumar Nadella, Devananda Uchoi and Pankaj Kishore

Email: nranjeetkumar@gmail.com

Foodborne illnesses are among the most common worldwide health issues, and because of the financial costs associated with morbidity and mortality, their implications for public health are widely acknowledged. One of the most prevalent foodborne illnesses with a high zoonotic potential that can cause serious and even deadly infections in both people and animals is Salmonellosis. *Salmonella* is regarded as the primary cause of gastroenteritis and is responsible for the global outbreak of human salmonellosis. Salmonellosis, or infection with *Salmonella*, can result in enteritis, nausea, stomachaches, digestive issues, and even potentially fatal conditions like typhoid and paratyphoid fever. Human adapted *Salmonella* serotypes like Typhi and Paratyphi

result in grave systematic diseases like typhoid fever, whereas, infections with non-typhoid *Salmonella* serotypes most often lead to self-limited acute gastroenteritis.

Salmonella belongs to the Enterobacteriaceae family, characterized as a nonsporulated gram-negative bacillus. It is routinely classified by serotype, based on the expression of three types of antigens: (O) somatic, (H) flagellar and (vi) capsular, according to the Kauffmann-White scheme. This current classification scheme is based on two main Salmonella species: *S. enterica* and *S. bongori*. ***S. enterica*** *subspecies enterica*- 1435 serovars; *S. enterica subspecies salamae*- 485 serovars; *S.enterica subspecies arizonae*- 94 serovars *; S.enterica subspecies diarizonae*- 321 serovars; *S.enterica subspecies houtenae*- 96 serovars and *S.enterica subspecies indica*- 11 serovars. ***S. bongori*- 17** serovars. Based on biochemical characteristics, Salmonella is grouped into three species. ***S. choleraesuis***: Have only one serovar, and affects swine. ***S. typhi***: Have only one serovar, and affects mainly human. ***S. enteritidis***: Contain about 2000 serovars, each of which is given a species name and includes all the serovars infecting animals and human nowadays.

Salmonella is a facultative anaerobic, and oxidase-negative, usually mobile, that produces gas from glucose. Its growth temperature ranges from 7°C to 46°C, with temperature optimum ranging from 35°C to 43°C and, growth pH ranging from 3.8 to 9.5, with optimum pH between 7.0 and 7.5. *Salmonella* is catalase and methyl red positive, and indole, vogues proskauer, malonate and urea negative. It produces hydrogen sulphide gas (H_2S) from the reduction of sulfur through cysteine desulphydrase and displays as metabolic characteristics decarboxylation capacity regarding the amino acids lysine and ornithine, nitrate to nitrite reduction and the use of citrate as the only carbon.

Isolation and identification of Salmonella

Isolation of Salmonella was performed as per as per ISO 6579-1: 2017 for seafood and soil. For water samples isolation of Salmonella was carried out as per as per ISO 19250: 2010.

Pre-enrichment in non-selective liquid medium

Each 25 g of sample (edible muscle and soil) was aseptically collected in a sterile filter bag and 225 mL of buffered peptone water was added (one to nine ratio) and homogenized using a stomacher blender for 2 min. The pre-enriched samples were incubated for 18 to 24 h at 37 °C. For water sample, 50 ml sample is added to the same volume of double strength BPW. The samples were mixed well and incubated at 37 °C for 18 to 24 h.

Selective enrichment

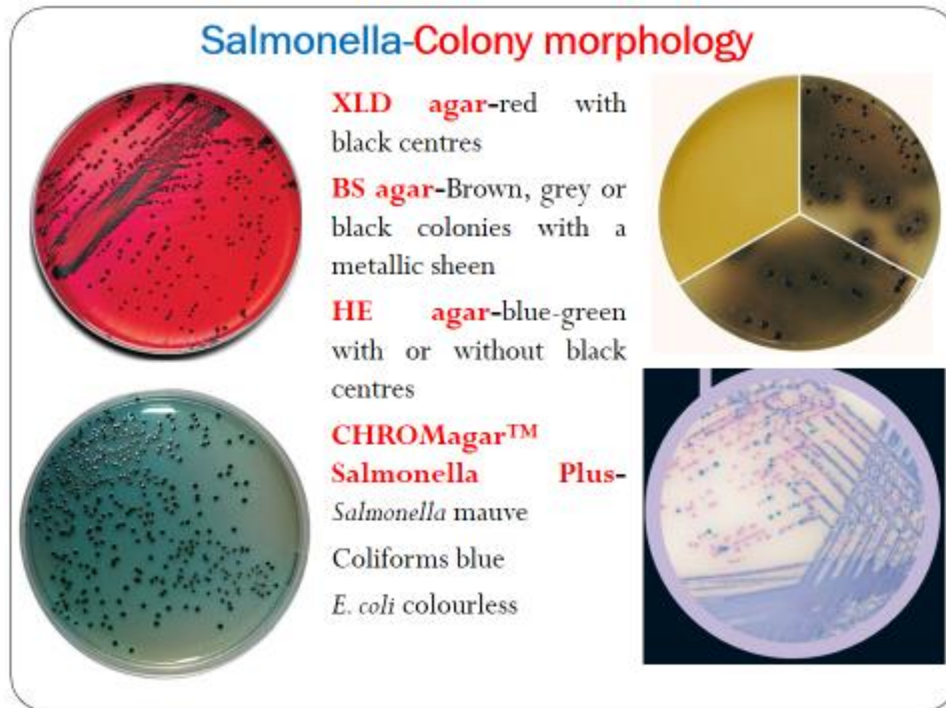
The pre-enrichment broth (1 mL and 0.1 mL) were transferred aseptically into 10 mL of MKTTn broth and 10 mL of RVS broth, mixed, and then were incubated for 18 to 24 h at 37 °C and 41.5°C, respectively.

Plating out

Following incubation, a loop-full of each culture was streaked onto the surface of XLD and BSA medium and incubated at 37 °C for 18 to 24 h. The XLD and BSA plates were examined for the presence of Salmonella colonies. If growth is slight or if typical colonies of Salmonella were not present, the plates were re-incubated for a further 18 to 24 h and reexamined for the presence of typical Salmonella colonies. The formation of red colonies with black centers and of black colonies with a brown hallow was inspected on XLD and BSA plates, respectively.

The characteristic individual colonies were selected for subculture and confirmation. If there is no individual colony, the suspected colonies were purified by plating on to a non-selective medium. Select up to four more suspect colonies ensuring that these colonies are sub-cultured from different selective enrichment/isolation medium combinations showing suspect growth.

Streak the selected colonies onto the surface of a pre-dried non-selective agar medium in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates at 37°C for 18 to 24 h. Alternatively, if well-isolated colonies (of a pure culture) are available on the selective plating media, the biochemical confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium.



Identification of the Bacterial cultures

Culture characteristics such as colony appearance of each strain were tested according to the standard methods.

Gram's staining

Gram staining is a method that differentiates bacteria in two large group Gram positive and Gram negative. This method differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, in gram positive it is present as a thick layer. A Gram positive results in a purple/blue color while a Gram negative results in a pink/red color. It was also used to find out the morphology of bacteria (rod, cocci, spiral etc).

Catalase test

Catalase is essential for the breakdown of H_2O_2 produced during respiration. In anaerobes, H_2O_2 inhibit their growth in the presence of oxygen because catalase is absent. If few drops of 3% H_2O_2 are added to a drop of broth culture or colony, oxygen gas so released can be seen as white effervescence.

Oxidase test

During aerobic respiration, oxidase enzyme plays a vital role in the operation of electron transport system. Cytochrome oxidase catalyses the oxidation of a reduced cytochrome by molecular oxygen and results in the formation of water and hydrogen peroxide

Biochemical testing

The sub-cultured colonies were picked and inoculated into the following biochemical test tubes for confirmation:

i. TSI (Triple Sugar Iron) test

Streak the agar slant surface and stab the butt. Incubate at 37°C for 18 to 24 h. The majority of the typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and (in about 90 % of the cases) formation of hydrogen sulfide (blackening of the agar).

ii. Urea Test

Streak the agar slant surface. Incubate at 37 °C for up to 24 h. If the reaction is positive, urea is hydrolyzed, liberating ammonia. This changes the colour of phenol red to rose-pink and later to deep cerise. The reaction is often apparent after 2 h to 4 h. Typical *Salmonella* cultures do not hydrolyze urea so that the colour of the urea agar will remain unchanged.

iii. L-Lysine decarboxylation (LDC) test

Inoculate just below the surface of the liquid medium. Incubate at 37 °C for 18 to 24 h. Turbidity and a purple colour after incubation indicates a positive reaction. A yellow colour indicates a negative reaction. The majority of the typical *Salmonella* cultures show a positive reaction in LDC.

iv. Indole test

Inoculate a tube containing tryptone/tryptophan medium with the suspected colony. Incubate at 37 °C for 24 h ± 3 h. After incubation, add 1 ml of the Kovacs reagent. The formation of a red ring (surface layer) indicates a positive reaction. A yellow-brown ring (surface layer) indicates a negative reaction.

Salmonella serovar Typhi:

A) TSI: Alkaline slant / Acid Butt / Trace H_2S / No Gas
B) Urea: Negative
C) LDM: Lysine Decarboxylase Positive
D) Indole reagent: Indole negative

Salmonella serovar Paratyphi A:

A) TSI: Alkaline slant / Acid Butt / No H2S /Gas
B) Urea: Negative
C) LDM: Lysine Decarboxylase Negative
D) Indole reagent: Indole negative

Most non-typhoidal serovars of *S. enterica*:

A) TSI: Alkaline slant / Acid Butt / H_2S Positive /Gas
B) Urea: Negative
C) LDM: Lysine Decarboxylase Positive
D) Indole reagent: Indole negative

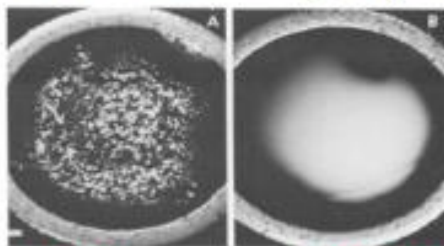
Serological testing

The pure colonies showing typical biochemical reactions for *Salmonella* are also tested for the presence of *Salmonella* O- and H-antigens by slide agglutination using polyvalent antisera. The pure colonies are cultured on a non-selective agar medium and tested for auto-agglutination. Strains that are auto-agglutinable cannot be tested for the presence of *Salmonella* antigens.

Place one drop of saline solution on a clean glass slide. Using a loop, disperse part of the colony to be tested in the saline to obtain a homogeneous and turbid suspension. Rock the slide gently for 5 s to 60 s. Observe the suspension, preferably against a dark background. If the bacteria have formed granules in the suspension, this indicates auto-agglutination and serological confirmation will become complicated. If it is non-auto-agglutinating pure colony continue the test by adding one drop of polyvalent anti-O sera in to the colony in the saline solution for examination of O-antigens. Similarly, an anti-H serum is used for the examination of H antigens. If agglutination occurs, this is considered a positive reaction in both cases.

Salmonella-Serological testing

- Salmonella O- and H- antigen; S. typhi- O- , H- and Vi- antigen by slide agglutination using polyvalent antisera
- Strains are tested for autoagglutination.



Positive Negative

Biochemical reactions	Auto-agglutination	Serological reactions	Interpretation
Typical	No	O- and H-antigens positive (and Vi positive if tested)	Strains considered to be *Salmonella*
Typical	No	O- and/or H-antigens negative	Presumptive *Salmonella*
Typical	Yes	Not tested because of auto-agglutination (see 9.5.4.2)	
No typical reactions	—	—	Not considered to be *Salmonella*

Diagram of procedure for detection of *Salmonella* in seafood and soil

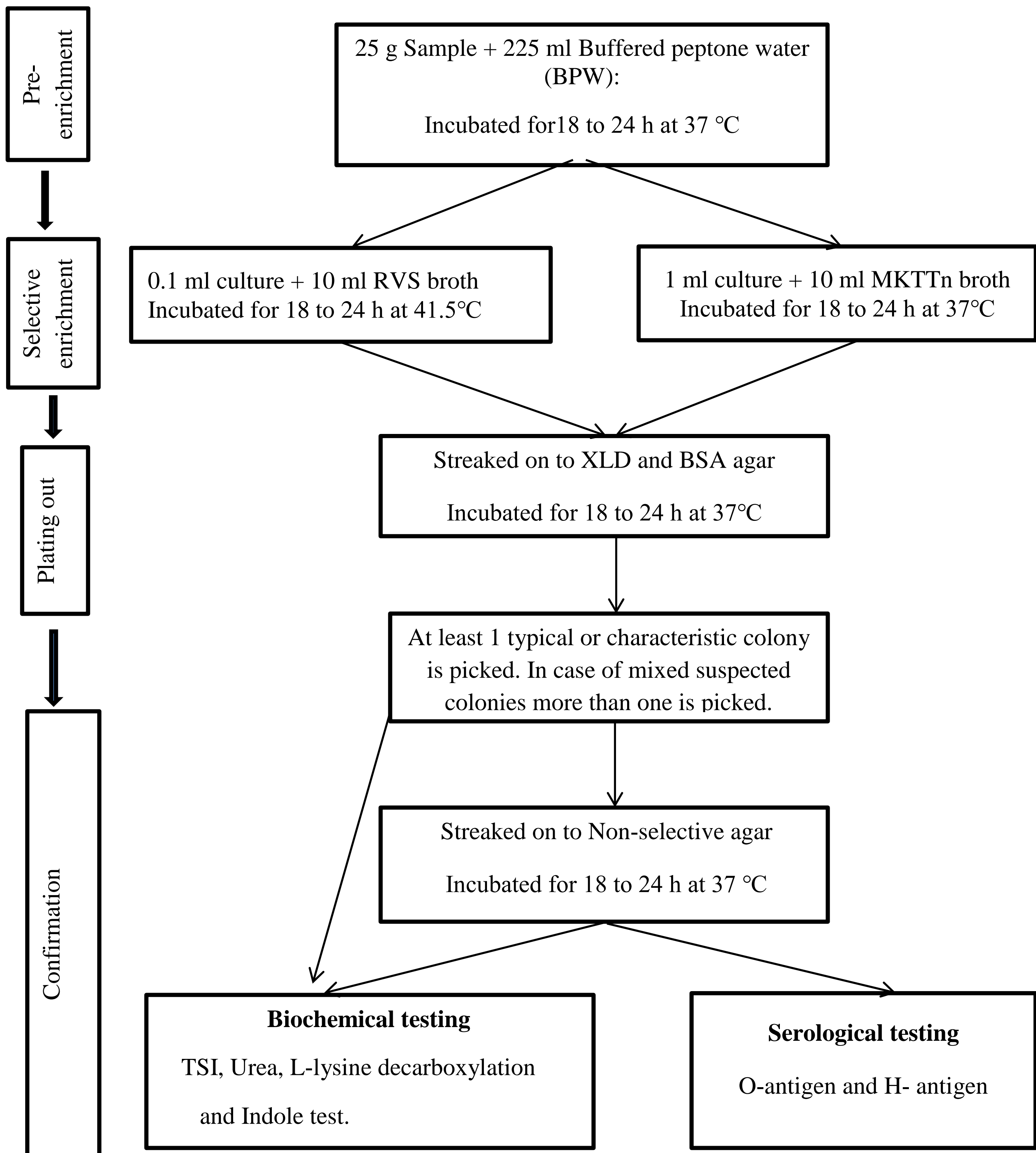
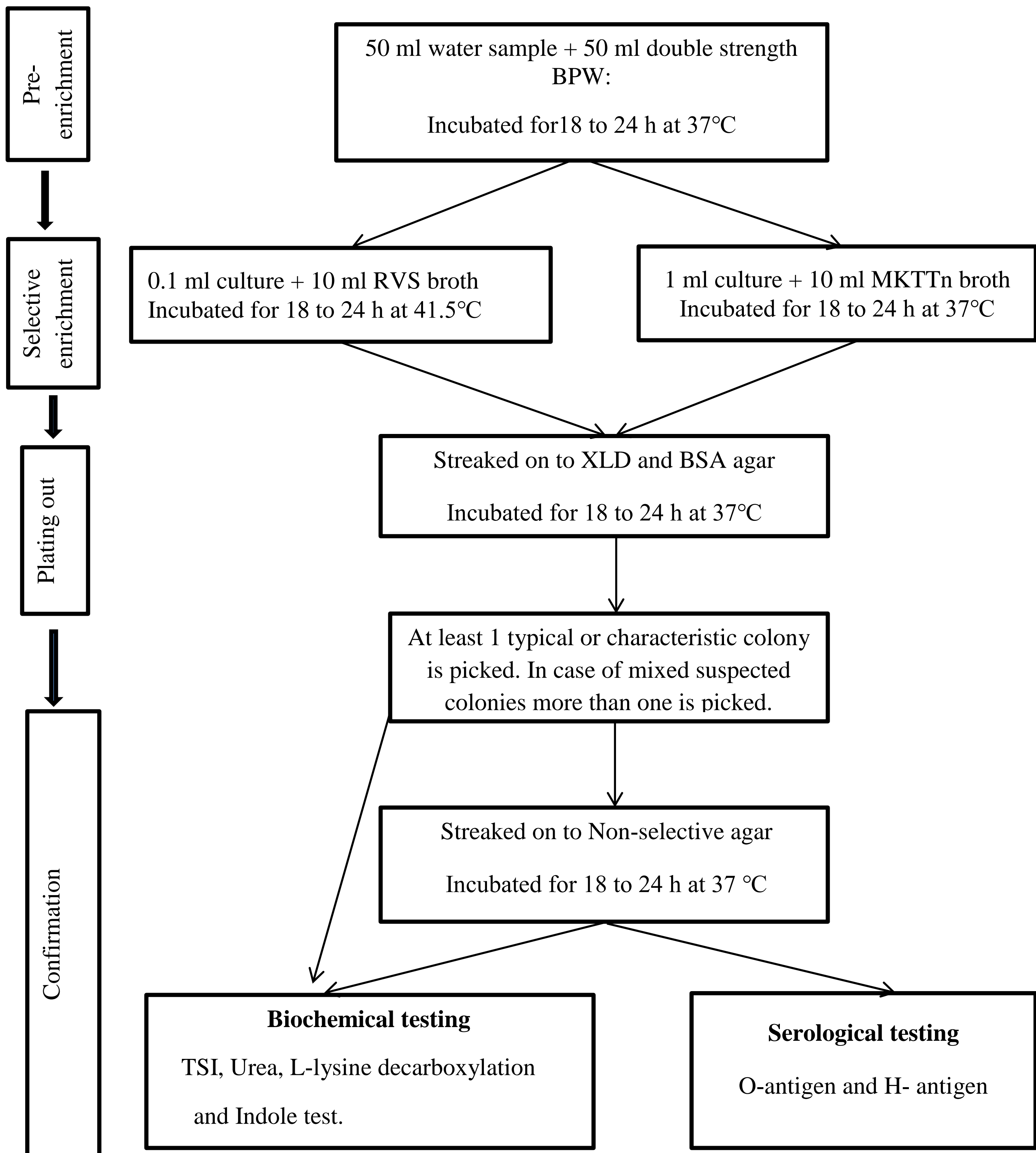


Diagram of procedure for detection of *Salmonella* in water



CHAPTER 7

Isolation and Identification of *Escherichia coli*

Femeena Hassan, Pankaj Kishore, Ranjit Kumar Nadella and Devananda Uchoi

Email: drfemeenahassan@gmail.com

Escherichia coli is one of the predominant species of facultative anaerobes in the human gut and usually harmless to the host; however, a group of pathogenic *E. coli* has emerged that causes diarrheal disease in humans. Referred to as Diarrheagenic *E. coli* or commonly as pathogenic *E. coli*, these groups are classified based on their unique virulence factors and can only be identified by these traits. Hence, analysis for pathogenic *E. coli* often requires that the isolates be first identified as *E. coli* before testing for virulence markers. The pathogenic groups includes enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and perhaps others that are not yet well characterized. Of these, only the first 4 groups have been implicated in food or water borne illness.

ETEC is recognized as the causative agent of travelers' diarrhea and illness is characterized by watery diarrhea with little or no fever. ETEC infections occurs commonly in under-developed countries but, in the U.S., it has been implicated in sporadic waterborne outbreaks as well as due to the consumption of soft cheeses, Mexican-style foods and raw vegetables. Pathogenesis of ETEC is due to the production of any of several enterotoxins. ETEC may produce a heat-labile enterotoxin (LT) that is very similar in size (86 kDa), sequence, antigenicity, and function to the cholera toxin (CT). ETEC may also produce a heat stable toxin (ST) that is of low molecular size (4 kDa) and resistant to boiling for 30 min. There are several variants of ST, of which ST1a or STp is found in *E. coli* isolated from both humans and animals, while ST1b or STh is predominant in human isolates only. The infective dose of ETEC for adults has been estimated to be at least 10^8 cells; but the young, the elderly and the infirm may be susceptible to lower levels. Because of its high infectious dose, analysis for ETEC is usually not performed unless high levels of *E. coli* have been found in a food. Also, if ETEC is detected, levels should also be enumerated to assess the potential hazard of the contaminated food. Production of LT can be detected by Y-1 adrenal cell assays (28) or serologically by commercial reverse passive latex agglutination assay and ELISA. The production of ST can also be detected by ELISA or by infant mouse assay. Both LT

and ST genes have also been sequenced and PCR and gene probe assays are available. Analysis of colonies on plating media using gene probe/colony hybridization also allows enumeration of ETEC in foods.

EIEC closely resemble Shigella and causes an invasive, dysenteric form of diarrhea in humans. Like Shigella, there are no known animal reservoirs; hence the primary source for EIEC appears to be infected humans. Although the infective dose of Shigella is low and in the range of 10 to few hundred cells, volunteer feeding studies showed that at least 10^6 EIEC organisms are required to cause illness in healthy adults. Unlike typical *E. coli*, EIEC are non-motile, do not decarboxylate lysine and do not ferment lactose, so they are anaerogenic. Pathogenicity of EIEC is primarily due its ability to invade and destroy colonic tissue. The invasion phenotype, encoded by a high molecular weight plasmid, can be detected by invasion assays using HeLa or Hep-2 tissue culture cells or by PCR and probes specific for invasion genes.

EPEC causes a profuse watery diarrheal disease and it is a leading cause of infantile diarrhea in developing countries. EPEC outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Through volunteer feeding studies the infectious dose of EPEC in healthy adults has been estimated to be 10^6 organisms. Pathogenesis of EPEC involves intimin protein (encoded by eae gene) that causes attachment and effacing lesions; but it also involves a plasmid-encoded protein referred to as EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells. Production of EAF can be demonstrated in Hep-2 cells and the presence of eae gene can be tested by PCR assays.

EHEC are recognized as the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome (HUS). EHEC are typified by the production of verotoxin or Shiga toxins (Stx). Although Stx1 and Stx2 are most often implicated in human illness, several variants of Stx1 and Stx2 exist. There are many serotypes of Stx-producing *E. coli* (STEC), but only those that have been clinically associated with HC are designated as EHEC. Of these, O157:H7 is the prototypic EHEC and most often implicated in illness worldwide. The infectious dose for O157:H7 is estimated to be 10 - 100 cells; but no information is available for other EHEC serotypes. EHEC infections are mostly food or water borne and have implicated undercooked ground beef, raw milk, cold sandwiches, water, unpasteurized apple juice and sprouts and vegetables. EHEC O157:H7 are phenotypically distinct from *E. coli* in that they exhibit slow or no fermentation of sorbitol and do not have glucuronidase activity; hence, these traits are often used to isolate this pathogen from foods. The production of

Stx1 and Stx2 can be tested by cytotoxicity assays on vero or HeLa tissue culture cells or by commercially available ELISA or RPLA kits. Gene probes and PCR assays specific for stx1 and stx2 and other trait EHEC markers are also available.

Enrichment for Pathogenic *E. coli*

The approach recommended here permits qualitative determination of the presence of pathogenic *E. coli*. Aseptically weigh 25 g of sample into 225 ml of BHI broth (dilution factor of 1:10). If necessary, sample size may deviate from 25 g depending on availability of the sample, as long as the diluent is adjusted proportionally. Blend or stomach briefly. Incubate the homogenate for 10 min at room temperature with periodic shaking then allow the sample to settle by gravity for 10 min. Decant medium carefully into a sterile container and incubate for 3 h at 35°C to resuscitate injured cells. Transfer contents to 225 mL double strength TP broth in a sterile container and incubate 20 h at 44.0 ± 0.2°C. After incubation, streak to L-EMB and MacConkey agars. Incubate these agars for 20 h at 35°C.

Selection of colonies

Typical lactose-fermenting colonies on L-EMB agar appear dark centered and flat, with or without metallic sheen. Typical colonies on MacConkey agar appear brick red. Lactose non-fermenting biotypes on both agars produce colorless or slightly pink colonies.

NOTE: EIEC do not ferment lactose and there may also be atypical non-lactose fermenting strains in the other pathogenic *E. coli* groups; therefore, as many as 20 colonies (10 typical and 10 atypical) should be picked for further characterization.

Conventional Biochemical Screening and identification

Use the IMViC procedures for biochemical and morphological identification of *E. coli*. However, because many enteric bacteria can also grow in the TP enrichment broth, plus anaerogenic, non-motile and slow or lactose non-fermenting strains of *E. coli* must also be considered, additional tests may need to be performed. Some of these new or modified reactions are discussed here.

Primary screening. Transfer suspicious colonies to TSI agar, BAB slant, tryptone broth, arabinose broth, and urea broth. Incubate 20 h at 35°C. Reject H_2S-positive, urease-positive, arabinose non-fermenting, and indole-negative strains. To test for the ONPG reaction, suspend growth from TSI in 0.85% saline to give detectable turbidity. Add an ONPG-impregnated disk and incubate 6 h at 35°C. Yellow color indicates positive reaction. Reject ONPG-negative,

aerogenic cultures. Some Alkalescens-Dispar strains (i.e., anaerogenic *Escherichia*) are ONPG-negative.

Secondary screening (48 h incubation at 35°C unless otherwise specified). To identify cultures, test additional reactions shown in Table 1, Chapter 4, to subdivide *Escherichia* spp. Since it is not known whether these additional species are of pathogenic significance to humans, strains giving typical reactions for *E. coli* should be further investigated. To differentiate *E. coli* from *Shigella*, examine anaerogenic, non-motile, slow lactose fermenters for lysine decarboxylase, mucate, and acetate reactions. *Shigella sonnei*, which may grow in the same enrichment conditions, is anaerogenic and non-motile. It also produces a negative indole reaction and shows slow or non-fermentation of lactose. Alternatively, use API20E or the automated VITEK biochemical assay to identify the organism as *E. coli*.

Tests for Enterotoxigenic *E. coli* (ETEC)

When *E. coli* levels in foods exceed 10^4 cells/g, perform enumeration for ETEC by colony hybridization analysis using DNA probes for LT and ST. If biological activity assays are necessary, LT can be detected by the Y-l tissue culture test and ST can be detected by the infant mouse test. There are also commercially available RPLA and ELISA tests to detect LT and ST toxins as well as PCR assays.

Tests for Enteroinvasive *E. coli* (EIEC)

If an isolate is suspected to be EIEC, the invasive potential of the isolates may be tested by the Sereny test or the Guinea pig keratoconjunctivitis assay. Invasive potential of the isolates can also be determined by the HeLa tissue culture cell assay as described, or with the *in vitro* staining technique using acridine orange to stain intracellular bacteria in HeLa monolayers. Alternatively, since the *invA* gene sequence of EIEC closely resembles that of *Shigella*, DNA probe and PCR assays for *inv* gene of *Shigella* will also work for EIEC.

Tests for Enteropathogenic *E. coli* (EPEC)

EPEC strains are identified based on 3 key traits: attachment and effacing lesion (A/E), localized adherence on cells and the lack of Shiga toxin (*Stx*) production. This last trait is also used to distinguished strains of EPEC from EHEC. Phenotypically, A/E and localized adherence are tested using Hep-2 or HeLa tissue cells. Absence of Stx can be determined using tests outlined for EHEC

(see below). There are also PCR and probes for the EAF plasmid that encodes for localized adherence and the *eae* gene that encodes for the intimin that causes the A/E phenotype.

Caution: There are several variants of *eae* gene and some EPEC strains carry *eae* variants identical to EHEC serotypes; hence, these tests will detect strains from both pathogenic groups.

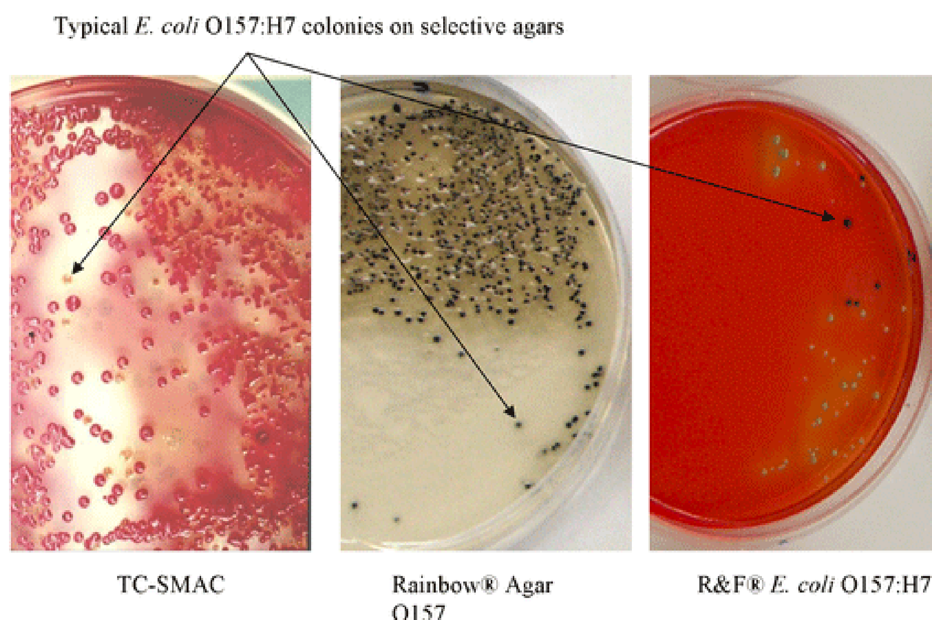
O157:H7 - Cultural Isolation and Presumptive Isolate Screening

For overnight enrichment samples that are found probable positive by the real-time PCR assay, cultural confirmation is required. Similarly, for samples that have not been screened by real-time PCR follow these procedures for culture isolation.

Isolation procedure.

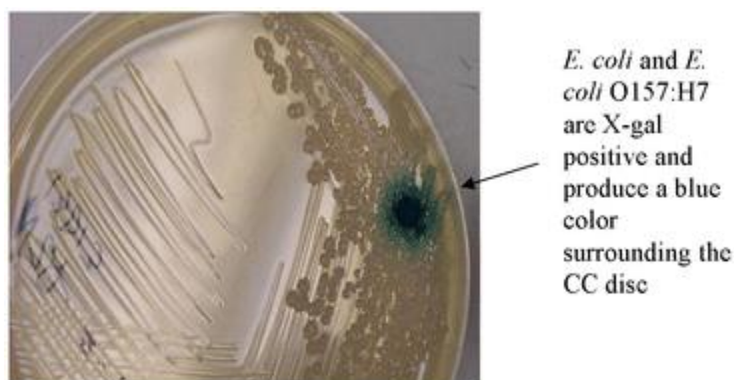
Serially dilute the overnight sample enrichment in phosphate buffer and spread-plate appropriate dilutions (usually 0.05 mL of 10^{-2} and 10^{-4} dilutions should yield approximately 100-300 isolated colonies) in duplicate onto TC-SMAC and one chromogenic agar (Rainbow® Agar O157 or R&F®*E. coli* O157:H7 agar). Optionally, a streak plate may also be included. Incubate plates at 37°C ± 1°C for 18 - 24 h. On TC-SMAC. typical O157:H7 colonies are colorless or neutral/gray with a smoky center and 1-2 mm in diameter. Sorbitol-fermenting bacteria such as most *E. coli* appear as pink to red colonies. On Rainbow® Agar O157 or R&F®*E. coli* O157:H7 agar, *E. coli* O157H7 colonies should appear as black to blue-black colonies.

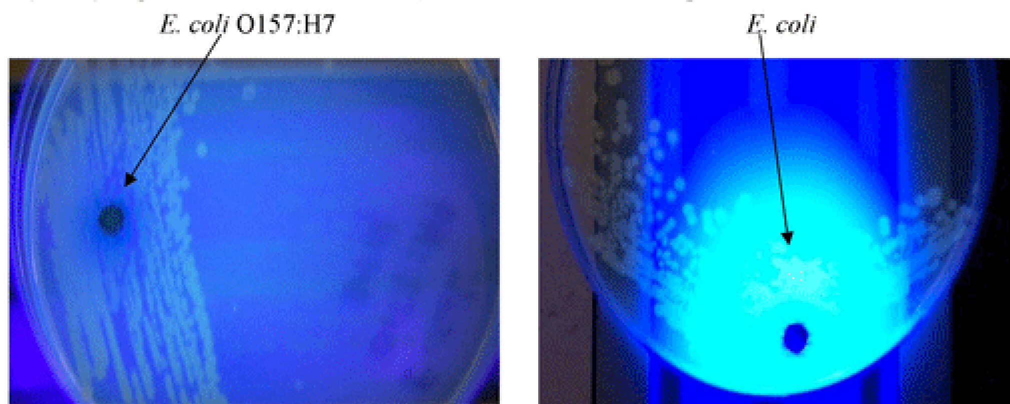
Figure: Appearance of typical *E. coli* O157:H7 on TC-SMAC, Rainbow® Agar O157 and R&F® *E. coli* O157:H7 agars.



Screen typical colonies by picking a portion of each isolated suspect colony from the isolation agar and testing for O157 antigen by latex agglutination (Remel kit). Pick all typical colonies that screen positive (up to 10, if >10 are present) from isolation agars and streak onto TSAYE plates to check for purity. Place a ColiComplete (CC) disc (BioControl, Bellevue, WA) in the heaviest streak area on the TSAYE plate. Prepare a similar TSAYE plate using a known MUG-positive *E. coli* strain as positive control. Incubate the plates 18-24 h at 37°C ± 1°C. CC has a chromogenic assay for galactopyranosidase (X-gal) and a fluorogenic assay for glucuronidase (MUG) on the same disc. The positive control should show blue color on and around the disc (indicative for coliforms) and blue fluorescence around the disc under long wave UV (365 nm) light (indicative of *E. coli*). Strains of O157:H7 are X-gal (+) but MUG (-).

Figure: Results of ColiComplete (CC) disc for *E. coli* and *E. coli* O157:H7





Spot Indole Test: Spot growth from TSAYE plate to a filter wetted with Kovac's reagent.
E. coli O157:H7 are indole positive.

CHAPTER 8

Isolation and Identification of *Listeria monocytogenes*

Pankaj Kishore, Ranjit Kumar Nadella and Devananda Uchoi

Email: pkishore2007@gmail.com

Introduction

Listeria is an important food-borne pathogen which affects the elderly, pregnant women, neonates and immuno-compromised populations. Listeria monocytogenes remains the main pathogenic species of the genus listeria. The genus Listeria comprises up to 21 species. L. monocytogenes, was originally described in 1926, but assigned the name in 1940. *Listeria monocytogenes* can be found in moist environments, soil, water, decaying vegetation and animals including fish. This can survive and even grow under refrigeration. When people eat food contaminated with *L. monocytogenes*, they may develop a disease called listeriosis. *L. monocytogenes* is generally transmitted when food is harvested, processed, prepared, packed, transported or stored in environments contaminated.

Listeria is a Gram-positive, facultative intracellular bacterial pathogen with the ability to adapt to a wide range of conditions of temperatures (2–4 °C), acidity and high-salt concentration. *Listeria* cells are slow growers and may be rapidly outgrown by competitors.

Isolation and characterization (BAM)

Qualitative detection from foods and environmental samples:

a. Individual subsample analysis: For solids, semi-solids, or liquids add 25 g representative portion to 225 ml BLEB containing pyruvate without selective additives (basal BLEB). Thoroughly homogenise the samples.
b. Aseptically add the three filter sterilized selective agents to achieve final concentrations of 10 mg/L acriflavin, 50 mg/L cycloheximide and 40 mg/L sodium nalidixic acid in the BLEB pre-enrichments.
c. Incubate at 30°C for 4 h.
d. Mix the enrichment with additives and continue incubation at 30°C for the remainder of the 24 to 48 h enrichment period.
e. A 50 g portion of the sample should be reserved for possible pathogen enumeration. Store it at 5°C if it is not frozen or, if frozen, in a non-defrosting freezer.

f. At 24 h and 48 h, streak BLEB enrichments onto one esculin-based and one chromogenic selective agar from each of the categories listed in Sections G.1.A and G.1.B. Incubate plates for up to 48 h. Check plates at both 24 h and 48 h.
g. Oxford agar (OXA) (18) (M118): After 24 h incubation at 35°C typical Listeria species colonies are approximately 1 mm diameter, gray to black colonies surrounded by a black halo. Following 48 h incubation typical Listeria species colonies are approximately 2-3 mm diameter, black with a black halo and sunken center. b. PALCAM (50) (M138a): Incubation conditions and appearance of Listeria species colonies are the same as for Oxford agar except that the background plate color is red. c. Modified Oxford Agar (MOX) (46) (M103a): Incubation conditions and appearance of Listeria species colonies are the same as for Oxford agar. CHROMagar™ Listeria (M40a): Incubation conditions and appearance of Listeria colonies are the same as for Agar Listeria according to Ottaviani and Agosti except that the background plate color is light blue (agars is indicative of phosphatidylinositol-specific phospholipase C (PI-PLC) activity. On these agars Listeria species with PI-PLC activity, L. monocytogenes and L. ivanovii, will appear blue-green and all other Listeria species will not develop the blue-green color and remain white in appearance. In the case of Agar Listeria according to Ottaviani and Agosti and CHROMagar™ the presence of a Listeria species is based on a specific β-glucosidase enzyme activity detected by the chromogen, therefore, all Listeria species will appear blue-green on these agars. The phospholipase activity specific for L. monocytogenes and L. ivanovii is determined by the additional opaque white halo surrounding the colony).
h. 2. Select up to 5 typical colonies from each esculin based agar and streak for purity to TSAye (M153) and incubate plates at 30°C for 24 to 48 h. Select up to 2 typical colonies for streaking if using L. monocytogenes-L. ivanovii differential chromogenic agars. The plates may be incubated at 35°C if colonies will not be used for wet-mount motility observations.
i. If isolated colonies are available use remaining colony growth to stab a 5% sheep blood agar (M135) plate. Incubate at 35°C for 24 to 48 h.

Table 1. Differentiation of Listeria species

Species	Mannitol	Rhamnose	Xylose	Virulence[a]	β-Hemolysis[b]
L. monocytogenes	-	+	-	+	+
L. ivanovii	-	-	+	+	+
L. innocua	-	V	-	-	-
L. welshimeri	-	V	+	-	-
L. seeligeri	-	-	+	-	+
L. grayi	+	V	-	-	-

a Mouse test

b Sheep blood agar stab

CAMP (Christie-Atkins-Munch-Peterson) test:

- Streak weakly β-hemolytic S. aureus (FDA strain ATCC 49444 (CIP 5710; NCTC 7428) or ATCC 25923) and R. equi (ATCC 6939; NCTC 1621) vertically on sheep blood agar.
- Separately streak test strains horizontally between the S. aureus and R. equi streaks without quite touching them. Incubate plate 24 to 48 h at 35°C.
- Examine plates for hemolysis in the zone of influence of the vertical streaks. Hemolysis of L. monocytogenes and L. seeligeri is enhanced near the S. aureus streak; L. ivanovii hemolysis is enhanced near the R. equi streak. Other species are non-hemolytic and do not react in this test

Buffered Listeria Enrichment Broth (BLEB):

Media Base

Trypticase soy broth	30 g
Yeast extract	6 g
Monopotassium phosphate (anhydrous)	1.35 g/liter
Disodium phosphate (anhydrous)	9.6 g/liter
Sodium Pyruvate (Sodium salt)	1.11 g/liter
Distilled water	1 liter

Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.1.

Selective Supplements

Acriflavin HCl	10 mg/liter
Nalidixic acid (sodium salt)	40 mg/liter
Cycloheximide	50 mg/liter

Oxford Medium:

Columbia blood agar base	39.0 g
Esculin	1.0 g
Ferric ammonium citrate	0.5 g
Lithium chloride	15.0 g
Cycloheximide	0.4 g
Colistin sulfate	0.02 g
Acriflavin	0.005 g
Cefotetan	0.002 g
Fosfomycin	0.010 g
Distilled water	1 liter

Sterilize by autoclaving at 121°C for 15 min

PALCAM Listeria Selective Agar:

Basal medium

Peptone	23 g
Starch	1 g
NaCl	5 g
Columbia agar	13 g
Mannitol	10 g
Ferric ammonium citrate	0.5 g
Esculin (aesculin)	0.8 g
Dextrose (glucose)	0.5 g
Lithium chloride	15.0 g
Phenol red	0.08 g
Distilled water	1000 ml

Sterilize by autoclaving at 121°C for 15 min.

Selective agents

Polymyxin B sulfate	10 mg
Acriflavin	5 mg
Ceftazidine	20 mg
Distilled water	2 ml

Modified Oxford Listeria Selective Agar:

Columbia Blood Agar Base (brand dependent)	39.0-44.0 g
Agar	2.0 g
Esculin	1.0 g
Ferric ammonium citrate	0.5 g
Lithium chloride (Sigma L0505 quality or equivalent)	15.0 g
Buffered colistin methane sulfonate (1 % w/v) solution	1.0 ml
Distilled water	1.0 L

Adjust pH to 7.2±0.1 if need be. Autoclave at 121° C for 10 min.

References:

Refer: https://www.microbiologyresearch.org/docserver/ fulltext/acmi/2/9/acmi000153.pdf?expires=1682128195&id=id&accname=guest&checksum=9AE0E616F28A348E80FD6DB4B469609D

Refere https://www.sciencedirect.com/topics/engineering/listeria

CHAPTER 9

Isolation and Identification of *Bacillus* sp. and *Clostridium* sp.

Ranjit Kumar Nadella, Pankaj Kishore and Devananda Uchoi

Email: nranjeetkumar@gmail.com

Inroduction

Bacillus (Latin "stick") is a genus of Gram-positive, rod-shaped bacteria, a member of the phylum Bacillota, with 266 named species. The term is also used to describe the shape (rod) of other so-shaped bacteria; and the plural Bacilli is the name of the class of bacteria to which this genus belongs. Bacillus species can be either obligate aerobes which are dependent on oxygen, or facultative anaerobes which can survive in the absence of oxygen. Cultured Bacillus species test positive for the enzyme catalase if oxygen has been used or is present. Bacillus can reduce themselves to oval endospores and can remain in this dormant state for years. The endospore of one species from Morocco is reported to have survived being heated to 420 °C. Endospore formation is usually triggered by a lack of nutrients: the bacterium divides within its cell wall, and one side then engulfs the other. They are not true spores (i.e., not an offspring). Endospore formation originally defined the genus, but not all such species are closely related, and many species have been moved to other genera of the Bacillota. Only one endospore is formed per cell. The spores are resistant to heat, cold, radiation, desiccation, and disinfectants. Bacillus anthracis needs oxygen to sporulate; this constraint has important consequences for epidemiology and control. In vivo, B. anthracis produces a polypeptide (polyglutamic acid) capsule that kills it from phagocytosis.

The genera Bacillus and Clostridium constitute the family Bacillaceae. Species are identified by using morphologic and biochemical criteria. Because the spores of many Bacillus species are resistant to heat, radiation, disinfectants, and desiccation, they are difficult to eliminate from medical and pharmaceutical materials and are a frequent cause of contamination. Not only are they resistant to heat, radiation, etc., but they are also resistant to chemicals such as antibiotics. This resistance allows them to survive for many years and especially in a controlled environment. Bacillus species are well known in the food industries as troublesome spoilage organisms. Ubiquitous in nature, Bacillus includes symbiotic (sometimes referred to as endophytes) as well as independent species. Two parasitic pathogenic species are medically significant: B. anthracis causes anthrax; and B. cereus causes food poisoning.

Clostridium is a genus of anaerobic, Gram-positive bacteria. Species of Clostridium inhabit soils and the intestinal tract of animals, including humans. This genus includes several significant human pathogens, including the causative agents of botulism and tetanus. It also formerly included

an important cause of diarrhea, Clostridioides difficile, which was reclassified into the Clostridioides genus in 2016.

Clostridium species are readily found inhabiting soils and intestinal tracts. *Clostridium* species are also a normal inhabitant of the healthy lower reproductive tract of females.

The main species responsible for disease in humans are:

- *Clostridium botulinum* can produce botulinum toxin in food or wounds and can cause botulism. This same toxin is known as Botox and is used in cosmetic surgery to paralyze facial muscles to reduce the signs of aging; it also has numerous other therapeutic uses.
- *Clostridium perfringens* causes a wide range of symptoms, from food poisoning to cellulitis, fasciitis, necrotic enteritis and gas gangrene.
- *Clostridium tetani* causes tetanus.

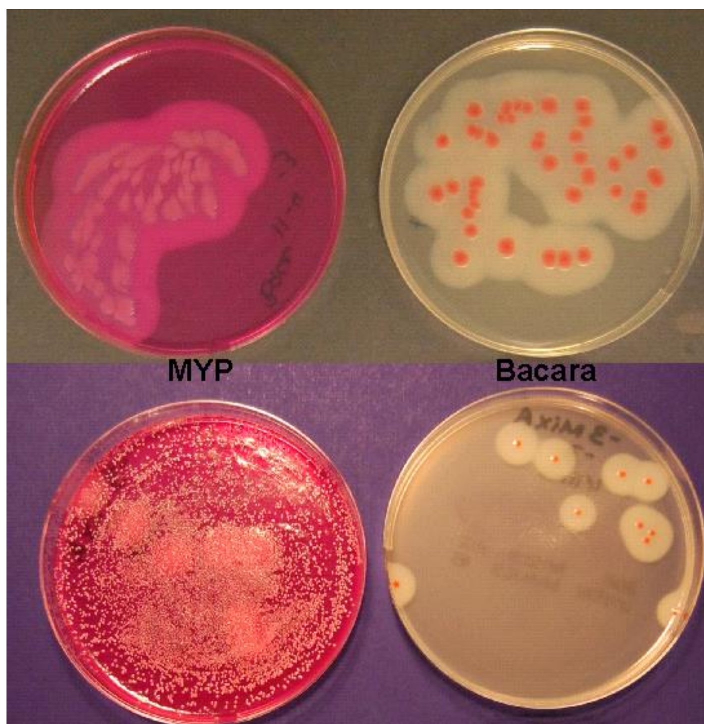
Several more pathogens were previously described in *Clostridium*, but have been moved to other genera with additional research

- *Clostridium difficile*, now placed in *Clostridioides*.
- *Clostridium histolyticum*, now placed in *Hathewaya*.
- *Clostridium sordellii*, now placed in *Paeniclostridium*, can cause a fatal infection in exceptionally rare cases after medical abortions.

Bacillus cereus

Bacillus cereus is an aerobic spore-forming bacterium that is commonly found in soil, on vegetables, and in many raw and processed foods. B. cereus food poisoning may occur when foods are prepared and held without adequate refrigeration for several hours before serving, with B. cereus reaching >106 cells/g. Foods incriminated in past outbreaks include cooked meat and vegetables, boiled or fried rice, vanilla sauce, custards, soups, and raw vegetable sprouts. Two types of illness have been attributed to the consumption of foods contaminated with B. cereus. The first and better known is characterized by abdominal pain and non-bloody diarrhea; it has an incubation period of 4-16 h following ingestion with symptoms that last for 12-24 h. The second, which is characterized by an acute attack of nausea and vomiting, occurs within 1-5 h after consumption of contaminated food; diarrhea is not a common feature in this type of illness1. The MYP agar has been the standard media for plating B. cereus, but it has little selectivity so background flora is not inhibited and can mask the presence of B. cereus. Bacara is a chromogenic selective and differential agar that promotes the growth and identification of B. cereus, but inhibits

the growth of background flora. The chromogenic agar has been suggested for the enumeration of B. cereus group as a substitute for MYP1,2. Typical colonies will grow as pink-orange uniform colonies surrounded by a zone of precipitation. The identification would include all species from the *B. cereus group: B. cereus, B. thuringiensis, B. anthracis,B. mycoides*, and *B. weihenstephanensis*. Biochemical testing will be necessary to delineate to the species level.



Colonies of B. cereus grown on MYP are pink and lecithinase positive, but other bacteria are not inhibited and can interfere with isolation of B. cereus. Colonies of B. cereus grown on Bacara are pink-orange and are lecithinase positive, but other organisms are inhibited.

B. cereus isolates can be identified by 1) produce large Gram-positive rods with spores that do not swell the sporangium; 2) produce lecithinase and do not ferment mannitol on MYP agar; 3) grow and produce acid from glucose anaerobically; 4) reduce nitrate to nitrite (a few strains may be negative); 5) produce acetylmethylcarbinol (VP-positive); 6) decompose L-tyrosine; and 7) grow in the presence of 0.001% lysozyme.

Bacillus anthracis

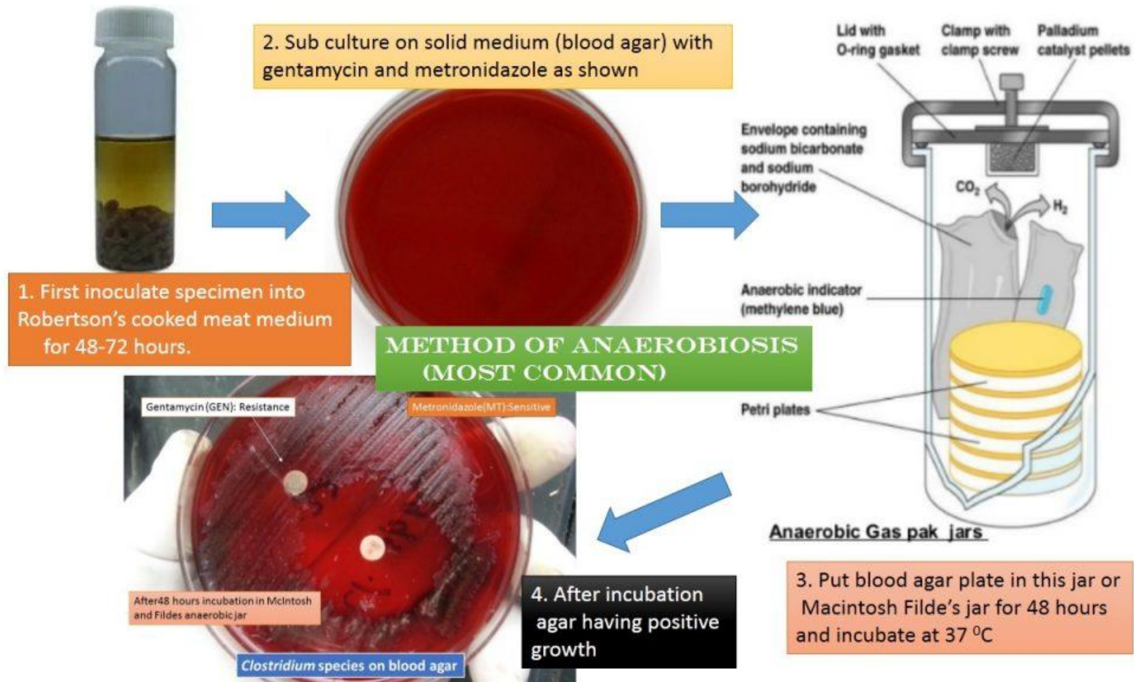
Anthrax is primarily a disease of herbivores. Bacillus anthracis has long been considered a potential agent for biological warfare or bioterrorism. Source: close contact with infected animals

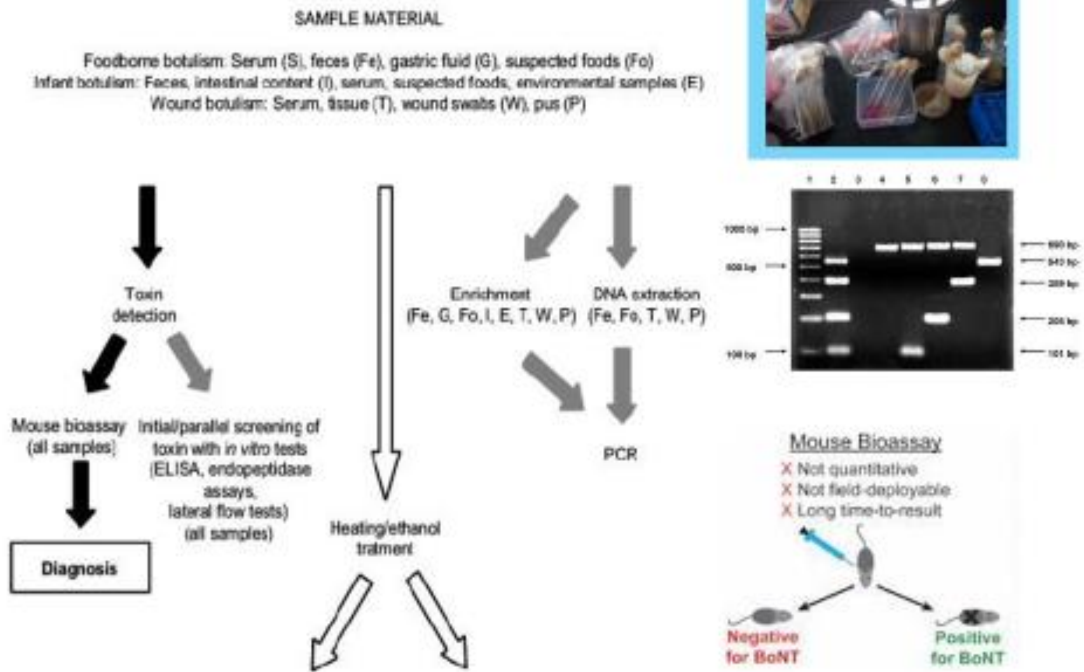
or their carcasses after death. It also causes Meningitis. The symptoms of oropharyngeal infections are fever, toxemia, inflammatory lesions in the oral cavity and oropharynx, cervical lymph node enlargement, and edema, and there is a high case-fatality rate.

Clostridium botulinum

Clostridium botulinum is an anaerobic, rod-shaped sporeforming bacterium that produces a protein with characteristic neurotoxicity. Under certain conditions, these organisms may grow in foods producing toxin(s). Botulism, a severe form of food poisoning results when the toxin-containing foods are ingested. Although this food illness is rare, its mortality rate is high; the 962 recorded botulism outbreaks in the United States from 1899 to 1990 involved 2320 cases and 1036 deaths. In outbreaks in which the toxin type was determined, 384 were caused by type A, 106 by type B, 105 by type E, and 3 by type F. In two outbreaks, the foods implicated contained both types A and B toxins. Due to a limited number of reports, type C and D toxins have been questioned as the causative agent of human botulism. It is suspected that these toxins are not readily absorbed in the human intestine. However, all types except F and G, which have not been as studied thoroughly, are important causes of animal botulism. *C. botulinum* is widely distributed in soils and in sediments of oceans and lakes. The finding of type E in aquatic environments by many investigators correlates with cases of type E botulism that were traced to contaminated fish or other seafoods. Types A and B are most commonly encountered in foods associated with soil contamination. In the United States, home-canned vegetables are most commonly contaminated with types A and B, but in Europe, meat products have also been important vehicles of foodborne illness caused by these types.

The mouse bioassay is a functional assay that detects biologically active toxin. The assay requires a three part approach: toxin screening, toxin titer, and finally toxin neutralization using monovalent antitoxins. The process requires two days of analysis at each step. Recently, rapid, alternative, in-vitro procedures have been developed for the detection of types A, B, E, and F botulinal toxin producing organisms and their toxins. The toxins generated in culture media can be detected using ELISA techniques such as the DIG-ELISA and the amp-ELISA. Biologically active and non-active toxins are detected since the assay detects the toxin antigen. The ELISA assays require one day of analysis. The toxin genes of viable organisms can be detected using the polymerase chain reaction technique and require one days of analysis after overnight incubation of botulinal spores or vegetative cells. In-vitro assays that are positive are confirmed using the mouse bioassay.


2. Sub culture on solid medium (blood agar) with gentamycin and metronidazole as shown
1. First inoculate specimen into Robertson's cooked meat medium for 48-72 hours.
METHOD OF ANAEROBIOSIS (MOST COMMON)
Lid with O-ring gasket
Clamp with clamp screw
Palladium catalyst pellets
Envelope containing sodium bicarbonate and sodium borohydride
CO_2
H_2
Anaerobic indicator (methylene blue)
Petri plates
Anaerobic Gas pak jars
Gentamycin (GEN): Resistance
Metronidazole(MT):Sensitive
After48 hours incubation in McIntosh and Fildes anaerobic jar
Clostridium species on blood agar
4. After incubation agar having positive growth
3. Put blood agar plate in this jar or Macintosh Filde's jar for 48 hours and incubate at 37 °C



Confirmation of Clostridium botulinum toxin by PCR assay and mouse bioassay

CHAPTER 10

Isolation and Identification of *Yersinia enterocolitica*

Pankaj Kishore, Ranjit Kumar Nadella and Devananda Uchoi

Email: pkishore@gmail.com

Introduction:

Yersinia enterocolitica is a particularly versatile food-borne pathogen which can survive in many habitats both within and external to host animals. Pathogenic Yersinia enterocolitica organisms are significant causes of human disease in many parts of the world including India. The association of human illness with consumption of Y. enterocolitica from contaminated food, animal wastes, and unchlorinated water is well reported. This organism may survive and grow during refrigerated storage. Because contamination is possible at the manufacturing site or in the home, refrigerated foods are potential vehicles.

Yersinia is a genus in the family of Enterobacteriaceae, which are psychrotrophic, oxidase negative, catalase positive and non-lactose fermenting bacilli. Species of these groups are differentiated based on biochemical traits, such as urease test, motility test and sugar fermentation reactions. The molar percentage of G+C content within the genus Yersinia is consistent with that of Enterobacteriaeceae and ranges from 46 to 50 and is consistent with that for Enterobactericeae species. Yersiniae are gram negative, facultatively anaerobic, non-spore forming straight rods or coccobacilli. They are often more active biochemically at 25°C than 37°C; most of them are motile when grown below 30°C with peritrichous flagella.

The genus Yersinia comprises eleven species, with *Y. enterocolitica*, Y. pseudotuberculosis, Y. pestis Y. rohdei, Y. ruckeri, Y. frederiksenii, Y. intermedia, Y.kristensenii, Y. mollaretii, Y. bercovieri, and Y. aldovae. Among these, *Y. enterocolitica*, Y. pseudotuberculosis, Y. pestis, and Y. ruckeri are known pathogens for humans and animals. *Y. enterocolitica* and Y. pseudotuberculosis are enteropathogenic organisms distributed worldwide that share common modes of transmission mainly through food and water. Y. pestis is a causative agent of the bubonic plague. Y. ruckeri is considered as causative agent of red enteric mouth disease in Salmonids. The most prevalent symptoms of Yersinia infections are abdominal pain and fever. However, other gastro-intestinal disorders such as diarrhea, nausea, headache, and vomiting may also be associated with the illness. The incubation period is about 24-36 hours which goes to a periods of up to 11 days have been reported.

Isolation of Y. enterocolitica:

The following procedure may be used for isolation Yersinia from food, water, and environmental samples -

1. Aseptically weigh 25 g sample into 225 ml PSBB. Homogenize 30 s and incubate at 10°C for 10 days (Don't freeze the samples).
2. If high levels of Yersinia are suspected in product, 0.1 ml may be spread plated on CIN agar before incubating broth. Also transfer 1 ml homogenate to 9 ml 0.5% KOH in 0.5% saline, mix for 2-3 seconds, and spread-plate 0.1 ml on MacConkey and CIN agars.
3. Incubate agar plates at 30°C for 1-2 days.
4. On day 10, remove enrichment broth from incubator and mix well. Transfer one loop-full of enrichment to 0.1 ml 0.5% KOH in 0.5% saline and mix for 2-3 s. Successively streak one loopful to MacConkey plate and one loopful to CIN plate.
5. Transfer additional 0.1 ml enrichment to 1 ml 0.5% saline and mix 5-10 s before streaking, as above. Incubate agar plates at 30°C for 1-2 days.

Isolation of Yersinia

6. Examine CIN plates after 1 day incubation. Select small (1-2 mm diameter) colonies having deep red center with sharp border surrounded by clear colorless zone with entire edge.

Fig. : Y. enterocolitica on YSA (CIN) agar

7. Inoculate each selected colony into LAIA (Lysine arginine iron agar) slant, Christensen's urea agar slant, and bile esculin agar plate or slant by stabbing with inoculation needle. Incubate 48 h at RT. Isolates giving alkaline slant and acid butt, no gas and no H2S (KA− −) reaction in LAIA, which are also urease-positive, are presumptive Yersinia. Discard cultures that produce H_2S and/or any gas in LAIA or are urease-negative.

Table 1. Biochemical Characteristics[(a)] of Yersinia species

Reaction	*Y. enterocolitica*	*Y. pseudo-tuberculosis*	*Y. pestis*	*Y. intermedia*
Lysine	-	-	-	-
Arginine	-	-	-	-
Ornithine	+	+[(c)]	-	+
Motility at RT (22-26°C)	+	+	+	+
35-37°C	-	-	-	-
Urea	+	+	+	+
Mannitol	+	+	+	+
Sorbitol	+	+	-	+
Cellobiose	+	+	-	+
Adonitol	-	-	-	-
Simmons citrate	+/-	-	-	
Voges-Proskauer	+	+/-(+)	-	+/-
Indole	+	+/-	-	+
Salicin	+	+/-	+/-	+
Esculin	+	+/-	+	+
				+

Table 2. Biotype scheme for Y. enterocolitica

	Reaction for biotypes						
Biochemical test	**1A**	**1B**	**2**	**3**	**4**	**5**	**6**
Lipase	+	+	-	-	-	-	-
Esculin/salicin (24 h)	+/-	-	-	-	-	-	-
Indole	+	+	(+)	-	-	-	-
Xylose	+	+	+	+	-	V	+
Trehalose	+	+	+	+	+	-	+
Pyrazinamidase	+	-	-	-	-	-	+
β -D-Glucosidase	+	-	-	-	-	-	-

	Reaction for biotypes						
Biochemical test	**1A**	**1B**	**2**	**3**	**4**	**5**	**6**
Voges-Proskauer	+	+	+	+/-	+	(+)	-

Composition:

Peptone Sorbitol Bile Broth

Na_2HPO_4	8.23 g
$NaH_2PO_4 \cdot H_2O$	1.2 g
Bile salts No. 3	1.5 g
NaCl	5 g
Sorbitol	10 g
Peptone	5 g
Distilled water	1 liter

Autoclave 15 min at 121°C. Final pH, 7.6 ± 0.2.

Cefsulodin-Irgasan Novobiocin (CIN) Agar or Yersinia Selective Agar (YSA)

A. **Basal medium**

Special peptone	20 g
Yeast extract	2 g
Mannitol	20 g
Pyruvic acid (Na salt)	2 g
NaCl	1 g
$MgSO_4 \cdot 7H_2O$ (10 mg/ml)	1 ml
Agar	12 g
Distilled water	756 ml

B. **Irgasan (Ciba-Geigy) solution and Novobiocin** solution

Irgasan 0.40% in 95% ethanol	1 ml
Novobiocin, [0.25 mg/ml]	10 ml
Cefsulodin[1.5 mg/ml]	10 ml

- May be stored at -20°C up to 4 weeks.

CHAPTER 11

Isolation and Identification of *Shigella* spp.

Ranjit Kumar Nadella, Pankaj Kishore and Devananda Uchoi

Email: nranjeetkumar@gmail.com

Introduction

Shigellosis, although commonly regarded as waterborne, is also a foodborne disease restricted primarily to higher primates, including humans. It is usually spread among humans by food handlers with poor personal hygiene. Foods most often incriminated in the transmission have been potato salad, shellfish, raw vegetables, and Mexican dishes. First discovered over 100 years ago by a Japanese scientist Kiyoshi Shiga. Shigellae are members of the family Enterobacteriaceae, and are genetically identical *to Escherichia coli* (*E. coli*) and are closely related to *Salmonella* and *Citrobacter* spp. The genus *Shigella* consists of four species: *S. dysenteriae* (subgroup A), *S. flexneri* (subgroup B), *S. boydii* (subgroup C), and *S. sonnei* (subgroup D). *Shigella* organisms may be very difficult to distinguish biochemically from *Escherichia coli.* Brenner considers *Shigella* organisms and *E. coli* to be a single species, based on DNA homology.

Shigella species are Gram-negative, facultatively anaerobic, nonsporulating, nonmotile rods (0.3 to 1 μm) in diameter and 1 to 6 μm in length, appearing singly, in pairs and in chains in the family *Enterobacteriaceae*. They do not decarboxylate lysine or ferment lactose within 2 days. They utilize glucose and other carbohydrates, producing acid but not gas. However, because of their affinity to *E. coli*, frequent exceptions may be encountered, e.g., some biotypes produce gas from glucose and mannitol. Neither citrate nor malonate is used as the sole carbon source for growth, and the organisms are inhibited by potassium cyanide. Typically, species of Shigella are oxidase negative, acetate and mucate negative, and do not produce gas from glucose. Additionally, Shigella are Voges-Proskauer negative and methyl-red positive, nor produce H_2S and are arginine dehydrolase and urease negative. *S. dysenteriae* type 1 strains produce a potent toxin known as Shiga toxin (STX). Three biological activities associated with STX are cytotoxicity, enterotoxicity, and neurotoxicity.

TABLE 1 Characteristics of *Shigella* spp.

Species	Serogroup	Serotypes	Geographic distribution	Distinguishing characteristics
S. dysenteriae	A	15	Indian subcontinent, Africa, Asia, Central America	Produce shiga toxin, causes most severe dysentery, high mortality rate if untreated
S. flexneri	B	6	Most common isolate in developing countries	Less severe dysentery
S. boydii	C	19	Indian subcontinent, rarely isolated in developed countries	Biochemically identical to *S. flexneri*, distinguished by serology
S. sonnei	D	1[a]	Most common isolate in developed countries	Mildest form of shigellosis

Enrichment

Two approaches are provided for the recovery of *Shigella*. The first approach is a conventional culture method that involves the use of a specially formulated medium, *Shigella* broth. Novobiocin is added to provide a selective environment. Sample enrichments are incubated as described below, and streaked to MacConkey agar. Typical colonies are biochemically and serologically confirmed as *Shigella* spp. The second approach uses DNA hybridization. The enzyme DNA gyrase induces negative supercoiling into closed circular DNA. It has been reported, however, that novobiocin inhibits DNA gyrase. Thus, the use of novobiocin in *Shigella* broth may cause this medium to be incompatible with DNA hybridization for detecting *Shigella*.

Conventional culture method

a) Enrichment of *Shigella sonnei*. Aseptically weigh 25 g sample into 225 ml *Shigella* broth to which novobiocin (0.5 µg/ml) has been added. Hold suspension 10 min at room temperature and shake periodically. Pour supernatant into sterile 500 ml Erlenmeyer flask. Adjust pH, if necessary, to 7.0 ± 0.2 with sterile 1 N NaOH or 1 N HCl. Place flask in anaerobic jar, insert anaerobic gas generating pouch/sachet (use number recommended by the anaerobic jar manufacturer, according to the volume of the jar), insert an anaerobic indicator, and tighten the lid. Incubate jars at 44.0°C in a forced air incubator for 20 h. Agitate enrichment culture suspension and streak on a MacConkey agar plate. Incubate 20 h at 35°C.

b) Enrichment of other *Shigella* species. Proceed as above, but use novobiocin at 3.0 µg/ml and incubate anaerobically at 42.0°C in a forced air incubator.

25 g sample into 225 ml *Shigella* broth with
3.0 ug/ml novobiocin (*S.sonnei*: 0.5 ug/ml)

↓ Incubate supernatant: 44°C for 20h, anaerobically

Streak enrichment culture onto
MacConkey agar plate

↓ Incubate 35°C for 20 h

Examine plates for characteristic *Shigella* colonies.
Pick suspect colonies and confirm with

↓ Biochemical test

↓ Serological test

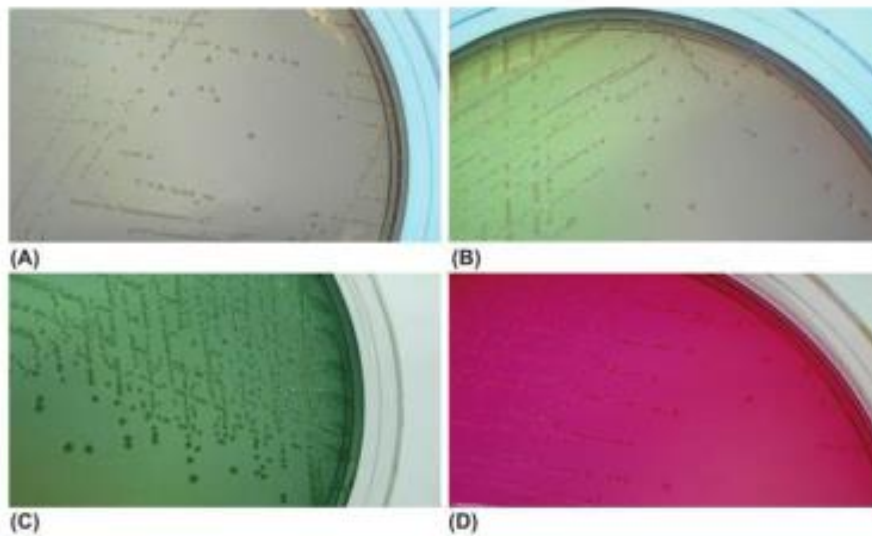
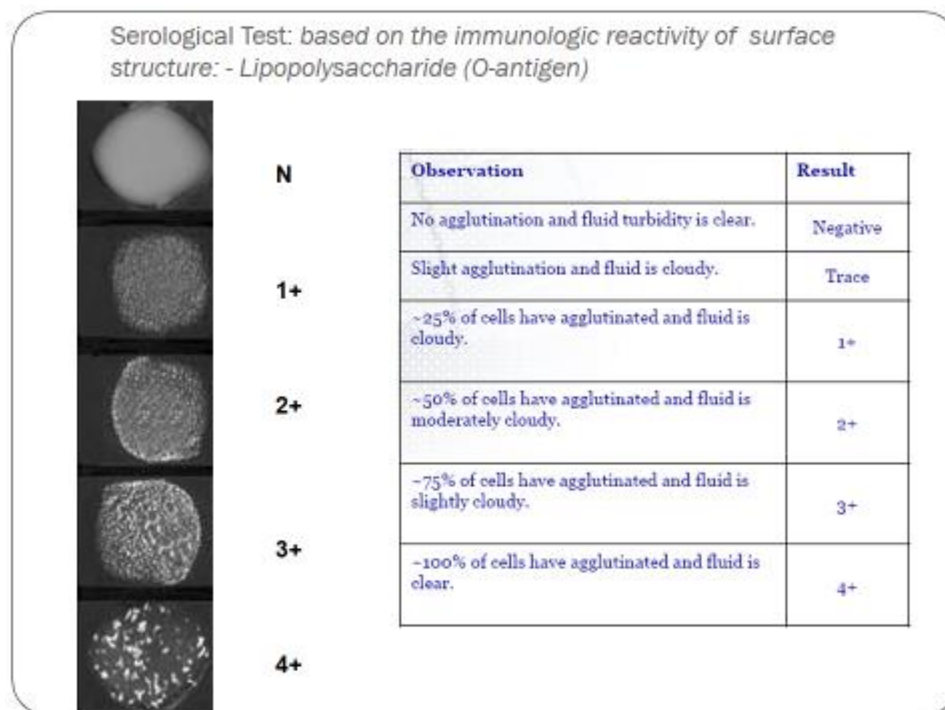


Figure 14.1 Appearance of *Shigella flexneri* colonies on: (A) MacConkey agar; (B) *Salmonella Shigella* (SS) agar; (C) Hektoen enteric (HE) agar; and (D) xylose lysine deoxycholate (XLD) agar plates.

Physiological characterization

Perform Gram stain and inoculate cultures giving satisfactory screening reactions to the other recommended biochemicals. The characteristics of *Shigella* are summarized as follows: Gram-negative rods; negative for H_2S, urease, glucose (gas), motility, lysine decarboxylase, sucrose, adonitol, inositol, lactose (2 days), KCN, malonate, citrate, and salicin; positive for methyl red. Pick isolates having positive reactions for *Shigella* to veal infusion agar slants. Use antisera for identification of serotype or compare with physiological behavior of the 32 serotypes. If serotype cannot be identified by these tests, two explanations are possible: 1) Several provisional serotypes have not been accepted by an international commission on the taxonomy of *Shigella* species. Proper interpretation of the mucate and acetate reactions should help. *Shigella* species tend to be negative in all these reactions, whereas anaerogenic *E. coli* tend to be positive in at least one of the reactions.



Observation	Result
No agglutination and fluid turbidity is clear.	Negative
Slight agglutination and fluid is cloudy.	Trace
~25% of cells have agglutinated and fluid is cloudy.	1+
~50% of cells have agglutinated and fluid is moderately cloudy.	2+
~75% of cells have agglutinated and fluid is slightly cloudy.	3+
~100% of cells have agglutinated and fluid is clear.	4+

Serological Identification of shigella sp.

CHAPTER 12

Isolation and identification of *Staphylococcus aureus*

Devananda Uchoi, Ranjit Kumar Nadella and Pankaj Kishore

Email: uchoidev514@gmail.com

Staphylococcus aureus is a Gram-positive spherically shaped bacterium, a member of the Bacillota, and is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin. It is often positive for catalase and nitrate reduction and is a facultative anaerobe that can grow without the need for oxygen. Although S. aureus usually acts as a commensal of the human microbiota, it can also become an opportunistic pathogen, being a common cause of skin infections including abscesses, respiratory infections such as sinusitis, and food poisoning. Pathogenic strains often promote infections by producing virulence factors such as potent protein toxins, and the expression of a cell-surface protein that binds and inactivates antibodies. S. aureus is one of the leading pathogens for deaths associated with antimicrobial resistance and the emergence of antibiotic-resistant strains, such as methicillin-resistant S. aureus (MRSA), is a worldwide problem in clinical medicine. Staphylococcus aureus is highly vulnerable to destruction by heat treatment and nearly all sanitizing agents. Thus, the presence of this bacterium or its enterotoxins in processed foods or on food processing equipment is generally an indication of poor sanitation. The presence of a large number of S. aureus organisms in a food may indicate poor handling or sanitation; however, it is not sufficient evidence to incriminate a food as the cause of food poisoning.

Methods used to detect and enumerate S. aureus depend on the reasons for testing the food and on the past history of the test material. Processed foods may contain relatively small numbers of debilitated viable cells, whose presence must be demonstrated by appropriate means. Analysis of food for S. aureus may lead to legal action against the party or parties responsible for a contaminated food.

Samples requiring enumeration of *S. aureus*

Add 225 ml of phosphate-buffered saline water to blender jar containing 25 g of sample and blend 2 min. This results in a dilution of 10^{-1}. Make dilutions of original homogenate promptly, using pipets that deliver required volume accurately. Prepare all decimal dilutions with 9 ml of sterile diluent plus 1 ml of previous dilution, unless otherwise specified. Shake all dilutions vigorously.

Not more than 15 min should elapse from the time sample is blended until all dilutions are in appropriate media.

Isolation and enumeration of *S. aureus*

For each dilution to be plated, aseptically transfer 1 ml sample suspension to 3 plates of Baird-Parker agar, distributing 1 ml of inoculum equitably to 3 plates (e.g., 0.4 ml, 0.3 ml, and 0.3 ml). Spread inoculum over surface of agar plate, using sterile bent glass streaking rod. Retain plates in upright position until inoculum is absorbed by agar (about 10 min on properly dried plates). If inoculum is not readily adsorbed, place plates upright in incubator for about 1 h. Invert plates and incubate 45-48 h at 35-37°C. Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of S. aureus.

Colonies of S. aureus are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasionally from various foods and dairy products, nonlipolytic strains of similar appearance may be encountered, except that surrounding opaque and clear zones are absent. Strains isolated from frozen or desiccated foods that have been stored for extended periods frequently develop less black coloration than typical colonies and may have rough appearance and dry texture.

Count and record colonies.

If several types of colonies are observed which appear to be S. aureus on selected plates, count number of colonies of each type and record counts separately. When plates of the lowest dilution contain <20 colonies, these may be used. If plates containing >200 colonies have colonies with the typical appearance of S. aureus and typical colonies do not appear at higher dilutions, use these plates for the enumeration of S. aureus, but do not count nontypical colonies. Select > 1 colony of each type counted and test for coagulase production. Add number of colonies on triplicate plates represented by colonies giving positive coagulase test and multiply by the sample dilution factor. Report this number as number of S. aureus/g of food tested.

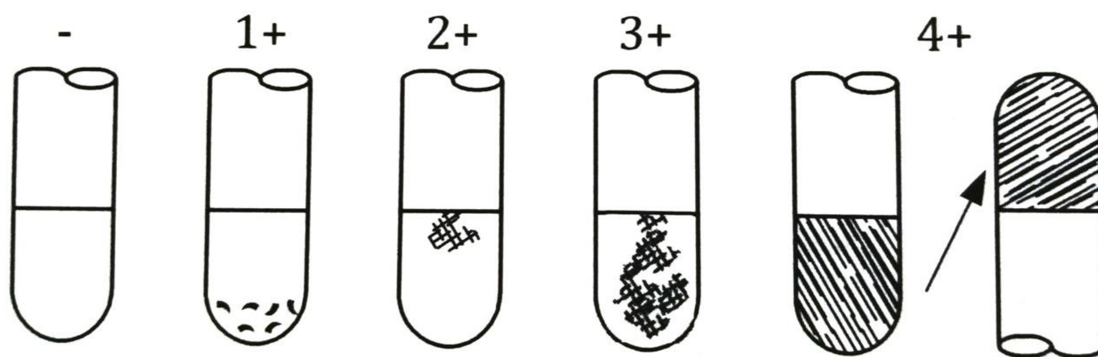
TYPICAL COLONY

ATYPICAL COLONY

TYPICAL COLONY CHARACTERISTICS	ATYPICAL COLONY CHARACTERISTICS
• Typical colonies are black or grey, shining, convex and are surrounded by a clear zone, which can be partially opaque.	• Shining black colonies with or without a narrow white edge; the clear zone is absent or barely visible and the opalescent ring is absent or hardly visible.
• 1 mm to 1.5 mm in dia after incubation for 24 h±**2** h and 1.5 mm to 2.5 mm dia after incubation for 48 ±4 h	• Atypical colonies have the same size as typical colonies.
• After incubation for at least 24 h, an opalescent ring immediately in contact with the colonies can appear in this clear zone.	• The colonies can be grey in colour free of clear zone.

Coagulase test

Transfer suspect S. aureus colonies into small tubes containing 0.2-0.3 ml BHI broth and emulsify thoroughly. Inoculate agar slant of suitable maintenance medium, e.g., TSA, with loopful of BHI suspension. Incubate BHI culture suspension and slants 18-24 h at 35-37°C. Retain slant cultures at room temperature for ancillary or repeat tests in case coagulase test results are questionable. Add 0.5 ml reconstituted coagulase plasma with EDTA (B-4, above) to the BHI culture and mix thoroughly. Incubate at 35-37°C and examine periodically over 6 h period for clot formation. Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for S. aureus. Partial clotting, formerly 2+ and 3+ coagulase reactions, must be tested further (4). Test known positive and negative cultures simultaneously with suspect cultures of unknown coagulase activity. Stain all suspect cultures with Gram reagent and observe microscopically.



Score	Observation	Coagulase interpretation at 24 h
-	No clot	Negative
1+	Small unorganized clot(s)	Intermediate shall be confirmed by another method
2+	Intermediate small organized clot	Intermediate shall be confirmed by another method
3+	Large organized clot	Positive
4+	Entire contents of the tube coagulates and is not displaced when the tube is inverted	Positive

CHAPTER 13

Molecular detection techniques for identification of bacterial pathogens

Pankaj Kishore, Ranjit Kumar Nadella and Devananda Uchoi

Email: pkishore2007@gmail.com

DNA ISOLATION

Heat breaks open bacterial cells and releases cell components such as DNA. Once DNA is released from the cells, PCR can be used to amplify any antibiotic resistance genes present in the sample DNA.

Procedure

1. Label the top and side of your clear PCR tube with your sample number.
2. Use the micropipette to add 1ml of sterile DNAase-free water to the tube.
3. Use a sterile pipette tip to 'touch' a series of bacterial colonies on your plate or overnight grown culture from slants. You can sample 10 colonies, but avoid picking up a large amount a bacterial cells. Too much cellular material will inhibit the PCR reaction.
4. Add 0.5 ml of Tris-EDTA buffer to the bacterial culture
5. Close the top of the tube and flick the bottom to mix your bacterial cells.
6. Heat the tube: Place the tube in the heating plate and heat the cells to 95°C for 5 minutes.
7. Immediately transfer the tubes to -20^{0}C.

POLYMERASE CHAIN REACTION

Polymerase chain reaction, or PCR, is an *in-vitro* technique used to amplify particular segments of DNA found in a sample. One of the most commonly used molecular-based method for the detection of foodborne bacterial pathogens is polymerase chain reaction (PCR). PCR was invented about 30 years ago and it allows the detection of a single bacterial pathogen that present in food by detecting a specific target DNA sequence. PCR operates by amplifying a specific target DNA sequence in a cyclic three steps process. Firstly, the target double-stranded DNA is denatured into single-stranded DNA at high temperature. Then, two single-stranded synthetic oligonucleotides or specific primers which are the forward and reverse primer will anneal to the DNA strands. This is followed by the polymerization process whereby the primers complementary to the single-stranded DNA are extended with the presence of deoxyribonucleotides and a thermostable DNA polymerase. The PCR amplification products are visualized on electrophoresis gel as bands by

staining with ethidium bromide. PCR have been used in the detection of numerous foodborne pathogens like *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Campylobacter jejuni*, *Salmonella* spp. and *Shigella* spp. PCR is used to rapidly produce billions of copies of one or more "target sequences" such as genes or repeating sequences of DNA. Once amplified, the target sequences can be used in a variety of ways. For example, DNA segments copied by PCR can be used for gene detection, gene sequencing, or even be inserted into the DNA of another organism. First, the DNA isolated from a particular sample is placed in a test tube with a "mastermix" containing 1) **Taq polymerase** (a heat-stable DNA polymerase), 2) **primers** specific to the target sequence, 3) **a supply of A, C, T, and G nucleotides**, and 4) **buffers** and **salts** necessary for Taq polymerase to function.

Then, the test tube is placed in a PCR machine, which performs the following steps.

1. **Denaturation**: DNA sample is heated, which breaks the hydrogen bonds and separates the DNA strands.
2. **Annealing**: DNA sample is cooled, which allows primers to hydrogen bond (anneal) with complementary sequences in DNA sample.
3. **Extension**: Once primers anneal to DNA sample, the sample is warmed again so Taq polymerase may add nucleotides to the 3' end of the primer according to base-pairing rules.

The PCR machine repeats these 3 steps for 20 or more cycles to yield over a billion copies of the target sequence ***if it is present*** in your DNA sample.

Procedure

1. Obtain one PCR tube containing the 22 µl PCR mastermix with primers that target the antibiotic reistant gene. Also obtain one PCR tube containing 22 µl PCR mastermix with primers that target the genes.
2. Use the micropipette to transfer 3 µl of template DNA to the mastermix PCR tube.
3. Keep your PCR tubes on ice tray.
4. The PCR tubes will go into the thermocycler, which will control the temperature shifts necessary for the PCR reaction. These shifts will be repeated 20-35 times to yield a large number of copies of the DNA region between the primers (if they are present).
5. After the PCR reactions are completed freeze store your samples for analysis.

GEL ELECTROPHORESIS

Gel electrophoresis is a technique that is used to separate fragments of DNA based on their sizes. DNA samples are loaded onto one end of an agarose gel that is immersed in buffer, and an electric current is used to pull the DNA through the gel. Because DNA is negatively charged, the DNA fragments will move away from the negatively-charged cathode and toward the positively-charged anode. Because the DNA fragments must pass through small pores within the agarose gel, the smaller fragments of DNA are able to move through the gel more easily and quickly. After a sufficient period of time has passed, the electric current will have separated the DNA fragments so that the larger fragments remain closer to the start point, while the smaller fragments have migrated farthest in the gel.

Multiplex PCR (mPCR)

Multiplex PCR offers a more rapid detection as compared to simple PCR through the simultaneous amplification of multiple gene targets. The basic principle of mPCR is similar to conventional PCR. However, several sets of specific primers are used in mPCR assay whereas only one set of specific primers are used in conventional PCR assay. Primer design is very important for the development of mPCR, as the primer sets should have similar annealing temperature in order to produce a successful mPCR assay. Besides, the concentration of primers is also important in mPCR. This is because interaction may occur between the multiple primer sets in mPCR that results in primer dimers, thus, the concentration of primers may need to be adjusted to ensure the production of reliable PCR products. Other important factors for a successful mPCR assay include the PCR buffer concentrations, the balance between magnesium chloride and deoxynucleotide concentrations, the quantities of DNA template, cycling temperatures and Taq DNA polymerase

Real-Time or Quantitative PCR (qPCR)

Real-time PCR or quantitative PCR is different from simple PCR whereby it does not require agarose gel electrophoresis for the detection of PCR products. This method is able to monitor the PCR products formation continuously in the entire reaction by measuring the fluorescent signal produced by specific dual labelled probes or intercalating dyes. The fluorescence intensity is proportional to the amount of PCR amplicons. Several fluorescent systems have been developed for qPCR and the most commonly used fluorescent systems include SYBR green, TaqMan probes and molecular beacons. SYBR green is a double-stranded DNA (dsDNA)-binding fluorescent dye. This non-sequence-specific intercalating dye emits little fluorescence and the fluorescence signal is enhanced when bound to the minor groove of the DNA double helix. TaqMan probes and molecular beacons are the common alternatives to SYBR green. TaqMan probes, also known as

double-dye probes, are oligonucleotides that contain a fluorophore as the reporter dye at the 5′-end and the quenching dye at the 3′-end. The reporter dye and the quenching dye are close to each other and this prevent the emitted fluorescence of the reporter. TaqMan probe is complementary to a specific nucleotide sequence in one of the strands of amplicon internal to both primers and the system depends on the 5′-3′ exonuclease activity of Taq DNA polymerase that cleaves the probe and separates both dyes in order to generate the fluorophore signal.

Loop-Mediated Isothermal Amplification (LAMP)

LAMP is a novel nucleic acid amplification method developed by Notomi et al. which provides a rapid, sensitivity and specific detection of foodborne pathogens. LAMP is based on auto-cycling strand displacement DNA synthesis carried out by Bst DNA polymerase large fragment under isothermal conditions between 59°C and 65°C for 60 min. In LAMP, four primers comprising two inner primers and two outer primers are used to target six specific regions of target DNA. Cauliflower-like DNA structures bearing multiple loops as well as stem-loop DNAs of different sizes are the final products of LAMP. Large amount of amplicons can be produced by LAMP within 60 min which is usually 103-fold or higher as compared to simple PCR. The LAMP amplicons can be detected by agarose gel electrophoresis or SYBR Green I dye.

CHAPTER 14

Spectral Fingerprinting of Pathogenic Bacteria by Fourier Transform Infrared Spectroscopy and Chemometric Models for Rapid Identification

Niladri Sekhar Chatterjee, Toms C. Joseph, Minimol V.A.

Email: niladri_icar@hotmail.com

Introduction

Identification and typing of pathogens employing rapid systems in the area of food safety, epidemiological surveillance and disease outbreak assumes more significance in the present scenario, as consumers are more concerned about the food that they consumed and the environment they live in (World Health Organization, 2008). The advanced microbiology techniques over conventional ones using biosensors and enzyme-based technologies ensures the rapid and accurate identification of pathogens within 6-9 h time (Franco-Duarte *et al*., 2019). Simultaneously, the DNA based molecular level identification system further eases the confirmation of specific bacteria or bacterial products (Adzitey *et al.,* 2013). DNA based techniques such as whole genome sequencing and next generation sequencing are widely used in routine diagnostics and surveillance studies where the most promising strain specific differences need to be studied (Besser *et al.,* 2018).

Similarly, the metabolomic fingerprinting can be used to monitor the conformation and composition of biochemical compounds in microbial cells and there by identification of microbes (Mester *et al*., 2018). Rapid analytical techniques for microbial identification include matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS), near-infrared Fourier transform (NIR FT) Raman spectroscopy and Fourier transform infrared (FTIR) spectroscopy (Dinkelacker *et al.,* 2018). The accuracy of test results from these techniques are comparable and similar to most widely used DNA-based methods such as pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and single nucleotide polymorphism (SNP) analysis of whole-genome sequencing (WGS) (Davis, 2012). The use of analytical techniques might provide useful information regarding the source or origin of contamination and their by necessary measures can be taken to reduce the contamination to safe level

Among the different analytical instruments, FTIR offers rapid discrimination, classification and identification of microbial cells by observing the vibration properties of chemical bonds present in the cell when excited by an infrared beam (Puzey et al., 2008). FTIR enables the identification of microbes upto subspecies level (Zarnowiec *et al.,* 2015) Use of FTIR spectroscopy has been successfully applied for the identification several pathogens including *Listeria, Bacillus,*

Staphylococcus, Clostridium, *Escherichia coli* and *Lactobacillus* (Dawson and Upton,2014; Li *et al.,* 2018)

Methicillin resistant strains of *Staphylococcus aureus* have emerged as an important nosocomial infective agent and several clinical and foodborne outbreaks have been reported in recent years (Lakhundi, 2018). Similarly, certain strains of *Vibrio cholerae i.e*., *V. cholerae* O1, *V. cholerae* O139 and several serovariants of them are encountered in recent foodborne outbreaks (Finkelstein, 1996). The differentiation of these bacteria by conventional biochemical methods is difficult. However, this can be achieved by using FTIR which is rapid and effective.

There are very few studies as on date on the use of FTIR for the identification and differentiation of pathogenic bacteria of seafood origin. Amiali et al. (2007) reported the rapid identification of glycopeptide intermediate *S. aureus* (GISA) from glycopeptidesusceptible (GSSA) among methicillin resistant *S. aureus* (MRSA) by using FTIR. They concluded that FTIR can be used as an alternative to conventional susceptibility test for the identification of GISA and GSSA among MRSA isolates. Similarly, Li et al. (2018) established a subtyping method using FTIR with principal component analysis and hierarchical cluster anlasis for epidemiological surveillance of pathogenic *V. parahaemolytius.* Hence, the present study was carried out to analyze the spectral responses of biochemical compounds present in the cell wall by FTIR for identification of *Staphylococcus aureus* as methicillin sensitive and methicillin resistant strains as well as *Vibrio* isolates.

Bacterial ioslates

In this study, bacterial isolates recovered from seafood orgin were used. A total of sixty isolates each comprising of fifteen nos. of *S. aureus,* fifteen nos. of Methicillin resistant *S. aureus* (MRSA), fifteen nos. of Methicillin resistant coagulase negative *S. aureus* (MRCoNS)*,* fifteen nos. of Coagulase negative *S. aureus* (CONS) isolated from retail fish markets and aquaculture farms of three district of kerala namely Ernakulam, Kottayam, and Alapuzha were selected for FTIR analyses. All *Staphylococcus* isolates were isolated during the period of 2012-2015 by Murugadas

et al. (2016). The identity of the isolates was further reconfirmed by following the procedure described in Bacteriological analytical manual for the isolation and identification of *Staphylococcus aureus* (Tallent et al., 2001). Briefly, the glycerol stock of each isolate was revived by inoculating a loopful of culture asceptically to trypticase soya broth and then streaked on to baired parker agar (Himedia, India) and incubated for 48 h at 37 ºC. Black colured colony from each BP agar plates were subjected to following biochemical tests such as Gram staining, catalase test and coagulase test. The methicillin susceptibility of the isolates was confirmed phenotypically by disc diffusion test (CLSI 2014). All the isolates from each category were maintained at -80º C in 30% glycerol until use.

Four species of *Vibrio* isolates comprising 60 nos each comprising of fifteen no's *V. cholerae,* fifteen no's *V. parahaemolyticus,* fifteen no's *V. mimicus, and* fifteen no's *V. vulnificus* were selected to anayse the discriminatory power of FTIR to differentiate the isolates to each category. All the *Vibrio* isolates were isolated from seafood samples from landning centres and retail markets of Cochin as a part of an ongoing project for the screening of seafood samples for the presence of emerging and reemerging foodborne pathogens by Microbiology, Fermentaion and Biotecnology Division, ICAR-CIFT (Unpublished data). Briefly, 190 seafood samples were screened for the presence of *Vibrio* species during the period of June 2018 to December 2019 and all the seafood samples were subjected to selective enrichment in alkaline peptone water (APW) and plating on selective agar (Thiosufate citrate bile salt sucrose agar (TCBS)) for the isolation of *Vibrio* species. Sucrose fermenting and non fermenting colonies were taken randomly from each plate, purified and subjected to a series of biochemical test for identification (Kaysner et al., 2004). Further all biochemically confirmed isolates from each category were further confirmed molecularly by polymerase chain reaction targeting their respective species specific prmers (Bej et al, 1999; Kumar and Lalitha, 2013; Guardiola- Avila et al., 2016; Canigral et al., 2010). All the confirmed isolates from each category were maintained at -80º C in 30% glycerol with 3% salt until use.

Measurement of FTIR spectral responses

The sample preparation was carried out as per the procedure of Grunert et al. (2013). The bacterial isolates were grown overnight at 37° C in trypticase soy broth (TSB). one ml of culture was taken and centrifuged at 5000 rpm for 5 min (Centrifuge 5430R, Eppendorf, India). The supernatant

was discarded and the bacterial pelled was washed with sretile water. The optical density (OD_{620}) of the bacterial suspension was adjusted to 0.5. Then, 30 µl of the suspension was placed on the ZnSe sample plate and dried at 37° C for 20-30 minutes. The transparent film obtained was taken for infrared measurement in Nicolet iS10 FTIR spectrometer, secondary Nicolet iZ10 module in 2010, loaded with Omni software for the data processing and anlysis (Thermo fisher scientific India PVT, LTD Mumbai). The individual spectra of each isolate were recorded in Absorbance/Transmission mode in the spectral range of 4000-400cm^{-1}. The spectral aqusition was carried out by averaging 50 scans for each sample and three replicate responses were recorded for each sample.

Principal of molecular spectroscopy

FTIR, FTNIR, Raman, and Hyperspectral imaging (HSI) techniques utilize the interaction of light with molecules in gas, liquid or solid matter to reveal crucial compositional details. When IR radiation is directed to a sample, some of it is absorbed, reflected back, or transmitted. The plot of %Absorbance/%Transmittance/%Reflectance against wavelength or wavenumber is the IR spectra which provides a molecular fingerprint of the sample. For FTIR spectra, the wavelength falls in the mid infrared region, typically 2500 to 25000 nm, whereas, the NIR spectral region is typically 800 to 2500 nm (Osborne, Fearn, & Hindle, 1993, Sun, 2009)

When IR light is pointed on a mater, the molecules within undergoes vibrational excitation. Infrared light is absorbed when its frequency matches with the frequency of energy transitions caused by the vibrational excitations. Molecular vibrations that cause a change in dipole moment of the molecules can only result in absorption of IR light. Hence, bonds with zero dipole moment show very weak absorbance. The IR light in mid-infrared region can cause fundamental transitions corresponding to stretching and bending vibrations, hence reveale crucial information on the functional group composition of the molecule. No two molecules have the same FTIR fingerprint and the unique fingerprints have important applications in food authenticity determination (Sun, 2009).

Traditionally, to obtain a good quality FTIR spectrum, sample preparation involving KBr palletization and Nujol mall is required. Spectra can be recorded by transmitting mid IR radiation through a sample. However, the thickness of a liquid or solid sample has a direct bearing on the intensity of the spectral features. This limits the use of FTIR spectroscopy in food analysis. Advent

of novel sampling accessories such as “Attenuated Total Reflectance (ATR)”, and “Diffuse Reflectance” has largely solved this problem of direct sample analysis.

The working principal of the ATR accessory is presented in the schematic Figure 1. When infrared light is pointed on an optically dense diamond crystal at a certain angle, the internal reflectance caused by the high refractive index creates an evanescent wave at the surface of the crystal and interacts with the sample placed in contact with the crystal. The sample absorbs energy from the evanescent wave thereby altering or attenuating it. The attenuated evanescent wave meets back with the IR beam, which then exits the opposite end of the crystal and reaches the detector. The detector system then generates the IR spectrum. For this technique to be successful, the sample should be in good contact with the crystal surface, since the evanescent waves do not extend a few microns beyond the crystal surface (Cocciardi, 2003).

The working principle of a diffuse reflectance accessory is presented in the schematic Figure 2. When light is illuminated on an uneven surface, the unevenness causes the light to reflect at all angles. This phenomenon is called diffuse reflectance. Fuller and Griffiths in 1978, first described the diffuse reflectance accessory for recording FTIR spectra in this particular mode (Fuller & Griffiths, 1978). Ever since, the accessory has been modified and adopted in commercial instruments. During measurements, the IR light passing through a lens system illuminates the sample at normal incidence. The diffused light is collected back through the lens system along the same axis as the incident IR beam. The plot of %Reflectance against the wavelength generates the diffuse reflectance spectra. This technique of FTIR analysis has recently found application in food analysis (Yang et al., 2021). High-throughput transmission (HTT) is another sampling technique where a transmission cell is used for holding liquid samples. This technique is particularly suitable for authenticity testing of oils and fats (Lerma-Garcia et al., 2010).

Chemometric data analysis tools and algorithms

An untargeted analysis of food even with a miniature spectrometer generates a large amount of data. Moreover, visual discrimination between the spectral fingerprints of an authentic vis-a-vis adulterated food sample is not straight forward. Such spectral dataset contains large number of variables depending upon the resolution of the spectrometer used. Meaningful interpretation of such food fingerprints need assistance from chemometrics. Broadly, chemometrics can be defined as the tool box of various statistical algorithms, machine learning, and cloud computing for

extracting chemically relevant information from food. During the last decade, the chemometrics aided detection of food fraud, adulteration, determination of quality, freshness, and traceability have seen rapid growth in innovations (Medina et al., 2019). Figure 5 presents a summary of various types of chemometrics techniques used in spectroscopy data analysis for food authentication.

The digital signals generated from a spectroscopy sensor may contain several unwanted information arising from electrical interferences, surrounding effects, and baseline shifts. To correct these aberrations in signal, several pre-processing techniques are recommended including peak alignment (Jellema, 2009; Savorani et al., 2010). Thankfully, spectroscopy signals are less prone to signal drift or horizontal shifts as compared to chromatographic signals. For chemometric analysis, the digital signals are arranged into a data matrix where the variables (usually wavelength or wavenumber) are assembled in a column and the corresponding signal values (usually, absorbance, % reflectance, transmittance etc.) are presented in a row. The pre-processing algorithms apply independently on each signal of the data matrix.

Smoothing, Baseline correction, Normalization, Signal correction, and Derivatization are the major pre-processing treatment, applied singly or in a chain to a data matrix. The major smoothing algorithms include "Savitzky-Golay", "Exponentially Weighted Moving Average" (EWMA), "Wavelet Denoise Spectral" (WDS), "Moving window", and "Asymmetric Least Square Smooting" (AsLS). Among the smoothing techniques, Savitzky-Golay is most commonly used where noise is removed by applying a moving polynomial to the data matrix (Rahman et al., 2019). WDS technique is particularly used for removing high frequency coefficients from the data set. For baseline correction, "Row-Center", "Offset correction", "Linear correction", and AsLS correction techniques are used. Row-Center technique that shifts each spectrum by subtracting the row mean from each row value is often successful for building chemometric models using spectroscopy data. The "Standard Normal Variate (SNV)" filter is commonly used for spectra normalization that subtracts the mean from each signal value and then divides by the standard deviation of the signal values (Mishra et al., 2021). Variation in spectroscopy signals due to different path-length and scatter effect is corrected using the "Multiplicative Signal Correction (MSC) technique. The MSC technique normalizes each spectrum by regressing against the average spectrum from a selected set of spectra (Mishra et al., 2021). Further, to reduce scatter effect first and second order derivatization of the spectra is performed. For new application

development, initially different pre-processing techniques are evaluated either singly or in combination to arrive at most optimum latent data set for model building (McVey et al., 2021).

Besides signal pre-processing, removal of redundant or uninformative variables is also important for creation of rugged chemometric models. Inclusion of so many variables from a complex spectrum may result in overfitting of the model. Unfortunately, for a new application, it is not known which part of the spectra will be most useful. For variable selection, initially, a full model is created including all the available variables. The model is optimized for all pre-processing treatments and classification/regression algorithms. Then, the variables that fall above a defined threshold of variable importance in projection (VIP) are selected (Chatterjee et al., 2019, Gomes et al. 2022). Hence, a subset using the selected variables are created which is further used to develop the final chemometric model. Variable selection is more important for Multiple Linear Regression (MLR), Linear Discriminant Analysis (LDA), and Quadratic Discriminant Analysis (QDA) methods which use a dataset of original registered variables and without any pre-processing treatments (Yun et al., 2013).

Chemometric methods are selected based on the objective of the study. The methods are broadly classified as unsupervised/exploratory methods and supervised methods. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) are the unsupervised multivariate analysis tools. PCA is highly useful for initial data structure exploration. A PCA scores plot reveals important insights on the intrinsic variability of the data set and natural groupings of samples. This in turn helps in selecting the important variables. However, one should keep in mind that PCA is strictly a data exploration technique and not to be used as a classification tool (Jiménez-Carvelo et al., 2019). HCA analysis, presented as dendrograms, shows the similarity or relationship between cluster of samples. Each node in the dendrogram denotes a cluster (Drab & Daszykowski, 2014).

Purpose of the supervised methods can be discrimination/classification, quantification or both. The major statistical tools for discrimination or class analysis are “k nearest neighbour (kNN)”, “Soft Independent Modelling of Class Analogies (SIMCA)”, “Partial Least Square Discriminant Analysis (PLSDA)”, “Orthogonal Partial Least Square Discriminant Analysis (OPLSDA)”, “Linear Discriminant Analysis (LDA)”, and “Artificial Neural Network (ANN)”. Essentially, different classification models define the class boundaries for sets of samples based on a training

set of representative samples comprising different classes (Oliveri, 2017). In case of kNN and SIMCA, the classification is strictly qualitative and works best for binary classification. On the other hand, the PLS algorithm-based analysis performs a multivariate regression and assigns a numeric value to each sample before classifying them into different classes (Brereton & Lloyd, 2014). There are merits and demerits of each of these classification models and they face several issues for application in regulatory control of food authenticity (Rodionova et al., 2016). However, recently one class classifier, particularly one class SIMCA (ocSIMCA) has been particularly successful in providing reliable results (Rodionova et al., 2016; Horn et al., 2018).

Newer algorithms, such as “Support Vector Machine (SVM)”, “Classification and Regression Tree (CART)”, and “Random Forest (RF)” are also finding application in the area of food authentication. The advantage of these algorithms is that it can be used for both classification and quantification models. Despite their advantage over conventional classification methods and successful applications in the field of metabolomics and ecology, examples of application in food authentication are still limited (Cutler et al., 2007; Gromski et al., 2015). On the other hand, purely quantitative algorithms such as “Principal Component Regression (PCR)”, “Partial Least Square Regression (PLSR)”, and “Multiple Linear Regression (MLR)” are widely used for non-destructive determination of a quantitative parameter in food material. Particularly, determination of the content of an adulterant in food non-destructively or with minimum sample preparation is important application of these quantitative statistical methods (Ejeahalaka et al., 2020).

Conclusion

Using chemometrics and molecular spectroscopy it is possible to identify microbial pathovers in real time. However, these chemometrics models are built initially using authentic isolates and comprehensive spectral datbases needs to be built. Once the chemometric algorithms are trained, they will be able to identify unknown sample in real time. These rapid methods can be highly accurate and saves lot of time in identification.

Reference:

- Adzitey, F., Huda, N. and Ali, G.R.R., 2013. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. *3 Biotech*, *3*(2), pp.97-107.

- Amiali, N.M., Mulvey, M.R., Berger-Bächi, B., Sedman, J., Simor, A.E. and Ismail, A.A., 2007. Evaluation of Fourier transform infrared spectroscopy for the rapid identification of glycopeptide-intermediate Staphylococcus aureus. *Journal of antimicrobial chemotherapy*, *61*(1), pp.95-102.
- Besser, J., Carleton, H.A., Gerner-Smidt, P., Lindsey, R.L. and Trees, E., 2018. Next-generation sequencing technologies and their application to the study and control of bacterial infections. *Clinical microbiology and infection*, *24*(4), pp.335-341.
- Dinkelacker, A.G., Vogt, S., Oberhettinger, P., Mauder, N., Rau, J., Kostrzewa, M., Rossen, J.W., Autenrieth, I.B., Peter, S. and Liese, J., 2018. Typing and species identification of clinical Klebsiella isolates by Fourier transform infrared spectroscopy and matrix-assisted laser desorption ionization–time of flight mass spectrometry. *Journal of clinical microbiology*, *56*(11), pp.e00843-18.
- Franco-Duarte, R., Černáková, L., Kadam, S., Kaushik, K.S., Salehi, B., Bevilacqua, A., Corbo, M.R., Antolak, H., Dybka-Stępień, K., Leszczewicz, M. and Relison Tintino, S., 2019. Advances in Chemical and Biological Methods to Identify Microorganisms—From Past to Present. *Microorganisms*, *7*(5), p.130.
- Grunert, T., Wenning, M., Barbagelata, M.S., Fricker, M., Sordelli, D.O., Buzzola, F.R. and Ehling-Schulz, M., 2013. Rapid and reliable identification of Staphylococcus aureus capsular serotypes by means of artificial neural network-assisted Fourier transform infrared spectroscopy. *Journal of clinical microbiology*, *51*(7), pp.2261-2266.
- Kumar, R. and Lalitha, K.V., 2013. Prevalence and molecular characterization of Vibrio cholerae O1, non-O1 and non-O139 in tropical seafood in Cochin, India. *Foodborne pathogens and disease*, *10*(3), pp.278-283.
- Lakhundi, S. and Zhang, K., 2018. Methicillin-resistant Staphylococcus aureus: molecular characterization, evolution, and epidemiology. *Clinical microbiology reviews*, *31*(4), pp.e00020-18.
- Li, Z., Chen, S., Xu, C., Ju, L. and Li, F., 2018. Rapid subtyping of pathogenic and nonpathogenic Vibrio parahaemolyticus by fourier transform infrared spectroscopy with chemometric analysis. *Journal of microbiological methods*, *155*, pp.70-77.

- Mester, P., Robben, C., Witte, A.K., Kalb, R., Ehling-Schulz, M., Rossmanith, P. and Grunert, T., 2018. FTIR Spectroscopy Suggests a Revised Mode of Action for the Cationic Side-Chain Effect of Ionic Liquids. *ACS combinatorial science*, *21*(2), pp.90-97
- Puzey, K.A., Gardner, P.J., Petrova, V.K., Donnelly, C.W. and Petrucci, G.A., 2008, April. Automated species and strain identification of bacteria in complex matrices using FTIR spectroscopy. In *Chemical, biological, radiological, nuclear, and explosives (CBRNE) sensing IX* (Vol. 6954, p. 695412). International Society for Optics and Photonics.
- World Health Organization, 2008. *Foodborne disease outbreaks: guidelines for investigation and control*. World Health Organization.
- Zarnowiec, P., Lechowicz, L., Czerwonka, G. and Kaca, W., 2015. Fourier transform infrared spectroscopy (FTIR) as a tool for the identification and differentiation of pathogenic bacteria. *Current medicinal chemistry*, *22*(14), pp.1710-1718.
- Cañigral, Irene, Yolanda Moreno, Jose L. Alonso, Ana González, and María A. Ferrús. "Detection of Vibrio vulnificus in seafood, seawater and wastewater samples from a Mediterranean coastal area." *Microbiological research* 165, no. 8 (2010): 657-664.
- Guardiola-Avila, I., Acedo-Felix, E., Sifuentes-Romero, I., Yepiz-Plascencia, G., Gomez-Gil, B. and Noriega-Orozco, L., 2016. Molecular and genomic characterization of Vibrio mimicus isolated from a frozen shrimp processing facility in Mexico. *PloS one*, *11*(1).
- Kaysner, C.A., DePaola, A.J. and US Food and Drug Administration, 2004. bacteriological analytical manual; methods for specific pathogens; Chapter 9 Vibrio.
- Bej, A.K., Patterson, D.P., Brasher, C.W., Vickery, M.C., Jones, D.D. and Kaysner, C.A., 1999. Detection of total and hemolysin-producing Vibrio parahaemolyticus in shellfish using multiplex PCR amplification of tl, tdh and trh. *Journal of microbiological methods*, *36*(3), pp.215-225.

CHAPTER 11

Automated Microbial Detection Techniques in Food Microbiology

Ranjit Kumar Nadella, Pankaj Kishore and Devananda Uchoi

Email: nranjeetkumar@gmail.com

Microbiology has always been very traditional and very labour intensive with the view that automation was for other disciplines but not suited for microbiology. Over the last few years, however, new and improved automated technologies have provided solutions to the challenges facing today's microbiology lab. The first stand-alone automation for the micro lab was introduced in the 1950s, with the initial systems primarily designed for studying human specimen samples such as blood cultures, tissue samples, urine samples antibiotic susceptibility, and biochemical based identification. It wasn't until 2006 that the first true bacteriology automation was introduced with barcoding of dishes, inoculation, moving tracks systems, automated incubation, and digital imaging. Like many other industry advancements, laboratory automation is designed to increase efficiency, streamline processes and deliver high-quality, consistent results in less time.

Today, automation is a complex integration of computers, robotics, liquid handling/processing, and other combined technologies. Automation of routine procedures such as dedicated workstations and software to program instruments has already impacted laboratories worldwide. With repetitive tasks such as pipetting, transporting plates, and various types of assay being the first to be automated. In last decade, automation has steadily spread throughout the analytical chemistry and clinical areas of medical diagnostic laboratories, microbiology laboratories have been excluded from this trend. In general automated microbial identification systems, and automated antimicrobial susceptibility testing systems are widely utilized in microbiology laboratories. In conventional microbiology, microbiology samples are collected and transported by utilizing a wide variety of devices and are processed by maceration, digestion, sonication prior to being plated, or plated directly, and analysis can be quantitative, semi-quantitative, or non-quantitative.

In most inoculation and streaking systems that are fully automated, the samples first need to be in a liquid format The common perception is that digital imaging can be used to make a determination. In fact, it is used to sort the plates, which may be of interest to do further work or sensitivity testing. The others can be sent to discard without being handled by a biomedical scientist. There will always be some plates that may require a visual check by the laboratorian prior to doing any further work being performed. With automation, a majority of manual processing of bacteriology is removed and reading using digital imaging is different and takes some getting used to by biomedical scientists. Automation changes the workflow of the lab by allowing continuous flow processing as opposed to batch processing. This is a move from the traditional approach of reading plates in the morning and setting up plates in the afternoon and is more compatible with a 24/7 operation. The centralized processing and reading gets away from the traditional specialized benches or areas, staff can easily access all the data from a particular sample and compare on one screen. It also frees trained, experienced staff from doing dull repetitive tasks they can be usefully employed in using their skills and knowledge where it is most needed - in the unusual results rather than the routine ones.

Prerequisites for automation in microbiology laboratory

The main factors for automation in microbiology laboratory are the continued pressure on reducing costs whilst increasing productivity, turnaround time, and result reliability. The current trend is towards merging smaller labs into large super labs, which are considered to be the most cost-effective and efficient way to process samples, and these have the advantage of creating centers

of excellence in terms of expertise. Automated systems are ideally suited to meet accreditation requirements by automatically monitoring each step of the analysis, retaining the data for later access. Recruiting and retaining qualified, experienced staff, especially with a trend towards 24/7 working, is also an issue for many labs, so again automation can step in. For automation in microbiology laboratory to be successful, it need to be flexible in design, embrace the human element, and adapt to the challenges of analysing diverse samples. Flexibility acknowledges that one size will not fit all and incorporates an open, expandable architecture that can be adapted to a laboratory's available space and potential future growth. Moreover, flexibility will also require that automation systems embrace diversity of equipment manufacturers. Microbiology must move as much as is practical to liquid-based transport devices to facilitate automated plating. The automated solutions must be able to accommodate the introduction of manually inoculated media into their systems.

Advantages of Lab Automation:

- Increased productivity, more samples processed per person
- A move away from batch processing to continuous, even 24/7 processing
- The ability to handle surge demands
- Remote reading and access to images of plates and organisms
- Assurance that the sample is processed correctly with the right plates and incubation conditions
- Ability to view the whole patient's plate set and historical plate sets
- Reduction in technical and transcription errors
- Improvement in traceability and fully audit trails including the reading process
- Images available for retrospective and training purposes

Process to be automated in microbiology laboratory

In microbiology laboratory several process are required for processing and analysis of samples. In this process automation is possible in many stages

a) Media Preparation:

Perhaps the most well established and long-standing area that can be automated is media preparation, labs will not see this as a core activity with all the associated validations and Quality Control protocols and will buy in ready to use media.

b) Specimen Preparation (Plating/Inoculation/Streaking):

Plates Most fully automated inoculation and streaking systems require liquid transport swabs or liquid samples. Specimens can be loaded into racks and then loaded onto the instrument; alternatively, samples can be added to a turntable for continuous loading. The sample is scanned, and the system will know how to process the specimen and what plates are required. After vortexing the required plates arrive ready barcoded so that they can be tracked and traced throughout the process. Plates are then planted/inoculated or streaked depending on what was specified for that particular specimen. A HEPA environment ensures no cross-contamination. Specific streaking patterns can be pre-programmed and achieved by robotic loop. This results in a consistent, reproducible inoculation and streaking pattern and produces single colonies more often than by a manual process. Systems will include a monitoring step to ensure that some sample has indeed been taken up by the pipette or loop. Inoculated plates can then be sorted according to required atmospheric conditions and temperature and transported by conveyor belt to the appropriate incubators. Any non-liquid or other specialized samples can be done in a semi-automated fashion whereby the technician prepares the plate, which then goes back into the system with the bulk of samples.

C. Incubation:

As each plate is barcoded, on the way to the incubator, it's scanned so incubation start time is registered and how long that plate will need to be incubated before going to the plate reader.

D. Plate Reading and Interpretation:

After incubation plates are automatically moved to the image analyzer for reading and may subsequently be returned to the incubator if necessary, this means plates get exactly the correct incubation time even if due for reading during the night if the lab is 24/7. The barcode on the plate contains information on which camera and lighting settings are required to take images for that particular plate. Even chromogenic plates, can be automatically read and interpreted. The whole plate set from a patient can be put together on one screen for viewing together in one place, so secondary plates such as antibiotic sensitivities can be seen with the primary plates, or the image from day 1 can be viewed with day 2. Images can be saved for later reference or auditing purposes.Looking at plates on a screen is probably one of the most significant changes that

automation brings for the biomedical staff who are used to holding a plate, seeing it in 3D, and maybe quickly doing some basic biochemical tests. But plates can always be called up to the workbench for examination by eye, and as staff gain more confidence in the digitized system they will most likely need to only call up those plates that are necessary, leaving the bulk routine plates to be handled by the instrument.

E. Antibiotic Sensitivity Testing:

The inoculation and streaking modules are able to produce seeded plates for sensitivities. However, the relevant antibiotic sensitivity discs need to be added using traditional disc dispensers. These plates can be returned to a workbench for the discs to be added.

F. Artificial Intelligence:

Artificial Intelligence can be applied to screening and interpretation of plates following incubation; algorithms can be adjusted to meet a particular lab's requirements to enable the automated screening of non-critical plates, depending on visual appearance, sample or patient histories, etc. This results in the vast majority of plates being automatically read and recorded without the need for any technician intervention.

Systems Available

Larger automated systems are modular and can be configured to fit into the available laboratory space. Quite often, the systems must be built to specific design specifications. However, the inoculation and streaking modules have a fixed footprint and are available off-the-shelf. Additional modules can be added on, which include the fully automated transport of plates to fully-automated incubators. Many of these systems will have a lead in time, however this allows time for the lab to prepare for the change and complete any enabling works. The following automated systems are widely used for identification of bacteria in microbiology laboratory.

A) API (Analytical Profile Index) KIT

API identification products are test kits for identification of Gram positive and Gram negative bacteria and yeast. API strips give accurate identifications based on extensive databases and are standardized, easy-to-use test systems. The kits include strips that contain up to 20 miniature biochemical tests which are all quick, safe and easy to perform.API (Analytical Profile Index) 20E is a biochemical panel for identification and differentiation of members of the family Enterobacteriaceae. It is hence a well-established method for manual microorganism identification

to the species level. The API range provides a standardized, miniaturized version of existing identification techniques, which up until now were complicated to perform and difficult to read. In the API 20E, the plastic strip holds twenty mini-test chambers containing dehydrated media having chemically-defined compositions for each test. They usually detect enzymatic activity, mostly related to fermentation of carbohydrate or catabolism of proteins or amino acids by the inoculated organisms. A bacterial suspension is used to rehydrate each of the wells and the strips are incubated. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. All positive and negative test results are compiled to obtain a profile number, which is then compared with profile numbers in a commercial codebook (or online) to determine the identification of the bacterial species.

The test kit enables the following tests:

ONPG: test for β-galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-b-D-galactopyranoside

ADH: decarboxylation of the amino acid arginine by arginine dihydrolase

LDC: decarboxylation of the amino acid lysine by lysine decarboxylase

ODC: decarboxylation of the amino acid ornithine by ornithine decarboxylase

CIT: utilization of citrate as only carbon source

H2S: production of hydrogen sulfide

URE: test for the enzyme urease

TDA (Tryptophan deaminase): detection of the enzyme tryptophan deaminase: Reagent- Ferric Chloride.

IND: Indole Test-production of indole from tryptophan by the enzyme tryptophanase . Reagent- Indole is detected by addition of Kovac's reagent.

VP: the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway

GEL: test for the production of the enzyme gelatinase which liquefies gelatin

GLU: fermentation of glucose (hexose sugar)

MAN: fermentation of mannose (hexose sugar)

INO: fermentation of inositol (cyclic polyalcohol)

SOR: fermentation of sorbitol (alcohol sugar)

RHA: fermentation of rhamnose (methyl pentose sugar)

SAC: fermentation of sucrose (disaccharide)

MEL: fermentation of melibiose (disaccharide)

AMY: fermentation of amygdalin (glycoside)

ARA: fermentation of arabinose (pentose sugar)

Method

Confirm the culture is of an Enterobacteriaceae. To test this, a quick oxidase test for cytochrome c oxidase may be performed. Pick a single isolated colony (from a pure culture) and make a suspension of it in sterile distilled water. Take the API20E Biochemical Test Strip which contains dehydrated bacterial media/bio-chemical reagents in 20 separate compartments. Using a pasteur pipette, fill up (up to the brim) the compartments with the bacterial suspension. Add sterile oil into the ADH, LDC, ODC, H_2S and URE compartments. Put some drops of water in the tray and put the API Test strip and close the tray. Mark the tray with identification number (Patient ID or Organism ID), date and your initials. Incubate the tray at 37oC for 18 to 24 hours.

Result interpretation

For some of the compartments, the color change can be read straightway after 24 hours but for some reagents must be added to them before interpretation.

Add following reagents to these specific compartments:

TDA: Put one drop of Ferric Chloride

IND: Put one drop of Kovacs reagent

VP: Put one drop of 40 % KOH (VP reagent 1) & One drop of VP Reagent 2 (α-Naphthol)

Get the API Reading Scale (color chart) by marking each test as positive or negative on the lid of the tray. The wells are marked off into triplets by black triangles, for which scores are allocated. Add up the scores for the positive wells only in each triplet. Three test reactions are added together at a time to give a 7-digit number, which can then be looked up in the codebook. The highest score possible for a triplet is 7 (the sum of 1, 2 and 4) and the lowest is 0. Identify the organism by using API catalog or apiweb (online).

<u>B. VITEK® 2 COMPACT</u>

The VITEK® 2 Compact system offers quality control testing solutions for fast and accurate microbial identification. The efficiency of the VITEK® 2 COMPACT instrument and VITEK® 2

PC software have the capacity to help improve therapeutic success and patient outcomes through reliable microbial identification (ID) and antibiotic susceptibility testing (AST). The instrument also lets you enhance laboratory efficiencies with reduced hands-on time and rapid reporting capabilities. All this, in a cost-effective, space-saving design.With technology that includes an extensive and robust identification database, rapid results, and minimal training time, it will streamline laboratory workflow for increased productivity. The system identifies the majority of microorganisms that contaminate production areas and finished products in a minimal amount of time. Identification cards presently available for product safety include: Gram-negative bacilli (time to result: 2 – 10 h); Gram-positive cocci (time to result: 2 – 8 hours); Yeast-like organisms (time to result: 18 hours); Anaerobic bacteria (time to result: 6 hours); Gram-positive spore forming bacilli (time to result: 14 hours) Coryneform bacteria (Time to result: 8 hours).

Testing using VITEK 2 can be performed as follows:

a. Select the appropriate card based on the Gram stain reaction and the organism's microscopic appearance. Allow the card to come to room temperature before opening the package liner.

b. Aseptically transfer at least 3 mL of sterile saline into a clear polystyrene 12×75 mm test tube. Using sterile cotton swabs, prepare a homogenous organism suspension by transferring several isolated colonies from the plates to the saline tube. Adjust the suspension to the McFarland standard required by the ID reagent. The required inoculum concentrations card McF range for differnent bacteria are as follows: GN 0.5-0.63; GP 0.5-0.63; ANC 2.7-3.3; BCL 1.8-2.2.

c. Place the prepared suspensions in the cassette

d. Insert the straw. The age of the suspension must not exceed 30 minutes before inoculating the cards.

e. Proceed to data entry. Enter the card data by scanning the car code on the card. The Cursor must be in the Bar Code space to be entered.

f. Filling the Cards: lace the cassette in the Filler box on the left side of the V2C unit and hit Start Fill button on the instrument. Filling the cards takes approximately 70 seconds for a cassette regardless of the number of cards in the cassette holder. The cassette must be placed inside the Loader Door within 10 minutes from the end of the filling cycle to avoid the cards being rejected. When the cards are finished filling, the Load Door is automatically unlocked.

g. Place the cassette in the Load Door. The V2C Instrument will verify the scanned barcodes against the Virtual Cassette (the information scanned in by the analyst). Cards are sealed, straws are cut and the cards are loaded automatically into the carousel. The V2C will beep once all cards are loaded into the cassette.
h. When the cards are loaded, remove the cassette and dispose of the tubes and straws in a biohazard container.
i. The V2C automatically processes the cards once all the cards are loaded.
j. When the cards are processed and results obtained, cards will be automatically ejected into the waste collection bin
k. Results are concurrently printed and the data sent to the Results View folder on the left side of the screen also called the Navigation Tree where the information is archived.
l. The VITEK system analyses the data results and determines the identity of the test microbes/QC organism based on colorimetric tests (biochemical reactions).

C. VIDAS

VIDAS® is a multiparameter, automated immunoanalyser. It includes an analytical module, a computer and a printer. The analytical module automatically performs all stages of the analysis. The VIDAS® system contains five independant compartments, each accepting up to 6 tests. The computer module is used to manage and print out the results. The VIDAS® system can manage up to two analytical modules simultaneously, giving the system a capacity of 60 tests per hour and is based on Enzyme Linked Fluorescent Assay (ELFA) based technology. VIDAS® reagents are optimized, ready-to-use and stem from an integration of antibody engineering, immuno-concentration, and phage recombinant protein technology. VIDAS® offers a wide range of next-day, simple protocols to answer the need of detecting *Salmonella*, *Listeria* spp., *Listeria monocytogenes*, *Escherichia coli* O157, *Campylobacter* and *Staphylococcal* enterotoxins.

The detection protocol can be broken down as follows:
a. Enrichment
b. Enzyme immunoassay
c. Cultural confirmation

D. ASSURANCE® Gene detection system

The Assurance® GDS genetic detection system combines the latest advancements in molecular detection technology and food microbiology to provide faster results with the increased accuracy required to meet today's food and environmental testing challenges. The Assurance® GDS system comprises three simple steps: Sample enrichment, Sample preparation assays utilizing our innovative GDS PickPen® immunomagnetic separation (IMS) device, and PCR analysis with the GDS Rotor-Gene® thermal cycler. GDS uses proprietary magnetic particles to capture the target organism from the enriched sample. The innovative GDS PickPen® concentration device quickly and easily collects and transfers the concentrated target – 8 samples at a time. It utilizes probes and primers which are highly conserved target gene sequences and ensures greater specificity with fewer indeterminate or false positive reactions. Also accompanied with multiplex platform allows for the simultaneous detection of multiple targets within each amplification tube.

It works on the combination of two different technologies such as immunomagnetic separation (IMS) and polymerase chain reaction (PCR) to create a single method. IMS is the use of paramagnetic particles coated with specific antibodies to capture and separate cells containing the target antigen from the surrounding environment (sample). This technique has been widely used by microbiologists to aide in the isolation and recovery of low levels of pathogenic organisms from problematic sample matrices and high background microflora environments. It can provide additional advantages when utilized in preparation of samples for PCR-based pathogen detection. Assurance GDS™ utilizes a novel intrasolution IMS method to prepare samples for analysis via PCR. In this method, the sample aliquot and particles are combined in a deep well plate. The magnetic tips of the Assurance GDS PickPen™ device are inserted directly into the wells to collect the particles and transfer them through a wash solution into a resuspension buffer. Once deposited in the buffer, the particles and the associated captured organisms are ready for analysis with the Assurance GDS system.

E. MALDI-TOF

Identification of microorganisms is typically performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). It works on the principle of protein based spectral identification of bacteria. One of the great advances in microbiology in recent years due to its speed of result together with a low cost per test it easily outperforms biochemical based approaches. Most MALDI-TOF will sit near or immediately next to an automated system, and

some systems can use a loop to seed the MALDI-TOF target plate automatically. The technology touts accurate, rapid, and inexpensive identification of microorganisms isolated from samples. MALDI-TOF procedures are highly amenable to automation because they are technically relatively simple and reproducible. Additionally, spotting of target plates and extraction of proteins can be standardized for most organisms and, when combined with automation, can be performed with minimal staffing.

The identification protocol includes

The sample for analysis by MALDI/MS is prepared by mixing or coating with solution of an energy-absorbent, organic compound called matrix. When the matrix crystallizes on drying, the sample entrapped within the matrix also co-crystallizes. The sample within the matrix is ionized in an automated mode with a laser beam. Desorption and ionization with the laser beam generates singly protonated ions from analytes in the sample. The protonated ions are then accelerated at a fixed potential, where these separate from each other on the basis of their mass-to-charge ratio (m/z). The charged analytes are then detected and measured using different types of mass analyzers like quadrupole mass analyzers, ion trap analyzers, time of flight (TOF) analyzers. For microbiological applications mainly TOF mass analyzers are used. During MALDI-TOF analysis, the m/z ratio of an ion is measured by determining the time required for it to travel the length of the flight tube. A few TOF analyzers incorporate an ion mirror at the rear end of the flight tube, which serves to reflect back ions through the flight tube to a detector. Thus, the ion mirror not only increases the length of the flight tube, it also corrects small differences in energy among ions. Based on the TOF information, a characteristic spectrum called peptide mass fingerprint (PMF) is generated for analytes in the sample. Identification of microbes by MALDI-TOF MS is done by either comparing the PMF of unknown organism with the PMFs contained in the database, or by matching the masses of biomarkers of unknown organism with the proteome database.

Problems/draw-backs with automated systems

Several factors have contributed to the current dearth of automation in microbiology labs. These include the ideas that microbiology is too complex to automate, no machine can replace a human in the microbiology laboratory, automation is too expensive for microbiology laboratories, and microbiology laboratories are too small to automate. Microbiology samples are more complex for analysis by conventional methods. Humans are generally considered capable of performing tasks

faster than machines and that machines cannot think. The perception that machines cannot exercise the critical decision-making skills required to process microbiology specimens has persisted. Specifically, human observation of organism growth on agar plates is still considered essential by many. Automation has historically been considered too expensive for microbiology. It simply has not been viewed as cost-effective. Although automation is justified for chemistry, the relative test volumes for microbiology are much smaller, making automation seemingly less attractive. Most microbiology laboratories have been considered to be too small for automation. Automation may have a place in the very largest microbiology labs, it does not have a place in the average-sized laboratory as these labs are small, automation would be underutilized. At last shortage of well trained personnel for operation of automated instruments also play an important role in automation of microbiology laboratory.

Glossary

Biochemical test in microbiology

Biochemical tests are performed on bacteria for their identification on the basis of their biochemical activities. The Biochemical tests are conventional but gold standard methods for the identification of microorganisms, usually performed for phenotypic identification. Biochemical tests are of different types on the basis of which the identification between different microorganisms is carried out. The results are based on the change in color of the medium as a result of the change in the pH of the medium in certain tests. The physiology of bacteria and other microorganisms differs from one another, which allows for the differentiation of such microorganisms.Biochemical tests are thus, essential as they are inexpensive and relatively simple to perform but has disadvantage as it takes longer time.

Some of the common biochemical tests performed routinely in the microbiology laboratories are as follows:

Oxidase Test

This test is used to identify microorganisms containing the enzyme cytochrome oxidase (important in the electron transport chain). Cytochrome oxidase transfers electrons from the electron transport

chain to oxygen (the final electron acceptor) and reduces it to water. In the oxidase test, artificial electron donors and acceptors are provided. When the electron donor is oxidized by cytochrome oxidase it turns a dark purple. This is considered a positive result. It is commonly used to distinguish between oxidase negative *Enterobacteriaceae* and oxidase positive *Pseudomadaceae*.

Catalase Test

This test is used to identify organisms that produce the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas.

$2H_2O_2$ $2H_2O + O_2$

The bubbles resulting from production of oxygen gas indicates positive. he *Staphylococcus* spp. and the *Micrococcus* spp. are catalase positive. The *Streptococcus* and *Enterococcus spp. are* catalase negative.

Sugar ferementation test

Different sugars like glucose, lactose, mannitol etc. are tested for organism to see the ability to ferment as well as its ability to convert the end product of glycolysis, pyruvic acid into gaseous byproducts. This is a test commonly used when trying to identify Gram-negative enteric bacteria, all of which are glucose fermenters but only some of which produce gas. The medium may contain different pH indicators such as bromocresol purple, phenol red etc. If an organism is capable of fermenting the sugar glucose, then acidic byproducts are formed and the pH indicator turns yellow. When glucose is used, the end product of glycolysis is pyruvate. Organisms that are capable of converting pyruvate to formic acid and formic acid to H_2(g) and CO_2 (g), via the action of the enzyme formic hydrogen lyase, emit gas. *Escherichia coli* and *Proteus mirabilis* are both gas producers. *Shigella dysenteriae* ferments glucose but does not produce gas. *Escherichia coli* is

capable of fermenting glucose as are *Proteus mirabilis* and *Shigella dysenteriae*. *Pseudomonas aeruginosa* is a non-fermenter.

Methyl Red / Voges-Proskauer (MR/VP)

This test is used to determine which fermentation pathway is used to utilize glucose. In the mixed acid fermentation pathway, glucose is fermented and produces several organic acids (lactic, acetic, succinic, and formic acids). The stable production of enough acid to overcome the phosphate buffer will result in a pH of below 4.4. If the pH indicator (methyl red) is added to an aliquot of the culture broth and the pH is below 4.4, a red color will appear. If the MR turns yellow, the pH is above 6.0 and the mixed acid fermentation pathway has not been utilized. The 2,3-butanediol fermentation pathway will ferment glucose and produce a 2,3 butanediol end product instead of organic acids.

In order to test this pathway, an aliquot of the MR/VP culture is removed and a-naphthol and KOH are added. They are shaken together vigorously and set aside for about one hour until the results can be read. The Voges-Proskauer test detects the presence of acetoin, a precursor of 2,3 butanediol. If the culture is positive for acetoin, it will turn "brownish-red to pink/brick red". If the culture is negative for acetoin, it will turn "brownish-green to yellow". *Escherichia coli* is MR positive and VP negative. In contrast, *Enterobacter aerogenes* and *Klebsiella pneumoniae* are MR negative and VP positive. *Pseudomonas aeruginosa* is a glucose non-fermenter and is thus MR negative and VP negative.

Simmon's Citrate Agar

This test is used to determine if an organism can use citrate as its sole carbon source. It is often used to differentiate between members of *Enterobacteriaceae*. In organisms capable of utilizing citrate as a carbon source, the enzyme citrase hydrolyzes citrate into oxaoloacetic acid and acetic acid. The oxaloacetic acid is then hydrolyzed into pyruvic acid and CO_2. If CO_2 is produced, it

reacts with components of the medium to produce an alkaline compound (e.g. Na_2CO_3). The alkaline pH turns the pH indicator (bromthymol blue) from green to blue. This is a positive result (the tube on the right is citrate positive). *Klebsiella pneumoniae* and *Proteus mirabilis* are examples of citrate positive organisms. *Escherichia coli* and *Shigella dysenteria*e are citrate negative.

Urease Test

This test is used to identify bacteria capable of hydrolyzing urea using the enzyme urease. It is commonly used to distinguish the genus Salmonella, *Proteus* etc. from other enteric bacteria. The hydrolysis of urea forms the weak base, ammonia, as one of its products. This weak base raises the pH of the media above 8.4 and the pH indicator, phenol red, turns from yellow to pink. *Proteus mirabilis* is a rapid hydrolyzer of urea. No change in color will be in negative.

Kliger's Iron Agar (KIA)

This is a differential medium. It tests for organisms' abilities to ferment glucose and lactose to acid and acid plus gas end products. It also allows for identification of sulfur reducers. This media is commonly used to separate lactose fermenting members of the family *Enterobacteriaceae* (e.g. *Escherichia coli*) from members that do not ferment lactose, like *Shigella dysenteriae*. These lactose nonfermenting enterics generally tend to be the more serious pathogens of the the gastrointestinal tract.

KIA tubes are also capable of detecting the production of H_2S. It is seen as a black precipitate. Sometimes the black precipitate obscures the butt of the tube. In such cases, the organisms should be considered positive for glucose fermentation (yellow butt). *Proteus mirabilis* is glucose positive organism, lactose negative, sulfur reducing enteric.

Mannitol Salt Agar (MSA)

This type of medium is both selective and differential. The MSA will select for organisms such as *Staphylococcus* species which can live in areas of high salt concentration. This is in contrast to *Streptococcus* species, whose growth is selected against by this high salt agar. The differential ingredient in MSA is the sugar mannitol. Organisms capable of using mannitol as a food source will produce acidic byproducts of fermentation that will lower the pH of the media. The acidity of the media will cause the pH indicator, phenol red, to turn yellow. *Staphylococcus aureus* is capable of fermenting mannitol while *Staphylococcus epidermidis* cannot ferment.

Blood Agar Plates for Hemolysis

This is a differential medium. It is a rich, complex medium that contains 5% sheep red blood cells. BAP tests the ability of an organism to produce hemolysins, enzymes that damage/lyse red blood cells (erythrocytes). The degree of hemolysis by these hemolysins is helpful in differentiating members of the genera *Staphylococcus*, *Streptococcus* and *Enterococcus*.

Beta-hemolysis is complete hemolysis. It is characterized by a clear (transparent) zone surrounding the colonies. *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus agalactiae* are b-hemolytic.

Partial hemolysis is termed alpha-hemolysis. Colonies typically are surrounded by a green, opaque zone. *Streptococcus pneumoniae* and *Streptococcus mitis* are a-hemolytic.

If no hemolysis occurs, this is termed gamma-hemolysis. There are no notable zones around the colonies. *Staphylococcus epidermidis*is gamma-hemolytic.

CAMP Test

CAMP factor is a diffusible, heat-stable protein produced by group B streptococci. This is a synergistic test between *Staphylococcus aureus* and *Streptococcus agalactiae*. *S. agalactiae* produces CAMP factor. *S. aureus* produces sphingomyelin C, which binds to red blood cell membranes. The two bacteria are streaked at 90° angles of one another. They do NOT touch.

The CAMP factor produced by *S. agalactiae* enhances the beta-hemolysis of *S. aureus* by binding to already damaged red blood cells. As a result, an arrow of beta-hemolysis is produced between the two streaks. The test is presumptive for *S. agalactiae* that produces CAMP factor.

In the picture here, *Streptococcus agalactiae* was streaked throughout the top region of the plate and brought down toward the center of the plate. *Staphylococcus aureus* was streaked in a straight line across the center of the plate. Rings of hemolysis are evident all around *S. aureus*, however the hemolysis if greatly enhanced (in an arrow shape) where the *S. agalactiae* crosses the hemolysis rings.

Bile Esculin Agar

This is a medium that is both selective and differential. It tests the ability of organisms to hydrolyze esculin in the presence of bile. It is commonly used to identify members of the genus *Enterococcus* (*E faecalis* and *E. faecium*). The first selective ingredient in this agar is bile, which inhibits the growth of Gram-positives other than enterococci and some streptococci species. The second selective ingredient is sodium azide. This chemical inhibits the growth of Gram-negatives. The differential ingredient is esculin. If an organism can hydrolyze esculin in the presence of bile, the product esculetin is formed. Esculetin reacts with ferric citrate (in the medium), forming a phenolic iron complex which turns the entire slant dark brown to black.

Nitrate Broth

This is a differential medium. It is used to determine if an organism is capable of reducing nitrate (NO_3^-) to nitrite (NO_2^-) or other nitrogenous compounds via the action of the enzyme nitratase (also called nitrate reductase). This test is important in the identification of both Gram-positive and Gram-negative species. After incubation, these tubes are first inspected for the presence of gas in the Durham tube. In the case of non-fermenters, this is indicative of reduction of nitrate to nitrogen gas. However, in many cases gas is produced by fermentation and further testing is

necessary to determine if reduction of nitrate has occurred. This further testing includes the addition of sulfanilic acid (often called nitrate I) and dimethyl-alpha-napthalamine (nitrate II). If nitrite is present in the media, then it will react with nitrate I and nitrate II to form a red compound. This is considered a positive result. If no red color forms upon addition of nitrate I and II, this indicates that either the NO_3^- has not been converted to NO_2^- (a negative result), or that NO_3^- was converted to NO_2^- and then immediately reduced to some other, undetectable form of nitrogen (also a positive result). In order to determine which of the preceding is the case, elemental zinc is added to the broth. Zinc will convert any remaining NO_3^- to NO_2^- thus allowing nitrate I and nitrate II to react with the NO_2^- and form the red pigment (negative). If no color change occurs upon addition of zinc then this means that the NO_3^- was converted to NO_2^- and then was converted to some other undetectable form of nitrogen (positive).

Starch Hydrolysis Test

This test is used to identify bacteria that can hydrolyze starch (amylose and amylopectin) using the enzymes a-amylase and oligo-1,6-glucosidase. This is often used to differentiate species from the genera *Clostridium* and *Bacillus*. Because of the large size of amylose and amylopectin molecules, these organisms cannot pass through the bacterial cell wall. In order to use these starches as a carbon source, bacteria must secrete a-amylase and oligo-1,6-glucosidase into the extracellular space. These enzymes break the starch molecules into smaller glucose subunits which can then enter directly into the glycolytic pathway. In order to interpret the results of the starch hydrolysis test, iodine must be added to the agar. The iodine reacts with the starch to form a dark brown color. Thus, hydrolysis of the starch will create a clear zone around the bacterial growth. *Bacillus subtilis* is positive for starch hydrolysis.

Motility Agar

This is a differential medium used to determine movement of an organism with flagella and thus capable of swimming away from a stab mark. The results of motility agar are often difficult to interpret, if not observed within time. Generally, if the entire tube is turbid, this indicates that the bacteria have moved away from the stab mark (are motile).

Coagulase Test

Coagulase is an enzyme that clots blood plasma. This test is performed on Gram-positive, catalase positive species to identify the coagulase positive *Staphylococcus aureus.* Coagulase is a virulence factor of *S. aureus.* The formation of clot around an infection caused by this bacteria likely protects it from phagocytosis. This test differentiates *Staphylococcus aureus* from other coagulase negative *Staphylococcus* species.

Sulfur, Indole, Motility (SIM) Media

This is a differential medium. It tests the ability of an organism to do several things: reduce sulfur, produce indole and swim through the agar (be motile). SIM is commonly used to differentiate members of *Enterobacteriaceae*.

Sulfur can be reduced to H_2S (hydrogen sulfide) either by catabolism of the amino acid cysteine by the enzyme cysteine desulfurase or by reduction of thiosulfate in anaerobic respiration. If hydrogen sulfide is produced, a black color forms in the medium. *Proteus mirabilis* is positive for H_2S production. The organism pictured on the far left is positive for hydrogen sulfide production. Bacteria that have the enzyme tryptophanase, can convert the amino acid, tryptophane to indole. Indole reacts with added Kovac's reagent to form rosindole dye which is red in color (indole +). *Escherichia coli* is indole positive. The organism pictured second from left is *E. coli* and is indole positive.

SIM tubes are inoculated with a single stab to the bottom of the tube. If an organism is motile than the growth will radiate from the stab mark and make the entire tube appear turbid. *Pseudomonas aeruginosa* and the strain of *Proteus mirabilis* that we work with are motile.