CONTAMINATION ASSESMENT

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ASSESMENT OF CONTAMINATION AND SPOILAGE

1. **Physical and Chemical change**
   - Change in Viscosity
   - pH
   - Stability
   - Surface Activity
2. **Sterility Test**
3. **Viable microorganism testing in non-sterile products**
4. **Estimation of Pyrogens**
   - Rabbit
   - LAL Test

Microbial Limit Test (MLT) or Total Viable Count (TVC)

Microbial limit test or total viable count was designed to perform quantitative or qualitative estimation of number of viable aerobic micro-organism present or detecting the presence of designated microbial species in pharmaceutical product.

Preliminary testing:

The method given here are invalid unless it is demonstrated that the test specimen to which they are applied do not themselves inhibit the multiplication under the test condition of micro- organism that can be present.

Therefore, inoculate diluted specimen of substance being examined with separate viable culture of

(1) ***E. coli***

(2) ***S. aureus***

(3) ***S. typhi***

(4) ***P. aeruginosa***

If organism fails to grow in medium the procedure should be modified by:

a) increasing the volume of diluents with quantity of test materials remain same,

or

b) increasing a sufficient quantity of inactivating agent in diluents

or

c) combining aforementioned modification so as to permit growth of organisms in media.

- If inhibitory substances are present in sample, 0.5% soya lecithin & 4% of polysorbate 20 may be added to the culture medium.

- Repeat the same procedure using fluid casin digest -soya lecithin -polysorbate-20 medium to demonstrate neutralization of preservative or other antimicrobial agent in test material.

- Where inhibitory substance are contained in product & latter is soluble, the Membrane filtration method may be used.

The microbiological quality of non-sterile pharmaceutical or cosmetic material can be controlled by using two methods

- Estimation of the total number of viable aerobic microorganisms in given sample (total viable count)

- Detecting the presence of specific microbial species in pharmaceutical substance.

This microbial limit tests are applied to raw material of pharmaceutical products of natural or biological origin.(starch, gum, gelatin) and some finished products (calamine lotion, dried aluminium hydroxide gel) etc.

Pathogenicity of specific Microorganisms I.P

- ✓ ***E. coli:*** **Enterotoxins/Diarrhoel diseases. Hence, exclude from pharmaceutical materials.**
- ✓ ***Salmonella species*:** **Initiate infections by ingestion/ Excluded from pharm. materials because they represent major infection.**
- ✓ ***S. aureus*:** **Originate on skin /Limit tests for *S.aureus* are most likely to be applied to topical products.**
- ✓ ***P. aeruginosa*:** **Pathogen infects vulnerable sites eg: eyes opportunist/immunity/topical products/resistant to preservatives**

METHODS

TOTAL AEROBIC MICROBIAL COUNT

- ❖ MEMBRANE FILTRATION METHOD

- ❖ PLATE COUNT METHOD
  - ✓ POURED PLATE
  - ✓ SPREAD PLATE

- ❖ MUTIPLE OR SERIAL DILUTION METHOD

TOTAL AEROBIC MICROBIALCOUNT

PREPARATION OF TEST FLUID:

Water soluble product: Dissolve 10g or 10ml of the sample in buffer or fluid medium & adjust volume to 100ml.

Product insoluble in water (non fatty): Take 10g of sample, grind to fine powder & suspend it in buffer or fluid medium & adjust the volume to 100ml. A suitable surface-active agent such as 0.1%w/v of polysorbate 80 may be added to assist the suspension of poorly wettable substance.

Fatty product: Homogenise 10g or 10ml of sample with 5g of polysorbate-20 or polysorbate-80.

- If necessary ,heat to not more then 40°c for 30 min.
- Add 85 ml of buffer or fluid medium.
- Adjust the PH to about 7.

Membrane filtration

Use membrane filter 50mm in diameter & having nominal pore size not greater than 0.45 µm or less.

Sterilize the filters, filteration apparatus, media & other apparatus used.

- Transfer 10ml or quantity of each dilution contain 1g of preparation being examined to each of two membrane filter & filter immediately.
- If necessary dilute the pretreated preparation so that 10-100 colony count may be expected.
- After filtration wash the each filter three or more time with appropriate fluid such as phosphate buffer, sodium chloride-peptone buffer or fluid medium.
- For fatty substance add polysorbate-20 or polysorbate-80 to washing.
- Transfer one of the membrane filter, intended for enumeration of bacteria to surface of plate of soyabean casein digest agar & intend for enumeration of fungi to surface if sabouraud dextrose agar with antibiotics.
- Incubate the plate for 5 days, unless more reliable count is obtained in shorter time, at 30 to 35°c in test for bacteria & 20 to 25°c in test for fungi.
- Count the number of colonies that are formed.
- Calculate the no of organism per gram or ml of preparation being examined.

POUR PLATE METHOD

FOR BACTERIA:

- ✓ Use Petri dish 9 to 10 cm diameter, add to each dish a mixture of 1ml of the pretreated preparation & about 15ml of liquefied soyabean casein digest agar at not more than 45°c.
- ✓ If necessary dilute the preparation as described above so that colony count not more than 300 may be expected.
- ✓ Incubate the plate at 30 to 35 °c for 5 days unless more reliable count is obtained in shorter time.
- ✓ Calculate the result using plate with greatest no. of colonies but taking 300 colonies per plate as maximum consistent with good evaluation.

FOR FUNGI:

- ❖ Use saboraud dextrose agar with antibiotics & incubate the plate at 20 to 25 °c for 5 days.
- ❖ Calculate the result using plate with not more than 100 colonies.

SPREAD PLATE METHOD

- ✓ Place 0.05-0.2 ml of test fluid on solidified dried surface of agar medium spread it uniformly using spreader.
- ✓ Proceed under same condition as for the pour plate method.

MULTIPLE TUBE OR SERIAL DILUTION METHOD

Use 12 test tubes : 9 containing 9 ml of soybean- casein digest medium each and 3 containing 10 ml of the same medium each for control. Prepare dilutions using the 9 tubes.

- ❑ First, add 1 ml of the test fluid to each of three test tubes and mix to make 10-times dilutions.(100µl)
- ❑ Second, add 1 ml of each of the 10-times dilutions to each of another three test tubes and mix to make 100- times dilutions.(10µl)
- ❑ Third, add 1 ml of each of the 100-times dilutions to each of the remaining three test tubes and mix to make 1,000- times dilutions (1µl)
- ✓ Incubate all 12 test tubes for at least 5 days at 30 - 35°c.
- ✓ No microbial growth should be observed for the control test tubes.
- ✓ If the determination of the result is difficult or if the result is not reliable, take a 0.1ml fluid from each of the9 test tubes and place it to an agar medium or fluid medium, incubate all media for 24 — 72 hours at 30 — 35°c, and check them for the absence or presence of microbial growth.
- ✓ Calculate the most probable number of microorganisms per ml or gram of the sample.

SERIAL DILUTION METHOD

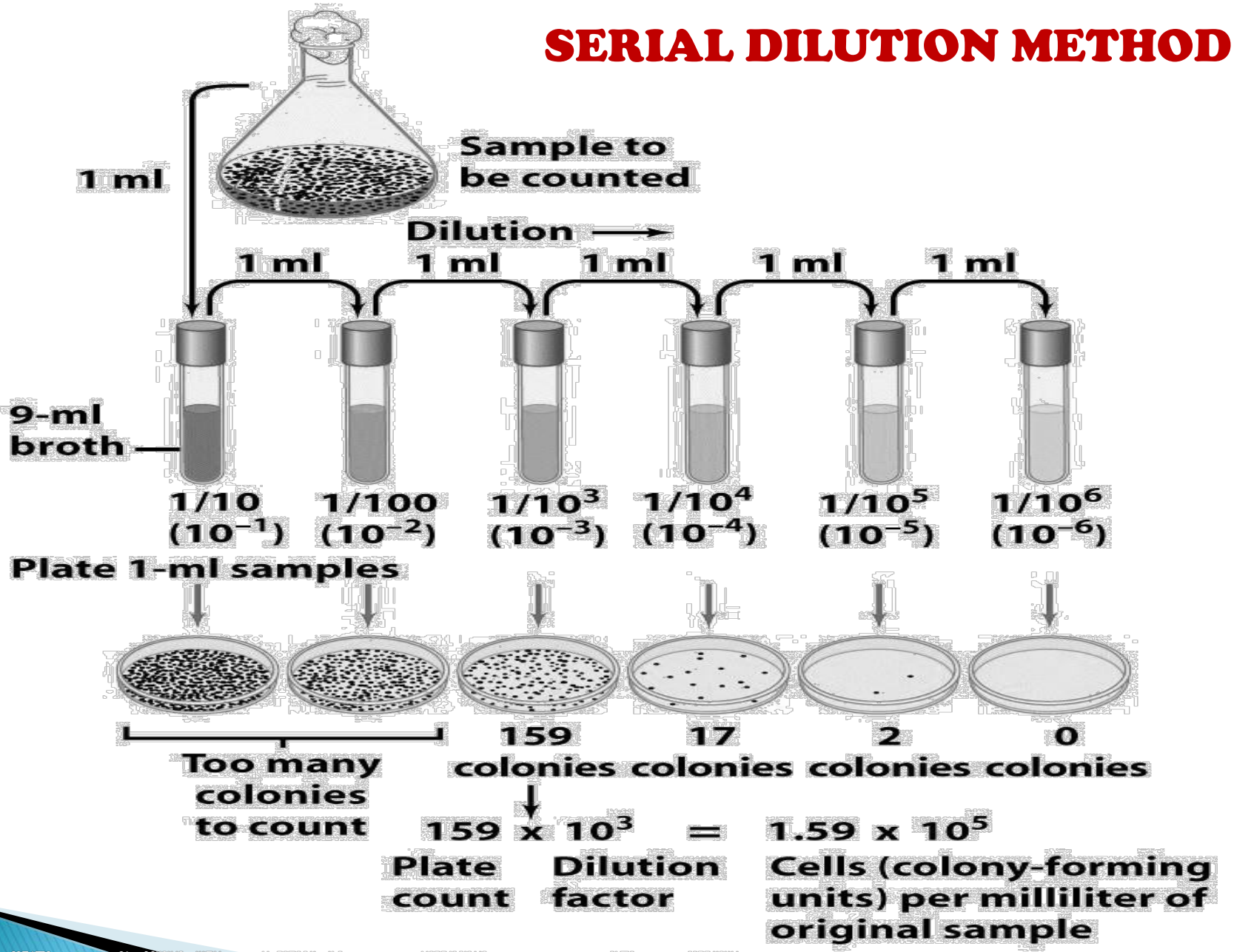

Figure 6.11 Brock Biology of Microorganisms 11/e
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Most probable number (MPN)

1. Take 14 test tubes of similar size place 9 ml of sterile fluid soyabean casein digest medium.

2. Arrange twelve of the tubes in four sets of 3 tubes each. One among serves as control.

3. Prepare stock solution of sample and dispense 1ml into one set each of three tubes of conc. named 100µg/ µl and into tube A and from it to tube B.

4. Now ,where the concentrations of tube B and second set is 10 µg/ µl and set three is 1µg/ µl.

5. Close well and incubate all tubes and examine the growth in each test tubes. The 3 tubes remain clear.

Interpret with reference table indicate MPN of Microorganisms per gm/ml of test sample

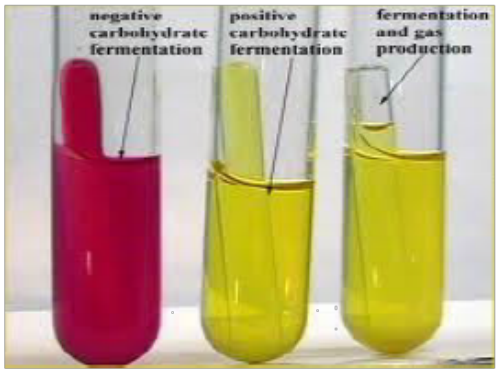
Most probable number of multiple tube or serial dilution method

0.1 ml	0.01 ml	0.001 ml	The most probable number per gram or ml of sample
3	3	3	>1100
3	3	2	1100
3	3	1	500
3	3	0	200
3	2	3	290
3	2	2	210
3	2	1	150
3	2	0	90
3	1	3	160
3	1	2	120
3	1	1	70
3	1	0	40
3	0	3	95
3	0	2	60
3	0	1	40
3	0	0	23

TEST FOR SPECIFIED MICROORGANISMS

DETECTION OF *E. coli*

Prescribed quantity of test sample + 50ml of nutrient broth



Shake and incubate at 37^{0}C/24 hrs

Perform primary test

1ml enrichment culture + 5ml of Mackonkey broth

Shake and incubate at 37^{0}C for 48 hours

Durham's Tube

If tube shows acid and gas formation then perform secondary test

0.1ml enrichment culture + 5ml Mac Conkey broth

incubate at 37^{0}C/24 hrs

Presence of acid and gas

0.1ml enrichment culture + 5ml peptone water

incubate at 37^{0}C/24 hrs

0.5ml of Kovacs regt.

Shake and detect presence of red colour Indole (+ve)

Indicates the presence of E.coli

DETECTION OF *Salmonella*

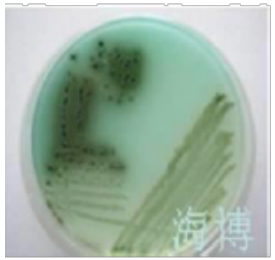
1gm or 1ml of the test sample+100 of NAB

Perform primary test

Shake &incubate at 37C/24hrs

1ml enrichment culture +10ml selenite F-broth

1ml of enrichment culture +10ml of tetrathionate bile-brilliant green broth



Incubate at 37^{0}C /48 hrs

Sub culture each of two cultures, on at least two of the following 4 agar media

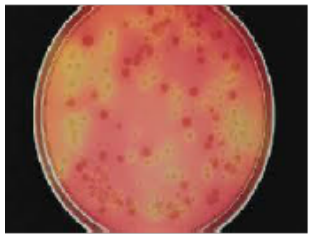
Bismuth sulphite agar (BSA)

Brilliant green agar BGA

Deoxycholate citrate agar (DCA)

Xylose lysine deoxycholate agar (XLDCA)

Incubate all plates at 37C/24hrs and observe the colony characteristics



Black or green

Small, tranparent and colourless or apaque,pinking or white

Colourless and opaque with or without black centers

Red with or without black centers

If none of the colonies confirm to the characteristics on the different media, the sample meets the requirements of the absence of the salmonella. If colonies are formed confirming on the basis discription, carrry out the secondary test.

Perform secondary test

Subculture any colonies showing the positive characteristics

Triple sugar iron agar Slant

TSIA (stab)

Urea broth

Incubate at 37^{0}C/24hrs

Absence of acidity

Formation of acid and gas

Absence of red colour

Indicates presence of Salmonella

DETECTION OF *P. aeruginosa*

Pre-heat &inoculate 100ml of fluid soyabean casein digest medium with a quantity of the 1gm(or as specified in monograph) test sample

Mix& incubate 37C°/48hrs

Examine the medium for growth, if growth is present, streak on the surface of cetrimide agar medium

incubate at 37°C for 24 hours

Greenish colonies

No greenish colonies
(Sample free from *p.aeruginosa)*

Permorm oxidase test

Streak representative colonies on the surfaces of Pseudomonas agar medium for detection of fluorescein and pyocyanin

Incubate at 37°C for not less than 3 days and examine under U.V light

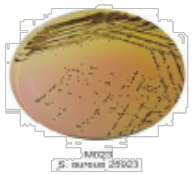
Examine the plates to confirming the colonies as yellowish (flurescein) and blue (pyocyanin) colour

If colony confirms for colour and growth, add growth, add 2-3 drops of 1%w/v solution of N,N,N1, N2 tetramethyl-4-phenylenediamine di-hydrochloride on filter paper and smear with the colony.

If there is no development of pink colour (changing to purple)

Absence of *pseudomonas aeruginosa*

DETECTION OF *S. aureus*



Pre-heat &inoculate 100ml of fluid soyabean casein digest medium with a quantity of the 1gm(or as specified in monograph) test sample

Mix& incubate 37C⁰/48hrs

Examine the medium for growth, if growth is present, streak on the surface of following medium

Vogel-Johnson agar

Black colonies surrounded by yellow zones

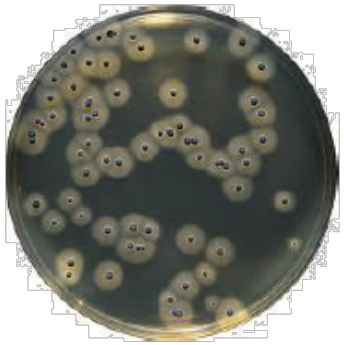
Mannitol-salt agar

incubate at 37C⁰/24hrs

Yellow colonies with yellow zones

Baired-Parker agar

Black shiny colonies surrounded by clear zones



If none of colonies have the characteristics given as above for the media used that indicates absence of *S.aureus*. If growth occurs and colony shows the above specific charecteristics, carry out coagulase test.

Transfer representative colonies from the agar surface into a test tube containing 0.5 ml of mammalian, preferably rabbit or horse plasma with or with out additives.

Incubate in water broth at *37C⁰* and examining the tubes at 3 hrs and subsequently at suitable intervals up to 24 hrs.

If no coagulation is observed that indicates absence of *S. aureus*

IMViC TEST OF COMMON ORGANISMS

Species	Indole	Methyl Red	Voges-Proskauer	Citrate
Escherichia coli	Positive	Positive	Negative	Negative
Shigella spp. [2]	Negative	Positive	Negative	Negative
Salmonella spp.	Negative	Positive	Negative	Positive
Klebsiella spp.	Negative	Negative	Positive	Positive
Proteus vulgaris [3]	Positive	Positive	Negative	Negative
Proteus mirabilis	Negative	Positive	Negative	Positive
Citrobacter freundii	Negative	Positive	Negative	Positive
Enterobacter aerogenes	Negative	Negative	Positive	Positive

Conclusion

In Microbial limit test, microorganisms are count and are compared with the ATCC/MTCC culture for detection of pathogenic bacteria.

The bacterial culture of ATCC/NCTC/MTCC used, are given below:

- ***Escherichia coli*** ***ATCC No. 8739***
- ***Pseudomonas aeruginosa*** ***ATCC No. 9027***
- ***Staphylococcus aureus*** ***ATCC No. 6539***

THANK YOU