TO THE POINT CLASS LECTURES

MILK AND MILK PRODUCTS INSPECTION

(MICRO 404)

PRACTICAL WORK

COMPLETE

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Delivered by:

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Milk is a complete food which obtained from the animals. Inspection of milk is necessary to control the transmissible diseases from milk to human beings.

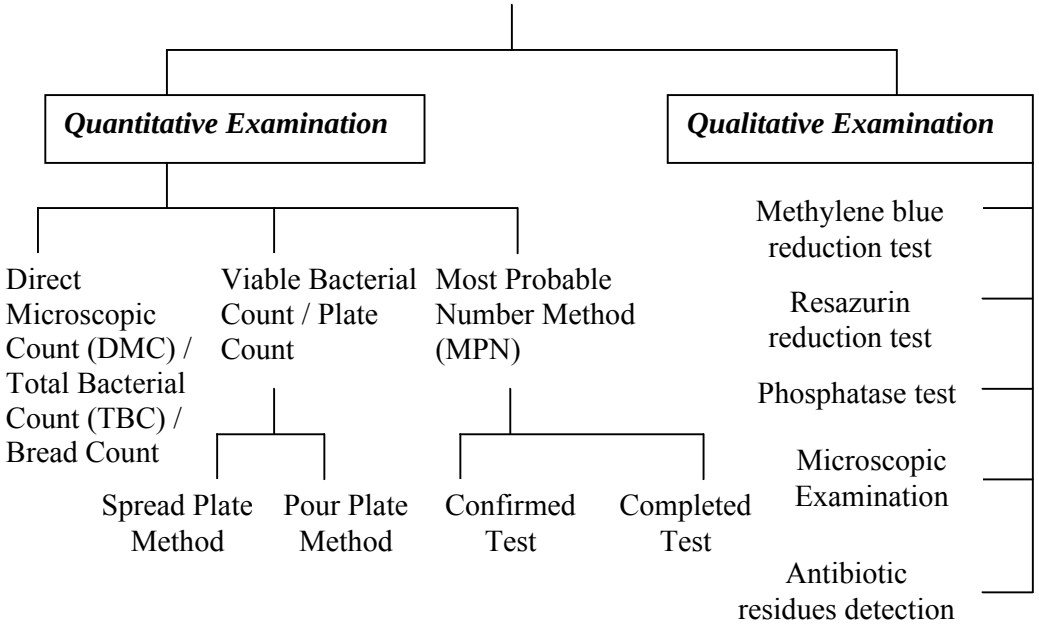
Objective:
To determine certain pathogens in the milk qualitatively and quantitatively.

Sources of Infection that Spoiling Milk

There are various sources which are responsible for contamination of milk.

a) **Animal itself:**
The important source of infection is animal itself – main disease is tuberculosis which is transferred from animals secretions to humans. Mycobacterium tuberculosis is an acid fast bacterium that can be killed by boiling of milk.
b) **Milkers**:
Transmission occurs through coughing, exhalation, and dirty hands (direct contact).
c) **Environment**:
If udders are not properly washed, it may be a source of infection.
Normally microbes are present on the skin of the udder. Lactobacillus is present in the milk. It produces sourness in the milk. It causes fermentation of lactose and converts it into lactic acid. It is beneficial in case of yogurt making and manufacturing of different fermented milk products.
d) **Equipments**:
If the utensils are not properly cleaned and disinfected or used again and again without disinfecting and proper cleaning, then pathogens also transmitted through uncleaned milking equipments and milking machines.

Milk and Milk Products Examination



Preparation of Heat Fixed Smears

Heat fixed smears are prepared when bacteria are to be stained.

a) Spread thinly a small drop of the bacterial suspension on the slide with the help of platinum loop.
b) Air-dry the smear (waving it about gently hastens drying).
c) Fix the air dried smear by passing over a low flame for a blink of a second.
d) Apply the stain (Gram stain, Methylene blue stain, etc.)
e) The organism will not be washed away during staining process.
f) Study the slide stained smear under bright field microscope.

QUANTITATIVE EXAMINATION

There are three methods under this category:

i) Direct Microscopic Count (DMC)
ii) Viable Plant Count
iii) Most Probable Number (MPN) method

Direct Microscopic Count (DMC)

Syn: Bread count, Total Bacterial Count (TBC)

Formula:

TBC / ml = MF x Average number of cells per microscopic field x dilution factor (i.e. 100)

Microscopic field:

A bright illuminating circular area under microscope is called a microscopic field.
Area of microscopic filed is denoted with "a".

Bread Area:

An area of 1 cm length and 1 cm with is marked on the slide as a bread area.
It is denoted with "A". It is determined as follows:

Length = 1 cm = 10^4 μm
Width = 1 cm = 10^4 μm

So, Bread Area will be:

A = 1 cm x 1 cm = 10^4 μm x 10^4 μm
A = 1 cm^2 = 10^8 μm^2

Microscopic Factor (MF):

It is defined as "Number of microscopic fields in a bread area".
It is determined as follows:

$$\text{Microscopic Factor (MF)} = \frac{\text{Bread Area}}{\text{Area of microscopic field}} \quad MF = A / a$$

a) Calculation for Microscopic factor (MF):

As we know: A = 10^8 μm^2, and now we determine the area of microscopic field "a":

area of a circle = $a = \pi r^2$.: $\pi = 3.14$ and radius = r = d/2

$a = 3.14 (d/2)^2$

- In order to know the diameter of a microscopic filed, we use "stage micrometer".
- Stage micrometer is a glass slide with small lines graduation.
- Distance between two larger division is 100 μm.

- Among two large divisions, there are further 10 small lines/ divisions.
- Distance between two smallest divisions is 10 µm.
- Suppose, total divisions in a bright area (microscopic filed) are 38.
- Then, diameter = d = 38 x 10 = 380 µm

Now, we calculating "a" area of microscopic field:

$a = 3.14 (d/2)^2$

$a = 3.14 (380/2)^2$

$a = 113354\ \mu m^2$

Hence, Microscopic factor (MF) will be:

$MF = A / a$

$MF = 10^8\ \mu m^2 / 113354\ \mu m^2$

$MF = 882$

b) Calculation for average number of bacteria per microscopic field:

Select 10 microscopic fields on the slide. Count the bacteria in each microscopic field one by one and then calculate the average number of bacteria in one microscopic field.

Suppose: bacterial count from 10 microscopic fields is 110. It means

Average number of bacteria / microscopic field = 110 / 10 = 11 bacteria

c) Calculation for Total Bacterial Count (TBC) / ml:

Since, standard inoculating loop takes 0.01 bacterial suspension when it is dipped.

As we calculated above; MF = 882 and

So, Total bacteria Count in 0.01 ml sample = MF x avg. bacteria per microscopic field

= 882 x 11 = 9702

Total Bacterial Count (TBC) in one ml = $9702 \times 100 = 9.702 \times 10^5$

TBC / ml = MF x average number of bacteria per microscopic field x dilution factor

= 882 x 11 x 100

= 9702 x 100

= 9.702×10^5

Viable Count

Syn: Plate Count

It is a quantitative method which is only applicable for counting the living cells/bacteria.

Requirements:

Glass ware (including test tubes, pipettes, petri plates), PBS (phosphate buffer solution) or Normal Saline, Nutrient agar (culture medium), incubator, inoculating loop

Procedure:

Prepare 10 fold serial dilution of milk sample as follows:

- Take 10 test tubes and 9 ml of any diluent i.e. PBS or normal saline in each test tube.
- 1 ml of milk sample is poured in first test tube by a pipette and it is then mixed thoroughly (Dilution 1/10 or 1:10)
- 1 ml from the first test tube is transferred to the second test tube by pipette. It is mixed again thoroughly. (Dilution 1/100 or 1:100)
- In the same pattern, dilution is done up to the last test tube. In this fashion, as dilution is increasing, bacterial number is decreasing in that particular test tube.

- 10 petri plates are prepared with general purpose "nutrient agar" in each test tube.
- Inoculation of 1 ml sample from each test tube is done in each respective plate by spread method with the help of a spreader or dispensor.
- Incubate these plates at 37 C for 24 hours.

Observations:
Dense colonies are formed in first two plates and then gradual decrease in intensity is observed. Count the number of colonies and select only one plate having 30-300 colonies.
Suppose; Plate 4 = 240 colonies
As a colony is formed by a single living cell, so a colony represents a living cell/bacteria.

Formula:
Viable count / ml = average no. of colonies x dilution factor

Calculation:
Average number of colonies = 240 Dilution factor = 10^4
So, viable count / ml = average no. of colonies x dilution factor
= 240 x 10^4 = 2.4 x 10^6 CFU (.: CFU = colony forming unit)

Merits:
- It is the only method used to count live cells.

Demerits:
- As culture medium, nutrient agar is used in viable count method, which is not suitable for growth of Mycobacterium and many other fastidious bacteria. Due to which, there may be formation of less number of colonies.
- Time consuming as 24 hours are required for incubation where as TBC takes only 5 minutes.
- Incubating period for some bacteria is more than 24 hours, so such bacteria may not give optimum growth during this period.
- More glass ware is required.
- It is a laborious method as well.
- Some bacteria i.e. anaerobic bacteria require anaerobic environment to grow which is not provided in this method.
- There may be aggregation of colonies and these falsely counted as one colony.

Comparison with DMC
In comparison to DMC,
- Viable count method is time consuming, laborious.
- More glass ware is required in this case.
- We can not differentiate into groups of bacteria as gram +ve or gram-ve.
- Contamination chances are also still higher in viable count.

Most Probable Number (MPN) Count

It is is a statistically designed, presumptive technique for quantitative method of examination
- Mostly performed for Coliform bacteria (*E.coli*) which ferment lactose, so lactose broth is used in it.
- Phenolphthalein is used as an indicator.

- Durham's tube is also used in order to detect the glass production

Procedure:

Prepare lactose broth (75 ml) in a beaker.
Take 15 glass test tubes and divide them into 3 groups/sets. (5 test tubes in each set)
Take 5 ml lactose broth in each test tube.
Then, a chemical indicator i.e. phenol red (0.001%) is added in each test tube which gives red color to the culture medium.
Place Durham's tube in each test tube in inverted form for detection of gas
In order to sterilize, place these tests tubes in autoclave at 121 C for 15 minutes.
After this, take 10 ml milk in each test tube of 1st set.
Take 1 ml milk in each test tube of 2nd set.
Take 0.1 ml milk in each test tube of 3rd set.
Then, incubate at 37 C for 48 hours.

Results:

If yellow color appear ---- indicate +ve result.
If yellow color + gas formation ----- indicates strongly +ve result
If no color change --------- indicates –ve result.

Interpretation:

E.coli (Colliform bacteria) ferment the lactose into lactic acid due to which there is decrease in pH --- > acidic pH ---- > results into change in color from red to yellow color. Accumulation of gas in Durham's tube indicates strongly +ve result.

Count +ve results by observing all the test tubes in each set and find results as: MPN / 100ml from MPN table. For example:

10 mL	1.0 mL	0.1 mL	MPN / 100mL
0	0	1	2
1	0	0	2
0	2	0	4

Confirmed Test

Water, milk and milk products which showed positive presumptive or MPN test may be further processed for Confirmed Test procedure which will elaborate the availability of coliform organism including *E.coli*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella* etc.

Water, milk and milk products are applied on the McConkey's agar plate and incubated at 37 C for 24 hours.

Results:

The appearance of growth on McConkey plate will indicate the presence of coliform organisms in the sample. The plates are examined for the development of pink colored colonies on the McConkey agar plates. Pink colored colonies; rounded, convex, shining in appearance, gram –ve rods will specifically indicate the presence of *E.coli* that may be further confirmed through the Completed Test procedure.

Completed Test

The specific pink colored colonies from MacConkey agar surface should be processed for detailed morphological examination using Gram's staining method and biochemical tests (sugar fermentation test) as follows:
Indole test, Methyl red test, Vogese prosker, Citrate utilization test, Catalase test, Nitrate reduction test etc.
Note: Water & milk acquired colliform organisms particularly from sewerage contamination.

QUALITATIVE EXAMINATION

Milk and milk products are examined on the basis of bacterial load present in the milk samples.
The enumeration of total bacterial load is elaborated with respect to the reduction time taken up by the milk and milk products to reduce the color of the dye.
Two methods are internationally recognized:

(i) Methylene blue reduction test
(ii) Resazurin test

Methylene Blue Reduction Test

The ability of bacteria to reduce the color of Methylene blue dye from the milk sample is utilized in this method. The dye reduction time is inversely proportional to the presence of total number of bacteria in the sample.
Hence, greater the bacterial population, shorter the dye reduction time.

Requirements:
Glass test tubes, water bath, Methylene blue, glass pipettes, glass beaker

Procedure

- Take10 ml milk sample in the test tube.
- Add 1 ml dye (Methylene blue) in the milk.
- Incubate at 37 C in the water bath for 2-8 hours.
- Record the change in color f the milk after every 30 minutes.

Results:

Color of the sample	Quality of sample
No change in color (blue color) in 8 hours	Excellent
Change in color in 6-8 hours (blue --> white)	Good
Change in color in 2-6 hours (blue -- > white)	Fair
Change in color within 2 hours (blue --> white)	Poor

Resazurin Test

The ability of bacteria to reduce the color of resazurin dye from the milk sample is utilized in this method. The dye reduction time is inversely proportional to the presence of total number of bacteria in the sample. Resazurin dye solution must be used freshly prepared.

Requirements:
Same as in Methylene blue reduction test.

Procedure:

- Take 10 ml of the milk sample in the test tube.
- Add 1 ml resazurin dye solution and properly stopper with aluminum foil.
- Incubate at 37 C in water bath for 1 hour.
- Record the change in color of sample after 1 hour.

Results:

Color of sample (within 1 hour)	**Quality of sample**
No color change (blue color)	Excellent
Change in color from blue to deep mauve	Good
Change in color from deep mauve to deep pink	Fair
Change in color from deep pink to whitish pink	Poor
Change in color from whitish pink to white	Bad

Comparison:

Methylene blue reduction test	**Resazurin test**
Require more time to complete(about 8 hours)	Less time is required (about 1 hour)
Result interpretation is easy	Difficult to interpret (result vary from individual to individual)
Dye not sensitive too light	Dye sensitive to light, it reduced when exposed to sunlight.

Detection of Antibiotic Residues in Milk
STAF Test (Swab Test on Animal Foods)

STAF test is used to detect the antibiotic residues secreted in the milk.

Requirements:

Spore suspension of *Bacillus subtilis* (non-pathogenic bacteria)
Nutrient agar (Melted)
Petri dishes
Sterilized cotton swabs
Standardize antibiotic disc e.g. Kenamycin (5 µg)
Milk or milk product i.e. sample

Procedure:

- Put melted nutrient agar in test tube
- Then, put 0.2 ml spore suspension in that test tube.
- Mix it and pour the whole mixture in a Petri dish.
- Wait for solidification
- Take kenamycin disc and place it with sterilized forceps on the surface of the nutrient agar. Also press the disk a bit.
- Now dip the sterilized cotton swab in the milk for few seconds and place on the surface of nutrient agar plate and press it gently with the help of forceps. Distance between disk and swab should be >1.5 cm.
- Then, incubate petri dish at 37 C for 16-20 hours.

Observations:
Clear zone of inhibition is seen around the kenamycin disk i.e. must be 14-16 mm
Now look at any other such zone around the cotton swabs
If it is more than 2 mm, antibiotics residues are more than the permissible limits.

Interpretation:

- If zone of inhibition around antibiotic disc is 14-16 mm, it means the test is valid or complete.
- If zone of inhibition is less than it then repeat the whole procedure.
- If swab zone is equal or more than 2 mm then the antibiotic residues are present and greater than permissible limits.
- If swab zone is less than 2 mm then the antibiotic residues are in permissible limits. Milk can be used without any antibiotic residue hazards.
- If no zone around swab, no antibiotic residue.

Examination of Milk for Different Bacteria

(*Salmonella*, *Staph. aureus*, *Clostridium*, *Fungi / Yeast*)

Procedure is same as that of Plant Count/ Viable Count; includes major steps:

- 10 fold serial dilution of milk sample
- Inoculation on the culture medium
- Incubation
- Select plate having 30-300 colonies

Viable count / ml = average no. of colonies x dilution factor
Inoculation done on selective medium for growth of each bacteria. Common culture medium used for scertain bacterial growth are given below:

Staph aureus ---------- Staph. 110 medium
Salmonella ---------- McConkey agar, SS agar
Clostridial spp. ---------- Meat broth
Fungi / yeast ---------- Sabaroud agar, Potato dextrose agar

Assignment: Up to Date: 10 Jan 2011 - Monday
Grading of Milk on Total Bacterial Count
Grading of Milk on Viable Count basis
Grading of Milk on MPN count basis