## Supplementary Material

**Annex I: - Checklist and Questionnaire**

**Socio demographic characters of interviewers**

1. Sex of milk handler a) male b) female
2. Marital status a) single b) married
3. Educational status a) illiteracy b) 1-8 c) 9-12 d) college and above

**Hygienic practices observed personally**

4. Wearing of clean overcoat a) yes b) No
5. Wearing head cover a) yes b) No
6. Nose picking habit during work a) yes b) No
7. Milk container used a) aluminium coated tunk b) plastic jercan

**Interview questions**

8. How many years do you providing this services(working on milk)? A) <1year b) 1 year c)> 1 year
9. **Do you know what hygienic practices mean?** a) yes b) no
10. **If yes,**
11. Do you wash your hand before and after handling milk
12. What you use for washing? A) using soap and water b) with water only
13. Do you wash milk container before and after using milk a) yes b) no
14. What you use to wash? A) using soap and water b) with water only
15. Have you trained on hygienic food handling practices a) yes b) no
16. Who trained_____
17. Have you check your health status regularly? a) yes b) no
18. How many times per year? A) once b) twice c ) more than
19. Have you ever checking normal and abnormal milk while working on it?

    a) yes b) no

17. How you know normal and abnormal milk? _____

18. have you encountered such kind of problems? a) yes b) no

18. Do you touch your nose while working on milk by any means? a) yes b) no

**Annex II :- Culture Media Preparation**

Maconkey Agar media preparation

Principle

MAC agars are slightly selective and differential plating media used for isolation and detection of gram negative organisms. Peptone provides nutrient to the organisms while lactose is the fermentable sugars. MAC agar is used for differentiating lactose fermenters from non-lactose fermenting gram negative enteric bacilli. When lactose is fermented due to production of acid, the local ph falls and methyl red (indicator) changes to red.

MAC agar contains crystal violet and bile salts that inhibit gram negative organisms and allow gram negative organisms too low.

Materials used

Mac conkey dehydrated powder, weighing balance, weighing boats, spatula, flask, hot plate with stirrer, sterile petridishes, autoclave, autoclave tape, water bath, and distilled water.

Procedures

- The MAC agar powder base weighed according to the instructions given on the label of the dehydrated powder. Then suspend the powder in distilled water in the Erlenmeyer flask. Heat with frequent agitation and boil for one minute to completely dissolve the powder.
- Autoclaved at 121°C for 15 minutes and Cooled the medium to 50°C. Aseptically poured into sterile petridishes (media dispensed in the BSC). After dispensed the media allowed to cool at room temperature to remove excess moisture.
- Plates labeled with media names, preparation date and expiration date
- The labeled plates Wrapped in plastic bags (10 plates per bag). Leave appropriate number of plates outside for quality control. Label media bags with media name, preparation date and expiration date, storage temperature.

- Media preparation recorded on appropriate form.
- Store wrapped plates at 2-8°C for up to 8 weeks
- Perform media quality control.

Quality Control

Prepared media is tested for sterility, ability to support growth and ability to produce appropriate biochemical reactions.

Quality Control Inoculating procedures.

The test organisms were inoculated over the quadrants of the plate. The test organisms used were *E. coli* and S. typhi streaking of the plate was with a four way isolation streak using sterile inoculating loop. Result is recorded in quality control form.

| QC | Media preparation date | Quality control result(growth/ no growth) | Test for sterility | QC passed/failed | Performing techn | Supervisor review |
|---|---|---|---|---|---|---|
| MAC | | Growth of *E. coli* and S. typhi seen | | passed | | |

Table12. Quality control of MAC Agar

Interpretation

Lactose fermenting organisms grow as pink to brick red colonies with or without the zone of precipitated bile. The bile precipitate is due to the local PH drop around the colony due to the lactose fermentation.

Lactose non fermenting organisms grow as colorless or clear colonies. When non lactose fermenters grow in proximity to coliform colonies, the surrounding medium appears as cleared areas. The gram positive organisms such as staphylococcus and streptococcus are inhibited.

1. TSI Agar media preparation

Principle

TSI is used for the differentiation of gram negative enteric organisms based on carbohydrate fermentation and production of hydrogen sulfide gas. TSI agar contains three sugars (dextrose ,lactose and sucrose), phenol red for detecting carbohydrate fermentation and ferrous ammonium sulfate for detection of hydrogen sulfide production(indicated by blackening in the butt of the tube). Carbohydrate fermentation is indicated by the production of gas and a change in the color of the ph indicator form red to yellow. To facilitate the detection of organisms that only ferment the dextrose, the dextrose concentration is one tenth the concentration of lactose or sucrose. The small amount of acid production in the slant of the tube during dextrose fermentation oxidizes rapidly, causing the medium to remain red or revert to an alkaline ph . in contrast, the acid reaction (yellow) is maintained in the butt in the tube because it is under lower oxygen tension. After depletion of the limited dextrose, organisms able to do so will begin to utilize the lactose or sucrose. To enhance the alkaline condition of the slant, free exchange of air must be permitted by closing the tube cap loosely. If the tube is tightly closed, an acid reaction (caused by solely dextrose fermentation) will also involve the slant.

Materials

TSI dehydrated powder weighing balance, weighing boats, spatula, flask, and hot plate with stirrer, sterile petridishes, autoclave, autoclave tape, and distilled water.

Procedures used

- Weigh the TSI base according to the instructions given on the label of the dehydrated powder. Then suspend the powder in distilled water in the Erlenmeyer flask. Heat with frequent agitation and boil for one minute to completely dissolve the powder.
- Dispense into the tubes and autoclave with caps loosened
- Cool in a slanted position so that deep butts are formed
- Tighten caps. Label tubes with media name and batch no
- Place tubes in a cartoon box label media box with media name, lot number and batch no, preparation date, expiration date

- Leave appropriate number of plates outside for quality control.
- Media preparation recorded on appropriate form.
- Store tubes at 2-8°C for up to 6months.
- Perform media quality control.

Quality Control

Prepared media is tested for sterility, ability to support growth and ability to produce appropriate biochemical reactions.

Quality Control Inoculation procedures.

To inoculate, carefully touch the center of an isolated colony on an enteric plated medium with a cool, sterile needle. Not use inoculating loop to inoculate a tube of TSI. Not take whole colony or go through colonies b/c of contamination. Stub into the medium in the butt of the tube and then streak back and forth along the surface of slant. Incubate with the caps loosened at 35 degree Celsius and examine after 18-24 hours for CHO fermentation, gas production and hydrogen sulfide production. Not incubate longer than 24hr b/c acid reaction in slant revert.

Interpretation

a. CHO fermentation is indicated by a yellow coloration of the medium. If butt =yellow(acidic), slant=red(alkaline), the organism being tested only ferments dextrose(glucose)
b. Yellow(acidic) in slant and butt indicates that orgs being tested ferments dextrose, lactose and or sucrose
c. Red (alkaline) in butt and slant indicates the orgs being tested is non-fermenter.
d. Hydrogen sulfide production is results in black precipitate in the butt of tube
e. Gas production is indicated by splitting and cracking of the medium.

2. SIM media preparation

Principle

SIM (Sulfide Indole Motility) Medium is a semisolid medium recommended for use in qualitative procedures for differentiation of microorganisms on the basis of hydrogen sulfide,

indole production, and motility. Casein and meat peptones supply nitrogenous compounds and amino acids necessary for the growth of enteric gram-negative bacilli. Sodium thiosulfate is a sulfur source. Ferric ammonium citrate, an indicator, reacts with the H2S produced by sulfate-reducing bacteria to form ferrous sulfide, a black precipitate. Organisms that possess the enzyme tryptophanase degrade tryptophan in the medium to form indole. Detection of indole is accomplished by addition of Kovacs' Reagent or Ehrlich's Reagent. Indole combines with ρ-dimethyl-aminobenzaldehyde to produce a red complex. The addition of agar results in a semisolid medium which is ideal for the detection of motility

Dehydrate culture medium preparation

1. Suspend 30 grams of medium in 1000 ml of demineralized water
2. Heating to boilwith frequent agitation inorder to completely dissolve the ingredients.
3. Dispense into tubes and sterilize by autoclaving at 121°C for 15 minutes.

Procedures

1. Consult current editions of appropriate references for the recommended procedure for sample preparation, inoculation, testing, and interpretation.
2. Lightly inoculate SIM Medium from a pure, 18-24 hour culture of the test isolate. Using an inoculating needle, stab down into the center of the medium to within the bottom ⅓ of the tube.
3. Incubate tube in ambient air with loosened cap at 33-37°C for 18-24 hours.
4. Examine tube for H2S production and motility (see Interpretation).
5. To detect indole production, add 3-4 drops of Kovacs' Reagent (REF R21227) or Ehrlich's Reagent (REF R21213) and observe medium for a red color development.

Interpretation of the test

| **Hydrogen Sulfide (S):** | |
|---|---|
| Positive Test - | Blackening along the line of inoculation |
| Negative Test - | No blackening of the medium |
| **Indole (I):** | |
| Positive Test - | Red color development in the upper |

| | |
|---|---|
| | portion of the medium |
| Negative Test - | No red color development |
| **Motility (M):** | |
| Positive Test - | Diffuse growth outward from the stab line or turbidity throughout the medium |
| Negative Test - | Growth only along the line of inoculation |

Quality control

Each lot number of SIM Medium has been manufactured, packaged, and processed in accordance with current Good Manufacturing Practice regulations. All lot numbers have been tested using the following quality control organisms and have been found to be acceptable. Testing of control organisms should be performed in accordance with established laboratory quality control procedures. If aberrant quality control results are noted, sample results should not be reported.

3. Mueller Hinton Agar

Principle

MHA is recommended for antimicrobial disk diffusion susceptibility testing of common ,rapidly growing bacteria by the Kirby-bauer method as standardized by the clinical laboratory institute.

MHA with 5% Sheep blood is recommended for antimicrobial disk diffusion susceptibility testing of streptococcus pneumonia and other streptococcus spp as standardized by the clinical laboratory standard institute.

Materials used

Mueller Hinton dehydrated powder, weighing balance, weighing boats,spatula, flask,hot plate with stirrer, sterile petridishes, autoclave, autoclave tape, water bath, distilled water, Thermometer,ph meter.

Procedures

- MHA agar weighed according to the instructions given on the label of the media powder. Then powder suspended in distilled water. Heated with frequent agitation and boiled for one minute to completely dissolve the powder. Not overheated
- Autoclaved at 121°C for 15 minutes and Cooled the medium to 50°C. Aseptically poured into sterile petridishes (media dispensed in the BSC to minimize contamination). The media allowed cooling at room temperature to remove excess moisture. (pour 60-70 ml for plates with diameter of 150mm and 25-30 ml for plates with diameter of 100 mm)
- The PH of each batch of MHA checked when the medium is prepared.
- Plates Labeled with media names, preparation date and expiration date.
- Plates wraped in plastic bags, 10 plates per bag. Leave appropriate number of plates outside for quality control. Media bags Label with media name, preparation date and expiration date, storage temperature.
- Store wrapped plates at 2-8°C for up to 8 weeks.
- Media preparation recorded on appropriate form
- Media quality control perform.

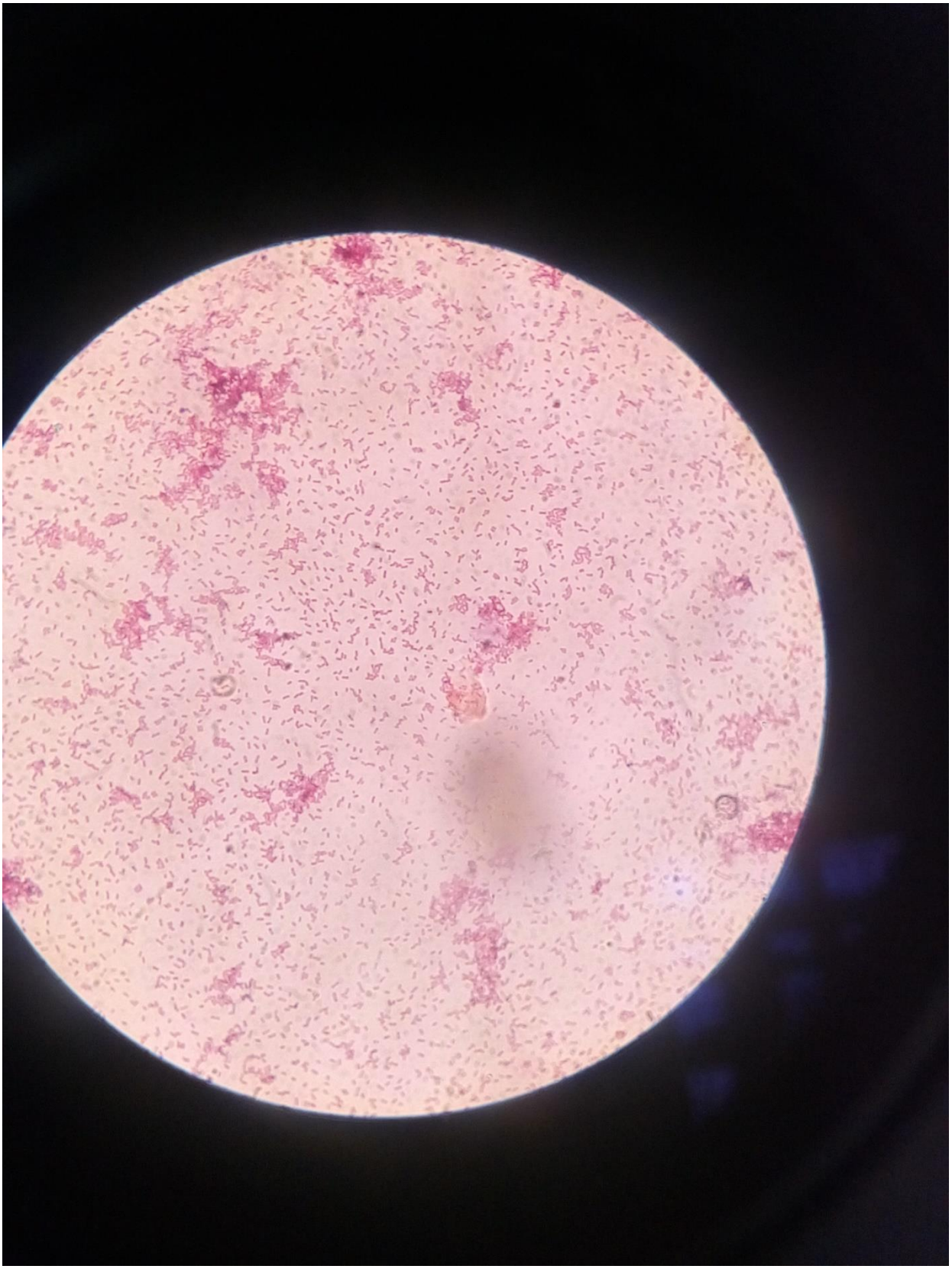
Quality Control

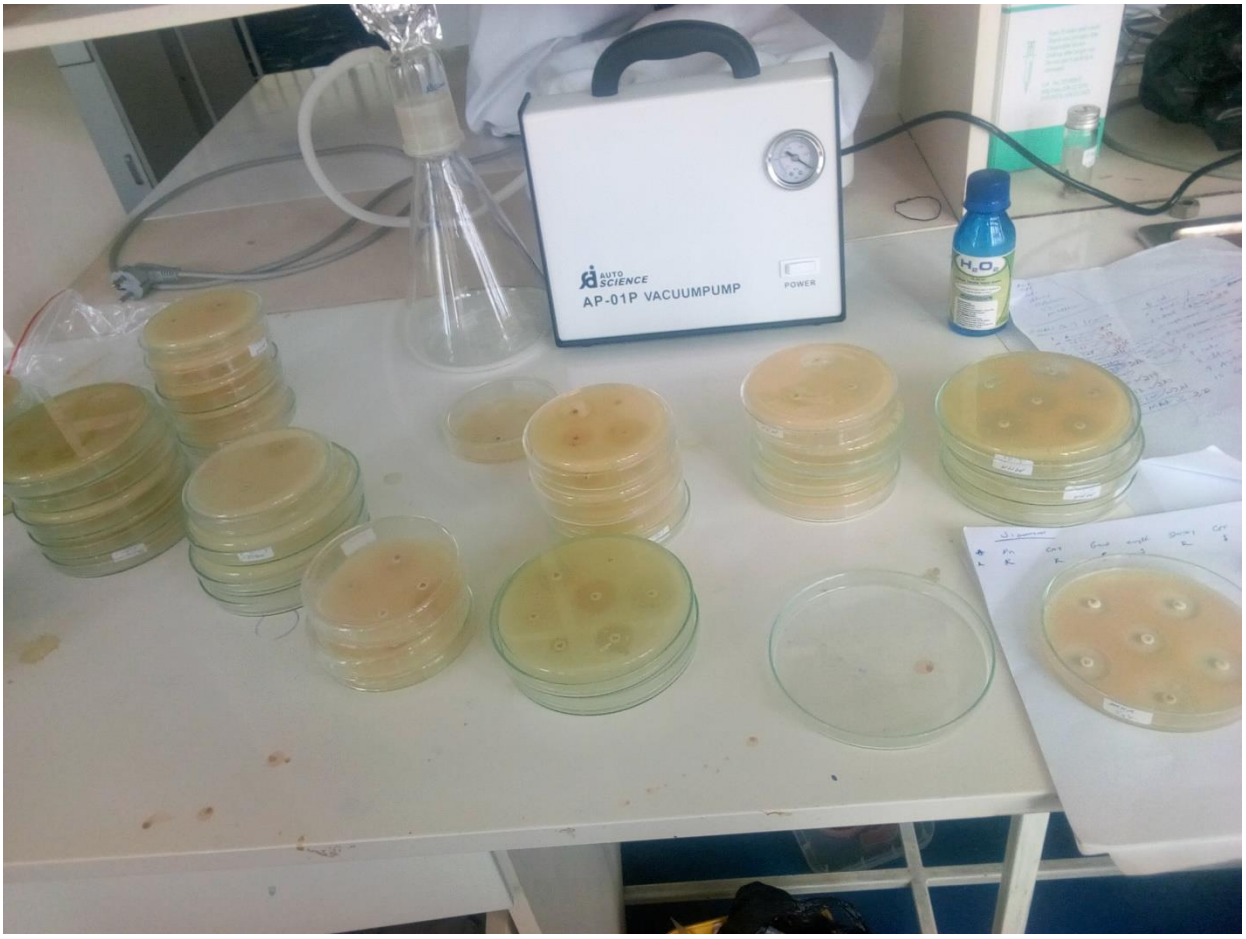
- One uninoculated agar plate incubated at least from each batch or lot overnight or longer to verify the sterility of the medium
- one agar plates or disk with the appropriate QC strains tested to determine if zone sizes obtained with the batch fall within expected range.

| QC | Media preparation date | Quality control result(growth/no growth) | Test for sterility | QC passed/failed | Performing techn | Supervisor review |
|---|---|---|---|---|---|---|
| Mueller hinton agar | | Growth and zone of inhibition | Sterile(no growth seen in | passed | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | detected. | uninoculated control media) | | | |

**Annex III; representative figures of the findings**

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