Standard Laboratory Protocol on Testing Milk Samples for Quality & Safety

Prepared under the

Assam Agribusiness & Rural Transformation Project (APART)

ARIAS Society, Khanapara, Guwahati

For

Dairy Development, Assam

Animal Husbandry & Veterinary Department, Govt. of Assam

By

International Livestock Research Institute (ILRI)

ARIAS Society
GOVERNMENT OF ASSAM
Assam Rural Infrastructure and Agricultural Services Society

ANIMAL HUSBANDRY & VETERINARY DEPARTMENT
Government of Assam

ILRI
INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE

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FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA
Inspiring Trust, Assuring Safe & Nutritious Food

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Preface

Milk has been recognized as an important part of our daily diet, across all cultures. It is considered to be a complete meal, as it contains all the essential nutrients and is consumed either directly or converted into desserts and sweets. However, it can also pose a serious threat to human health, if contaminated with microorganisms or adulterated with materials detrimental to health. Therefore, it is of prime importance that proper guidelines of milk testing are followed by all the value chain actors, at different levels, so that milk is safe for consumption to the end-user.

I would like to appreciate the sincere efforts of the International Livestock Research Institute (ILRI), a knowledge partner to the Department of Animal Husbandry, Veterinary and Dairy (AHVD), Govt. of Assam under the World Bank aided, Assam Agribusiness and Rural Transformation Project (APART), in developing the protocol for milk testing, to maintain safety and quality of the milk which, in turn, would ensure better health of the milk consumers.

The protocol, which includes all the tests as recommended by the Food Safety and Standard Authority of India (FSSAI), will be of great help to all the officers and technicians involved in milk testing, to follow a uniform guideline.

I am hopeful that this manual on '**Standard Laboratory Protocol on Testing Milk Samples for Quality & Safety**' will be of great help to everyone involved in the entire process of the milk value chain.

(Laya Madduri)

Rajesh Prasad, IAS
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Foreword

Milk is a good source of nutrients for human health but if it is adulterated with other external materials by milk value chain actors or if it is contaminated with germs or contains residues of antimicrobials, pesticides etc. it may cause health hazard to the consumers. Hence, it is important to test the milk supplied by both formal and informal sector for ensuring quality and safety of milk. Keeping this in perspective, the Dairy Development, Assam, under the World Bank aided Assam Agribusiness & Rural Transformation Project (APART), has been strengthening few laboratories that are lying attached with its dairy plants.

To support this initiative, International Livestock Research Institute (ILRI), a knowledge partner to Dairy Development, Assam under APART project, is coming up to train the laboratory officers and technicians to conduct the milk quality and safety tests as recommended by Food Safety and Standard Authority of India (FSSAI). In conducting the tests, it is important to ensure the quality and trustworthiness of the tests across the laboratories. In doing so, a test protocol becomes necessary so that all laboratory officers/ technicians across laboratories follow the same process of conducting the tests. In line of these requirements, ILRI has developed this test protocol of all the FSSAI recommended tests explaining the test procedures in simple steps in flow charts and stating clearly the method of interpretation of test results. This test protocol will help in avoiding individual level variation of conducting tests and interpreting test results across the laboratories.

I am sure this test protocol will be an important document for all the laboratories under Dairy Development, Assam. I congratulate ILRI for coming up with this test protocol.

(Rajesh Prasad)

Acknowledgement

We sincerely thank and acknowledge the guidance and support that we received from the Agriculture Production Commissioner (APC) to the Govt. of Assam; Commissioner & Secretary to the Govt. of Assam, Animal Husbandry & Veterinary Department (AHVD); State Project Director, ARIAS Society; Director, Nodal Officer (APART) and other officials of the Dairy Development (DDD), Assam and concerned officials of the ARIAS Society without which the compilation of tests protocol would not have been possible to complete.

We also gratefully acknowledge the support received from the local FSSAI officials at different stages of drafting the tests protocol.

Our sincere thanks also go to those mentioned under reference whose documents helped us in developing this test protocol.

Team Leader and Resident Consultant, APART-ILRI
International Livestock Research Institute (ILRI)

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Abbreviation

APART	: Assam Agribusiness & Rural Transformation Project
BTB	: Bromothymol Blue
CFU	: Colony Forming Unit
CB	: Crossbred
CLR	: Corrected Lactometer Reading
CMT	: California Mastitis Test
DDD	: Directorate Dairy Development
DMAB	: Dimethyl Amino Benzaldehyde
DPA	: Dipi Colinic Acid
FSSAI	: Food Safety and Standards Authority of India
FSSA	: Food Safety and Standards Act
GC	: Gas Chromatography
HCL	: Hydrochloric Acid
LOQ	: Limit of Quantification
MBRT	: Methylene Blue Dye Reduction Test
MFT	: Multiple Fermentation Tube
MRT	: Milk Ring Test
PCA	: Plate Count Agar
ROSA	: Rapid One Step Assay
RSM	: Reconstituted Skimmed Milk
SNF	: Solids-Not-Fat
TCA	:Trichloroacetic Acid
TYMC	:Total Yeast and Mold Counts

1. Introduction

Milk and milk products constitute an important source of nutrition for human. It contains several nutrients, including carbohydrates, lipids, protein, minerals, vitamins, etc. in a balanced form for building up and maintaining human and animal body. Milk is a product that can easily be converted into a number of different milk products or can be used as ingredient for other food items. However, being a highly perishable product its storage for long duration is constrained. It is an ideal medium for growth and proliferation of micro-organisms and in liquid form it is very easily contaminated by microbes. If it contains beneficial group of microbes i.e. *Lactobacillus* spp., it forms delicious curd but harmful microbes may cause milk spoilage and various foodborne diseases.

It is often seen that the milk value chain actors do not follow proper clean and hygienic measures for milk production, handling, storing and marketing. Besides, some of them also may adulterate the milk with various, sometimes harmful, substances or with substances with no nutritional value in lure of earning more profit out of the milk business. Some of them may also use preservatives for increasing shelf life of milk. Moreover, unknowingly or with the temptation of avoiding losses milk producers even extract milk from diseased animals/ animals under treatment. Similarly feed may be contaminated with different substances. In such situations milk can not only transmit diseases of microbial origin including zoonotic diseases (e.g. tuberculosis, brucellosis, leptospirosis, Q-fever etc.) but also may pass antimicrobial residue, pesticide residues etc. to human body. In other words, it may cause a serious hazard to human health.

Milk in the upper part of the udder of a healthy cow generally contains lower microbial load than the opening of teats. Usually, total bacterial count in milk from a healthy is below 50,000 per ml. It may go up to several millions because of poor and unhygienic conditions during milking and milk handling and/or in milk from diseased cow. Milk generally becomes sour 4-6 hours after keeping it at room temperature post milking. The onset of souring depends on quantity and quality of contamination and on milk temperature. The higher the number of lactic acid bacteria in milk, shorter the onset of souring (lowering of pH) and vice versa.

In the wake of potential health hazards through consuming poor quality and unsafe milk, it is of utmost importance to conduct different tests for assessing physical, microbiological and chemical quality of milk. Keeping this in view, the Food Safety and Standard Authority of India (FSSAI) has recommended conducting about 32 milk tests of which some are found very essential in Assam's context while some others are not. Also, to conduct some tests, good laboratory infrastructure and facilities are required while for some other tests minimum laboratory infrastructure and facilities are required.

To perform each test uniformly and perfectly by all laboratories under DDA, a standard test protocols required to be followed by each laboratory. Therefore, this protocol has been developed under the World Bank aided Assam Agribusiness and Rural Transformation Project (APART) to guide the Lab Manager as well as Lab Technicians in performing the tests and interpreting the test results.

This test protocol explains the procedure of conducting all the tests as mentioned in Table 1. For ease of understating of the test procedures, each step of conducting the tests are presented in the form of a flow chart.

Table-1: List of tests to be conducted for milk quality and safety

		Recommended by FSSAI	Milk quality relevance	Food safety relevance	Degree of difficulties to conduct
A. Physical tests					
1.	Sight and smell test/ Organoleptic test	√	++		+
2.	Extraneous matter	√	++		+
3.	Determination of fat in milk	√	++		++
4.	Determination of SNF in milk (Validated Method)	√	++		++
5.	Salt detection test	√	+		+
6.	Sugar detection test	√	+		+
7.	Maltodextrin detection test Enzymatic Method	√	+		+
8.	Starch detection test	√	+		+
9.	Urea detection test	√	+		+
10.	Neutralizers (Rosalic) detection test	√	+		+
11.	Method of Detection of Melamine in Milk and Milk Products	√		+	++++
12.	Detection of mineral oil in ghee (Holde's test)	√			+
13.	Butyrorefractometer (BR) Reading for Detection of Vegetable Fat	√			++
14.	Ammonium Sulphate detection test	√			+
15.	Synthetic milk detection test				+
16.	Glucose test (Validated Method)				+
17.	Test for presence of Boric acid and Borates (Validated Method)				+
B. Chemical tests					
18.	Determination of pH of milk				+
19.	Determination of Temperature of milk	√	++	++	+
20	Determination of Titratable Acidity as Lactic Acid	√	++		+
21.	Clot on boiling test	√	+		+
22.	Phosphatase test for pasteurized milk	√	+		++
C. Food safety controls					
23.	Test for presence of Hydrogen Peroxide (H_2O_2)	√	+	-	+
24.	Detection of Detergent residues	√		+	+

		Recommended by FSSAI	Milk quality relevance	Food safety relevance	Degree of difficulties to conduct
25.	Rapid test for detection of of aflatoxin M1 in milk	√		+	++
26.	Detection of heavy metal in milk	√		++	+
27.	Detection of Insecticide & pesticide residues in milk	√		+	++++
28.	Formalin test	√		+	+
D. Additional tests					
29.	Test for presence of skimmed milk powder in natural milk		+		++
30.	Test for Detection of Antibiotic residues		+	+	+++
E. Microbiological test procedures					
31.	Total Plate count or Standard plate count	√	++	+	++
32.	Yeast and Mould counts				++++
33.	Coliform count	√	++	++	+++
34.	*E. coli*	√	++	++	++++
35.	*Staphylococcus aureus*	√		+++	++++
36.	*Salmonella*	√			+++
37.	*Listeria monocytogenes*	√		+++	++++
38.	*Enterobacter sakazakii*				++++
39.	*Sulfite reducing clostridia*				++++
40.	*Bacillus cereus*				++++
42.	Milk ring test (MRT) for Brucellosis			+++	+
42.	California Mastitis Test (CMT) for Mastitis			+++	+
43.	MBR (Methylene Blue Dye Reduction) test for raw milk and pasteurized milk				+

Note:

Degree of relevance in regards to food safety:
Weak relevance= +
Medium relevance= ++
Strong relevance= +++

Degree of difficulties to conduct:
Least difficult=+
Difficult= ++
Fairly difficult= +++
Highly difficult= ++++

Milk tests are generally conducted by conventional methods that requires different equipment, consumables, chemicals, glassware, etc. These conventional methods are labour intensive and time consuming. Despite the fact, most of the milk testing laboratories follow conventional methods as per unit cost of the test is relatively lower and it is perceived that results derived from the conventional methods are more accurate. In this protocol, all milk tests are explained in conventional method under three different sub-heads as stated below.

- Reagents required
- Test procedure to be followed
- Interpretation of the results

In addition, some of the tests could be conducted by use of compact electronic machine (e.g. electronic milk analyser) or by rapid test using ready-made kits. These tests are conducted following the manufacturers' instructions. These tests give the results quickly. Only disadvantage is, laboratory becomes more dependent on outside supply of every test kit and cost of conducting each test may be relatively higher. Under this protocol, these rapid tests have also been mentioned in appropriate section.

Quality control of laboratory tests

This test protocol will also help in ensuring quality of the lab tests. If all the laboratories, under DDA follow the same test protocol, it is expected that the test results should be comparable. To crosscheck the test results from time to time, all the laboratories should use duplicate samples of the same sample to perform different tests. If all the laboratory shows similar results for each test, it can be considered that all the laboratories are performing well. If any laboratory shows significantly diverse result, it can be anticipated that there should be some problem in handling or performing the tests or interpreting the test result in that particular laboratory. In that case, the said laboratory should take necessary measures to correct their mistakes with support of the other laboratories.

Similarly, each Lab Manager can also check the quality of lab test in his/her laboratory by making duplicate samples of the same sample and can assign different identification no. to the duplicate samples. The Lab Technicians should be asked to conduct the test of each duplicate samples without informing him/her about the duplicity. If the results of all the duplicate samples are almost same, it can be interpreted that the Lab Technicians are performing the test properly. If any, significant deviation observed among the test results, it can be interpreted that there is some problem in conducting the test. Lab Manager should explore the reason behind of this deviation and address the problem.

Further, the Lab Manager should randomly conduct some tests using the same protocol to verify the test results. If he/she finds the same results with the Lab Technician it can be interpreted that the Lab Technician has performed the test well. If any deviation is observed between both the results, both the Lab Manager and Lab Technician should sit together to resolve the issue.

In addition, certain cases, DDA may send some samples outside DDA laboratories, e.g. to FSSAI laboratory, to verify the test results or may invite any professional agency to evaluate the lab system and suggest necessary changes.

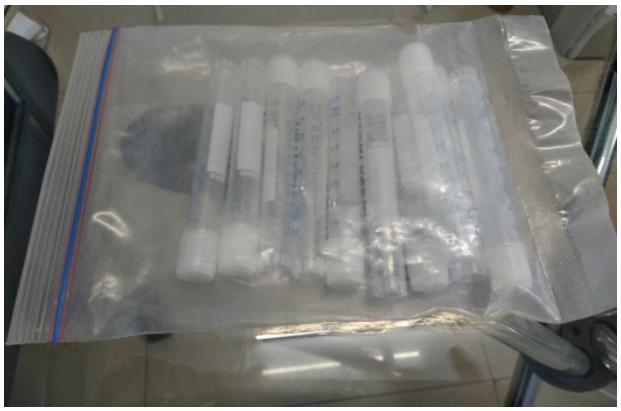
2. Method of sample collection, labeling, storing, and dispatching

The first step of lab operation is collection, transportation, handling and storage of milk samples following a standard process in order to maintain the quality of milk samples that is fit for testing purpose. In the following sections, a standard process has been explained for the lab and field staff.

2.1. General requirement for sample collection

Make arrangement of all necessary materials before going to sample collection. Please take the following items for collection of milk samples.

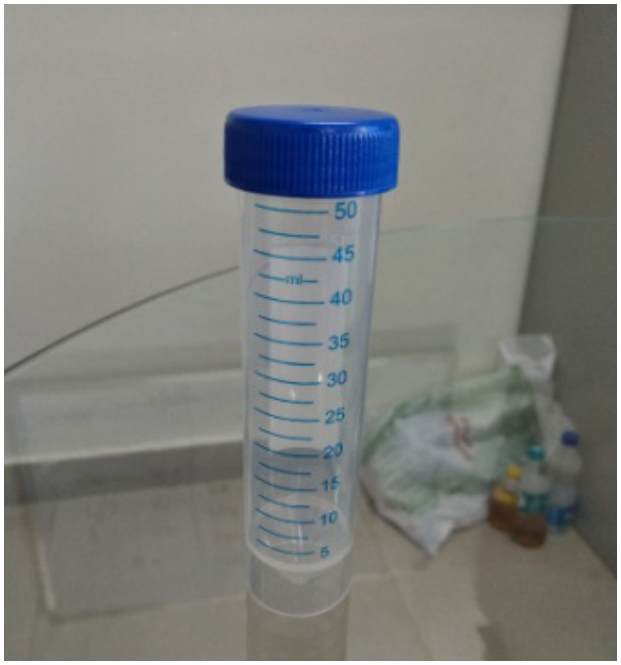
- Clean, dry, leak-proof, sterile container (mainly plastic) with graduation/calibration on the body and polythene zip bag;
- Glass Beakers , 100 ml;
- A plunger/ dipper;



Sterile containers in polythene zip bag

Marker

Glass Beakers



Leak-proof sterile container

Face mask

Apron

A milk sampling plunger

A milk sampling dipper

- A cool box/ thermos flask to carry the sample;
- Required ice/gel packs in cool box to keep the sample cool during transportation;
- Personal protective clothing like apron, gloves, mask, etc.;
- Sticker tags, marker, note pad, mask, sanitizers and biohazard bag;
- A disposal bag for carrying disposable materials like leftover milk, gloves, mask, etc.;
- A hand sanitizer to sanitize hands of the sample collector;
- A small weighing balance to weigh if milk products (e.g. paneer, curd, etc.) are to be collected;

2.2 Information to be collected along with the sample

The following information need to be collected from the household/source at the time of sample collection:

- Type of the sample (e.g. milk/curd /cream/others);
- Species: Cow/Buffalo;
- Type of animal: Exotic/ CB/Non descript;
- If milk sample is collected from the cow: Quarter of udder: L (left)/R(right): F(fore)/R(rear);
- If bulk milk is collected: Time of milking;
- Weight/ volume of the sample;

- Place of collection;
- Date and time of collection;
- Name and designation of the collector;
- Purpose of collecting the samples;
- Name, address and thumb impression/signature of the person from whom the sample has been taken.
- All samples should be marked with a unique sample number
- The above information shall be recorded against the specific sample number allocated to each sample collected and part of the information shall be supplied with the sample to the lab.

2.3 General considerations in sample collection, handling and storage

- The samples should never be touched with bare hands. Gloves and mask should always be used in the process of collection.
- Knife/dippers/plungers, instruments used for cutting, removing and manipulating samples (e.g. paneer, sweets, etc.) should be sterilized with hot water before and after use.
- Sample should not be exposed to dirty materials/environment after collection and should not be mixed with other biological samples.
- Temperature and pH shall be recorded at the collection stage and after transporting to the laboratory.
- Disinfect the surface of the work area before opening the samples for measuring, packaging, etc. at the laboratory
- Sample should preferably be measured directly in the sterile container with graduation;
- Gloves, mask and other materials in contact with the sample must be disposed properly.
- The stopper/cover of the container shall be securely fastened to prevent leakage of the contents in transit.

2.4 Method of milk sample collection from milk container

In order to collect milk sample for testing purpose, following methods should be followed

- Agitate the liquid milk thoroughly before sample is taken in order to make the contents of a milk container as homogenous as possible for obtaining a representative sample.
- Never agitate too vigorously because air bubbles, if dispersed in milk, will change its physical properties and disturb the analysis.
- Use a plunger or a dipper having a handle long enough for doing this and immediately take the sample of required volume into a sample bottle and close it.
- In order to make sure that a sample will well represent the whole contents of milk can take the half of the required sample from the lower portion and another half from upper part of the milk can.
- To take sample from a smaller milk container, turn the container upside down few times before sampling ensuring the container is closed well.
- Agitate the sample carefully again before the sample start to analysis in a laboratory.

2.5 Sampling from several containers

If milk needs to be collected from several containers, the following procedure shall be followed -

- Mix the content of each container thoroughly, take equal volume of milk from each container and pour into a small container.
- Take a sample after mixing the combined sample.

2.6 Sampling from storage tanks and rail and road milk tankers

If milk needs to be collected from a large tanker, the following procedure shall be followed-

- The method of sampling of milk from storage tanks and rail and road tankers is largely governed by storage/transport conditions.
- In all cases, the milk in the tank/tanker shall be thoroughly mixed by a sufficiently large plunger, a mechanical agitator or by compressed air; the uniformity of the samples being determined, when necessary, by mixing till such time as complete agreement is obtained between samples taken at the manhole and at the outlet cock in respect of fat and total milk solids.

2.7 Collecting milk sample directly from cow

To collect milk sample directly from a cow, the following procedure shall be followed-

- Ask the owner of the cow to clean the udder and teats of the cow thoroughly with water.
- Put on the clean gloves, face mask, apron, etc.

  Strip two to three streams of milk from each teat in order to flush the teat canal and thereby to reduce contamination risk.
- Dry teats thoroughly with an individual cloth towel, paying close attention particularly to the teat end.

- While holding the top of the teat steady, wipe the end of the teat well with an alcohol-soaked cotton ball. Use as many cotton balls as necessary until the cotton ball still looks clean after using.

- Open the milk vial and immediately take the sample, making sure not to touch the inside of the tube or bottom part of the lid. Hold the milk vial about 3 inches from the teat end and fill the tube half to three-quarters full of milk. Hold the vial at a 45 degree angle to prevent dirt from falling into the vial.

- Close the lid immediately and label the top with the date, cow number, and quarter sampled.

- Put the sample in cool box immediately.

Note: Do not place the teat inside the vial when sampling.

2.5 Sample collection, labelling and storing

- About 50-100 ml or gm of milk and milk products should be collected for testing purpose.
- Separate sterile container should be used for each sample.
- Immediately after collection of each sample, it should be properly labeled stating sample no., date of collect, time of collection etc.
- If sample is collected for regulatory purpose (as advised by Food safety Officer), a paper slip of the size that goes round completely from the bottom to top of the container, bearing the signature of the Designated Officer and number of the sample, shall be pasted on the wrapper, the signature or thumb impression of the person from whom the sample has been taken, shall be affixed in such a manner that the paper slip and the wrapper both carry a part of this signature or the thumb impression. The outer covering of the packet shall also be marked with the same number of the sample.
- The labeled container should immediately be transferred to the cool box/ thermos flask filled with ice packs.
- The collected container shall be properly secured and sealed so that no tempering is possible after collection. To ensure this, signature of the milk producer/trader/sweet maker and a witness should be taken on the sealed pack.
- All samples should be transported to the laboratory by maintaining cold chain in a cool box/thermos flask with gel/ice packs.
- After arriving the sample at the laboratory, a separate code should be assigned to each sample at the reception desk. The sample should be processed in the laboratory and results should be mentioned against that code only.
- No personal details of the owner of the sample should be supplied to the laboratory technicians who conduct the tests to avoid any potential pre-judgment by the lab technicians.
- The sample should be stored at 4°C if milk is tested within a day or two (maximum 96 hours).
- In case of preserving the sample for longer duration, samples need to be stored at (-)20°C

A deep freezer

2.6 Packaging and dispatching of samples to other laboratories

- For dispatching the samples to other laboratories, sample with their details should be put in a thermo-cool box. Adequate quantity of cool pack/gel pack should be put in the box to keep the sample cool during the time of transportation.

A cool box

Gel pack

- The outside of the thermo-cool box should be wrapped up with white paper and address of 'From' and 'To' should be clearly written on it preferably in all capital letters.
- A certificate should also be enclosed with the box stating the nature of the materials and purpose of sending.
- Packages should be marked clearly to provide information about the contents of package and, nature of the hazard, if any.
- Sample should be sent by the mode of transportation that can deliver the sample at the quickest possible time in the destination. If the transportation time is more, the ice/ gel pack may come to normal temperature and the sample may get spoiled. In order to avoid this, adequate no. of ice/ gel pack should be put in the thermos-cool box. There shall not be empty space inside the box as it will allow movement of samples as well as allow the gel/ice pack to get melted early.
- The thermo-cool box should be marked with 'Handle with care' and an 'Arrow mark' showing upside of the box, in order to guide the handlers during handling and transportation.

3. Physical Test

3.1 Sight and smell test (organoleptic test)

The sight and smell test of raw milk and milk products is done using normal senses of sight and smell in order to observe and record the overall quality. We get an instant result where and when it is carried out. If used correctly, it is very useful to do rapid screening of physical quality of milk. It is applicable on farms, during milk collection, at milk reception and at the milk processing plant. It is the first and basic test for judgement of qualities of milk and various milk products. This test, of course, should be complemented by further laboratory tests. When milk is tested by taste to judge the quality of milk there is a risk of disease transmission, and this is not recommended from a health point of view.

The sight and smell test should be carried out immediately after opening the lid of the milk can/ container by following means-

- Observe the colour, appearance, and cleanliness of milk
- Smell the milk just above the milk surface immediately after removal of the lid.
- Taste of milk is more permanent and easy to define than smell. Before tasting the milk, ensure that the raw milk is from healthy dairy animal. Do not perform the test using raw milk if it is not very essential because of risk associated with milk born zoonosis.

The following are the abnormalities that can be detected by organoleptic testing:

- Abnormal colour/consistency/ visible dirt and interpretation

Colour/consistency/ visible dirt	Interpretation
Pink colour	Contaminated with blood
Yellowish creamy colour	Colostrum or late milk
Thin creamy colour	Adulterated by adding water
Large clots or flakes	Sour milk or milk from cow suffering from mastitis
Small white clots or grains	Milk from cow suffering from mastitis or milk adulterated with flour and skim milk powder. Can also be early spoiling.
Visible dirt and impurities (fragments of straw, cow dung, etc.)	Milk produced under unhygienic conditions

- Off-flavours from feeds
  - ➢ Garlic, onion, beets, bad silage, certain plants and pastures can cause off-flavours to milk
- Absorption of off-flavours from air, milk containers etc.
  - ➢ It is well known that milk and cream can absorb smelling compounds from the air. This is caused by the ability of butter fat to absorb, especially after milking when the milk is warm, strong smells like paint, phenol, cresol, lysol, petroleum, etc. Strongly smelling paints, disinfectants and other chemicals should not be handled and stored in places where the dairy animals are kept and milked.

- Storage of milk together with fruits and fish also causes off-flavours to milk

- Abnormal smell and/or taste and interpretation

Smell and/or taste	Interpretation
Souring	Lactose fermenting, acid producing bacteria
Bitter	Peptonising of milk by *Streptococcus liquefaciens*
Blue souring	unpleasant sweet and sour smell, thin and waterish appearance caused by bacterial activity and storage in a closed container without ventilation
Fruit aroma	Pseudomonas producing esters
Slimy milk	Indicates capsule forming bacteria, e.g. *Aerobacter aerogenes* and *Alcaligenes viscosus*
Bubbles, coagulation and whey separation	Fermentation by yeast

Organoleptic test

In order to conduct organoleptic test, a test panel of milk tasters should be employed. The milk tasters should have good sense of sight, smell and taste of milk. No equipment is required for organoleptic testing. The milk tasters should test the milk and make observation on its taste and smell. Since raw milk testing may pose risk of zoonotic disease to human health, it is advisable to avoid organoleptic testing of raw milk, if it is not very essential.

3.2 Extraneous matter

Procedure

In order to find out extraneous matter in milk, the following method should be used

- Strain the milk sample;
- Allow the milk to settle in the container and observe at the top fatty layer as well as at the bottom of the container;
- The milk sample can also be centrifuged for few minutes to get the extraneous matter

Observations and interpretation

- Feed particle in milk: indicates poor management of feed and fodder
- Dung particle in milk :indicates uncleaned cow
- Dust particle in milk: indicates milk got exposed to dirty environment

3.3 Determination of fat in milk {Ref: IS-1224 (Part-I): 1997}

Fat is the most important component of milk. It provides more energy than the energy provided by carbohydrate (lactose) and proteins taken together. It imparts soft texture and creamy taste to milk products. It is the source of essential fatty acids and carrier for fat-soluble vitamins. Due to these reasons the producers and/ or sellers of milk and milk products are paid for their product on the fat basis. Therefore, determination of fat in milk and its products is an important exercise. *(Agrimoon,2015)*

Fat in milk is tested by two methods:

A. Conventional method by using Gerber centrifuge

B. Using a Digital milk analyzer

A. Conventional method by using Gerber centrifuge

Apparatus and reagents required

- Sulphuric acid (Specific Gravity 1.807 – 1.812 g/ml at 27^{0}C, colourless).
- Amyl alcohol (Specific Gravity 0.810 to 0.812) conforming to grade 1 of IS: 360:1964.
- Butyrometer 10% Scale
- Stoppers and shaker stands for butyrometers made from a suitable grade of rubber or plastics.
- 10 ml Acid pipette for sulphuric acid (with rubber suction device).
- 10.75 ml pipette for milk.
- 1 ml automatic measure for amyl alcohol
- Centrifuge, electric or hand driven (1400± 70 RPM)
- Water bath at 65 + 2^{0}C

A Butyrometer

Procedure

Transfer 10 ml of sulphuric acid into a butyrometer using a 10 ml acid pipette.

↓

Fill the 10.75 ml pipette with milk and deliver the sample into butyrometer.

↓

Add 1 ml of amyl alcohol using a 1 ml pipette and close.
Shake the butyrometer in the shaker stand until no white particles are seen and invert it a few times.

↓

Put the butyrometer in the water bath for 5 min.

↓

Take it out and dry with a cloth, put it in the centrifuge, placing two butyrometers diametrically opposite, centrifuge at maximum speed for 4 minutes.

↓

Transfer the butyrometers, stoppers downwards into water bath for 3-10 minutes.

↓

Bring lower end of fat column on to a main graduation mark by slightly withdrawing stopper.

Points to be considered:

- The colour of the fat should be straw yellow;
- The ends of the fat column should be clear and sharply defined;
- The fat column should be free from specks and sediment;
- The water just below the fat column should be perfectly clear;
- The fat should be within the graduation.

Interpretation

Note down the upper and lower scale readings corresponding to the lowest point of fat meniscus and surface of separation of fat and acid. The difference between the two readings gives the percentage by mass of fat in milk. The reading has to be done quickly before the milk cools.

Note: *The butyrometers should be emptied into a special container for the very corrosive acid-milk liquid, and the butyrometers should be washed in warm water and dried before the next use. Fat testing is often carried out on composite or random samples in order to reduce time and costs involved in testing.*

B. Using a Digital milk analyzer Milk fat is also measured by using a Digital milk analyzer. Put the milk sample in a container that fits into the Digital milk analyzer and place it in the machine. The analyzer will give instant results for fat percentage. Digital milk analyzer is also used for assessing SNF%, salt, sugar, added water, etc. in milk.

A Digital milk analyzer

Advantage of Digital milk analyzer:

- Gives instantaneous result
- A printed test results can be obtained in some machines
- Recurring cost is minimal
- Easy to perform the test

Disadvantage of Digital milk analyzer:

- Needs to buy a milk analyzer

3.4 Determination of SNF in milk

Solids not fat (SNF) are the solid contents present in milk excluding the fat content. Because of adoption of some unethical practices, like adding water to increase the volume of milk, the SNF content decreases in milk. So, in order to raise the SNF level in milk, cane sugar, starch, sulphate salt, etc. are added to milk. *(Agrimoon,2016)*

SNF in milk is tested by two methods as stated below:

A. Conventional method using Lactometer

B. Using a Digital milk analyzer

A. Conventional method using Lactometer

Apparatus required

- Lactometer
- Lactometer Jar
- Dairy thermometer

Procedure

Mix the milk sample thoroughly.

↓

Pour it into a dry cylinder which enables the lactometer to float without touching the sides.

↓

Let the lactometer into the cylinder.

↓

Allow the lactometer to remain steady in the milk. Take the reading from the lactometer as soon as it becomes stationary (within about 30 seconds).

↓

Note the corrected lactometer reading (CLR).

Calculation of SNF

$$SNF\% = \frac{CLR}{4} + 0.2F + 0.36$$

Where

CLR = Corrected lactometer reading.
F= Fat percentage in the milk sample.

B. Using a Digital milk analyzer

Procedure

Same as stated in estimation of fat % in milk at 3.3.B

3.5 Salt detection test (Ref: DGHS Manual)

Salt or sugar is used to mask extraneous water added to milk or to elevate total solids in milk. So it is important to detect presence of salt in milk.

Presence of salt in milk can be detected by two methods as stated below-

A. Conventional method

B. By using Milk Adulterants Kit

A. Conventional method

Apparatus and reagents required

- Test tubes
- 5% potassium chromate
- 0.1N silver nitrate
- Milk sample

Procedure

Take 2.0 ml of milk in a test tube

↓

Add 1.0 ml of 5% potassium chromate to the milk

↓

Add 2.0 ml of 0.1N silver nitrate to the test tube

Inference

Appearance of red precipitate indicates the absence of dissolved chloride in milk

Appearance of yellow colour indicates presence of dissolved chloride

B. By using Milk adulterants kit

These kits are commercially available in the market

Procedure

As instructed by the manufacturer.

Advantage of using Milk adulterants kit:

- It gives an instant result
- Easy to perform

3.6 Sugar detection test

The common sugar present in milk is lactose. Sometimes, table sugar like sucrose is added to the milk to increase the carbohydrate content of the milk and allow adulteration with water without detection during the lactometer test.

(vlab.amrita.edu, 2011).

Presence of sugar in milk can be detected by two methods as stated below:

A. Conventional method

B. By using Milk adulterants kit

A. Conventional method

Apparatus and reagents required

- Test tubes
- Concentrated hydrochloric acid
- Resorcinol
- Hot water bath
- Dilute HCl
- Milk sample

Under conventional method, there are two procedures to follow

Procedure -1: Concentrated hydrochloric acid method:

- Take 15ml of milk in a test tube and 1 ml of concentrated hydrochloric acid and 0.1 gm of resorcinol and mix.
- Place the tube in boiling water-bath for five minutes.

Inference

Brick red colour formation indicates sugar test positive

Procedure -2: Dilute hydrochloric acid method:

- Take 3 ml of milk in a test tube and add 5 ml dilute HCl (1:2) containing resorcinol (0.1 gm. resorcinol dissolved in 100 ml dilute HCl).
- Mix well and keep the test tube in boiling water for 5 minutes.

Inference

Brick red colour formation indicates sugar test positive

B. By using Milk adulterants kit

These kits are commercially available in the market.

Procedure

As instructed by the manufacturer.

Advantage of using Milk adulterants kit:

- It gives an instant result
- Easy to perform

3.7 Maltodextrin detection test:

Maltodextrin is a polysaccharide that is used to increase the volume of the foods like yoghurts, sauces, puddings, ice-cream, milk powders, cheeses, and also in indigenous milk products such as Burfi. Maltodextrin can cause several health problems. It is emphasized that milk should be tested for the presence of various adulterants including maltodextrin for the safety of the *consumers* Presence of Maltodextrin in milk can be detected by employing three different methods as stated below:

A. By using Conventional method (Enzymatic method),

B. By using Mid-infrared and ultrasound milk analyzer

C. By using reagent strips

A. Conventional Method (Enzymatic Method)

Apparatus and reagents required

- Glass beaker
- Lactic acid solution-1 ml (10% w/v to adjust the pH at 4 to 4.5).
- 1.0 ml of enzyme (0.20%w/v-200 mg/100 ml solution)
- Hot water bath
- Milk sample

Procedure

Take 20 ml milk in a beaker. Add lactic acid solution-1 ml (10% w/v).

↓

Add 1.0 ml of enzyme (0.20%w/v-200 mg/100 ml solution) and keep for 5 min at 62^{0}C.

↓

Immerse diastic in the curdled milk for 1 to 2 seconds and wipe of excess milk from the strip.

↓

Wait for 30 seconds and compare reagent area with colour chart and report the result as present or absent.

Inference :

Change in color of glucose strip indicating +ve (+, ++, +++ and ++++ as depicted on glucose testing strip bottle) after enzyme treatment, indicates presence of maltodextrin

B. By using Mid-infrared and ultrasound milk analyzer

Procedure: As instructed by the manufacturer

Advantage of using Mid-infrared and ultrasound milk analyzer:

- It gives instant result
- Easy to perform

C. By using reagent strips.

These strips are commercially available in the market.

Procedure

As instructed by the manufacturer

Interpretation

Visual observation of change in colour in the test strip as indicated by the manufacturer

Advantage of using Reagent strips**:**

- Reagent strips start working rapidly when dipped and gives result within 5-10 min.
- Easy to perform

3.8 Starch detection test: (IS-1479 (Part-I): 1997)

Starch is sometimes added to adulterate milk. Starch being cheaper, is sometimes added in the milk by adulterators to raise the S.N.F of milk.

(vlab.amrita.edu,2011).

Presence of Starch in milk can be detected by employing two different methods as stated below:

A. By using Conventional method

B. By using Mid-infrared and ultrasound milk analyzer

A. Conventional Method

Apparatus and reagents required

- Test tube
- 1% Iodine solution
- Milk sample

Procedure

Take 3 ml milk in a test tube, boil and cool under tap water.

↓

Add a drop of 1% Iodine solution.

Inference

Presence of starch is indicated by the appearance of a blue colour, which disappears when the sample is boiled and re-appears on cooling.

B. By using Mid-infrared and ultrasound milk analyzer

This milk analyzer is commercially available in the market.

Procedure

As instructed by the manufacturer

Advantage of using Mid-infrared and ultrasound milk analyzer:

- It gives an instant result
- Easy to perform

3.9 Urea detection test

Many unscrupulous vendors adulterate milk with urea which have harmful effect on human health like- indigestion, malfunctions of kidney, even cancers, etc. Urea is added in raw milk to enhance the nitrogen content resulting in a false appearance of a higher level of protein. The most widely practiced approach of adulterating milk is to add water in it and subsequently adding urea to raise SNF%. In addition, a small amount of urea is generally added into pure milk in order to increase shelf life of milk. In India, the addition of external urea to the milk is not permitted legally under the FSSA (2006).

(Researchgate,2000)

Presence of Urea in milk can be detected by employing three different methods as stated below:

A. By using conventional method

B. By using Milk adulterants kit

C. By using reagent strips

A. Conventional Method

Apparatus and reagents required

- Test tube
- Dimethyl aminobenzaldehyde (DMAB) solution
- Milk sample

Procedure

- Take 2 ml milk in a test tube, add 2 ml Dimethyl aminobenzaldehyde (DMAB) solution and mix the contents.

Inference:

Appearance of yellow colour indicates the presence of urea making the milk unacceptable

B. By using Milk adulterants kit

Procedure

As instructed by the manufacturer.

Advantage of using Milk adulterants kit:

- It gives an instant result
- Easy to perform

C. By using reagent strips

These reagent strips are commercially available in the market.

Procedure

As instructed by the manufacturer

Interpretation

Visual observation of change in colour in the test strip as indicated by the manufacturer
Advantage of using reagent strips**:**

- Reagent strips start working rapidly when dipped and gives result within 7-8 min. Development of pink color indicates the presence urea.
- Easy to perform

3.10 Neutralizers (Rosalic) detection test (IS-1479 (Part-I): 1997)

There is development of acidity in milk due to microbial growth, which increases under unhygienic conditions, poor milk quality, lack of proper facilities to transport milk from production centres to the processing plants soon after milking and non availability of cold storage ,etc. which reduces the shelf life of milk. Informal milk market actors add neutralizers like sodium carbonate, sodium bicarbonate, sodium hydroxide, calcium hydroxide, etc. which improve the shelf life of milk by neutralizing the developed acidity. Addition of neutralizers can cause increased mineral concentration in body fluids and soft organs leading to kidney stone development and commercial preparation of neutralizers might even be contaminated with heavy metals like arsenic, lead, etc. Continuous use of such milk and milk product may cause serious health hazards.

(J Food Sci Technol. 2015)

Presence of Neutralizers in milk can be detected by three methods as stated below:

A. Conventional method

B. By using Milk adulterants kit

C. By using reagent strips

A. Conventional method

Apparatus and reagents required

- Test tube
- Alcohol
- 1% (w/v) alcoholic solution of rosalic acid
- Rosalic acid solution (0.05% in 60:40 ethyl alcohol and distilled water, sp. gr. 0.91 g/ml)
- Milk sample

Procedure-1

Take 5 ml of milk in a test tube.

↓

Add 5 ml of alcohol into it.

↓

Add few drops of 1 % (w/v) alcoholic solution of rosalic acid and mix.

Procedure-2

Take 2 ml rosalic acid solution (0.05% in 60:40 ethyl alcohol and distilled water, sp. gr. 0.91 g/ml) in a test tube.

↓

Add 2 ml of milk.

Inference

If neutralizer is present, a rose red colour appears whereas pure milk shows only a brownish colouration

B. By using Milk adulterants kit

These strips are commercially available in the market.

Procedure

As instructed by the manufacturer

Advantage of using Milk adulterants kit:

- It gives an instant result
- Easy to perform

C. By using reagent strips

These strips are commercially available in the market.

Procedure

As instructed by the manufacturer

Interpretation

Visual observation of change in colour in the test strip as indicated by the manufacturer

Advantage of using reagent strips**:**

- Reagent strips start working rapidly when dipped and gives result within 5-10 min.
- Easy to perform

3.11 Method of Detection of Melamine in Milk and Milk Products

Melamine is a toxic triazine compound used as an adulterant in milk & milk related products to increase the protein content. Milk adulteration may lead to series of health problem like kidney failure and even death in many cases.

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Presence of Melamine in milk can be detected by two methods as stated below:

A. Conventional method

B. By using rapid test kit for melamine detection

A. Conventional method

This method should only be used after adequate training in an institute with experience on the machine.

Chemicals and reagents required

- Melamine (MEL). CAS #: 108-78-1.
- Acetonitrile (ACN.) LC grade.
- Formic acid. Reagent grade >95%.
- Water. LC grade, or purified by Millipore Milli-Q system to>18 Mohm resistivity, or equivalent.
- Ammonium Formate. Purity> 97%.

Preparation of solutions

- 0.1% Formic acid in water. 1ml formic acid is transferred to 1L graduated flask and diluted to volume with LC water.
- Mobile Phase A. 0.1% Formic acid in Acetonitrile (5:95 v/v). Mix 50 ml of 0.1% formic acid in water with 950 ml ACN in a 1 L solvent bottle.
- Mobile Phase B. 20 mM Ammonium Formate in Acetonitrile (50:50 v/v). Mix 500 ml of 20 mM ammonium formate and 500 ml of acetonitrile in a 1 L solvent bottle.
- 2.5% Formic acid in water. 25 ml formic acid is transferred to 1 L volumetric flask and diluted to volume with LC grade water.
- 20mM Ammonium formate. 0.63 gm of ammonium formate is weighed and dissolved in 0.5 L LC grade water.

Equipments required

- Liquid chromatograph. Binary LC pump is recommended for accurate mixing at low flow rate and rapid response to mobile phase gradient.
- Liquid chromatography column. ZIC-HILIC, 2.1 X 150mm, 5µm, 200 A
- Mass Spectrometer. Triple quadrupole capable of meeting system suitability.
- Centrifuge. Capable of 4000 RPM with 50 ml tubes.
- Microcentrifuge. Capable of 13,000 RPM with 1.5 or 2 ml tubes. (vi) Mixers and shakers. Single and multi tube vortex mixers (VWR), platform shaker.

- Utrasonic bath. Including timer and heater
- Centrifuge tubes. 50ml disposable polypropylene with caps, with graduations from 5 to 50 ml and 1.5 ml microcentrifuge tubes. (ix) Syringe Filters. Polyvinylidene fluoride (PVDF), 13mm, 0.22um (x) Syringes. Three ml polypropylene.

Procedure

i. Standard preparation

Individual stock solutions, melamine, approximately 100 µg/ml. Weigh approximately 10 mg of standard using a weigh boat to nearest 0.1 mg and transfer to a 100 ml glass volumetric flask.

↓

Add 70 ml 0.1% formic acid in water and sonicate for 10 minutes. Maintain the volume as 100ml with 0.1% formic acid in water and mix thoroughly

↓

Calculate exact concentration, correcting for purity

↓

Standard mixture dilution, 50 µg/ml is used for fortification and matrix calibration standards. Transfer 5.00 ml of each stock standard into a 20 ml glass scintillation vial.

ii. Sample preparation

Sample powder (2.0 ± 0.1gm) is weighed in a 50 mL polypropylene centrifuge tube

↓

Pre-fortify control and matrix calibration standards

↓

14 mL of 2.5% Formic acid in water is added to samples. Tube is tightly sealed. Dissolve sample by shaking for 15-30 seconds (vortex as needed), then sonicate in ultrasonic bath and mix on multi vortex mixer for 30 minutes each.

↓

Centrifuge at 4000 rpm for 10 minutes at room temperature.

↓

Approximately 1.4 mL of the supernatant is transferred into a 1.5 mL micro centrifuge tube

↓

Centrifuge at 13,200 rpm (16100 gm) for 30 minutes

↓

Load aqueous extract into a plastic 3 ml syringe and force through a 13mm, 0.22um PVDF filter into a micro centrifuge tube. Possible stopping point: aqueous extracts can be stored at 5-10°C for future dilutions

↓

Vortex mix for 30 seconds and centrifuge at 13200 rpm (16100gm) for 30 minutes

↓

Supernatant is transferred to a 2 mL autosampler vial, avoiding the precipitate

Instrumental analysis

- The column is equilibrated in Mobile Phase A at 0.4 ml/min for 30-60 min.
- It is necessary to evaluate system suitability. To do this, solvent blank (1x) and mixed standard are injected at 7.0 ng/ml (3-4x).
- Data should meet the signal-to-noise and ion ratio criteria before continuing.
- It is recommended to inject the standards and sample in following sequence: (i) solvent blank (Mobile Phase A), (ii) extracted matrix standards from 0.25 to 5 µg/g, (iii) solvent blank, (iv) control extracts, (v) post-fortified extracts and solvent standards for calculation of recoveries and matrix effects, (vi) solvent blank, (vii) unknown samples, and (viii) continuous calibration standards (an extracted matrix standard as well as solvent standard at 7 ng/ml), to verify that instrument response was maintained during the run.

Calculations

- Use external standard calibration. The calibration curve should not include the origin, but does include a matrix blank with a concentration of 0.
- Export the processed data into Microsoft Excel or equivalent spreadsheet program for further calculations:
- Recovery (%) = calculated from extracted calibration curve
- Matrix effect (%) = 100 x Post-fortified sample / solvent standard (same cone)
- The limit of quantification (LOQ) for each analyte is defined as the concentration of the lowest calibration standard used, or the lowest calibration standard which shows > 10-fold higher response than background signals in negative control sample.

Calculations for Confirmatory Analysis

Calculate ion ratios as percent relative abundances. The melamine ion ratio is m/z 68/85.

B. By using rapid test kit for melamine detection

This kit is commercially available in the market.

Procedure

As instructed by the manufacturer

Advantage of using melamine rapid test Kit:

- It gives an instant result
- Easy to perform

3.12 Detection of mineral oil in ghee (Holde's test)

Milk fat is a highly valuable and costlier product consumed throughout the world as it imparts good sensory and nutritional properties and also adds economy to the milk and other milk products. Therefore it is highly prone to adulteration with cheaper and inedible mineral oils etc.

Apparatus required Flat-bottom flask: 250 ml capacity fitted with reflux condenser

Reagents required

Alcoholic potassium hydroxide (4%):

> Dissolve 4 g of potassium hydroxide in approximately 10 ml water and make the final volume to 100 ml with ethanol (95%, v/v). The solution should be colourless or very pale yellow. Keep in a dark place.

Procedure

Take 2 ml rosalic acid solution (0.05% in 60:40 ethyl alcohol and distilled water, sp. gr. 0.91 g/ml) in a test tube.

↓

Cool and add 100 ml hot (just boiled) distilled water.

Inference

Appearance of turbidity indicates the presence of mineral oil

3.13 BR reading for detection of vegetable fat IS: 1479(part–1): 1997

Milk fat is a highly valuable and costlier product consumed throughout the world as it imparts good sensory and nutritional properties and also adds more value to milk and milk products. Therefore it is highly prone to adulteration with cheaper oils/fats such as vegetable oils, animal depot fats, hydrogenated fats and inter esterified fats.

Presence of vegetable fat in milk can be detected by two methods as stated below:

A. Conventional method

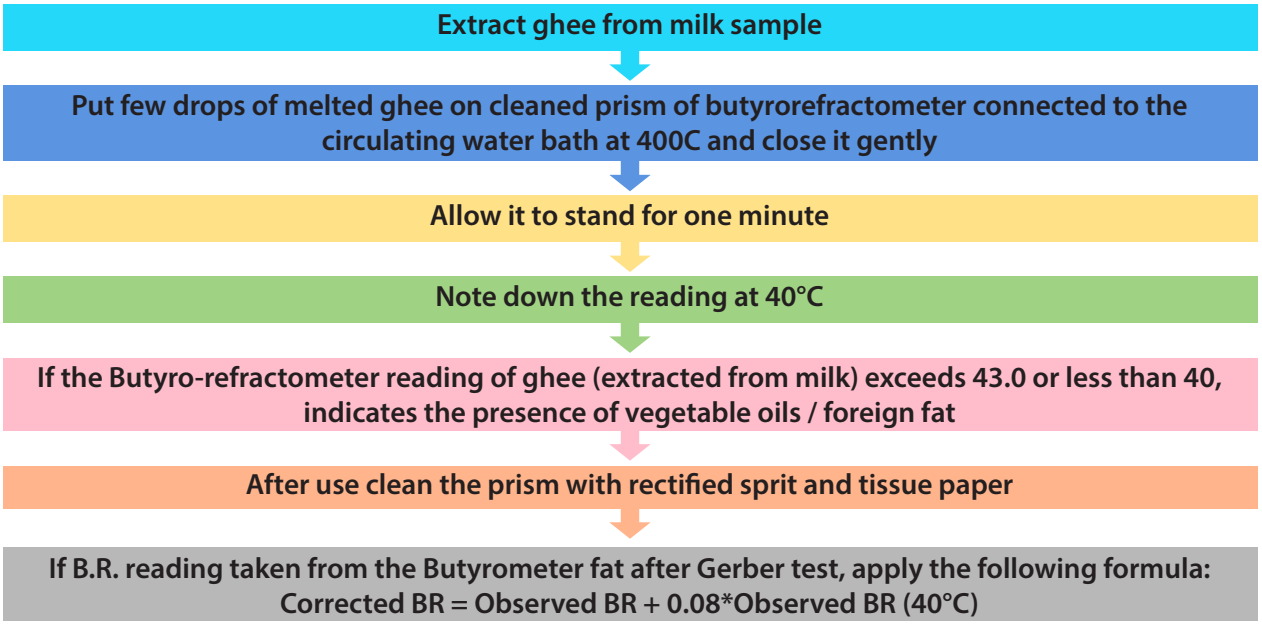
B. By using Milk adulterants kit

A. Conventional method

Apparatus and reagent required

- Butyrorefractometer
- Hot water bath
- Milk sample

Procedure



Note:

For homogenized milk, fat has to be extracted by the Gerber method using open-ended butyrometer. Observed B.R reading is to be corrected as follows to nullify hydrolytic effect of H_2SO_4 on the fat.

Corrected B.R. = Observed BR + 0.08 * observed BR

B. By using Milk adulterants kit

This kit is commercially available in the market.

Procedure

As instructed by the manufacturer

Advantage of using Milk adulterants kit:

- It gives an instant result.
- Easy to perform.

3.14 Ammonium sulphate detection test

Bovine milk contains around 80% casein and 20% whey proteins of high biological value. In general, fraudulent producers and traders of milk tries to increase the volume produced and delivered to the market by adding water, which alters its composition and reduces its nutritional quality. The reduction in protein concentration is one of the most significant effects. As a consequence, unethical producers and traders add nitrogen-rich compounds like ammonium sulphate to correct the apparent milk protein content. This practice is very dangerous for consumers leading to serious health hazards.

Presence of ammonium sulphate in milk can be detected by two methods as stated below:

A. By using barium chloride

B. By using Milk adulterants kit

A. By using barium chloride

Reagents and apparatus required:

- Barium chloride ($BaCl_2.2H_2O$) solution: 5% (w/v, aq.)
- Trichloroacetic acid (TCA): 24% (w/v, aq.).
- Milk Sample
- 50 ml stoppered test tube
- Whatman filter paper Grade 42

Procedure:

Take 10 ml of milk in a 50 ml stoppered test tube and add 10 ml of TCA solution

↓

Filter the coagulated milk through Whatman filter paper Grade 42

↓

Take 5 ml of clear filtrate and add 5 ml of barium chloride solution.

Inference:

Formation of milky-white precipitates indicates the presence of added sulfates like ammonium sulfate, sodium sulfate, zinc sulfate, magnesium sulphate, etc. to milk.

B. By using Milk adulterants kit

This kit is commercially available in the market.

Procedure

As instructed by the manufacturer

Advantage of using Milk adulterants kit:

- It gives an instant result
- Easy to perform

3.15 Synthetic milk detection test

Some milkmen remove milk fat and replace it with vegetable oil. However, vegetable oil is not miscible with milk, so they put detergent in it to make it miscible. The milk thus prepared is called synthetic milk.

Conventional method:

Reagents required

- One test tube
- Urease (20 mg. per ml)
- 0.5% BTB solution (bromothymol blue)

Procedure

Take 5 ml milk in a test tube and add 0.2ml urease (20 mg. per ml)

↓

Shake well and then add 0.1 ml of BTB solution (0.5%)

↓

The methods for detection of urea and synthetic milk are same; the only difference is appearance of dark blue colour in case of synthetic milk and yellow colour in case of urea.

Inference

Appearance of dark blue colour indicates the presence of synthetic milk

3.16 Glucose test (Validated Method)

Presence of Glucose in milk can be detected by three methods as stated below:

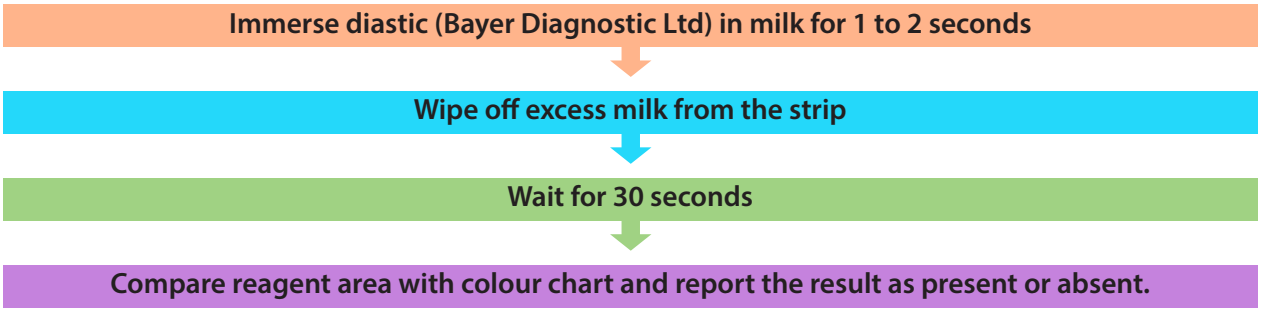
A. Conventional method

B. By using Milk Adulterants Kit

C. By using reagent strips

A. Conventional method

Procedure



B. By using Milk adulterants kit

This kit is commercially available in the market.

Procedure

As instructed by the manufacturer

Advantage of using Milk adulterants kit:

- It gives an instant result
- Easy to perform

C. By using reagent strips

These strips are commercially available in the market.

Procedure

As instructed by the manufacturer

Interpretation

Reagent strips start working rapidly when dipped in milk and gives a result within 3-4 min. Visual observation of change in colour in the test strip as indicated by the manufacturer

Advantage of using reagent strips:

- It gives an instant result
- Easy to perform

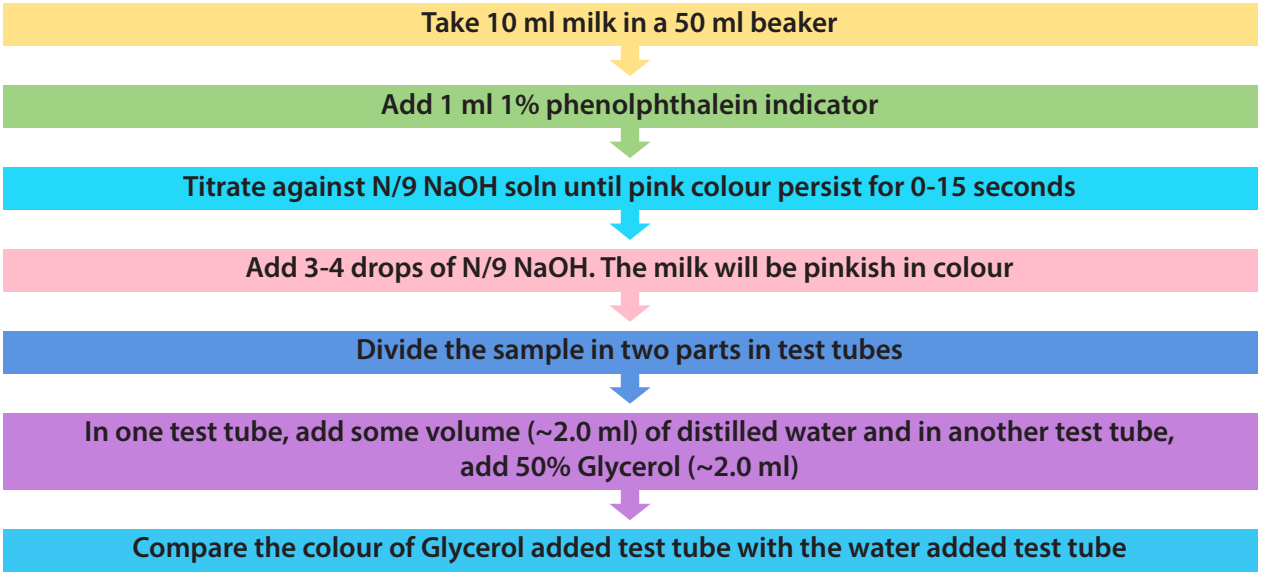
3.17 Test for presence of boric acid and borates (Validated Method)

Presence of boric acid and borates can be detected in milk by conventional method.

Apparatus and reagents required

- 50 ml beaker
- Two test tubes
- 1% phenolphthalein indicator
- N/9 NaOH solution
- Distilled water
- 50% Glycerol
- Milk sample

Procedure (Glycerol method)



Inference

The test tube added with glycerol will be faded or pink colour will disappear indicates the presence of boric acid i.e. boric acid Positive

Detection Level: 0.02% (200 ppm)

4. Chemical Tests

4.1 Determination of pH in milk

The measurement of pH in milk is important in testing for impurities, spoilage, and signs of mastitis infection. Milk is slightly acidic or close to neutral pH. For all species, milk with colostrum has a lower pH and mastitic milk has a higher pH. Fresh milk has a pH value of 6.7. When the pH value of the milk falls below pH 6.7, it typically indicates spoilage by bacterial degradation. Eventually, when the milk reaches an acidic enough pH, coagulation or curdling will occur along with the characteristic smell and taste of "sour " milk. Mastitis is an ever-present challenge with dairy milking cows. A pH measurement offers a quick way to screen for infection of the udder

Determination of pH of milk can be me done by two methods as stated below:

A. By using indicator strips

B. By using pH meter

A. By using indicator strips

Procedure

Indicator paper strips are made by soaking strips of absorbent paper in a suitable indicator and drying them

↓

A rough estimate of pH is obtained by dipping a strip of the prepared paper in milk and observing the colour

↓

Bromocresol purple (pH range 5.2 to 6.8 – colour changes from yellow to purple) and bromothymol blue (pH range 6.0 to 7.6 – colour changes from straw-yellow to bluish-green) are commonly used as indicators

↓

Both narrow and wide range ready-made indicator papers are available commercially over the pH range 2.0 to 10.5

Note: *Indicator paper strips shall always be kept in a closed glass bottle and in a dry condition. These strips are commercially available in the market.*

B. By using pH meter

Take 50 ml of milk sample at 30°C in a 100 ml glass beaker

↓

Measure the pH of milk with help of a calibrated pH meter (calibrated with a standard buffer of known pH value i.e. pH 7.0 or 9.2) by dipping the electrode in the beaker

↓

Read the pH of the milk after 30 sec

Inference

- On an average cow milk gives a pH of 6.7and buffalo milk 6.8.
- Milk of pH below 6.7 should be considered suspected as an indication of some diseases of the udder or of late lactation milk.
- Alkaline pH of the milk may also indicate neutralization of milk by NaOH, Na2CO3, and NaHCO3 etc.

4.2 Determination of temperature of milk

Materials required

- Standard calibrated thermometer
- Milk sample

Procedure

Determine the temperature of milk with a standard calibrated thermometer. Bulk raw milk, when received from a chilling centre in the dairy factory shall not have temperature above 7°C.

4.3 Determination of titratable acidity as lactic acid (Ref: 79 (Part-I): 1997)

The titratable acidity test is employed to ascertain if milk is of such a high acidity so as to reduce its keeping quality and heat stability. Generally the acidity of milk means the total acidity (Natural + developed) or titratable acidity. It is determined by titrating a known volume of milk with a standard alkali .

Determination of titratable acidity of milk as Lactic Acid can be me done by two methods as stated below:

A. Conventional method

B. By using paper strip test with color comparator

A. Conventional method Apparatus and materials required

- 100 ml conical flask
- Distilled water
- Phenolphthalein indicator
- N/10 NaOH
- Milk sample

Procedure

Take 10 ml milk in 100 ml conical flask, add 10 ml distilled water

↓

Add 1ml phenolphthalein indicator and titrate against N/10 NaOH till a faint pink colour appears

↓

Calculate the acidity % as volume of NaOH used×0.09

Calculation

Titratable acidity % (as lactic acid) = $9V_1N/V_2$

Where

- V_1 = Volume in ml of the standard sodium hydroxide required for titration,
- N = Normality of the standard sodium hydroxide solution, and
- V_2 = Volume in ml of milk taken for the test

B. By using paper strip test with color comparator

These strips are commercially available in the market.

Procedure: As per the instruction given within the kit

Advantage of using Milk Adulterant Kit :

- It gives an instant result.
- Easy to perform.

4.4 Clot on boiling test (IS-1479 (Part-I): 1997) *

This test is done for assessment of keeping quality of milk. Formation of clot means milk is no longer marketable.

Apparatus and materials required

- Test tube
- Water bath
- Spirit lamp
- Milk sample

Procedure

Transfer 5 ml of the sample to the test tube and smell for any acidic flavour

↓

Place the tube in a boiling water bath and hold for about 5 minutes, and smell again for any acidic flavour

↓

Remove the tube from the water bath and rotate it in an almost horizontal position and examine the film of milk or side of the test tube for any precipitated particles

↓

Alternately, take 5 ml of milk in a test tube, boil on the flame of a spirit lamp and examine the film of milk or side of the test tube for any precipitated particles

Inference

Formation of clots in the test tube indicates COB positive milk and is unacceptable

4.5 Phosphatase test for pasteurized milk IS: 1479 (Part II): 1961

Pasteurisation is an essential process of making milk safe and free from pathogens. Alkaline phosphatase is an enzyme which is naturally present in milk, but is destroyed at a temperature just near to the pasteurization temperature. Alkaline phosphatase test is used to indicate whether milk has been adequately pasteurised or whether it has been contaminated with raw milk after pasteurisation. The test is not applicable to sour milk and milk preserved with chemical preservatives.

Apparatus required

- Lovibond All-Purpose Comparator with stand
- Standard Discs - giving 0, 6, 10, 18, 42 or 0, 6, 10, 14, 18, 25, 42 readings.
- Fused Glass cells - 25 mm.
- Test-Tubes - 15 x 1.9 cm, fitted with rubber stoppers.

Reagents required

- **Buffer solution** - 3-5 g of sodium carbonate analytical reagent grade (see IS: 296-1951), and 1.5 g of sodium bicarbonate analytical reagent grade (see IS: 491-1954) dissolved in 1ltr of water.
- **Substrate** - disodium p-nitrophenylphosphate not less than 95 percent pure.
- **Buffer substrate** - Transfer 0.15 g of the substrate into a 100 ml measuring cylinder or stoppered graduated flask and make up to the mark with the buffer solution. The solution should not be stored for long periods but may normally be kept in a refrigerator for up to one week.

The solution is practically colourless; when viewed through a 25 mm cell in the all-purpose comparator, it should give a reading of less than 10 on the disc.

Procedure

Take 10 ml of the buffer substrate solution into test-tubes marked at 10 ml and bring to 37 to 38°C in a water-bath

↓

Add 2 ml (one ml if 5 ml of buffer substrate are used) of the milk to be tested, close the tubes with rubber stoppers and invert to mix

↓

Prepare in the same way a blank from a boiled milk of the same type as that under test. Incubate all the tubes at 37-38°C

↓

Read the yellow colour after 30 minutes, return to the bath, and take a second reading after incubation for a further 90 minutes

↓

The yellow colour is read in a Lovibond all-purpose comparator on a resazurin stand, fitted with the disc calibrated in microgram p-nitrophenol

↓

Blank should be placed on the left of the stand and the sample on the right

↓

Readings are taken by looking down on to the two apertures with the comparator facing a good source of north daylight; the disc is revolved until the sample is matched; readings falling between two standards are recorded to the nearest reading

Interpretation of results

Disc reading after 30 minutes incubation	Interpretation
0 or trace 6	Properly pasteurized Doubtful
10 or over	Under pasteurized
Disc reading after 2 hours incubation	
0 to 10	Properly pasteurized
Over 10	Under pasteurized

The 30-minute test will reveal any serious fault in pasteurization, but to enable minor errors to be detected, readings shall be taken after further incubation for 90 minutes.

5. Food Safety Control Tests

5.1 Test for presence of hydrogen peroxide (H_2O_2)

(Ref: A.O.A.C 17th edn, 2000 Official Method 957.08 Hydrogen Peroxide in milk)

Hydrogen peroxide is used as a sanitizing of milk handling equipment. The presence of hydrogen peroxide can contaminate the milk. The purpose of the test is to detect any traces inside the product. This test also allows to check the possible addition of hydrogen peroxide in raw milk, before pasteurization, to increase its shelf-life.

Presence of hydrogen peroxide in milk can be detected by three methods as stated below:

A. Rapid test for detection of presence of hydrogen peroxide (H2O2)

B. By using potassium iodide and starch method

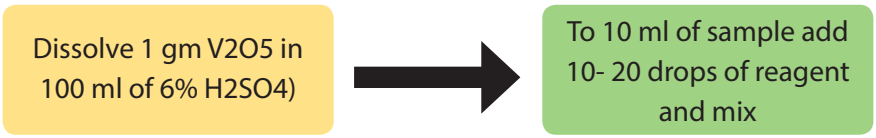
C. By using reagent strips

A. Rapid test for detection of presence of hydrogen peroxide (H_2O_2)

Apparatus and reagents required

- Beaker
- Venedium pentoxide (V_2O_5)
- 6% H_2SO_4

Procedure



Inference

The development of pink or red colour indicates presence of H_2O_2

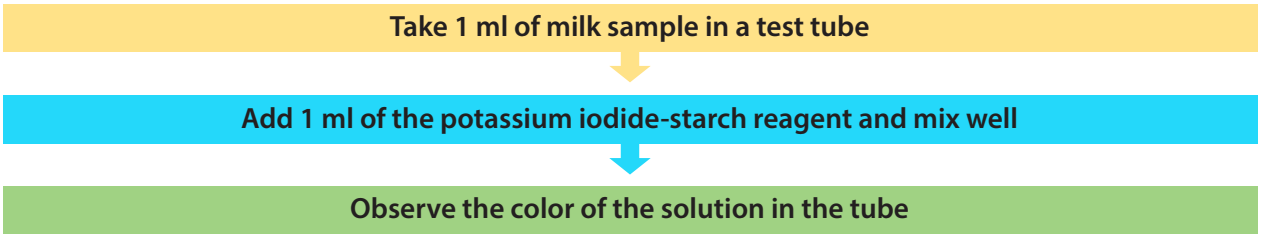
B. By using potassium iodide and starch method

Preparation of milk sample for analysis

Reagents required

- Potassium iodide solution: Weigh 20 g of potassium iodide and dissolve it in distilled water to obtain a 100 ml solution.
- Starch solution: Take 1 g starch powder and dissolve it in distilled water by heating and make up the volume to 100 ml.
- Potassium iodide–starch reagent: Mix equal volumes of 20% potassium iodide solution and 1% starch solution.

Procedure



Inference

Blue color will be developed in the presence of H_2O_2, whereas pure milk sample remain white in color

C. By using reagent strip

These strips are commercially available in the market.

Procedure

As instructed by the manufacturer

Interpretation

Reagent strips start working rapidly when dipped in milk and gives a result within 30-60 seconds. Visual observation of change in colour in the test strip as indicated by the manufacturer

Advantage of using reagent strips:

- It gives an instant result
- Easy to perform

5.2 Detection of detergent residues

Detergents are used for a different purpose. Some milk value chain actors remove milk fat and replace it with vegetable oil. However, vegetable oil is not miscible with milk, so they put detergent in it to make it miscible. The milk thus prepared is called synthetic milk. Detergents are used most widely in such milk-like preparations due to their low cost and ease in availability.

Presence of detergent in milk can be detected by two methods as stated below:

A. Conventional method employing methylene blue test

B. By using detergent detector

A. Conventional method employing methylene blue test

Reagents required:

- Methylene blue dye
- Chloroform

Reagent(s) preparation:

- Methylene blue dye - 12.5 mg is dissolved in 100 ml of distilled water. Protect the solution against direct sunlight.
- Chloroform (Inflammable and toxic on inhalation. Mouth pipetting is not recommended).

Procedure

Pipette 1 ml of suspected milk sample into a 15 ml test tube

↓

Add 1 ml of dye solution followed by addition of 2 ml chloroform

↓

Vortex the contents for about 15 sec and centrifuge at about 1100 rpm for 3 min

↓

Note the intensity of blue color in lower and upper layer

Inference

Relatively, more intense blue color in lower layer indicates presence of detergent in milk. Relatively more intense blue color in upper layer indicates absence of detergent in milk (as mentioned in below Fig.).

Limits of detection:

The method can detect presence of 12.5 mg of laboratory grade detergent (labolene) in 100 ml of milk sample. Thus limit of detection of method is 0.0125% labolene in milk.

Fig. : Comparison of developed color in presence and absence of detergent in milk. Relatively more blue color in lower layer (right side tube) than upper layer indicates presence of detergent in milk.

Note: *The method is capable of detecting all the commonly available detergents in different brand names such as Ezee, Safe Wash, Super Nirma, Rin Advanced, Rin Shakti, Rin (Powder), Tide (Powder), Nip (Powder), Clinic Plus, Sunsilk, Pentene, Head & Shoulder etc. The efficacy of the method is not affected in presence of other additives viz., urea, sucrose, glucose, starch, formalin, hydrogen peroxide and neutralizers etc. In presence of high concentration of neutralizers, lower layer may appear pinkish. It has been observed that defatted milk provides better clarity in results. Defatted milk can be prepared by centrifuging milk at 15°C for 10 minute followed by removal of fat accumulated at top surface of milk present in centrifuge tube. In the literature, bromocresole purple test has been mentioned. However, the test has not worked in authors' laboratory even in the presence of 1% detergent in milk.*

B. By using detergent detector

This detector is commercially available in the market.

Procedure

As instructed by the manufacturer

Advantage :

- It gives an instant result
- Easy to perform

5.3 Rapid test for detection of aflatoxin M1 in milk

Aflatoxin is a kind of toxins produced by certain fungi (*Aspergillus* spp.) which are generally found in agricultural crops and by products like maize, wheat bran, rice bran, rice polish, hay, paddy straw, etc., mainly when feeds and fodder are stored in a damp condition and gets mouldy. When dairy animals are fed with these crops, crop residues and by-products contaminated with aflatoxins, the toxin is secreted through milk, eventually affecting the consumers of this contaminated milk. The toxin is carcinogenic in nature, which means they can cause cancer. Very high doses of aflatoxins cause acute aflatoxicosis, which leads to acute liver failure, jaundice, lethargy and nausea, eventually leading to death, according to a World Health Organization (WHO) study.

Presence of Aflatoxin in milk can be detected by multiple methods, of which the three easiest methods are:

A. By using Charm EZ platform

B. By using Rapid One Step Assay (ROSA)

C. By using rapid test kit

A. Using Charm EZ platform

Charm EZ is very small portable machine and it can give quantitative results of presence of aflatoxins within 10-60 min depending on types of aflatoxins present in the milk sample.

This machine is a complete simultaneous incubator and analyzer system specially designed for use in the new ultra-rapid EZ tests, and is also compatible with most Charm 3 and Charm Rosa test strips. Results are interpreted based on a numerical scale and are stored in the Charm EZ memory. Data can be printed, displayed on the Charm EZ screen or, downloaded to computer system in a real time or post analysis mode.

It is a platform connecting with a customized printer in which printed form of results can be obtained instantly. Manufacturer/Supplier can send their technical person to train the laboratory staff on how to operate the machine.

Requirement

- Charm EZ platform
- Calibrator
- Aflatoxin M1 specific Strips (It is a readymade one and needs to be procured from the manufacturer). No other consumables are required for the test.

Precaution

- Sample must be maintained at 2-80 C
- Milk fat in the sample must be kept at below 5%
- Strips should be brought to room temperature before use
- Calibration check must be done
- Look for spillage of milk in the compartment where strip is put

Calibration of the machine

Calibration to be done before conducting any test

Calibration check

Daily performance monitoring is to verify that the equipment is performing properly.

Procedure

Step 1:

Go to the main menu → Press the Perf Mon button

Step 2:

Insert the calibrator strip with the Low calibrator (Dark Pink) side facing down

Close the door

Press the OK button on the screen

Step 3:

After evaluating the strip, the Charm EZ will report the result with the high and low limits → Press the OK button when ready to proceed

Step 4:

Insert the calibrator strip with the high calibrator side (Light Pink) side facing down

Close the door

Press the OK button on the screen

Step 5:

After evaluating the strip, the Charm EZ will report result with the high and low limits → Press the OK button when ready to proceed

Step 6:

When completed, Press the OK button when ready to proceed

Step 7:

If calibrator strip fails to read within specified range, see "Cleaning the Reader Lens" section or contact the Technical Services Staff at Charm Sciences.

Temp adjustment

The temperature requirement is specific to aflatoxin M1 residue, hence the temperature must be adjusted prior to conducting the test .

Actual Procedure

Before starting the test, put any one of the strips in the machine without closing the door

↓

Check the incubation temperature

↓

Orange colour indicator turns to green after sometime and show the incubation temperature in green

↓

Then, start the work by putting water in the strip

↓

This is required to check the incubation temperature , especially , if it is different from the each other as the machine needs to be adjusted with the specific temperature required for aflatoxin M1

Results

The results will be displayed on the monitor of the machine. As it can be connected to the printer and the print out can be taken out for record keeping.

B. By using Rapid One Step Assay (ROSA)

Apparatus and Reagents

- Rosa incubator
- ROSA strip
- Dilution buffer
- Milk sample

Test procedure

Place the strip in the incubator and the peel off the tape

↓

For testing of aflatoxin M1 in milk, mix equal amount of milk sample and dilution buffer and kept at 4 oC

↓

Add 300 µl of milk sample carefully into Sample loading space and release the tape

↓

Close the lid and tighten the latch; incubate the sample for 8 minutes

↓

Remove the strip after incubation and inspect the C (control) line visually for even development and afterwards , read the strips on respective channel

↓

Quantify antibiotic residues in milk sample based on the standard curve

C. By using rapid test kit

This kit is commercially available in the market.

Procedure

As instructed by the manufacturer

Advantage of using rapid test kit:

- It gives an instant result.
- Easy to perform.

5.4 Detection of heavy metal in milk

Heavy metal pollution is very much a concern because of their toxicity for animal and human beings and their lack of biodegradability. Heavy metal toxicity is linked with a number of diseases, mainly due to chronic exposures. Heavy metals in milk, which is the basic food item of vulnerable age group of people is of particular concern. Contamination of environment leads to contamination of food chain, which is the main route of entry of heavy metal in the animal body, which ultimately causes contamination of milk and animal originated food.

Testing is done by conventional method, and lab technicians needs adequate training on the instrument.

Acid digestion of milk

Apparatus and reagents required

- Test tubes
- 10% HNO3 solution
- Distilled water
- Hydrogen peroxide (H2O2)
- Hot plate
- Flame Atomic Absorption Spectrophotometer

Procedure

All glassware shall be first cleaned with 10% HNO3 solution and then further washed with the distilled water

↓

Digest 10 mL milk with 1:3 of H2O2 and HNO3 on a hot plate

↓

Heat the samples on hot plate until their volume reduces to 2 ml

↓

Dilute this 2 mL with 20 mL of distilled water and make a clear solution of it and brought to the required volume with distilled water

↓

Examine by Flame Atomic Absorption Spectrophotometer

The method covers Fe, Ni, Cu, Cd, Zn, Mn, Cr and Pb.

3 different concentrations, 1.0 ppm, 1.5 ppm and 2.0 ppm, shall be prepared to calibrate the Flame AAS.

The resultant calibration curve of well-prepared standard concentrations results in a linear curve by Atomic Absorption Spectrophotometric Analysis

Data analysis

Data was gathered and ordered in tables. The concentrations of milk samples in ppm yielding positive results for the occurrence of heavy metals were transformed into concentration in mg/kg (ppm).

5.5 Detection of insecticide & pesticide residues in milk

A pesticide is a substance used for killing pests (insects, fungi, herbs and nematodes) harmful to cultivated crops. Residues of pesticides and their metabolites can be found in milk due to contamination of water, use of pesticides in the control of ectoparasites directly in the animal, consumption of contaminated pastures and/or ration with pesticides and these cause a wide range of toxic effects in human beings. The presence of these compounds in milk may pose a potential threat to public health.

Paper strip for rapid detection of pesticide residues in milk (Patent Reg. no 2213/DEL/2014)

A. Extraction of pesticide from milk

Extraction of pesticide from milk: Pesticide are extracted from spiked reconstituted skimmed milk (RSM) / natural milk sample as per following protocol:

Procedure

Mix equal quantity of milk sample and organic solvent, vortex and centrifuge @ 10000 rpm for 5 min at 37 oC

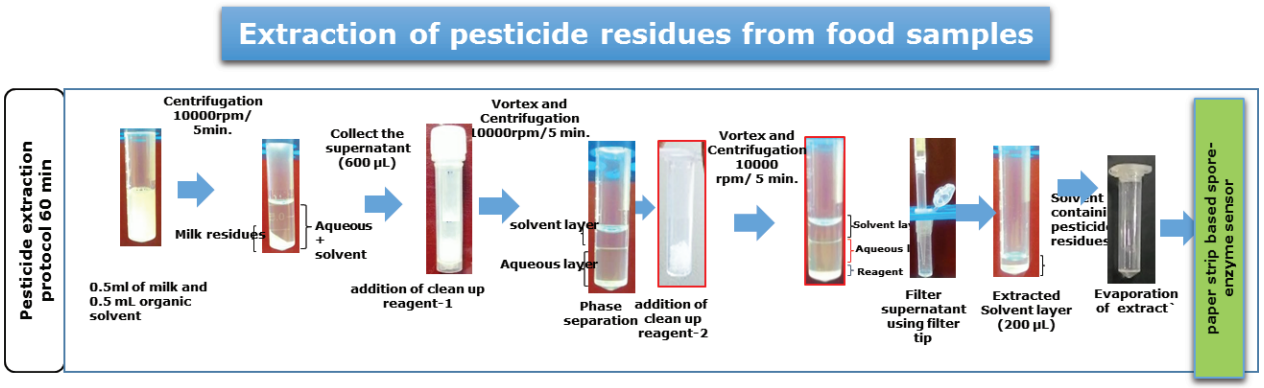
Mix supernatant with clean up reagent (I), vortex and centrifuge @ 10000 rpm for 5 min at 37°C

Transfer solvent layer carefully to a tube containing cleanup reagent (II), vortex and centrifuge @ 10000 rpm for 5 min at 37°C

Separate out upper organic solvent layer and filter through specific filter tips

Evaporate filtrate using block heater at 80°C for 40 min

The tube containing pesticide residue (Tube-2) is used to carry out paper-strip assay.



Stepwise extraction procedure for pesticide residues in milk

B. Paper-strip protocol

Enzyme pesticide interaction: Transfer reconstituted spores from Tube-1 to Tube-2 containing evaporated pesticide residues from extracts of spiked / natural milk sample and incubate in dry block heater at 37 °C for 40 min and vortex for 25 sec

Addition of paper strip: Add paper-strip functionalized with chromogen to test and control tube and incubate in dry block heater at 37°C for 15-20 min. After incubation, paper strips were air dried for 5 min after color development in control tubes

Result interpretation

Development of sky blue color on paper strip, indicates absence of pesticide and no blue color indicates presence of pesticide in milk as depicted in below mentioned figure.

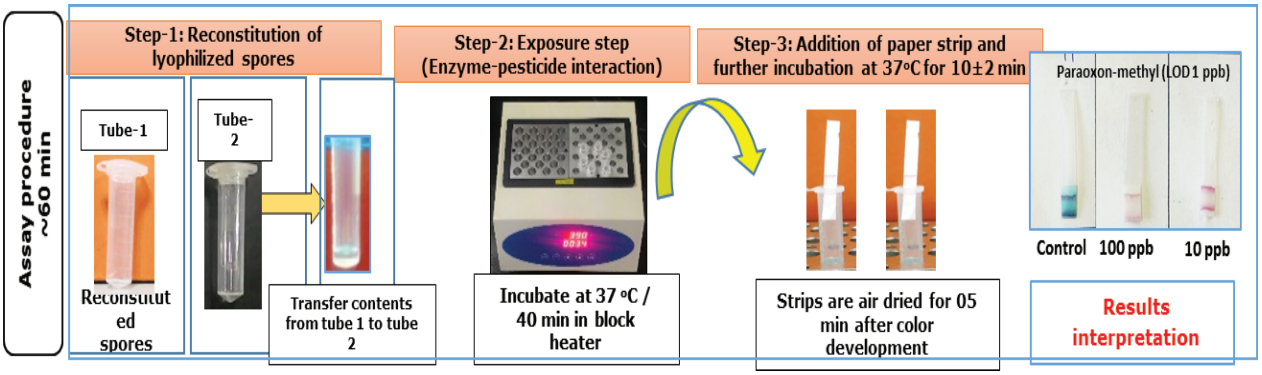


Fig: Stepwise assay procedure of paper strip assay for detection of pesticide residues in milk

GC procedure for confirmation / quantification of pesticide residues in milk

Sample preparation protocol for GC (Gas Chromatography)

This method should only be used after adequate training in an institute with experience on the machine.

5mL of milk sample was taken in dry mortar, add 20g of silica gel and 15g of anhydrous sodium sulphate and mix using pestle to make free flowing powder

Prepare a glass column by plugging glass wool and add 40mL of Dichloromethane (DCM)

Transfer the prepared content from step-1 into glass column. Allow to stand for 90 min

Elute DCM drop wise @ 20 drops/ min. Again eluate the column using 150ml of acetone and DCM in the ratio of 2:1 (v/v)

Elute the solvent again and evaporate by keeping in fume board till the volume get concentrated

Dissolve the concentrate obtained using n-hexane by thorough shaking and again evaporate by keeping in fume board to ensure complete removal of DCM

The obtained extract or concentrate is reconstituted using 5mL of n-hexane and acetone in the ratio of 1:1 and used for further GC analysis

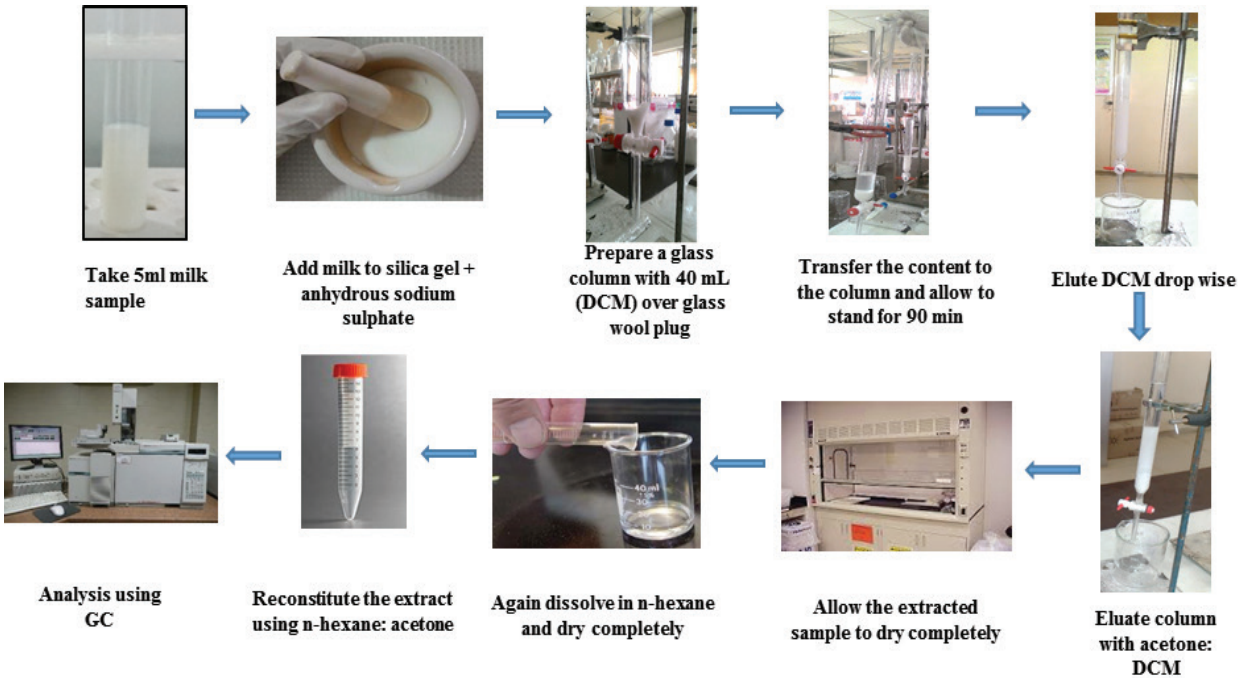


Fig: Sample preparation for Gas chromatography analysis of milk samples

Equipment and Conditions for GC

- Gas chromatographic (GC) methods are suitable for the separation and quantitative determination of compounds which are volatile or semi-volatile and thermally stable at the temperature of the measurement. Estimation of pesticide residues is done using GC equipped with electron capture detector (ECD) and flame thermionic detector (FTD), like Shimadzu 2010 Plus.
- The GC oven temperature for ECD was programmed for an initial temperature of 170°C with holding time of 13 min, and then increased to 270°C at a rate of 3°C/min with the hold time of 20 min.
- Detection was carried out by electron capture at 310°C.The chromatographic conditions were: injector, 280°C; sample volume 1 ml; carrier gas, nitrogen at a pressure of 117.4 kPa and a flow-rate of 1 ml/min.
- Whereas for FTD oven temperature was programmed for an initial temperature of 180°C with holding time for 2 min, then increased to 270°C at a rate of 10°C/min with holding time of 3 min and finally to 280°C at a rate of 5°C/min with holding time of 5 min.
- The injection port temperature was kept at 280°C and the detectors temperature at 310°C.

5.6 Formalin test

Formalin is not permitted to add in any edible food products. It is also not allowed in milk meant for regular use, but it is the only legally permitted preservative for milk and milk products samples meant for analytical purposes in India.

Test for presence of formalin in milk by Hehner's Test

Apparatus and reagents required

- Test tube
- 90 percent H_2SO_4 containing traces of ferric chloride
- Formaldehyde free milk
- Milk sample

Procedure

Take 2 ml of milk sample in a test tube

↓

Add 2 ml of 90 percent H2SO4 containing traces of ferric chloride from the side of the test tube slowly

↓

If sucrose is present, distil the milk sample (25 ml) and then carry out the test on the distillate by taking 2-3 ml of distillate and adding 2 ml of formaldehyde free milk

Inference

Formation of purple ring at the junction indicates formaldehyde is present in milk. The violet coloration does not appear usually when relatively large quantities of formaldehyde are present. The milk supplies the tryptophane that must be present for the test to operate.

6. Additional Tests

6.1 Test for presence of skimmed milk powder in natural milk

By conventional method: (Journal of Food Science and Technology, 1985)

Apparatus and Reagents Required

- Centrifuge machine with centrifuge tube
- Acetic acid:4%.
- Phosphomolybdic acid: 1% solution in water
- Distilled water
- Milk sample

Procedure

Take 50 ml of milk in a 60 ml centrifuge tube

↓

Place the tube in the centrifuge and balance it properly

↓

Centrifuge at 5000 rpm for 15 minutes. Decant the supernatant creamy layer carefully

↓

Add 0.5 ml of 4% acetic acid to skim milk portion for coagulation of protein

↓

Centrifuge the tubes at 5000 rpm for 5 min. Decant the supernatant and wash the precipitate with distilled water twice. Discard the washings

↓

Then, add 2 ml of 1% phosphomolybdic acid to the washed precipitates

↓

Mix the contents thoroughly and heat in a water bath at boiling temperature for 15 minutes and then cool

Inference:

The curd obtained from pure milk shall be greenish in colour whereas the curd of sample containing skimmed milk powder shall be bluish in colour

The intensity of bluish colour depends on the amount of the skimmed milk powder present in the sample

6.2 Test for detection of antibiotic residues

Presence of Antibiotic residues in milk can be detected by three methods as stated below:

A. Rapid test for detection of antimicrobial residue in milk by using Charm EZ platform

B. By employing DPA (dipicolinic acid) based test

C. By employing paper strip based test

D. By employing Charm assay for detection of antibiotic residues in milk

E. By employing Rapid One Step Assay (ROSA)

F. By using broad spectrum screening test kit

A. Rapid test for detection of antimicrobial residue in milk by using Charm EZ platform

Charm EZ is very small portable machine and it can give results of presence of antimicrobial residues within 10-60 min depending on types of antimicrobials present in the milk sample.

This machine is a complete simultaneous incubator and analyzer system specially designed for use in the new ultra-rapid EZ tests, and is also compatible with most Charm 3 and Charm Rosa test strips. Results are interpreted based on a numerical scale and are stored in the Charm EZ memory. Data can be printed, displayed on the Charm EZ screen or, downloaded to computer system in a real time or post analysis mode.

It is a platform connecting with a customized printer in which printed form of results can be obtained instantly.

Manufacturer/supplier sends their technical person to train the laboratory staff on how to operate the machine.

Requirement

- Charm EZ platform
- Calibrator
- Antimicrobial specific strips (These are antibiotic specific and each needs to be procured from the manufacturer). No other consumables are required for the test.

Precaution

- Sample must be maintained at 2-8°C
- Milk fat in the sample must be kept at below 5%
- Strips should be brought to room temperature before use
- Calibration check must be done
- Look for spillage of milk in the compartment where strip is put

Calibration of the machine

Calibration to be done before conducting any test

Calibration check

Daily performance monitoring is to verify that the equipment is performing properly.

Step 1:

Go to the main menu → Press the Perf Mon button

Step 2:

Insert the calibrator strip with the Low calibrator (Dark Pink) side facing down

Close the door

Press the OK button on the screen

Step 3:

After evaluating the strip, the Charm EZ will report the result with the high and low limits → Press the OK button when ready to proceed

Step 4:

Insert the calibrator strip with the high calibrator side (Light Pink) side facing down

Close the door

Press the OK button on the screen

Step 5:

After evaluating the strip, the Charm EZ will report result with the high and low limits → Press the OK button when ready to proceed

Step 6:

When completed, Press the OK button when ready to proceed

Step 7:

If calibrator strip fails to read within specified range, see "Cleaning the Reader Lens" section or contact the Technical Services Staff at Charm Sciences.

Temp adjustment

The temperature requirement is specific to particular antimicrobial residue, hence the temperature must be adjusted prior to each test as required by the particular antimicrobial residue test.

Actual Procedure

Before starting the test, put any one of the strips in the machine without closing the door

↓

Check the incubation temperature

↓

Orange colour indicator turns to green after sometime and show the incubation temperature in green

↓

Then, start the work by putting water in the strip

↓

This is required to check the incubation temperature, especially , if it is different from the each other as the machine needs to be adjusted with the specific temperature required for each type of antimicrobials. Say, for Chloramphenicol-39.60 C, for Quinolone-59.0 C, and so on

Results

The results will be displayed on the monitor of the machine. As it can be connected to the printer and the print out can be taken out for record keeping.

B. By employing DPA (dipicolinic acid) based test (patent No. 264145)

Stepwise procedure:

- **Instrument settings:** Calibration with known standards provided with the kits
- **Analytical methods:** The following analytical methods were used for detection of antibiotic residues in milk samples procured during 2018-19 from Rajasthan, Punjab, and Haryana.

Pipette out 100 µl of milk sample to be tested and transfer to labeled tubes slowly straight to the agar medium. Negative reconstituted skimmed milk (RSM) is also kept as negative control along with test sample

↓

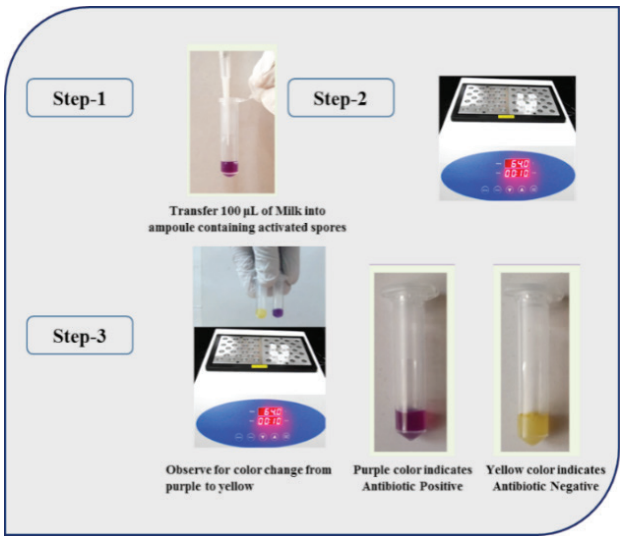
Incubate negative / test samples in a pre-set incubator at 64±0.5°C for 3:00 hours

↓

After incubation, Observe for color change from purple to yellow within time period as specified above

Results Interpretation

- Yellow color indicates the complete absence of antibiotic Residues in milk sample.
- A Purple color indicates the presence of antibiotic Residues in milk sample at MRL or >MRL level



C. By employing paper strip based test

Stepwise procedure:

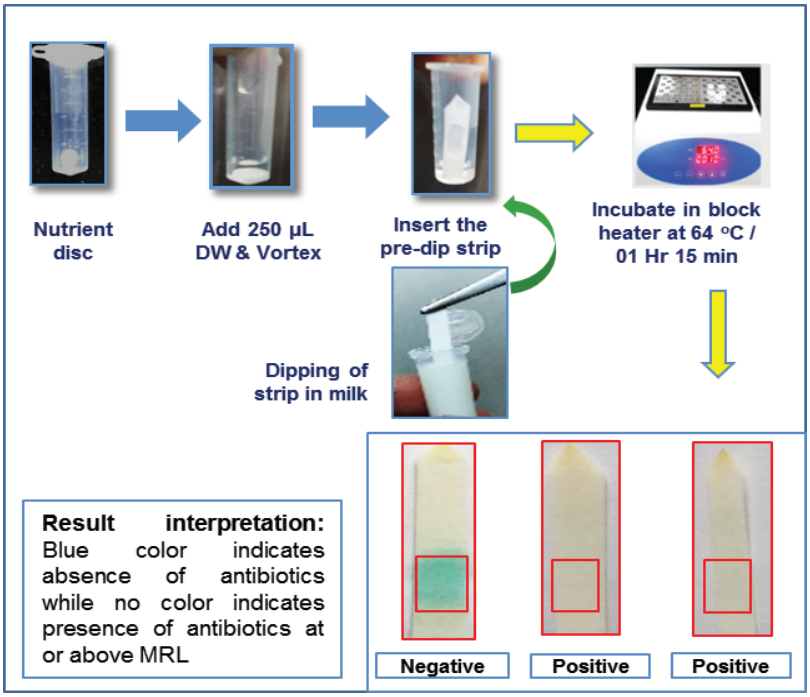
Results Interpretation

Appearance of blue color on paper strip indicates absence of antibiotic

Add 250 µl of distilled water to the tube containing a nutrient disc to hydrate it and vortex for 25 seconds

Impregnate the paper strip with spore, dip chromogenic substrate into the milk sample and insert into the tube containing rehydrated nutrient media

Incubate the preparation at 64°C for 1 hr. and 15 min in dry block heater, then observe for sky blue color development



residues in milk while no color development indicates presence of antibiotic residues in milk at or above MRL as depicted in below figure

Step-wise working procedure of paper strip test

D. By employing Charm assay for detection of antibiotic residues in milk

Test procedure

Same as in case of 5.3 Rapid test for detection of aflatoxin M1 in milk

E. By employing Rapid One Step Assay (ROSA):

Test procedure

Same as in case of 5.3 Rapid test for detection of aflatoxin M1 in milk

F. By using broad Spectrum screening test kit .

Procedure

As per the instruction given within the kit.

It covers 15 groups of antibiotics and detect more than 40 antibiotic residues. It complies with the detection limit of antibiotic residues in different types of milk at MRL (maximum residue limit) levels recommended by the Codex / EU limits/FSSAI. Kit can be used for routine monitoring of raw milk, pasteurized milk and dried milk products. It is suitable to implement in the system at each segment of milk supply value chain to monitor drug residues in milk for routine monitoring as well as for regulatory compliance of requirements set by FSSAI.

Advantage**:** A cost effective and affordable way of testing for antibiotic residues.

7. Microbiological Test Procedures

Milk , being a good medium for growth and proliferation of a variety of microorganisms such as –bacteriamoulds & yeast and their toxins, needs to be stringently screened before use in order to prevent transmission of these microbes to consumers through milk. The most commonly used tests recommended by FSSAI and the test recommended by this assessment for state and district laboratories are given below:

7.1 Total plate count or Standard plate count

IS 5402:2012 (RA-2018)/ISO 4833:2003.

Total plate count results reflect the number of colonies that can emerge under the given physical and chemical conditions (atmosphere, temperature, pH, available nutrients, and presence of growth inhibitory compounds). Colonies are aggregates of living microbial cells, and hence, the results cannot be compared with those from direct counts. Plate counts underestimate the presence of microorganisms, as quiescent, viable but not culturable, and non-culturable microorganisms are omitted from the count

[Rebhun's Diseases of Dairy Cattle (Second Edition),2008]

Materials Required

- Diluent: 0.1% Peptone or Phosphate buffer (90 or 99 ml, 9 ml)
- Media: Plate count agar (PCA) medium (pH 7.0 at 25° C); autoclaved at 121° C for 15 minutes
- Pestle & mortar, Petri dishes, pipettes, incubator (35° C) & Marker

Procedure

Sampling: Collect the sample randomly from entire lot. According to FSSAI sampling 5 sachets (500 ml) of samples will be collected from entire lot. Sample will be stored at refrigeration temperature (2-7°C) until analysis.

Preparation of the test sample: 100-150 ml of the sample will be poured into the sterile sample bottle from each sachet. Opened sachets will be sealed and kept at refrigeration temperature (2-7°C).

Serial dilution and plating

Mark the tubes and petri dishes for batch no., sample no., parameter, dilution etc.

↓

Pipette out 10 or 11 mL of sample from sample bottle into 90 or 99 mL of diluent bottle (1:10 dilution)

↓

Pipette out 1 mL of diluted sample from 1:10 dilution bottle into 9 mL of diluent tube

↓

Subsequently go for further dilutions if required by pipetting out from previous dilution into 9 mL of diluent tube

↓

Pipette out 1 mL from each dilution into respectively marked dilution plates

↓

Pour the PCA (50oC) medium into the plates and allow it to solidify

↓

Incubate the plates at 35 ± 2oC for 48–72 hrs

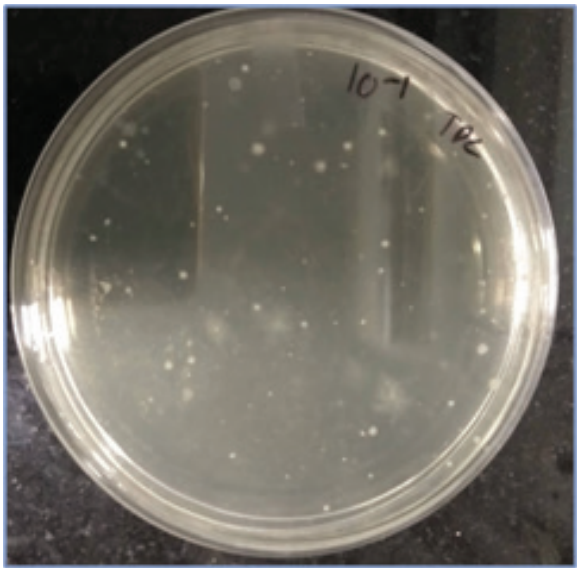


Fig: Typical colonies on plate count agar

Counting & Calculation

After incubation, retain dishes containing not more than 300 colonies at two consecutive dilutions. It is necessary that one of these dishes contains at least 15 colonies. Calculate the number N of micro-organisms per millilitre or per gram of product, depending on the case, using the following equation:

$$N = \frac{\Sigma C}{(n1 + 0.1n2)d}$$

Where

∑C is the sum of colonies counted on all the dishes retained; n_1 *is* the number of dishes retained in the first dilution; n_2 is the number of dishes retained in the second dilution; *d is* the dilution

factor corresponding to the first dilution. Round the result calculated to two significant figures. Take as the result the number of micro-organisms per millilitre or per gram of product, expressed as a number between 1.0 and 9.9 multiplied by 10^{x} where x is the appropriate power of 10.

7.2 Yeast and mould counts

(IS 5403:1999 (RA 2013) /| ISO 7954:1987)

Total Yeast and Mold Counts (TYMC) are used to detect and quantify the amount of fungal growth and allow for identification of viable yeast and mold species present. The amount of fungi is reported as the number of colony forming units (CFUs).

Materials Required:

- Diluent: 0.1% peptone or Phosphate buffer (90 or 99 ml, 9 ml)
- Media: Yeast Extract-Dextrose Chloramphenicol (YEDC) Agar (pH 5.4); Autoclaved at 121°C for 15 minutes
- 10% tartaric acid solution/ 1% lactic acid
- 0.25 % of sterile sodium propionate solution
- Pestle & mortar, Petri dishes, Glass pipettes, Incubator (25°C) and Marker

Procedure

Sampling: Collect 100 – 150 gm. of milk and milk product from the entire lot randomly and store the sample at refrigeration temperature (27°C) until analysis.

Serial dilution & plating

Weigh 10 of milk and milk product sample aseptically and add into pestle containing 90 ml potassium phosphate buffer (pH 7.0) or 0.1% peptone water

Triturate the sample in pestle by using mortar along with diluent for few minutes and pour it back into the dilution bottle (1:10 dilution/ 1st Dilution) for further dilution

Pipette out 1 ml of diluted sample from 1st Dilution into 9 ml of diluent and it is treated as (1:100 or 2nd dilution).

At the same time pipette out 1 ml diluted sample from respective dilution 1:10/ 1: 100) dilution into respectively marked petri dishes

If required you can go for further serial dilution and plating

Pour about 15 ml of the YEDCA medium, previously melted and maintained at 45±1oC in a water-bath, from a culture bottle into each petri dish.

The time elapsing between the end of the preparation of the initial suspension (or of the 10-1 dilution if the product is liquid) and the moment when the medium is poured into the dishes shall not exceed 15 min. At the time of addition the pH of the medium should be reduced to 3.5 using 10% tartaric acid or 1% lactic acid

Carefully mix the inoculum with the medium and allow the mixture to solidify by leaving the petri dishes to stand on a cool - horizontal surface. Prepare a control plate, with 15 ml of the medium, to check its sterility

Invert the plates and place them in the incubator at 25 ± 1°C

Make a separate count of the yeast colonies, which usually will be characterized as smooth, moist, elevated or surface colonies.

After counting the typical yeast colonies, count the mould colonies. Mould colonies are easily recognized by their profuse growth of hyphae. If only yeast counts are required, add 0.25 % of sterile sodium propionate solution to the plate at the time of pouring to inhibit the growth of moulds

If necessary, carry out a microscopic examination in order to distinguish, according to their morphology, the colonies of yeasts and moulds from colonies of bacteria. Generally, it is desirable to differentiate between moulds and yeasts. It is advisable to examine the plates at the end of three days for yeast colonies as they are likely to be overgrown by mould growth.

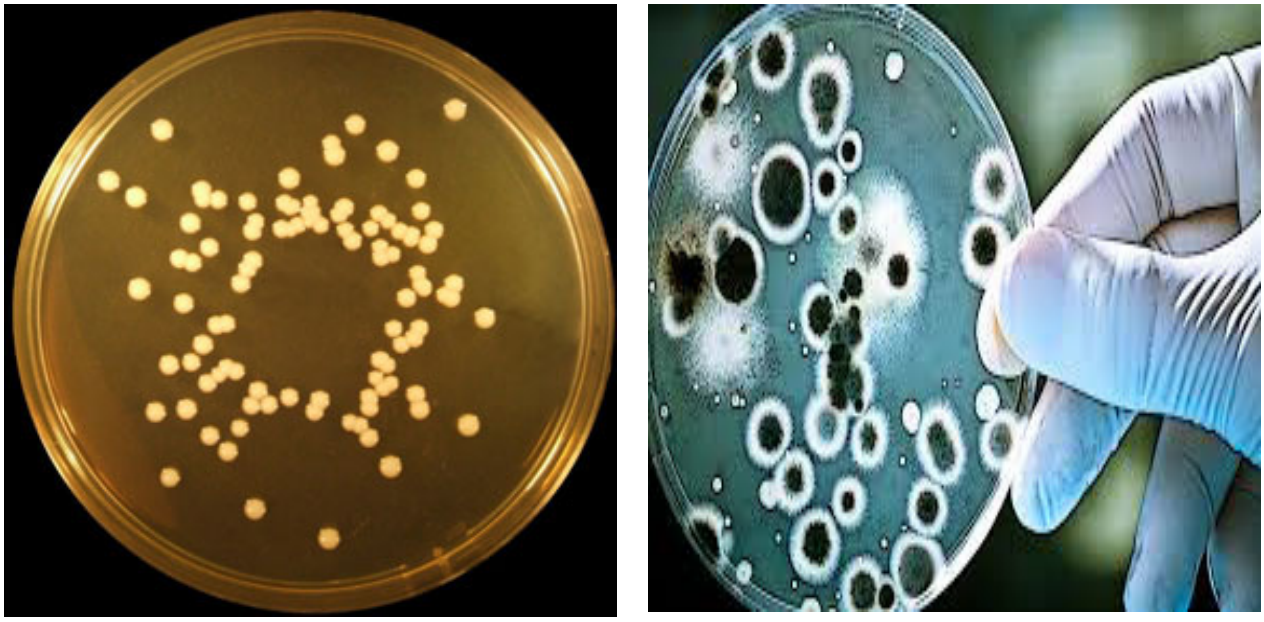


Fig: Plates showing yeast and mould colonies

Interpretation

Count the colonies on each plate after 3, 4 and 5 days of incubation. After 5 days, retain those plates containing fewer than 150 colonies. If parts of the plates are overgrown with moulds, or if it is difficult to count well-isolated colonies, retain the counts obtained after 4 or even 3 days of incubation. In this event, record the incubation period of 3 or 4 days in the test report.

Expression of results

Use counts from plates containing fewer than 150 colonies. The number of yeasts and moulds per gram or per millilitre is calculated according to the equation described in D.16. If there are no colonies on plates from the initial suspension, if the initial product is solid, the number of yeasts and moulds per gram of product should be reported as fewer than 10. If there are no colonies on plates from the test sample. If the initial product is liquid, the number of yeasts and moulds per millilitre of product should be reported as less than 1.

7.3 Coliform count (IS 5401: 2002/ ISO 4833: 1991 (RA-2012)

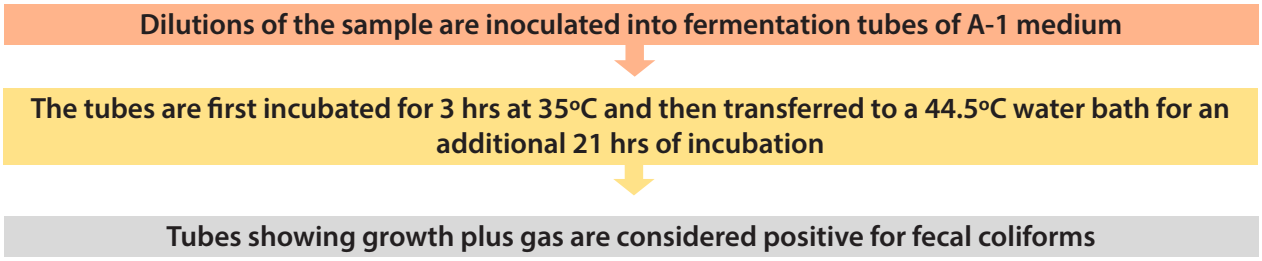
Coliforms are a group of Gram-negative rod-shaped bacteria that have similar biochemical characteristics and are not a single species of microorganism. They are used to monitor the quality of milk being able to ferment lactose with the production of acid and gas within 48 hr at 35°C and grow with or without oxygen. These are usually present in small number in raw milk. It is a simple test and easy to conduct.

Absence of coliforms in 1:100 dilutions in raw milk and in 1:10 dilution of pasteurized milk is accepted as a satisfactory quality. The presence of *E. coli* is a proof that contamination from excreta has occurred.

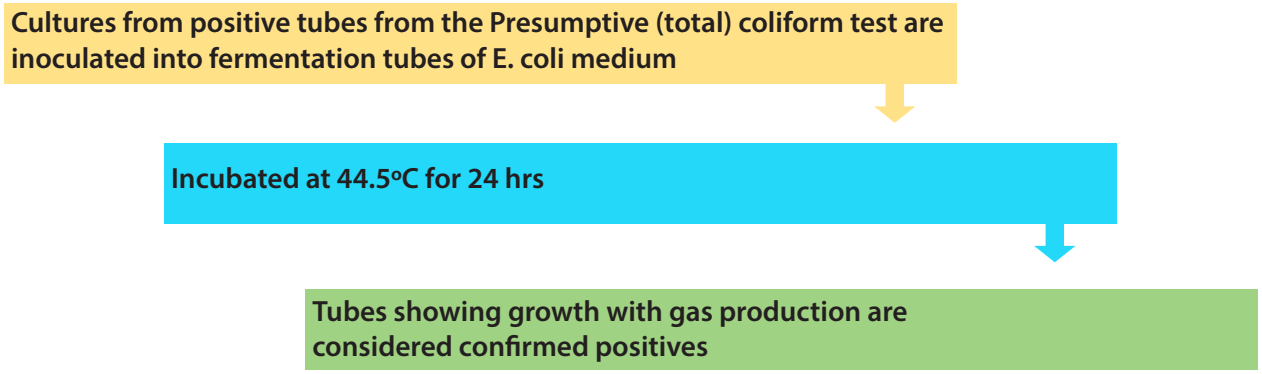
Materials required

A-1 Medium, EC medium, m-FC medium, Membrane filtration unit; Test tubes/ Sampling bottle; Incubator (37°C and 45°C)

Procedure-1: Single-step procedure



Procedure-2: Multiple Fermentation Tube (MFT) Technique



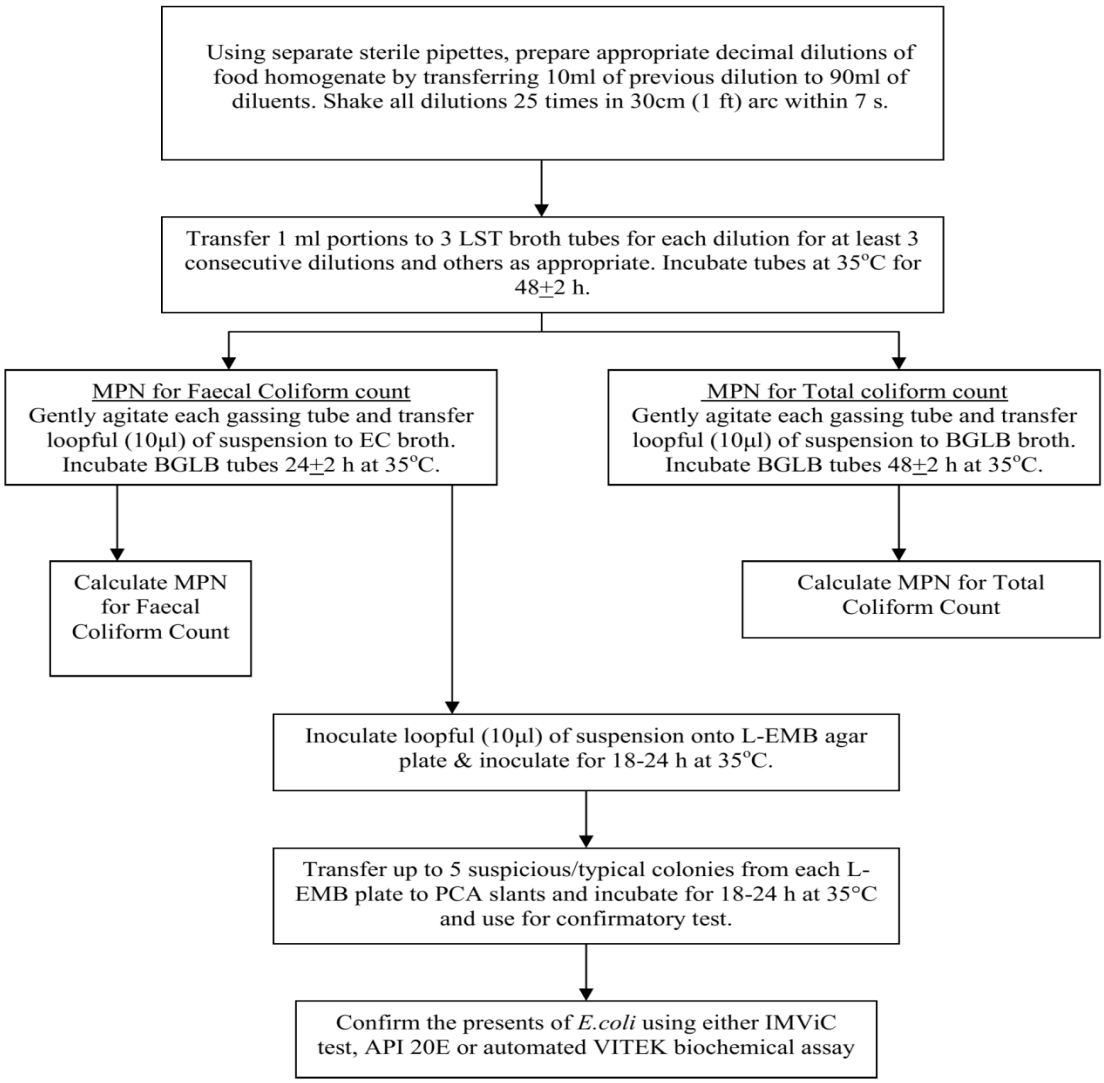
Procedure-3: Membrane Filtration (MF) technique

Samples are filtered onto membranes as in the total coliform test; the membranes are placed onto plates of m-FC medium, sealed in water-tight plastic bags, and submerged in a 44.5°C water bath incubator for 24 hrs. Colonies with a characteristic faecal coliform appearance are then counted and faecal coliform density is computed.

Fig: Typical colonies ion VRBL Agar

FLOW CHART SHOWING

ESCHERICHIA COLI and TOTAL COLIFORM ANALYSIS



Flow chart for E. coli and coliforms

7.4 Escherichia coli (E. coli)

IS 5887 Part I 1976 (RA-2013)

Conventional method for the enumeration of *E. coli*

Media *and* reagents *:*

- Lauryl tryptose (LST) broth
- Levine's eosin-methylene blue (L-EMB) agar
- MR-VP broth
- Butterfield's phosphate-buffered water
- Kovacs' reagent, Voges-Proskauer (VP) reagents
- Methyl red indicator ,Violet red bile agar (VRBA)
- Peptone Diluents, 0.1% & Brilliant Green Lactose Bile Broth

Materials

- Test sample
- 1% peptone
- MacConkey broth medium
- MacConkey agar medium
- Eosin methylene blue lactose agar
- Tergitol-7 agar
- Kovac's reagent
- Methyl red
- alpha-naphthol
- Potassium hydroxide
- Incubator
- Pipette

Procedure

Blend the sample in a sterile blender jar for 2 minutes or macerate with sterile sand in a sterile mortar using approximately 200 mL of diluting fluid per 25 g of the sample

↓

The diluting fluid for preparing the homogenate should be at 1% peptone (ISO 6887 solution in water

↓

Inoculate 1 mL of the blended or macerated sample into 10 mL of single strength MacConkey broth medium. If the numbers of organisms are assumed to be very small, inoculate 10 mL of double strength MacConkey broth medium.

↓

Also streak loop full on to MacConkey agar medium, eosin methylene blue lactose agar, and if available Tergitol-7 agar

↓

Incubate all the inoculated media at 37°C overnight

↓

If there is growth with fermentation of lactose in the MacConkey broth medium streak out a loop full on to each of the solid media, and incubate at 37°C overnight

Test for Identification: Pick out and mark as many suspected colonies from the solid media as possible, but not less than 5, to investigate. The suspect colonies are smooth and are lactose fermenting on Mac Conkey agar and on eosin ethylene blue lactose agar, and are yellow colonies surrounded by yellow zones on Tergitol-7 agar medium.

(1) **Test for Indole** - Inoculate peptone water medium as in with a loopfull of 24 hour growth in nutrient broth and incubate at 37°C for 48 hours. Add 0.5 ml of Kovac's reagent, prepared by dissolving 10 g p-dimethyl-aminobenzaeldehyde in 150 ml amyl alcohol or isoamyl alcohol and to which 50 ml of concentrated hydrochloric acid is slowly added. Prepare the reagent in small quantities and store in refrigerator. After adding Kovac's reagent, shake the tube gently, the appearance of a red colour indicates the presence of indole.

(2) **Test with Methyl Red**- Inoculate the glucose peptone water medium and incubate at 37°C for 2 days. Add 2 drops of methyl red solution prepared by dissolving 0.04 g of methyl red in 40 ml of absolute ethanol and diluting with water to make up to 100 ml. A positive reaction is indicated by red colour and a negative reaction by yellow colour.

(3) **Test for Voges-Proskauer Reaction** - Inoculate the glucose peptone water medium, and incubate at 37°C for 2 days. To 1 ml of the growth add 0.6 ml of alpha-naphthol solution prepared as 5 percent solution in ethanol. Shake and add 2 ml of 40 percent aqueous solution of potassium hydroxide. Shake and slope the tube and observe for up to 4 hours for the appearance of a pink colour which indicates a positive reaction.

(4) **Test for Citrate Utilization**- Inoculate the strain on to Simmion's Citrate Agar medium with a young nutrient agar slant culture using a straight wire. Incubate at 37°C for up to 4 days for growth of the organism.

Procedure for enumeration:

Preparation of Sample

Take 25 to 50 g of the sample in a, sterile blender jar and to this add diluting fluid (0.1% Peptone) to have dilution of 10-1

→

Blend at 8 000 to 10000 rev/min for 2 minutes. Alternatively macerate with the diluting fluid in a sterile mortar with sterile sand, Make serial ten-fold dilutions with the diluting fluid, in duplicate series, up to 10-6

Plate Count:

Spread out 0.1 mL from each dilution tube, evenly on to Tergitol-7 agar, and incubate at 37°C for 24 hours

→

Enumerate the colonies of E. coli, which are yellow in colour surrounded by a yellow zone, and confirm these as being Escherichia coli by IMViC tests

The number of viable colonies of *E. coli* per gram of sample shall be determined by multiplying by the dilution factor(s) and dividing by the mass of the sample. If Tergitol-7agar is not in use, then MacConkey agar plates or eosin methylene blue lactose agar plates may be used.

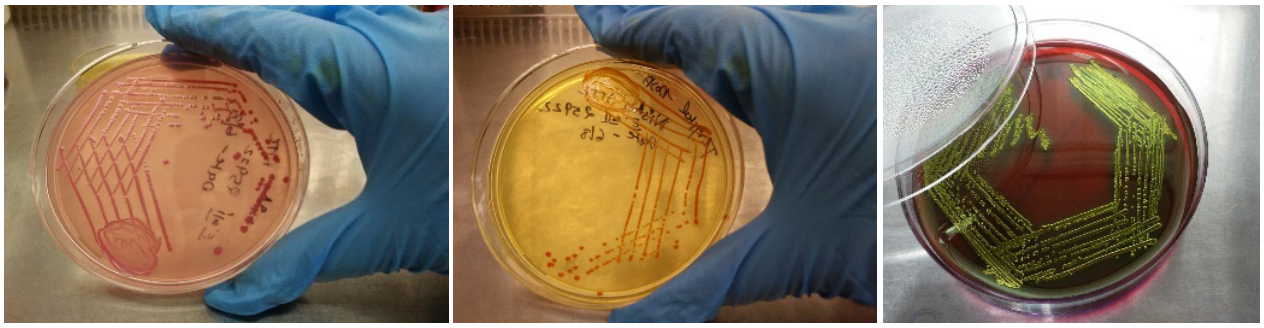


Fig: E. coli on MacConkey Agar, Tergitol 7 Agar and EMB Agar

7.5 Staphylococcus aureus

Highly pathogenic strains of *Staphylococcus aureus* can cause disease in both humans and animals. In animal species, including ruminants, *S. aureus* may cause severe or sub-clinical mastitis. Dairy animals with mastitis frequently shed *S. aureus* into the milk supply which can lead to food poisoning in humans.

Materials required

- Incubator,
- Drying cabinet or incubator,
- Water bath,
- Test tubes,
- Flasks or bottles with screw caps,
- Petri dishes, straight wire and Pasteur pipette,
- Total-delivery graduated pipettes,
- Spreaders,
- pH Meter.

Test portion, initial suspension and dilutions:

Agitate the sample thoroughly by inverting the sample container 25 times

↓

Pipette out 1mL or 10 mL of the sample with a sterile pipette and add to 9mL or 90 mL mL of diluents

↓

Shake this primary dilution with a movement of about 300 mm for 7s manually or using a mechanical agitator to obtain a 10-1 dilution

Inoculation:

Transfer 0.1 ml of the test sample if liquid, or 0.1 ml of the initial suspension (10-1 dilution) in the case of other products, to each of two Baird Parker agar (BPA) plates.

↓

Repeat the procedure for the 10-2 dilution and for further decimal dilutions, if necessary.

↓

If, for certain products, it is desirable to count low numbers of coagulase-positive staphylococci, the limits of detection can be raised by a factor of 10 by inoculating 1.0 ml of the test sample if liquid, or 1.0 ml of the initial suspension for other products, either on the surface of one large agar plate (140 mm) or on the surface of three small agar plates (90 mm). In both cases, prepare duplicates by using two large plates or six small ones.

↓

Carefully spread the inoculum as quickly as possible over the surface of the agar plate, trying not to touch the sides of the dish, using the spreader. Allow the plates to dry with their lids on for about 15 min at laboratory temperature.

Incubation

Invert the plates prepared and incubate them for 24 h ± 2 h then re-incubate for a further 24 h ± 2 h in the incubator at 35°C or 37°C).

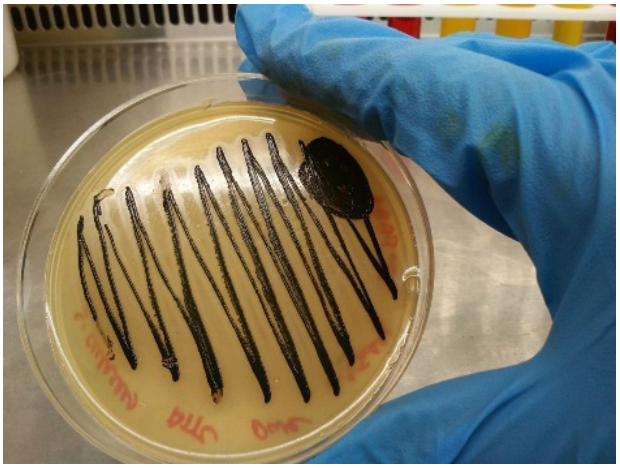


Fig: Staph. aureus on Baird Parker Agar

Selection of plates and interpretation

- After incubation for 24 h ±2 h, mark on the bottom of the plates the positions of any typical colonies present
- Re-incubate all plates at 35°C or 37°C for a further 24 h ± 2 h, and mark any new typical colonies. Also mark any atypical colonies present. Take for enumeration only those plates that contain at the maximum 300 colonies with 150 typical and/or atypical colonies at two successive dilutions. One of the plates shall contain at least 15 colonies.
- Select for confirmation a given number A (in general 5 typical colonies if there are only typical colonies, or 5 atypical colonies if there are only atypical colonies, or 5 typical colonies and 5 atypical colonies if both types are present, from each plate).

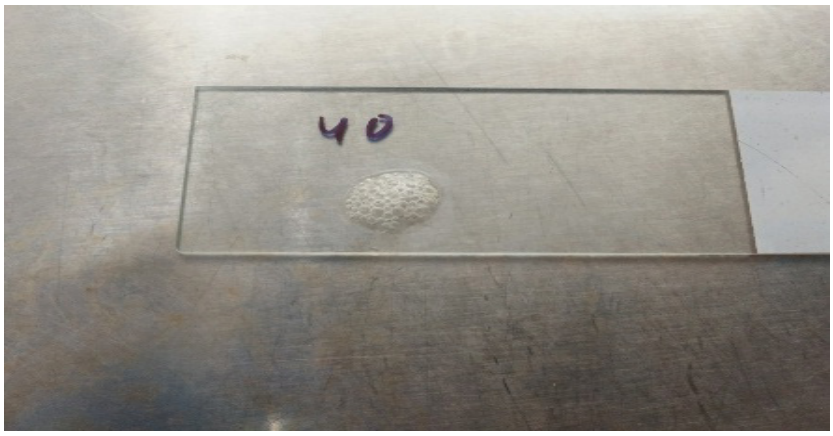


Fig: Catalase test (+ve indication)

Catalase reaction: Take an isolated colony and suspend it in a drop of hydrogen peroxide solution (3%) on a slide. The immediate formation of gas bubbles indicates a positive reaction.

Confirmation (coagulase test)

From the surface of each selected colony, remove an inoculum with a sterile wire and transfer it to a tube or bottle of brain-heart infusion broth

Incubate at 35°C or 37°C for 24 h ± 2 h. aseptically add 0.1 mL of each culture to 0.3 mL of the rabbit plasma (unless other amounts are specified by the manufacturer) in sterile haemoliysis tubes or bottles, and incubate at 35°C or 37°C).

By tilting the tube, examine for clotting of the plasma after 4 h to 6 h of incubation and, if the test is negative, re-examine at 24 h of incubation, or examine at the incubation times specified by the manufacturer

Consider the coagulase test to be positive if the volume of clot occupies more than half of the original volume of the liquid. As a negative control, for each batch of plasma, add 0.1 mL of sterile brain-heart infusion broth to the recommended quantity of rabbit plasma and incubate without inoculation

For the test to be valid, the control plasma shall show no signs of clotting

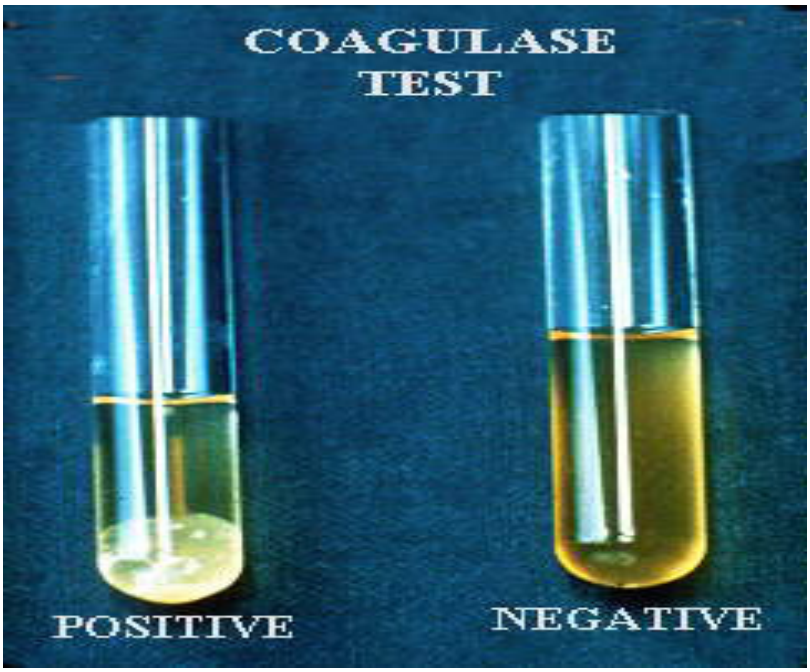


Fig: Interpretation of Coagulase test

7.6 Salmonella spp IS 5887 Part 3: 1999 (RA 2013)

Contamination of raw milk and products with *Salmonella* spp. is mostly due to infected persons, fecal contamination from the cow, and contamination of the environment, since natural infections of the udder are rare. Deficient hygiene in dairies, especially those from developing countries, has often been considered as one of the major reasons for contamination of milk with both spoilage and pathogenic bacteria. Raw milk and milk products are increasingly becoming important sources of human infection.

Pre-enrichment

Twenty-five (25) grams or ml of sample is added to 225 ml of buffered peptone water and incubated at 37°C. In case of Salmonella 2 ml of brilliant green (0.1% sol.) is added and incubation is done for 24 hours.

Selective Enrichment:

Transfer 1ml portion from pre-enrichment step to each 10ml of serenity cosine broth & tetrathionate broth and incubated at 37°C for overnight

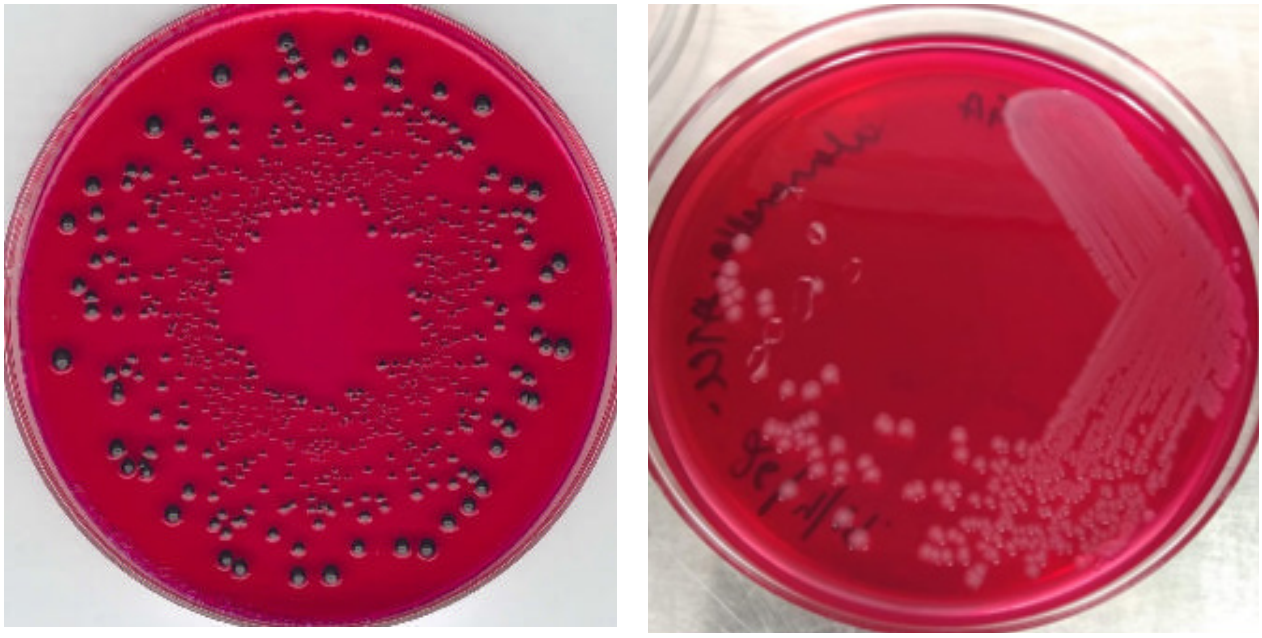
↓

After required incubation time, contents of both tubes are mixed and a loopful from the mixed contents is streaked on to the Xylose lysine deoxycolate agar (XLD), and Bismuth Sulfite Agar (BSA) plate and hektoen enteric agar

↓

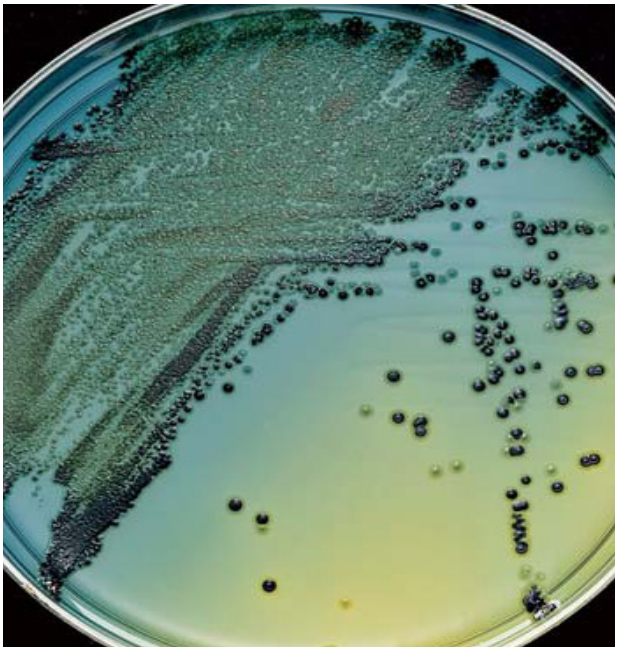
These plates are incubated at 37°C for 24 hrs. The incubation may be continued up to 72 hrs before report as nil.

Observation:



Pink and black color colony of Salmonella on XLDA and BGA

Black color colony of Salmonella on BS agar



Blue-green to bluish colony of Salmonella on HE agar

Red color colony of Salmonella on Rambach agar (Chromogenic media)

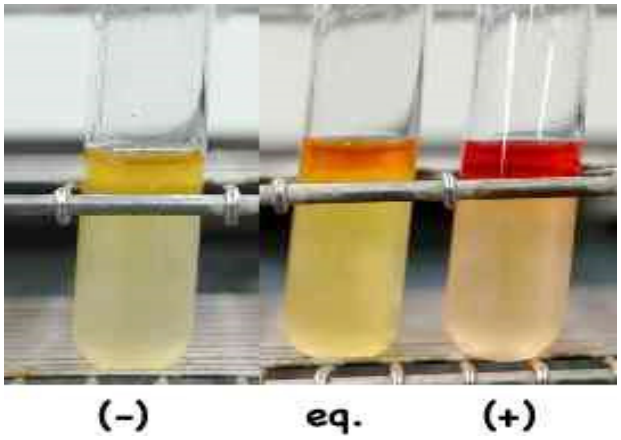
Biochemical test for detection of Salmonella

Materials required:

- Lysinedecarboxylase broth,
- Phenol red dulcitol broth,
- SI test,
- Tryptone broth,
- Potassium cyanide (KCN) broth,

- Malonate broth,
- Indole test, P
- henol red sucrose broth or Purple sucrose broth,
- MR-VP broth etc.

Observation:



Indole test

Urease Test

Methyl Red Test

Triple Sugar Iron Test

Observation for TSI slants

Reaction	Fermentation
• Acid butt (yellow), alkaline Glucose fermented Slant (red)	• Glucose fermented
• Acid throughout medium, butt Lactose or sucrose or and slant yellow both fermented	• Lactose or Sucrose or both fermented
• Gas bubbles in butt, medium sometimes split	• Aerogenic culture
• Blackening of the butt	• Hydrogen sulfide Produced
• Alkaline slant and butt(Medium entirely red	• None of the three sugars fermented

Observation for biochemical tests

Test or substrate	Result		Salmonella species reaction
	Positive	Negative	
▪ Glucose (TSI)	Yellow Butt	Red Butt	+
▪ Lysine decarboxylase (LIA)	Purple butt	Yellow butt	+
▪ H2S (TSI)	Blackening	No blackening	+
▪ Urease	Purple-red coor	No color change	-
▪ Lysine decarboxylase broth	Purple color	Yellow color	+
▪ Phenol red dulcitol broth	Yellow color and/or gas	No gas; no color change	+
▪ KCN broth	Growth	No growth	-
▪ Malonate broth	Blue color	No color change	-
▪ Indole test	Violet color at surface	Yellow color at surface	-
▪ Phenol red lactose broth	Yellow color and/or gas	No gas; no color change	-
▪ Phenol red sucrose broth	Yellow color and/or gas	No gas; no color change	-
▪ Voges-Proskauer test	Pink-to-red color	No color change	-
▪ Methyl red test	Diffuse red color	Diffuse yellow color	+

7.7 Listeria monocytogenes

Listeria monocytogenes is an omnipresent bacterium which causes listeriosis in humans, a serious infectious disease that occurs on consumption of milk contaminated with this pathogen. *Listeria monocytogenes* most commonly occurs as a consequence of post-pasteurization contamination. *L. monocytogenes* has the ability to multiply and grow at low temperatures (4 ° C) and to survive even on freezing temperatures, and as such poses risk for health of consumers, if found in milk, cheese, ice-cream and other dairy products.

Materials required:

- Test sample
- Half Fraser broth
- Fraser broth
- PALCAM agar
- Incubator
- Pipette

Procedure:

Test portion and initial suspension: In general, to prepare the initial suspension, add a test portion of x g or x ml to 9x ml or 9x g of the selective primary enrichment medium (half Frazer broth), to obtain a ratio of test portion to selective primary enrichment medium of 1/10 (mass to volume or volume to volume).

Primary enrichment: A, food samples of 25 g are homogenized in 225 ml, respectively, of half Fraser broth and incubated for 24 h at 30°C.

Secondary enrichment: A volume of 0.1 ml of the primary-enriched sample is used to inoculate 10 ml of Fraser broth and incubated 24 h at 37°C.

Plating out and identification:

- From the primary enrichment culture incubated for 24 h ± 3 h at 30°C, take, by means of a loop or glass rod, a portion of the culture and inoculate the surface of the first selective plating medium, *Agar Listeria according to Ottaviani and Agosti* (ALOA), so that well-separated colonies are obtained. Proceed in the same way with the second selective plating-out medium
- From the secondary enrichment medium incubated for 48 h ± 2 h at 35°C or 37°C, repeat the procedure with the two selective plating-out media.
- Invert the dishes and place them in an incubator set at 37°C for **ALOA** and at the appropriate temperature for the second selective medium (PALCAM Agar). If a commercial medium is used for the second selective medium, follow the manufacturer's instructions.
- After incubation for 24 h ± 3 h (and for an additional 24 h ± 3 h if the growth is weak of if no colony is observed after 24 h incubation) for **ALOA** or for the appropriate time (second selective agar), examine the dishes for the presence of colonies presumed to be *Listeria* spp."
    - **a.** **ALOA**: Consider as *L. monocytogenes* the green-blue colonies surrounded by an opaque halo (typical colonies). If growth is slight, or if no colony is observed, or if

no typical colony is present after 24 h ± 3 h of incubation, re-incubate the plates for further 24 h ± 3 h.

b. Second selective medium: Examine after the appropriate time to check for the presence of colonies which, from their characteristics, are considered to be presumptive *Listeria* spp. or *monocytogenes*, depending on the type of medium used."

Confirmation of *Listeria* spp.

Selection of colonies for confirmation:

- Take from each plate of each selective medium, select up to five colonies presumed to be Listeria spp.
- Streak the selected colonies onto the surface of pre-dried plates of TSYEA (tryptone soya yeast extract agar).
- Place the plates in the incubator set at 35°C or 37°C for 18 h to 24 h or until growth is satisfactory.
- Typical colonies are 1 mm to 2 mm in diameter, convex, colorless and opaque with an entire edge

Catalase reaction: Take an isolated colony and suspend it in a drop of hydrogen peroxide solution (3%) on a slide. The immediate formation of gas bubbles indicates a positive reaction.

Gram staining: Perform the Gram stain on a colony separated *Listeria* spp. are revealed as Gram-positive slim, short rods.

Motility test*:*

- Take an isolated colony and suspend it in a tube containing TSYEB. Incubate in the incubator set at 25°C for 8 h to 24 h until a cloudy medium is observed.
- Deposit a drop of the above culture using a loop onto a clean glass microscope slide. Place a coverslip on top and examine it with the microscope.
- Listeria spp. appears as slim, short rods with tumbling motility.
- Cultures grown above 25°C may fail to exhibit this motion.

Carbohydrate utilization:

- Inoculate using a loop each of the carbohydrate utilization broths with a culture from TSYEB. Incubate at 35°C or 37°C for up to 5 days.
- Positive reactions (acid formation) are indicated by a yellow colour and occur mostly within 24 h to 48 h.

Table -1 Differentiation of *Listeria* species

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

7.8 Enterobacter sakazakii

Enterobacter sakazakii causes infections in mainly neonates leading to development of bacteraemia, necrotizing enterocolitis (NEC) and infant meningitis. It is an emerging disease.

Pre-enrichment in non-selective liquid medium: The pre-enrichment medium i.e. buffered peptone water is inoculated with the test portion and incubated at 37°C±1°C for 16- 20 h.

Enrichment in selective liquid medium: The selective enrichment medium is inoculated with the culture obtained in Pre-enrichment in non-selective liquid medium and incubated at 44°C±0.5°C for 22-26 hrs.

Plating out and identification: A chromogenic agar is inoculated with the enrichment culture and incubated at 44°C±1°C for 22-26 hrs.

Confirmation: Typical colonies are selected from the chromogenic agar, and isolates producing a yellow pigment on tryptone soya agar are biochemically characterized.

Materials required

Diluent: Buffered peptone water

Media for growth

- Modified lauryl sulfate tryptose broth (mlST)/vancomycin medium
- Enterobacter sakazakii isolation agar (ESIATM)
- Tryptone soya agar (TSA)
- L-Lysine decarboxylation medium
- L-Ornithine decarboxylation medium
- L-Arginine dihydrolation medium and
- Simmons citrate medium

Media for fermentation of carbohydrates

- Peptone water with phenol red, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose and amygdaline); Carbohydrate solutions (D-sorbitol, L-rhamnose, D-sucrose, D-melibiose or amygdaline), 80 mg/ ml
- Oxidase test reagent: N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride ($C1_0H_{16}N_2 \cdot 2HCl$)

Equipment and other requirements

- Total delivery pipettes, having a nominal capacity of 1 ml;
- Water bath, capable of being maintained at 44°C±0.5°C.
- Petri dishes made of glass or plastic, of diameter 90 mm to 100 mm.
- Incubators, capable of operating at 25°C±1°C, 30°C±1°C and 44°C±1°C, respectively.
- Loop, made of platinum-iridium or nickel chromium, of diameter approximately 3 mm, or disposable loops.
- Test tubes, of diameter 18 mm and length 160 mm (plugged or with screw caps).
- pH meter, accurate to 0.1 pH unit at 25°C±1°C.

Procedure

Test portion

To prepare the primary dilution, add x g of the test sample to 9 times x ml of pre-enrichment medium, which is the ratio of test sample to pre-enrichment medium specified in this method.

Allow dry samples to disperse in the liquid without stirring. If a sample has not been dissolved completely after 30 min, then mix it gently with the medium.

Pre-enrichment: Incubate the inoculated pre-enrichment medium at 37°C±1°C for 18 ± 2 hrs.

Selective enrichment

After incubation of the inoculated pre-enrichment medium, transfer 0.1 ml of the obtained culture into 10 ml of mlST/ vancomycin medium.

Incubate at 44°C±0.5°C for 24±2 hrs. It is recommended to use either a water bath or a forced-air incubator to ensure that the maximum temperature (44.5°C) is not exceeded.

Isolation of presumptive *E. sakazakii:* After incubation of the inoculated mlST/ vancomycin medium, streak a loopful (ca. 10 µL) onto the surface of the *Enterobacter sakazakii* isolation agar plate. Incubate the plate at 44°C±1°C for 24±2 hrs. After incubation, examine the chromogenic plate for the presence of typical colonies of presumptive *E. sakazakii*.

NOTE Typical colonies are small to medium sized (1 mm to 3 mm) green to blue-green colonies. Non-typical colonies are often slightly transparent and violet colored.

Confirmation: Production of a yellow pigment

Selection of colonies: Select one to five of the typical colonies of presumptive *E. sakazakii* examined on the incubated chromogenic plate.

Incubation:

- Streak the selected colonies onto the surface of the TSA plate so that after incubation separate colonies can be observed.
- Incubate the plate at 25°C±1°C for 44-48 hrs. After incubation, examine the TSA plates for the presence of yellow-pigmented colonies.
- When only one colony is selected and transferred to the TSA plate and after incubation no yellow pigmented colonies can be seen, select four more typical colonies.
- If there are fewer than five typical colonies, select all of them.

Biochemical confirmation: Miniaturized biochemical identification kits, currently available commercially and permitting the identification of *Enterobacter sakazakii*, may be used.

Selection of colonies: Select one yellow pigmented colony from each tryptone soya agar plate for following biochemical characterization.

Oxidase disposable

- Using a glass rod or inoculation needle, take a portion of each selected characteristic colony.
- Streak the taken portion on a filter paper moistened with the oxidase reagent or on a commercially available disc.

- Do not use a nickel/ chromium loop or wire. Consider the test to be negative when the color of the filter paper has not changed to mauve, violet or deep blue within 10 s.

L-Lysine decarboxylase

- Using a loop, wire or glass rod, inoculate the L-lysine decarboxylation medium with each of the selected colonies just below the surface of the liquid medium.
- Incubate the tubes at 30°C±1°C for 24±2 hrs.
- A violet color after incubation indicates a positive reaction. A yellow color indicates a negative reaction.

L-Ornithine decarboxylase

- Using a loop, wire or glass rod, inoculate the L-ornithine decarboxylation medium with each of the selected colonies just below the surface of the liquid medium.
- Incubate the tubes at 30°C±1°C for 24±2 hrs.
- A violet color after incubation indicates a positive reaction. A yellow color indicates a negative reaction.

L-Arginine dihydrolase

- Using a loop, wire or glass rod, inoculate the L-arginine dihydrolation medium with each of the selected colonies just below the surface of the liquid medium.
- Incubate the tubes at 30°C±1°C for 24±2 hrs.
- A violet color after incubation indicates a positive reaction. A yellow color indicates a negative reaction.

Fermentation of various sugars

- Using a loop, wire or glass rod, inoculate each carbohydrate fermentation medium with each of the selected colonies just below the surface of the liquid medium.
- Incubate the tubes at 30°C±1°C for 24±2 hrs.
- A yellow color after incubation indicates a positive reaction. A red color indicates a negative reaction.

Utilization of citrate

- Using a loop, wire or glass rod, streak the selected colonies onto the slant surface of Simmons citrate medium.
- Incubate the tubes at 30°C±1°C for 24±2 hrs.
- The reaction is positive if the medium turns blue.

Interpretation

Confirmatory test	+/ - ve reaction	Observation
Production of a yellow pigment	+	
Oxidase	–	
L-Lysine decarboxylase	-	
L-Ornithine decarboxylase	+	
Arginine dihydrolase	+	

Acid from	+/ - ve reaction	Observation
Fermentation of D-sorbitol	-	
Fermentation of L-rhamnose	+	
Fermentation of D-sucrose	+	
Fermentation of D-melibiose	+	
Fermentation of amygdaline	+	
Hydrolysis of citrate	+	

7.9 Sulfite reducing clostridia

Clostridia spores are omnipresent in the farming environment. It has been recommended to use sulphite reducing clostridia as a hygiene indicator for dairy plant systems.

Materials required

- Culture medium and Saline peptone diluent
- Plate count medium: Iron sulfite agar
- Homogenization equipment, for samples of solid food
- Water bath, capable of being maintained at between 44oC & 47oC
- Anaerobic jars, with equipment for generating an anaerobic atmosphere, and including a system to check the anaerobic conditions;
- Incubator, capable of being maintained at 37 oC ± 1oC and, if necessary, at 50oC ± 1oC;
- Test tubes, of dimensions 16 mm × 160 mm, and flasks or bottles of capacity 500ml.

Procedure

Sampling: Collect 100 – 150 gm. of milk and milk product sample from the entire lot randomly and store the sample at refrigeration temperature (2-7^{0}C) until analysis

Serial dilution & plating

Weigh 10 gm. of sample aseptically and add into pestle containing 90 mL potassium phosphate buffer (pH 7.0)

Triturate the sample in pestle by using mortar along with diluent for few minutes and pour it back into the dilution bottle (1:10 dilution/ 1st Dilution)

Heat treatment of the initial suspension may be necessary to eliminate vegetative forms of spore-forming bacteria and/or non-sporeforming bacteria.

Temperatures and heating times vary according to the actual need, from combinations producing a definite pasteurization effect at a moderate heat activation effect (e.g. 75o C for 20min), to boiling for several minutes. In this case, results could be given as number of spores of sulfite reducing bacteria growing under anaerobic conditions

Pipette out 1 mL of diluted sample from 1st Dilution into 9 mL of diluent and it is treated as 1:100 or 2nd dilution

At the same time pipette out 1 mL diluted sample from respective dilution 1:10/ 1: 100) dilution into respectively marked petri dishes

For the test to be valid, the control plasma shall show no signs of clotting

Pour into each Petri dish approximately of iron sulfite agar which has been cooled to 44°C - 47°C in the water bath. The time elapsing between inoculation of the Petri dishes and addition of the agar should not exceed 15 min. carefully mix the inoculum with the medium by horizontal movements and allow the medium to solidify

After the medium has solidified, pour 10 to 15 mL of the same medium into the dish as an overlay

If tubes are used, inoculate a 1 mL volume from each dilution into each of two tubes of medium kept at to 44oC to 47oC. Mix gently without forming bubbles, and leave the medium to solidify in a cold water bath. After the medium has has solidified, pour to of the same medium into each tube as an overlaysolidified, pour to of the same medium into each tube as an overlay

After solidification, incubate the Petri dishes in anaerobic jars at 37°C ± 1°C for 24-48 hrs. If thermophilic bacteria are suspected, prepare a second set of Petri dishes. Incubate this set at 50°C ± 1°C. In the case of tubes, incubation in anaerobic jars is not necessary

Counting of the colonies

- Read the results after 24 and 48 hrs, depending on the degree of black color and the growth rate of the microorganisms. Black colonies, possibly surrounded by a black zone, are counted as sulfite-reducing bacteria.

Note 1: Diffuse, unspecific blackening of the medium may occur, especially when inoculation is performed in agar tubes instead of Petri dishes.

- The growth of anaerobic bacteria, which only produce hydrogen (not H2S), may also reduce the sulfite present and lead to a general blackening of the medium.
- Count colonies of sulfite-reducing bacteria in each dish containing less than 150 typical colonies and less than 300 total colonies.
- When the number of colonies is high, some tubes may be unreadable. In this case, only tubes where the colonies are clearly separate should be considered for counting.

Note 2: This International Standard may be used to enumerate only *Clostridium*. After obtaining characteristic colonies, pick five of them from each dish, and confirm the genus *Clostridium* with confirmation tests (e.g. respiratory tests, spore forming tests).

Expression of results

Calculate the number N of mesophilic lactic acid bacteria present in the test sample, as the weighted mean from two successive dilutions, using the equation:

$$N = \frac{\Sigma C}{V(n1 + 0.1n2)d}$$

Where

- ∑C is the sum of the colonies counted on all the dishes from two successive dilutions, at least one of which contains at least 15 colonies; V is the volume of inoculum applied to each dish, in milliliters; n1 is the number of dishes retained in the first dilution; n2 is the number of dishes retained in the second dilution; d is the dilution factor corresponding to the first dilution.
- Round the result calculated to two significant figures. Take as the result the number of mesophilic lactic acid bacteria per milliliter (liquid products) or per gram (other products), expressed as a number between 1.0 and 9.9 multiplied by the appropriate power of 10.

7.10 Bacillus cereus

Bacillus cereus causes of two types of food borne disease: (i). an emetic intoxication and (ii). diarrhoeal infection. The enterotoxins are heat-labile and sensitive to acid conditions or proteolysis. They are destroyed during cooking or gastro-intestinal digestion, however the spores are highly resistant. Milk containing too many cells will are not destroyed by digestion, but instead colonize the gut of the host and produce sufficient enterotoxin to cause disease.

Materials required

- Drying cabinet or incubator (37°C ± 1°C and 55°C ± 1°C),
- Incubator, capable of operating at 30°C±1°C,
- Water baths, capable of being maintained at 45°C±0.5°C and 50 °C±1°C.
- Loops, made of platinum/iridium or nickel/chromium (3mm),
- pH-meter, Test tubes, (18 mm diameter and length 180 mm, and culture flasks, Petri dishes (90 mm to 100 mm),
- Graduated pipettes.

Test portion, initial suspension and dilutions

- Agitate the sample thoroughly by inverting the sample container 25 times.
- Pipette out 10 ml of the sample with a sterile pipette and add to 90 ml of diluents.
- Shake this primary dilution with a movement of about 300mm for 7s manually or using a mechanical agitator to obtain a 10^{-1} dilution.

Inoculation and incubation

Transfer 0.l ml of the test sample, if the product is liquid, or of the initial suspension in the case of other products, to each of two agar plates (MYP Agar). Repeat the procedure using further decimal dilutions if necessary

↓

Carefully spread the inoculum as quickly as possible over the surface of the agar plate without touching the sides of the dish with the spreader

↓

Use a fresh sterile spreader for each plate. Leave the plates with the lids on for about 15 mm at ambient temperature for the inoculum to be absorbed into the agar

↓

Invert the prepared plates and incubate them for 18 h to 24 h in an incubator) set at 30°C. If colonies are not clearly visible, incubate the plates for an additional 24 h before counting

Counting of the colonies

- After the period of incubation, select plates, preferably at two successive dilutions, containing less than 150 colonies.
- Count the presumptive B. cereus colonies on each plate. The presumptive colonies are large, pink (indicating that mannitol fermentation has not occurred, and generally surrounded by a zone of precipitation (indicating the production of lecithinase).
- If there are less than 15 characteristic colonies on plates inoculated with the liquid product or the lowest dilution for other products.

- Calculate the arithmetic mean m of the colonies of B. cereus on both dishes.

Confirmation

Select five presumptive colonies from each plate selected. If there are less than five colonies on the plate, take all presumptive colonies present. Confirm these colonies by glucose utilization, VP test and nitrate reduction test. If the plates are overcrowded and it is not possible to select well-isolated colonies, streak five presumptive colonies on plates with complete medium. Incubate in an incubator set at 30°C for 18 h to 24 h.

Biochemical confirmation

Glucose utilization

- Inoculate selected colonies, using a stab inoculation wire, centrally into tubes containing freshly heated glucose agar. Incubate for 24 h in an incubator set at 30°C.
- A yellow colour throughout the whole tube indicates a positive reaction.

Voges-Proskauer test

- Inoculate selected colonies into tubes containing VP medium. Incubate for 24 h in an incubator set at 30°C.
- Transfer from each tube 1 ml of the culture to a clean tube to test for acetylmethylcarbinol.
- Add 0.2 ml of potassium hydroxide solution, 0.6 ml of α-naphthol solution and a few crystals of creatine. Shake vigorously and leave to stand for 1 h.
- Formation of an eosin pink colour indicates a positive reaction.

Nitrate Reduction Test

- Inoculate selected colonies into tubes containing nitrate medium. Incubate for 24 h in an incubator set at 30°C.
- Test for the reduction of nitrate to nitrite by adding 0.2 ml to 0.5 ml of nitrite reagent to each tube with a pipette equipped with a rubber bulb.
- In all other cases, calculate the number of B. cereus from the percentage of B. cereus obtained after counting which are confirmed. Formation of a red colour indicates the reduction of nitrate to nitrite.
- If no red colour is formed within 15 min, add a small amount of zinc dust and leave for 10 min. If after the addition of zinc dust a red colour is formed, the confirmatory test is negative.
- If no red colour is formed after the addition of zinc dust, then the test is positive.

7.11 Milk ring test for Brucellosis

Brucellosis is a communicable disease of zoonotic importance which causes mainly abortion in cattle and undulant fever and sterility in humans. Milk is a good medium for transmission of this organism from animal to humans

Materials Required

- 5 ml glass tubes
- *B. abortus* Bang Ring Antigen (hematoxylin-stained antigen manufactured by the State Biological Laboratory, Institute of Veterinary Preventive Medicine, Ranipet, India).

Procedure

Take sufficient quantity of milk samples so as achieve approximately 25 mm height

↓

Add 30 µl (0.03 ml) of B. abortus Bang Ring Antigen carefully

↓

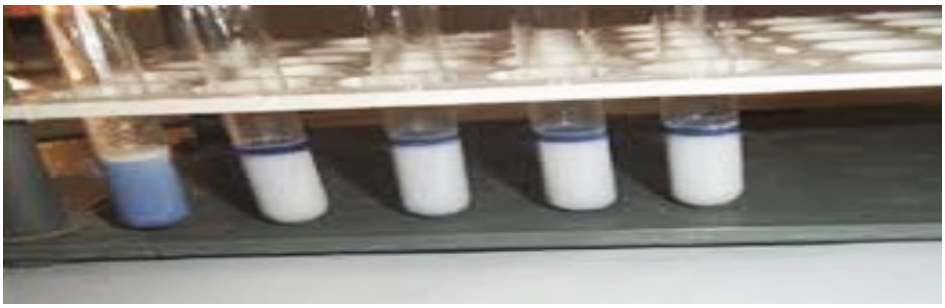
Incubate the preparation at 37°C for 1 hr

↓

Also run negative and positive controls simultaneously

Observation

- A strongly positive reaction is indicated by formation of a dark blue ring above a white milk column.
- The test is considered negative if the color of the underlying milk exceeded that of the cream layer and when the cream layer is normal.
- Samples are read as negative, 1+, 2+, 3+ and 4+ depending on the intensity of color in the cream layer.



Milk ring test for detection of brucellosis in milk samples

Inference

The criteria of reactions are as follows:

- Negative reaction (-): cream ring white, skim milk fraction blue white;
- Suspicious reaction (1+): cream ring pale blue but less colored than the skim milk fraction;
- Suspicious reaction (2+): the pink color of the cream ring equal to that of the skim milk fraction;
- Positive reaction (3+): color of cream ring deeper blue than that of the skim milk fraction;
- Positive reaction (4+): cream ring pink, skim milk fraction white.

7.12 California Mastitis Test for mastitis

Milk is a very good medium for growth and proliferation of the microbial organisms. Milk, when milked from a cow infected with subclinical mastitis, seems to be quite normal in appearance but it harbours millions of pathogenic germs which quickly multiply and may cause diseases in humans on consumption of the same. It is a rapid and easy test for detection of subclinical mastitis.

Materials Required

- Stripping cup
- CMT paddle
- Reagent
- Gloves

Procedure

After discarding the first stream of milk, draw the next milk into the shallow cups on the paddle, keeping the quarters separate

↓

Drain Excess milk: The ideal amount of milk is that which remains in the cup when the paddle is tilted to an almost vertical position

↓

Add an equal amount of the reacting solution

↓

Form pools of milk in cups, as shown, by tilting paddle

↓

Squirt test solution over milk. Avoid making bubbles

↓

Proportion of solution to milk should be at least one to one

↓

Mix the reagent and milk

↓

Gently rotate the paddle in horizontal plane, swirling the mixture for 10-30 seconds

↓

Positive reactions occur and can be graded during this rotary motion

↓

Always assume the same position when holding the paddle under the udder to keep track of the quarters when interpreting results

Leukocyte count per milliliter	Test appearance	CMT score	Description	Percent infected
Below 200,000	Mixture liquid, no precipitate	negative	Negative	25
150,000 to 500,000	Slight precipitate, tends to disappear with paddle movement	T	Trace	50
400,000 to 1,500,000	Distinct precipitate but does not gel with paddle movement	1	Weak positive	75
800,000 to 5,000,000	Distinct gel formation	2	Distinct positive	90
Over 5,000,000	Strong gel formation that tends to adhere to paddle. Forms distinct central peak	3	Strong positive	95-100

7.13 MBR (Methylene Blue Dye Reduction) test for raw milk and pasteurized milk

Methylene Blue Dye Reduction Test, commonly known as MBR test is used as a quick method to assess the microbiological quality of raw and pasteurized milk. This test is based on the fact that the blue colour of the dye solution added to the milk get decolourized when the oxygen present in the milk get exhausted due to microbial activity. The sooner the decolourization, more inferior is the bacteriological quality of milk assumed to be. This test is widely used at the dairy reception dock, processing units and milk chilling centres where it is followed as acceptance/ rejection criteria for the raw and processed milk

Materials and Reagents Required

- One sterilized test tube
- MBR dye solution
- Sterilized cork
- Serological water bath
- Milk sample

Procedure

The test has to be done under sterile conditions.

Take 10 ml milk sample in sterile MBRT test tube

↓

Add 1 ml MBRT dye solution (dye concentration 0.005%)

↓

Stopper the tubes with sterilized rubber stopper and invert the tube to mix contents properly

↓

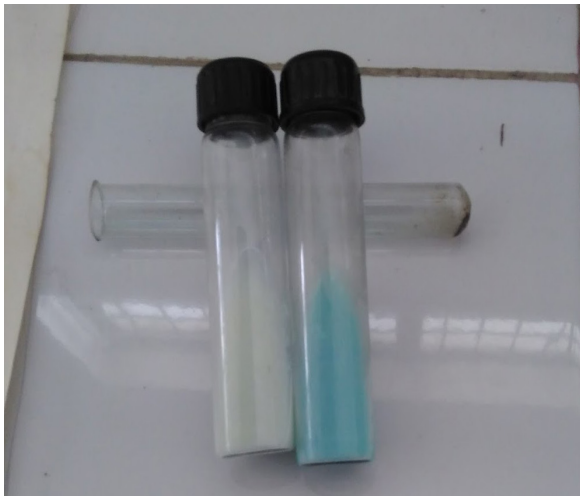
Carefully place them in a test tube stand dipped in a serological water bath maintained at 37±1^{o}C

↓

Record this time as the beginning of the incubation period

↓

Decolorization is considered complete when only a faint blue ring (about 5mm) persists at the top.



Results of MBR Test

Recording of Results

During incubation, observe colour changes as follows:

- If any sample is decolourized on incubation for 30 minutes, record the reduction time as MBRT - 30 minutes.
- Record such readings as, reduction times in whole hours. For example, if the colour disappears between 0.5 and 1.5 hour readings, record the result as MBRT - 1 hour; similarly, if between l.5 and 2.5 hours as MBRT - 2 hour and so on.
- Immediately after each, reading, remove and record all the decolourized samples and then gently invert the remaining tubes if the decolourization has not yet begun.

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