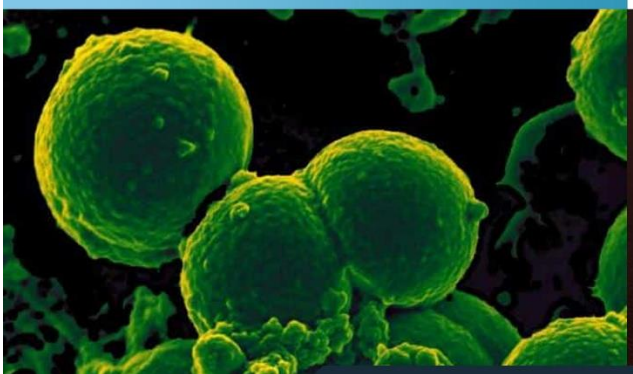

Standard Operating Procedures

ICMR Foodborne Pathogens Survey and Research Network (NE Region)

Institutional Support

ICMR - NICED, Kolkata
Dr. Shanta Dutta, Director

C-DAC, Kolkata
Shri Aditya Kumar Sinha, Scientist 'G' & Director

Foreword
Dr. Rajiv Bahl
Secretary DHR, Director General, ICMR
Dr. Shanta Dutta
Director, ICMR - NICED, Kolkata

Indian Council of Medical Research,
Ansari Nagar, New Delhi – 110029

Revised Edition - 2024

Standard Operating Procedures

ICMR Foodborne pathogen survey and research Network (North-East India)

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2024

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FOREWORD

Foodborne infections are caused by different harmful bacteria, viruses, fungi and parasites that affect several countries of the world. Many of these pathogens have broad hosts, including human, animals and the environment, which needs to be monitored and controlled under One Health approach. Contamination of food may occur at any stage of the food-chain, and hence investigation of the causative pathogens is important in prevention and the spread of the disease. With several simple and sensitive diagnostic techniques, it is now possible to precisely detect the implicated pathogen(s).

ICMR is making constant efforts to generate data collection on foodborne infection events and their sources through the ICMR-FoodNet project that includes thirteen Institutions located in the North-eastern States of India. In addition, ICMR-National Institute of Cholera and Enteric Diseases, and Centre for Development of Advanced Computing, Kolkata functioning as External Quality Assessment Services & Training partner, and Data Management, respectively. This FoodNet activity facilitates the collaborating centres to link phenotypic and molecular characteristics of the pathogens and antimicrobial resistance profiles from foodborne illness cases and several food items.

Laboratory activities are important and integral parts of the public health, as they play a pivotal role in the diagnosis, monitoring of diseases and provides quality health care delivery. The use of standard operating procedures (SOP) in laboratory testing is one of the most crucial components in achieving quality as well as generating comparable surveillance data. The SOP for the FoodNet project has been methodically developed to improve the skills of the scientists and other laboratory personnel working in the collaborating Institutions. The current edition of the SOP includes additional zoonotic pathogens and toxigenic fungi so as to generate comprehensive information under One Health activity.

This SOP manual will be useful in achieving its purpose of improving the quality of laboratory services and contribute towards the successful implementation of the ICMR-FoodNet project and other similar research activities in the country.

Rajiv Bahl

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WHO COLLABORATING CENTRE FOR RESEARCH AND TRAINING ON DIARRHOEAL DISEASES

Dr. Shanta Dutta, MD, PhD, MAMS, FWAST, FNASc, FNAMS
Director & Scientist-G

FOREWORD

It is a great pleasure to welcome all participants to the hands-on laboratory training course organized by ICMR-NICED during 27-31 May, 2024 under the ongoing ICMR-task force Phase II project on "Surveillance of foodborne disease pathogens from Northeast India". In this respect revised version of SOP (Standard Operating Procedures) is going to be released. I am happy to contribute and forward the SOP and dedicate it for the people of India.

Foodborne illness continues to be a major cause of morbidity and mortality across countries including India. Approximately 600 million people worldwide are affected by foodborne illnesses every year, and children under 5 years of age are the most vulnerable group, accounting about 40% of all foodborne illnesses, leading to around 125,000 deaths annually. There is no common cause of foodborne diseases or organisms, as they can vary depending on the region, food habits, nature and processing of foods. Hence, rapid detection of foodborne pathogens becomes most important to contain the spread of the pathogen before it may cause a serious outbreak.

Detection and diagnostics rely on culture-based methods, immunological methods such as enzyme-linked immunosorbent assays (ELISA) and molecular biology-based methods. The aim has always been to apply a simple, rapid, sensitive, specific and cost-effective method.
ICMR-NICED has always been a strong ally to combat enteric infections with special reference to food/ waterborne pathogens and also part of the global foodborne pathogens network. This Institute is functioning as the National repository of foodborne enteric pathogens and antimicrobial resistant bacteria. Some of the recent activities include the Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) based bacterial identification and whole genome characterization of specific pathogens.

As a part of our ongoing efforts to expand our understanding of foodborne pathogens causing illnesses in different regions of India, it is our privilege to organize this training program that will highlight crucial and contemporary aspects of detecting foodborne pathogens. I gratefully acknowledge the generous funding support from ICMR to organize this training program. I take this opportunity to thank the project investigators, NICED scientists and staff involved in this training program.

I am confident that this hands-on laboratory training course will help in useful skill development along with fruitful outcomes, which will be of benefit for the entire nation. I wish a grand success of this training program.

S. Dutta

25th May, 2024

Dr. Shanta Dutta

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ICMR Delhi, Regional Medical Research Centre, Dibrugarh& National Institute of Cholera and Enteric Diseases, Kolkata	Standard operating Procedure (SOP)	SOP No	03
		Revision No	5
		Date of issue	14.05.2024
	"Surveillance of Food Borne Pathogens (FBP) from North-East India"	Prepared by	MD, UD, PJB
		Reviewed by	TRM
		Approved by	IK, RC

1. PURPOSE

To be used as a standard operating procedure for isolation and identification of foodborne pathogens from different food sources, clinical specimens as well as environmental sources.

2. SCOPE: To find out the burden of foodborne pathogen disease burden

The methods described below outline the cultural procedures to isolate and identify *foodborne pathogens* as well as to characterize the isolates in respect of its antimicrobial susceptibility, serotype, genotype or toxin production.

3. ASSOCIATED DOCUMENTS

Flow chart-1	Processing of milk for isolation of foodborne pathogens
Flow chart-2	Processing of meat for isolation of foodborne pathogens
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Figure S4a	STEC colony morphology in selective media
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Figure S5b and S5c	DEC Biochemical test results and multiplex PCR
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Figure S7	Growth and biochemical test results of *Listeria monocytogens*
Figure S8	Growth and biochemical test results of *Bacillus cereus*
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Figure S10	Growth and biochemical test results of *Yersinia enterocolitica*
Figure S11	Growth of *Clostridioides difficile* in CCFA medium
Figure S12	Flocculated milk powder in treated water samples for the detection of HAV and HEV

4. SAFETY

- Carry out all procedures in accordance with the local codes of safe practice
- Universal precautions such as barrier protection, hand washing, safe techniques, safe handling of sharp items & specimen are used.
- All potentially contaminated materials (culture tubes, plates, glass slides, gloves, tips etc.) are disposed after decontamination. Any spills should be wiped up thoroughly using Lysol solution.
- Wear gloves and don't touch your eyes, nose, or other exposed membranes or skin with gloved hand.
- Wash hands thoroughly with soap and running water immediately after work. If gloves are worn, wash your hands with soap and water after removing the gloves.
- Wear laboratory coat, when working in the laboratory. Remove this protective clothing before leaving the laboratory. While handling stool specimens, use personal protective equipment (PPE).
- Never pipette by mouth.
- Care should be taken when using sharp or pointed instruments.

- Broken glass should be picked up with a brush and pan. Hands must never be used. Chipped or cracked glassware should not be used.
- In case of a wound or cut in hand, do not perform the test.
- Do not eat, drink, smoke, and apply cosmetics or store food or personal items in the laboratory where specimens or kit reagents are being handled.
- Paperwork should not be done on a potentially contaminated surface/working table in the testing laboratory.
- Disinfect work surfaces when procedures are completed at the end of each working day.

5. METHODOLOGY

5.1 LIST OF SAMPLES AND THE CORRESPONDING PATHOGENS TO BE SCREENED

S. No	Food items/Animal sources/environment	Pathogens to be screened
1	Raw Milk (locally produced and supplied) /ice cream/Dry milk powder	*Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni* *Diarrhoeagenic Escherichia coli* (DEC) [ETEC, EPEC, EAEC, EIEC, STEC and DAEC] *Staphylococcus aureus* *Salmonella* spp. *Shigella* spp., *Brucella* spp., *Mycobacterium bovis* Hepatitis A Virus (HAV) Hepatitis E Virus (HEV) Norovirus
2	Raw pork, beef, mutton, poultry meat, bush meat	*Diarrhoeagenic Escherichia coli* (DEC) [ETEC, EPEC, EAEC, EIEC, STEC and DAEC] *Staphylococcus aureus* *Salmonella* spp*., Shigella* spp *Yersinia enterocolitica*, *Clostridium perfringens*, *Listeria monocytogenes* *Campylobacter* spp., *Clostridioides dificile* *Brucella* spp., *Leptospira* spp., *Mycobacterium bovis* Parasite ova, cyst HAV, HEV Norovirus
3	Fish (raw fish/tinned fish)	Pathogenic *Vibrio parahaemolyticus* *Vibrio cholerae* serogroups O1 and O139

		Diarrhoeagenic Escherichia coli (DEC) [ETEC, EPEC, EAEC, EIEC, STEC and DAEC] *Salmonella* spp. *Staphylococcus aureus* *Listeria monocytogenes* *Clostridium botulinum* *Clostridium perfringens* *Campylobacter jejuni* HAV HEV Norovirus
4	Raw vegetables	*Salmonella* spp. *Shigella* spp. *Diarrhoeagenic Escherichia coli* (DEC) [ETEC, EPEC, EAEC, EIEC, STEC and DAEC] *Vibrio cholerae* serogroups O1 and O139 *Salmonella spp.* *Listeria monocytogenes* HAV HEV Norovirus Helminth ova, cyst
5	Environment (water)	*Diarrhoeagenic Escherichia coli* (DEC) [ETEC, EPEC, EAEC, EIEC, STEC and DAEC] *Staphylococcus aureus* *Salmonella* spp.*, Shigella* spp. *Yersinia enterocolitica*, *Clostridium perfringens* *Listeria monocytogenes* *Campylobacter* spp. *Vibrio cholerae* serogroups O1 and O139 *Staphylococcus aureus* *Leptospira* spp. HAV, HEV Norovirus Parasite ova, cyst
6	Food items of suspected hepatitis patient	HAV HEV Norovirus

During outbreaks

S.No	Source	Sample	Pathogens to be screened
1	Human (cases)	Stool, vomitus,	*Diarrhoeagenic Escherichia coli* (DEC) [ETEC, EPEC, EAEC, EIEC, STEC and DAEC] *Salmonella* spp. *Shigella* spp. *Yersinia enterocolitica*, *Campylobacter spp.* *Vibrio cholerae* serogroups O1 and O139 Pathogenic *Vibrio parahaemolyticus* HAV, HEV Norovirus Parasite ova, cyst
2	Human (control)	Stool	
3	Food handler's	skin swabs, nasal swabs etc.	*Diarrhoeagenic Escherichia coli* (DEC) [ETEC, EPEC, EAEC, EIEC, STEC and DAEC]
4	Human case and control	Urine/serum	*Leptospira* spp.
5	Environment	Food (cooked rice as targeted sampling) Water (as targeted sampling) Equipment (slicers, grinders, cutting boards, knives, storage containers)	*Diarrhoeagenic Escherichia coli* (DEC) [ETEC, EPEC, EAEC, EIEC, STEC and DAEC] *Staphylococcus aureus* *Salmonella* spp., *Shigella* spp. *C. difficile, Yersinia enterocolitica*, *Clostridium perfringens* *Listeria monocytogenes* *Campylobacter spp.* *Vibrio cholerae* serogroups O1 and O139 Pathogenic *Vibrio parahaemolyticus* *Staphylococcus aureus,* *Leptospira* spp HAV, HEV Norovirus Parasite ova, cyst

5.2 SAMPLE COLLECTION AND TRANSPORTATION

Procedures and equipment for specimen collection

FOOD SAMPLES (Following the sampling protocol mentioned in Bacteriological Analytical Manual, FDA)

General

The samples will be collected aseptically. It will be put into sterile jars or plastic bags to avoid any cross-contamination

Obtain samples of approximately 20-50 gm or 20 ml.

Sampling Unit (SU): 100 gm; A sample unit may consist of more than one container when containers are smaller than 100 gm (*e.g.,* four 25 gm containers could constitute a sample unit, these four 25 gm containers are considered as sub-samples of a sample).

The packaged foods will be taken to the laboratory in their original containers/wraps.

The original packages or containers will be checked and recorded for code numbers that can be used to identify the place and time of processing. Include any unopened packages or cans belonging to the same batch.

The samples of perishable foods shall be refrigerated at 4°C until they can be examined. [Do not freeze food samples as certain pathogens (*e.g.,* Gram-negative bacteria, vegetative forms of *Clostridium perfringens*) die off rapidly when frozen, but foods that were frozen when collected should be kept frozen until examined].

Meat or fish

A portion of food (usually SU: 100 gm of meat or SU: 250 gm of fish) shall be cut or separated out using a sterile knife or other utensil if necessary. It will be collected aseptically and put into a

sterile plastic bag or wide-mouth jar. Collection will be done from top centre and elsewhere of the samples, as necessary, and refrigerated.

Liquid food or beverages

At first samples are to be stirred or shaken. Collection of samples will be done using one of the following methods:

Using a sterile utensil, transfer approximately SU: 20 ml into a sterile container; refrigerate.

Frozen foods

They should be kept frozen, using dry ice as necessary. Transportation shall be done in an insulated container.

Dried foods

Samples shall be collected using a sterile spoon, spatula, or similar utensil and transferred to a sterile water- and airtight container.

Environmental swabs from food, equipment, pipes, slicers, grinders, cutting boards, knives, storage containers filter etc.

Moisten swabs with 0.1% peptone water or buffered distilled water and wipe over contact surfaces of equipment or environmental surfaces. Place in enrichment broth.

WATER

Water samples can be collected in narrow mouthed glass sterile bottles. The appropriate volume of the sample should be about SU: 2-4 liters. Transport of samples should be made in ice chests and processed within 2 hrs after collection. Use sterile membranes for filtering the water samples

using a vacuum pump and filtration assembly (Millipore). Avoid leakage of water while processing.

1) Water samples should be collected in the narrow-mouthed heat sterilized glass bottles containing freshly prepared sodium thiosulphate (1.8% w/v) to neutralise any chlorine contamination (1.0 ml per 1lit) of water collected.
2) The water is allowed to drain for 2-3 min before sample collection.
3) For collection from stream/lakes/ponds etc., the collection of the sample should be done at a depth of 30 cm, with the mouth of the bottle facing the current direction of running water.
4) The stopper/lid to be put into the mouth of the bottle and properly labelled with full details of place, source, time and date of collection.
5) The collected samples need to be transported to the laboratory within 6 hrs under cold condition (4°C) using ice/gel pack.

STOOL SAMPLES

Two containers of about 25 ml capacity, screw capped, wide mouthed plastic bottle, preferably with spoon shall be used to collect 1-2 ml of faeces. The specimen shall be transported immediately to the laboratory. If delay is unavoidable in one of the containers the faeces shall be collected in a container holding about 6 ml buffered glycerol transport medium for isolation of bacteria. To permit diagnosis of certain viral agents the other container shall be immediately refrigerated at 4°C (do not freeze) and sent as soon as possible to the laboratory.

If faecal samples cannot be obtained rectal swabs shall be collected

Procedure for collection of rectal swabs

1. Insert swab into Cary-Blair medium to moisten it
2. Insert swabs 3-5 cm into rectum and rotate gently
3. Remove swab and examine it to ensure that the cotton tip is stained with faeces.

4. Insert swabs immediately into tube of transport medium (Cary-Blair)
5. Push the swabs to the bottom of the tube
6. Break off the top parts of the sticks and tighten screw cap firmly.

For isolation of *V. cholerae* use Cary-Blair transport medium kept at room temperature (24-30°C).

SKIN LESIONS *(boils, lesions, abscesses, secretions)*; Only during Outbreak Investigation

Clean the skin with normal saline or weak disinfectant. Apply pressure to the lesion using sterile gauzes and collect specimens on sterile swab, trying to obtain as much secretion as possible.

Transport immediately to laboratory at ambient temperature. If this is not possible, the specimen can be left for up to 24 hrs, at which time the swab should be placed in a container of ice.

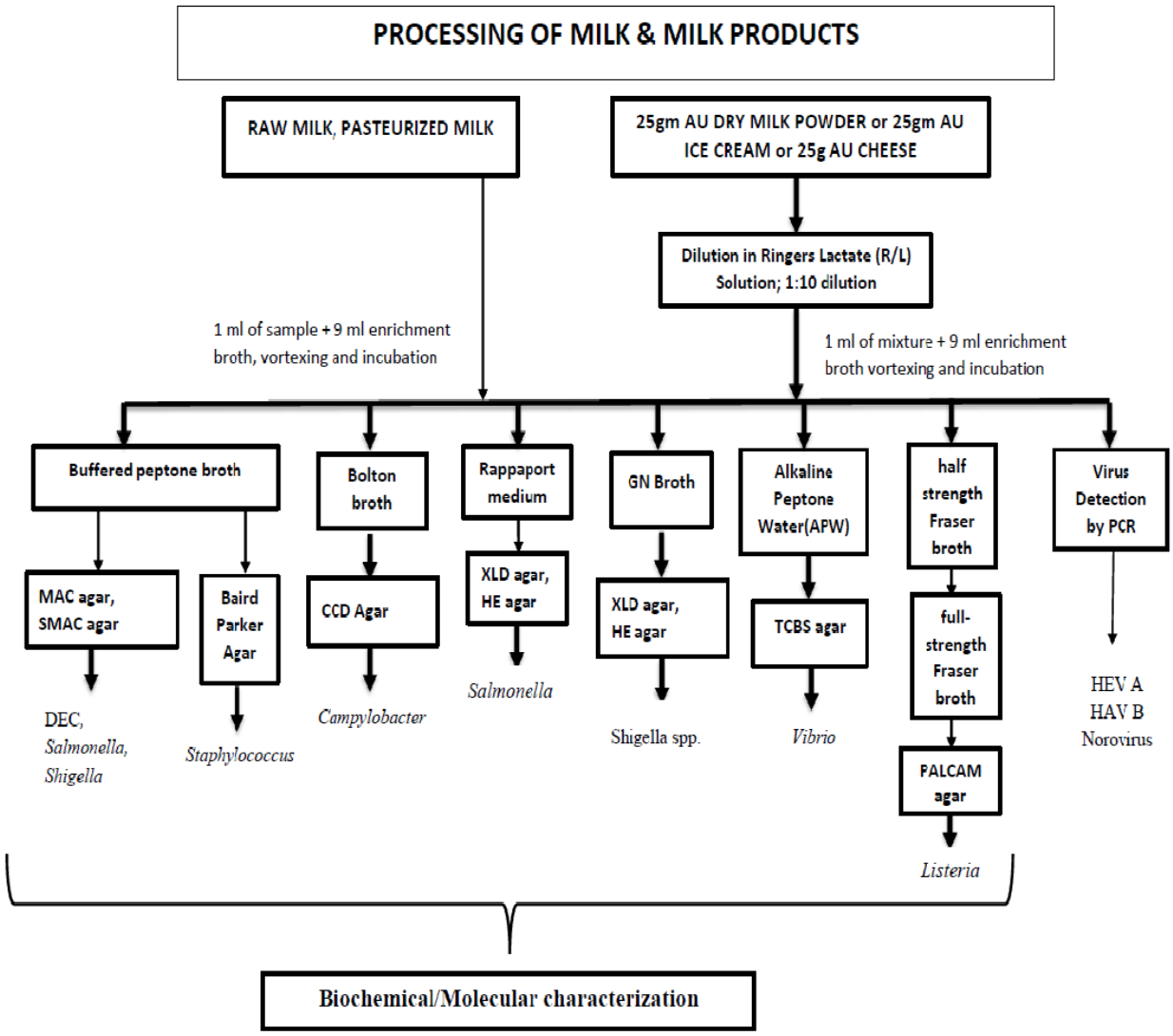
NASAL SWABS; Only during Outbreak Investigation

Collect specimen with a sterile swab and immediately place in transport medium (*e.g.,* Stuart's).

Transport immediately to laboratory at ambient temperature. If this is not possible, the specimen can be left for up to 24 hrs, at which time the swab should be placed in a container of ice.

5.3 PROCESSING OF DIFFERENT SAMPLES

PROCESSING OF RAW MILK, PASTEURISED MILK, Dry Milk Powder and Milk Products

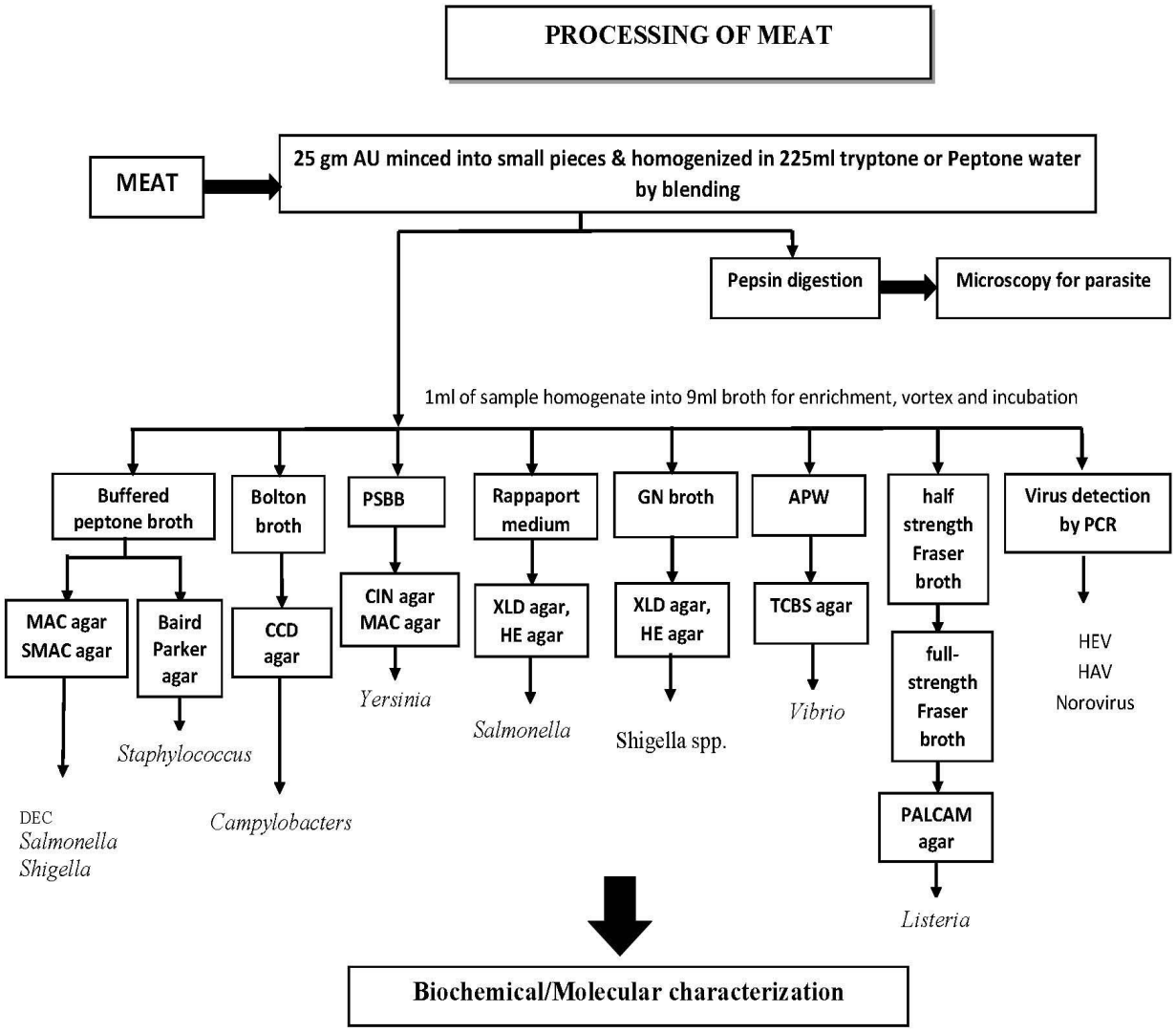


Flow chart-1: Processing of milk for isolation of foodborne pathogens

Procedure

1. From the SU, weigh aseptically 25 gm AU Dry milk powder, cheese shall be diluted in 225 ml Ringer's lactate in 1 in 10 dilutions
2. One ml of the diluted dry milk powder or cheese or 1 ml of raw milk, pasteurized milk shall be inoculated into 9 ml each of the following broths for enrichment: buffered peptone broth for enrichment of DEC, *Salmonella*, *Shigella*; Selenite-F broth for enrichment of *Shigella*; Rappaport-Vassiliadis (RV) medium for enrichment of *Salmonella*; APW for enrichment of vibrios and half strength Fraser broth for enrichment of *Listeria*. Mix gently by vortexing.
3. After overnight incubation at 37°C, the sample shall be inoculated from Buffered peptone broth to a plate of MAC, SMAC, Baird Parker and CCD agar for isolation of DEC, *Salmonella*, *Shigell*a, *Staphylococcus aureus* and *Campylobacter*, respectively.
4. Following 18-24 hrs of incubation, the growth from Rappaport-Vassiliadis (RV) medium and Selenite-F broth is plated to HE (or XLD). The plates are incubated for 18-24 hrs at 37°C in a non-CO_2 incubator for isolation of *Salmonella* and *Shigella*.
5. Following an incubation of 4-6 hrs at 37°C for enrichment, the growth from APW is subcultured on TCBS for isolation of vibrios.
6. For isolation of *Listeria*, with 0.1 ml of pre-enriched Fraser broth content in 10 ml of Fraser broth containing selective supplements for 48 hrs at a 37°C incubation temperature will be done. The inoculum will be plated on PALCAM agar and incubated for 48 hrs at 37°C for isolation.
7. Virus will be detected by RT-PCR directly after extraction of RNA from the diluted sample.

PROCESSING OF RAW MEAT



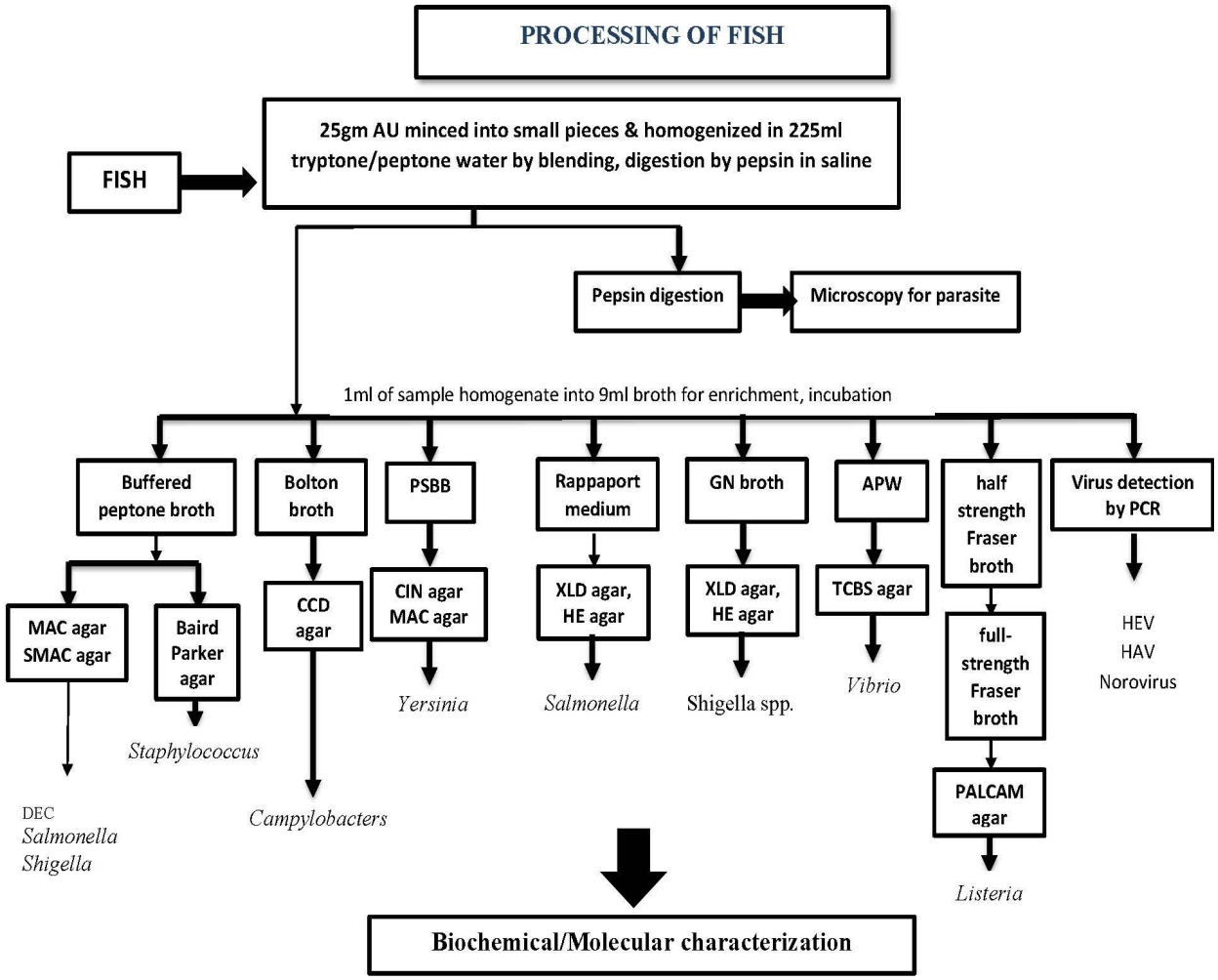
Flow chart-2: Processing of meat for isolation of foodborne pathogens

Procedure

1. From 100gm SU of meat 25 gm AU will be minced into small pieces by a sterile scissor and homogenized in 225 ml tryptone or peptone water by blending.
2. One ml of the sample homogenate shall be inoculated into each of the following broth (9.0 ml) for enrichment: buffered peptone broth for enrichment of DEC, *Salmonella*, *Shigella*; Selenite-F broth for enrichment of *Shigella*; Rappaport-Vassiliadis (RV) medium for enrichment of *Salmonella*; PSBB for enrichment of *Yersinia*; RCM, APW for enrichment of vibrios and half strength Fraser broth for enrichment of *Listeria*. Mix gently by vortexing.
3. After overnight incubation at 37°C, the sample shall be inoculated from Buffered peptone broth to a plate of MAC, SMAC, Baird Parker and CCD agar for isolation of DEC, *Salmonella*, *Shigell*a, *Staphylococcus aureus* and *Campylobacter,* respectively.
4. Following 18-24 hrs of incubation, the growth from Rappaport-Vassiliadis (RV) medium and Selenite-F broth is plated to HE (or XLD). The plates are incubated for 18-24 hrs at 37°C in a non-CO_2 incubator for isolation of *Salmonella* and *Shigella*.
5. Following an incubation of 4~6 hrs at 37°C for enrichment, the growth from APW is subcultured on TCBS. The plates are incubated overnight for 18-24 hrs at 35°±2°C in a non-CO_2 incubator for isolation of vibrios.
6. The PSBB is incubated at 10°C for 10 days, after which it is streaked on CIN and MacConkey plates for isolation of *Yersinia*.
7. For isolation of *Listeria*, with 0.1 ml of pre-enriched Fraser broth content in 10 ml full strength Fraser broth containing selective supplements for 48 hrs at a 37°C incubation temperature will be done. The inoculum will be plated on PALCAM agar and incubated for 48 hrs at 37°C for isolation.
8. Virus will be detected by RT-PCR directly after extraction of RNA from the diluted sample.

9. For parasite microscopic detection homogenization is done with saline and digestion in a mixture of pepsin in saline. After centrifugation the deposit is observed for parasite ova or cyst.

PROCESSING OF FISH AND OTHER EDIBLE ANIMALS

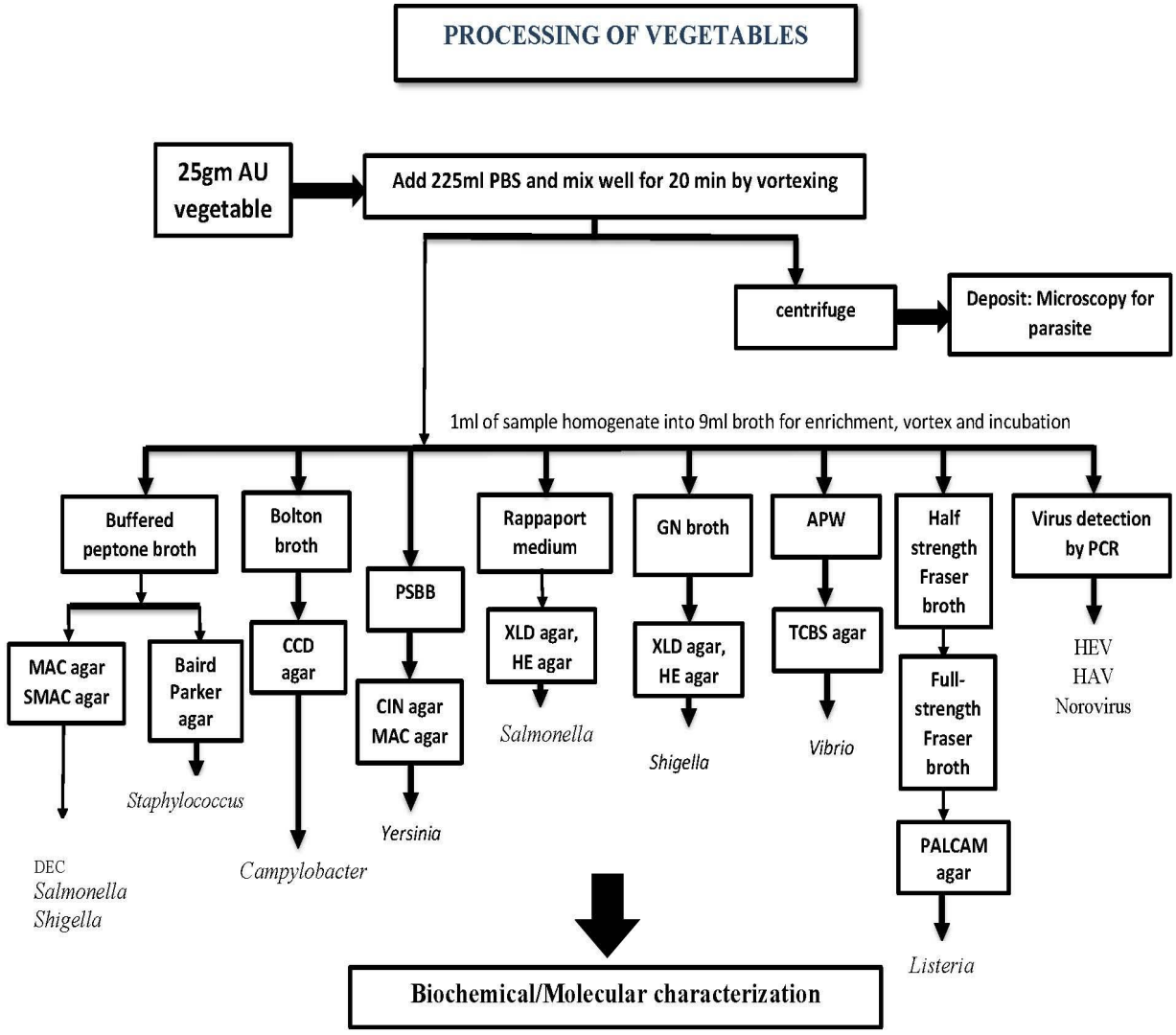


Flow chart-3: Processing of fish for isolation of foodborne pathogens

Procedure

1. Aseptically weigh 25 gm AU of fish and other edible animals (frog, crab, snail, etc.,) from SU and out into a sterile wide mouth container will be minced into small pieces by a sterile scissor and homogenized in 225 ml tryptone or peptone water or PSBB by blending.
2. One ml of the sample homogenate shall be inoculated into each of the following broth (9.0 ml) for enrichment: buffered peptone broth for enrichment of DEC, *Salmonella*, *Shigella*; Selenite-F broth for enrichment of *Shigella*; Rappaport-Vassiliadis (RV) medium for enrichment of *Salmonella*; PSBB for enrichment of *Yersinia*; APW for enrichment of vibrios and half strength Fraser broth for enrichment of *Listeria*. Mix gently by vortexing.
3. After overnight incubation at 37°C, 0.1 ml of the sample shall be inoculated from Buffered peptone broth to a plate of MAC, SMAC, Baird Parker and CCD agar for isolation of DEC*, Salmonella*, *Shigell*a, *Staphylococcus aureus* and *Campylobacter,* respectively.
4. Following 18-24 hrs of incubation, the growth from Selenite-F broth and Rappaport-Vassiliadis (RV) medium is plated to HE (or XLD). The plates are incubated for 18-24 hrs at 37°C in a non-CO_2 incubator for the isolation of *Shigella* and *Salmonella*.
5. Following an incubation of 4~6 hrs at 37°C for enrichment the growth from APW is subcultured on TCBS. The plates are incubated overnight for 18-24 hrs at 35°±2°C in a non-CO_2 incubator for isolation of vibrios.
6. The PSBB is incubated at 10°C for 10 days, after which it is streaked on CIN and MacConkey plates for isolation of *Yersinia*.
7. For isolation of *Listeria*, with 0.1 ml of pre-enriched Fraser broth content in 10 ml full strength Fraser broth containing selective supplements for 48 hrs at a 37°C incubation temperature will be done. The inoculum will be plated on PALCAM agar and incubated for 48 hrs at 37°C for isolation.
8. Virus will be detected by RT-PCR directly after extraction of RNA from the diluted sample.
9. For parasite microscopic detection homogenization is done with saline and digestion in a mixture of pepsin in saline. After centrifugation the deposit is observed for parasite ova or cyst.

PROCESSING OF VEGETABLES

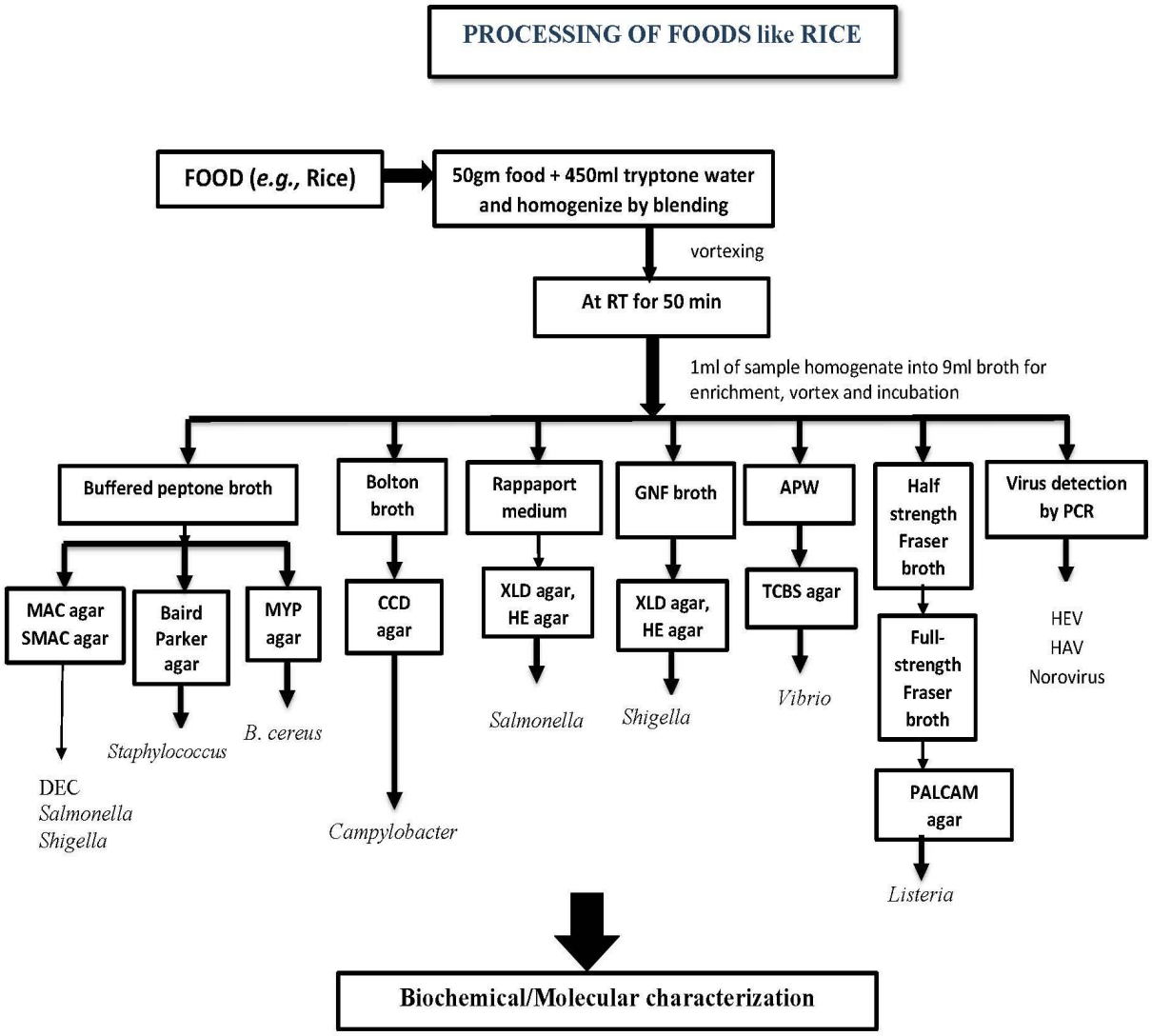


Flow chart-4: Processing of vegetables for isolation of foodborne pathogens

Procedure

1. From 100 gm SU of vegetables, aseptically weigh 25 gm AU will be mixed with 225 ml PBS and mixed well by swirling using sterile glass rod/spoon for 20 min. Incubate at 35º ± 2.0º C for 24 ± 2.0 hrs.
2. One ml of the sample homogenate shall be inoculated into each of the following broth (9.0 ml) for enrichment: buffered peptone broth for enrichment of DEC, *Salmonella*, *Shigella*; Selenite-F broth for enrichment of *Shigella*; Rappaport-Vassiliadis (RV) medium for enrichment of *Salmonella*; PSBB for enrichment of *Yersinia*; APW for enrichment of vibrios and half strength Fraser broth for enrichment of *Listeria*. Mix gently by vortexing.
3. After overnight incubation at 37°C, 0.1 ml of the sample shall be inoculated from Buffered peptone broth to a plate of MAC, SMAC, Baird Parker agar for isolation of DEC*,* *Salmonella*, *Shigell*a, *Staphylococcus aureus,* respectively.
4. Following 18-24 hrs of incubation, the growth from Selenite-F broth and Rappaport-Vassiliadis (RV) medium is plated to HE (or XLD). The plates are incubated for 18-24 hrs at 37°C in a non-CO_2 incubator for the isolation of *Shigella* and *Salmonella*.
5. Following an incubation of 4~6 hrs at 37°C for enrichment the growth from APW is subcultured on TCBS. The plates are incubated overnight for 18-24 hrs at 35°±2°C in a non-CO_2 incubator for isolation of vibrios.
6. The PSBB is incubated at 10°C for 10 days, after which it is streaked on CIN and MacConkey plates for isolation of *Yersinia*.
7. For isolation of *Listeria*, with 0.1 ml of pre-enriched Fraser broth content in 10 ml full strength Fraser broth containing selective supplements for 48 hrs at a 37°C incubation temperature will be done. The inoculum will be plated on PALCAM agar and incubated for 48 hrs at 37°C for isolation.
8. Virus will be detected by RT-PCR directly after extraction of RNA from the diluted sample.
9. For parasite microscopic detection each vegetable sample was eluted by vigorous agitation in a water bath in 1 L of sterile phosphate-buffered saline (pH 7.4), to which 50 ml of 0.01% Tween 80 will be added. The eluent was filtered through gauze and then dispensed into clean centrifuge tubes and centrifuged at 2000*g* for 30 min. The centrifuged deposit will be observed for parasite ova or cyst.

PROCESSING OF FOODS: RICE

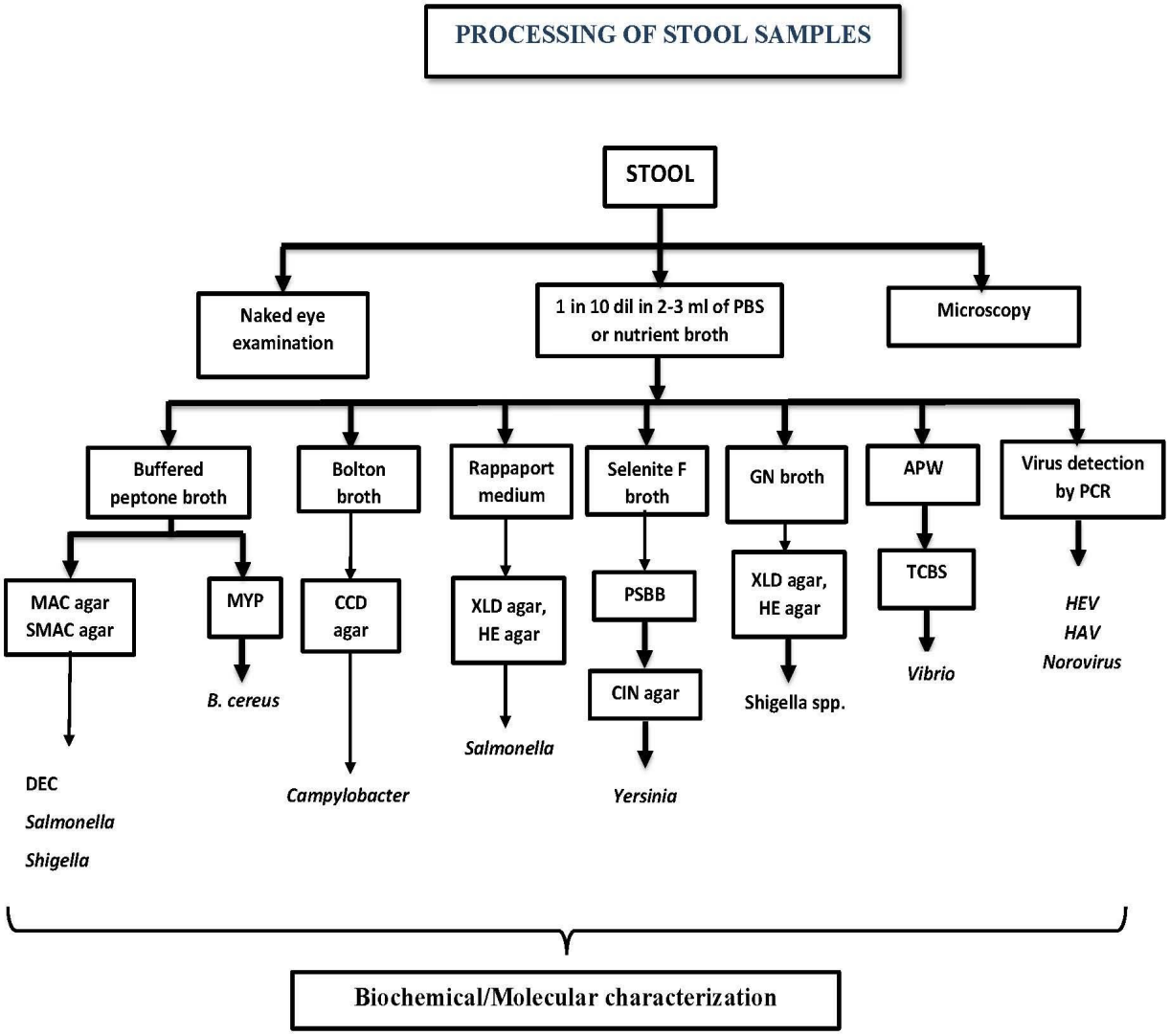


Flow chart-5: Processing of dried foods like rice for isolation of foodborne pathogens

Procedure

1. Fifty gm AU of food like rice will be mixed with 450 ml of tryptone water, homogenize and mixed well by blending for 50 min at room temperature.
2. One ml of the mixture homogenate shall be inoculated into each of the following broth (9.0 ml) for enrichment: buffered peptone broth for enrichment of DEC, *Salmonella*, *Shigella, B. cereus*; Selenite-F broth for enrichment of *Shigella*; Rappaport-Vassiliadis (RV) medium for enrichment of *Salmonella*; APW for enrichment of vibrios and half strength Fraser broth for enrichment of *Listeria*. Mix gently by vortexing.
3. After overnight incubation at 37°C, 0.1 ml of the sample shall be inoculated from Buffered peptone broth to a plate of MAC, SMAC, Baird Parker, CCD, MYP agar for isolation of DEC, *Salmonella*, *Shigell*a, *Staphylococcus aureus, Campylobacter* and *Bacillus cereus,* respectively.
4. Following 18-24 hrs of incubation, the growth from Selenite-F broth and Rappaport-Vassiliadis (RV) medium is plated to HE (or XLD). The plates are incubated for 18-24 hrs at 37°C in a non-CO_2 incubator for the isolation of *Shigella* and *Salmonella*.
5. Following an incubation of 4-6 hrs at 37°C for enrichment the growth from APW is subcultured on TCBS. The plates are incubated overnight for 18-24 hrs at 35°±2°C in a non-CO_2 incubator for isolation of vibrios.
6. For isolation of *Listeria*, with 0.1 ml of pre-enriched Fraser broth content in 10 ml full strength Fraser broth containing selective supplements for 48 hrs at a 37°C incubation temperature will be done. The inoculum will be plated on PALCAM agar and incubated for 48 hrs at 37°C for isolation.
7. Virus will be detected by RT-PCR directly after extraction of RNA from the diluted sample.

PROCESSING OF STOOL SAMPLES

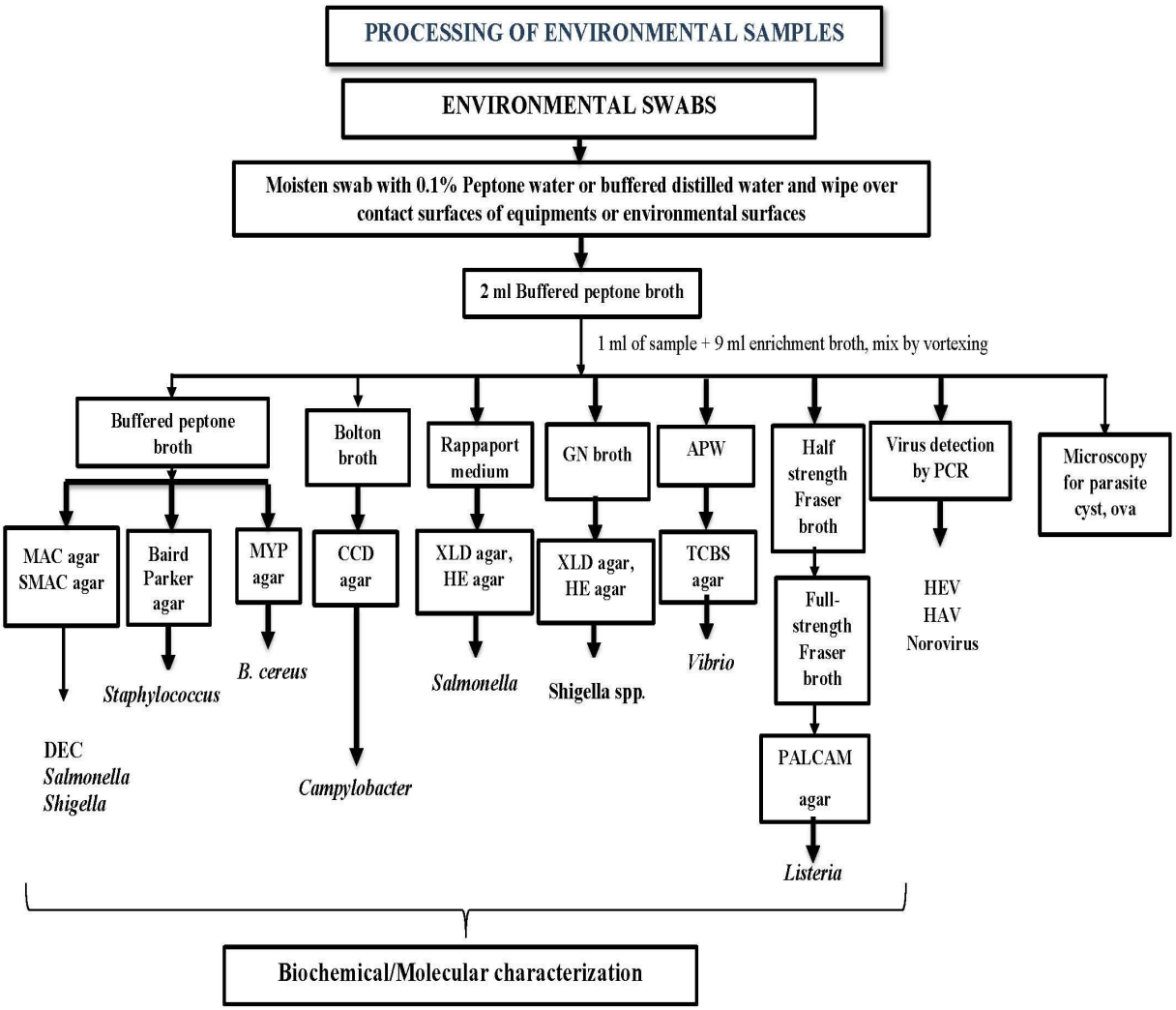


Flow chart-6: Processing of stool for isolation of foodborne pathogens

Procedure

1. The stool will be examined microscopically for consistency, presence of blood, or any helminth
2. A smear of stool will be also examined microscopically for parasitic ova or cyst.
3. For bacterial isolation, first the sample will be diluted 1-10 dilutions in 2-3 ml PBS or nutrient broth.
4. For direct plating: Using an applicator swab, collect a small amount of feces. Collect from areas with visible blood or mucous, if present. The swabs are then to be rolled over the first quadrant of the MacConkey & Hektoen plate (or MacConkey & XLD plate). Then, using a sterile 1 µl inoculating loop the plates are to be streaked for isolation. Insert a new, sterile swab into the sample and then drop the swab into the tube of Selenite-F broth. Loosely cap the tube. Incubate all media at 36°C (+/- 1°C) in a non-CO_2 incubator for 18-24 hrs (overnight). Following overnight (18-24 hrs) incubation, plates are examined for *Salmonella*-like or *Shigella*-like colonies:
5. One ml of the diluted sample shall be inoculated into each of the following broth for enrichment: buffered peptone broth, Selenite-F broth, APW and half strength Fraser broth.
6. After overnight incubation at 37°C, 0.1 ml of the sample shall be inoculated from Buffered peptone broth to a plate of MAC, SMAC, CCD, and MYP for isolation of *E. coli* pathogroups*, Salmonella*, *Shigell*a, *Staphylococcus aureus, Campylobacter* and *Bacillus cereus,* respectively.
7. Following 18-24 hrs of incubation, the growth from Selenite-F broth is plated to HE (or XLD). The plates are incubated for 18-24 hrs at 37°C in a non-CO_2 incubator for isolation of *Salmonella* and *Shigella*.
8. Following an incubation of 4-6 hrs at 37°C for enrichment the growth from APW is subcultured on TCBS for isolation of vibrios.
9. The PSBB is incubated at 10°C for 10 days, after which it is streaked on CIN and MacConkey plates for isolation of *Yersinia*.
10. For isolation of *Listeria*, with 0.1 ml of pre-enriched Fraser broth content in 10 ml full strength Fraser broth containing selective supplements for 48 hrs at a 37°C incubation temperature will be done. The inoculum will be plated on PALCAM agar and incubated for 48 hrs at 37°C for isolation.
11. Virus will be detected by RT-PCR directly after extraction of RNA from the diluted sample.

PROCESSING OF ENVIRONMENTAL SAMPLES



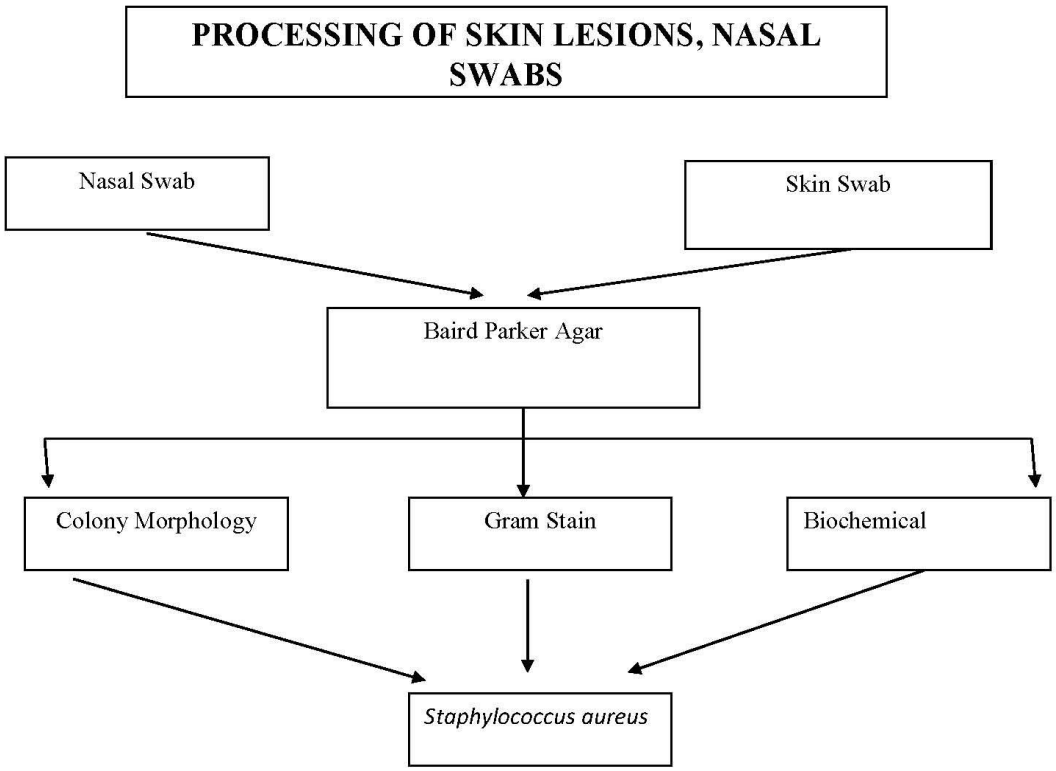
Flow chart-7: Processing of environmental swabs for isolation of foodborne pathogens

Procedure

1. Swab will be moistened with 0.1% peptone water or buffered distilled water and wiped over different surfaces.
2. The swab will be inoculated into 2 ml of buffered peptone water. The sample will be carried to the laboratory.
3. One ml of the diluted sample shall be inoculated into each of the following broth (9.0 ml) for enrichment: buffered peptone broth for enrichment of DEC, *Salmonella*, *Shigella, B. cereus*; Selenite-F broth for enrichment of *Shigella*; Rappaport-Vassiliadis (RV) medium for enrichment of *Salmonella*; PSBB for enrichment of *Yersinia;* APW for enrichment of vibrios and half strength Fraser broth for enrichment of *Listeria*. Mix gently by vortexing.
4. After overnight incubation at 37°C, 0.1 ml of the sample shall be inoculated from Buffered peptone broth to a plate of MAC, SMAC, CCD, and MYP for isolation of DEC*, Salmonella*, *Shigell*a, *Staphylococcus aureus, Campylobacter* and *Bacillus cereus* respectively.
5. Following 18-24 hrs of incubation, the growth from Selenite-F broth Rappaport-Vassiliadis (RV) medium is plated to HE (or XLD). The plates are incubated for 18-24 hrs at 37°C in a non-CO_2 incubator for the isolation of *Shigella* and *Salmonella*.
6. Following an incubation of 4-6 hrs at 37°C for enrichment the growth from APW is subcultured on TCBS for isolation of vibrios.
7. The PSBB is incubated at 10°C for 10 days, after which it is streaked on CIN and MacConkey plates for isolation of *Yersinia*.
8. For isolation of *Listeria*, with 0.1 ml of pre-enriched Fraser broth content in 10 ml full strength Fraser broth containing selective supplements for 48 hrs at a 37°C incubation temperature will be done. The inoculum will be plated on PALCAM agar and incubated for 48 hrs at 37°C for isolation.
9. Virus will be detected by RT-PCR directly after extraction of RNA from the diluted sample.

10. Parasites can be detected directly from the diluted sample by microscopy following centrifugation

PROCESSING OF SKIN LESIONS, NASAL SWABS (Only during outbreak)



Flow chart-8: Processing of skin and nasal swabs from food handlers for isolation of foodborne pathogens

Procedure

1. Nasal and skin swabs will be directly plated onto Baird parker agar plate and incubated overnight at 37°C.
2. *Staphylococcus aureus* will be identified based on colony morphology, Gram stain and biochemical tests.

5.4 PROCEDURE FOR ISOLATION OF PATHOGENS

SALMONELLA & SHIGELLA

Steps for isolation from stool

1. Using an applicator swab, collect a small amount of faeces. Collect from areas with visible blood or mucous, if present. The swabs are then to be rolled over the first quadrant of the MacConkey & Hektoen plate (or MacConkey & XLD plate).
2. Then, using a sterile 10 µl inoculation loop the plates are to be streaked for isolation.
3. Insert a new, sterile swab into the sample and then drop the swab into the tube of Selenite-F broth. Loosely cap the tube. Incubate all media at 36°C (+/- 1°C) in a non-CO_2 incubator for 18-24 hrs (overnight). Following overnight (18-24 hrs) incubation, plates are examined for *Salmonella*-like or *Shigella*-like colonies.
4. Insert a new, sterile swab into the sample and then drop the swab into the tube of Rappaport-Vassiliadis (RV) medium. Loosely cap the tube. Incubate all media at 36°C (+/- 1°C) in a non-CO_2 incubator for 18-24 hrs (overnight). Following overnight (18-24 hrs) incubation, plates are examined for *Salmonella*-like or *Shigella*-like colonies.

Colony morphology of *Salmonella* & *Shigella* in selective media

Medium	**Colony morphology**
MacConkey Agar (MAC)	Both *Salmonella* and *Shigella* produce colourless (lactose negative) colonies (2-4 mm) on MAC
Hektoen Enteric Agar (HE)	*Salmonella* typically produces clear colonies with distinct black centres (hydrogen sulphide: H_2S^+) on HE. Colonies of *Salmonella* ser. Typhi are typically clear with pinpoint black centres and colonies of *Salmonella* ser. Paratyphi A are typically clear (no H_2S) (**Fig. S1a**). *Shigella* spp. typically produce colonies on HE, which range in colour from clear to white/pale-green (**Fig. S2a**)

Xylose Lysine Desoxycholate Agar (XLD)	*Salmonella* typically produces clear to light pink colonies with distinct black centres on XLD. Colonies of *Salmonella* ser. Typhi are typically clear with pinpoint black centres and colonies of *Salmonella* ser. Paratyphi A are typically clear (no H_2S) (**Fig. S1a**) *Shigella* spp. typically produce colonies on XLD which range in colour from clear to white/pale-red (**Fig. S2a**)

Broth Enrichment:

Following 18-24 hrs of incubation, the Selenite-F broth and Rappaport-Vassiliadis (RV) medium is plated to HE (or XLD) using the same technique as above. The plates are incubated for 18-24 hrs at 36°C in a non-CO_2 incubator.

Following overnight incubation, the plate is examined as described above, suspect colonies must be biochemically confirmed.

Steps for isolation from foods and water:

1. Mix the incubated Rappaport-Vassiliadis (RV) medium (in tube) by vortexing. Culture from the broth is plated to MacConkey, HE (or XLD) agar. The plates are incubated at 36°C (+/- 1°C) in a non-CO_2 incubator for 18-24 hrs (overnight). Following overnight incubation, the plate is examined for *Salmonella*-like colonies as described above.
   Suspect colonies must be biochemically confirmed.
2. Mix the incubated Selenite-F broth (in tube) by vortexing. Culture from the broth is plated onto MacConkey, HE (or XLD) agar. The plates are incubated at 36°C (+/- 1°C) in a non-CO_2 incubator for 18-24 hrs (overnight). Following overnight incubation, the plate is examined *Shigella*-like colonies as described above.
   Suspect colonies must be biochemically confirmed.

Biochemical Identification:

Pickup well separated colony grown in selective media (use five separated colonies for each sample) and sub-culture on nutrient agar (NA) plates, incubate at 37°C for 18-24 hrs. Biochemical tests is only performed from the bacterial colonies grown in NA medium.

Biochemical Identification of *Salmonella* & *Shigella* (WHO-GFN laboratory protocol) (Figs. S1b and S2b)

Test	***Salmonella* (majority)**	***Salmonella* serovar Typhi**	***Salmonella* serovar Paratyphi**	***Shigella* spp.**
TSI (slant)	K	K	K	K
TSI (butt)	A	A	A	A
TSI (H_2S)	+	Trace amount	Negative	Negative
TSI (gas)	+	No gas	+	- (most)
LIA	+	+	-	-
MIO (Motility)	+	+	+	-
MIO (Ornithine)	+	+	+	*S. dysenteriae, S. flexneri, & S. boydii*: - *S. sonnei*: +
MIO (Indol)	-	-	-	Varies by species / serotype
Urea	-	-	-	-
Citrate (Simmons)	+	-	-	-

Scheme for identification of *Salmonella enterica*

Isolate identification- Based on colony morphology and non-lactose fermentation, the isolates are identified using standard biochemical tests. The *Enterobacteriaceae* with biochemical indicating *Salmonella* Typhi or Paratyphi A, B or C will be further confirmed by slide agglutination test using the *Salmonella* antisera.

Serologic identification of *Shigella*: rapid slide agglutination test

1. If the test for urease is negative on non-motile cultures with TSI reactions suggestive of *Shigella* species, we shall do rapid slide agglutination tests, with the appropriate sera, according to indole test and mannitol fermentation test results.

2. *Shigella* is divided based on mannitol fermentation

Mannitol negative- *Shigella dysenteriae* (Group A)

Mannitol positive- *Shigella flexneri* (Group B)

Shigella boydii (Group C)

Shigella sonnei (Group D)

3. Group A (10 serotypes) is divided on the indole production into

Indole negative serotypes 1, 3, 4, 5, 6, 9, 10

Indole positive serotypes 2, 7, and 8

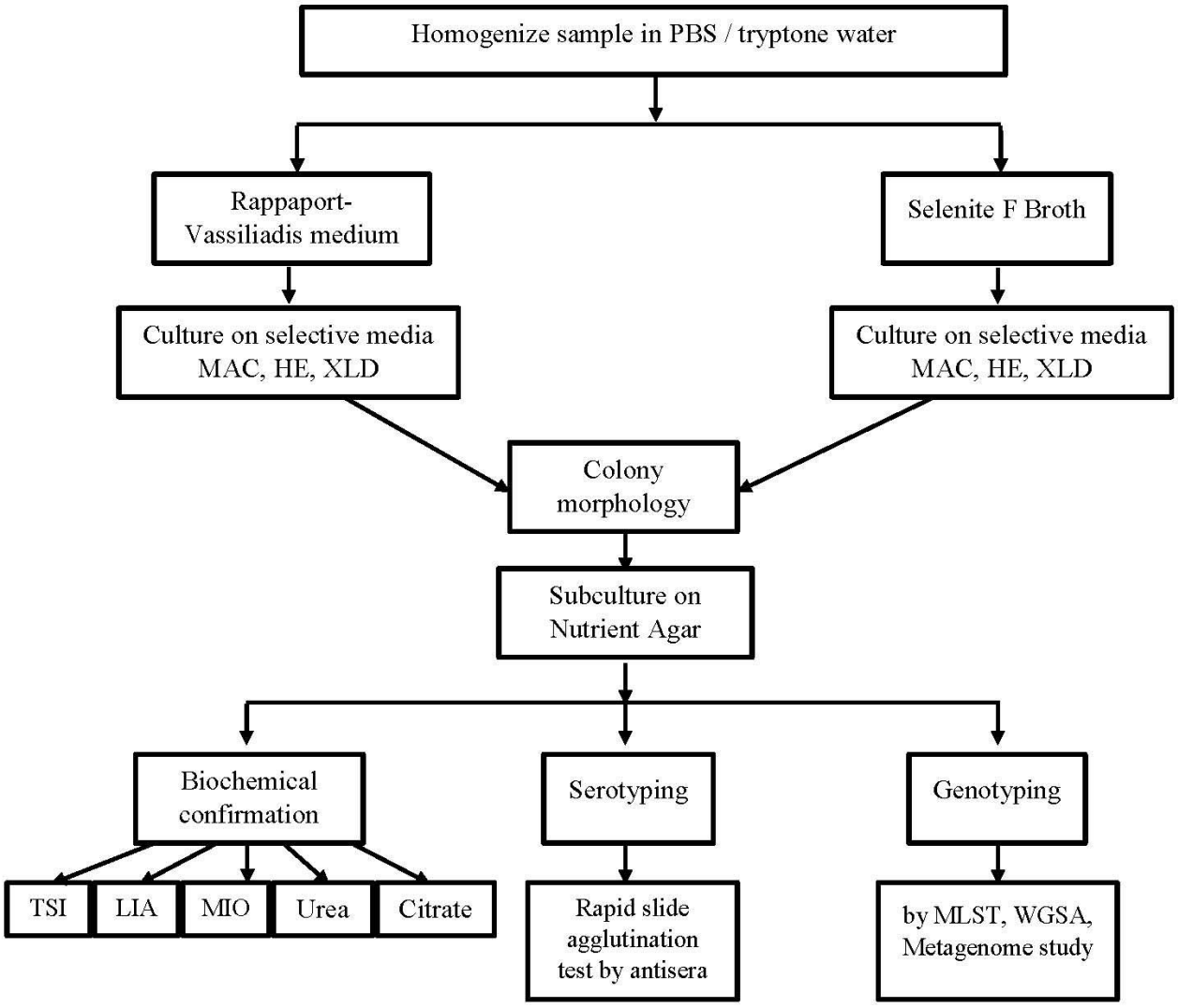
4. Groups B, C, D are based on lactose fermentation and indole production

5. Group D is the only late lactose fermenter; it doesn't produce indole and has only one serotype.

6. Between Group B (6 serotypes) and Group C (15 serotypes); only serotypes 6 of Group B is indole negative along with serotypes 1, 2, 3, 4, 6, 8, 10, 12, 14 of group C and serotypes 1-5 of group B with serotypes 5, 7, 9, 11, 13, 15 of group C are indole positive.

7. Suspend growth from cultures, which appear to be *Shigella*, but which do not react with *Shigella* antisera in saline, heat at 100°C for 30 min, and retest.

Isolation & identification of *Salmonella* & *Shigella* from food and environmental samples



Flow Chart-9: Isolation & identification of *Salmonella* and *Shigella* from food & human faeces

CAMPYLOBACTER SPP.

Steps for isolation of thermotolerant *Campylobacter* from faeces, food or water

Procedure for faeces

Day 1: Selective enrichment with charcoal cefoperazone deoxycholate (CCD) agar plates

Pick faeces by a swab, and streak it onto CCD agar plate. Incubate the plate at 42°C for 1-5 days under microaerophilic conditions.

Day 3: Spreading on Columbia agar plates containing 5% cattle blood

Characteristic growth from CCD-agar plates is transferred to a blood agar plate in a way that single colonies can be expected. Incubate under microaerophilic conditions overnight at 42°C.

Procedure for food

Day 1: Enrichment in selective medium

Transfer 10 gm of food to a flask containing 90 ml of Preston broth. Incubate the enrichment broth at 42°C for 24 to 48 hrs. The flask must be equipped with a cotton plug and incubated under microaerophilic conditions.

Day 2: Isolation on solid selective medium, CCD-agar

Using a 10 µl loop, transfer material from the incubated enrichment broth to a CCD agar plate. Incubate under microaerophilic conditions at 42ºC for 1-5 days. Observe for white smooth colonies (**Fig. S3a**).

Day 3: Spreading on Columbia agar plates containing 5% sheep blood

Characteristic growth from CCD-agar plates is transferred to a blood agar plate in a way that single colonies can be expected. Incubate under microaerophilic conditions overnight at 42°C. In the blood agar, *Campylobacter* spp will grow as non-haemolytic colonies with typical growth along the streak line (**Fig. S3a**).

Procedure for water

Day 1: Enrichment in selective medium

Transfer 10 ml of the water to a flask containing 90 ml of Preston broth or more in general to filtrate a portion of water, do not let filter become completely dry. Immediately transfer finished filter aseptically to broth using a sterile forceps. Enrich the filter in 10-25 ml Preston broth. Incubate the enrichment broth at 42°C for 24-48 hrs. The flask must be equipped with a cotton plug and incubated under microaerophilic conditions.

Day 2: Isolation on solid selective medium, CCD-agar

Using a 10 µl loop, transfer material from the incubated enrichment broth to a CCD agar plate. Incubate under microaerophilic conditions at 42°C for 1-5 days.

Day 3: Spreading on Columbia agar plates containing 5% sheep blood

Characteristic growth from CCD-agar plates is transferred to a blood agar plate in a way that single colonies can be expected. Incubate under microaerophilic conditions overnight at 42°C.

Identification

Biochemical characterization:

Generally, they are inert organisms. The morphology on Gram's stain is confirmatory.

Reactions are as follows.

Oxidase-positive, nitrate reduction positive, indole negative, urease negative, methyl red negative, Voges-Proskauer negative. *C. jejuni* is hippurate test-positive, whereas, *C. coli* is-negative (**Fig. S3b**).

Multiplex PCR for the detection of *C. jejuni* and *C. coli*

Shall be carried out using published primers

Primers

Primer Col1: 5'-AGGCAAGGGAGCCTTTAATC-3'

Primer Col2: 5'-TATCCCTATCTACAAATTCGC-3'

Primer Jun3: 5'-CATCTTCCCTAGTCAAGCCT-3'

Primer Jun4: 5'-AAGATATGGCACTAGCAAGAC-3'

Procedure

1. One ml of PBS is transferred to a 1.5 ml Eppendorf tube. Using a disposable inoculation loop a loop full of bacteria is picked from a plate and transferred to the Eppendorf tube.
2. Centrifuge at 14,000 rpm for 5 min. Supernatant is discarded and the pellet is re-suspended in 100 µl TE 10:1.
3. Boil the suspension (or heat at 95˚C) for 5-10 min and transfer directly to ice. Dilute the lysed DNA 10-fold in TE 10:1
4. Check the number of samples and calculate the amount of PCR master mix needed.
5. Prepare the PCR master mix in a tray of crushed ice.
6. Aliquot the PCR master mix to the required number of PCR tubes (24 µl per tube). Depending on the PCR machine that is used, one drop of mineral oil should be added.
7. Add 1 µl of water to the negative control tube and close the lid.
8. Add 1 µl of sample to the sample tube and close the lid.
9. Finish the procedure by adding the positive control DNA and close the lid.
10. Place the tubes into the PCR thermocycler
11. Program the PCR thermocycler
12. Run the program as given below

PCR Program

5 min. 94°C

1 min. 94°C, 1 min. 64°C, 1 min 72°C, 2 cycles

1 min. 94°C, 1 min. 62°C, 1 min 72°C, 2 cycles

1 min. 94°C, 1 min. 60°C, 1 min 72°C, 2 cycles

1 min. 94°C, 1 min. 58°C, 1 min 72°C, 2 cycles

1 min. 94°C, 1 min. 56°C, 1 min 72°C, 2 cycles

1 min. 94°C, 1 min. 54°C, 1 min 72°C, 30 cycles

10 min. 72°C

Hold 4°C

Preparation of the agarose gel

1. Assemble the gel tray and make a proper set-up.
2. Prepare a 1% agarose solution by boiling the solution a few min till completely solved by the use of a water bath or microwave oven.
3. Cool the agarose to 40-50°C in a water bath and poor the agarose into the gel tray.
4. Let the gel solidify for 15-30 min.
5. Prepare a staining bath containing a final concentration of 5 µg/ml ethidium bromide.

Strains for Positive control

C. jejuni ATCC 29428: Positive control

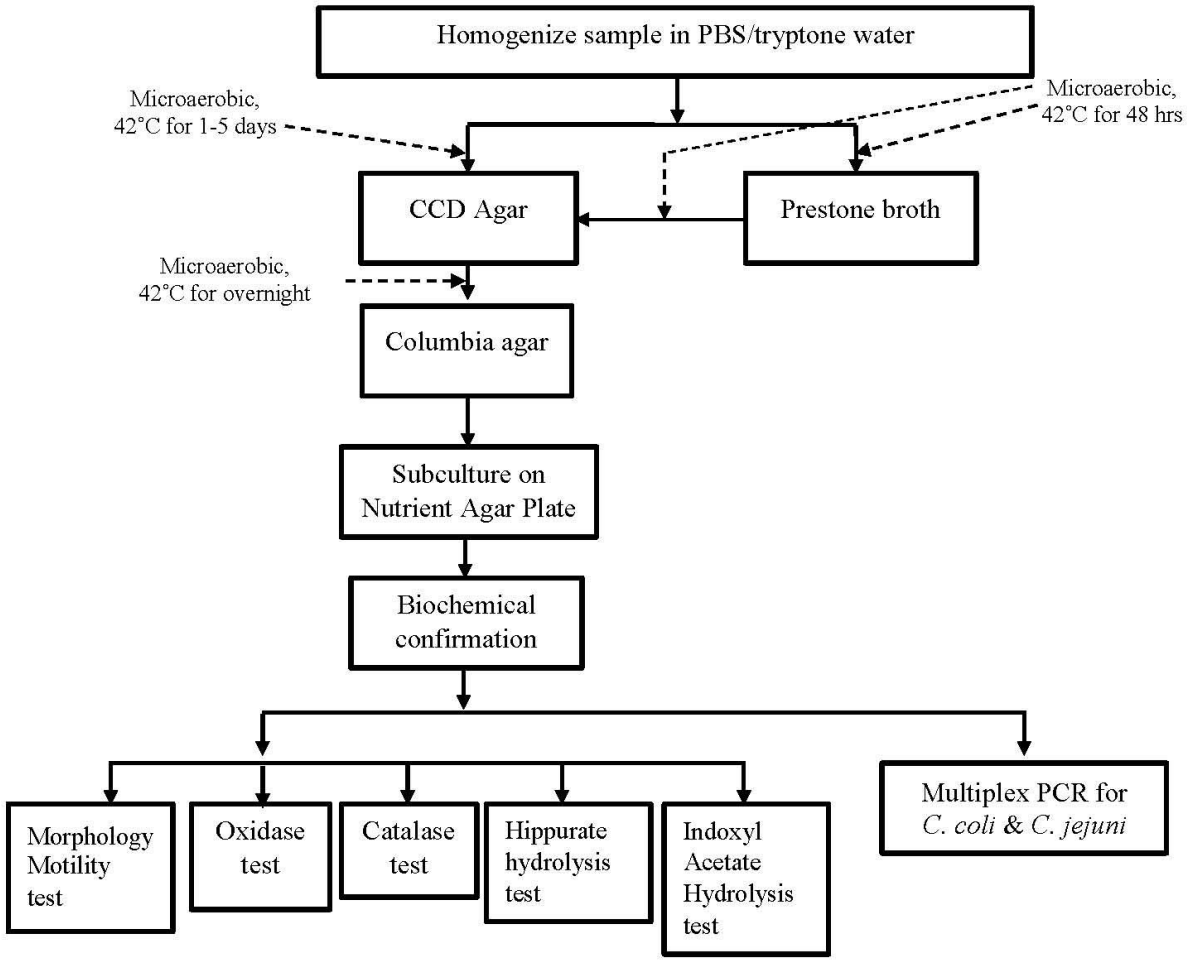
C. coli ATCC 33559: Positive control

C. lari ATCC 35221: Negative control

Detection

Look for the presence of specific bands. For *C. jejuni* this should be 773 bp and for *C. coli* 364 bp.

Identification and characterization of *Campylobacter*



Flow chart-10: Isolation & identification of Campylobacter from food & human faeces

ESCHERICHIA COLI O157:H7

Steps for isolation and identification of *Escherichia coli* O157:H7 from faeces and food

Day 1: Enrichment

Shall be carried out in buffered peptone water

Spread on selective agar plates:

Spread-inoculate two plates of each dilution to Cefixime Tellurite Sorbitol MacConkey (CT-SMAC agar) and one plate of MacConkey agar. Incubate at 37°C for 18 hrs. Typical growths in MaC and SMAC media are shown in **Fig. S4a**.

Day 2: Subculturing of presumptive colonies of *Escherichia coli* O157:H7.

Observe the CT-SMAC plates.

Isolate five presumptive sorbitol negative colonies from each agar plate and inoculate onto Nutrient agar plates. Incubate at 37°C for 18-24 hrs.

Escherichia coli O157:H7 on CT-SMAC plates. The colonies are transparent/colourless with a weak, pale brownish appearance because the bacterium does not ferment sorbitol (**Fig. S4a**).

Observe the MacConkey agar for other DEC (**Fig. S5a**). Typical biochemical test results are shown in **Figs. S4b** and **S5b**.

Pick five *Escherichia coli* O157:H7 suspect colonies from the CT-SMAC agar plates and streak the colonies onto non-selective medium, e.g. nutrient agar plates for biochemical confirmation and agglutination. Incubate the plates at 37°C for 18-24 hrs. Continue with the biochemical confirmation on pure cultures.

Day 3 & 4: Biochemical confirmation.

From a pure culture on nutrient agar plates, carry out the indole and other tests (**Figs. S3b** and **S4b**).

Escherichia coli O157:H7 should then be agglutinated. If negative, test for the other major STEC serogroups (O26, O103, O111, and O145).

Conventional agglutination of *Escherichia coli* O157:H7

Shall be carried out using latex kit

MULTIPLEX PCR FOR THE DETECTION OF *E. COLI* PATHOTYPES (EAEC, EPEC, EHEC, ETEC, EIEC, DAEC)

Primers		**Size**
DEC multiplex PCR		(Vidal *et al.,* 2005)
EHEC		
stx1	Forward 5'-CAG TTA ATG TGG TGG CGA AGG-3' Reverse 5'-CAC CAG ACA ATG TAA CCG CTG-3'	348 bp
stx2	Forward 5'-ATC CTA TTC CCG GGA GTT TAC G-3' Reverse 5'-GCG TCA TCG TAT ACA CAG GAG C-3'	584 bp
EPEC		
eae	Forward 5'-TCA ATG CAG TTC CGT TAT CAG TT-3' Reverse 5'-GTA AAG TCC GTT ACC CCA ACC TG-3'	482 bp
bfp	Forward 5'-GGA AGT CAA ATT CAT GGG GGT AT-3' Reverse 5'-GGA ATC AGA CGC AGA CTG GTA GT	300bp
ETEC		
elt	Forward 5'-GCA CAC GGA GCT CCT CAG TC-3' Reverse 5'-TCC TTC ATC CTT TCA ATG GCT TT-3'	218 bp
est	Forward 5'-AAA GGA GAG CTT CGT CAC ATT TT-3' Reverse 5'-AAT GTC CGT CTT GCG TTA GGA C	129 bp
EIEC		
virF	Forward 5'-AGC TCA GGC AAT GAA ACT TTG AC-3' Reverse 5'-TGG GCT TGA TAT TCC GAT AAG TC-3'	618 bp
ipaH	Forward 5'-CTC GGC ACG TTT TAA TAG TCT GG-3' Reverse 5'-GTG GAG AGC TGA AGT TTC TCT GC-3'	933 bp

DAEC		
daaE	Forward 5'-GAA CGT TGG TTA ATG TGG GGT AA-3' Reverse 5'-TAT TCA CCG GTC GGT TAT CAG T-3'	542 bp
EAEC		
aafII	Forward 5'-CAC AGG CAA CTG AAA TAA GTC TGG-3' Reverse 5'-ATT CCC ATG ATG TCA AGC ACT TC-3'	378 bp
EAEC		
astA	Forward 5'-TGCCATCAACACAGTATATCCG-3' Forward 5'-ACGGCTTTGTAGTCCTTCCAT-3'	102 bp

EAEC multiplex PCR (Cerna *et al.*, 2003)

aap	Forward 5'-CTT GGG TAT CAG CCT GAA TG-3' Reverse 5'-AAC CCA TTC GGT TAG AGC AC-3'	310 bp
aggR	Forward 5'-CTA ATT GTA CAA TCG ATG TA-3' Reverse 5'-AGA GTC CAT CTC TTT GAT AAG-3'	457 bp
AA probe	Forward 5'-CTG GCG AAA GAC TGT ATC AT-3' Reverse 5'-CAA TGT ATA GAA ATC CGC TGT T-3'	629 bp

Procedure

1. One ml of BHI is transferred to a 1.5 ml Eppendorf tube. Using a disposable inoculation loop a loop full of bacteria is picked from a plate and transferred to the Eppendorf tube.
2. Centrifuge at 15,000g for 3 min. Supernatant is discarded and wash the pellet twice in physiological saline.
3. Resuspend the pellet in 100 µl TE, boil the suspension (or heat at 95˚C) for 5-10 min and transfer directly to ice. Dilute the lysed DNA 10-fold in TE 10:1. Store at -20˚C.
4. Check the number of samples and calculate the amount of PCR master mix needed.
5. Prepare the PCR master mix in a tray of crushed ice
6. Aliquot the PCR master mix to the required number of PCR tubes (24 µl per tube). Depending on the PCR machine that is used.
7. Add 1 µl of water to the negative control tube and close the lid.
8. Add 1 µl of sample to the sample tube and close the lid.
9. Finish the procedure by adding the positive control DNA and close the lid.
10. Place the tubes into the PCR Thermocycler

11. Program the PCR Thermocycler
12. Run the program as given below

<u>The PCR protocol</u>:

DEC multiplex PCR thermal profile

5 min at 94°C
1.5 min at 94°C
1.5 min at 60°C } 35 cycles of
1.5 min at 72°C, followed by a
final extension step at 72°C for 7 min.
hold at 4°C

ETEC PCR thermal profile

5 min at 94°C
1 min at 94°C
1 min at 60°C } 30 cycles of
2 min at 72°C
final extension step at 72°C for 5 min.
hold at 4°C

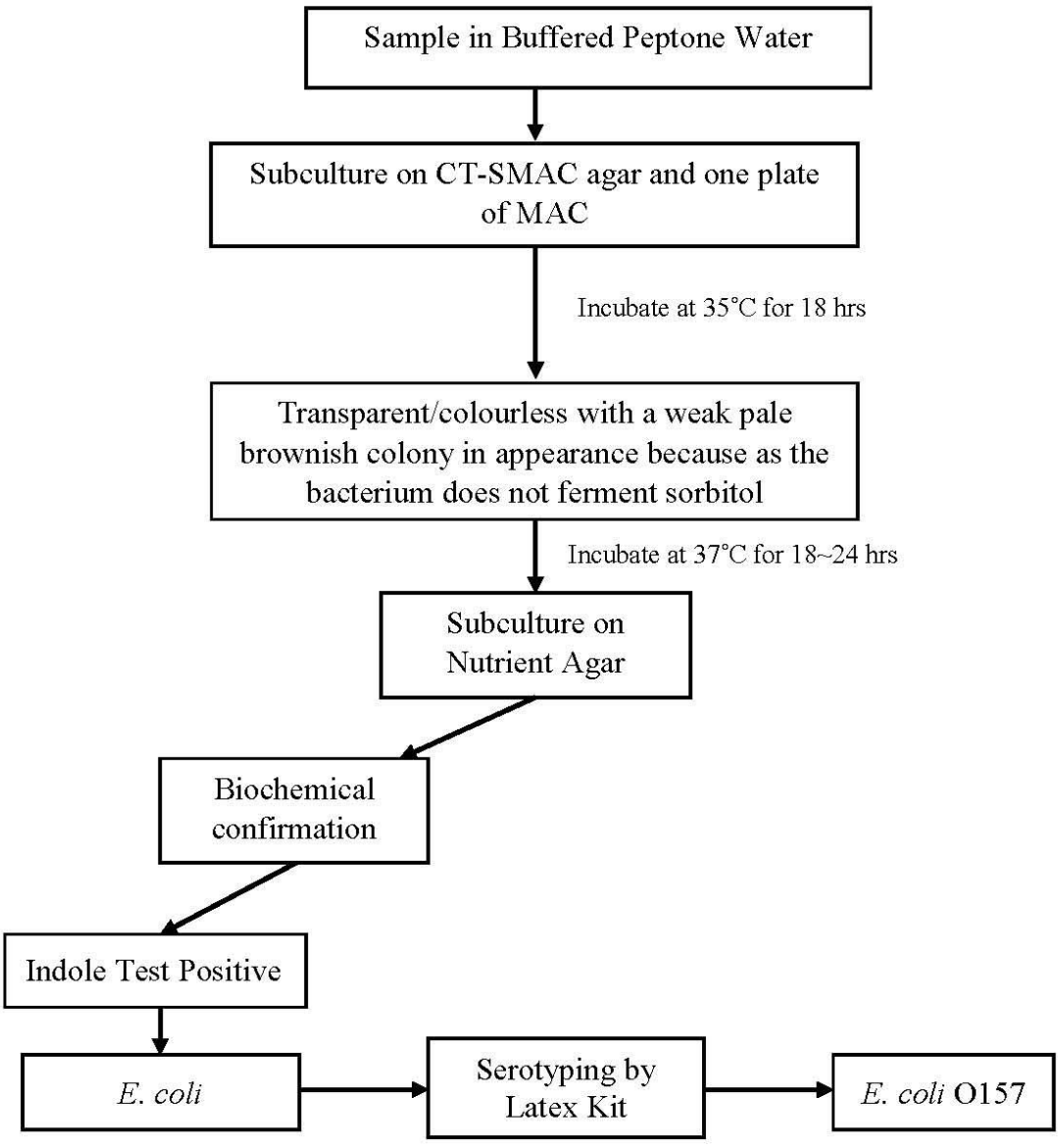
EAEC PCR thermal profile

5 min at 95°C
45 Sec at 94°C
45 Sec at 55°C } 30 cycles of
45 Sec at 72°C
final extension step at 72°C for 10 min and at hold at 4°C.

<u>Detection</u>

The multiplex PCR protocol will detect the 6 pathotypes *E. coli* and the presence of the virulence factors. For example, multiplex PCR amplicons of the most common DEC are shown in **Fig. S5c.**

Flow diagram for isolation and identification of enterohaemorrhagic *Escherichia coli* O157 from food and faeces



Flow Chart 11: Isolation and identification of enterohaemorrhagic *Escherichia coli* O157 from food and faeces

VIBRIO CHOLERAE

Identification of *Vibrio cholerae* O1 and O139 strains from clinical specimens as well as environmental sources

Enrichment, isolation and presumptive identification of V. cholerae

Procedure for processing faecal specimens

Isolation of *V. cholerae* O1/O139 from faecal specimens is accomplished with the use of highly selective media that suppress the growth of other organisms. Alkaline peptone water (APW, pH 8.0) is suggested as an enrichment broth, and thiosulfate citrate bile salts sucrose (TCBS) agar is the selective agar medium. APW enrichment for 4-6 hrs at 37°C and a selective plating medium on TCBS should always be used.

Procedure for processing water samples

For water samples, use the whole membrane for enrichment in 10-20 ml of APW. The membrane should be well immersed in the APW enrichment medium and incubated for 4-6 hrs at 37°C.

Using a sterile loop, a small portion from the surface of the enriched culture to be streaked onto TCBS agar plate. After overnight incubation at 37°C, observe the plates for the presence of well isolated 2-4 mm diameter umbilical shaped yellow coloured colony, which is the indicator for tentative presence of *V. cholerae* in the test samples (**Fig. S6a**).

Characterisation of presumptively identified V. cholerae:

Select well-separated yellow-coloured colonies from TCBS plate (use five separated colonies for each sample) and inoculate on nutrient agar plates and incubate at 37°C for 18 hrs. Isolates that showed oxidase positivity are identified as *V. cholerae*. Typical biochemical test results are shown in **Figs. S6b**.

Serotyping of *V. cholerae*:

Cultures that were presumptively identified as *V. cholerae* will be tested for positivity in the oxidase test followed serological agglutination with polyvalent anti-O1 and anti-O139 sera. Positive agglutination with either O1 or O139 antisera signifies the presence of *V. cholerae* strains of the respective serogroup.

Strains that were identified as *V. cholerae* by the property of giving yellow coloured colony on TCBS, gave oxidase positivity, but not agglutinable with either of the anti-O1 or -O139 sera should be considered as *V. cholerae* strains belonging to non-O1, non-O139 serogroups.

Strains that were identified, as O1 can be further tested with monoclonal anti-Ogawa or anti-Inaba sera for placing these strains in either of the serotype based on their agglutination result.

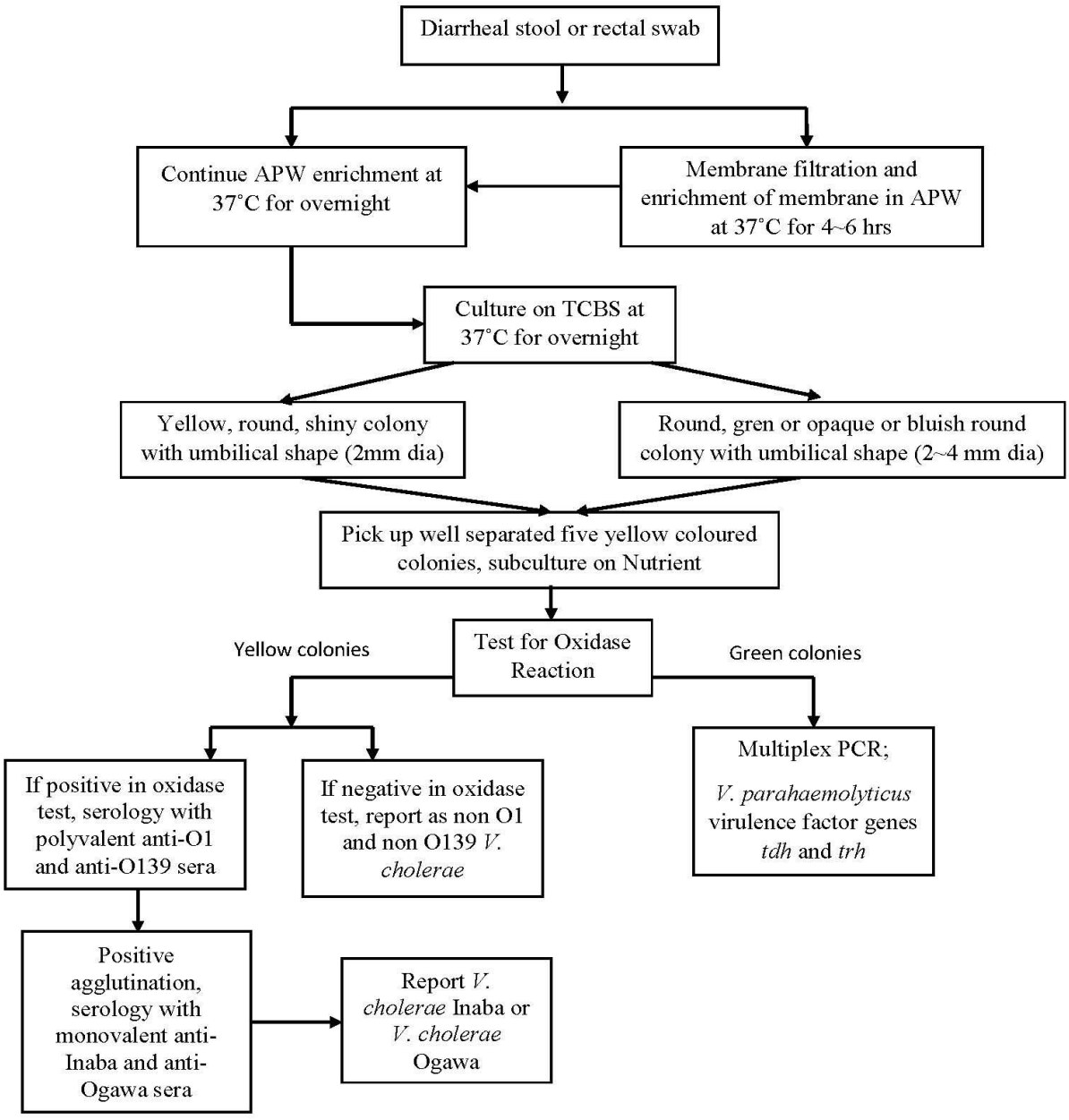
Interpretation:

Isolates that produced yellow coloured colony on TCBS and showed positive oxidase reaction should be tested for serological character.

V. cholerae strains do not produce gas, so any indication related to gas production to be considered as the presence of observation.

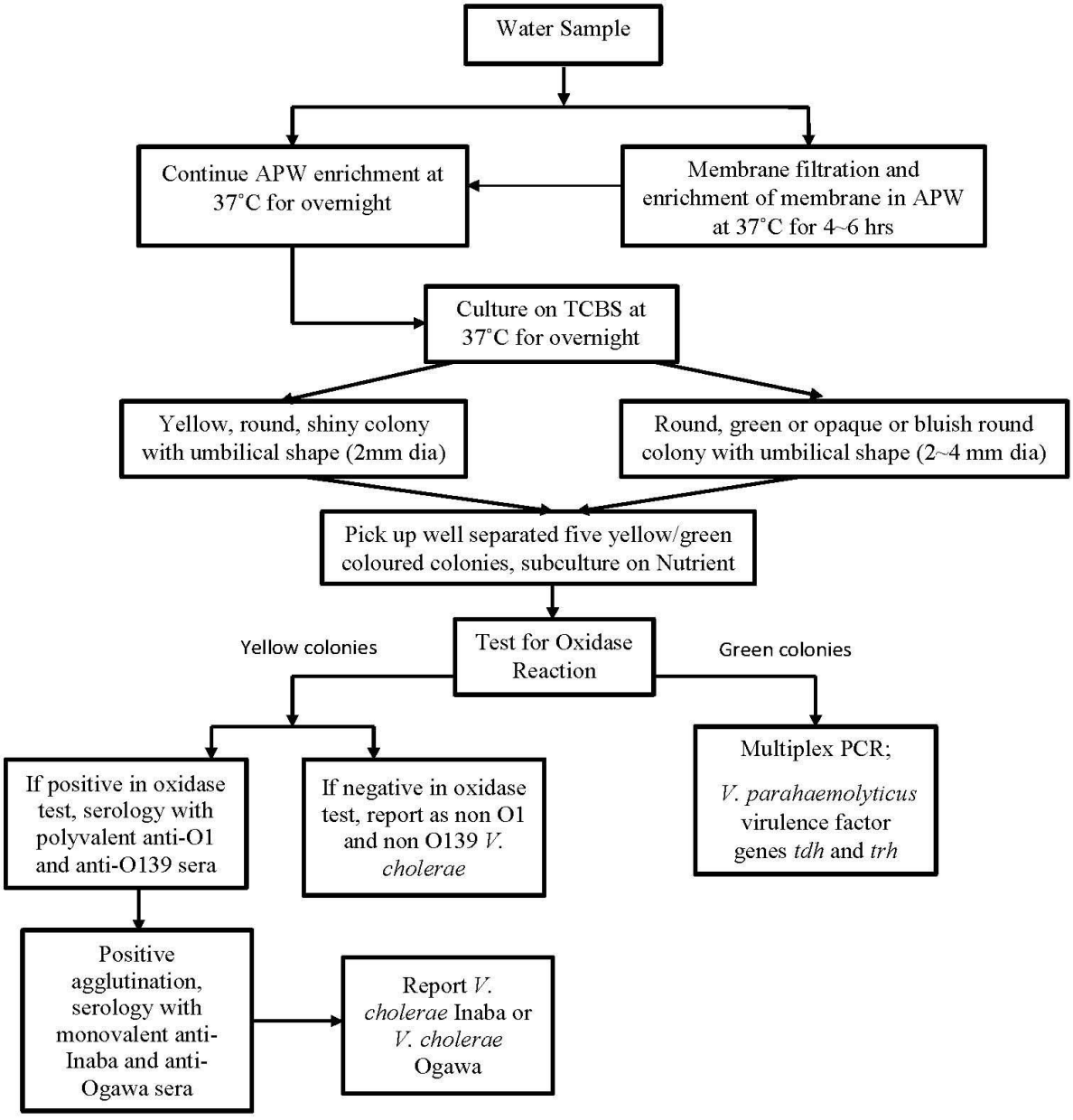
Interpretation should be based on the results obtained from serological tests and to confirm the presence or absence of *V. cholerae* strains belonging to either O1 or O139 serogroups.

Flow diagram for Isolation and Identification *Vibrio Cholerae /Vibrio parahaemolyticus* from stool sample



Flow Chart 12: Isolation and Identification Vibrio Cholerae /Vibrio parahaemolyticus from stool sample

Isolation and Identification *Vibrio Cholerae /Vibrio parahaemolyticus* from Water sample



Flow Chart 13: Isolation and Identification Vibrio Cholerae /Vibrio parahaemolyticus from Water sample

Detection of Cholera toxin gene (*ctx* gene) in *V. cholerae*

A genotypic assay such as PCR amplification of the *ctx* gene is recommended.

Shall be carried out using published primers

Primers

Primer ctx F: 5'-TGAAATAAAGCAGTCAGGTG-3'

Primer ctx R: 5'-GGTATTCTGCACACAAATCAG-3'

Procedure

1. One ml of APW is transferred to a 1.5 ml Eppendorf tube. Using a disposable inoculation loop a loop full of bacteria is picked from a plate and transferred to the Eppendorf tube.
2. Boil the suspension (or heat at 95˚C) for 5-10 min and transfer directly to ice. Dilute the lysed DNA 10-fold in TE 10:1. Store at -20˚C.
3. Check the number of samples and calculate the amount of PCR master mix needed.
4. Prepare the PCR master mix in a tray of crushed ice
5. Aliquot the PCR master mix to the required number of PCR tubes (24 µl per tube). Depending on the PCR machine that is used.
6. Add 1 µl of water to the negative control tube and close the lid.
7. Add 1 µl of sample to the sample tube and close the lid.
8. Finish the procedure by adding the positive control DNA and close the lid.
9. Place the tubes into the PCR thermocycler
10. Program the PCR thermocycler
11. Run the program as given below

PCR Program

3 min. 94°C
1 min. 94°C
1 min. 55°C } 35 Cycles
2 min. 72°C
3 min. 72°C
Hold 4°C

Preparation of the agarose gel

1. Assemble the gel tray and make a proper set-up.
2. Prepare a 1% agarose solution by boiling the solution a few min till completely solved by the use of a water bath or microwave oven.
3. Cool the agarose to 40-50°C in a water bath and poor the agarose into the gel tray.
4. Let the gel solidify for 15-30 min.
5. Prepare a staining bath containing a final concentration of 5 µg/ml ethidium bromide.

Detection

Look for the presence of specific bands, the amplified *ctx* gene should be 777 bp.

VIBRIO PARAHAEMOLYTICUS

Enrichment, isolation and presumptive identification of V. parahaemolyticus:

A loop-full of diarrhoeal stool or the swab stick containing clinical specimen will be used to inoculate APW broth for selective enrichment of *V. parahaemolyticus*. Allow an incubation of 4~6 hrs at 37°C for enrichment.

Using a sterile loop, a small portion from the surface of the enriched culture to be streaked onto TCBS agar plate. After overnight incubation at 37°C, observe the plates for the presence of well-isolated 2~4 mm diameter umbilical shaped green or opaque, or bluish coloured colony, which is the indicator for the tentative presence of *V. parahaemolyticus* (**Fig. S6a**).

Characterization of presumptively identified V. parahaemolyticus

Pickup well separated green coloured colony from TCBS plate (use five separated colonies for each sample) to inoculate on nutrient agar plate and incubate at 37°C for 18 hrs.

Isolates that showed oxidase positivity, presumptively to be identified as *V. parahaemolyticus.* Typical biochemical test results are shown in **Fig. S6b**.

Serotyping of V. parahaemolyticus

Cultures that were presumptively identified as *V. parahaemolyticus w*ill be tested for positivity in the oxidase test and send to NICED, Kolkata for serotyping.

Multiplex PCR for the detection of *V. parahaemolyticus* virulence factors by amplification of *tdh* and *trh* genes (BAM protocol)

Shall be carried out using published primers

Primers

Primer L-TL: 5'-AAA GCG GAT TAT GCA GAA GCA CTG-3'
Primer R-TL: 5'-GCT ACT TTC TAG CAT TTT CTC TGC-3'
Primer VPTRH-L: 5'-TTG GCT TCG ATA TTT TCA GTA TCT-3'
Primer VPTRH-R: 5'-CAT AAC AAA CAT ATG CCC ATT TCC G-3'
Primer VPTDH-L: 5'-GTA AAG GTC TCT GAC TTT TGG AC-3'
Primer VPTDH-R: 5'-TGG AAT AGA ACC TTC ATC TTC ACC-3'

Procedure

1. One ml of PBS is transferred to a 1.5 ml Eppendorf tube. Using a disposable inoculation loop a loop full of bacteria is picked from a plate and transferred to the Eppendorf tube.
2. Centrifuge at 15,000g for 3 min. Supernatant is discarded and wash the pellet twice in physiological saline.
3. Resuspend the pellet in 100 µl TE, boil the suspension (or heat at 95˚C) for 5-10 min and transfer directly to ice. Dilute the lysed DNA 10-fold in TE 10:1. Store at -20˚C.
4. Check the number of samples and calculate the amount of PCR master mix needed.

5. Prepare the PCR master mix in a tray of crushed ice
6. Aliquot the PCR master mix to the required number of PCR tubes (24 µl per tube). Depending on the PCR machine that is used.
7. Add 1 µl of water to the negative control tube and close the lid.
8. Add 1 µl of sample to the sample tube and close the lid.
9. Finish the procedure by adding the positive control DNA and close the lid.
10. Place the tubes into the PCR thermocycler
11. Program the PCR thermocycler
12. Run the program as given below

PCR Program

3 min. 94°C
1 min. 94°C
1 min. 60°C } 25 Cycles
2 min. 72°C
3 min. 72°C
Hold 4°C

Preparation of the agarose gel

1. Assemble the gel tray and make a proper set-up.

2. Prepare a 1% agarose solution by boiling the solution a few min till completely solved by the use of a water bath or microwave oven.

3. Cool the agarose to 40-50°C in a water bath and poor the agarose into the gel tray.

4. Let the gel solidify for 15-30 min.

5. Prepare a staining bath containing a final concentration of 5 µg/ml ethidium bromide.

Detection

Look for the presence of specific bands, the amplified *trh, tdh* and *tlh* gene should be 500 bp, 270 bp and 450 bp respectively.

LISTERIA MONOCYTOGENES

Isolation and identification of *Listeria* spp

Pre-enrichment:

1. Shall be done in half strength Fraser broth containing selective supplements for 24 hrs at 30°C.

Second enrichment

2. Add 0.1 ml of pre-enriched Fraser broth content in 10 ml full strength Fraser broth containing selective supplements and incubate for 48 hrs at 37°C.
3. The inoculum will be plated on PALCAM agar and incubated for 48 hrs at 37°C.
4. The gray-green colonies surrounded by diffuse black zone on PALCAM agar will be picked up and further purification on Tryptone Soya Yeast Extract agar (TSYEA) will be done.
5. The pinpoint colonies in TSYEA will be subjected for Gram staining to look for positive, coccobacillary or short rod-shaped bacterial cells. Typical colony morphology in PALCAM medium and biochemical test results are shown in **Fig. S7.**
6. Catalase test and oxidase test will be done. Colonies showing catalase positive and oxidase negative will be subcultured in Brain-Heart Infusion broth at 25°C for 12-18 hrs.
7. Motility test can be done by hanging drop preparation. Cultures showing typical tumbling motility will be considered as "presumptive" listeria isolates.

Detection of *Listeria monocytogenes* virulence-associated gene by PCR

- The *L. monocytogenes* isolates will be screened for the presence or absence of virulence-associated gene(s) by using the standard PCR protocols for the detection of *act*A, *hly*A, and *iap* genes.

Primers:

Gene	Primer sequence (5'-3')	Amplicon
*act*A	F: CGC CGC GGA AAT TAA AAA AAG R: ACG AAG GAA CCG GGC TGC TAG	839 bp
*hly*A	F: GCA GTT GCA AGC GCT TGG AGT GAA R: GCA ACG TAT CCT CCA GAG TGA TCG	456 bp
iap	F: ACA AGC TGC ACC TGT TGC AG R: TGA CAG CGTGTG TAG TAG CA	131 bp

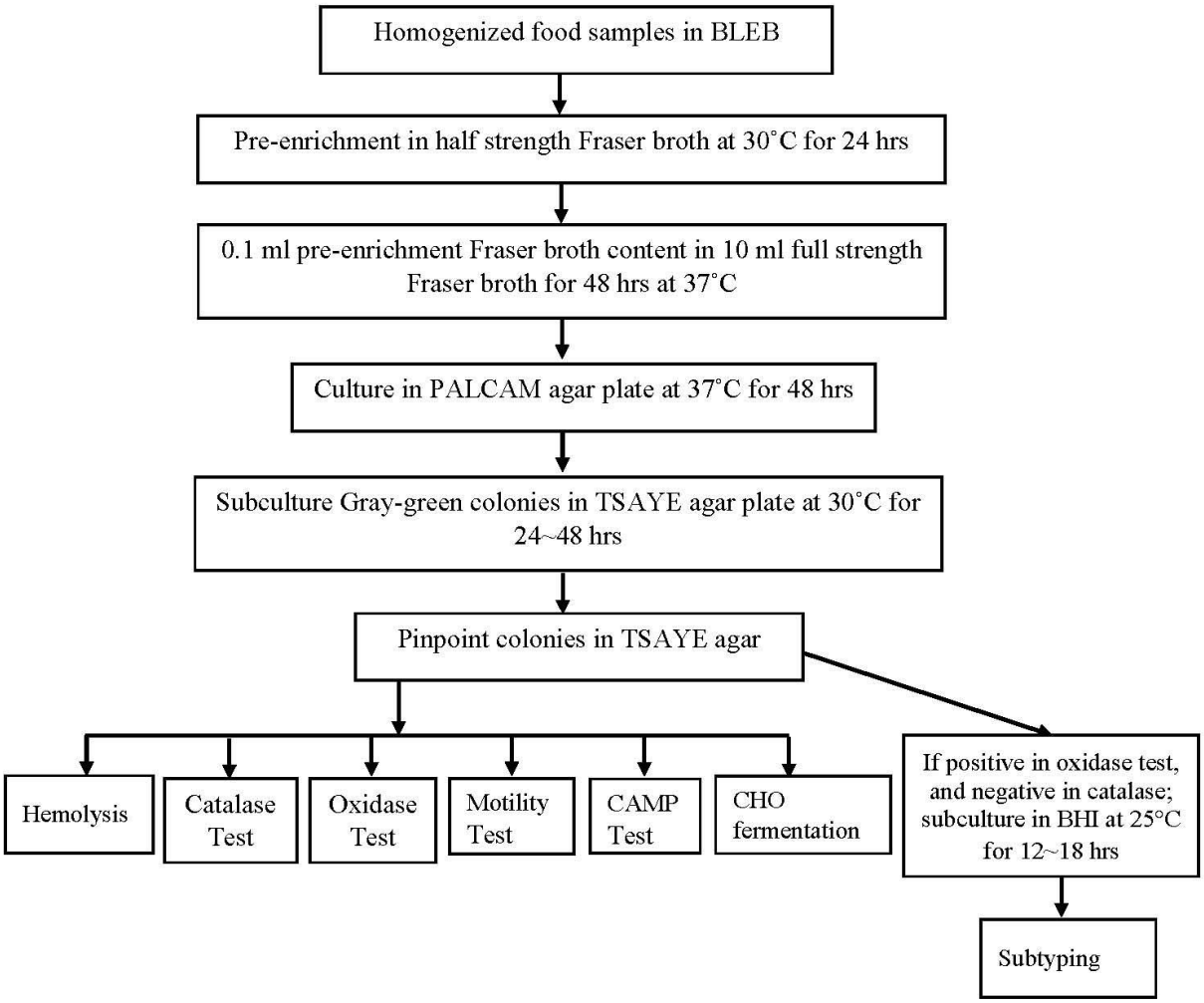
The PCR protocol:

Initial denaturation 95°C for 2 min

Denaturation 95°C for 15 sec
Annealing 60°C for 30 sec
Extension at 72°C for 1 min 30 sec
} 35 cycles

Final extension 72°C for 10 min

Isolation and identification of Listeria monocytogenes from faeces and food



Flow Chart 14: Isolation and identification of *Listeria monocytogenes* from faeces and food

BACILLUS CEREUS

Procedure of isolation

- Serial dilutions will be prepared, and 2 µl of each diluted sample will be streaked in MYP agar medium.
- Plates will be incubated for 24 hrs at 30°C.
- Typical colonies of pink-orange, surrounded by a zone of precipitation indicating lecithinase production (**Fig. S8**).
- These colonies will be presumptively identified to be *B. cereus*. Typical biochemical test results are shown in **Fig. S8.**
- From each sample, a typical colony presumed to belong to the *B. cereus* group will be subcultured on brain heart infusion-yeast extract (BHI-YE) agar and incubated for 24 hrs at 30°C.

Molecular identification

1. Detection of *Bacillus cereus* group

Procedure

- *B. cereus*-like isolates will be grown overnight at 30°C on brain heart infusion-yeast extract (BHI-YE)
- Genomic DNA will be extracted by using commercially available bacterial DNA isolation kit.
- Spore structural protein gene (*sspE*) sequence specific to the *B. cereus* group will be targeted for PCR amplification using published primers.

Primers	**Amplicon**	**Reference**
Forward: 5′-GAAAAAGATGAGTAAAAAACAACAA-3′	71 bp	(Kim et al., 2005)
Reverse: 5′-CATTTGTGCTTTGAATGCTAG-3′		

The PCR protocol:

4 min at 95°C
30 sec at 95°C
30 sec at 59°C } 30 cycles
1 min at 72°C
Final extension step at 72°C for 7 min
Hold at 4°C

Detection

Look for the presence of specific bands, the amplified *sspE* gene should be 71 bp.

2. Detection of Emetic strains of *B. cereus*

Method-1

Primers	**Amplicon**	**Reference**
CER1: 5'-ATCATAAAGGTGCGAACAAGA-3'	188 bp	(Horwood et al., 2004)
EMT1: 5'-AAGATCAACCGAATGCAACTG-3'		

Procedure

1. Check the number of samples and calculate the amount of PCR master mix needed.
2. Prepare the PCR master mix in a tray of crushed ice
3. Aliquot the PCR master mix to the required number of PCR tubes (24 µl per tube). Depending on the PCR machine that is used
4. Add 1 µl of water to the negative control tube and close the lid
5. Add 1 µl of sample to the sample tube and close the lid
6. Finish the procedure by adding the positive control DNA and close the lid.
7. Place the tubes into the PCR thermocycler
8. Program the PCR thermocycler
9. Run the program as given below

PCR Program

10 min 94°C
1 min 94°C
1 min 52°C } 35 Cycles
1 min 72°C

7 min 72°C
Hold 4°C

Preparation of the agarose gel

1. Assemble the gel tray and make a proper set-up.

2. Prepare a 1% agarose solution by boiling the solution a few min till completely solved by the use of a water bath or microwave oven.

3. Cool the agarose to 40-50°C in a water bath and poor the agarose into the gel tray.

4. Let the gel solidify for 15-30 min.

5. Prepare a staining bath containing a final concentration of 5 µg/ml ethidium bromide.

Detection

Look for the presence of specific bands, a 188 bp amplicon confirms the presence of emetic strains of *Bacillus cereus*.

OR

Method-2

Primers	**Base pair**	**Reference**
EM1F: 5′-GACAAGAGAAATTTCTACGAGCAAGTACAAT-3′	635 bp	(Ehling-Schulzet al., 2004)
EM1R: 5′-GCAGCCTTCCAATTACTCCTTCTGCCACAGT-3′		

Procedure

1. Check the number of samples and calculate the amount of PCR master mix needed
2. Prepare the PCR master mix in a tray of crushed ice
3. Aliquot the PCR master mix to the required number of PCR tubes (24 µl per tube). Depending on the PCR machine that is used.
4. Add 1 µl of water to the negative control tube and close the lid
5. Add 1 µl of sample to the sample tube and close the lid
6. Finish the procedure by adding the positive control DNA and close the lid
7. Place the tubes into the PCR thermocycler
8. Program the PCR thermocycler

9. Run the program as given below

PCR Program

15 min	95°C	
30 sec	95°C	30 Cycles
30 sec	60°C	
1 min	72°C	
5 min	72°C	
Hold	4°C	

Preparation of the agarose gel

1. Assemble the gel tray and make a proper set-up

2. Prepare a 1% agarose solution by boiling the solution a few min till completely solved by the use of a water bath or microwave oven

3. Cool the agarose to 40-50°C in a water bath and poor the agarose into the gel tray

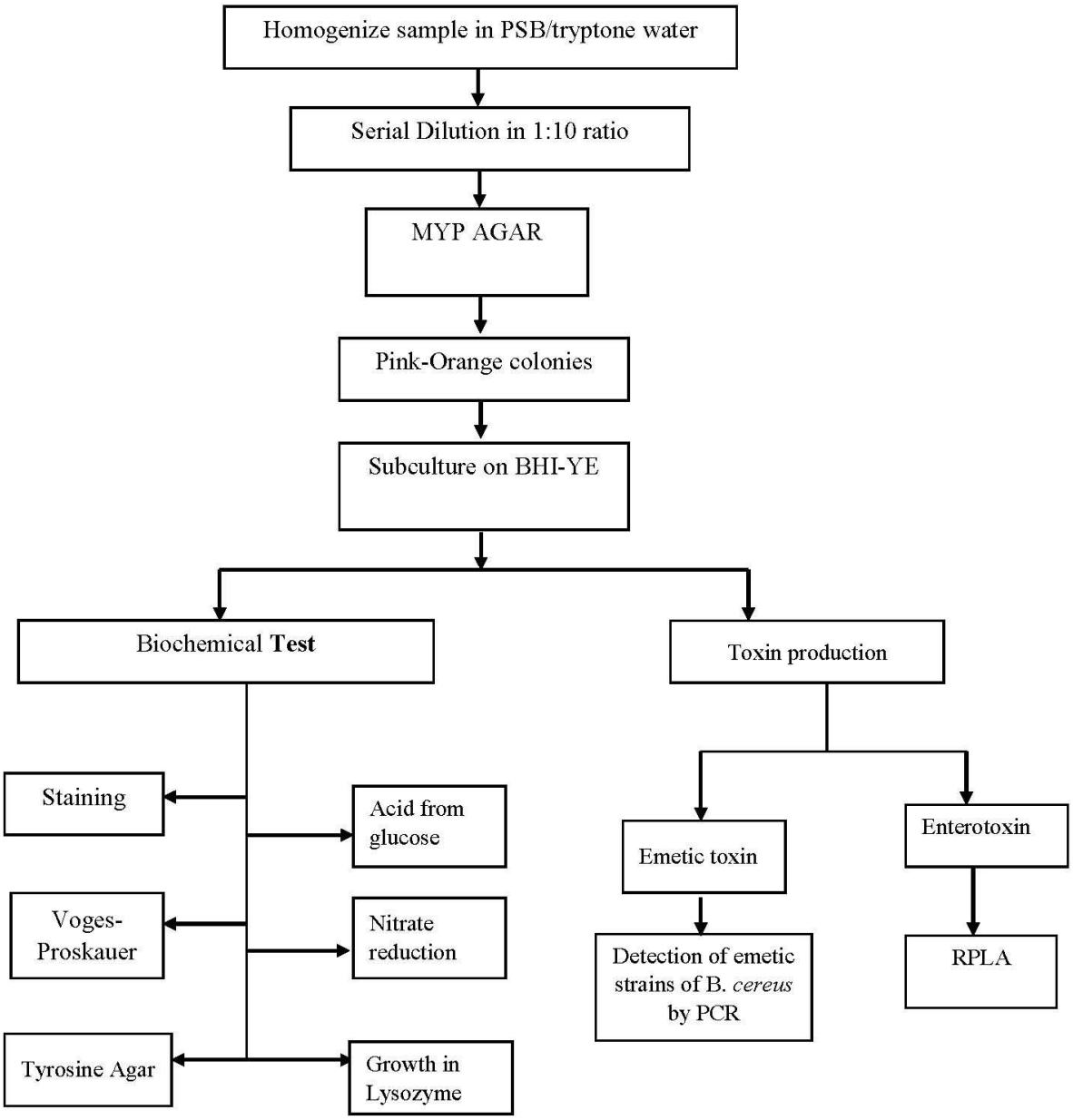
4. Let the gel solidify for 15-30 min

5. Prepare a staining bath containing a final concentration of 5 μg/ml ethidium bromide

Detection

Look for the presence of specific bands, a 635 bp amplicon confirms the presence of emetic strains of *Bacillus cereus*.

Isolation and identification of *Bacillus cereus* from food like rice



Flow Chart 15: Isolation and identification of *Bacillus cereus* from food like rice

STAPHYLOCOCCUS AUREUS

Steps for isolation and identification of *Staphylococcus aureus* from food samples (routine surveillance), nasal swabs and skin lesions (Outbreak investigation)

Enrichment

For food samples enrichment shall be carried out in Peptone Water (PW) culture on selective media.

The selective medium used for isolation of *S. aureus* will be Baird Parker Agar (BPA). A loopful of inoculum from enrichment media will be streaked on Baird-Parker agar and incubated for 48 hrs at 37°C.

Identification

Characteristic appearance of jet-black colonies surrounded by a white halo will be presumptively identified as *Staphylococcus aureus* (**Fig. S9**).

Subculture shall be done on nutrient agar for biochemical characterization. Typical biochemical test results are shown in **Fig. S9.**

Biochemical identification

The following tests shall be carried out-

A. Additional Tests

(i) Gram staining for Gram-positive cocci in cluster

(ii) Catalase test: *S. aureus* will be positive

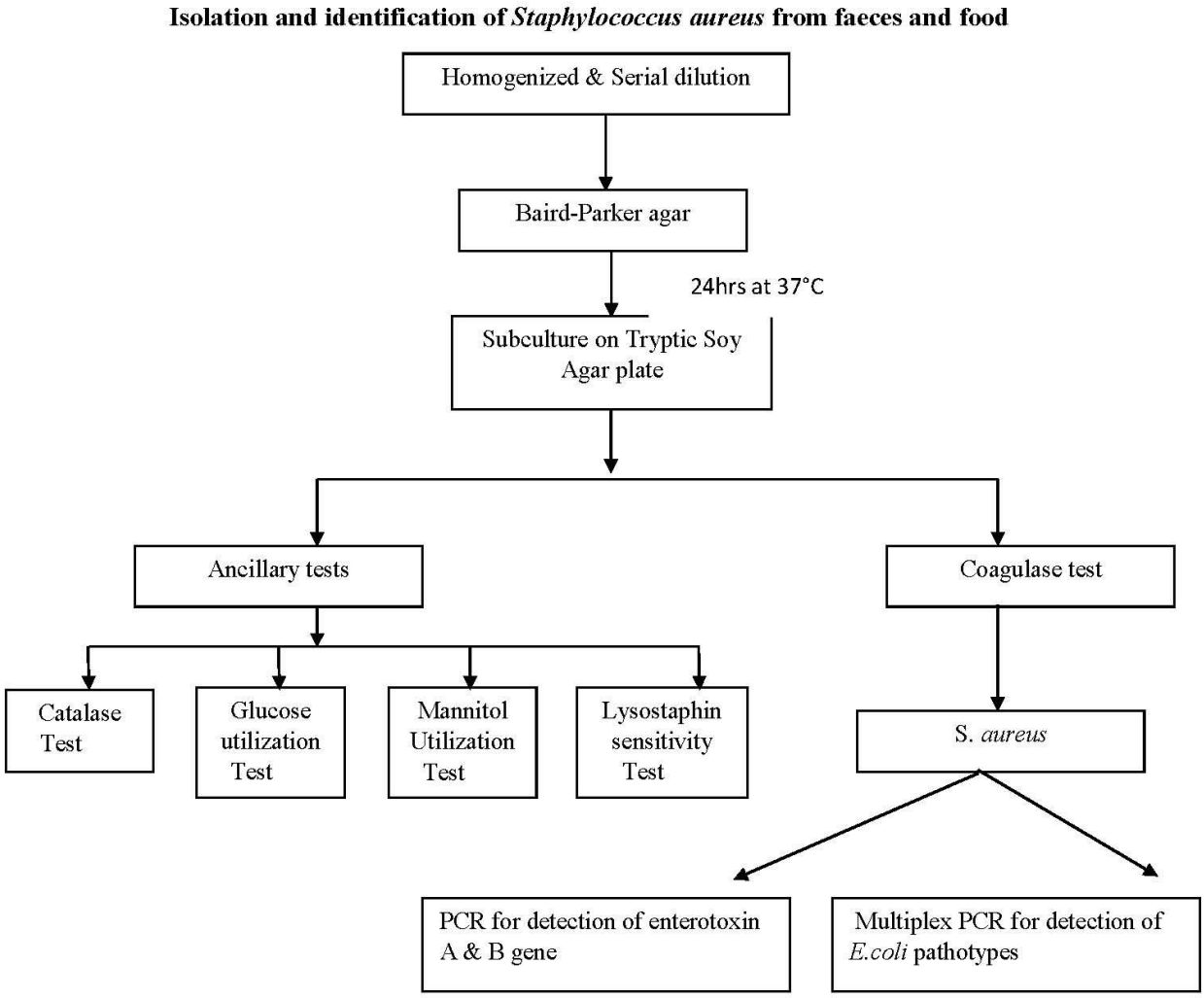
(iii) Anaerobic utilization of glucose: Acid production anaerobically and change in the colour to yellow throughout the tube confirms the presence *S. aureus*

(iv) Anaerobic utilization of mannitol: Acid production anaerobically and change in the colour to yellow confirms the presence *S. aureus*

(v) Lysostaphin sensitivity test: *S. aureus* is generally positive

B. Coagulase Test

S. aureus will be both tube and slide coagulase positive



Flow Chart 16: Isolation and identification of *Staphylococcus aureus* from faeces and food

Multiplex PCR for the detection of genes encoding staphylococcal enterotoxins A to E

Primers **Reference**

Primer GSEAR-1: 5'-GGT TAT CAA TGT GCG GGT GG-3' Mehrotra *et al.,* 2000
Primer GSEAR-2: 5'-CGG CAC TTT TTT CTC TTC GG-3'
Primer GSEBR-1: 5'-GTA TGG TGG TGT AAC TGA GC-3'
Primer GSEBR-2: 5'-CCA AAT AGT GAC GAG TTA GG=3'
Primer GSECR-1: 5'-AGA TGA AGT AGT TGA TGT GTA TGG-3'
Primer GSECR-2: 5'-CAC ACTT TTA GAA TCA ACC G-3'

Primer GSEDR-1: 5'-CCA ATA ATA GGA GAA AAT AAA AG-3'
Primer GSEDR-2: 5'-ATT GGT ATT TTT TTT CGT TC-3'
Primer GSEER-1: 5'-AGGT TTT TTC ACA GGT CAT CC-3'
Primer GSEDR-2: 5'-CTT TTT TTT CTTC GGT CAA TC-3'

Procedure

1. One ml of BHI is transferred to a 1.5 ml microfuge tube. Using a disposable inoculation loop a loop full of bacteria is picked from a plate and transferred to the Eppendorf tube
2. Centrifuge at 15,000g for 3 min. Supernatant is discarded and wash the pellet twice in physiological saline
3. Resuspend the pellet in 100 µl TE, boil the suspension (or heat at 95˚C) for 5-10 min and transfer directly to ice. Dilute the lysed DNA 10-fold in TE 10:1. Store at -20˚C
4. Check the number of samples and calculate the amount of PCR master mix needed.
5. Prepare the PCR master mix in a tray of crushed ice
6. Aliquot the PCR master mix to the required number of PCR tubes (24 µl per tube). Depending on the PCR machine that is used
7. Add 1 µl of water to the negative control tube and close the lid
8. Add 1 µl of sample to the sample tube and close the lid
9. Finish the procedure by adding the positive control DNA and close the lid
10. Place the tubes into the PCR thermocycler
11. Program the PCR thermocycler
12. Run the program as given below

The PCR protocol:

5 min at 94°C
2 min at 94°C
2 min at 59°C } 35 cycles
1min at 72°C
Final extension step at 72°C for 7 min.
Hold at 4°C

Preparation of the agarose gel

1. Assemble the gel tray and make a proper set-up.
2. Prepare a 1% agarose solution by boiling the solution a few min till completely solved by the use of a water bath or microwave oven.
3. Cool the agarose to 40-50°C in a water bath and poor the agarose into the gel tray.
4. Let the gel solidify for 15-30 min.
5. Prepare a staining bath containing a final concentration of 5 µg/ml ethidium bromide.

Detection

Look for the presence of specific bands, the amplified *sea, seb, sec, sed,* and *see* genes should be 102 bp, 164 bp and 451 bp, 278 bp and 209 bp respectively.

YERSINIA ENTEROCOLITICA

Sample processing

- Analyze samples promptly after receipt, or refrigerate at 4°C. (Freezing of samples before analysis is not recommended, although *Yersinia* have been recovered from frozen products).
- Aseptically weigh 25 gm sample into 225 ml PSBB. Homogenize 30 sec and incubate at 10°C for 10 days.

Enrichment

- If high levels of *Yersinia* are suspected in product, spread-plate 0.1 ml on MacConkey agar and 0.1 ml 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 sec, and spread-plate 0.1 ml on MacConkey and CIN agars. Incubate agar plates at 30°C for 1-2 days.
- On day 10, remove enrichment broth from the 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 sec. Successively streak one loopful to MacConkey plate and one loopful to CIN plate.
- Transfer additional 0.1 ml enrichment to 1 ml 0.5% saline and mix 5-10 sec before streaking, as above. Incubate agar plates at 30°C for 1-2 days.

Isolation of *Yersinia*

- Examine CIN plates after incubation for one day. Select small (1-2 mm diameter) colonies having a deep red center with sharp border surrounded by clear colorless zone with entire edge (**Fig. S10**).

- Examine MacConkey agar plates after 1 to 2 days incubation. Select small (1-2 mm diameter) flat, colorless, or pale pink colonies.

- Inoculate each selected colony into LAIA slant, Christensen's urea agar plate or slant, and bile esculin agar plate or slant by stabbing with inoculation needle.

- Incubate 48 hrs at RT. Isolates giving alkaline slant and acid butt, no gas and no H_2S (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. Give preference to typical isolates that fail to hydrolyze esculin.

- Using growth from LAIA slant, streak culture to one plate of TSAYE and incubate at RT. Use growth on TSAYE to check for culture purity and do the oxidase test and Gram stain. Typical biochemical test results are shown in **Fig. S10.**

Biochemical tests for biotyping

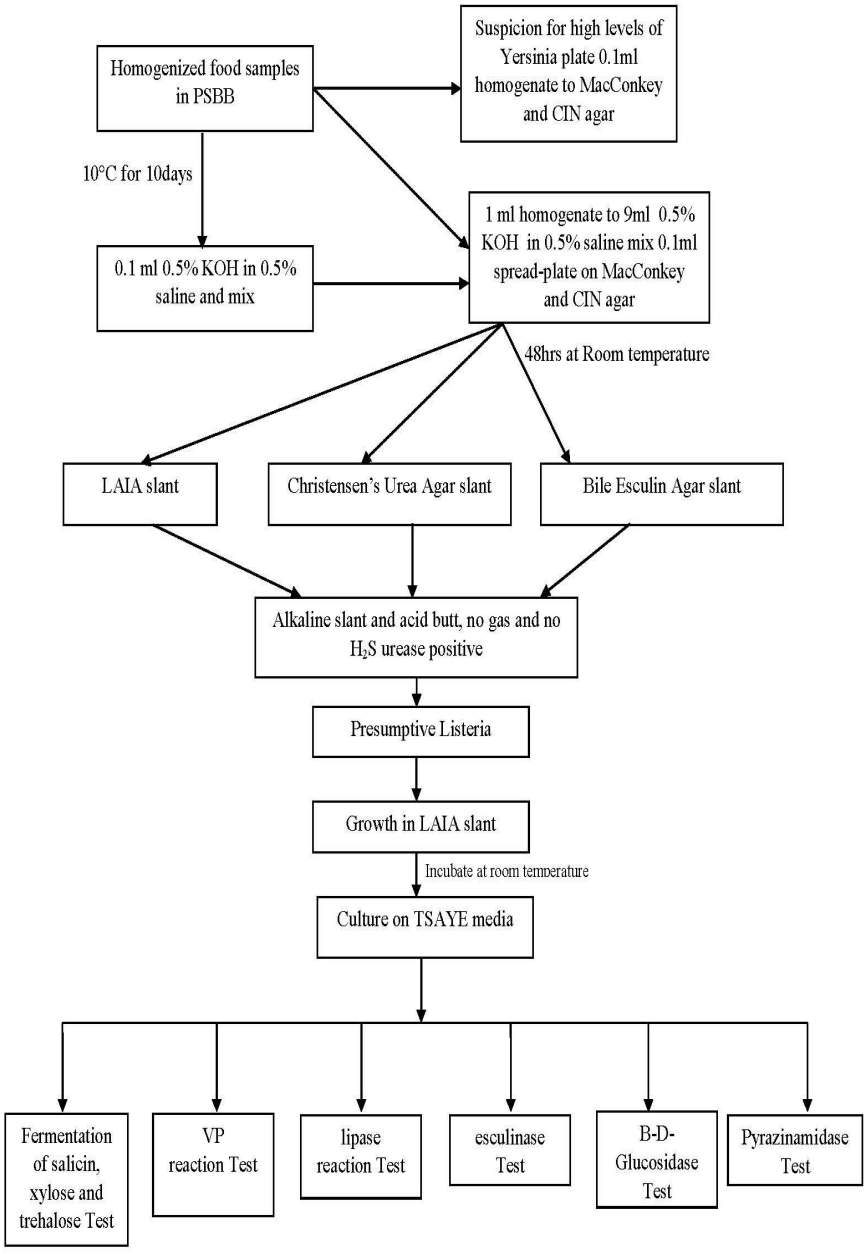
From colonies on TSAYE, inoculate an agar medium containing egg yolk such as anaerobic egg yolk (AEY) agar for lipase reaction (at 2-5 days, incubated aerobically at RT). Also, inoculate the following biochemical test media and incubate all at RT for 3 days (except one motility test medium and one MR-VP broth, which are incubated at 35-37°C for 24 hrs).

1. Fermentation of salicin, xylose and trehalose.
2. VP reaction: Add 0.6 ml α-naphthol and shake well. Add 0.2 ml 40% KOH solution with creatine and shake. Read results after 4 hrs. Development of pink-to-ruby red color in medium is a positive test.
3. Lipase reaction: From colonies on TSAYE, inoculate an agar medium containing egg yolk such as Anaerobic egg yolk (AEY) agar for lipase reaction (at 2-5 days, incubated aerobically at RT).
4. Esculin
5. **β-D-glucosidase test***:* Add 0.1 g 4-nitrophenyl-β-D-glucopyranoside to 100 ml 0.666 M NaH_2PO_4 (pH 6). Dissolve; filter-sterilize. Emulsify culture in physiologic saline to

McFarland Turbidity Standard No. 3. Add 0.75 ml of culture to 0.25 ml of test medium. Incubate at 30°C overnight. A distinct yellow color indicates a positive reaction.

6. **Pyrazinamidase test***:* After growth of culture on slanted pyrazinamidase agar at RT, flood 1 ml of 1% freshly prepared ferrous ammonium sulfate over slant. Development of pink color within 15 min is a positive test, indicating presence of pyrazinoic acid formed by pyrazinamidase enzyme.

Steps for isolation and identification of *Yersinia enterocolitica* from faeces and food



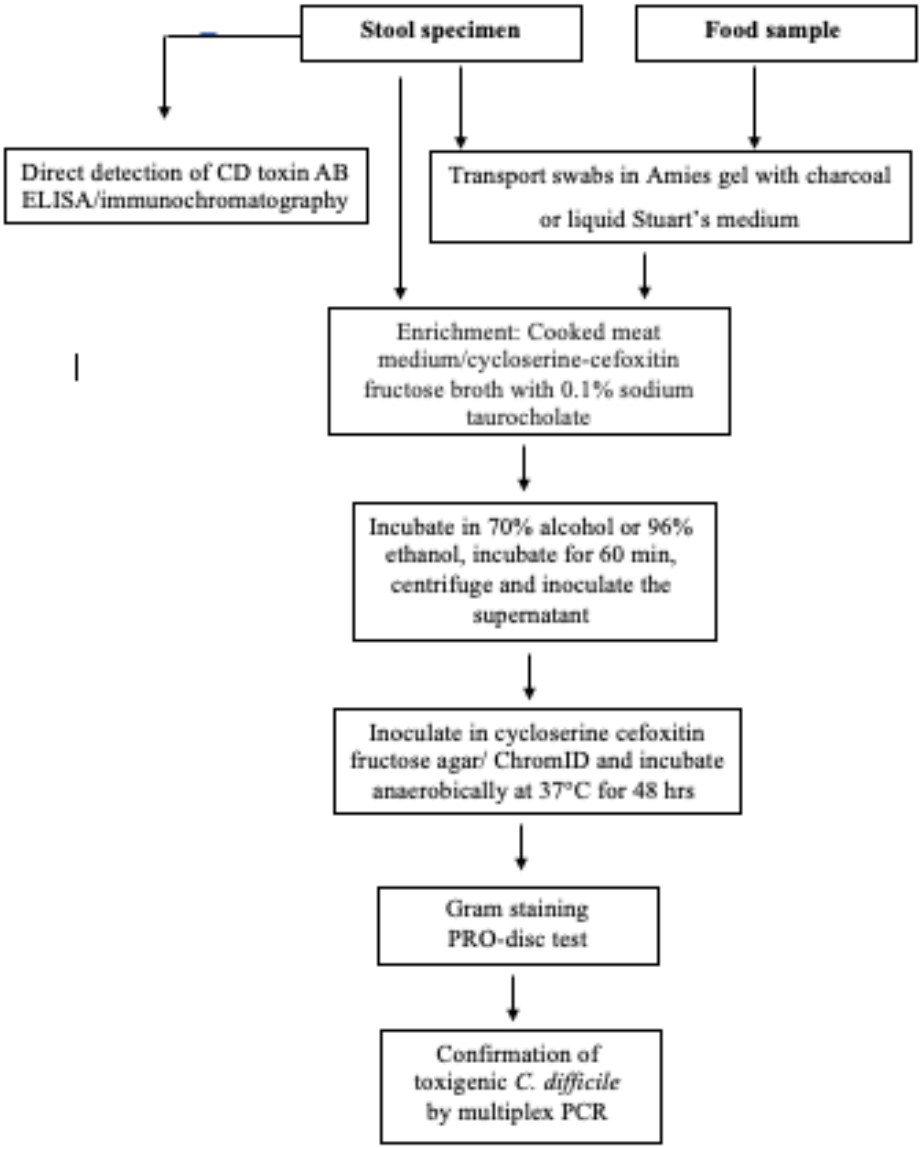
Flow Chart 17: Steps for isolation and identification of *Yersinia enterocolitica* from faeces and food

CLOSTRIDIOIDES DIFFICILE

Clostridioides difficile (Clostridium difficile) is an important cause of nosocomial and antimicrobial-associated diarrhea worldwide. Foodborne outbreaks of *C. difficile*-associated diarrhea (CDAD) are not uncommon. *C. difficile* is an anaerobe, spore forming, Gram-positive bacterium. It is an environmental organism and the spores can survive in the environment for a very long time and are resistant to drying, alcohol and detergents. Rapid diagnosis of CDAD involves direct detection its toxin in stools by commercial enzyme immunoassays. Due to difficulties in working with this anaerobic pathogen, isolation and identification of are not routinely made. The loss of viability during sample handling, submission delays, and suboptimal storage and shipping conditions decrease the detection rate of *C. difficile*. Swabs samples can be placed in either Amies gel with charcoal or liquid Stuart's medium for transportation.

There are various selective culture media for *C. difficile*. Cycloserine cefoxitin fructose agar (CCFA) with taurocholate or lysozyme have been proposed to improve sensitivity. Growth of *C. difficile* exhibit a characteristic yellow, ground-glass colonial morphology (**Fig. S11**). Pre-treatment of stool with alcohol (70%) shock can enhance the sensitivity of culture, as spore-forming organisms can survive this procedure, eliminating the growth of non-spore forming faecal organisms. Typical colonies from selective media can be tested for toxigenicity, such as cell culture cytotoxin neutralization, toxin EIA, or by PCR. Chromogenic medium for *C. difficile*, ChromID *C. difficile* agar [CDIF]) can detect β-glucosidase-producing *C. difficile* within 24 hrs based on the presence of grey-to-black colonies with irregular or smooth borders. Colonies grown on CDIF agar should not be tested in an automated analyser (VITEK 2) for identification.

Thermo Scientific™ Remel™ PRO Kit identifies *C. difficile* by detecting the enzyme L-Proline aminopeptidase. This kit can be used as a single colony screening test. The PRO disk contains L-proline-β-naphthylamide. This substrate is hydrolysed by an aminopeptidase releasing β-naphthylamine. After addition of Cinnamaldehyde reagent, β-naphthylamine reacts with ρ- dimethyl-aminocinnamaldehyde to form a red-coloured complex.



Flow chart 18. Steps for isolation and identification of *C. difficile* from stool and food samples

Multiplex PCR for the detection virulence encoding genes in *C. difficile*

Following isolation of bacterial DNA from suspected colonies or cell lysates, the specific target DNA can be amplified using a multiplex PCR. The *gluD* gene codes for the metabolic enzyme glutamase dehydrogenase, which confirms the identification of *C. difficile*. The two major toxins of *C. difficile* are the enterotoxin A and the cytotoxin B. The *tcdA* gene, encoding the enterotoxin A, is present in all most all the toxin-producing *C. difficile* strains. The *tcdB* gene, encoding for cytotoxin B, is present in all toxin-producing *C. difficile* strains and present in about 5% of the strains that do not produce TcdA. The binary toxin encoded by *cdtA* and *cdtB* which encode for the two enzymatic parts.

Table. Primers and PCR conditions for the identification of toxigenic *C. difficile.*

Gene target	Primer	Primer sequence (5'-3')	Amplicon (bp)
gluD	GluD-F	GTCTTGGATGGTTGATGAGTAC	158
	GluD-R	TTCCTAATTTAGCAGCAGCTTC	
tcdA	Tcd-F	GCATGATAAGGCAACTTCAGTGGTA	629
	Tcd-R	AGTTCCTCCTGCTCCATCAAATG	
tcdB	TcdB-F	CAAARTGGAGTGTTACAAACAGGTG	410
	TcdB-RA	GCATTTCTCCATTCTCAGCAAAGTA	
	TcdB-RB	GCATTTCTCCGTTTTCAGCAAAGTA	
cdtA	CdtA-FA	GGGAAGCACTATATTAAAGCAGAAGC	221
	CdtA-FB	GGGAAACATTATATTAAAGCAGAAGC	
	Cdt-R	CTGGGTTAGGATTATTTACTGGACCA	
cdtB	CdtB-F	TTGACCCAAAGTTGATGTCTGATTG	262
	CdtB-R	CGGATCTCTTGCTTCAGTCTTTATAG	

PCR reaction mixture

Hot start master mix	12.5 µl
TcdA-F	1.0 µl
TcdA-R	1.0 µl
TcdB-F	0.4 µl
TcdB-RA	0.05 µl
TcdB-RB	0.05 µl
CdtA-FA	0.05 µl
CdtA-FB	0.05 µl
CdtA-R	0.01 µl
CdtB-F	0.01 µl
CdtB-R	0.01 µl
GluD-F	0.1 µl
GluD-R	0.1 µl
H_2O	7.17 µl
Total reaction volume	22.5 µl

Primer concentration: 50 pmol/µl

PCR Condition: Initial denaturation: 94°C for 15 min

Denaturation: 94°C for 45 sec
Anneal: 50°C for 45 sec. } 35 cycles
Extension: 72°C for 1 min
Extension: 72°C for 30 min
Hold: 15°C

After the PCR, transfer the PCR products to 1.0% agarose gel, stain with EtBr and using a Gel documentation system visualise the bands under UV.

LEPTOSPIRA SPP

Leptospirosis is caused by several species and serovars of *Leptospira*. This zoonotic infection is transmitted either directly or indirectly from animal to human. *Leptospira* spp. resides in renal tubules in animals, and shed through urine into the environment. Humans and animals can be infected through mucous membranes/skin when they encounter this pathogen. Consumption of contaminated food or water is sometimes responsible for the outbreak of leptospirosis.

Several methods have been established for the diagnosis of *Leptospira*. Identification of *Leptospira* spp. by culture requires months of incubation. PCR using the extracted DNA directly from the clinical or animal samples or from the enriched cultures often used in the quick diagnosis of *Leptospira*. Microscopic agglutination test (MAT) and ELISA using serum samples are generally used in the seroepidemiological studies. An outline of identification of *Leptospira* spp presented in Flow chart 18.

Procedure for processing the water samples:

1. Collect 300 to 500 ml of water in a sterile container.
2. Filter the sample using 0.22 μM sterile membrane filter (*Leptospira* can pass through the filters retaining the other bacteria).
3. Centrifuge the sample 4000g at 4°C for 20 min.
4. Suspend the pellet in 1 ml of sterile PBS.
5. Inoculate 1 ml of the homogenate into 9 ml of Ellinghausen–McCullough–Johnson–Harris (EMJH) broth supplemented with 5-Fluorouracil (5FU) and incubate at 28-30°C.
6. Examined weekly by dark field microscopy, for up to 3 months.

Procedure for processing tissue samples:

1. Preferred tissue sample is the kidney.
2. Clean the sample surface rectified spirit (70% ethanol in sterile distilled water).
3. Collect the tissue sample in 10 mL sterile PBS.

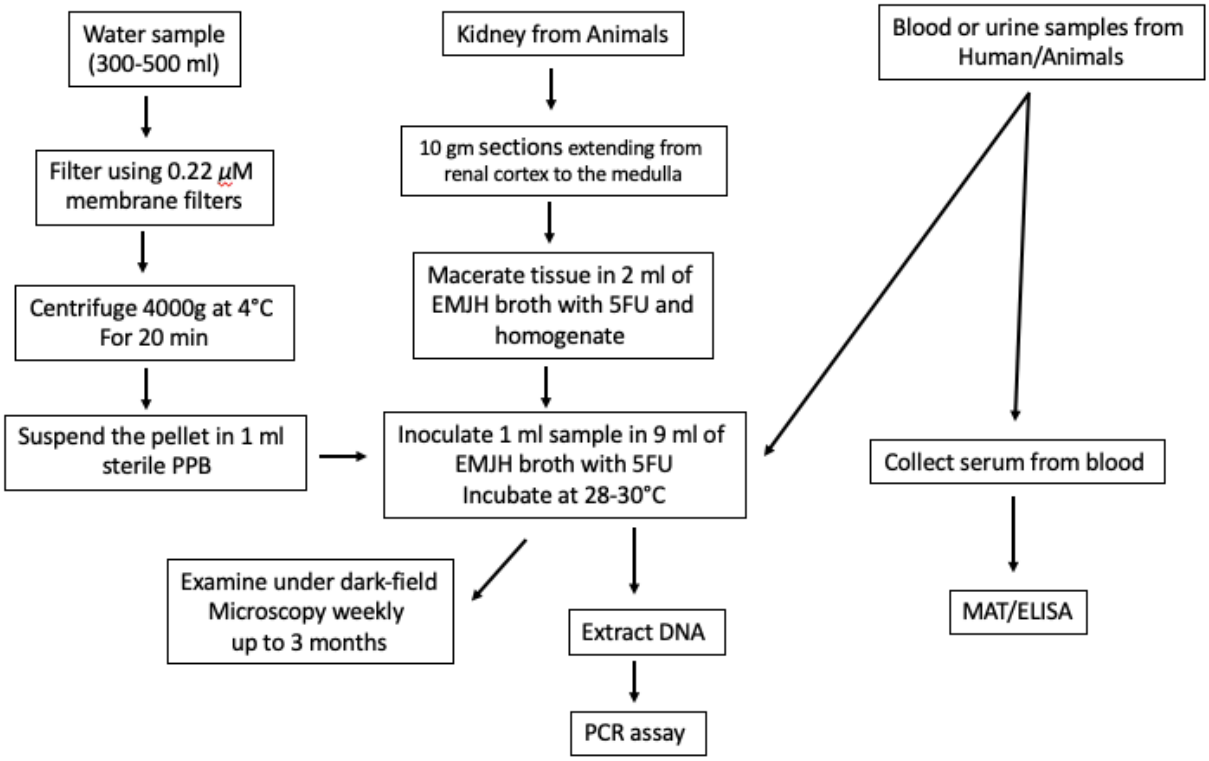
4. Take about 10 gm section extending from the renal cortex to the medulla severed in fine pieces using a sterile scalpel.
5. Placed the sample into 10 ml sterile PBS, and homogenized in a stomacher for 10 min.
6. Inoculate 1 ml of the homogenate into 9 ml of EMJH broth supplemented with 5FU and incubate at 28-30°C.
7. Examine the culture weekly by dark field microscopy, for up to 3 months.

Procedure for processing blood/urine samples:

1. Collection of blood/urine samples from the symptomatic cases is generally recommended
2. Inoculate 1 ml of the sample into 10 ml of EMJH medium supplemented with 5FU and incubate at 28-30°C.
3. Examined the culture weekly by dark field microscopy, for up to 3 months.

PCR assay

Extract DNA from the enriched EMJH broth and perform the PCR.



Flow chart 19. Steps for isolation and identification of *Leptospira* spp from water, humans and animals.

MICROSCOPY

- They may be seen in wet preparations by dark-field microscopy using a 40X objective with 10X eyepiece and bright illumination. Under low power magnification (200X) bacterial cell looks like a small piece of thread and hooked ends are visualized as dense dot like structures. The fine coils are seen only under high power magnification (1000X).
- *Leptospira* spp. can also be visualized by electron microscopy.

STAINING

Silver impregnation technique

Silver impregnation staining techniques are used for the tissues

Leptospira staining kit contains the following four reagents.

• Reagent 1: Fixative

• Reagent 2: Absolute alcohol

• Reagent 3: Mordant

• Reagent 4: Silver stain

Staining Protocol

- Take a clean grease free glass slide. Wipe it with the alcohol and allow it to dry.
- Label the slide towards one corner and cello tape it to avoid the wetting of the label.
- Take 25-30 µl of the culture to be stained in the middle of the slide and make a smear. Allow it to air dry completely.
- Pour the fixative in a coupling jar and place the smeared slide. Allow it to get fixed for 2 min. Care should be taken to immerse the slide completely. Wipe the underneath of the slide and allow it to air dry completely.
- Place the smeared slide in the coupling jar containing absolute alcohol for 3 min. Dip it in the same jar for 2 to 3 times and remove the slide. Wipe the underneath of the slide and allow it to air dry.
- Immerse the smeared slide for 1 minute in the preheated mordant (at 80ºC for 1 min) wipe the underneath of the slide and dip it in the distilled water for 3 to 5 times. Allow the smear to air dry completely.
- Finally, place the smear in the silver stain which is preheated for 1 min at 80ºC. Allow the slide to get stained for 1 min. Wash the slide in the fresh distilled water and air dry it completely and mount in Canada balsam or Dibutylphthalate polystyrene xylene (DPX).
- It is essential that the specimen be mounted in balsam under a cover slip before examination, as some immersion oils cause the film to fade at once. The spirochetes are stained brownish black on a brownish-yellow background.

- Observe under light microscope at 400X and oil immersion for brownish pink colored, both ends hooked like spirochete with coiled morphology with a yellowish-brown background. Note the size and shape of the microorganism. Stained debris or fibrin materials or artifacts will look fibrous and can be differentiated.

CULTURE

Preparation of EMJH culture medium

(i) Basal media preparation

EMJH Medium Base (Difco, USA)

Media type per 100 ml	Difco EMJH Medium Base	Millipore autoclaved H_2O	Agarose
Liquid medium	0.23 g	95 ml	
Semi Solid medium	0.23 g	95 ml	0.1 g

- Autoclave at 121ºC for 30 min.
- After cooling add 1. 25 ml of filtered 5FU (1% stock solution filtered)
- Aliquot 4.5 ml of this media into sterile 15 ml disposable centrifuge tubes.
- Check sterility at 37°C for 2 days and store at 30°C till use.

(ii) Difco Enrichment Medium:

- Difco enrichment Medium (Difco, catalogue no. 279510, BD, Difco).
- Before use aliquot 10 ml of this medium into sterile 15 ml disposable centrifuge tubes
- Check sterility at 37°C for 2 days and store at 4°C till use.

Preparation of 5FU

The pyrimidine analogue, 5FU exerts a strong inhibitory effect on several heterotrophic bacteria, thereby supporting the growth of leptospirae at the concentration of 1,000 g/ml. 5FU has been used in the selective isolation of leptospirae from mixed bacterial populations (Johnson and Rogers, 1964).

- Add 1g of 5FU in 50 ml Millipore autoclaved H_2O.
- Add 1 ml 2N NaOH gently with stirring in the 56ºC hot water bath for 2 hrs.
- Cool to room temperature and adjust pH to 7.4 with 1N HCl.
- Make up the volume to 100 ml and Filter with the Millipore 0.22 µm filter.
- Make aliquots and Store at 4ºC.
- Allow it to come to room temperature when it is at use.

{5FU is added at the rate of 1g in 100 ml Millipore autoclaved H_2O. Since 125 to 250 µg/ml of the medium is the required}.

It is necessary to add this 5FU stock solution at the following rate:

Total volume of the EMJH medium	Quantity of the stock solution to be added
100 ml	1.25 ml
250 ml	3.125 ml
500 ml	6.25 ml
1000 ml	12.50 ml

For EMJH Transport Medium

Total volume of the EMJH medium	Quantity of the stock solution to be added
100 ml	2.5 ml
250 ml	6.25 ml
500 ml	12.5 ml
1000 ml	25.0 ml

For Tissue sample

The specimens collected will depend on the resources available and cultural restrictions. Tissue samples should be collected aseptically and as soon as possible; they should also be inoculated into culture medium. The samples should be stored and transported at 4°C.

CULTURE METHOD

- The medium use in current practice is Difco EMJH medium base, Difco *Leptospira* enrichment medium and agarose for semisolid media (as described earlier). Growth of other bacterial contaminants can be inhibited by the addition of 5FU in the medium.
- The medium is prepared in sterile conditions and semisolid medium is aliquoted into screw cap borosilicate bottles and liquid medium into plastic tubes/vials of 15 ml for subsequent use. The medium is kept for a sterility check for three days at 37°C and then store at 30°C until use.
- All cultures received from the reference laboratory from semisolid stock are revived and maintained in liquid media. Weekly subculture has to be made for reviving cultures from stock and maintained in liquid medium for regular use in MAT. Liquid medium can be used for the regular weekly subculture maintenance.

- About 0.6 ml of the original 5-7 days grown culture (2x108/ml) is taken in plastic dropper (sterile Pasteur Pipette) and inoculated into the 6 ml of liquid medium and culture is allowed to grow at 30°C for 5-7 days. These 5 to 7 days old cultures have to be used as live *Leptospira* antigen in the microscopic agglutination test **(**MAT).
- Semi solid medium is used for the backup storage of culture and sub-cultured once in every 3 months for the revival in liquid medium and subsequent storage in semi-solid medium. This preservation method is labour-intensive and at high risk for contamination.
- One ml of the of the original culture (2x108 /ml) is taken and inoculated into the 10 ml of semi solid media and culture is allowed to grow at 30°C for one week and later on store at room temperature for long term storage.

Storage of *Leptospira* isolates

Confirmed isolates can be maintained in tubes of semi-solid media and stored in dark at room temperature. *Leptospira* colures are viable for 3 months to one year and subcultures have to be confirmed again for subsequent storage. *Leptospira* isolates can be stored for years in liquid nitrogen either with 5% glycerol or 5% di-methyl sulfoxide (DMSO) as a cryoprotectant.

MAT for animal serum samples

Principle: Antibodies in the test serum react with antigens on the surface of the bacteria and agglutinate them.

Materials: Micro U-Bottom plastic plates; Universal reagent reservoir (TARSONS-T524091); Incubator (30°C); Glass slides (0.1mm thickness); Dark Field Microscope (Trinocular Microscope Model NI-U) (equipped with objective 20X and 40X dark field condenser and 10x eyepieces); Micro pipettes (1000 µl, 100 µl and 10 µl); Multichannel pipettes; Marker pens.

Reagents: Pure live reference *Leptospira* cultures (5-10 days old); Sterile PBS (pH 7.2)

Procedure: Sero-screening of the samples by MAT helps to determine the frequency distribution of serovars and its prevalence.

Live *Leptospira* culture antigen preparation:

Maintain different serovars received from the referral laboratory Regional Medical Research Centre in the EMJH liquid and semisolid medium. For MAT, 5-8 days old culture with high motility is good. As the culture gets old, there will be auto agglutination and hence would be difficult in taking the MAT reading. Good growth culture taken for the MAT is approximately 2*108 /ml bacterial culture. This can be counted using the Cell counter with the grid or can be simply assumed the approximate when seen under the DFM. The field focused has to show motile organism.

- EMJH Liquid and semi solid media are prepared using Difco (279410) EMJH medium Base (0.23gm/100ml medium) + 10 ml of Difco *Leptospira* enrichment (279510) + 125 mg/ml 5 FU (Sigma-F6627) (optional 0.2 % Agarose for the Semi solid medium).
- The medium is kept for a sterility check for one week and then used for sub –culturing.
- Aliquot 15 ml of the medium in sterile condition into glass tubes or screw cap borosilicate bottles or the plastic vials.
- Semi solid medium can be used for the backup culture, whereas the liquid medium is used for the regular weekly culture maintenance.
- Use 0.5 ml of the original culture and inoculate into the 5 ml liquid or semi-solid medium and allow to grow at 30˚C for 5 days. This culture can be used in the MAT. Semisolid medium is used for backup and is revived and sub cultured once in every 3 months.

1. Take 490 µl of the sterile PBS (pH 7.2) to 12 rows of the Universal reagent reservoir.
2. Add 10 µl of the serum to be tested for each of the 11 rows and 10 µl PBS for the 12th row (i.e., for antigen control). (i.e., 1:50 dilution of serum of animal samples; for human serum samples 1:10 dilution of serum has to be made (50 µl of serum in 450 µl of the PBS).
3. Mix thoroughly-Universal reagent reservoir and U- bottomed micro-plates.

4. Take 50 µl of each sample and add to the Micro 'U' bottom test plates in such a way that each row has one sample and the last row is the control. i.e., A row from 1 to 11 diluted 1:50 sera samples, and 12th column is antigen control. Similarly, add diluted sera in the row B, C,…… H as stated above.
5. Add 50 µl of the well grown live *Leptospira* antigen (8 different serovars) culture of 5-8 days old antigen row-wise. For e.g., A row for antigen Australis, B row for Autumnalis, C row for Canicola and another serovars, etc.

(i.e., each plate can accommodate 8 serovars antigen only).

Similarly, for other serovars, another plate can be used.

Finally, one serum sample to be tested for "n" of live *Leptospira* serovars antigen for determining the specific reactivity with different serovars.

6. Mix thoroughly by tapping for a few seconds and cover the plates with the aluminium foil.
7. Keep the plate at 30°C in an incubator for 2-4 hrs.

Reading:

After incubation of 2 hrs, the serum-antigen mixtures are examined under a dark field microscope for agglutination. This can be done by transferring one drop of mixture to a microscope slide. The endpoint (titer) is taken as that dilution which gives 50% agglutination, leaving 50% of the cells free when compared with a control suspension of *Leptospira* is considered positive at 1:100 dilutions. No agglutination should be seen in the control row.

List of Animal *Leptospira* serovars

Leptospira species	Serogroup	Serovar	Strain
L. interrogans	Australis	australis	Ballico
L. interrogans	Autumnalis	autumnalis	Akiyami
L. borgpetersenii	Ballum	ballum	Mus 127
L. interrogans	Bataviae	bataviae	Swart
L. interrogans	Canicola	canicola	Hond Utrecht IV
L. weilli	Celledoni	celledoni	Celledoni
L. kirschneri	Cynopteri	cynopteri	3522 C
L. kirschneri	Grippotyphosa	grippotyphosa	Moskva V
L. borgpetersenii	Mini	mini	Sari
L. interrogans	Hebdomadis	hebdomadis	Hebdomadis
L. interrogans	Icterohaemorrhagia e	icterohaemorrhagia e	RGA
L. borgpetersenii	Sejroe	hardjobovis	117123
L. borgpetersenii	Javanica	javanica	Veldrat Batavia 46
L. interrogans	Pomona	pomona	Pomona
L. interrogans	Pyrogenes	pyrogenes	Salinem
L. interrogans	Sejroe	hardjo	Hardjoprajitno
L. borgpetersenii	Tarassovi	tarassovi	Perepelitsin

Enzyme Linked Immuno-Sorbent Assay (ELISA)

Materials required, but not provided in the Kit

• Distilled water

• Adjustable single and multichannel micropipettes

• Micropipette tips

• Incubator set at 37ºC, with or without shaker

• ELISA 96-well microplate reader with 450 nm filter (preferably with 630 nm reference filter).

• Container for dilution of wash buffer solution

• Microtiter plate

• Plate sealers, reservoirs, foil.

Materials provided in the kit

• Pre-coated ELISA plates

• Wash Buffer (20X concentrated)

• Sample Diluent

• Positive Milk control

• Negative Milk control

• Positive Sera control

• Negative Sera control

• Substrate (3,3′,5,5′-Tetramethylbenzidine, TMB)

• Stop solution (1M H_2SO_4)

• Peroxidase conjugated antibody 1000 X concentrate), Ready to use

Preparation

- Determine the total number of samples and controls to be tested. At least 3 replicates of each control (milk and/or sera) are recommended for each test run. For the strip kit, remove any strips not required from the frame and store in the resealable bag at 4ºC.
- Crystals in the concentrated wash buffer can be dissolved by heating to 37°C. Dilute the wash buffer 1:20 with distilled water.
- Dilute the concentrated conjugate 1:1000 in the diluted wash buffer just before use. The diluted conjugate is light sensitive and should be protected from exposure to light.

Procedure

- Dilute serum and control serum samples in sample diluents in the ratio of 1:50 in a separate plate. Take undiluted 150 µl milk sample in another plate.
- Mix the above contents thoroughly and transfer 100 µl of samples into the test wells. Cover the wells with a plate sealer.

- Incubate the plate at 37°C for 40 min with shaking (1 hour without shaking).
- After incubation, wash the test wells with diluted wash buffer at least 200 µl/well for four times. Following the final wash, remove residual wash buffer by inverting the plate and blotting firmly on absorbent paper.
- Add 100 µl of the diluted conjugate to each well, cover the wells with a plate sealer and incubate in the dark at 37°C for 30 min with shaking (40 min without shaking).
- Wash the wells with wash buffer four times.
- Add 100 µl of substrate to each well. Incubate the plate at room temperature in the dark for 10 min.
- Add 50 µl of stop solution to each well at the same order as the substrate was added.
- Read at a wavelength of 450 nm or with a corrected OD using a reference filter (630 nm).

Calculations for milk & sera samples

S/P ratio (Sample value related to Positive Control value) can be calculated using the following formula

The S/P ratio must be calculated using the respective controls for the sample type.

Interpretation of results

$$\frac{\text{Mean Sample OD} - \text{Mean Negative control OD}}{\text{Mean Positive control OD} - \text{Mean Negative control OD}}$$

A. Milk samples

Result	If the ratio is:	Interpretation
Negative	≤ 0.03	Naive and/or unvaccinated
Low Positive	≥ 0.04 ≤ 0.10	A few seropositive cows
Medium Positive	>0.10 ≤ 0.50	Mix of infected and susceptible animals
High Positive	>0.50	Heavily infected/vaccinated

B. In case of individual animal milk/sera

Result	If the ratio is:	Interpretation
Negative	Sera ≤ 0.05 Milk ≤ 0.03	Naive and/or unvaccinated
Inconclusive	Sera ≥ 0.06 ≤ 0.12 Milk ≥ 0.04≤ 0.10	A retest is recommended after a few weeks. If the sample is inconclusive after retest, the animal can be considered negative depending on the herd history
Positive	Sera> 0.12 Milk >0.10	Exposed to infection/vaccinated

PCR Diagnosis of *Leptospira* spp

G1/G2 primers amplify *secY* gene that encodes housekeeping pre-protein translocase from all the serovars of *Leptospira* spp. except *L. kirschneri*. The second primer pair B64I/B64II that encodes a class B polypeptide subunit of the periplasmic flagella (*flaB*) is used for the identification of *L. kirschneri.* LipL primers that target the virulence specific lipoprotein *Lipl32* present only in pathogenic leptospires.

Pure culture template

Isolation and amplification of leptospiral DNA from cultures and clinical samples.

An overnight culture of *Leptospira* spp. was harvested by centrifugation at 13,000g for 10 min at 4°C. The pellet was resuspended in 800 µl sterile distilled water. Then, 200 µl of 0.1 M EDTA (pH 8.0) and 0.5% formaldehyde solution was added and the mixture was centrifuged at 13,000g for 10 min at 4°C. The pellet was washed with 1 ml 1 mM EDTA (pH 8.0), and again with 1 ml of sterile distilled water, leaving 100 µl of liquid to resuspend the pellet. The DNA was released by incubation at 100°C for 10 min, and was used directly in PCR, or stored at -20°C until further use.

DNA isolation from tissues

Materials required

Centrifuge, microcentrifuge tubes, Pestle and mortar, ice-water bath, -20°C deep freezer, liquid nitrogen, Lysis buffer (10 mM Tris HCl pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% SDS; 10 mg/ml Proteinase K, 20 µg/ml DNAse free from RNAse); 0.5M Tris-HCl saturated phenol and Ethanol Phenol:chloroform (1:1), Ethanol/acetate solution (95% ethanol/0.12 M sodium acetate); TE buffer (10 mM Tris-HCl, pH 7.6-8.0, 0.1 mM EDTA).

Procedure

Freeze the tissue samples using liquid nitrogen. Tissue samples weighing ~1gm should be reduced to powder using a mortar and pestle before lysing the sample and homogenizing with Phenol. To get a good amount of genomic DNA, they should be first lysed with lysis buffer properly before homogenization with 0.5M Tris-HCl saturated phenol and Ethanol precipitation of DNA for elution of genomic DNA. Then an equal volume of buffer-saturated phenol:chloroform (1:1) to the sample lysate is added and mixed well. This mixture can be vortexed for 10 sec except for high molecular weight DNA, which should be gently rocked. Spine the sample mixture in a microcentrifuge for 3 min and the aqueous layer should be carefully removed to a new tube, avoiding the interface (these steps can be repeated until the white interface is no longer visible). To remove traces of phenol, an equal volume of chloroform can be added to the aqueous layer. Then, 2.5 to 3 volumes of an ethanol/acetate solution are added to the DNA sample in a microcentrifuge tube, which is placed in an ice-water bath for at least 10 min. Alternatively, this precipitation is performed by incubation at -20°C overnight. To recover the precipitated DNA, the tube is centrifuged, the supernatant is discarded, and the DNA pellet is rinsed with a more dilute ethanol solution. After a second centrifugation, the supernatant again is discarded, and the DNA pellet is dried and then dissolved in TE buffer.

It is advisable to aliquot the purified DNA into several small volumes (0.5 ml) in microcentrifuge tubes in a deep freezer. Avoid repeated freezing and thawing. Isopropanol can also be used instead of ethanol. However, the precipitation efficiency of the isopropanol is higher making one volume enough for precipitation. Isopropanol is less volatile than ethanol and needs more time to air-dry in the final step. The pellet might also adhere less tightly to the tube while using isopropanol

Polymerase Chain Reaction (PCR)

Multiplex PCR Primers

G1-Forward: 5'- CTG AAT CGC TGT ATA AAA GT-3'
G2-Reverse: 5'- GGA AAA CAA ATG GTC GGA AG-3'

B64I-Forward: 5'- CTG AAT TCT CAT TCT AAC TC-3'
B64II-Reverse: 5'- GCA GAA ATC AGA TGG ACG AT-3'

Simplex PCR Primers

LipL32 Forward: 5'-ATC TCC GTT GCA CTC TTT GC-3'

LipL32 Reverse: 5'-ACC ATC ATC ATC AT CGT CCA-3'

Deoxynucleoside triphosphates (dNTPs): Standard PCRs contain equimolar amounts of dATP, dTTP, dCTP and dGTP. Concentrations of 200-250 µM of each dNTP are recommended for reaction containing 1.5 mM $MgCl_2$.

DNA Polymerase: A thermostable DNA polymerase, routinely Taq polymerase is used to catalyse template-dependent synthesis of DNA. Usually, 0.5 to 2.5 units per standard 25-50 µl reaction is used.

Template DNA: The DNA can be extracted directly from the blood samples from livestock and human. It is also possible to extract DNA from milk, *cerebrospinal fluid* (CSF) and tissues. DNA extracted from confirmed cultures can be used as a positive control.

Equipment /plastic ware

a. Thermal cycler
b. Micropipettes
c. PCR tubes

Reagents

a. Oligonucleotide primers: The oligonucleotide primers synthesized and supplied in lyophilized form from the company needs to be reconstituted to 100 pmol/µl stocks in sterile TE buffer. Then working primers should be made to 10 pmol/µl in sterile nuclease free water (NFW).
b. dNTP (10 mM)
c. Taq DNA polymerase (1 U/µl)
d. PCR buffer (10X) e. $MgCl_2$ (25 mM) (If PCR buffer contains $MgCl_2$, no need of adding separately).

PCR reaction

(i) Multiplex PCR is to be carried in a 50 µl reaction mixture in PCR tube. The PCR reaction mixture includes:

PCR buffer (10X) : 5.0 µl

dNTP (10 mM) : 1.0 µl

G1-Forward primer (10 pmole/µl) : 1.0 µl

G2-Reverse primer (10 pmole/µl) : 1.0 µl

B64I-Forward primer (10 pmole/µl) : 1.0 µl

B64II-Reverse primer (10 pmole/µl) : 1.0 µl

Taq DNA polymerase (1U/µl) : 1.0 µl

Template DNA : 5.0 µl

Nuclease free water : 34.0 µl

Total volume : 50.0 µl

(ii) Simplex PCR is to be carried in a 50 µl reaction mixture in PCR tube. The PCR reaction mixture includes:

PCR buffer (10X) : 5.0 µl

dNTP (10 mM) : 1.0 µl

LipL32-Forward (10 pmole/µl) : 1.0 µl

LipL32 Reverse (10 pmole/µl) : 1.0 µl

Taq DNA polymerase (1U/µl) : 1.0 µl

Template DNA : 5.0 µl

Nuclease free water : 36.0 µl

Total volume : 50.0 µl

The DNA amplification should be carried out by keeping PCR tube along with reaction mixture in a thermal cycler using the following conditions.

Multiplex PCR condition

Steps	**Temperature**	**Duration**	**No of cycles**
Initial denaturation	94°C	3-5 min	
Denaturation	94°C	1.0 min	30 cycles
Annealing	55°C	1.5 min	
Extension	72°C	2.0 min	
Final extension	72°C	5-9 min	

285 bp fragment amplified by G1 & G2 (all genospecies except *L.* Krischneri)
563 bp fragment amplified by B641 & II (*L.* Krischneri)

Simplex PCR condition

Steps	**Temperature**	**Duration**	**No of cycles**
Initial denaturation	94°C	5.0 min	
Denaturation	94°C	30 sec	35 cycles
Annealing	58°C	30 sec	
Extension	72°C	1.0 min	
Final extension	72°C	5 min	

A fragment of 474 bp is amplified by LipL32 primers.

After PCR cycles are over, the amplified product can be checked in 1 or 1.5 % agarose gel and the gel image is captured in the gel documentation under the UV Trans illuminator.

Agarose gel electrophoresis

Equipment

a. Weighing balance

b. Horizontal electrophoresis apparatus with power pack

c. Microwave oven

d. UV transilluminator/ gel documentation system

Reagent

a. Agarose

b. TBE buffer (Tris - Borate EDTA buffer) (10X, pH 8.2)

Tris-base	: 108.0 g
Boric acid	: 55.0 g
EDTA disodium salt	: 8.3 g
Double distilled water up to	: 1000.0 ml

The stock solution has to be sterilized by autoclaving and made to 0.5X before use.

c. Gel loading dye (6X)

Bromophenol blue	: 0.25% (w/v)
Xylene cyanol	: 0.25% (w/v)
Sucrose	: 40% (w/v) in distilled water

Stored at 4°C.

d. Ethidium bromide (10 mg/ml)

Ethidium bromide (Biogene, USA)	: 100 mg
Double distilled water	: 10 ml

The suspension was stirred to ensure that the dye was completely dissolved. The container was then wrapped in aluminum foil and stored at 4°C until use.

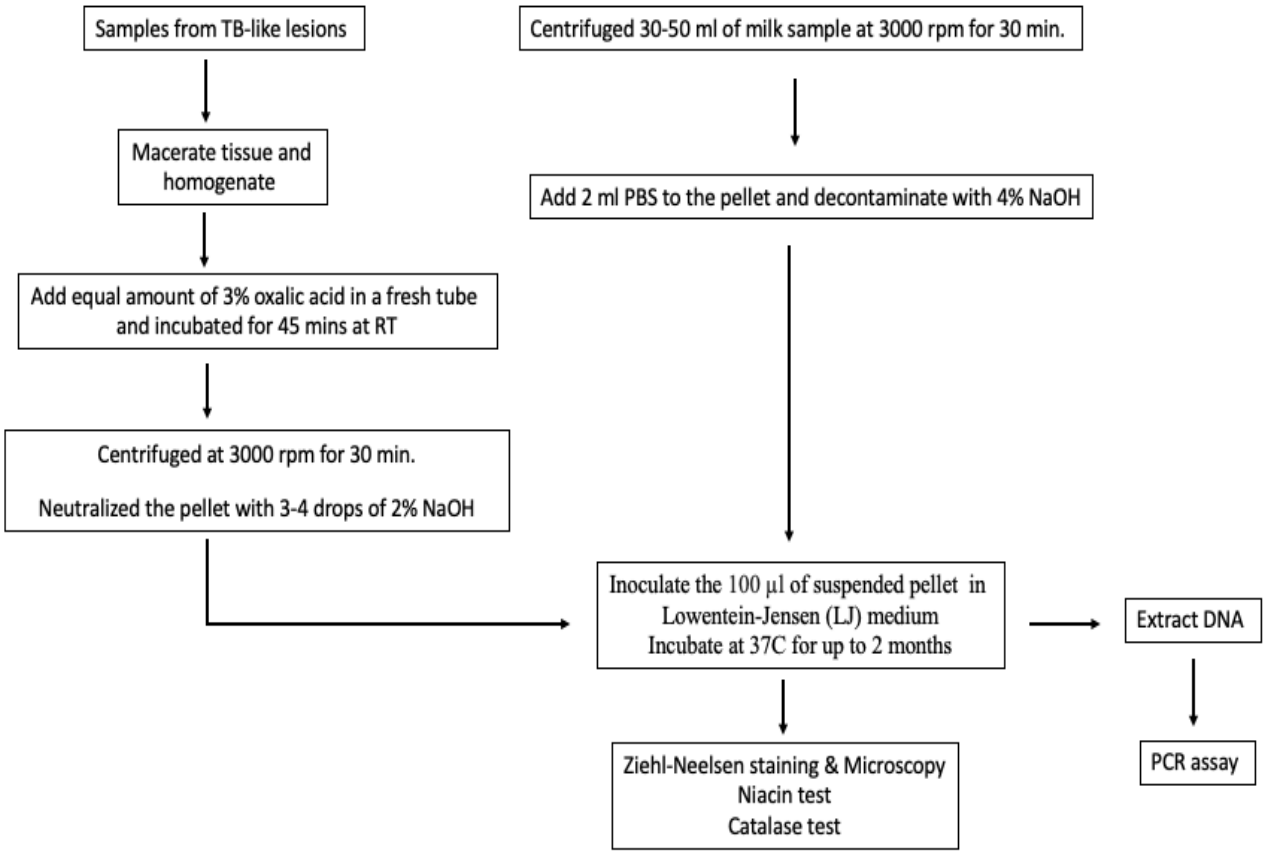
Procedure

- The edges of a clean, dry, gel casting tray to be sealed at both the ends using adhesive tape. An appropriate comb should be placed to form a sample slot in the gel.
- Agarose solution is prepared by dissolving required quantity of agarose in a proportionate volume of 0.5X TBE buffer and melted in a microwave oven for one min.
  (For 0.8% gel, 0.32 g agarose in 40 ml of 0.5X TBE buffer).
- Once the molten gel cooled, 0.5 µg/ml of ethidium bromide to be added and mixed thoroughly by gentle swirling.
- Warm agarose solution is then to be poured into the gel casting tray avoiding formation of air bubbles and allowed to solidify.
- Once agar gets solidified, a small amount of electrophoresis buffer is to be poured on the top of the gel to remove the comb. Then the buffer to be poured off and the tape removed.
- The gel casting tray is to be mounted in the electrophoresis tank and the electrophoresis buffer is to be added just enough to cover the gel to a depth of 1 mm.
- Five µl of sample DNA to be mixed with 1 µl volume of 6X gel loading dye and slowly loaded into the slots of submerged gel using a micropipette.
- The gel tank to be closed with the lid and electrical leads attached so that the DNA will migrate towards the anode.
- The electrophoresis to be carried out at 5V/cm at room temperature until the bromophenol blue dye migrated to an appropriate distance through the gel.
- Following electrophoresis, the gel/bands need to be visualized at 300 nm wavelengths using a UV trans-illuminator and recorded in a gel documentation unit.

MYCOBACTERIUM BOVIS

Zoonotic tuberculosis is caused by several subspecies of *Mycobacterium tuberculosis* complex. The most common subspecies is *M. bovis*. Bovine tuberculosis (bTB) associated with *M. bovis*, presents significant public health. Slaughterhouses may serve as sentinel sites may provide insight into the prevalence of bTB. Infection of *M. bovis* is caused by consuming contaminated, unpasteurized dairy products. Infection might occur during slaughter of animals infected with *M. bovis.* Transmission from animals to humans through the air is rare.

Methods for isolation and identification of *M. bovis* is presented in Flow chart 19.



Flow chart 20. Steps for isolation and identification of *M. bovis* from the milk and tissue samples.

Procedure for processing tissue samples:

1. Collect tissue samples from TB-like lesions and/or prediction sites (mediastinal, retropharyngeal, pre-crural and pre-scapular lymph nodes, lungs and liver).
2. Collect fine-needle aspirate (FNA) of specimens from enlarged cervical lymph nodes with a 21-gage needle attached to a 10 ml syringe and transfer into cryo-tubes containing 1 ml of PBS. Collect sputum specimens in sterile 50 ml plastic tubes. Transport samples on ice (4°C).
3. Finely chop the tissue sample of using sterile scalpel blades and forceps.
4. Homogenized the tissue pieces for 2 min in a stomacher bag with 1-5 ml of sterile PBS.
5. Transfer the homogenates into sterile tubes and add an equal amount of 3% oxalic acid, and incubated at room temperature for 45 min with occasional shaking.
6. Centrifuge at 3000 rpm for 15 min. and neutralize the sediments with 3-4 drops of 4% NaOH solution. Add 3-4 drops of 0.1% phenol red indicator to control the pH.
7. Mixed the sediments well and inoculate 100 µl on Lowenstein-Jensen (LJ) medium containing pyruvate or glycerol, incubate the slants at 37°C and observe for colony growth weekly up to 2 months.
8. Typical M. *bovis* grow as yellowish and creamy colonies in the LJ medium.

Procedure for processing milk samples:

1. Centrifuged 30-50 ml of milk sample at 3000 rpm for 30 min 4°C.
2. Reconstitute the sediment in 2 ml normal saline and decontaminate with equal volume of 4% NaOH (modified Petroff's method).
3. The obtained pellet can be used (100 µl) for inoculation onto LJ medium and incubate at 37°C for eight weeks and inspect weekly for visible growth. The cells from colonies that suggested the growth of mycobacteria are examined microscopically after Ziehl-Neelsen staining.

4. The samples are considered negative, if no visible growth was detected after eight weeks of incubation.

PCR assay

Suspend the typical colonies in PBS, extract DNA and perform the PCR.

Microscopy

Mycobacterium bovis can be demonstrated microscopically on direct smears from clinical samples lymph nodes and tissues with lesions by Ziehl-Neelsen staining.

Ziehl-Neelsen Staining

Materials required

- Sterile 1 oz. universal containers with identification number engraved cap.
- Wire loop with an inner diameter of 5 mm.
- Clean new, washed microscopy slide (no grease and no scratches on the slide).
- Diamond marker to enter identification number on the microscopy slide.
- Forceps to hold slide with sputum smear.
- Bunsen burner to fix smear.
- Metal waste bin with disinfectant (5% phenol solution) to discard infected material.
- Staining rack to hold the slides.
- Slide rack to place stained smear slides to dry in the air.
- 1% Carbol-fuchsin.
- 25% H_2SO_4.
- 0. 1% Methylene blue.
- Tap Water.

Staining Procedure

- Place the slides on a staining rack in batches (maximum 12) with the smeared side facing up. Ensure that the slides do not touch each other.
- Flood entire slide with filtered 1% Carbol-fuchsin.
- Heat each slide slowly until it is steaming. Do not boil. Maintain steaming for five min by using intermittent heat.
- Rinse each slide individually in a gentle stream of running water until all free stain is washed away.
- Flood the slide with the 25% H_2SO_4 solution for 2-3 min.
- Rinse the slide thoroughly with water. Drain off excess water from the slide.
- Flood the slide with 0.1% Methylene blue for 30 sec.
- Rinse the slide thoroughly with water. Drain excess water from the slide. Allow the smear to air dry. Do not heat or use blotting paper.

Examination and Reporting (Ziehl-Neelsen staining & microscopy)

- View the sample using 100X.
- Apply one drop of Liquid paraffin oil (heavy) /immersion oil to the left edge of the stained smear.
- Scan the stained smear carefully from left to right side.
- Count acid fast bacilli (AFB) in low positive smears for quantification.
- Grade the smear according to WHO guidelines (Table below).
- Place the slide smear-down on a piece of absorbent paper (absorbent tissue paper) after examination; let the oil soak in and do not rub.
- Clean the objective lens at the end of each day using a lens or soft tissue.

Grading Chart for Ziehl-Neelsen staining & Microscopy

(100X oil immersion objective and 10X eye piece)

Ziehl-Neelsen staining grading	Reporting /Grading
>10 AFB/field after examination of 20 fields	Positive, 3+
1-10 AFB/field after examination of 50 fields	Positive, 2+
10-99 AFB/100 field	Positive, 1+
1-9 AFB/100 field	Positive, Scanty
No AFB per 100 fields	Negative

Culture method

Media preparation

LJ MEDIUM (DRUG FREE)

Homogenization of egg

- Select eggs not older than 7 days for the preparation of egg fluid.
  (Note: Hens should be fed on food without antibiotics).
- Fresh eggs for minimum air space are checked for viability and is done by candling method.
- Clean eggs with soap water; place in a basin and wash in running water until the water is clear, then rinse with distilled water and then again immerse finally in 70% alcohol for 5 min; place the eggs on a clean towel to dry.
- Break the eggs individually and transfer into a stainless-steel beaker and transfer the egg fluid into 2 liters round flat-bottomed flask.
- Homogenize the egg fluid using a mechanical egg churner.
- Filter the egg fluid using a sterile gauze and a funnel.
- Measure one liter of egg fluid using a sterile measuring cylinder and transfer into a 3 or 5 lit conical flask.

- Transfer 600 ml of the sterilized mineral salt malachite green solution to the egg fluid
- Gently shake to mix thoroughly.
- Fix the pourer to the mouth of the conical flask and distribute approximately 6 ml of medium in a McCartney bottle.

Coagulation of medium

- Pour distilled water into the Inspissator tank through the side opening up to the mark.
- Place the bottles in the Inspissator to coagulate the media for 60 min at 85°C-90°C.
- Remove after 60 min from the Inspissator and leave at room temperature.
- Record the Inspissator temperature periodically in a notebook (every 15 min).
- Reinspissate the bottles at 85^0C-90^0C for 30 min on the consecutive day after overnight storage at room temperature.
- Label the medium with batch number and date of preparation. The same should be recorded in the Media Preparation Register.

Sterility check

After inspissation randomly the whole batch of the medium should be incubated at 37^0C for 24 hrs. Select 2 bottles of plain LJ for sterility check and record in the Media Sterility register.

Processing of Tissue and Biopsy Specimen for smear and culture

- Cut into small pieces using sterile scissors.
- Transfer into a sterile tissue grinder tube and add 5 ml of sterile distilled water and homogenize with a sterile Teflon grinding rod.
- Make a direct smear from the homogenate.
- Centrifuge the homogenate at 3500 rpm for 15 min.
- Decant the supernatant carefully.
- To deposit add 1 ml sterile distilled water.

- Add one drop to the direct smear, dry and stain.
- Add 1 ml 5% H_2SO_4.
- Mix well and let stand for 15 min.
- Fill up bottle with sterile distilled water.
- Centrifuge at 3500 rpm for 15 min.
- Discard supernatant carefully.
- To deposit add 0. 2 ml sterile distilled water.
- Inoculate onto two slopes each of LJ.

CULTURE READING

- Cultures are examined starting from 8th week cultures.
- Typical colonies of *M. bovis* are rough, buff, tough, non-pigmented (cream coloured). The organism grows slowly at 37°C but does not grow at 22°C or 45°C.

NaOH method/modified Petroff's procedure

Material required

- Incubator set at 37°C.
- Aerosol containment Centrifuge (Megafuge-1, Megafuge-11), capable of speed up to at least 3000g, fitted with rotor to take at least 12 McCartney bottles.
- Pan balance.
- Bio-safety cabinet.
- One culture bin.
- Two 5 mm wire loops (Nichrome wire of 27 SWG).
- One lysol bin with lid for disposable waste.
- Wire baskets or racks to hold 12 McCartney tubes.
- Wire racks for holding 150 McCartney bottles.
- Stock of clean, sterile McCartney bottles.

- Stock of LJ slopes.
- Sterile 4% NaOH solution (as per SOP).
- Sterile distilled water in 500 ml conical flask.
- Diamond marker pencils.
- Timer.

Processing by 4% NaOH method

- Add double the volume of sterile 4% NaOH.
- Tighten the caps of the McCartney bottles and mix it well by hand for 1 min.
- Invert each bottle to ensure that the NaOH solution contacts all the sides and inner portion of caps.
- In case of Leakage, change the caps of McCartney bottle.
- Place the bottles in shaker for 10-15 min.
- At the end of 15 min, Insert the bottles in centrifuge bucket.
- Balance the buckets properly in pan balance before keeping for centrifuge at 3000 rpm for 15 min. At the end of 15 min, remove the McCartney bottles from the centrifuge without shaking.
- Discard the supernatant fluid into a lysol bin.
- Add sterile distilled water up to the neck of the McCartney bottles.
- Mix it well and centrifuge at 3000 rpm for 15 min.
- At the end of 15 min remove McCartney bottles from the centrifuge without shaking.
- Discard the supernatant fluid slowly into a lysol bin.
- From the sediment, inoculate two slopes of LJ medium.
- Use one loopful of sediment for each slope (using a sterile twisted wire loop made up of Nichrome wire.
- Incubate the bottles in rack.
- Label the rack with study name and the rack number.

Sterility checks

- Inoculate one loop of randomly selected distilled water flask or 2 LJ slopes and write the date on the bottles.
- Incubate all the LJ medium slopes at 37°C.
- Keep them in a rack in which that particular batch number was used.
- Check the growth weekly for eight weeks.

Processing by Cetyl pyridinium chloride (CPC) method

Materials required

- Vortex mixer, Same as modified Petroff's method except 4% NaOH

Reagents required

CPC solution

- 1% CPC (weigh 10 gm of CPC and 20 gm of NaCl and dissolve in 1 liter of distilled water).
- Distribute 5 ml quantity in bottles and autoclave in 121°C for 15 min.

Procedure

CPC containing specimens should be processed as described below:

- To the specimen with CPC, add 10-15 ml sterile distilled water (to reduce the viscosity).
- Tighten cap of container and mix well by inversion.
- Centrifuge at 3000 rpm for 15 min.
- Carefully pour off the supernatant in discarding bin containing Lysol and vortex for 2-3 min.
- Add approximately 20 ml sterile distilled water and resuspend the sediment.
- Centrifuge again at 3000 rpm for 15 min.

- Decant supernatant, prepare the deposit smear and inoculate the deposit on to two slopes of LJ medium.
- Incubate all the LJ medium slopes at 37°C.

CULTURE READING

- Cultures are examined starting from 8th week cultures.
- Typical colonies of *M. bovis* are rough, buff, tough, non-pigmented (cream coloured) and slow- growers, i.e., colonies appearing after one to two weeks after inoculation.

NIACIN TEST

Equipment and materials

- Test culture on LJ medium (A culture must be at least three to four weeks old and must have sufficient growth of at least 2+ growths).
- O-toluidine - 1. 5% (Weigh 1. 5 g of O-toluidine and add 100 ml of ethanol; mix in an amber-coloured bottle; prepare fresh weekly).
- Cyanogen bromide 10%.

Procedure

- Select 4 weeks old LJ culture (from all studies after drug sensitivity testing reading) with minimum 2+ growth and include positive and negative controls.
- Enter the Lab number in the niacin register.
- Mark the corresponding serial number on the slopes.
- Check for water of condensation in the culture tube.
- If needed, add 0. 5 or 1 ml of sterile water to the tube accordingly.
- Place the bottles in the autoclave at 121ºC for 30 min.
- Cool to room temperature.
- Switch on the fume hood cabinet.

- Remove 0. 25 ml of the water of condensation to a clean tube (75 x 12 mm).
- Add 0. 25 ml of 1. 5% O-toluidine using micropipette.
- Add 0. 25 ml of 10% Cyanogen bromide and mix well.
- Observe for color development within 5 min.

Pink color (Niacin-positive)

White precipitate (Niacin-negative)

Discard the tubes into the bin containing 4% NaOH solution

CATALASE TEST

Reagents

- 0.067 M phosphate buffer solution, pH 7.0.
- Hydrogen peroxide, 30% solution. Store in the refrigerator.
- Tween-80, 10%.
- Complete catalase reagent (Tween-peroxide mixture):

Immediately before use, mix equal parts of 10% Tween-80 and 30% hydrogen peroxide. Allow 0.5 ml reagent for each strain to be tested.

Procedure

- With a sterile pipette, aseptically add 0.5 ml of 0.067 M phosphate buffer to 16 x 125 mm screw capped test tubes.
- Suspend a loopful of test culture in the buffer solution, using a sterile loop.
- Place the tubes containing the emulsified culture in a previously heated water bath at 68^0C for 20 min. Time & temperature are critical.
- Remove the tubes from heat and cool to room temperature.
- Add 0.5 ml of freshly prepared Tween-peroxide mixture to each tube.

- Observe the formation of bubbles appearing on the surface of the liquid. Do not shake the tubes because Tween-80 may also form bubbles when shaken, resulting in false positives.
- Hold negative tubes for 20 min before discarding.

Identification of *M. bovis*

- Growth rate slow
- Optimal growth temperature 35-37℃ only
- No pigmentation
- Niacin-negative
- Catalase-positive

MOLECULAR IDENTIFICATION

PCR assay

Rapid identification of isolates to the level of the *M. tuberculosis* complex can made by multiplex PCR. A loop full of the isolate is taken from the Lowenstein-Jensen slant and transferred to the 1.5 ml tube with 200 µl of 0.1% Triton X-100. The resuspended bacteria are heated in a dry bath at 90°C for 40 min and centrifuged at 10,000g for 10 min. The supernatant is used as target DNA.

The primer sequences, targets and PCR condition are shown in the table.

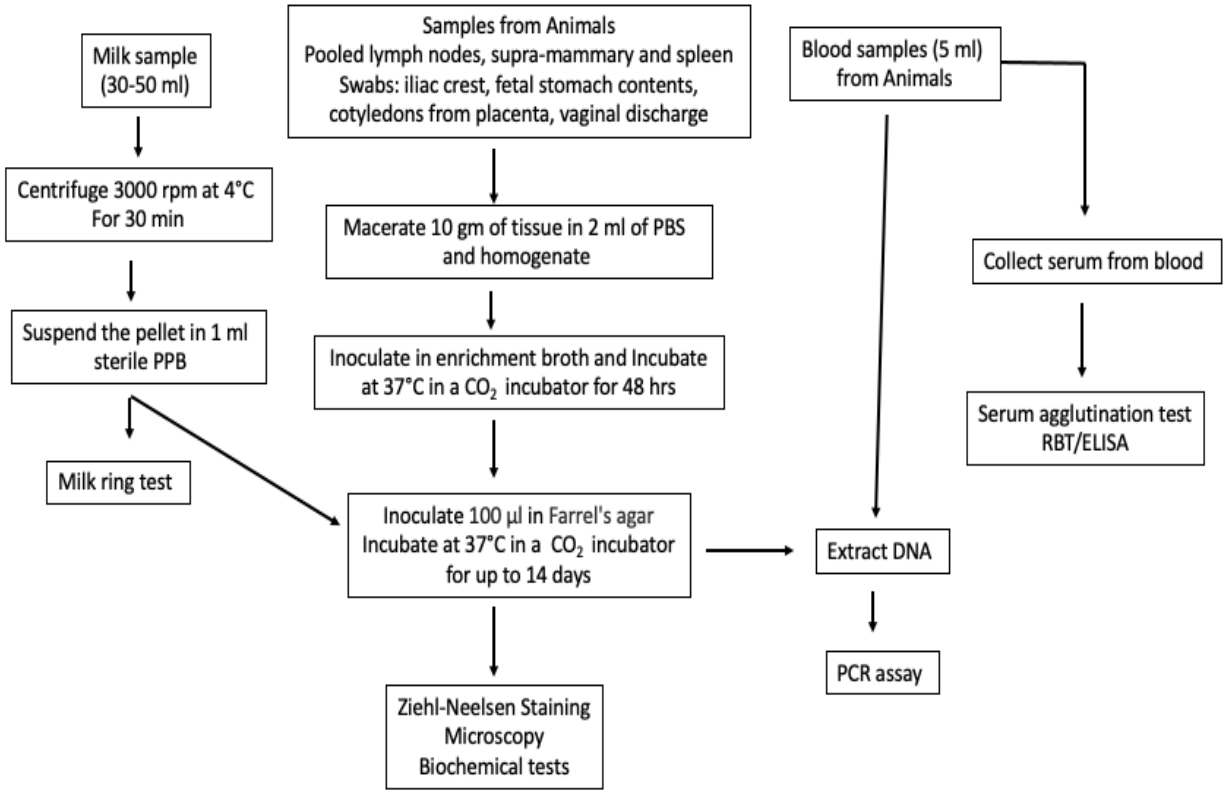
Multiplex PCR for detecting *Mycobacterium* spp.

Primer	Primer sequence (5'-3')	Target	Amplicon (bp)	PCR condition
CSB1-F	TTCCGAATCCCTTGTGA			Denaturation at 95°C for 5 min Denat: 95°C for 45 sec Ann: 57°C for 45 sec Ext: 72°C for 1 min Final Ext: 72°C for 10 min
CSB2-R	GGAGAGCGCCGTTGTA	*M. bovis*	168	
CSB3-R	AGTCGCGTGGCTTCTCTTTTA	*M. tuberculosis*	262	
16S rRNA-F	ACGGTGGGTACTAGGTGTGGGTTTC	*Mycobacterium s*pp.	575	
16S rRNA-R	TCTGCGATTAGCGACTAAGACTTCA			

The PCR reaction mixture (25 μl) consisted of 5 pmol of each primer, 0.5 μl of Taq DNA polymerase (5 U/μl, Takara, Otsu, Japan), 2.5 μl of 25 mM MgCl2, 2 μl of 2.5 mM dNTP mixture and genomic DNA. The amplified PCR products can be separated in a 1.5% agarose gel electrophoresis and visualized by UV-transilluminator and documented using a gel documentation system.

BRUCELLA SPP.

Brucellosis is a zoonosis infection that present major public health risks. The etiological agents are bacteria of the genus *Brucella*, and the most important species include *B. melitensis*, *B. abortus*, *B. ovis*, and *B. suis*. In animals, the disease is characterized by abortion, sterility, and other reproductive disorders. Brucellosis in humans is transmitted through the contact with or inhalation of contaminated or infected faetal material, vaginal discharges, consumption of contaminated milk, feed and water. In humans, this pathogen is a one of the major food safety and occupational hazard, with dairy products, and contact with infected animals like cattle, goats/sheep. Methods for isolation and identification of *Brucella* sp. presented in Flow chart 20.



Flow chart 21. Steps for isolation and identification of *Brucella* spp. from the milk, tissue and blood samples.

Procedure of sample preparation and inoculation:

- The most important samples include vaginal secretions (swabs), aborted fetuses (stomach contents, spleen and lung), faetal membranes, and milk, semen and arthritis or hygroma fluids. From animal carcasses, the preferred tissues for culture are those of the reticulo-endothelial system (i.e., lymph nodes and spleen), the pregnant or early post-parturient uterus, and the udder.
- Primary isolation of the organisms from may be enhanced using tryptose broth, brain heart infusion broth, or *Brucella* broth in biphasic bottles.
- The tissues may be ground manually or homogenised in a blender or stomacher with a small proportion of sterile PBS, and inoculated onto trypticase soy agar containing 5% sheep blood agar, *Brucella* agar with 5% serum, or serum dextrose agar.
- Milk samples should be allowed to stand overnight at 4℃ before centrifuging at 3000 rpm for 30 min. The cream and the deposit are spread onto the surface of at least three plates of solid selective medium.
- If contamination of the sample by other microorganisms is a strong possibility, selective media should be employed for primary isolation, e.g., Farrel's agar medium supplemented with antibiotics (bacitracin, polymyxin B, nalidixic acid, vancomycin, cyclohexamide, and nystatin.
- The plates are incubated at 37°C in an atmosphere of 5-10% CO_2. After 48–72 hrs of incubation (extend up to 14 days, if negative).
- After 48-72 hrs of incubation, smooth *Brucella* organisms form a circular, convex colonies, 1-3 mm in diameter, with a smooth glistening surface.

MICROSCOPY

Smears of blood, tissues, iliac crest, faetal stomach contents, cotyledons from placenta, vaginal discharge of the suspected animals may be stained using modified Ziehl-Neelsen method of staining.

Ziehl-Neelsen staining & Microscopy

Materials required

- Container to collect specimen.
- Sterile 1 oz. universal containers with identification number engraved cap.
- Wire loop with an inner diameter of 5 mm to spread specimen on the slide.
- Clean new, washed microscopy slide (no grease and no scratches on the slide).
- Diamond marker to enter identification number on the microscopy slide.
- Forceps to hold slide with specimen smear.
- Bunsen burner to fix smear.
- Metal waste bin with disinfectant (5% phenol solution) to discard infected material.
- Staining rack to hold the slides.
- Slide rack to place stained smear slides to dry in the air.
- 1% Carbol-fuchsin.
- 25% H_2SO_4.
- 1% Methylene blue.
- Tap Water.

Staining Procedure

- Place the slides on a staining rack in batches (maximum 12) with the smeared side facing up. Ensure that the slides do not touch each other
- Flood entire slide with filtered 1 % Carbol-fuchsin.
- Heat each slide slowly until it is steaming. Do not boil. Maintain steaming for five min by using intermittent heat.
- Rinse each slide individually in a gentle stream of running water until all free stain is washed away.
- Flood the slide with the 0.5% acetic acid solution for 2-3 min.

- Rinse the slide thoroughly with water. Drain off excess water from the slide.
- Flood the slide with 0. 1% Methylene blue for 30 sec.
- Rinse the slide thoroughly with water. Drain excess water from the slide. Allow the smear to air dry. Do not heat or use blotting paper.

Examination and Reporting (Microscopy)

- Use the objective 100X.
- Apply one drop of Liquid paraffin oil (heavy) immersion oil to the left edge of the stained smear.
- Scan the stained smear systematically from left to right side.
- Count AFB in low positive smears for quantification.
- Place the slide smear-down on a piece of absorbent paper (absorbent tissue paper,) after examination; let the oil soak in and do not rub.
- Clean the objective lens at the end of each day using a soft tissue.

RESULTS

Small (0.5–0.7 by 0.6–1.5 μm), non-motile, non-spore-forming, Gram-negative coccobacilli

CULTURE

- The most valuable samples include vaginal secretions (swabs), aborted fetuses (stomach contents, spleen and lung), faetal membranes, and milk, semen and arthritis or hygroma fluids. From animal carcasses, the preferred tissues for culture are those of the reticulo-endothelial system (i.e. head, mammary and genital lymph nodes and spleen), the pregnant or early post-parturient uterus, and the udder.
- Primary isolation of the organisms from may be enhanced using tryptose broth, brain heart infusion broth, or *Brucella* broth in biphasic bottles.

- The tissues may be ground manually or homogenised in a blender or stomacher with a small proportion of sterile water, and inoculated onto trypticase soy agar containing 5% sheep blood agar, *Brucella* agar with 5% serum, or serum dextrose agar.
- Milk samples should be allowed to stand overnight at 4°C before lightly centrifuging. The cream and the deposit are spread onto the surface of at least three plates of solid selective medium.
- If contamination of the sample by other microorganisms is a strong possibility, selective media should be employed for primary isolation, e.g., Farrel's agar medium supplemented with antibiotics (bacitracin, polymyxin B, nalidixic acid, vancomycin, cyclohexamide, and nystatin.
- The plates are incubated at 37°C in an atmosphere of 5-10% CO_2. After 48-72 hrs of incubation.
- After 48-72 hrs of incubation, smooth *Brucella* organisms form a circular, convex colonies, 1-3 mm in diameter, with a smooth glistening surface.

SEROLOGICAL IDENTIFICATION

Serum agglutination test

- The test is performed in clear glass or plastic tubes of approximately 1-2 ml total volume by placing 0.8 ml of phenol saline (0.5% phenol in 0.15 M sodium chloride) into the first tube and 0.5 ml volumes of phenol saline in the remaining tubes of a series of five or ten tubes.
- A volume of 0.2 ml serum is added to the first tube, mix, and then 0.5 ml is transferred to the next tube. Further volumes of 0.5 ml are transferred to subsequent tubes to give a series of doubling dilutions.
- An equal volume of standard *Brucella* agglutination suspension, dilute to working strength in phenol saline, is then added to each tube, and the tubes are incubated at 37°C for 20 hrs.

- The tests are read against opacity standards prepare by diluting the working strength antigen 1 in 4, 2 in 4, and 3 in 4, to correspond to 25%, 50% and 75% agglutination. Phenol saline is used as the 100% control, and the undiluted working strength antigen as the 0% control. The results will score as the degree of agglutination (1+ = 25%, 2+ = 50%, 3+ = 75%, 4+ = 100%) over the serum dilution. In each set of tests, a positive control serum calibrated against the International Standard for *B. abortus* antiserum (ISABS) must be included.
- For cattle, titres equivalent to 50 IU or more for unvaccinated animals and 100 IU or more for vaccinates are regarded as indicative of infection. Microagglutination methods using a stain antigen may be performed in microtitre plates instead of tubes.

Coombs antiglobulin agglutination test

The serum agglutination test is performed according to the recommended procedure.

- Following incubation at 37°C, the cells are pelleted by centrifugation, preferably in a refrigerated centrifuge at 4°C.
- The supernatant liquid is then discarded and the cell deposit is washed by resuspension in 0.15 M NaCl follow by centrifugation.
- This process is performed at least twice. The cell deposit is then finally re-suspended in 0.5 ml volumes of anti-IgG serum diluted to working strength.
- The tubes are then re-incubated at 37ºC overnight.
- Agglutination is then score as for the agglutination test. A positive serum control and a saline negative control should be included in the series. If IgA antibodies are detected, a broad specificity anti-immunoglobulin reagent should be used. This test has now been largely superseded by the ELISA.

Indirect ELISA

Test procedure

- Lipopolysaccharide (LPS) antigen is diluted in coating buffer to a concentration determine by checkerboard titration, usually approximately 1 µg/ml, and dispensed to all microwells in 100 µl volumes. The microplates are then incubated at 37°C for two hrs or at 4°C overnight. As this is a solid-phase ELISA technique, the microplate wells require intervening washes between each assay step to remove unbound or unreacted reagents. Three to four wash cycles using the washing buffer, are sufficient. Prior to the addition of the next reagent, the plates should be inverted and slapped onto a lint-free absorbent surface to discharge any residual contents.
- Test sera and controls are diluted 1/200 in diluent buffer and added into appropriate wells in 100 µl volumes. Cover and seal the plates and place on an orbital plate shaker and incubate at 37°C for one hour with continuous shaking. Wash the plate as above.
- The enzyme conjugate is diluted in diluent buffer and added to all the wells in 100 µl volumes. Cover and seal the plates and place on an orbital plate shaker and incubate at 37°C for one-hour continuous shaking. The optimal dilution of conjugate should be such that when reacting with the strong positive control under standard conditions, it will result in an average absorbance value of between 1.0 and 1.4 absorbance units. A known positive Reference Serum should be used. Wash the plate as above.
- Fresh substrate/chromogen solution is prepared by adding 60 µl of a 3% H_2O_2 stock solution to 12 ml of phosphate/citrate buffer containing 3.6 mM 2,2′-azino-di-3-ethylbenzthiazoline sulfonic acid (ABTS) buffer. The substrate/chromogen solution applies to all wells in 100 µl volumes. The plates are transferred to an orbital plate shaker and incubate at 37°C for precisely 15 min with continuous shaking. After 15 min incubation, the stopping solution is added to all wells in 100 µl volumes and the plate is shaken briefly on the plate shaker to ensure thorough mixing. All wells now contain a total volume of 200 µl.
- The colour development is read with a microplate photometer using a 405 or 414 nm interference filter.

- The data may be express in a number of ways, but it is recommended that test serum reactivity be expressed as percent positivity of a standardized strong positive control serum. The strong positive control serum should be such that, when prediluted in negative serum, it exhibits an antibody activity that lies on the linear portion of the dose/response curve of the original high-titred serum, just below the plateau phase.

The Milk Ring Test (MRT)

A simple and effective method, can only be used with cow's milk.

- A drop of haematoxylin-stained antigen is mixed with a small volume of milk in a glass or plastic tube.
- If specific antibody is present in the milk it will bind to the antigen and rise with the cream to form a blue ring at the top of the column of milk.
- The test is reasonably sensitive but may fail to detect a small number of infected animals within a large herd. Non-specific reactions are common with this test, especially in brucellosis free areas.

Rose Bengal plate test (RBT)

- Serum samples might be screened using the Rose Bengal plate agglutination test or card test Serum (0.03 ml) is mixed with an equal volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter.
- The mixture is agitated gently for 4 min at ambient temperature, and then observed for agglutination.
- Any visible reaction is considered to be positive.
- The test is very sensitive and positive samples should be checked by the complement fixation test (CFT) or by an IgG specific procedure such as ELISA. False-negative reactions occur especially in the early stages of acute infection. The RBT can be used in all animal species, but positive results should be confirmed by a quantitative test. False

positive results occur in vaccinated animals. False negative results are common in sheep, goats and pigs.

MOLECULAR IDENTIFICATION

PCR assay

DNA extraction and determination of purity and yield of DNA samples:

- DNA from all blood samples and bacterial strains can be extracted using a commercial purification system with columns (QIAamp Blood Midi; QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.
- From *Brucella* cultures grown overnight in *Brucella* selective broth at 37°C, extract DNA with the QIAamp DNA mini-Kit (Qiagen, Germany) after inactivation for 2 hrs at 80°C.
- Estimate the purity and concentration of the genomic DNA extracted with a Nanodrop spectrophotometer (Thermoscientific, USA).
- Concentration of DNA calculated using absorbance at 260nm (A_{260} of 1.0 = 1.0 = 50µg/ml for pure dsDNA)
- Purity of DNA determined from the ratio of absorbance at 260nm and 280nm (A_{260}/A_{280} ~1.8 is generally considered pure DNA).
- DNA extracted from these samples is used as the template for PCR.

Species specific PCR assay: (This technique is useful only to detect the *Brucella* at the genus level. It cannot differentiate the different species under the genus *Brucella* or their biovars. The World Organization of Animal Health does not recommend any specific PCR technique for confirmatory diagnosis of brucellosis in animals).

- The PCR assay will be carried out for gene *bcsp31* encoding an immunogenic outer membrane protein of 31 kDa of *B. abortus*, which is conserve in all *Brucella* spp.

- Using the specific primers BSCP31-F and BSCP31-R according to the protocol of Baily *et al.* 1992.
- The initial PCR assay denaturation was done at 95°C for 3 min followed by 35 cycles at 95°C for 1 min, 60°C for 2 min, 72°C for 2 min and finally at 72°C for 5 min
- Amplified PCR products corroborate through 1% agarose gel and document the gel profile using the Geldoc 1000 System-PC (BioRad, USA)

Primers used in PCR

Primer designation	Sequence 5′-3′	Amplicon size (bp)
BSCP31-F	TGGCTCGGTTGCCAATATCAA	224
BSCP31-R	CGCGCTTGCCTTTCAGGTCTG	

HEPATITIS 'A' AND 'E' VIRUSES

The hepatitis A virus (HAV) and hepatitis E virus (HEV) are enterically transmitted viruses, typically through contaminated water or food. HAV has no animal reservoirs. HAV causes several food-related viral outbreaks worldwide, while HEV is recognised as an emerging pathogen with a global health burden. Transmission of these viral infections may occur via person-to-person. Food contamination can occur at any stage of the food chain, especially when infected/asymptomatic individuals handle raw foods.

HAV and HEV are single-stranded positive-sense RNA viruses that belong to Hepeviridae family. The gold standard for the diagnosis of these viruses is ribonucleic acid detection using nucleic acid amplification testing. After the extraction of RNA from the suspected samples, HAV and HEV can be detected by polymerase real-time reverse transcription chain reaction (RT-PCR).

Detection of hepatitis A and E viruses by RT-PCR

Requirements:

Buffers and Reagents:

a) Sea Salts (Sigma Cat. No S9883-500G)

b) Milk Power (Nestle)

c) HCl (Qualigens Cat. No 29505)

d) QIAamp Viral RNA Mini Kit (250) Qiagen, Hilden, Germany, (Cat. No. REF 52906)

e) Premium Grade Ethanol 500 ml (Hayman, Cat. No Un:1170200-578-6)

f) HAV and HEV specific Forward and Reverse Primers and Taqman Probes

g) SuperScript III Platinum One- Step qRT-PCR kit (Invitrogen, Cat. No.11732-088)

h) Nuclease Free Water

Equipment:

a) 6400 Funnel separating Pear shape with glass stop cock & interchangeable stopper (Borosil, Cat. No.6400030)

b) Laminar Flow Cabinet/Class II Type A2 Biological Safety Cabinet

c) Weighing balance

d) Vortex

e) Refrigerated Centrifuge

f) Micropipettes (100-1000 µl, 20-200 µl, 0.5-10 µl, 2-20 µl)

g) Real Time PCR Machine (7500 Fast Real Time PCR System Applied Biosystems by Thermo Fisher Scientific or any other system compatible with FAM and VIC dyes)

Consumables:

a) Sterile, nuclease-free microcentrifuge tubes (1.5 ml)

b) PCR reaction tubes/strips with caps/reaction plates and plate sealer

c) Micropipette tips (aerosol barrier tips 10-1000 µl)

d) Tube racks

e) Mini cooler

f) PCR microplate transfer tray

g) 50 ml centrifuge tubes

Viral hepatitis refers to infection caused by viruses that affect the liver. Viral hepatitis is caused by five distinct viruses that include hepatitis A to E. Hepatitis A is a picornavirus, while Hepatitis E is a hepevirus. These two viruses are made of single stranded RNA. HAV and HEV are transmitted through the faecal-oral route. In Asia and other developing courtiers HAV epidemics are caused by consumption of contaminated water, dairy products, uncooked vegetables or meat. Hepatitis virus from water samples can be concentrated by using adsorption-elution followed by re-concentration methods. The viral RNA can be detected by RT-PCR using specific primers and probes.

A. Processing of meat samples for RNA isolation for Hepatitis A & E screening by RT-PCR

1. Aseptically collect 100-150 mg of animal organ tissue such as liver and transport to the laboratory maintaining cold chain at 4°C*.
2. Take a 50-100 mg tissue, add 750 µl of TRIZOL TS reagent (Invitrogen, Carlsbad, CA), and homogenize tissue with homogenizer/probe/ mortar-pestle.
3. Incubate for 10 min at room temperature (RT)
4. Add 200 µl Chloroform, vortex and keep on ice for 15 min. Repeat vortexing in between.
5. Centrifuge for 15 min at 14000 rpm at 4°C.
6. The mixture separates into a lower, an interphase, and a colourless upper aqueous phase. Transfer the aqueous phase in a new microcentrifuge tube and add equal volume of 70% ethanol.
7. Load this onto QIAamp Viral RNA Mini Kit column as follows-
   Assemble the column with collection tube and apply above solution to the column & centrifuge at 8000 rpm for 1 min at room temperature (RT).
8. Discard the filtrate and collection tube and repeat same procedure with remaining 630 µl solution.
9. Add 500 µl of buffer AW1 to the column and centrifuge at 8000 rpm for 1 min at RT.
10. Discard filtrate and collection tube and add 500 µl of buffer AW.
11. Centrifuge at 14000 rpm for 3 min at RT.
12. Discard filtrate and collection tube and place the column in a fresh collection tube and give a dry spin at 14000 rpm for 1 min at RT.
13. Discard collection tube and place column in 1.5 ml microcentrifuge tube.
14. Add 50 µl of buffer AVE, close the cap and incubate at RT for 2 min.
15. Centrifuge at 8000 rpm for 1 min at RT to collect the eluate containing RNA

**If samples cannot be processed on the same day, collect tissues in tubes containing ~5 volumes of RNAlater (ThermoFisher), ~0.5 ml/ sample, store at 25°C overnight and then transfer to -20°C for long time storage. For processing, thaw the tissue, remove from RNAlater and process similar to fresh sample.*

B. Processing of multicomponent/ composite foodstuffs for RNA isolation for Hepatitis A & E screening by RT-PCR

1. Take 25 gm of the collected food material in a 400 mL polypropylene bag containing a filter compartment soaked in 40 mL of elution buffer (Tris-HCl 100 mM, glycine 50 mM, 1% beef extract at pH of 9.5.
2. Remove the rinsing fluid via the bag's filter compartment and centrifuge at 10,000 rpm for 30 min at 4°C to pellet the food particles.
3. Adjust the pH of the decanted supernatant to adjusted to 7.2 ± 0.2 by adding 5 N HCl while swirling the fluid constantly.
4. Supplement the neutralized supernatant with 10% (w/v) poly-ethylene glycol (PEG) 8000 and 0.3 M NaCl and incubate with constant shaking for 1 h at 4°C.
5. Concentrate the viruses by centrifuging the solution at 10,000 rpm for 30 min at 4°C.
6. Discard the supernatant and re centrifuge the sediment again at 10,000g for 5 min at 4°C to compact the pellet.
7. Resuspend the pellet in 500 µl of PBS and vortex.
8. Add 500 µl of chloroform: butanol, 1:1 (v/v) and incubate for 5 min at RT.
9. Centrifuge at 8000 rpm for 15 min at 4°C.
10. Proceed for nucleic acid extraction using the upper aqueous phase containing the virus.

Processing of Water samples for RNA isolation for Hepatitis A & E screening by RT-PCR

1. Add 33.33 g of sea salts in one liter of 1:1 mix of distilled water and tap water.
2. Prepare 1% Milk powder suspension in artificial sea water and adjust pH to 3.5 using 1N HCl. Flocculation of milk powder is observed at pH 3.5.
3. Take 2 lit of water sample to be tested, adjust pH of water to 3.5 by using 1N HCl and transfer water to the glass separating funnel.
4. Add 20 ml of pre-flocculated milk powder suspension to the pH adjusted water sample, mix thoroughly and keep overnight at RT.

5. Next day, collect ~ 5 ml of the precipitate settled at the bottom of the separating funnel in a 50 ml conical centrifuge tube (**Fig. S12**).
6. Centrifuge at 5000 rpm for 10 min to pellet down the precipitate and gently discard the supernatant without disturbing the pellet.
7. Resuspend the pellet in 1.0 ml 1X PBS, mix it by vertexing, and take 200 µl of this suspension for RNA extraction.

Common Procedure for RNA Extraction

Note: Prepare and reconstitute all the reagents in the Kit as per the manufacturer's instructions.

1. Take 200 µl of dissolved precipitate in 1.5 ml microcentrifuge tube.

2. Add 565.6 µl AVL buffer containing carrier RNA (560 µl AVL + 5.6 µl Carrier RNA), mix by pulse vertexing for 15 sec and incubate at room temperature (RT) for ~10 min.

3. Centrifuge briefly to remove drops from inside of the lid, add 560 µl of ethanol (96-100%) and mix by pulse vertexing for 15 sec and centrifuge briefly.

4. Assemble the column with collection tube and apply 630 µl of above solution to the column & centrifuge at 8000 rpm for 1 min at RT.

5. Discard filtrate and collection tube and repeat same procedure with remaining 630 µl solution.

6. Add 500 µl of buffer AW1 to the column and centrifuge at 8000 rpm for 1 min at room temperature.

7. Discard filtrate and collection tube and add 500 µl of buffer AW2.

8. Centrifuge at 14000 rpm for 3 min at RT.

9. Discard filtrate and collection tube and place the column in a fresh collection tube and give a dry spin at 14000 rpm for 1 min at RT.

10. Discard collection tube and place column in 1.5 ml microcentrifuge tube.

11. Add 50 µl of buffer AVE, close the cap and incubate at room temperature for 2 min.

12. Centrifuge at 8000 rpm for 1 min at RT to collect the eluate containing RNA.

Viral RNA extraction using QIAamp VIRAL RNA MINI KIT

Reagents Provided in the Kit:

- QIAamp mini spin columns
- Collection tubes
- Carrier RNA
- RNase-free buffers (Lysis buffer/AVL, Wash buffers (AW1, AW2) and Elution buffer/ AVE)

Reconstitution of carrier RNA

Take 310 µl AVE Buffer + 310 µg carrier RNA (Solution of 1µg/µl), make aliquots and store at 20˚C.

Preparation of AVL Buffer

Add 5.6 µl carrier RNA to 560 µl of AVL buffer (for one sample)

Preparation of AW1 Buffer

Add 125 ml of absolute ethanol to AW1 concentrate

Preparation of AW2 Buffer

Add 160 ml of absolute ethanol to AW2 concentrate

Primers for HAV Real-time PCR

Forward Primer 5'-AACAGCGGCGGATATTGG-3'
Reverse Primer 5'-AATGCATCCACTGGATGAGAG-3'
Probe VIC- 5'-AAAAACCATTCAACGCCG-3'-MGB

Primers for HEV Real-time PCR

Forward Primer 5'-AGTGCTYGACCTGACAAATTCAAT-3'
Reverse Primer 5'-GGCGCAGCARAAGACATGTT-3'
Probe FAM- 5'-TCGGGTGGAATGAA-3'-MGB

5.0 Protocol for Real-Time RT-PCR:

PCR Mix for HAV: Ag Path system

2X Agpath buffer (master mix)	12.5 µl
HAV Forward primer (10 µM)	1.25 µl
HAV Reverse primer (10 µM)	1.25 µl
HAV Probe (10 µM)	0.75 µl
Agpath Enzyme mix	1.0 µl
Nuclease free water	3.25 µl
Sample RNA	5.0 µl
Total	25.0 µl

PCR Mix for HEV:

2X master mix	12.5 µl
HEV Forward primer (10 µM)	0.75 µl
HEV Reverse primer (10 µM)	0.75 µl
HEV Probe (10 µM)	0.75 µl
Agpath Enzyme mix	1.0 µl
Nuclease free water	4.25 µl
Sample RNA	5.0 µl
Total	25.0 µl

Positive control (PC): Take either HAV/ HEV positive stool and process similar to test sample, starting from the extraction till RNA detection or take in vitro transcripts of HAV/ HEV in respective qRT-PCR set up.

Negative control (NC): Process tap water similar to test water from the step of virus concentration onwards for detecting false positivity. Include additional non template control (NTC), i.e. water, in the qRT-PCR set up.

Cycling Conditions for HAV and HEV Real-Time PCR

48°C	30 min	
95°C	10 min	
95°C	15 sec	45 cycles
60°C	1 min	

Results/ Interpretation: Ct<38 should be considered as positive for HAV or HEV. Ct above 38 should be considered as negative.

NOROVIRUS

Detection of Norovirus Genogroup I and II by multiplex real time RT-PCR

From stool samples: The stool samples will be subjected to viral RNA extraction using the QIAamp Viral RNA mini kit (QIAGEN, Hilden, Germany) followed by a multiplex real time RT-PCR using a specific MGB probe for simultaneous detection of both the genogroups. The primer and probe sequences used for the multiplex real time RT-PCR is listed in the Table below.

Primers and probes used for one tube multiplex real-time RT-PCR

Genogroup	Primer	Sequences (5'-3')
GI	NV192 (s)	GCYATGTTCCGCTGGATGC
	NV193 (as)	CGTCCTTAGACGCCATCATCA
	TM9-MGB probe	**VIC-**TGGACAGGAGATCGC-**MGB-NFQ**
GII	NV107a (s)	AGCCAATGTTCAGATGGATG
	NV107c (s)	AICCIATGTTYAGITGGATG
	NV119 (as)	TCGACGCCATCTTCATTCAC
	TM3A probe	**6'FAM-**TGGGAGGGCGATCGCAATCTGGC**-NFQ**

The single-tube multiplex real time RT-PCR will be carried out in PCR tubes or plates using a TaqMan® 7700. The reaction will be performed in 12 µl volumes using the Probe RT-PCR mix containing the Omniscript and Sensiscript reverse transcriptases, 0.2 µM of each primer as described in table 1; 80 nM GI TM9 MGB probe;160 nM GII TM3A probe. Two microliters of sample RNA preparation or standard DNA will be added to each reaction.

Thermal cycling for the TaqMan 7700 will be performed as follows: 30 min at 50°C for reverse transcription, 15 min at 95°C for heat inactivation of the reverse transcriptases and the initial activation of the HotStar polymerase, 45 cycles of 20 sec at 94°C and 30 sec at 60°C. The fluorescence data were collected at the end of the 60°C step. To generate a standard curve 10-fold serial dilution of plasmid-DNA containing the appropriate GI or GII sequences will be used.

PARASITES

Meat, fish

Sampling and sample preparation

From a sample weighing 1 kg, take a subsample (100 gm) of beef, pork, or poultry, or 250 gm of fish. Subsamples of most mammalian meat, poultry, or fish require no further preparation. They may be torn or separated into 5 or more pieces to increase the surface area. A 100 gm sample blended in 750 ml saline can improve digestion.

Digestion, sedimentation, and examination

Adjust incubator-shaker or water bath to 37 ± 0.5°C. Prepare digestion fluid in 1500 ml beaker by dissolving 15 gm pepsin in 750 ml saline, add sample, and adjust to pH 2 with concentrated HCl (about 3 ml). Place in an incubator or water bath and stir (about 100 rpm) after equilibration for about 15 min; check and adjust pH again. Cover the beaker with aluminum foil (if using stirrer, pierce hole for stirring rod) and continue incubating until digestion is complete. The time required for digestion will vary but should not exceed 24 hrs.

Carefully pour beaker contents through sieve into tray. Rinse remains with 250 ml saline and add to digest. Examine rinsed contents of sieve and record results. Larger parasites will remain on sieve.

Carefully transfer the contents of the tray to a centrifuge tube (50 ml capacity) and centrifuge at (2000 rpm for 30 min). The sediment will be mixed and examined for detection of parasitic eggs, cysts and larva.

Vegetable preparation

The vegetable samples will be transported to laboratory in plastic bags. They can be immersed immediately in tap water inside a sink/large container and left approximately 6-7 min for

sedimentation of mud and dust. Each vegetable sample can be eluted by vigorous agitation of each specimen for 30 min in 1 lit of sterile phosphate-buffered saline (pH 7.4), to which 50 ml of 0.01% Tween 80 to be added. The agitation can be carried out in a shaking water bath. The eluent can then be filtered through gauze and then dispensed into clean centrifuge tubes and centrifuged at 2000 rpm for 30 min. The supernatant to be discarded into disinfectant jar, and the pellet will be washed of Tween 80 by centrifugation (2000 rpm for 30 min) with sterile phosphate-buffered saline.

The precipitate will be mixed and examined for detection of parasitic eggs, cysts and larva:

Simple smear: a drop of the sediment will be applied on the center of a clean grease-free slide. A clean cover slip will be placed gently. The preparation will be examined under a light microscope using ×10 and ×40 objectives.

Iodine smear: a drop of the sediment will be mixed with a drop of Lugol's Iodine solution and examined as in simple smear.

Simple and iodine smears will be used for detection of parasitic eggs, cysts and larva. The process will be systematically repeated until the mixture in each test tube is exhausted.

Eggs, cysts and oocysts of parasites found under the light microscope will be identified

Staining of sediment smear can be performed by Modified Ziehl–Neelsen and modified trichrome to detect protozoal parasitic oocysts and spores of *Microsporidium* spp.

Each parasite's eggs, cysts or oocysts present in each sample will be enumerated and densities of each species will be expressed as

"many" (>three oocytes per high-power field; >20 eggs per low-power field);

"moderate" (two oocytes per high-power field; 10–19 eggs per-low power field);

"few" (one oocyte per high-power field; three to nine eggs per low-power field);

"rare" (two to five cysts and <two eggs per 100μ of sediment).

For simplification, numerical values were assigned to each density: many, 4; moderate, 3; few, 2; rare, 1; and none, 0.

6. ANTIBIOTIC SUSCEPTIBILITY TESTING

Disc diffusion testing: Basic Procedure

Inoculum: Isolated colonies of each type of organism that may play a pathogenic role should be selected from primary agar plates.

Preparation of inoculum

Direct colony suspension method – Prepare a saline suspension of the isolate from an overnight-incubated agar plate (use a non-selective medium, such as blood agar) to obtain 0.5 McFarland turbidity (1.5 x 10^8 CFU/ ml of *E. coli* ATCC25922).

Growth method – With a sterile straight wire touch the top of each of four to five colonies of the same morphological type, and inoculate MHB or any suitable broth. Incubate tube at 35ºC till turbidity of 0.5 McFarland tube or more is achieved. Then, with sterile normal saline adjust turbidity to exactly 0.5 McFarland.

Inoculating test plates - MHA plate should be inoculated within 15 min after the inoculum has been adjusted. A sterile cotton swab is dipped into the suspension, rotated several times, and gently pressed onto the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab will then be streaked over the entire surface of the agar plate three times, with the plate rotated approximately 60°each time to ensure even distribution of the inoculum. A final sweep of the swab will be made around the agar rim. The lid may be left ajar for 3 to 5 min but no longer than 15 min to allow any excess surface moisture to be absorbed before the drug-impregnated discs are applied.

Application of antimicrobial discs to MHA plate – Ideally, this should be done within 15 min of inoculation of plates. The selected antimicrobial discs will be dispensed evenly onto the agar plate with the help of a forceps/sterile needle/surgical blade. Flame the tips of the applicator intermittently. Each disc must be pressed down to ensure complete contact with the agar surface.

1. Ordinarily no more than 12 discs are applied on a 150 mm plate or 5 discs on a 100 mm plate, keeping at least a distance of 24 mm between discs. Dispensing too near to the edge of the plate should be avoided. Because some of the drugs diffuse instantaneously, a disc should not be relocated once it has come in contact with the agar surface.
2. It is advisable to place discs that give predictably small zones like aminoglycosides, next to those discs that give larger zones like cephalosporins.
3. Disc containers should be removed from the refrigerator or freezer one to two hrs before use, so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold disks.
4. Only those discs within the manufacturer's expiration date stated on the label will be used.

Incubation. No longer than 15 min after the discs are applied, the plates will be inverted and incubated at 35º ± 2ºC in ambient air.

Interpretation and reporting of results – Each plate will be examined after overnight incubation (16-18 hrs), for confluent growth and circular zones of inhibition. The diameters of the zones of complete inhibition, including the diameter of the disc, will be measured to the nearest whole millimeter with callipers or a ruler. With unsupplemented MHA, the measuring device is held on the back of the inverted petri dish, which is illuminated with reflected light located a few inches above a black, nonreflecting background. Zone margin should be considered the area showing no obvious visible growth detectable with the unaided eye. Faint growth of tiny colonies visible only by lens should be ignored. Zone sizes should be measured from the upper inoculated surface of opaque media like MHA with added blood, illuminated with reflected light, with the cover removed. In case of presence of discrete colonies within the clear zone of inhibition, repeat test with a subculture of a single colony/pure culture from the primary culture plate. With trimethoprim, the sulfonamides, and combinations of the two agents, antagonists in the medium may allow some minimal growth; therefore, the zone diameter should be measured at the obvious margin, and slight growth (20% or less of the lawn of growth) should be disregarded.

The sizes of the zones of inhibition are interpreted by referring to Clinical & Laboratory Standards Institute (CLSI) guidelines and the organisms are reported as either susceptible, intermediate, or resistant to the agents that have been tested. (Refer Tables S1-S3). Use *E. coli*ATCC25922, *P. aeruginosa* ATCC27853, *S. aureus* ATCC25923) for quality control with known susceptibility standards (**Table S4**).

Preparation of 0.5 McFarland Standard (Bailey and Scott, 1994)

Measure 0.05 ml of 1.175% (w/v) solution of barium chloride dehydrate (Bacl 2 2h2O) (HiMedia Laboratories Limited, Mumbai, India) and added slowly with constant agitation in to 9.95 ml of 1% H_2SO_4 (Merck, India Ltd, Mumbai) to make a total volume of10 ml in a 15 ml screw capped test tube. The tube is to be stored in dark at room temperature.

Antibiotic susceptibility testing of *Mycobacterium* spp

1. The Absolute Concentration Method

Procedure

Following is the drug containing media used for the absolute concentration method-

Drugs-

- First line- Isoniazid, Rifampicin, Ethambutol
- Second line- Ofloxacin, Kanamycin, Ethionamide

Inoculum preparation

- With 3 mm internal diameter loop (24 SWG nichrome wire) take 2/3 loop full of a representative sample (approximately 4 mg of moist weight) from the primary culture and place on the side wall of a Bijou bottle containing 0.3 ml double sterile distilled water.
- Vortex the bottle for 20–30 sec.
- Add 0. 7 ml of double sterile distilled water.
- Wait for 10 min.
- With 3 mm external diameter loop (27 SWG nichrome wire) inoculate the whole medium containing the graded concentration of first- and second-line drug from lower to higher concentration.

First line AST: 12 slopes to be inoculated (Isoniazid-3, Rifmapicin-3, Ethambutol-3, plain LJ-2 and PNB-1).

Second line AST: 16 slopes to be inoculated (Ofloxacillin-3, Kanamycin-4, Ethionamide-6, plain LJ-2 and PNB-1).

2. Resistance Ratio (RR) Method

Drug- Streptomycin

Inoculum preparation

- Same as Absolute concentration method (MIC)
- Inoculate only LJ 16 µg/ml for RF samples

3. Proportion method

Drug Concentrations added to LJ medium and critical proportion for Interpretation for economic variant of Proportion Method are:

Drug	Media Concentration
Streptomycin (dihydro-streptomycin sulfate)	4 µg/ml
Isoniazid	0.2 µg/ml
Rifampicin	40 µg/ml
Ethambutol	2 µg/ml

Materials required

1. Drug-free LJ medium bottles -5 per test culture.
2. Drug media- Streptomycin (4 µg/ml), Isoniazid (0.2 µg/ml), Rifampicin (40 µg/ml), Ethambutol (2 µg/ml)- two bottles each for test culture.
3. Calibrated inoculation loops: 3 mm internal diameter loop made with 24 SWG nichrome wire. This delivers 0.01 ml of inoculum. Delivery volume must be verified by weighing 10 loopfuls of distilled water deposited on a filter paper.
4. Bijou bottles (5 per culture).
5. McCartney bottles with 1ml sterile distilled water and six 5mm glass beads, sterile (1 per culture). And empty McCartney bottle, sterile.

6. Bijou bottles with one ml distilled water, sterile (2 per culture).
7. Sterile distilled water – 10 ml per culture.
8. Wire meshes to hold McCartney bottles and bijou bottles.

Preparation of McFarland Nephelometer Barium chloride Standards

- Prepare 1% aqueous barium chloride (100 mg of Barium chloride (anhydrous) in 10 ml of sterile distilled water.
- Prepare 10 ml of 1% sulphuric acid solution (99ml of distilled water and 1 ml of concentrated H_2SO_4).
- Add 0.1 ml of 1% Barium chloride solution to 9.9 ml of 1% H_2SO_4 solution to obtain the McFarland standard, which matches with 1 mg/ml of *M. tuberculosis*.
- Seal the tubes (wrap with parafilm) and label as No. 1 McFarland standard tube with the date of preparation.
- Once prepared, standard can be stored & used for up to 4 months.

Inoculum preparation

1. With a 3 mm wire loop, a representative sample of approximately 4-5 mg (loop full) is taken from the primary culture and placed on the side wall of a McCartney bottle containing 1 ml SDW and 6 glass beads of diameter 3 mm.
2. Emulsify the bacterial inoculum, (with a loop of water, if required), onto the side wall of McCartney bottle in round rotatory movements with inoculation loop, till the bacterial mass is emulsified, (this is visible by a reduction in the clumpy hydrophobic to aqueous hydrophilic nature of suspension).
3. Let the emulsified suspension be fully dissolved in the 1ml of sterile distilled water.
4. Vortex the bottle for 20-30 sec.
5. 4 ml of distilled water is added slowly.
6. Allow the coarse particles to settle down (leave it to stand for approximately 5 min).
7. Decant the *Mycobacterium* solution carefully into another clear, sterile McCartney bottle.

8. Match the opacity/turbidity of inoculum with McFarland standard No.1, against a black background. This is the neat bacterial suspension, standardized at 1 mg/ml, close to the 10^7 to 10^8 CFU/ml. Make sure that no clumps are taken.
9. If required, the opacity of the bacterial suspension is then adjusted by the addition of distilled water to obtain a concentration of 1 mg/ml of tubercle bacilli by matching with McFarland's standard No.1.
10. Make further two log dilutions to achieve 10^2 and 10^4 dilutions as given below-
    a. The dilution 10^2 is produced by discharging two loopfuls of the neat bacterial suspension, into a small tube containing 2 ml of distilled water, and shaking.
    b. Similarly, the dilution 10^4 is produced by discharging two loopfuls of the dilution 10^2 into a small tube containing 2 ml of distilled water, and shaking.

Neat: 1 ml SDW with six 3 mm glass beads + 1 loop-full (3 mm loop) of culture

↓

Vortex for 20 – 30 sec

↓

Add 4 ml of SDW to the above

↓

Adjusted turbidity with McFarland Std. No. 1 with SDW

↓

S2-10^2 two loop-full of neat + 2 ml of SDW, vortex

↓

S2- 10^4 two loop-full of S2 + 2 ml of SDW

Precautions:

- Avoid touching the medium while picking the colonies.
- Cool down the loop sufficiently before picking the colonies.
- Try to take loop-full of colonies in one sweep, by touching all colonies on the LJ slope.
- Avoid touching the water of condensation while scrapping the colonies

Drug susceptibility testing:

Label the medium slopes with lab number of culture, and serially arrange in the wire mesh-

- Heat the loop to red hot (incandescence) in flame for each dilution separately, ensure the loop is cooled by touching the insides of medium slope, before using the loop.
- Inoculate a loop-full (using 3 mm calibrated loop) of each dilution on to media slopes. Should inoculate uniform suspension into all slopes.

Inoculum	**Drugfree medium**	**Strep (4 ug/ml) medium**	**INH (0.2 ug/ml) medium**	**Rif (40 ug/ml) medium**	**ETB (2 ug/ml) medium**	**PNB (500 ug/ml) medium**
Neat (~10^8 CFU/ml)						
10^2						
10^4						
Total bottles						

Interpretation of the test-

- First reading is taken at 28th day after inoculation.
- Count the colonies only on the slopes seeded with the inoculum that have produced exact readable counts or actual counts (up to 100 colonies on the slope). This inoculum may be the same for the control slopes and the drug-containing slopes, or it may be the low

inoculum (10^4 dilution) for the control slopes and the high inoculum (10^2 dilution) for the drug-containing slopes.

- The average number of colonies obtained for the drug-containing slopes indicates the number of resistant bacilli contained in the inoculum.
- Dividing the number of colonies in drug containing slopes by that in drug free slopes gives the proportion of resistant bacilli existing in the strain. Below a certain value – the critical proportion – the strain is classified as sensitive; above that value, it is classified as resistant. The proportions are reported as percentages.
- If, according to the criteria indicated below, the result of the reading made on the 28th day is "resistant", no further reading of the test for that drug is required: the strain is classified as resistant. If the result on the 28th day is "sensitive", a second reading is made on the 42nd day only for the sensitive strain. The final definitive results for all the four drugs should be reported on 42nd day. If the strain is resistant to all the four drugs on the 28th day, then the report can be given on the same day. Otherwise, incomplete reports should not be given before 42nd day.
- In case growth on the control media is poor even after six weeks (i.e., few or no colonies on the 10^{-4} bacterial dilution), the test should be repeated.

Criteria of Resistance

Any strain with 1% (the critical proportion) of bacilli resistant to any of the four drugs – Rifampicin, Isoniazid, Ethambultol, and streptomycin – is classified as resistant to that drug. For calculating the proportion of resistant bacilli, the highest count obtained on the drug free and on the drug-containing medium should be taken (regardless of whether both counts are obtained on the 28th day, both on the 42nd day, or one on the 28th day and the other on the 42nd day).

7. FOODBORNE DISEASE OUTBREAK INVESTIGATION

Outbreak investigation will be done with the following objectives:

- Identification of specific risk factors related to the host, the agent and the environment
- Identification of factors that contributed to the contamination, growth, survival and dissemination of the suspected agent
- Prevention of future outbreaks and strengthening of food safety policies
- Acquisition of epidemiological data for risk assessment of foodborne pathogens

Investigation of a foodborne disease outbreak will include

- Epidemiological
- Environmental and Food investigation
- Laboratory Investigation (Microbiological)

A. Epidemiological:

Investigation of a potential outbreak will start with the assessment of all available information. This assessment will include with

- Checking the validity of the information
- Obtaining reports of applicable laboratory tests that have been performed
- Identifying cases and obtaining information about them
- Ensuring the collection of appropriate clinical specimens and food samples.

Investigation of food establishments

During a foodborne disease outbreak, investigation of a food establishment will be done by interviewing managers; employees, reviewing hygiene and illness of the employees, including, specimens for analysis, food and environmental sampling, assessment of the water system and supply.

Investigation of a suspect food

The complete processing and preparation history will be reviewed, including source and ingredient, persons who handled the specific foods, the procedures and equipment used, potential source of contamination, and time and temperature conditions to which foods were exposed.

B. Food sampling

Food samples collection and testing will include ingredients used to prepare implicated foods, leftover foods from a suspect meal/food from a menu that has been implicated epidemiologically, foods known to be associated with the pathogen in question.

Environmental samples

Samples will be taken from work surfaces, food contact surfaces of equipment, containers, and other surfaces such as refrigerators, door handles, etc. Environmental samples may also include clinical specimens (such as faecal specimens, nasal swabs) from food workers and water used for food processing.

Food-handlers

Stool specimens or rectal swabs, nasal, skin will be collected from food-handlers for laboratory analysis to identify potential carriers or sources of contamination.

C. Laboratory investigations will be done as per protocol

8. QUALITY CONTROL

A. Internal quality control (IQC)

The aim of a quality control program would be to monitor the following:

- Performance of the media and reagents used in the test.
- The precision (repeatability) and accuracy of the susceptibility test procedure
- The performance of persons
- All the participating laboratories would test all media, discs and reagents to be used for the study.
- Sterility: Each batch of medium should be tested for sterility. A plate would be selected at random and incubated at 37ºC for 24-48 hrs. The media should be sterile before inoculation. Presence of surface or subsurface colonies warrants discarding of the media.
- Growth: The ability of the medium to support the growth is determined by inoculating the medium with a typical stock culture isolate. Diluted inoculum is used for this purpose. Appropriate growth is observed and recorded after incubation of the medium.
- Biochemical reactions: Media used for biochemical reactions are tested with strains, which give both positive and negative reactions.
- Negative controls (non-spiked autoclaved distilled water) and positive controls will be incorporated with each set of test samples and subjected to RNA extraction and PCR assays.

B. External quality assessment (EQA)

An EQA ensures approved centre staff members are making proper identification that are consistent with the qualification learning/activity outcomes and assessment criteria. For this purpose, ICMR-National Institute of cholera and Enteric Diseases (NICED), Kolkata will send 5 unknown bacterial strains once in year, following all the biosafety measures. Each participating center has to identify and send the results in a format that will be communicated along with the strains.

9. DATA RECORDING AND DOCUMENTATION

The results shall be recorded on the CRF and logbook. An online or offline mobile based application may be used rapid collection of data during routine surveillance and foodborne outbreak surveillance.

In the ICMR-FoodNet, transfer data to the computer and analytical tool/software.

10. REPORTS AND SENDING OF STRAINS/DNA/RNA

The results from all the centers shall be submitted to RMRC, Dibrugarh on a monthly basis.

The report from all the NE states shall be compiled at ICMR-RMRC, Dibrugarh and sent to ICMR, Delhi for analysis on a quarterly basis.

Bacterial strains and DNA/RNA extracted from samples shall be shared with ICMR-RMRC, Dibrugarh on a monthly basis.

ANNEXURE-1

Tests for identification of *Salmonella* & *Shigella*

Inoculation and Interpretation of Simmons Citrate Agar

Procedure

A small amount of growth is harvested with a sterile (1 µl) loop.

Lightly inoculate the surface of the agar slant.

Do not use a heavy inoculum.

Tubes are incubated under aerobic conditions at 36°C (+/- 1°C) with caps loosened.

Tubes should be examined and results recorded at 24 hrs, 48 hrs, and 3-5 days.

Interpretation/Results/Reporting

Positive - intense blue colour (initially the colour change may only occur on the agar slant)

Negative - agar remains green

Quality Control

Quality control testing is performed with each new lot and shipment of media.

Prior to use, the media are tested for sterility: uninoculated media should have no growth following 48 hrs of incubation at 36°C (+/- 1°C). Each new lot / shipment of media must also produce expected reactions with QC organisms. The following reactions must be observed:

Positive: *Enterobacter aerogenes*

Negative: *Escherichia coli*

Reference: WHO GFN Laboratory Protocol: "Biochemical Identification of *Salmonella* and *Shigella*, Using an Abbreviated Panel of Tests" – version 002; October 2015

Inoculation and Interpretation of Lysine Iron (LIA) Agar

Procedure

A small amount of growth is harvested with a sterile inoculating needle.

Lightly inoculate the surface of the agar slant.

Make a single stab into the butt of the tube.

Tubes are incubated under aerobic conditions at 36°C (+/- 1°C) with caps loosened.

Tubes should be examined and results recorded at 24 hrs, 48 hrs, and 5-7 days (unless H_2S production occurs sooner).

Interpretation/Results/Reporting

A. H_2S production:

Positive - black colour along the streak or throughout the medium

Negative - no black colour

B. Lysine Decarboxylase (LDC):

Decarboxylation of lysine is detected in the butt of the tube.

LDC positive organisms will turn the agar in the butt of the tube purple.

LDC negative organisms will turn the agar in the butt of the tube yellow.

C. Lysine Deamination:

Lysine Deamination is detected on the agar slant.

Lysine deaminase positive organisms will turn the agar slant red.

Lysine deaminase negative organisms will turn the agar slant purple

Quality Control

Quality control testing is performed with each new lot and shipment of media.

Prior to use, the media are tested for sterility: uninoculated media should show no growth following 48 hrs of incubation at 36°C (+/- 1°C). Each new lot / shipment of media must also produce expected reactions with QC organisms. The following reactions must be observed.

Bacteria	**Slant**	**Butt**	**H_2S**
Proteus mirabilis	Red	Yellow	-
Salmonella Typhimurium	Purple	Purple	+
Shigella flexneri	Purple	Yellow	-

Inoculation and Interpretation of Motility-Indol-Ornithine Agar (MIO Agar)

Procedure

A small amount of growth is harvested with an inoculating needle.

Make a single stab into the tube of MIO agar. The stab should be made straight into the agar and stop approximately 1 cm from the bottom of the tube.

Do not make multiple stabs into the agar and do not twist the needle into the media.

Tubes are incubated under aerobic conditions at 36°C (+/- 1°C) with caps loosened.

Tubes should be examined and results recorded following overnight (18-24 hrs) incubation.

Interpretation/Results/Reporting

Motility

Positive: Visible growth extending away from the stab line. Typically the agar will become visibly turbid.

Negative: Growth only along the stab line. The agar remains clear. Isolates which only produce small tufts of growth along the stab line (similar to bristles on a brush) are considered non-motile.

Ornithine Decarboxylase

Positive: The agar in the middle of the tube turns a light, purple colour. These tubes are distinctly purple; however, they will be a lighter shade of purple than their uninoculated counterparts.

Negative: The agar in the middle of the tube turns yellow. Only the colour of the agar in the middle of the tube should be noted. Oxidation may cause the agar on the surface of the tube to turn purple this is not significant.

Quality Control

Quality control testing is performed with each new lot and shipment of media.

Prior to use, the media are tested for sterility: Uninoculated media should show no growth following 48 hrs of incubation at 36°C (+/- 1°C). Each new lot/shipment of media must also produce expected reactions with QC organisms. The following reactions must be observed:

Motile/Ornithine Positive/Indol Positive: *E. coli.*

Non-motile/Ornithine Negative/indol Negative: *Shigella flexneri*

Inoculation and Interpretation of Triple Sugar Iron (TSI) Agar

Procedure

A small amount of growth is harvested with a sterile inoculating needle.

Lightly inoculate the surface of the agar slant.

Make a single stab into the butt of the tube.

Tubes are incubated under aerobic conditions at 36°C (+/- 1°C) with caps loosened.

Tubes should be examined and results recorded at 24 hrs, 48 hrs, and 5-7 days (unless H_2S production occurs sooner).

Interpretation:

A. Carbohydrate fermentation:

Alkaline slant/alkaline butt- no sugars fermented

Alkaline slant/acid butt- only glucose fermented

Acid slant/acid butt- glucose fermented along with lactose and/or sucrose

B. Gas production:

Positive- gas bubbles in agar or splitting of agar

Negative- no bubbles or splitting of agar

C. H_2S production:

Positive - black colour along the streak or throughout the medium

Negative - no black colour

Quality Control

Quality control testing is performed with each new lot and shipment of media.

Prior to use, the media are tested for sterility: uninoculated media should show no growth following 48 hrs of incubation at 36°C (+/- 1°C). Each new lot/shipment of media must also produce expected reactions with QC organisms. The following reactions must be observed:

Bacteria	Slant	Butt	Gas
Enterobacter aerogenes (e.g. strain CDC 659-66)	Acid	Acid	+
Citrobacter freundii	Alkaline	Acid	+
Pseudomonas aeruginosa (e.g. ATCC 27853)	Alkaline	Alkaline	-

Recording Results:

TSI results are recorded using the following notations:

Acidification is indicated with the capital letter "A"

Alkalization is indicated with the capital letter "K"

Gas production is indicated with a lower-case letter "g"

Hydrogen sulphide production is indicated as follows:

"Tr" = Trace amounts of hydrogen sulphide

"+" = Small to moderate amount of hydrogen sulphide

"+++" = Large amounts of hydrogen sulphide

The fermentation reactions on the slant and butt are recorded. The reactions are separated by a diagonal line. The gas production is noted in the subscript and H_2S production is noted in subscript

ANNEXURE-2

Tests for identification of *Campylobacter*

The media and reagents are available from companies like Oxoid, Merck and Difco. Also, the media should be prepared according to the manufacturer's description if it differs from the description given here.

Preston Broth

Composition

Lab-Lemco meat extract	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Sodium pyruvate	0.25 g
Sodium metabisulphite	0.25 g
Ferrous sulphate	0.25 g
Water	1000 ml
Preston selective supplement	
Polymyxin B	5000 i.u.
Trimethroprim	10.0 mg
Rifampicin	10.0 mg
Cycloheximide (instead: Amphotericine-B)	100.0 mg
Lysed horse blood	50 ml

Preparation:

Dissolve the dehydrated medium in the water by heating if necessary. Transfer into a bottle and autoclave at 121°C for 15 min. Allow the media to cool to below 50°C before adding the selective (and growth) supplements and the lysed horse blood as appropriate.

CCD (Charcoal cefoperazone deoxycholate) agar

Composition

Campylobacter Blood-Free Selective Agar Base	45.5 gm
Meat extract	10.0 gm
Enzymatic digest of animal tissues	10.0 gm
Sodium chloride	5.0 gm
Charcoal	4.0 gm
Casein hydrolysate	3.0 gm
Sodium deoxycholate	1.0 gm
Ferrous sulphate	0.25 gm
Sodium pyruvate	0.25 gm
Agar	18.0 gm
Water	1000 ml

2 vials of CCDA Selective Supplement consisting of

Cefoperazone	32 mg
Amphotericin-B	10 mg

Preparation

Dissolve Campylobacter Agar Base in water by heating if necessary. Autoclave at 121^{o}C for 15 min. Add to each of the 2 vials 2 ml of sterile water. Dissolve gently. Add the selective supplement to the 50^{o}C warm Campylobacter Agar Base. Pour plates with about 15-20ml melted medium in each Petri dish.

Columbia-agar

Composition

Columbia agar base	45 gm
Water	1000ml

Preparation

Dissolve the Agar Base in water, and let it stand for 15 min. Boil the solution for 15 min and adjust pH~7.1-7.5. The medium is poured into 1000 ml flasks and autoclaved at 121°C for 15 min.

Gram-staining

Composition & Preparation

Crystal violet

Crystal violet	2.0 gm
Ethanol 95% (vol/vol)	20.0 ml
Ammonium oxalate	0.8 gm
Distilled water	80.0 ml

The crystal violet is first dissolved in the ethanol, then the ammonium oxalate is dissolved in the distilled water. The two solutions are added together. To aid the dissolving process, both mixtures are agitated in a bath of hot water.

Gram's iodine

Iodine crystals	1.0 gm
Potassium iodide	2.0 gm
Distilled water	200 ml

The iodine crystals and the potassium iodine are ground together in a mortar and the distilled water is added slowly. If necessary, the mixture can be agitated in a bath of hot water to aid dissolution.

Destaining

Ethanol 95% (vol/vol)

Carbol fuchsine (counterstain)

Concentrated carbol fuchsine	10.0 ml
Distilled water	90.0 ml

10% (wt/vol) Indoxylacetate solution

Composition & Preparation

Indoxylacetate ($C_{10}H_9NO_2$) 10 gm

Acetone (C_3H_6O) 90 ml

Dissolve the chemical in acetone. Stored at +4°C in a dark bottle.

Oxidase solution

Composition & Preparation

L(+)-Ascorbicacid	0.03 gm
N,N,N′,N′- Tetramethyl-p-Phenylendiamine Dihydrochloride ($C_{10}H_{16}N_2$ • 2HCl)	0.03 gm
Sterile water	30 ml

Dissolve the chemicals in water, and store the solution in a dark bottle at +5°C for 3 weeks.

3.5% Ninhydrin solution

Composition & Preparation

Ninhydrin ($C_9H_6O_4$)	3.5 gm
Acetone (C_3H_6O)	50 ml
Butanol ($C_4H_{10}O$)	50 ml

Dissolve the chemical in the solutions. Stored at + 5°C in dark bottles of 20 ml.

1% Hippurate solution

Composition & Preparation

Natriumhippurat ($C_9H_8NNaO_3$)	1.0 gm
PBS	99 ml

Dissolve the chemical with the solutions. Stored at -20°C in tubes of 15 ml.

Test for catalase

Put a colony at a small spot on a slide. Put one drop of 3%-H_2O_2 on the spot with the bacterial material. Examine immediately for evolution of gas, which indicates catalase activity.

Oxidase test

Add 1% tetra-methyl-p-phenylenediamine dihydrochloride in water. Store refrigerated in a dark bottle no longer than 1 week. Transfer one colony to a filter paper. Soak the filter in an oxidase solution. Appearance of a blue color within 10 sec indicates a positive result.

Test for hippurate hydrolysis

Suspend a loopful of a growth from an 18-24 hrs Columbia agar plate containing 5% cattle blood culture in 400 µl of a 1%-hippurate solution. Incubate at 37 for 2 hrs. Slowly add 200 µl 3.5%-ninhydrin solution to the side of the tube to form an overlay. Re-incubate at 37°C for 10 min, and read the reaction.

Positive reaction: dark purple/blue. Negative reaction: clear or gray.

Hydrolysis of indoxyl acetate

Add 50 µl of a 10% (w/v) solution of indoxyl acetate in acetone to an absorbent paper disc 6 mm in diameter and allow to dry in air. Apply growth from a Campylobacter colony directly to disc and then wet with a drop of sterile distilled water. Appearance of a blue-green color within 5-10 min indicates a positive result.

Candle jar

Purpose:

The candle jar creates an atmosphere with reduced oxygen and elevated levels of carbon dioxide. This condition enhances the growth of microaerophiles.

Principle:

The flame of the candle within a closed environment will use up a certain percentage of the oxygen. When the available oxygen is reduced and elevated carbon dioxide created by the flame is increased, the flame will be extinguished. The plated medium within this atmosphere will show enhanced growth of certain bacteria. The candle jar will usually be incubated at 42°C.

ANNEXURE-3

Tests for identification of *Escherichia coli* O157:H7

Kovacs reagent for indole reaction
Composition and preparation

4-Dimethylaminobenzaldehyde	5 gm
Hydrochloric acid = 1.18 - 1.19 g/ml	25 ml
2-Methylbutan-2-ol	75 ml

Mix the components.

Tryptone/tryptophane medium for indole reaction
Composition and preparation

Tryptone	10 gm
Sodium chloride	5 gm
DL-Tryptophane	1 gm
Water	1000 ml

Dissolve tryptone and chemicals in the water at 100°C. Adjust pH to ~ 7.5 after sterilisation. Dispense 5 ml of medium into tubes and autoclave at 121°C for 15 min.

Indole test Description

When Kovacs reagent containing amyl alcohol and p-dimethylaminobenzaldehyde is added, indole can be extracted into the amyl alcohol layer by shaking a little. Indole and p-dimethylaminobenzaldehyde produces a red/pink colour.

ANNEXURE-4

Tests for identification of vibrios

Alkaline peptone water

Composition and preparation

Peptone	10 gm
Sodium Chloride	20 gm
Water	1000 ml

Add the ingredients in 1 litre of distilled water. Mix well, distribute into final containers and sterilize by autoclaving at 121°C for 15 min.

Thiosulfate-Citrate-Bile Salts-Sucrose agar

Composition and preparation

Proteose peptone	10.0 gm
Yeast extract	5.0 gm
Sodium thiosulphate	10.0 gm
Sodium citrate	10.0 gm
Bile	8.0 gm
Sucrose	20.0 gm
Sodium chloride	10.0 gm
Ferric citrate	1.0 gm
Bromo thymol blue	0.04 gm
Thymol blue	0.04 gm
Agar	15.0 gm
Water	1000 ml
Final pH (at 25°C)	

Suspend 89.08 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Cool to 45-50°C. Mix well and pour into a sterile Petri dish.

ANNEXURE-5

Tests for identification of *Listeria monocytogens*

Buffered *Listeria* Enrichment Broth (BLEB)

Composition and preparation

Media Base

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

Weigh ingredients and dissolve in water. 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

Prepare acriflavin and nalidixic acid supplements as 0.5% (w/v) stock solutions in distilled water. Prepare cycloheximide supplement as 1.0% (w/v) stock solution in 40% (v/v) solution of ethanol in water. Filter-sterilize stock solutions and store at 4°C, protect acriflavin from light.

Aseptically add the 3 selective supplements to enrichment after 4 hrs incubation at 30°C. The final volume per supplement stock solution determined by total volume of BLEB used in enriched sample.

Fraser broth

Composition and preparation

Peptic digest of animal tissue	5.0 gm
Casein enzymic hydrolysate	5.0 gm
Yeast extract	5.0 gm
Meat extract	5.0 gm
Sodium chloride	20.0 gm
Disodium hydrogen phosphate.2H2O	12.0 gm
Potassium dihydrogen phosphate	1.35 gm
Esculin	1.0 gm
Lithium chloride	3.0 gm
Final pH (at 25°C)	7.2±0.2

Suspend 54.92 gm (equivalent weight of dehydrated medium per litre) in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. Cool to 45-50°C and aseptically add rehydrated contents of 1 vial of Fraser Selective Supplement and 2 vials of Fraser Supplement to 1000 ml medium for primary enrichment or 1 vial of each to 500 ml medium for secondary enrichment. Mix well and dispense as desired.

PALCAM agar

Composition and preparation

Basal medium

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

Selective agents

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

To make 500 ml of medium, weigh 34.4 gm basal medium powder (all ingredients except the three selective agents) and suspend in 500 ml distilled water. Sterilize by autoclaving at 121°C for 15 min. Dissolve the selective agent supplement mixture in sterile distilled water at 17.5 mg/ml and filter sterilize. Add 1 ml selective agent supplement solution to 500 ml sterile basal medium that has been cooled to 50°C. Mix gently and pour plates. Final pH, 7.2 ± 0.1.

Trypticase Soy Agar with 0.6% Yeast Extract (TSAYE)

Composition and preparation

Trypticase soy agar	40 gm
Yeast extract	6 gm
Distilled water	1 liter

Weigh ingredients, add water, mix, and autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2. After autoclaving, swirl to disperse molten agar.

Motility Test Medium

Composition and preparation

Beef extract	3 gm
Peptone or gelysate	10 gm
*NaCl	5 gm
Agar	4 gm
Distilled water	1 liter

Heat with agitation and boil 1-2 min to dissolve agar. For use with *Salmonella*, see the instructions listed below. Dispense 8 ml portions into 16 × 150 screw-cap tubes. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2.

ANNEXURE-6

Tests for identification of *Bacillus cereus*

Mannitol-Egg Yolk-Polymyxin (MYP) Agar

Composition & Preparation

Base

Beef extract	1 gm
Peptone	10 gm
Mannitol	10 gm
NaCl	10 gm
Phenol red (1% solution in 95% ethanol)	2.5 ml
Agar	15 gm
Distilled water	900 ml

Heat with agitation to dissolve agar. Adjust pH so that the value after sterilization is 7.2 ± 0.2. Dispense 225 ml portions to 500 ml Erlenmeyer flask. Autoclave 15 min at 121°C. Cool to 50°C. MYP agar is commercially available from Difco.

Polymyxin-B solution, 0.1%

Dissolve 500,000 units polymyxin B sulfate in 50 ml distilled water. Filter-sterilize and store in the dark at 4°C until needed.

Egg yolk emulsion, 50%

Also available from commercial suppliers.

Final medium

To 225 ml melted base add 2.5 ml polymyxin B solution and 12.5 ml egg yolk emulsion. Mix and dispense 18 ml portions to sterile 15 × 100 mm petri dishes. Dry plates at room temperature for 24 hrs before use.

MRVP Broth

Composition& preparation

Buffered peptone	7.0 gm
Glucose	5.0 gm
Dipotassium phosphate	5.0 gm

Dissolve ingredients in water and adjust pH if necessary. Dispense 5 ml portions into 20 × 150 mm tubes. Autoclave 10 min at 121°C. Final pH, 6.5 ± 0.2.

Voges-Proskauer Reagent A: Barritt's reagent A

Alpha-Naphthol, 5%	50 ml
Absolute Ethanol	1000 ml

Voges-Proskauer Reagent A: Barritt's reagent B

Potassium Hydroxide	400 gm
Deionized Water	1000 ml

Phenol Red Glucose Broth

Composition & Preparation

Proteose peptone No. 3	10 gm
NaCl	5 gm
Beef extract (optional)	1 gm
Dextrose	5 gm
Phenol red (7.2 ml of 0.25% solution)	0.018 gm
Distilled water	1 liter

Dispense 2.5 ml portions into 13 × 100 mm tubes. Autoclave 10 min at 118°C.Final pH, 7.4 ± 0.2.

Nitrite Detection Reagents

Composition & Preparation

A. Sulfanilic acid reagent

Sulfanilic acid	1 gm
5 N acetic acid	125 ml

B. N-(l-naphthyl) ethylenediamine reagent

N-(l-naphthyl) ethylenediamine dihydrochloride	0.25 gm
5 N acetic acid	200 ml

C. α-Naphthol reagent

α-Naphthol reagent	1 gm
5 N acetic acid	200 ml

To prepare 5 N acetic acid, add 28.75 ml glacial acetic acid to 71.25 ml distilled water.
Store reagents in glass-stoppered brown bottles.

Motility Medium (for *Bacillus cereus*)

Composition & Preparation

Trypticase	10 gm
Yeast extract	2.5 gm
Dextrose	5 gm
Na_2HPO_4	2.5 gm
Agar	3 gm
Distilled water	1 liter

Heat with agitation to dissolve agar. Dispense 100 ml portions to 170 ml bottles. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Cool to 50°C. Aseptically dispense 2 ml portions to sterile tubes. Store at room temperature 2 days before use.

Nitrate Broth

Composition & Preparation

Beef extract	3 gm
Peptone	5 gm
KNO_3 (nitrite-free)	1 gm
Distilled water	1 liter

Dissolve ingredients. Dispense 5 ml portions into tubes. Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2.

Lysozyme Broth

Composition & Preparation

Prepare nutrient broth as recommended. Dispense 99 ml portions to 170 ml bottles. Autoclave 15 min at 121°C. Cool to room temperature before use.

Lysozyme solution

Dissolve 0.1 gm lysozyme in 65 ml sterile 0.0l N HCl. Heat to boiling for 20 min. Dilute to 100 ml with sterile 0.0l N HCl. Alternatively, dissolve 0.1 g lysozyme in 100 ml distilled water. Sterilize by filtration through 0.45 µm membrane. Test for sterility before use. Add 1 ml lysozyme solution to 99 ml nutrient broth. Mix and dispense 2.5 ml portions to sterile 13 × 100 mm tubes.

Tyrosine Agar

Composition & Preparation

Base

Prepare Nutrient agar. Dispense 100 ml portions into 170 ml bottles. Autoclave 15 min at 121°C. Cool to 48°C.

Tyrosine suspension

Suspend 0.5 gm L-tyrosine in 10 ml distilled water in 20 × 150 mm culture tube. Mix thoroughly with Vortex mixer. Autoclave 15 min at 121°C.

Final medium

Combine 100 ml base with sterile tyrosine suspension. Mix thoroughly by gently inverting bottle 2 or 3 times. Aseptically dispense 3.5 ml into 13 × 100 mm tubes with frequent mixing. Slant tubes and cool rapidly to prevent separation of tyrosine.

Biochemical tests for *Bacillus cereus*

1. **Acid from glucose**: Inoculate 3 ml broth with 2 mm loopful of culture. Incubate tubes anaerobically 24 hrs at 35°C (GasPak anaerobic jar). Shake tubes vigorously and observe for growth as indicated by increased turbidity and color change from red to yellow, which indicates that acid has been produced anaerobically from glucose.

2. **Nitrate reduction:** Inoculate 5 ml broth with 3 mm loopful of culture. Incubate tubes 24 hrs at 35°C. To test for nitrite, add 0.25 ml each of nitrite test reagents A and C to each culture. An orange color, which develops within 10 min, indicates that nitrate has been reduced to nitrite.
3. **VP test:** Inoculate 5 ml medium with 3 mm loopful of culture and incubate tubes 48 ± 2 hrs at 35°C. Test for production of acetylmethyl-carbinol by pipetting 1 ml culture into a tube and adding 0.6 ml alpha-naphthol solution and 0.2 ml 40% potassium hydroxide. Shake, and add a few crystals of creatine. Observe results after holding for 1 h at room temperature. The test is positive if pink or violet color develops.
4. **Tyrosine decomposition:** Inoculate entire surface of tyrosine agar slant with 3 mm loopful of culture. Incubate slants 48 hrs at 35°C. Observe for clearing of medium near growth, which indicates that tyrosine has been decomposed. Examine negative slants for obvious signs of growth, and incubate for a total of 7 days before considering as negative.
5. **Growth in Lysozyme broth**. Inoculate 2.5 ml of nutrient broth containing 0.001% lysozyme with 2 mm loopful of culture. Also inoculate 2.5 ml of plain nutrient broth as positive control. Incubate tubes 24 hrs at 35°C. Examine for growth in lysozyme broth and in nutrient broth control. Incubate negative tubes for additional 24 hrs before discarding.

ANNEXURE-7

Tests for identification of *Staphylococcus aureus*

Baird-Parker Medium, pH 7.0

Composition and preparation

Basal medium

Tryptone	10 gm
Beef extract	5 gm
Yeast extract	1 gm
Sodium pyruvate	10 gm
Glycine	12 gm
Lithium chloride·$6H_2O$	5 gm
Agar	20 gm

Autoclave 15 min at 121°C.Final pH, 7.0 ± 0.2. If desired for immediate use, maintain the melted medium at 48-50°C before adding enrichment. Otherwise, store solidified medium at 4 ± 1°C up to 1 month. Melt medium before use.

Enrichment

Bacto EY tellurite enrichment.

Complete medium

Aseptically add 5 ml prewarmed (45-50°C) Bacto EY tellurite enrichment to 95 ml melted base. Mix well (avoiding bubbles) and pour 15-18 ml portions into sterile 15 × 100 mm petri dishes. The medium must be densely opaque. Dry plates before use. Store prepared plates at 20-25°C for up to 5 days.

Trypticase (Tryptic) Soy Agar

Composition and preparation

Trypticase peptone	15 gm
Phytone peptone	5 gm
NaCl	5 gm

Agar	15 gm
Distilled water	1 liter

Heat with agitation to dissolve agar. Boil 1 min. Dispense into suitable tubes or flasks. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

Brain Heart Infusion (BHI) Broth

Calf brain, infusion from	200 gm
Beef heart, infusion from	250 gm
Proteose peptone (Difco) or polypeptone (Bioquest)	10 gm
NaCl	5 gm
Na_2HPO_4	2.5 gm
Dextrose	2.0 gm
Distilled water	1 liter

Dissolve ingredients in distilled water with gentle heat.
Dispense broth into bottles or tubes for storage. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2.
Commercially available BHI is acceptable.

To prepare brain heart infusion agar, add 15 gm of agar to 1 liter BHI broth. Heat to dissolve agar before dispensing into bottles or flasks. Autoclave 15 min at 121°C.

ANNEXURE-8

Tests for identification of *Yersinia enterocolitica*

CIN agar

Composition and preparation

Basal Medium

Special peptone	20.0 gm
Yeast extract	2.0 gm
Mannitol	20.0 gm
Pyruvic acid (Na salt)	2.0 gm
NaCl	1.0 gm
$MgSO_4 \cdot 7H_2O$ (10 mg/ml)	1.0 ml
Agar	12.0 gm
Distilled water	756 ml

Irgasan (Ciba-Geigy) solution

May be stored at -20°C up to 4 weeks.

Desoxycholate solution

Sodium desoxycholate	0.5 gm
Distilled water	200 ml

Bring to boil with stirring; cool to 50-55°C.

A. Sodium hydroxide, [5 N] 1 ml
B. Neutral red, [3 mg/ml] 10 ml
C. Crystal violet, [0.1 mg/ml] 10 ml
D. Cefsulodin (Abbott Labs), [1.5 mg/ml] 10 ml
E. Novobiocin, [0.25 mg/ml] 10 ml
F. Strontium chloride, [10%; filter-sterilized] 10 ml

May be stored at -70°C. Thaw to room temperature just before use.

Preparation: Add ingredients for basal medium to water and bring to boil with stirring. Cool to about 80°C (10 min in 50°C water bath). Add Irgasan solution and mix well. Cool to 50-55°C. Add solution desoxycholate; solution should remain clear. Add solutions A through E. Slowly add solution F with stirring. Adjust pH to 7.4 with 5 N NaOH. Dispense 15-20 ml into each petri dish. Commercially prepared dehydrated Yersinia selective agar (Difco) with supplements may be substituted. Follow manufacturer's instructions for preparation.

Peptone Sorbitol Bile Broth

Composition and preparation

Na_2HPO_4	8.23 gm
$NaH_2PO_4 \cdot H_2O$	1.2 gm
Bile salts No. 3	1.5 gm
NaCl	5.0 gm
Sorbitol	10.0 gm
Peptone	5.0 gm
Distilled water	1 liter

Dispense 100 ml into Wheaton bottles. Autoclave 15 min at 121°C.Final pH, 7.6 ± 0.2.

Bile Esculin Agar

Composition and preparation

Beef extract	3.0 gm
Peptone	5.0 gm
Esculin	1.0 gm
Oxgall	40.0 gm
Ferric citrate	0.5 gm

Agar	15.0 gm
Distilled water	1 liter

Heat with agitation to dissolve. Dispense into tubes, autoclave 15 min at 121°C, and slant until solidified. Final pH, 6.6 ± 0.2.

Christensen's Urea Agar

Composition and preparation

Base

Peptone	1.0 gm
NaCl	5.0 gm
Dextrose	1.0 gm
KH_2PO_4	2.0 gm
Phenol red (6 ml of 1:500 solution)	0.012 gm
Agar	15.0 gm
Distilled water	900 ml

Dissolve all ingredients except urea in 900 ml water (basal medium). For halophilic *Vibrio* spp., add extra 15 gm NaCl (final NaCl concentration, 2%). Autoclave 15 min at 121°C. Cool to 50-55°C.

Urea concentrate

Urea	20.0 gm
Distilled water	100 ml

Filter-sterilize; add aseptically to cooled basal medium. Mix. Final pH, 6.8 ± 0.1. Dispense to sterile tubes or petri dishes. Slant tubes for 2 cm butt and 3 cm slant.

Anaerobic Egg Yolk Agar

Composition and preparation

Agar base

Yeast extract	5.0 gm
Tryptone	5.0 gm
Proteose peptone	20.0 gm
NaCl	5.0 gm
Agar	20.0 gm
Distilled water	1 liter

Autoclave 15 min at 121°C. Adjust pH to 7.0 ± 0.2.

2 Fresh eggs: Treatment of eggs
Wash 2 fresh eggs with stiff brush and drain. Soak eggs in 70% ethanol for 1 h. Crack eggs aseptically. Retain yolks. Drain contents of yolk sacs into sterile stoppered graduate and discard sacs. Add yolk to an equal volume of sterile 0.85% saline. Invert graduate several times to mix. Egg yolk emulsion (50%) is available commercially.

Preparation of medium

To 1 liter melted medium (48-50°C) add 80 ml yolk-saline mixture (available from Difco as Bacto Egg Yolk Enrichment 50%), and mix. Pour plates immediately. After solidification dry 2-3 days at ambient temperature or at 35°C for 24 hrs. Check plates for contamination before use. After drying, plates may be stored for a short period in the refrigerator.

Lysine Arginine Iron Agar

Peptone	5.0 gm
Yeast extract	3.0 gm
Glucose	1.0 gm
L-Lysine	10.0 gm
L-Arginine	10.0 gm

Ferric ammonium citrate	0.5 gm
Sodium thiosulfate	0.04 gm
Bromcresol purple	0.02 gm
Agar	15.0 gm

Adjust pH to 6.8. Heat to boiling and dispense 5 ml into each 13 × 100 mm screw-cap culture tube. Autoclave at 121°C for 12 min. Cool tubes in slanted position. (This medium may also be prepared by supplementing Difco lysine iron agar (LIA) with 10 gm L-arginine per liter).

Pyrazinamidase Agar

Tryptic soy agar	30.0 gm
Yeast extract	3.0 gm
Pyrazine-carboxamide	1.0 gm
0.2 M Tris-maleate, pH 6.0	1 liter

Heat to boiling; dispense 5 ml in 16 × 125 mm tubes. Autoclave at 121°C for 15 min. Cool slanted.

ANNEXURE-9

Long-term cold storage of bacterial strains

Materials

- Cryolabel and a permanent marker
- Log phase liquid bacterial culture (culture grown for about 8–12 hrs)
- Micropipette and sterile tips
- Sterile cryovial with screw-cap
- Sterile glycerol (autoclaved)
- DMSO
- Cryo-box

Procedure

1. Grow the bacterial strains to log phase culture (~8 hrs) in 1.5 ml of Luria-Bertani (LB) broth or Tryptic Soy broth at 37°C.
2. Using the cryolabel, mark a sterile 1.5 ml cryovial with screw-cap tube with the date and strain number
3. Using a micropipette, add 150 µl of sterile glycerol to the tube.
4. Using a new tip transfer 850 µl of the bacterial culture to the same cryovial.
5. Cap the cryovial and mix well by inverting it several times. It is very important that glycerol should be mixed well the bacterial culture before freezing.
6. Arrange the bacterial strains in a cryobox and store in a -80°C freezer.
7. To recover bacterial strains from the glycerol stock, open the cryovial and use a sterile loop or pipette tip to scrape some of the frozen bacteria from the top. Streak the bacterial strain onto an LB agar or blood agar plate.
8. Place the strains back in the freezer. Do not let the glycerol stock unthaw, as frequent freeze and thaw cycles reduce shelf life of strains. Placing the glycerol stock on ice bath while streaking onto agar plates will prevent it from thawing completely and will improve the storage life. Do not to freeze/thaw your glycerol stock too many times.
9. For long term storage of *Leptospira* spp, store the fresh cultures in liquid nitrogen with 5% either glycerol or 5% DMSO.

ANNEXURE-10

List of control strains

Bacillus cereus ATCC 14579 or M15149
Brevibacillus laterosporus ATCC 64
Campylobacter jejuni ATCC 29428
C. coli ATCC 33559
C. lari ATCC 35221
Clostridioides difficile IDH-8564
Escherichia coil CCUG 29889
Enteroaggregative *Escherichia coli* (EAEC) IDH-4832
Enteropathogenic *Escherichia coli* (EPEC) MICRO-0052
Enteroinvasive *Escherichia coli* (EIEC) IDH-10275
Escherichia coli O157:H7 ATCC 35150 (EHEC) ATCC 35150
Escherichia coil ATCC 25922 (for AMR testing)
Pseudomonas aeruginosa ATCC 27853 and ATCC 27853
Listeria monocytogens ATCC 35152
Salmonella enterica Typhimurium ATCC 14028
Shigella flexneri ATCC 12022
S. sonnei NK4010
S. boydii NK 2379
Staphylococcus aureus ATCC 25923 or ATCC 43300
Vibrio cholerae O1 Ogawa O395
V. cholerae O1 Inaba N16961
V. cholerae O139 MO10
Vibrio parahaemolyticus ATCC 17802
Yersinia enterocolitica ATCC 23715
Brucella melitensis
Mycobacterium bovis
Leptospira interrogans
Leptospira biflexa
Leptospira krischneri

ANNEXURE-11

Over view of bacterial culture media and other tests for characterization

Bacteria	Enrichment	Culture medium	Biochemical tests with media requirement	Serology (antisera)	Remarks
General medium		Nutrient agar Cary Blair and Stuart's transportation media			
Salmonella spp.	Rappaport-Vassiliadis (RV) medium	Xylose lysine desoxycholate (XLD) agar Hektoen Enteric (HE) agar MacConkey (MAC) Agar	Triple Sugar-Iron (TSI) agar Simmons Citrate agar Christensen urea agar Indole production Urease LIA, MIO tests	O and H polyvalent antisera	Serotyping will be done at the NICED
Shigella spp.	Selenite-F broth	MAC agar XLD agar HE agar	TSI agar	Species-specific antisera	Serotyping will be done at the NICED
Campylobacter spp.	Preston broth	Charcoal cefoperazone deoxycholate agar Columbia agar plates with 5% sheep blood	Oxidase, nitrate reduction, indole, urease methyl red Voges-Proskauer and Hippurate tests		Species confirmation by PCR assay

Escherichia coli		Cefixime Tellurite Sorbitol MacConkey agar MAC agar		Serology with O157:H7 antiserum	Serotyping will be done at the NICED
Vibrio cholerae *Vibrio parahaemolyticus*	Alkaline peptone water (pH 8.0)	Thiosulfate-citrate-bile salts-sucrose (TCBS) agar	Oxidase test	Serology with O1 and O139 antisera	Serotyping of *V. parahaemolyticus* will be done at the NICED
Listeria spp.	Fraser broth	Polymyxin-Acriflavin-Lithium chloride-Ceftazidime-Aesculin-Mannitol (PALCAM) Agar Tryptone Soya Yeast Extract (TSYEA) agar Brain-Heart Infusion broth	Catalase, oxidase, haemolytic, Christie-Atkinson-Munch-Peterson (CAMP) and motility tests		Confirm virulence-associated genes by PCR
Bacillus cereus		Mannitol Egg Yolk Polymyxin (MYP) agar	VP, tyrosine, nitrate reduction, lysozyme growth tests		Confirm virulence-associated gene by PCR
Clostridium perfringens	Robertson's cooked-meat broth with gentamicin	Blood agar with gentamicin			Confirm virulence-associated gene by PCR

Clostridium botulinum	Cooked meat medium Tripticasopeptone glucose broth (TPGY)	Liver- veal-egg yolk agar Anaerobic egg yolk agar			Confirm virulence-associated genes by PCR
Staphylococcus aureus	Peptone water	Baird Parker Agar (BPA)	Catalase, use of glucose & mannitol, lysotaphine and coagulase tests		Confirm enterotoxins A and B genes by PCR
Yersinia enterocolitica	Peptone Sorbitol Bile Broth (PSBB)	MAC agar, Cefsulodin-Irgasan-Novobiocin (CIN) agar	VP, lipase, esculinase, β-D glucosidase, pyrazinamidase tests, fermentation of salicin, xylose, and trehalose		
Leptospira spp.	EMJH broth with 5FU	EMJH base medium + fetal bovine serum + sodium pyruvate + superoxide dismutase			Confirm pathogenic subspecies by multiplex PCR
M. bovis		Lowenstein-Jensen (LJ) medium containing pyruvate or glycerol	Microscopy after Ziehl-Neelsen staining Niacin test Catalase test		Species confirmation by PCR
Brucella spp.		Farrle's agar *Brucella* agar	Microscopy after Ziehl-Neelsen staining Milk ring test Coombs antiglobulin agglutination test Rose Bengal plate test	Serum agglutination test	Generic PCR assay

HAV and HEV	Viral concentration from water samples by Skim milk ppt	Dulbecco's Modified Eagle Medium (DMEM)			RT-PCR
Antibiotic susceptibility test		Mueller Hinton (MH) broth and MH agar (MHA)			

ANNEXURE-12

Equipment & Standards

Anaerobic jar
Autoclave
Electronic digital balance
Biosafety cabinet-BSL-2
Bunsen burner
Centrifuge
CO_2 incubator
Electrophoresis unit
Freezer (-80°C)
Homogenizer
Incubator at 37°C and 41.5°C
Incubator (10°C)
Microwave
Nanodrop spectrophotometer
PCR Thermocycler
PFGE
Pipettes
Refrigerator (4°C)
Real-time PCR
Separation funnel (1 lit) with stopcock
UV-transilluminator or gel doc
Vacuum pump and filtration assembly (Millipore)
Water bath (50°C)

ANNEXURE-13

Abbreviations

ABA	Anaerobic blood agar
ABTS buffer	2,2′-azino-di-3-ethylbenzthiazoline sulfonic acid buffer
AEYA	Anaerobic egg yolk agar
APW	Alkaline peptone water
AU	Analytical Unit
BA	Blood Agar
BHI	Brain heart infusion
BLEB	Buffered *Listeria* Enrichment Broth
BPA	Baird Parker Agar
BPB	Buffered peptone broth
CA	Columbia agar
CCD	Charcoal cefoperazone deoxycholate
CCFA	Cycloserine-cefoxitin-fructose agar
CFT	Complement fixation test
CIN	Cefsulodin-Irgasan-Novobiocin
CT-SMAC	Cefixime Tellurite Sorbitol MacConkey agar
CPC	Cetyl pyridinium chloride
dNTPs	Deoxynucleoside triphosphates
DMSO	Di-methyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMJH	Ellinghausen–McCullough–Johnson–Harris medium
FB	Fraser Broth
5FU	5-Fluorouracil
HE	Hektoen Enteric
LIA	Lysine Iron Agar
LPS	Lipopolysaccharide
LJ medium	Lowenstein-Jensen medium
LVEYA	Liver Veal egg yolk agar
MAC	MacConkey agar
MAT	Microscopic agglutination test
MIO	Motility-Indol-Ornithine Agar
MYP	Mannitol-Egg Yolk-Polymyxin
NA	Nutrient Agar
NB	Nutrient Broth
PALCAM	Polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol agar
PB	Preston Broth
PEG	Poly-ethylene glycol
PSBB	Peptone Sorbitol Bile Broth

RBT	Rose Bengal plate test
RCM	Robertsons cooked meat broth
RL	Ringers lactate
SCA	Simmons Citrate Agar
SFB	Selenite-F broth
SMAC	Sorbitol-MacConkey agar
SU	Sampling Unit
TCBS	Thiosulfate-citrate-bile-sucrose agar
TBE buffer	Tris-borate-ethylenediaminetetraacetic acid buffer
TE buffer	Tris- ethylenediaminetetraacetic acid buffer
TPGY	Trypticase-Peptone-Glucose-Yeast Extract Broth
TSA	Trypticase (Tryptic) Soy Agar
TSAYE	Trypticase Soy Agar with 0.6% Yeast Extract
TSI	Triple Sugar Iron
TW	Tryptone Water
UBEA	Urease, bile esculin agar
XLD	Xylose lysine desoxycholate

ANNEXURE-14

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Table. S1. List of antimicrobials for susceptibility testing of foodborne pathogens

Antimicrobial	**Pathogen**													
	Salmonella	***Shigella***	***Campylobacter***	***C. difficile***	**DEC**	***Vibrio cholerae***	***Vibrio parahaemolyticus***	***Listeria monocytogens***	***Bacillus cereus***	***Staphylococcus aureus***	***Yersinia enterocolitica***	***Leptospira* spp**	***Mycobacterium bovis***	***Brucella spp***
Gentamicin	X	X	X		X			X	X	X	X			X
Chloramphenicol	X	X			X	X	X	X	X	X	X	X		
Imipenem	X	X			X	X	X	X	X		X			
Meropenem	X	X			X	X	X				X			
Cefoxitin	X	X			X					X	X			
Cefatoxime	X	X			X				X		X	X		
Cefepime	X	X			X					X	X			
Ceftazidime	X	X			X				X	X	X			
Ceftriaxone	X	X			X						X	X		
Colistin	X	X			X						X			
Azithromycin	X	X			X	X	X			X	X			
Ampicillin	X	X	X		X	X	X	X	X		X			
Ciprofloxacin	X	X	X		X	X	X	X		X	X	X		
Nalidixic acid	X	X	X		X	X	X	X			X			
Trimethoprim-sulfamethoxazole	X	X			X	X	X	X		X	X	X		X
Tetracycline	X	X	X		X	X	X	X		X	X	X		X
Streptomycin			X			X	X						X	X
Erythromycin			X					X	X	X		X		
Vancomycin				X					X	X				
Clindamycin			X	X					X	X				
Linezolid									X	X				
Penicillin								X		X		X		
Rifampicin								X	X	X			X	
Cefuroxime								X						
Isoniazid													X	
Ethambutol													X	
Pyrazinamide													X	
Kanamycin													X	
Levofloxacin													X	
Ofloxacin													X	
Metronidazole				X										

For *Salmonella* spp. and *Shigella* spp., aminoglycosides, first- and second-generation cephalosporins, and cephamycins may appear active *in vitro* but are not effective clinically and should not be reported as susceptible. **X** indicates the antibiotics to be tested for the corresponding pathogen.

Table S2. Zone diameter (in mm) and MIC breakpoints for Enterobacterales and *Campylobacter*

Antimicrobial (Disk content in µg)	**Enterobacterales**			***Campylobacter*$**		
	S	I	R	S	I	R
Gentamicin (10)	≥15	13-14	≤12			
Chloramphenicol (30)	≥18	13-17	≤12			
Imipenem (10)	>23	20-22	<19			
Meropenem (10)	>23	20-22	<19			
Cefoxitin (30)	≥18	15–17	≤14			
Cefatoxime (30)	≥26	23–25	≤22			
Cefepime (30)	≥25	19-24	<18			
Ceftazidime	>21	18-20	<17			
Ceftriaxone (30)	≥23	20–22	≤19			
Azithromycin (15)	≥13 ≥16*	- 11-15*	≤ 12 ≤8*			
Ampicillin (10)	≥ 17	14-16	≤13			
Ciprofloxacin (5)	>26 ≥31#	20-25 21-30#	<21 <30#	>24	21-23	≤20
Nalidixic acid (30)	≥19	14–18	≤13			
Trimethoprim-sulfamethoxazole (1.25/23.75)	≥ 16	11-15	≤10			
Tetracycline (30)	≥15	12-14	≤11	≥26	23-25	≤22
Streptomycin (10)	≥15	12-14	≤11			
Erythromycin (15)				>16	13-15	<12

*for *Shigella* spp only; # for *Salmonella* spp; $Modified disk diffusion incubation conditions 42°C for 24 hrs.

Table S3. Zone diameter (in mm) and MIC breakpoints (MIC µg/ml) for non-Enterobacterales

Antimicrobial (Disk content in µg)	***Vibrio* spp**			***Listeria monocytogens***			***Bacillus cereus***			***Staphylococcus aureus****		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Gentamicin (10)	≥15	13-14	≤12				≤4	8	≥16	≥15	13-14	≤12
Chloramphenicol (30)	≥18	13-17	≤12				≤8	16	≥32	≥18	13-17	≤12
Imipenem (10)	≥23	20-22	≤19				≤4	8	≥16			
Meropenem (10)	≥23	20-22	≤19	≤0.25			≤4	8	≥16			
Cefoxitin (30)	≥18	15-17	≤14							≥22		≤21
Cefatoxime (30)	≥26	23-25	≤22									
Cefepime (30)	≥25	19-24	≤18									
Ceftazidime (30)	≥21	18-20	≤17									
Azithromycin (15)										≥18	14-17	≤13
Ampicillin (10)	≥10	14-16	≤13	≤2			≤0.25		≥0.5			
Ciprofloxacin (5)	≥21	16-20	≤15				≤1	2	≥4	≥21	16-20	≤15
Nalidixic acid												
Trimethoprim-sulfamethoxazole (1.25/23.75)	≥16	11-15	≤10	≤0.5/9.5			≤2/38		≥4/76	≥16	11-15	≤10
Tetracycline (30)	≥15	13-14	≤11				≤4	8	≥16	≥19	15-18	≤14
Erythromycin (15)							≤0.5	1-4	≥8	≥23	14-22	≤13
Vancomycin							≤4					
Clindamycin (2)							≤0.5	1-2	≥4	≥21	15-20	≤14
Methicillin/Oxacillin										≥2	-	≤4
Penicillin				≤2			≤0.12		≥0.25			
Rifampicin (5)							≤1	2	≥4	≥20	17-19	≤16
Streptomycin**												

*Vancomycin MIC µg /ml: ≥2, 4-8 and ≥16 for S, I and R, respectively

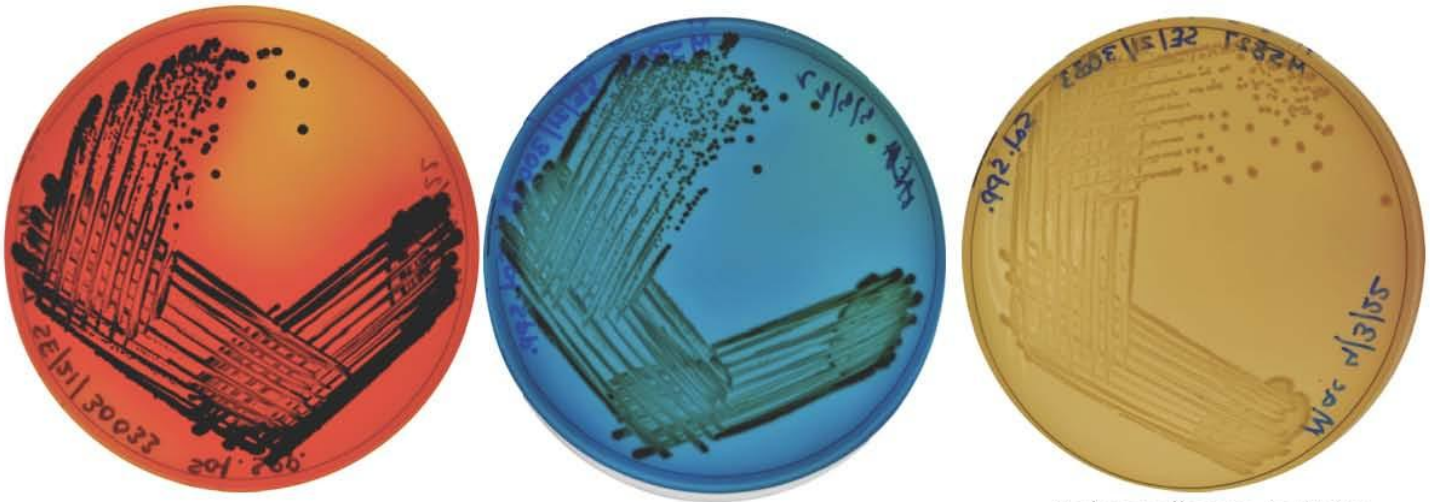
**The streptomycin-susceptible breakpoint for *Brucella* spp is ≤ 16 MIC µg/ml when the test is incubated in CO_2 and ≤ 8 MIC µg/ml when incubated in air

ICMR Delhi, Regional Medical Research Centre, Dibrugarh& National Institute of Cholera and Enteric Diseases, Kolkata	Standard operating Procedure (SOP)	SOP No	03
		Revision No	5
		Date of issue	14.05.2024
	"Surveillance of Food Borne Pathogens (FBP) from North-East India"	Prepared by	MD, UD, PJB
		Reviewed by	TRM
		Approved by	IK, RC

Table S4. Disk diffusion QC ranges for non-fastidious organisms and antimicrobial agents

Antimicrobial (Disk content in µg)	**Zone diameter (in mm) of QC Strain**		
	***Escherichia coli* ATCC25922**	***Pseudomonas aeruginosa* ATCC27853**	***Staphylococcus aureus* ATCC25923**
Ampicillin (10)	15-20	-	27-35
Azithromycin (15)	-	-	21-26
Cefotoxime (30)	29-35	18-22	25-31
Cefepime (30)	31-37	25-31	23-29
Cefoxitin (30)	23-29	-	23-29
Ceftazidime (30)	25-32	22-29	16-20
Ceftriaxone (30)	29-35	17-23	22-28
Cefuroxime (30)	20-26	-	27-35
Chloramphenicol (30)	21-27	-	19-26
Ciprofloxacin (5)	29-38	25-33	22-30
Clindamycin (15)	-	-	26-32
Erythromycin (15)	-	-	22-30
Gentamicin (10)	19-26	17-23	19-27
Imipenem (10)	26-32	20-28	-
Linezolid (30)	-	-	25-32
Meropenem (10)	28-35	27-33	29-37
Nalidixic acid (30)	22-28	-	-
Penicillin (10)	-	-	26-37
Rifampicin (5)	8-10	-	26-34
Streptomycin (10)	12-20	-	14-22
Tetracycline (30)	18-25	-	24-30
Trimethoprim-sulfamethoxazole (1.25/23.75)	23-29	-	24-32
Vancomycin (30)	-	-	17-21

Supplement Figures



Salmonella spp. in XLD | *Salmonella* spp. in HEA | *Salmonella* spp. in MAC

Fig. S1a. *Salmonella enterica* colony morphology in different selective media

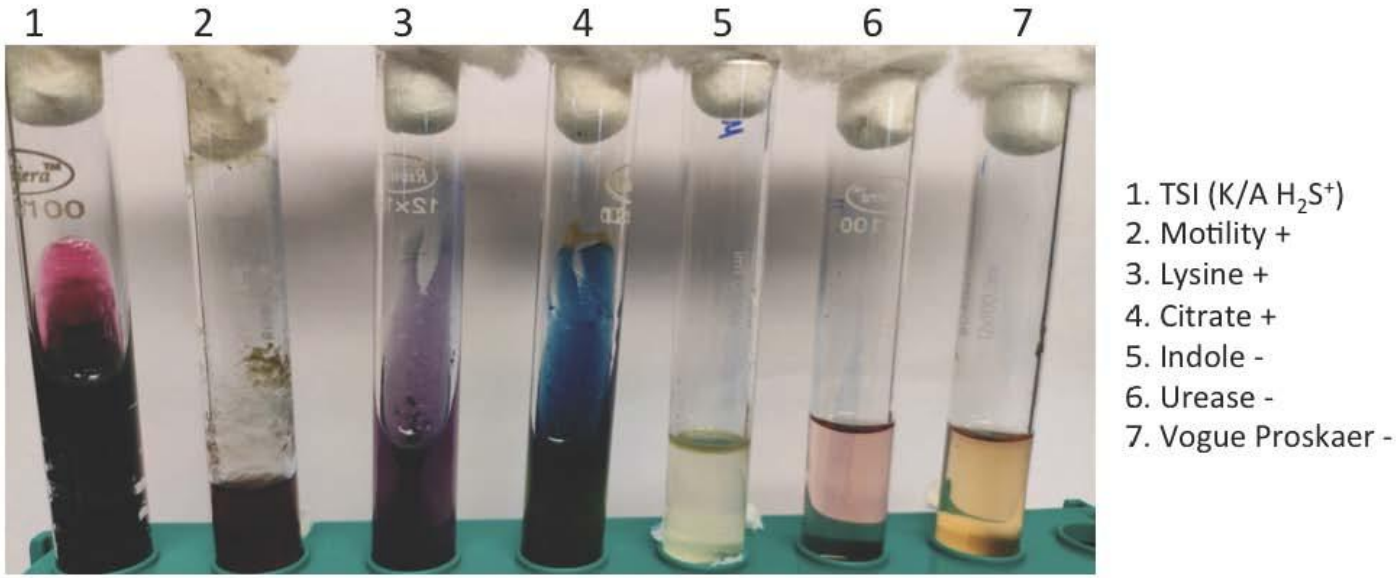
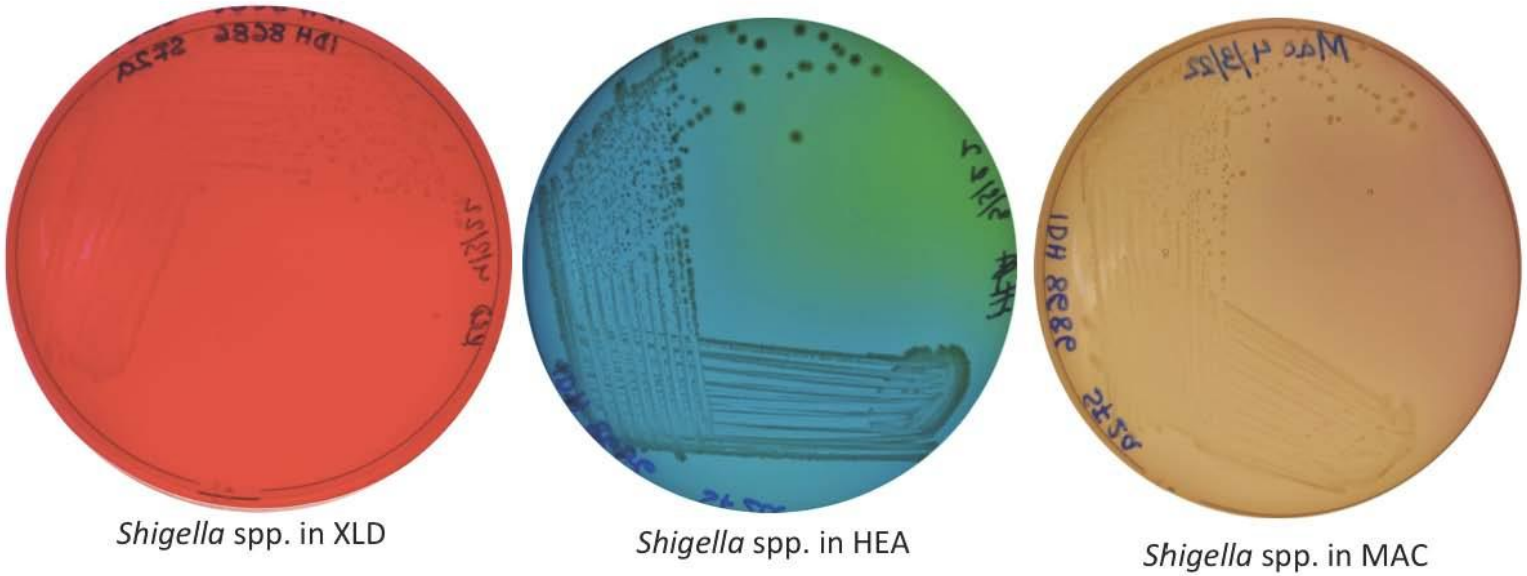


Fig. S1b. *Salmonella enterica* biochemical test results

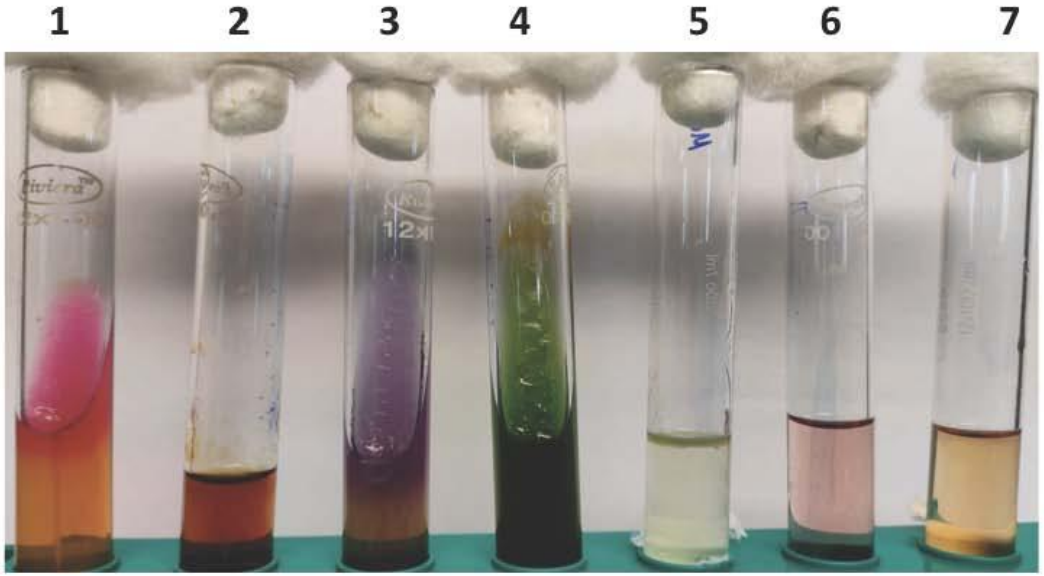


Shigella spp. in XLD

Shigella spp. in HEA

Shigella spp. in MAC

Fig. S2a. *Shigella* spp. colony morphology in different selective media



1. TSI K/A
2. Motility -
3. Lysine -
4. Citrate -
5. Indole -
6. Urease -
7. Vogue Proskaer -

Fig. S2b. *Shigella* spp. biochemical test results

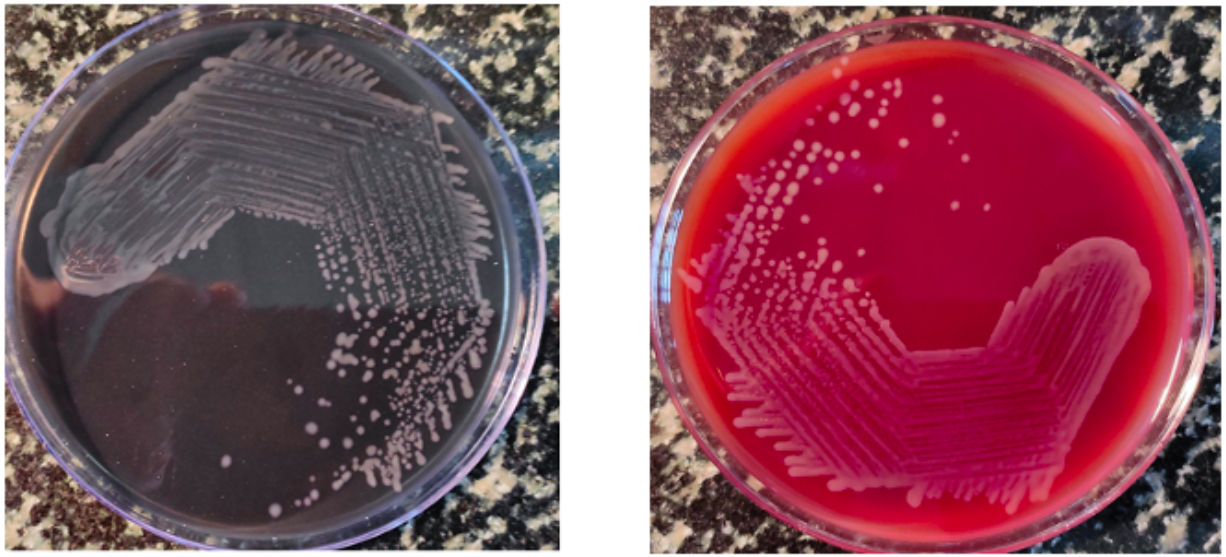


Fig. S3a. *Campylobacter* spp. in charcoal agar and blood agar

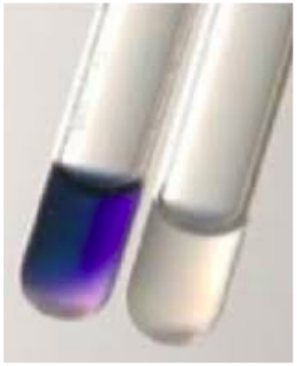
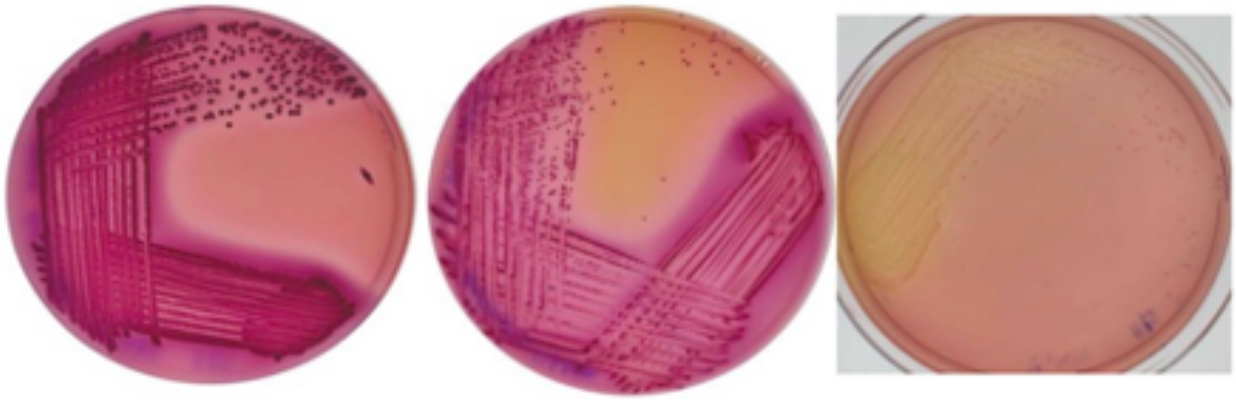
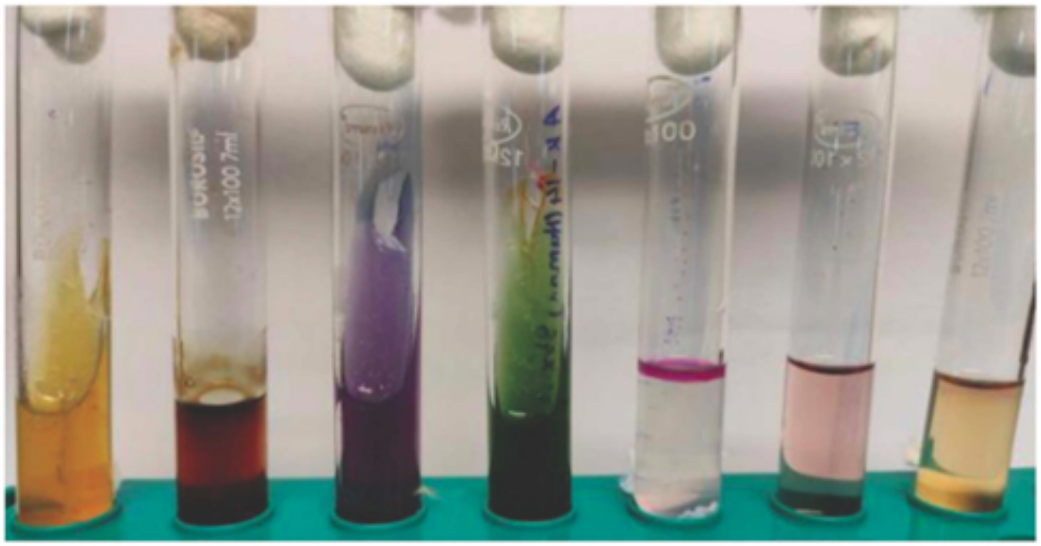


Fig. S3b. Hippurate test for *Campylobacter* spp.



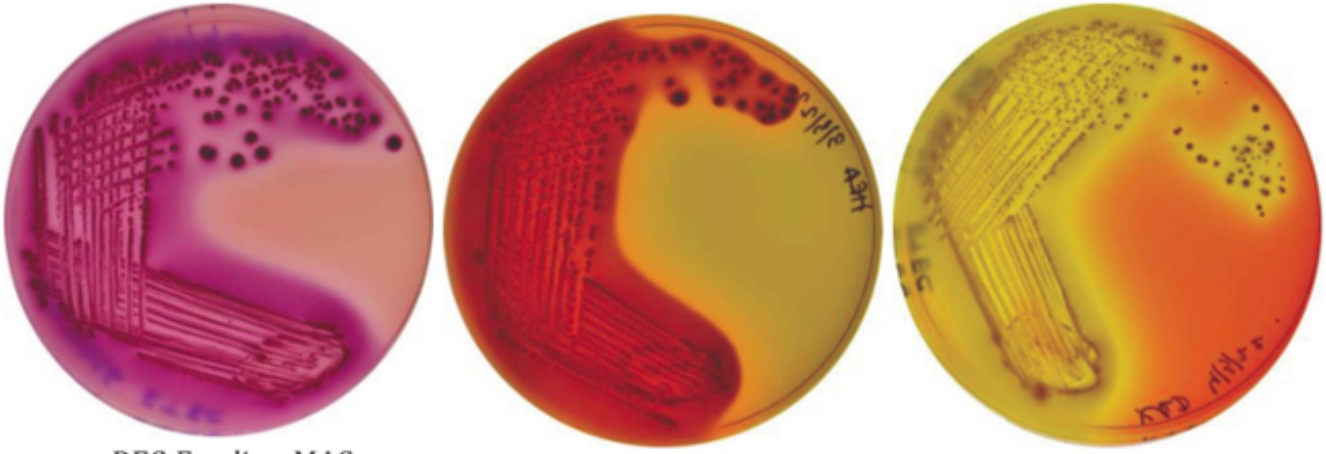
STEC on MAC STEC on S-MAC STEC O157:H7 on S-MAC

Fig. S4a. STEC colony morphology in MacConkey and sorbitol MacConkey media



1. TSI A/A
2. Motility +
3. Lysine +
4. Citrate -
5. Indole +
6. Urease -
7. Vogue Proskaer -

Fig. S4b. STEC biochemical test results

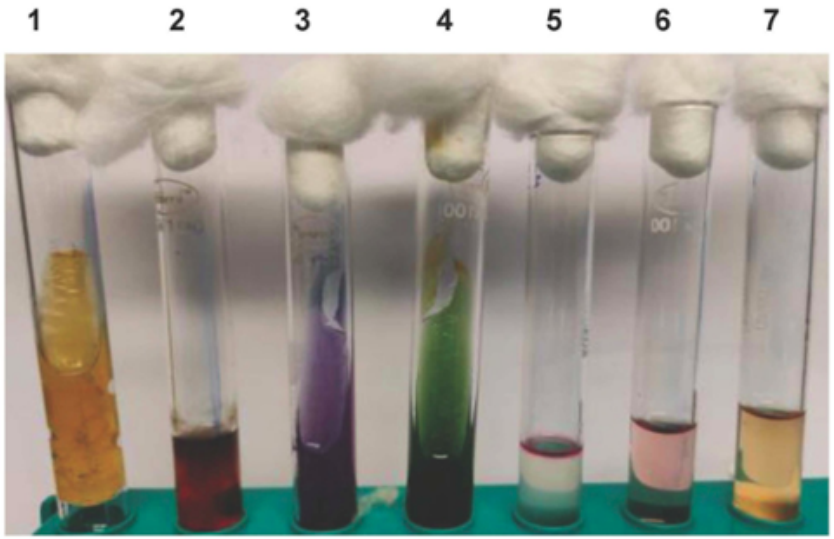


DEC *E. coli* on MAC

DEC *E. coli* on HEA

DEC *E. coli* on XLD

Fig. S5a. DEC colony morphology in Mac, HEA and XLD media



1. TSI A/AG
2. Motility +
3. Lysine +
4. Citrate -
5. Indole +
6. Urease -
7. Vogue Proskaer -

Fig. S5b. DEC biochemical test results

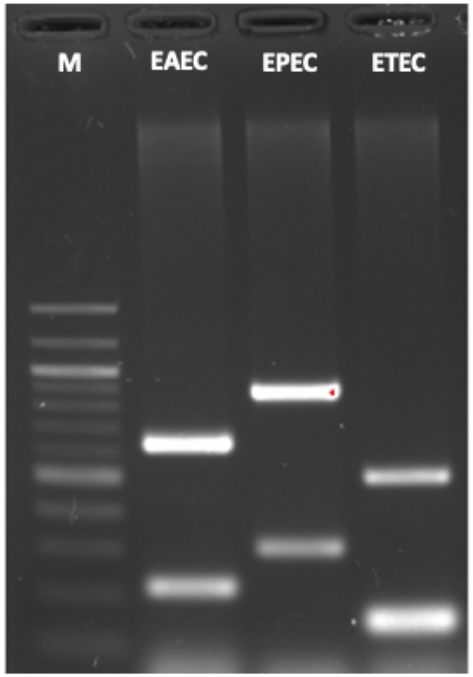


Fig. S5c. DEC Multiplex PCR amplicons

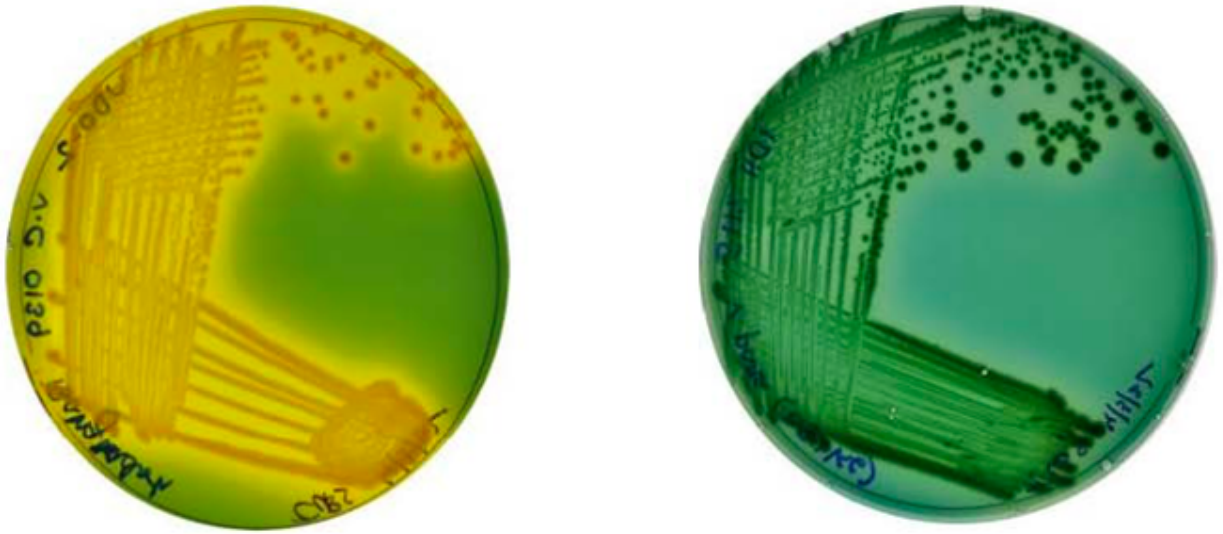
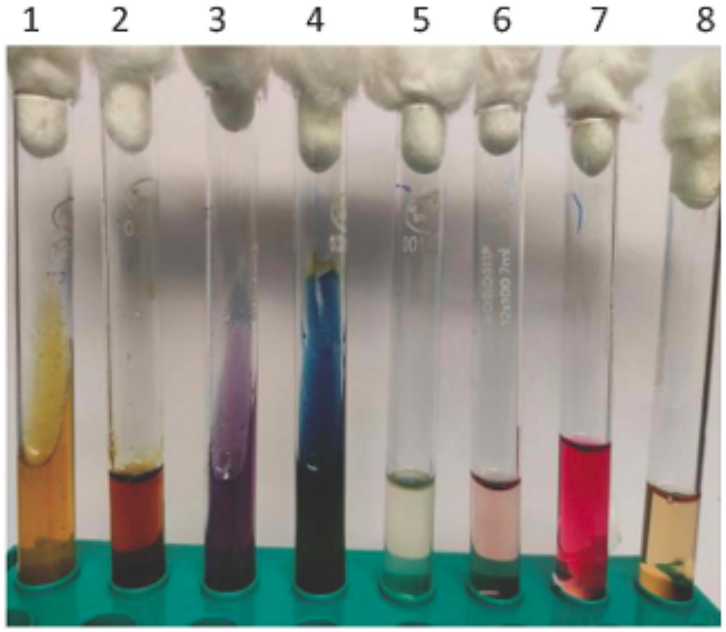
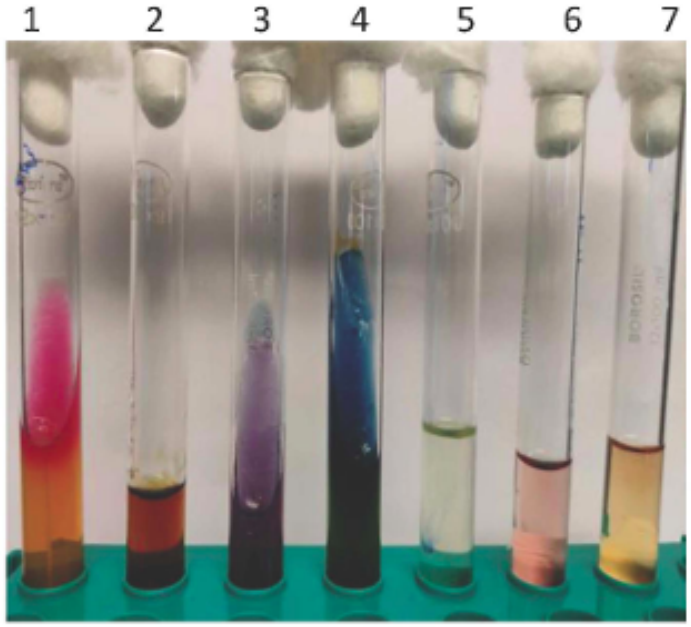


Fig. S6a. Growth of *V. cholerae* (yellow colonies) and *V. parahaemolyticus* (green colonies on TCBS agar



V. cholerae

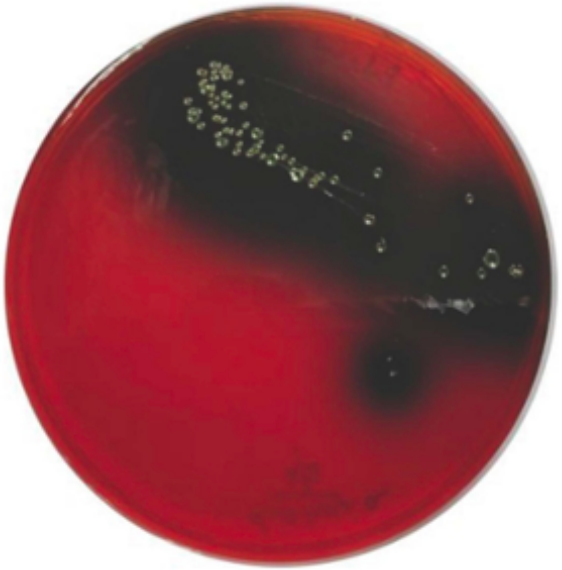
1. TSI A/A
2. Motility +
3. Lysine +
4. Citrate +
5. Indole +
6. Urease -
7. Vogue Proskaer (+); 8: Vogue Proskaer (-)



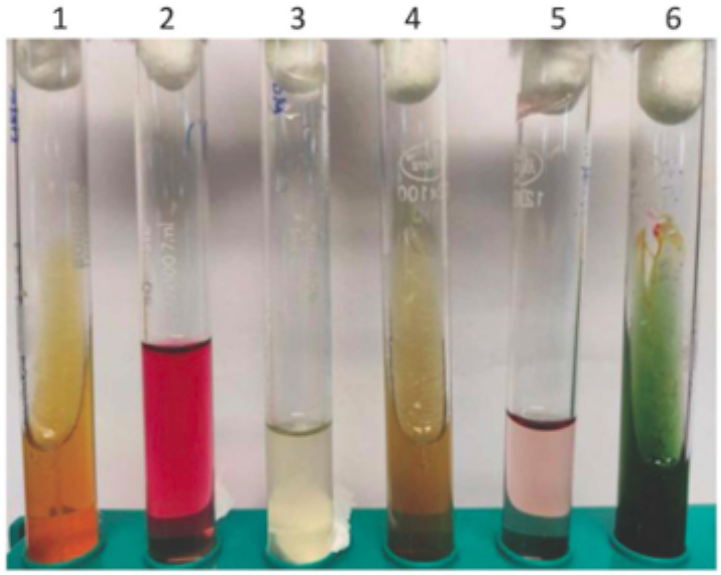
V. parahaemolyticus

1. TSI K/A
2. Motility +
3. Lysine +
4. Citrate +
5. Indole +
6. Urease Variable (*trh*-positive strains are +)
7. Vogue Proskaer -

Fig. S6b. *V. cholerae* and *V. parahaemolyticus* biochemical test results



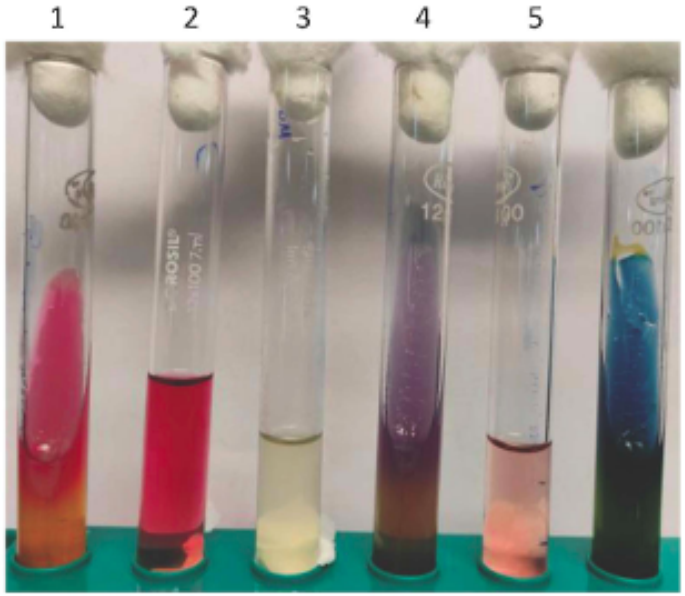
Growth of *Listeria monocytogenes* in PALCAM Agar



1. TSI A/A
2. Vogue Proskaer +
3. Indole -
4. Lysine -
5. Urease -
6. Citrate -

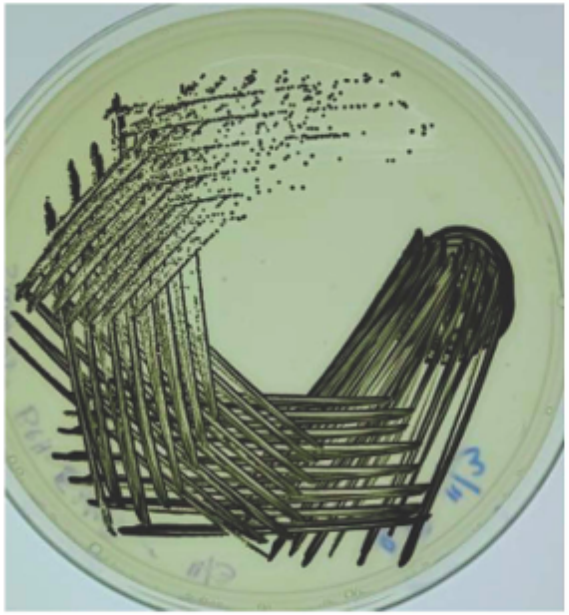
Fig. S7. Growth and biochemical test results of *Listeria monocytogens*

Growth of *Bacillus cereus* in MYP Agar

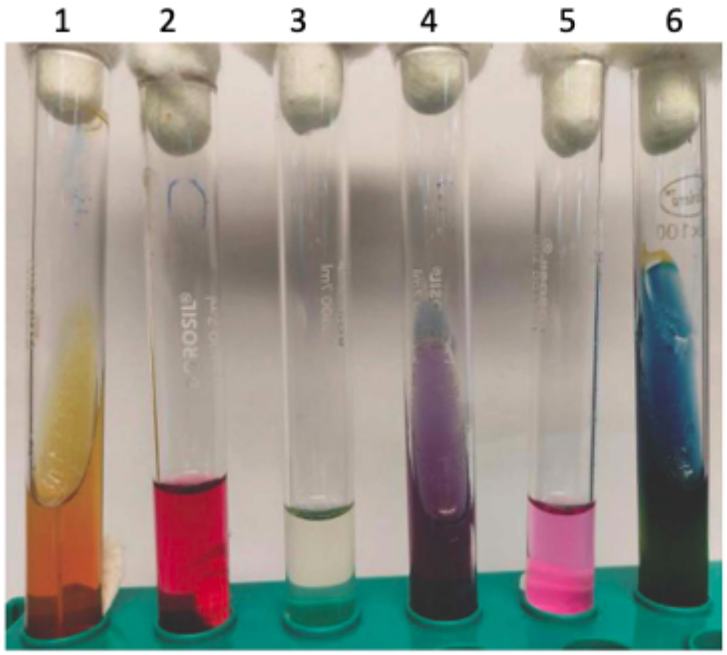


1. TSI K/-
2. Vogue Proskaer +
3. Indole -
4. Lysine -
5. Urease -
6. Citrate +

Fig. S8. Growth and biochemical test results of *B. cereus*

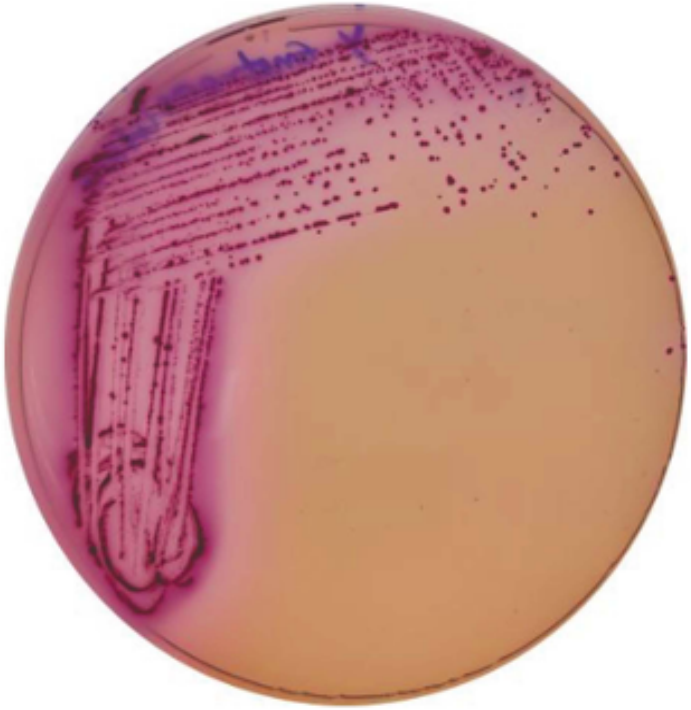


Growth of *Staphylococcus aureus* in Baird Parker Agar

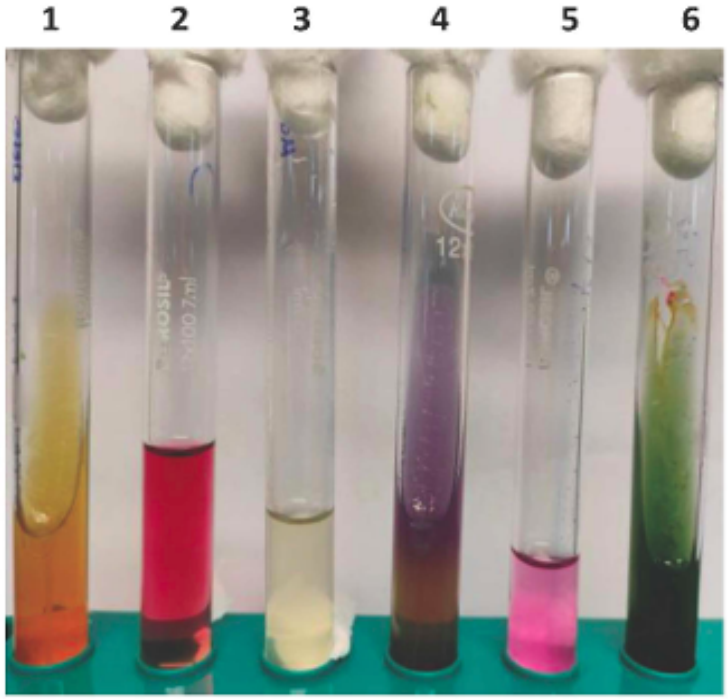


1. TSI -
2. Vogue Proskaer +
3. Indole -
4. Lysine +
5. Urease +
6. Citrate +

Fig. S9. Growth and biochemical test results of *S. aureus*



Growth of *Yersinia enterocolitica* in ClN Agar



1. TSI A/A
2. Vogue Proskaerv +
3. Indole -
4. Lysine -
5. Urease +
6. Citrate -

Fig. S10. Growth and biochemical test results of *Y. enterocolitica*

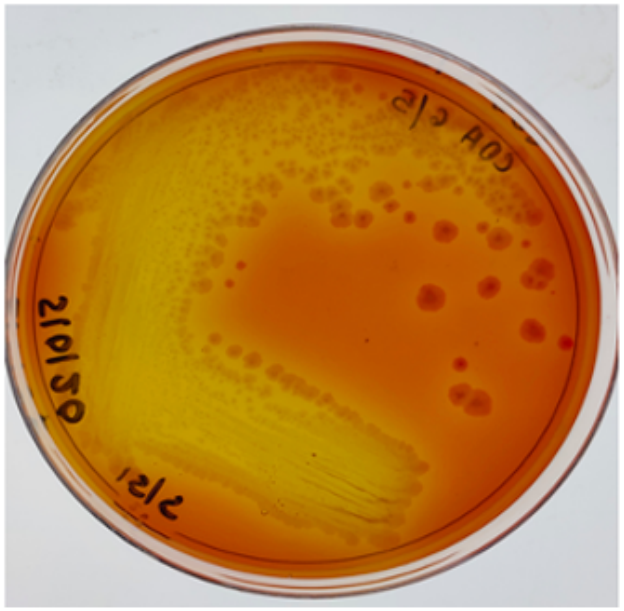


Fig. S11. Growth of *C. difficile* in CCFA medium

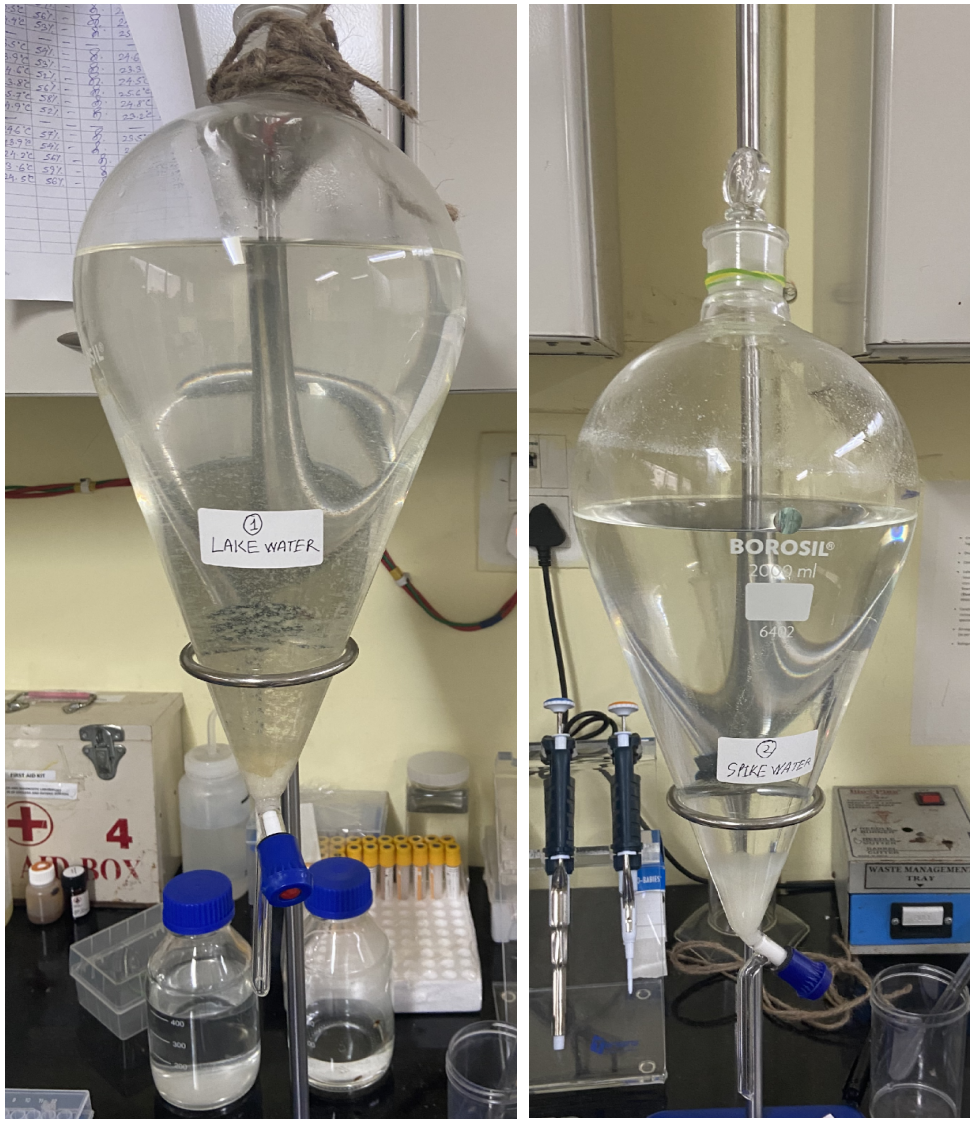


Fig. S12. Flocculated milk powder in treated water samples

DETECTION OF YEASTS, MOLDS AND MYCOTOXIN BY HPLC

Introduction

The dilution plating and the direct plating methods may be used to detect fungi in foods. The direct plating method is more efficient than the dilution plating method for detecting individual mold species, including most of the toxin producers, but it is less effective in detecting yeasts. It is also used to determine whether the presence of mold is due to external contamination or internal invasion. Methodology for testing the ability of isolates of toxigenic mold species to produce mycotoxins on sterile rice water substrate is included here.

Enumeration of Yeasts and Molds in Food--Dilution Plating Technique Equipment and materials

Basic equipment (and appropriate techniques) for preparation of sample homogenate

Equipment for plating samples

Incubator, 25°C Arnold

steam chest pH meter

Water bath, 45 ± 1° C

Media and Reagents

Media Dichloran rose Bengal chloramphenicol (DRBC) agar (M183) Dichloran 18% glycerol (DG18) agar (M184)

Plate count agar (PCA), standard methods (M124); add 100 mg chloramphenicol/liter when this medium is used for yeast and mold enumeration. This medium is not efficient when "spreader" molds are present.

Malt agar (MA)(M185) Malt extract agar (Yeasts and Molds) (MEAYM) (M182) Potato dextrose agar (PDA), dehydrated; commercially available (M127) Antibiotic solutions

Antibiotics are added to mycological media to inhibit bacterial growth. **Chloramphenicol** is the antibiotic of choice, because it is stable under autoclave conditions. Therefore, media preparation is easier and faster due to the elimination of the filtration step. The recommended concentration of this antibiotic is 100 mg/liter medium. If bacterial overgrowth is apparent, prepare media by adding 50 mg/liter chloramphenicol before autoclaving and 50 mg/liter filter sterilised chlortetracycline when the media have been tempered, right before pouring plates.

Prepare stock solution by dissolving 0.1 gm chloramphenicol in 40 ml distilled water; add this solution to 960 ml medium mixture before autoclaving. When both chloramphenicol and chlortetracycline are used, add 20 ml of the above chloramphenicol stock solution to 970 ml medium before autoclaving. Then, prepare chlortetracycline stock solution by dissolving 0.5 g antibiotic in 100 ml distilled water and filter sterilize. Use 10 ml of this solution for each 990 ml of autoclaved and tempered medium. Refrigerate in the dark and re-use remaining stock solutions for up to a month. Stock solutions should be brought to room temperature before adding to tempered medium.

Procedures:

Sample preparation

Analyze 25-50 gm from each subsample; generally, larger sample sizes increase reproducibility and lower variance compared with small samples. Test individual subsamples or composite according to respective Compliance Program for the food under analysis. Add appropriate amount of 0.1% peptone water to the weighed sample to achieve 10-1 dilution, then homogenize in a stomacher for 2 min. Alternatively, blending for 30-60 sec can be used but is less effective. Make appropriate 1:10 (1+9) dilutions in 0.1% peptone water. Dilutions of 10-6 should suffice.

Plating and incubation of sample

Spread-plate method. Aseptically pipet 0.1 ml of each dilution on pre- poured, solidified DRBC agar plates and spread inoculum with a sterile, bent glass rod. DG18 is preferred when the water activity of the analyzed sample is less than 0.95. Plate each dilution in triplicate.

Pour-plate method. Use sterile cotton-plugged pipet to place 1.0 ml portions of sample dilution into prelabeled 15 × 100 mm Petri plates (plastic or glass), and immediately add 20-25 ml tempered DG18 agar. Mix contents by gently swirling plates clockwise, then counterclockwise, taking care to avoid spillage on dish lid. After adding sample dilution, add agar within 1-2 min; otherwise, dilution may begin to adhere to dish bottom (especially if sample is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate.

From preparation of first sample dilution to pouring or surface-plating of final plate, no more than 20 min (preferably 10 min) should elapse. Note: Spread plating of diluted sample is considered better than the pour plate method. When the pour plate technique is used, fungal colonies on the surface grow faster and often obscure that underneath the surface, resulting in less accurate enumeration. Surface plating gives a more uniform growth and makes colony isolation easier. **DRBC agar should be used for spread plates only**.

Incubate plates in the dark at 25°C. **Do not stack plates higher than 3 and do not invert**. Note: Let plates remain undisturbed until counting.

Counting of plates

Count plates after 5 days of incubation. If there is no growth at 5 days, re-incubate for another 48 h. Do not count colonies before the end of the incubation period because handling of plates could result in secondary growth from dislodged spores, making final counts invalid. Count plates containing 10-150 colonies. If mainly yeasts are present, plates with 150 colonies are usually countable. However, if substantial amounts of mold are present, depending on the type of mold, the upper countable limit may have to be lowered at the discretion of the analyst. Report results in colony forming units (CFU)/g or CFU/ml based on average count of triplicate set. Round off counts to two significant figures. If third digit is 6 or above, round off to digit above (e.g., 456 = 460); if 4 or below, round off to digit below (e.g., 454 = 450). If third digit is 5, round off to digit below if first 2 digits are an even number (e.g., 445 = 440); round off to digit above if first 2 digits are an odd number (e.g., 455 = 460). When plates from all dilutions have no colonies, report mold and yeast counts (MYC) as less than 1 times the lowest dilution used.

Isolate individual colonies on PDA or MA, if further analysis and species identification is necessary.

Enumeration of Molds in Foods--Direct Plating Technique for Foods That Can Be Handled with Forceps (Dried Beans, Nuts, Whole Spices, Coffee and Cocoa Beans, etc.)

Equipment and materials

Freezer, -20° C

Beakers, sterile, 300 ml

Forceps, sterile

Arnold steam chest

Water bath, 45 ± 1° C

Incubator, 25° C

Media and Reagents

Dichloran rose Bengal chloramphenicol (DRBC) agar (M183)

Dichloran 18% glycerol (DG18) agar (M184)

Antibiotic solutions (see previous section)

NaOCl (commercial bleach) solution, 10%

Sterile distilled water

Analysis of non-surface-disinfected (NSD) foods

Sample and media preparation

Before plating, hold sample at -20° C for 72 hrs to kill mites and insects that might interfere with analysis.

Prepare DRBC agar as described in the appendix. If DRBC is not available, or the water activity of the analyzed sample is less than 0.95, use DG18 agar. Media should be prepared no more than 24 hrs prior to use.

Plating and incubation of sample

From each sample, transfer about 50 gm into a sterile 300 ml beaker. Using 95% ethanol-flamed forceps place intact food items on surface of solidified agar, 5-10 items per plate (depending on size of food item) 50 items total per sample.

Flame forceps between plating of each item. Use several forceps alternately to avoid overheating. Do not plate visibly moldy or otherwise blemished items.

Align 3-5 plates in stacks and identify with sample number plus date of plating. Incubate stacks, undisturbed in the dark at 25°C for 5 days. If there is no growth at 5 days of incubation, reincubate for another 48 h to allow heat- or chemically-stressed cells and spores enough time to grow.

Reading of plates

Determine occurrence of mold in percentages. If mold emerged from all 50 food items, moldiness is 100%; if from 32 items, moldiness is 64%. Determine percent occurrence of individual mold genera and species in like manner. Experienced analysts may identify Aspergillus, Penicillium and most other foodborne mold genera directly on medium with low power (10-30X) magnification.

Analysis of surface-disinfected (SD) foods

Perform disinfection in clean laboratory sink, not stainless steel, free from any acid residues, with tap water running (precautions against chlorine gas generation). Wear rubber gloves and transfer about 50 g of sample into a sterile 300 ml beaker. Cover with 10% chlorine (commercial bleach) solution for 2 min, while swirling beaker contents gently but constantly in clockwise and counterclockwise motion. Decant 10% chlorine solution and give beaker contents two 1-min rinses with sterile distilled water. Prepare plates; plate sample, incubate, and read plates as in non-surface disinfected direct plating method, above. Compare NSD and SD results from the same sample to determine if moldiness was due mainly to surface contamination or to internal invasion and growth. Isolate individual colonies on PDA or MA.

Fluorescence Microscopy Procedure for Quantitation of Viable and Nonviable Yeasts in Beverages (if available)

Methods for counting viable yeasts by plating are described above. A direct microscopic procedure for counting nonviable and viable yeasts in beverages and other liquid samples is presented here. Quantitating yeast cells by microscopy eliminates the need for extended incubation, thus reducing the analytical time required. All yeasts can be counted, and living and dead yeast cells can be differentiated.

Millipore disk filter holders for standard syringes

Millipore filters: AABG, 0.8 µm, black, gridded; 25 mm diameter

Syringes, disposable

Pipets

Forceps

Bibulous paper

Microscope slides and 24 × 24 mm coverslips

Fluorescence microscope: blue excitation; l0X eyepieces with Howard mold count or other eyepiece grid; 20× or 40× objective

Reagents

Aniline blue; 1% in M/15 K2HPO4 (M/15 is equivalent to 11.6 g/L), adjusted to pH 8.9 with K3PO4. A stock solution can be made; age improves fluorescence.

NaOH; 25 g in 100 ml water

Sample preparation for filterable liquids (e.g. water and grape juice)

Filter aliquot (usually 10 ml) of sample through Millipore filter (AABG, 0.8 µm, black, gridded), (Portion size can be increased or decreased, depending on level of contamination). Use Millipore disk filter holder which attaches to standard syringe. Make sure that syringe is accurate. If not, remove plunger, attach syringe to filter holder, and pipette 10 ml into syringe. Press all of sample through filter. Do this with air cushion of about 3 ml between plunger and sample. Keep filter holder vertical to ensure even distribution of sample over filter. Remove filter from filter holder and place on microscope slide; grid should be parallel to edges of slide to facilitate counting.

Sample preparation for non-filterable liquids that clog the filter (e.g. orange juice)
To suppress background interference in fluorescence microscope, mix 4 ml sample with 1 ml sodium hydroxide (25 m in 100 ml water). Shake well and wait 10 min. Place Millipore filter (AABG, 0.8 μm, black, gridded) on a piece of bibulous paper and spread 0.1 or 0.01 ml (depending on level of contamination) of sample over filter. When filter surface is dry, place filter on microscope slide, keeping grid parallel to edges of slide to facilitate counting.

Microscopic counting procedure
Cover filter with a drop of aniline blue, 1% in M/15 (11.6 g/L) K_2HPO_4, adjusted to pH 8.9 with K_3PO4. Spread aniline blue stain over whole filter with glass rod or coverslip without touching filter itself. Wait about 5 min; then cover filter with 24 × 24 mm coverslip.

Count yeasts, using fluorescence microscope with blue excitation. Use 10X eyepiece with Howard mold count or other eyepiece grid, and 20X (or 40X) objective. Count 3 squares of eyepiece grid in each field of filter not covered by gasket. Count budding yeasts as 1 cell if daughter cell is obviously smaller than mother cell. If they are approximately equal in size, count them as 2 cells. Count all yeasts located completely within an eyepiece square and all yeasts touching left and lower border of eyepiece square. Do not count yeasts touching right and upper borders.

This method also differentiates dead (heat- or formaldehyde-killed) and living yeast cells. Dead cells show fairly uniform fluorescence, and plasma may be granular. In living cells, the cell wall stains brighter and is more defined than the plasma, which is less prominent and uniformly stained.

Calculations to determine number of yeasts per ml
Determine area of filter covered by 1 square of eyepiece grid, using objective (stage) micrometer. For filtered samples, the working area of the Millipore filter (portion not covered by the gasket) is 380 mm2. For nonfiltered samples, it is the entire filter, or 491 mm2, since no gasket is used.

NOTE: For non-filterable liquids, volume includes only net amount used and not volume of NaOH added (i.e., 80% of total volume applied to filter).

Methods for Determining Toxin Production by Molds

A. Equipment and materials

Erlenmeyer flasks, 300 ml, wide-mouth

Cotton, nonabsorbent

Funnels, short-stem glass, 90-100 mm diameter

Filter paper, 18 cm diameter, folded (Schleicher & Schuell No. 588)

Boiling chips, silicon carbide

Fume hood equipped with steam bath; air-flow rate, 100 cubic ft/min

Blender, high speed, explosion-proof

Thin layer chromatographic apparatus or high-performance liquid chromatograph

Incubator, 22-25°C

B. Media and reagents

Long or short grain polished rice

Chloroform for extraction of aflatoxins, ochratoxins, sterigmatocystin, xanthomegnin, luteoskyrin, patulin, penicillic acid, citrinin, T-2 toxin, zearalenone

Methanol for extraction of deoxynivalenol

Appropriate mycotoxin standards

NaOCl solution, 5%

Toxin production

Into 300 ml wide-mouth Erlenmeyer flask, add 50 g rice and 50 ml distilled water. Plug flasks with cotton and autoclave 20 min at 121°C and 15 psi. Aseptically multispore-inoculate separate cooled flasks with individual mold isolates. Incubate inoculated flasks at 22-25°C until entire surface is covered with growth, and mycelium has penetrated to bottom of flask (15-20 days). To each flask, add 150 ml chloroform (150 ml methanol if toxin in question is deoxynivalenol), using short-stem glass funnel inserted alongside unremoved cotton plug (to minimize mold spore dissemination). Heat flask contents in fume hood on steam bath until solvent begins to boil. (Conduct all subsequent steps in fume hood.) With spatula, break up moldy rice cake and transfer flask contents into explosion-proof blender and blend at high speed for 1 min. Filter blender contents through filter paper inserted into short-stem glass funnel. Collect filtrate in 300 ml Erlenmeyer flask. Return rice cakes to blender, add 100 ml unheated solvent and blend 1 min at high speed. Filter as above and combine filtrates. Add boiling chips to flask containing filtrates and evaporate with steam to 20-25 ml. If analysis is not to follow immediately, evaporate to dryness and store flask in the dark. Rinse all glassware, etc., used for extraction in 5% NaOCl solution before soap and water cleansing. Submerge rice cake in 5% NaOCl solution for 72 h before autoclaving and disposal.

Toxin analysis

Appropriate mycotoxin standards are required for both qualitative and quantitative analysis of toxin. Use either thin layer chromatography as described elsewhere (FSSAI Standards) or high-

performance liquid chromatography, as described below to determine mycotoxins extracted from mold cultures. Naturally occurring mycotoxins in foods or feeds can best be determined by methods described in Official Methods of Analysis.

Media Dichloran 18% glycerol (DG18) agar (M184)

Reagent	**Quantity**
Glucose	10.0 gm
Peptone	5.0 gm
KH2PO4	1.0 gm
$MgSO_4 \cdot 7H_2O$	0.5 gm
Dichloran (2,6-dichloro-4-nitroaniline) solution (0.2% (w/v) in ethanol)	1.0 ml
Chloramphenicol	0.1 gm
Agar	15.0 gm
Distilled water	800 ml

Mix above items and steam to dissolve agar, then bring volume to 1000 ml with distilled water. Add 220 gm glycerol (analytical reagent grade), and sterilize by autoclaving at 121°C for 15 min. Temper medium to 45° C and pour plates under aseptic conditions. The final aw of this medium is 0.955. DG18 agar is used as a general-purpose mold enumeration medium and is preferred when the aw of the analyzed food is less than 0.95. The low water activity of this medium reduces interference by bacteria and fast-growing fungi. When both yeasts and molds must be enumerated, DRBC agar should be used.

Dichloran rose Bengal chloramphenicol (DRBC) agar (M183)

Reagent	**Quantity**
Glucose	10.0 gm
Bacteriological peptone	5.0 gm
KH2PO4	1.0 gm
$MgSO_4 \cdot 7H_2O$	0.5 gm
Rose Bengal (5% aqueous soln., w/v)	0.5 ml
Dichloran (0.2% in ethanol, w/v)	1.0 ml

Chloramphenicol 0.1 gm

Agar 15.0 gm

Distilled water 1.0 liter

Final pH should be 5.6

Mix ingredients, heat to dissolve agar and sterilize by autoclaving at 121°C for 15 min. Temper to 45 ± 1° C in a water bath and pour plates.

Notes: DRBC agar is especially useful for analyzing samples containing "spreader" molds (e.g. Mucor, Rhizopus, etc.), since the added dichloran and rose Bengal effectively slow down the growth of fast-growing fungi, thus readily allowing detection of other yeast and mold propagules, which have lower growth rates.

Media containing rose Bengal are light-sensitive; relatively short exposure to light will result in the formation of inhibitory compounds. Keep these media in a dark, cool place until used. DRBC agar should be used for spread plates only.

Malt Agar (MA) (M185)
Reagent Quantity

Malt extract, powdered 20.0 gm

Agar 20.0 gm

Distilled water 1.0 liter

Mix ingredients, steam to dissolve agar and sterilize for 15 min at 121° C. Temper medium to 45° C and pour plates under aseptic conditions. To prepare slants dispense 5-6 ml of steamed medium (before autoclaving) into each of several 16 × 125 mm screw-cap tubes, loosely cap tubes and sterilize as above. After autoclaving lay tubes in a slanting position and let them cool. This medium is recommended as a general maintenance medium.

Malt Extract Agar (Yeasts and Molds) (MEA) (M182)
Reagent Quantity

Malt extract, powdered 20.0 gm

Glucose 20.0 gm

Peptone 1.0 gm

Agar 20.0 gm

Distilled water 1.0 liter

Mix ingredients, heat to dissolve agar and sterilize at 121° C for 15 min. Temper media to 45° C and pour plates under aseptic conditions. Dehydrated MEA is commercially available, but since more than one MEA formula exists, check for the appropriate composition. This medium is recommended for the identification of Aspergillus and Penicillium.

Mycotoxin Analysis by HPLC Method

This procedure describes the process for the Estimation of Aflatoxins contamination in peanut and peanut products etc.

Procedure:

1 Chemicals and Reagents:

i. Gradient grade Methanol

ii. Acetic acid (Glacial)

iii. Sodium chloride

iv. Ammonium formate

v. Potassium chloride

vi. Potassium dihydrogen phosphate

vii. Anhydrous disodium hydrogen phosphate

viii. Hydrochloric acid

ix. Sodium hydroxide

x. Distilled, reverse osmosis or deionized water

xi. Dissolve 0.2 gm Potassium chloride, 0.2 gm Potassium dihydrogen phosphate, 0.93 gm and anhydrous disodium hydrogen phosphate and 8 gm Sodium chloride in 900 ml HPLC grade water. Then adjust the pH to 7.4(±0.1) with 0.1M HCl or 0.1 M NaOH.

Reference standards:

Use Aflatoxin mix standard solution with known concentration (as an example: Sigma Aldrich Aflatoxin Mix Solution 33415 containing Aflatoxin B1 1 µg/mL, Aflatoxin G1 1 µg/mL, Aflatoxin B2 .3 µg/mL, Aflatoxin G2 .3 µg/mL).

Apparatus:

i. Mixer and grinder (20 L capacity)

ii. Homogenizer/Blender
iii. Analytical and precision balance
iv. HPLC-Fluorescence
v. Pipettes
vi. Test tubes
vii. Vacuum manifold
viii. Immunoaffinity column
ix. Fluted filter paper (24 cm), funnel and Erlenmeyer flask
x. Glass microfiber filter GF/A (11 cm) and funnel
xi. Syringe (glass or other material compatible with methanol)
xii. Bottles of 250 ml

Calibration Standard Preparation:

Prepare an intermediate mixture of 1µg/ml by pipetting appropriate volume of all four aflatoxins. Prepare a set of calibration standards in range of 0.25, 0.5, 1, 2, 5, 10 ng/ml for linearity as below.

i. Preparation of 1 µg/mL of Aflatoxin intermediate stock standard:

a. Prepare 1 µg/ml intermediate mix of std. from 20µg/ml purchased stock standard by taking 50 µl in 1 ml volumetric flask and making up to 1 ml by adding 950 µl of methanol.
b. Store stock standard solution at -18^{o}C. Equilibrate to room temperature before use.

ii. Preparation of 100 ng/mL of Aflatoxin intermediate standard:

Prepare 100 ng/mL intermediate std. by taking 100 µl from 1 µg/ml standard solution in 1 ml volumetric flask and making up to 1 ml by adding 900 µl of methanol.

iii. Working Aflatoxin Standard Solutions:

a. From 100 ng/mL, prepare working standard solutions of 10 ng/ml, then make serial dilutions for 5 ng/ml, 2 ng/ml, 1 ng/ml, 0.5 ng/ml, 0.25 ng/ml.
b. Prepare daily 6 calibration standards in separate 1ml volumetric flasks according to Table 1

Preparation of Working Aflatoxins calibration solutions by serial dilution

Working standard solutions	Standard (ng/ml)	Volume required (µl)	Final volume (to 1 ml) with methanol
1	0.25	500 µl of 0.5 ng/ml	500 µl
2	0.5	500 µl of 1 ng/ml	500 µl
3	1	500 µl of 2 ng/ml	500 µl
4	2	400 µl of 5 ng/ml	600 µl
5	5	500 µl of 10 ng/ml	500 µl
6	10	100 µl of 100 ng/ml	900 µl

Sample Extraction:

Individual samples of ground nuts be divided into two 25 gm portions. One portion is to be kept as a blank. The other one is spiked with aflatoxin standards at the regulatory levels. Both portions were then carried through the Immunoaffinity column cleanup procedure.

i. Homogenize the sample with water in equal amount (1:1) to have paste.
ii. Weigh 25 gm sample in 250 ml capacity centrifuge bottle followed by addition of 100ml extraction solvent methanol: water (80:20) and 5g sodium chloride.
iii. Keep the centrifuge bottle on orbital shaker for 30min with speed of 200 rpm.
iv. Then centrifuge the sample at 5000 rpm for 5 min. Filter with fluted filter paper.
v. Take 3 ml supernatant in 50 ml centrifuge tube and dilute with water (for Aflatest, VICAM) or PBS Solution (Aflaprep, R-Biopharmimmunoaffinity column) to 15 ml. Filter through glass micro fiber filter paper.

Determination of Aflatoxins:

HPLC –Fluorescence:

i. HPLC column: BEH 18 (100×2.1mm, 1.7µm)
ii. Column oven: 40°C
iii. Mobile phase: Methanol: Acetonitrile: Water (18:18:64)
iv. Flow rate: 0.4ml /min
v. Injection volume: 10 µl
vi. Detector: Emission 429 nm, 456 nm and excitation 360 nm.

Calculation:

Concentration of Aflatoxin (ng/g) = $[(X-Y)/m] \times [(B/A) \times (D/C)]$

Where, X=Area of sample,

Y=Intercept value,

m= slope of calibration line,

B=Volume of extraction solvent,

A= Weight of sample (gm),

C= Volume of extract taken for cleanup

D= Final volume of extract.

Direct analysis of Aflatoxins (AF) and Ochratoxin A (OTA) in cereals and their processed products by Ultra-High-Performance Liquid Chromatography with fluorescence detection

Precautions:

As AFs and OTA are toxic as well as carcinogenic in nature, use nitrile gloves while handling these substances. Prior to sample extract disposal, the solutions must be treated with 5-6% sodium hypochlorite. All glassware exposed to the residues of these toxins must be rinsed with methanol and 1% sodium hypochlorite solution and then washed.

Principle

A reverse phase based HPLC separation of the AFs and OTA and their detection by fluorescence. The AFs and OTA are extracted with methanol-water. The extract is cleaned by using immunoaffinity columns.

Chemicals

1. Methanol (HPLC gradient grade),

2. Glacial Acetic acid,

3. Sodium chloride

4. Sodium hydroxide)

5. HPLC grade water (18.2 MΩ cm)

6. Monoclonal antibody-based immune-affinity columns (AFLAOCHRA PREP IAC (3 mL; R-Biopharm AG, Darmstadt, Germany)

7. Phosphate-buffered saline (PBS)

8. Reference standards: Individual AF standards (B1, B2, G1, and G2) with >95% purity OTA with purity of 98%.

Reagents

1. Sodium hydroxide, 0.2M; Dissolve 8 gm NaOH in 1 L water.

2. Phosphate buffered saline (PBS): Dissolve 8 gm NaCl, 1.16 gm Na_2HPO_4,

0.2 gm KH_2PO_4 and 0.2 gm KCl (j) in 1 L water. Adjust pH to 7.4 with 0.2M NaOH.

3. HPLC mobile phase A (v/v): Mix 1 parts glacial acetic acid with 99 parts water and 2 parts CH_3COOH; filter through 0.22 µm filter B and degas.

4. HPLC mobile phase B(v/v): Mix 1 parts glacial acetic acid with 99 parts methanol; filter through 0.22 µm filter B and degas

Preparation of standards

Stock standard: Dissolve 5 mg each standard in 10 mL methanol in an amber-colored vial. The stock solutions containing 500 µg/mL of each AF and OTA is stored at –20°C.

Intermediate standard: Dilute the stock solutions in methanol.

Calibration standards: Make serial dilutions of the intermediate solutions to obtain 0.02–10 ng/mL for each AF and 0.1–10 ng/mL for OTA in 1:1 ratio of methanol: water (plus 0.2% acetic acid, v/v).

Preparation of Samples

Test Grinding

Cereal grains and processed products (are thoroughly milled and allowed to pass through a No. 20 sieve.

Extraction

Add 12.5 gm of finely ground dry matrix to 12.5 g distilled water to make a slurry. Mix the slurry with 100 ml of extraction solvent (methanol– water, 8+ 2, v/v) and NaCl (5 gm). Shake for 30 min, 200 rpm), and then centrifuge (5000 rpm, 5 min). Take an aliquot (3 ml) and dilute with 15 mL PBS and add 50 µl NaOH (2 M) solution.

IAC cleanup

Load the diluted sample onto IAC connected to a vacuum manifold and allow to pass without any vacuum. Wash with 10 mL PBS. Elute with methanol (2 × 0.5 ml). Slowly evaporate the final extract (1 ml) to dryness. Reconstituted in 0.5 ml methanol–water (acidified with 0.2% acetic acid, 1:1), and finally inject 10 µl into the UHPLC-FLD instrument.

Chromatography conditions

1. Column: C18 column (2.1 × 50 mm, 1.7 µm).
2. Column temperature: 40°C,
3. Flow rate: 0.2 ml/min
4. Injection volume: 10 µl.
5. The mobile phases: (A) 1% acetic acid in water and (B) 1% acetic acid in methanol.
6. Detector:
7. Excitation wavelength 365 nm up to 8 min and subsequently switched to 333 nm and continued up to 15 min.

8. Emission wavelength: 456 nm
9. Linear Gradient program

Time (mins)	**% A**	**% B**
Initial	90	10
0.25	90	10
2.50	58	42
6.00	58	42
7.00	20	80
11.50	20	80
12.00	90	10
15.00	90	10

Calculation

Prepare a calibration curve for 0.02–10 ng/g for each AF and 0.1–10 ng/g for OTA using the working standard. From the equations determine the concentration of the unknown.

LOD and LOQ

The LODs are 0.02 ng/g (S/N > 3) and 0.1 ng/g (S/N > 10) for AFs and OTA, respectively

Reference:

Simultaneous Direct Analysis of Aflatoxins and Ochratoxin A in Cereals and Their Processed Products by Ultra-High-Performance Liquid Chromatography with Fluorescence Detection‘ Dhanshetty, M and Banerjee, K (2019) Journal of AOAC International, 102(6) 1666-1672.

PCR-BASED DETECTION OF MYCOTOXIN-PRODUCING GENES

Materials

Standard strains for use as positive controls

- *A. carbonarius* NRRL 368
- *A. flavus* NRRL3518
- *A. fumigatus* NRRL 62427
- *A. niger* NRRL 328
- *A. parasiticus* NRRL6111
- *A. terreus* NRRL 269
- *A. tubingensis* NRRL 66281
- *A. ochraceus* NRRL 35018

1) *F. avenaceum* NRRL A-28073
2) *F. culmorum* NRRL 13320
3) *F. graminearum* NRRL 3376
4) *F. poae* NRRL 36300
5) *F. proliferatum* NRRL 31866
6) *F. roseum* NRRL 6469
7) *F. solani* NRRL 13416
8) *F. sporotrichioides* NRRL3299
9) *F. verticillioides* NRRL 25457
10) *P. digitatum* NRRL 1202
11) *P. expansum* NRRL 976
12) *P. roqueforti* NRRL 849
13) *P. verrucosum* NRRL 965
14) *P. viridicatum* NRRL 5571

DNA Isolation:

1) Mycelial mats to be grown overnight in YPD medium.
2) Genomic DNA isolated from the Mycelial mats using lysis buffer containing hexadecyltrimethylammonium bromide (CTAB).
3) Lyophilized mycelial mats should be pulverized with 5 ml of 3-mm-diameter glass beads in a disposable 50-m conical centrifuge tube.
4) Ten millilitres of DNA extraction buffer (l.0 M Tris/HCl, pH 7.5; 1% (*w*/*v*) CTAB; 5 M NaCl; 0.5 M EDTA; 1% (*v*/*v*) 2-mercaptoethanol; and proteinase K at 0.3 mg/ml) to be added to the powdered mycelia and mixed gently, and incubated at 65 °C for 30 min.
5) The extracts are cooled prior to the addition of an equal volume of chloroform, gently mixed and centrifuged at 6000 rpm for 10 min.

6) The aqueous supernatant is recovered and the nucleic acids are precipitated with an equal volume of 2-propanol.
7) Gentle mixing results in the formation of high-molecular-mass DNA, which is precipitated by centrifugation at 4800g for 5 min.
8) The DNA is then resuspended in TE buffer solution (Tris-EDTA, pH 8.0) containing RNase A at 10 μg/ml, and further purified by phenol-chloroform extraction (A260/A280 ratio of 1.8–2.0).
9) The DNA is precipitated with 100% ethanol containing 3 M sodium acetate, rinsed in 70% (*v*/*v*) ethanol and resuspended in TE buffer.
10) The purity of the extracted DNA was assayed with a spectrophotometer.

Multiplex PCR:

Multiplex PCR is to be performed in a 25-μl reaction mix containing 20–50 ng of each genomic DNA as a template, 2 × multiplex PCR master-mix with 3 mM MgCl2 (QIAGEN, Hilden, Germany) and each primer at 0.2 μM. The PCR sequence comprised: initial denaturation of DNA polymerase at 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55–60 °C (Table 1 and Table 2) for 90 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. PCR products were electrophoresed on a 1% agarose gel with a 100-bp DNA size marker at 96 V for 1 h. (Primer sequences as given in Table S1)

Thermal Profile for the detection of Mycotoxin gene targets

Set No.	Species	DNA/Gene Target	Amplicon Size (bp)	Annealing Temperature
I	*A. tubingensis*	*caM*	505	60 °C
	A. parasiticus	*caM*	430	
	A. carbonarius	*caM*	371	
	A. fumigatus	*pep*	250	
	A. flavus	*pepO*	200	
II	*F. culmorum*	RAPD [2] marker	570	55 °C
	F. graminearum	RAPD marker	400–500	
	F. sporotrichioides	*tri13*	332	
	F. poae	RAPD marker	220	
III	*F. avenaceum*	RAPD marker	920	55 °C
	F. verticillioides	*caM*	578	
	A. niger	*caM*	357	
	F. solani	AFLP	175	
IV	*F. proliferatum*	*caM*	585	55 °C
	P. expansum	*IDH*	480	
	F. oxysporum	*ITS*	340	
	P. digitatum	*Cyp*51	250	
V	*P. verrucosum*	*Otanps*	750	60 °C
	P. paneum	*IDH*	482	
	A. terreus	Topoisomerase II	386	
	P. roqueforti	ITS1-5.8S-ITS2	300	

Primer sets for genes involved in biosynthesis of mycotoxins

Set No.	Mycotoxin	Gene Target	Amplicon Size (bp)	Annealing Temperature
VI	Aflatoxins	*avfA*	950	58 °C
		aflR1	798	
		ver1	452	
		nor1	397	
VII	Fumonisin/Trichothecene/Zearalenone	*fum13*	988	55 °C
		fum1	798	
		tri6	546	
		tri5	450	
		pks13	351	
VIII	Ochratoxin A	*otanps*	788	58 °C

Table S1 : Primer sets for mycotoxin gene detection

Set no.	Species	Primer name	Sequence (5'-3')	Size (b/p)	Annealing temperature	References
I	*A. fumigatus*	PEX1	TATGTCTTCCCCTGCTCC	250	60 °C	Logotheti *et al.*, 2009
		PEX2	CTATGCCTGAGGGGCGAA			
	A. flavus	PepO1	CGACGTCTACAAGCCTTCTGGAAA	200		Logotheti *et al.*, 2009
		PepO2	CAGCAGACCGTCATTGTTCTTGTC			
	A. parasiticus	PAR1	GTCATGGCCGCCGGGGGCGTC	430		Sardinas *et al.*, 2010
		PAR2	CCTGGAAAAAATGGTTGTTTTGCG			
	A. tubingensis	TUB1	TCGACAGCTATTTCCCCCTT	505		Susca *et al.*, 2007
		TUB2	TAGCATGTCATATCACGGGCAT			
	A. carbonarius	CARBO1	AAGCGAATCGATAGTCCACAAGAATAC	371		Perrone *et al.*, 2004
		CARBO2	TCTGGCAGAAGTTAATATCCGGTT			
II	*F. graminearum*	Fg16F	CTCCGGATATGTTGCGTCAA	400-500	55 °C	Nicholsons *et al.*, 1998
		Fg16R	GGTAGGTATCCGACATGGCAA			
	F. culmorum	Fc01F	ATGGTGAACTCGTCGTGGC	570		Nicholsons *et al.*, 1998
		Fc01R	CCCTTCTTACGCCAATCTCG			
	F. poae	Fp82F	CAAGCAAACAGGCTCTTCACC	220		Parry and Nicholsons, 1996
		Fp82R	TGTTCCACCTCAGTGACAGGTT			
	F. sporotrichioides	AF330109CF	AAAAGCCCAAATTGCTGATG	332		Demeke *et al.*, 2005
		AF330109CR	TGGCATGTTCATTGTCACCT			
	F. verticillioides	VER1	CTTCCTGCGATGTTTCTCC	578		Mule *et al.*, 2004
		VER2	AATTGGCCATTGGTATTATATAC			
III	*F. avenaceum*	FaF	CAAGCATTGTCGCCACTCTC	920	55 °C	Doohan *et al.*, 1998
		FaR	GTTTGGCTCTACCGGGACTG			
	F. solani	FS1	GCAGGTATGGCTTTTTGGAA	175		Casasnovas *et al.*, 2013
		FS2	AGTAAACTCCGACAGGTGCAA			
	A. niger	An F	GATTTCGACAGCATTTTCCAGAA	357		Palumbo *et al.*, 2015
		An R	GATAAAACCATTGTTGTCGCGGTCG			
	F. proliferatum	PRO1	CTTTCCGCCAAGTTTCTTC	585		Mule *et al.*, 2004
		PRO2	TGTCAGTAACTCGACGTTG			
	F. oxysporum	FOF1	ACATACCACTTGTTGCCTCG	340		Mishra *et al.*, 2003
		FOR1	CGCCAATCAATTTGAGGAACG			
IV	*P. expansum*	PE1	AATGTGTACTGACTGGTCGCAG	480	55°C	Dombrink-Kurtzman & Mcgovern, 2007
		PE2	CAACCAACATATTCGTGCCTGAC			Hamamoto *et al.*, 2000
	P. digitatum	Pri 207	TAGCTCCAAAACAAATCGTCTGGC	250		
		Pri 38c	CACTTGATCTGCCCTGTTAACA			Dombrink-Kurtzman & Mcgovern, 2007
V	*P. paneum*	PP1	GAATACACACTGACTGGC	482	60°C	
		PP2	TCAACCAACACATTCGTACCAGAC			
	P. verrucosum	otanpsF	AGTCTTCGCTGGGTGCTTCC	750		Bogs *et al.*, 2006
		otanpsR	CAGCACTTTTCCCTCCATCTATCC			
	P. roqueforti	ITS183	CTGTCTGAAGAATGCAGTCTGAGAAC	300		Pedersen *et al.*, 1997
		ITS401	CCATACGCTCGAGGACCGGAC			
	A. terreus	ATRF81	TACCTTCAAGCCTGACTACG	386		Kanbe *et al.*, 2002
		ATRR120	ACCTGCTCGGCCAGTTTGCTG			

Set no.	Mycotoxin	Primer	Sequence (5'-3')	Size	Annealing temperature	References
VI	Aflatoxins	aflR1F	AACCGCATCCACAATCTCAT	798	58°C	Manonmani *et al.*, 2005
		aflR1R	AGTGCAGTTCGCTCAGAACA			
		NorF	ACCGCTACGCCGGCGCTCTCGGCAC	397		Priyanka *et al.*, 2014
		NorR	GTTGGCCGCCAGCTTCGACACTCCG			
		avf723F	ATGGTCACATACGCCCTCCTCGGG	950		Yu *et al.*, 2000
		avf1675R	GCCTCGCATTCTCTCGGCGACCGAA			
		ver1	GCCGCAGGCCGCGGAGAAAGGTGGT	452		Skory *et al.*, 1992
		ver2	CCGCAGTCAATGGCCATGCAGCG			
VII	Fumonisin/ Trichothecene/ Zearalenone	Fum1F	ATTATGGGCATCTTACCTGGAT	798	55°C	Ramana *et al.*, 2011
		Fum1R	ACGCAAGCTCCTGTGACAGA			
		Fum13F	AGTCGGGGTCAAGAGCTTGT	988		Ramana *et al.*, 2011
		Fum13R	TGCTGAGCCGACATCATAATC			
		tri5F	GAGAACTTTCCCACCGAATAT	450		Ramana *et al.*, 2011
		tri5R	GATAAGGTTCAATGAGCAGAG			
		tri6F	GATCTAAACGACTATGAATCACC	546		Ramana *et al.*, 2011
		tri6R	GCCTATAGTGATCTCGCATGT			
		ZEA13F	CATTCTTGGTCTTGTGAGGA	351		Priyanka *et al.*, 2015
		ZEA13R	CCTTATGCTCATCGACATG			
VIII	Ochratoxin A	Aolc35F	GCCAGACCATCGACACTGCATGCTC	536	58°C	Priyanka *et al.*, 2015
		Aolc12R	CGACTGGCGTTCCAGTACCATGAGC			
		otanpsF	AGTCTTCGCTGGGTGCTTCC	750		Bogs *et al.*, 2006
		otanpsR	CAGCACTTTTCCCTCCATCTATCC			

PCR for identification of fungal isolates:
The ITS regions 1 and 2 of ribosomal DNA may be used to compare the ITS1-ITS2 nucleotide sequences. The universal ITS primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) to be used for amplifying ITS regions by PCR.

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