
isoclean

- **Sterilization**
- **A validated process used to completely eliminate or destroy all forms of microbial life — including bacteria, fungi, spores, and viruses — from a product, surface, or environment.**
- Sterilization results in a product that meets a **Sterility Assurance Level (SAL)**, typically **10^{-6}**, meaning the probability of one non-sterile unit is ≤ 1 in 1,000,000.

- **Aseptic Manufacturing**
- **A controlled manufacturing process in which sterile components (products, containers, closures) are assembled and filled in an environment that maintains sterility throughout the entire operation.**
- It relies on:
- Sterile materials
- Sterile equipment
- HEPA-filtered air
- Controlled cleanroom behavior
- Continuous environmental monitoring

- **Aseptic Technique**
- **A set of practices performed in a highly controlled manner to prevent contamination by microorganisms during handling, processing, and filling of sterile products.**
- Includes: proper gowning, restricted movements, disinfection, material transfer procedures, etc.

Counting, Detecting and identifying microorganisms

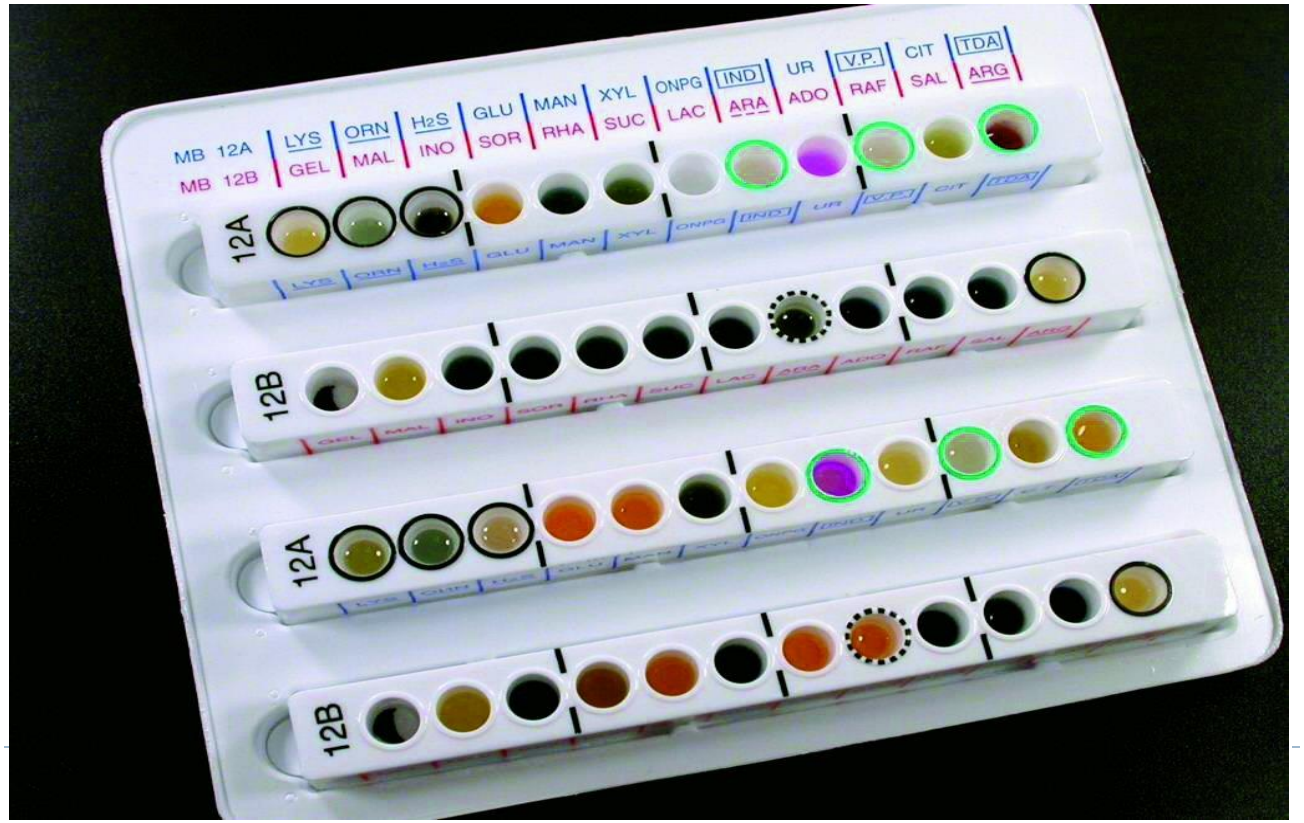
Chapter 15&18

Key Facts

- **Bioburden**: the number and type of microorganisms (MO) present in or on a pharmaceutical raw material, medicine, or medical device
- **Pharmacopeias describe procedures for**:
- Procedures for counting microorganisms
- Procedures for confirming the absence of named objectionable MO
- Specifications for the max permitted levels of bacteria and fungi for the different categories of nonsterile medicine
- Requirements for the absence of one or more of the major objectionable MO: E.coli, Salmonella species, Staph aureus, Pseudomonas aeruginosa, and Candida albicans

Key Facts

- Commercially available test kits and automated instruments are available for identifying different categories of bacteria and yeasts



Key Facts

- Objectionable:
- Because it is a pathogen so its presence in a medicine may cause an infection (E.coli & Staph aureus are pathogens→ health hazards)
- Its presence may be indicative of poor-quality raw materials or poor manufacturing procedures

Gelatin: test for absence of E. coli and Salmonella

Bioburden

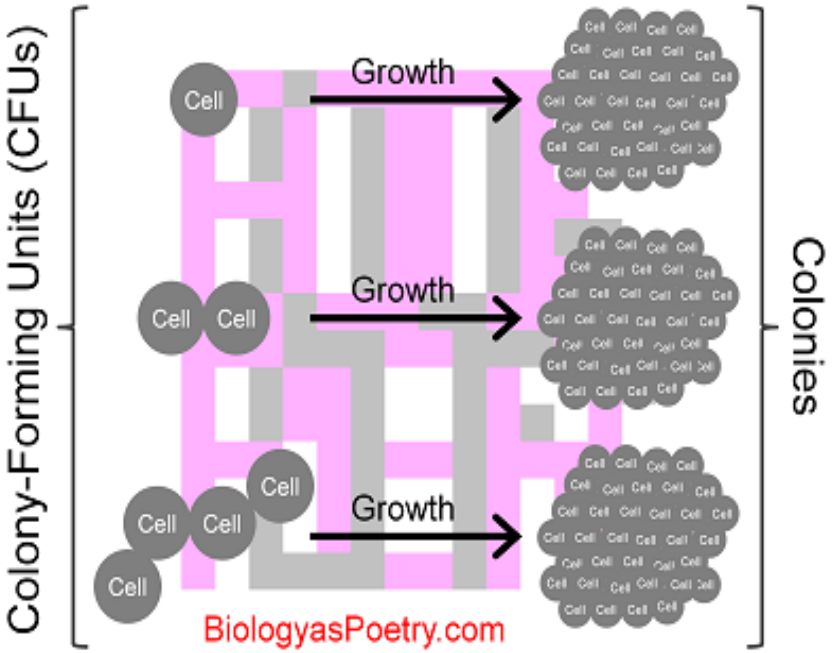
- Determination of bioburden quantitatively: to count the organisms present → Total Viable Count (TVC)
- Bioburden test → the most common microbiological test undertaken in the pharmaceutical industry:

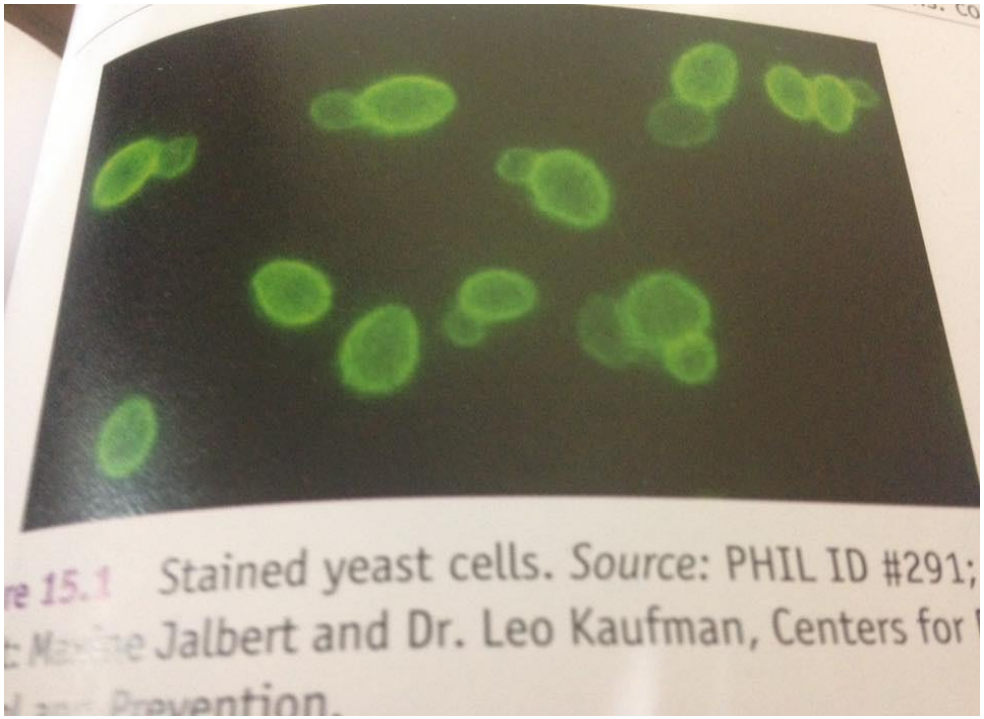
- Raw material (including water)
- Finished products
- Various stages during manufacturing process

Traditional counting methods:

- Place a sample of the material to be tested onto or into gelled culture medium in a petri dish (plate) and counting the visible colonies that arise after incubation
- A single colony may develop from an individual cell or from a group of cells attached or clumped together→ count is expressed as colony forming unit (CFU) and not cells per ml or gram
- USP states that petri dishes containing between 25-250 colonies produce reliable bioburden results
- High Bioburden materials:
- Mined minerals (talc, kaolin, bentonite)
- Vegetable origin (starches, gums, thickening agents)
- Animal origin (gelatin)

Traditional counting methods



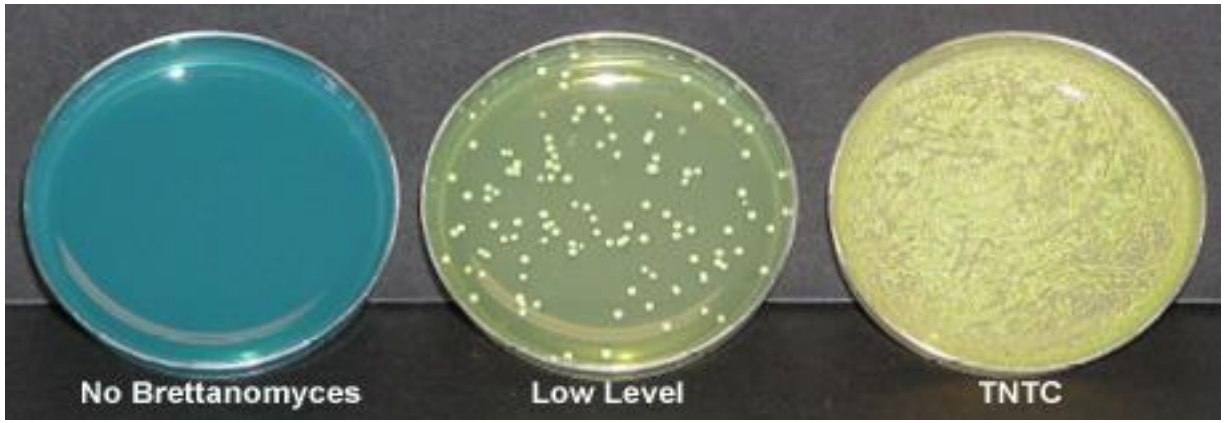


Traditional counting methods

- Dilution is necessary for material with a high bioburden, why?

to achieve a countable number of colonies on the plate

Perform a series of dilution (tenfold increments)



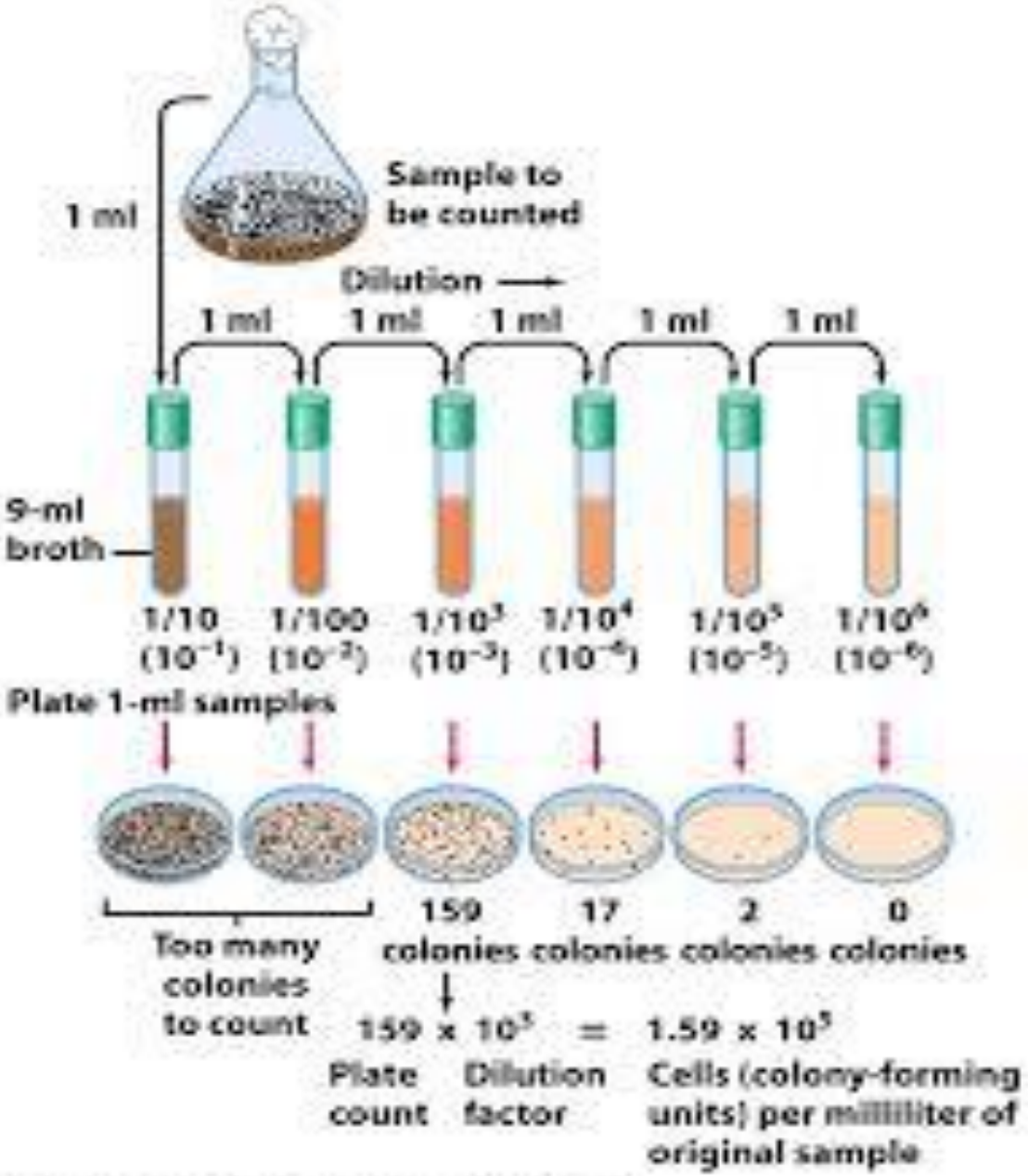
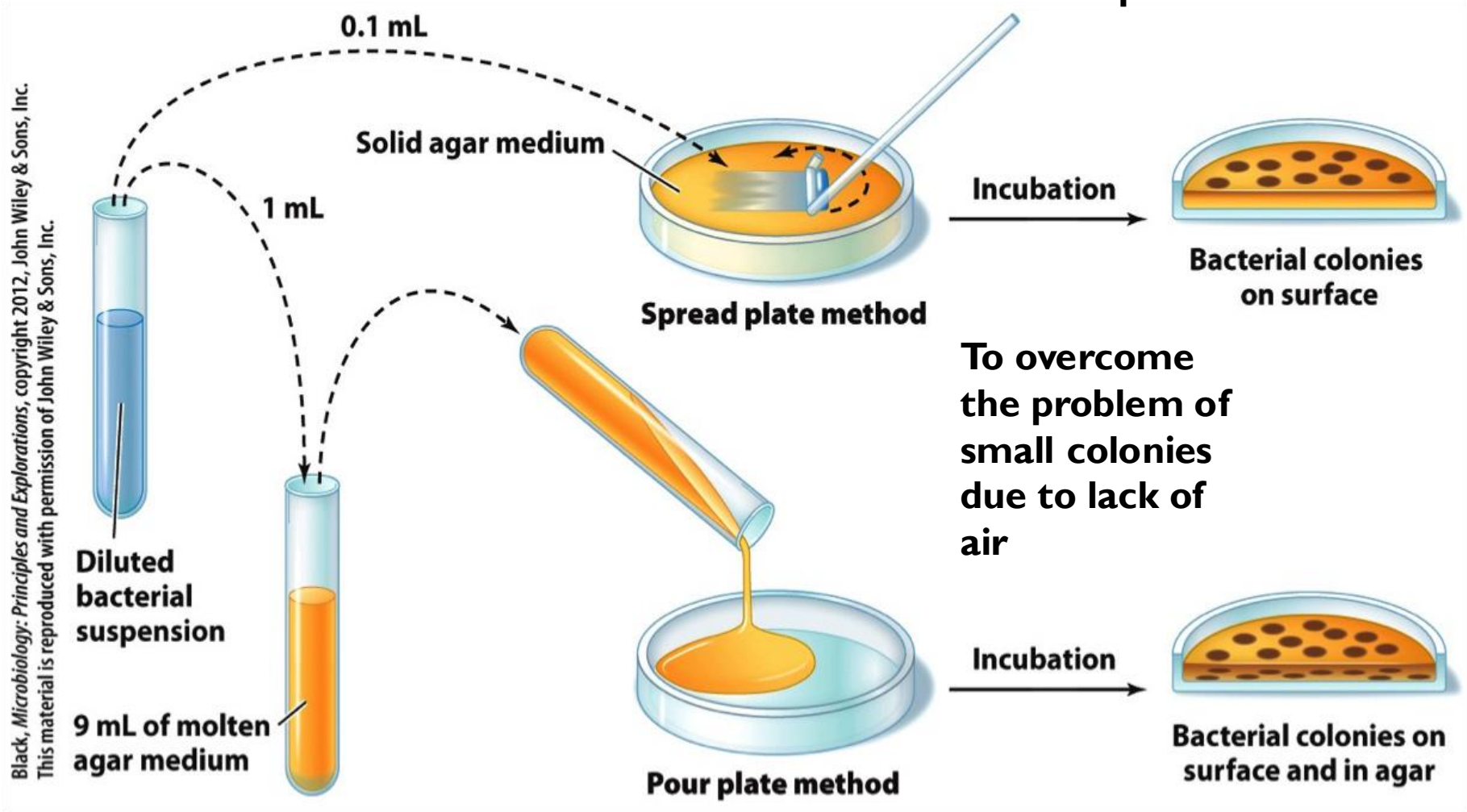


Figure 6-11 Brock Biology of Microorganisms 11/e

Traditional counting methods:

1. Pour plates (most popular)
2. Spread plates
3. Membrane filter method
4. Most probable number method

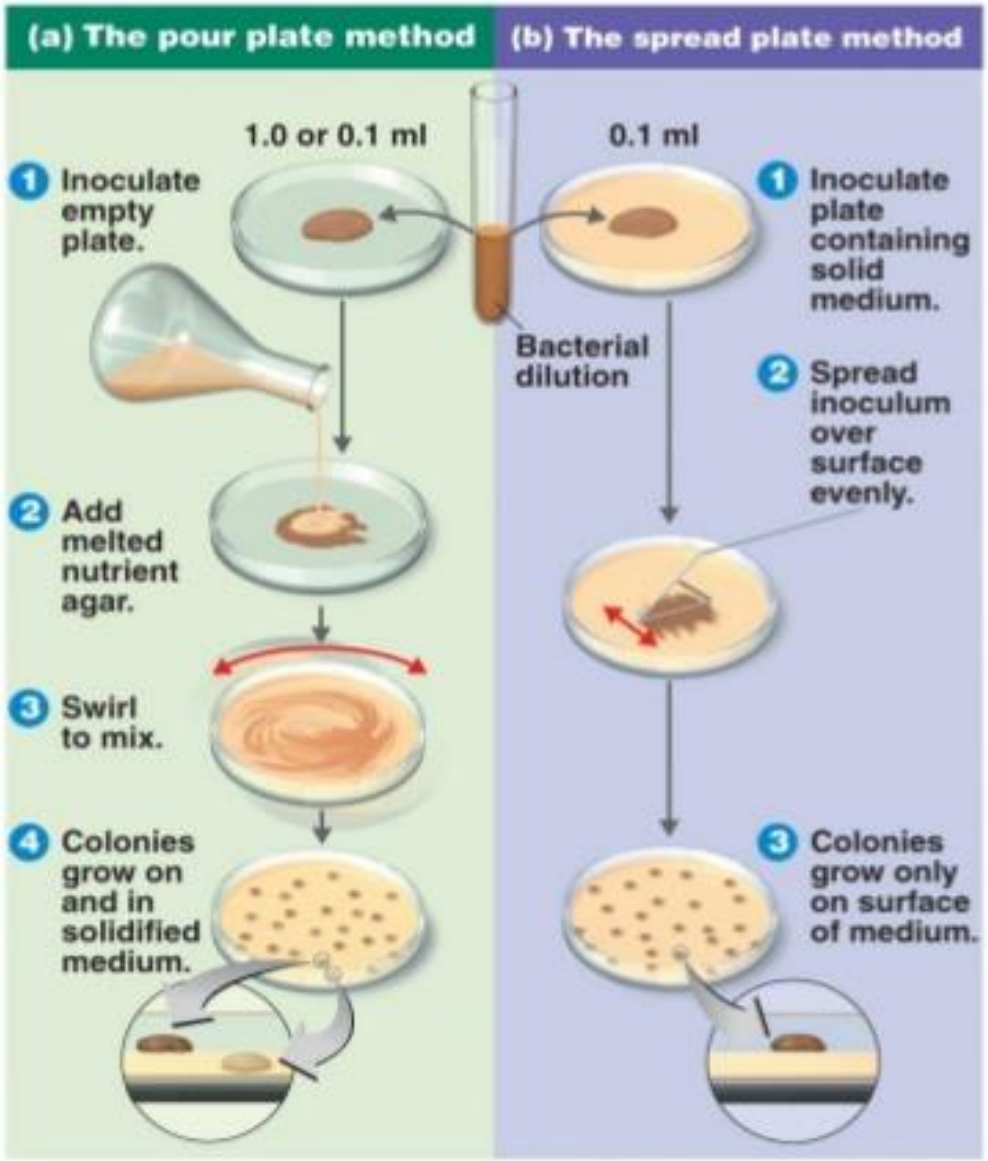
Sterile glass or plastic "hockey-stick" spreader

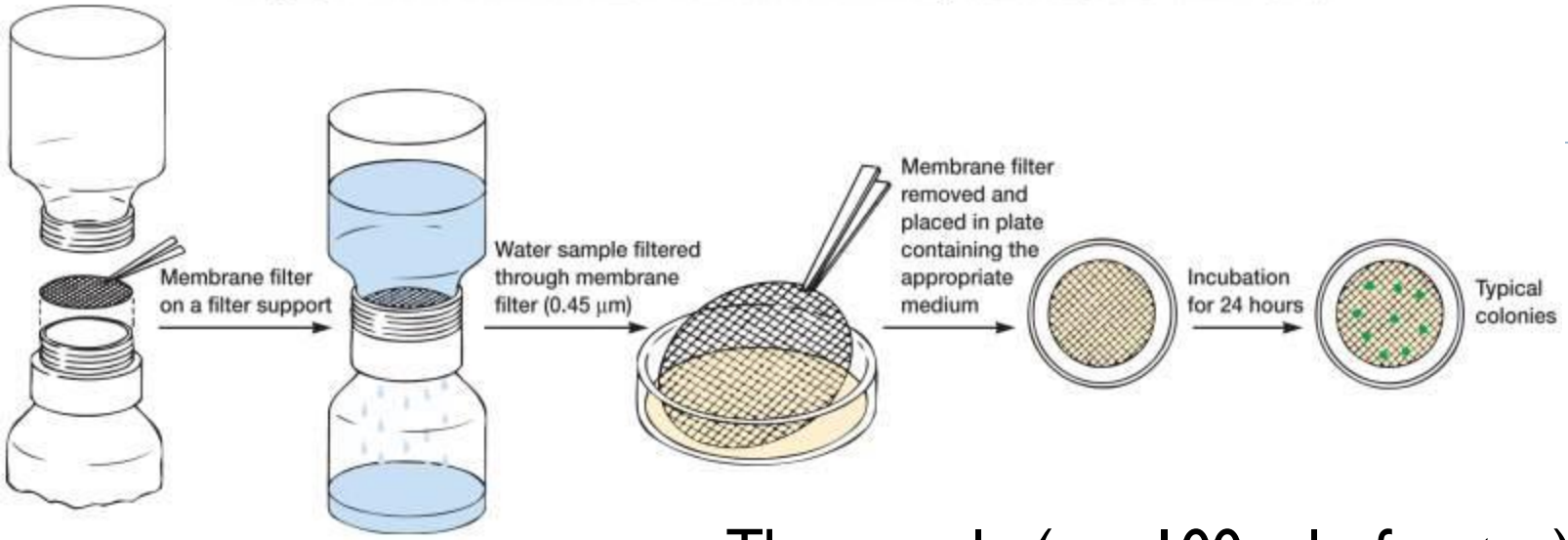


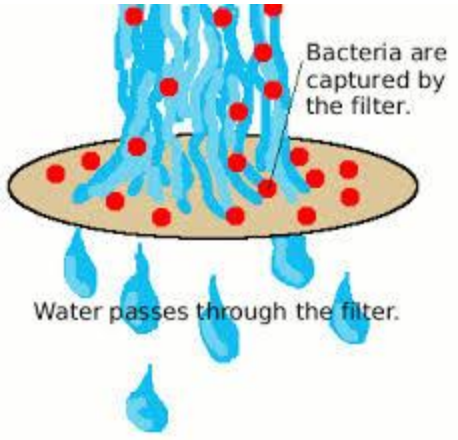
To overcome the problem of small colonies due to lack of air

- Plating serial dilutions of the specimen

- **Pour plate method**
- **Spread plate method**







- The sample (e.g. 100 ml of water) is passed under vacuum through a sterile filter membrane with pore size that is small to retain all contaminating MO on its surface (usually 0.45 μm)

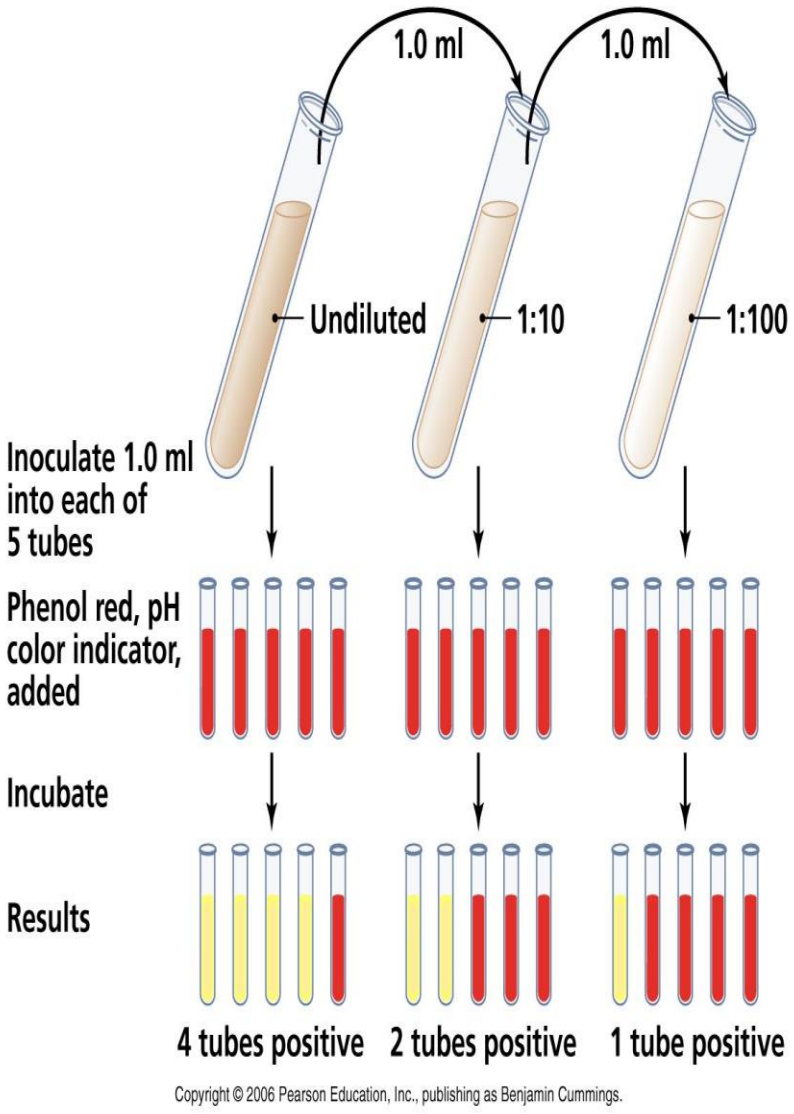
Membrane filter method

- Advantages:

- For low bioburden material like Purified water EP → required to have no more than 100 CFU/ml
- Can be used to determine concentration of organisms in oils or ointments (after dispersing it in oil)
- The best method if the sample is likely to contain antimicrobial chemicals (preservatives)

Antimicrobial agent	inactivator
Quaternary ammonium compounds, parabens and chlorhexidine	Lecithin with or without Polysorbate (Tween) 80
Thimerosal and other mercurials	Sodium thioglycolate
Beta-Lactam antibiotics	Beta-lactamase
Phenols, alcohols and weak acid preservatives	Dilution alone

MPN is used when the sample contains insoluble material that would interfere with plate counts as in herbal products



INDIRECT MEASUREMENTS: MPN

- Multiple Tube Fermentation Test as measured in MPN or Most probable Number
- Count positive tubes and compare to statistical MPN table.

Combination of Positives	MPN Index/ 100 ml	95% Confidence Limits	
		Lower	Upper
4-2-0	22	9	56
4-2-1	26	12	65
4-3-0	27	12	67
4-3-1	33	15	77
4-4-0	34	16	80
5-0-0	23	9	86
5-0-1	30	10	110
5-0-2	40	20	140
5-1-0	30	10	120
5-1-1	50	20	150
5-1-2	60	30	180
5-2-0	50	20	170
5-2-1	70	30	210
5-2-2	90	40	250
5-3-0	80	30	250
5-3-1	110	40	300
5-3-2	140	60	360

Parungao-Balolong 2011

Calculation of concentration of MOs in a sample

Table 15.3 Specimen results from a viable count.

Dilution	Dilution factor	Colony count 1	Colony count 2	Colony count 3
A	10^1	TNTC[a]	TNTC	TNTC
B	10^2	TNTC	TNTC	TNTC
C	10^3	453	521	419
D	10^4	85	79	81
E	10^5	7	6	8
F	10^6	0	1	0

[a]TNTC = too numerous to count

Viable count of original sample=

$$\frac{Mean\ colony\ count}{Volume\ of\ dilution\ used} \times dilution\ factor$$

Detection of objectionable MOs

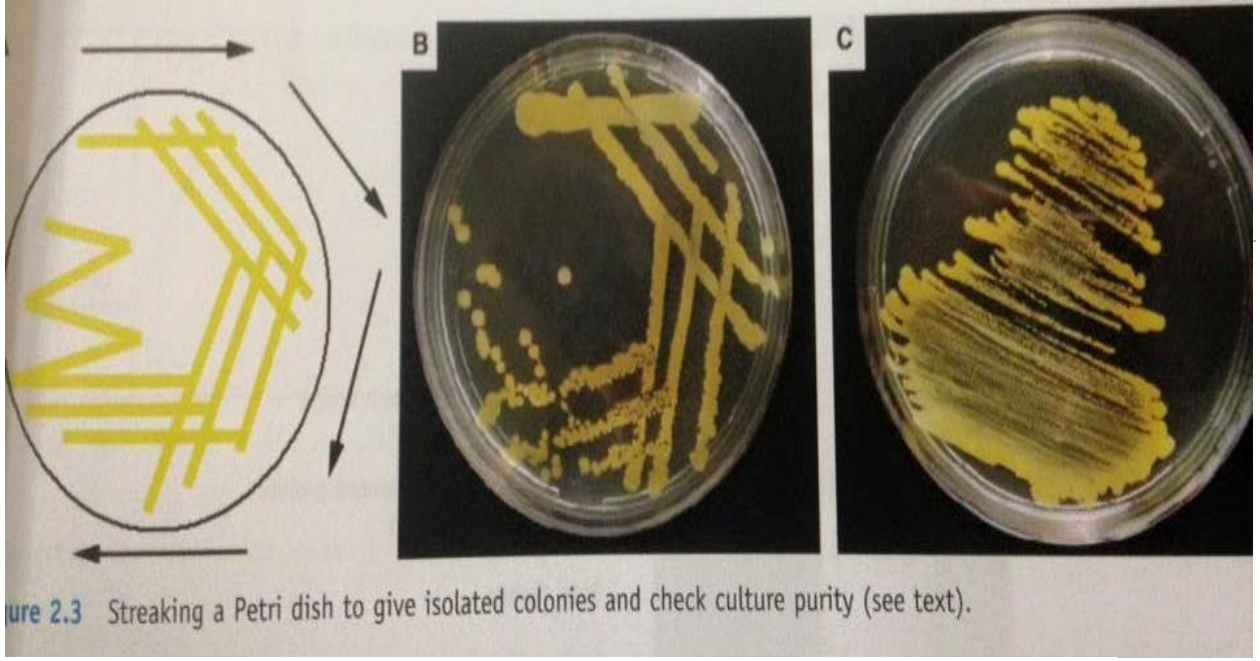
- Non sterile dosage forms may contain some MOs
- The quality of non sterile products is controlled by pharmacopeia in two ways:

1. Limit on total number or concentration of MO that may be present
2. Particular objectionable organisms must be absent in a specified weight of material:

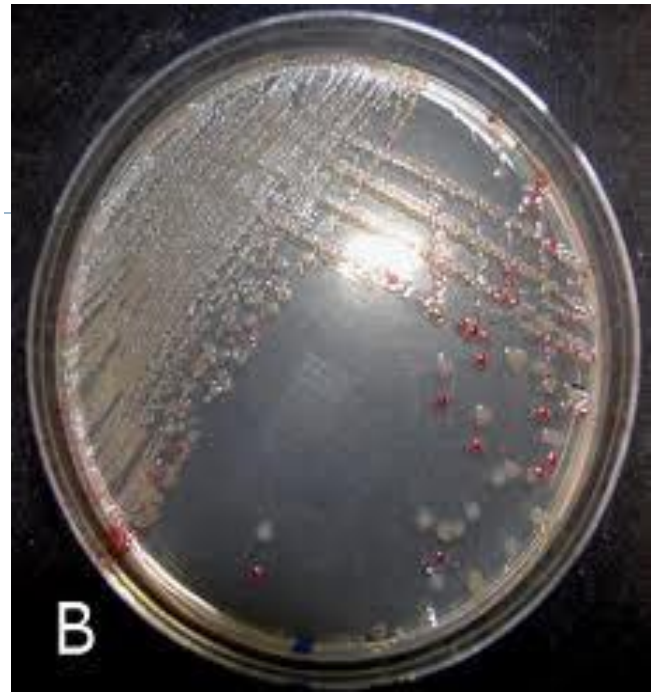
e.g. EP quality gelatin→ Salmonella should be absent in a 10g sample and E.coli absent in 1 g sample

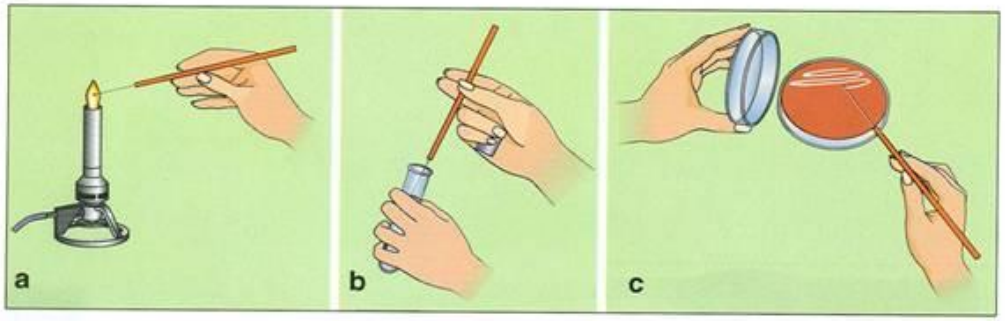
Detection of objectionable MOs

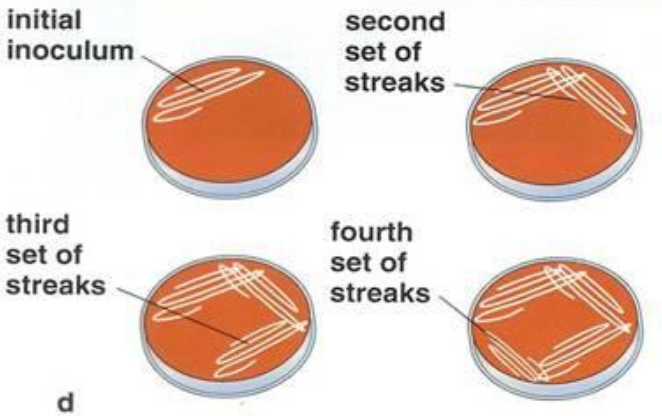
1. Dissolution or dispersal of the sample in a suitable liquid culture medium and , where necessary, inactivation of any substances that might inhibit the growth of the organism under test
2. Enrichment: increasing the relative concentration of the test organism by growing in a liquid medium that inhibit other contaminants but allows free multiplication of the organism of interest
3. Streaking liquid cultures from step 2 onto **selective agar media** that usually permit easy recognition of any colonies of the test organism that might arise
4. The use of specific biochemical or immunological confirmatory tests (test kits)

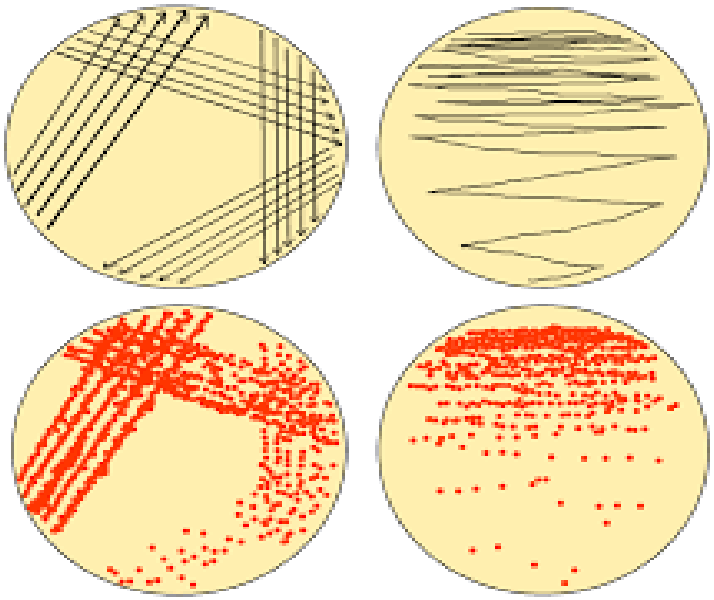


ure 2.3 Streaking a Petri dish to give isolated colonies and check culture purity (see text).









Detection of objectionable MOs

- Selective agar media recommended in the pharmacopeias:
- MacConkey's agar for E. coli
- XLD agar for Salmonella species
- Mannitol salt agar for Staphylococcus aureus
- Cetrimide agar for Pseudomonas aeruginosa

Students are required to read about each type of agar mentioned

Hemolyses of *Streptococcus* spp. (left) α-hemolysis (*S. mitis*); (middle) β-hemolysis (*S. pyogenes*); (right) γ-hemolysis (= nonhemolytic, *S. salivarius*)

General bacterial media [edit]

- Bile esculin agar (BEA)

  BEA is used for the isolation of *Enterococcus*, as well as Group D *Streptococcus* species

- CLED agar

  Cysteine, lactose, electrolyte-deficient agar (CLED) agar is used to isolate and differentiate urinary tract bacteria, since it inhibits *Proteus* species swarming and can differentiate between lactose fermenters and nonfermenters.

- Granada medium

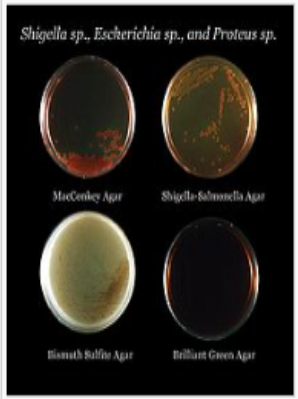
  Granada medium is used to isolate and differentiate Group B streptococcus (GBS), *Streptococcus agalactiae* from clinical samples. GBS grows in granada medium as red colonies and most of accompanying bacteria are inhibited.

- Hektoen enteric agar (HEA)

  HE agar is designed to isolate and recover fecal bacteria belonging to the Enterobacteriaceae family. It is particularly useful in isolating *Salmonella* and *Shigella*.

- Lysogeny broth (LB)
- MacConkey agar (MAC)

  MAC is a selective and differential medium used to differentiate between Gram-negative bacteria while inhibiting the growth of Gram-positive bacteria. The addition of bile salts and crystal violet to the agar inhibits the growth of most Gram-positive bacteria, making MacConkey agar selective. Lactose and neutral red are added to differentiate the lactose fermenters, which form pink colonies, from lactose nonfermenters that form clear colonies. An alternative medium, eosin methylene blue (EMB) serves a similar purpose.



Four types of agar plate demonstrating differential growth depending on bacterial metabolism

- Mannitol salt agar (MSA)

  MSA is also a selective and differential medium. The mannitol indicates organisms that ferment mannitol: mannitol fermentation produces lactic acid, lowering the pH and turning the plate yellow. The salt is to select for halophiles; organisms that cannot withstand a high salt content are unable to grow well.

- Mueller-Hinton agar (MHA)

  MHA contains beef infusion, peptone, and starch, and is used primarily for antibiotic susceptibility testing. It can be in a form of blood agar.

- Nutrient agar

  Nutrient agar is usually used for growth of nonfastidious organisms and observation of pigment production. It is safe to use in school science laboratories because it does not selectively grow pathogenic bacteria.

- Önöz agar

  Önöz agar allows more rapid bacteriological diagnosis, as *Salmonella* and *Shigella* colonies can be clearly and reliably differentiated from other Enterobacteriaceae. The yields of *Salmonella* from stool samples obtained, when using this medium, are higher than those obtained with LEIFSON agar or *Salmonella–Shigella* agar (SSA).

- Phenylethyl alcohol agar (PEA)

  PEA selects for *Staphylococcus* species while inhibiting Gram-negative bacilli (e.g., *Escherichia coli*, *Shigella*, *Proteus*, etc.).

Fungi (ascomycetes) growing in axenic cultures, each of which is a

Detection of objectionable MOs

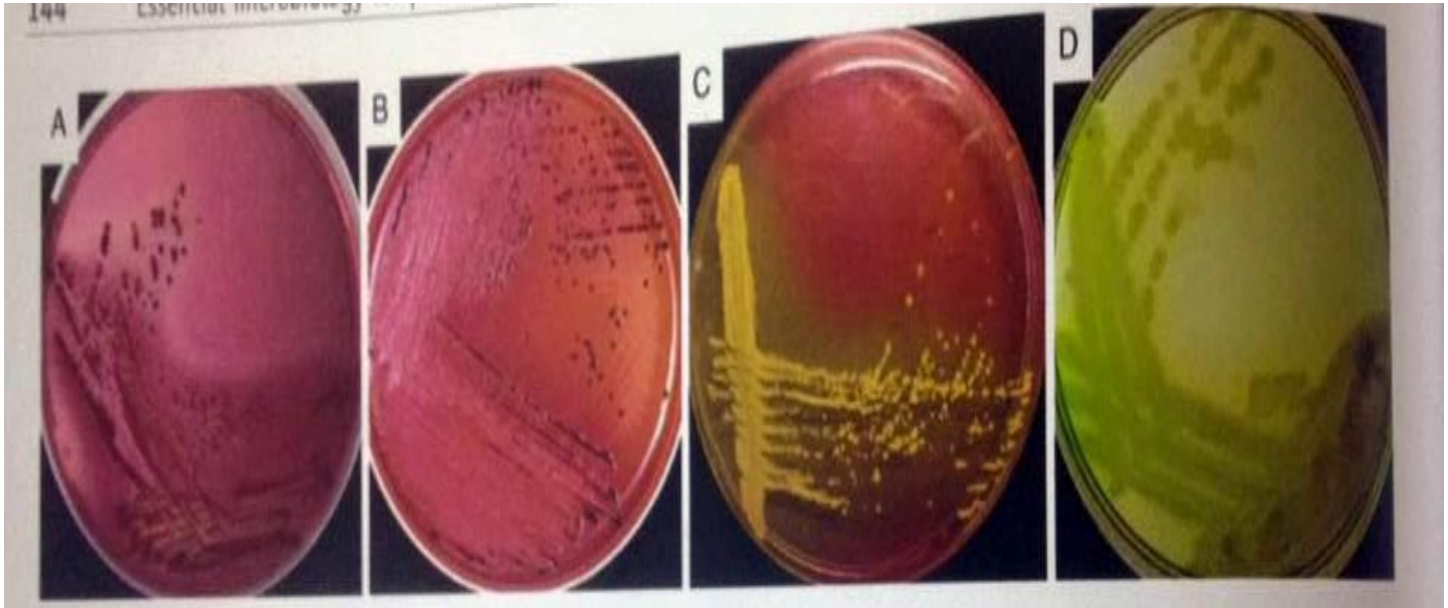


Figure 15.6 The appearance of 'objectionable organisms' on selective media. A. *E. coli* on MacConkey's agar, showing characteristic red colonies and precipitation of bile as a result of acid production; B. *Salmonella* species on XLD agar, showing alkali and hydrogen sulphide production indicated by pink agar and black precipitate in the colonies respectively; C. *Staphylococcus aureus* on mannitol salt agar showing yellow colonies that have produced acid as a result of mannitol fermentation; D. *Pseudomonas aeruginosa* on cetrimide agar, showing the characteristic green pigmentation.

Automated bioburden determinations

- Bases of the methods
- Limited application
- Look for pg 144

Bioburden specifications in the pharmacopeias

Table 15.4 Quality criteria for nonsterile medicines.

Route of administration[a]	Maximum total aerobic microbial count CFU/g or ml	Maximum total yeast and mould count CFU/g or ml	Specified microorganisms absent in 1 g or 1 ml
Nonaqueous oral products	10^3	10^2	Absence of *E. coli*
Aqueous oral products	10^2	10^1	Absence of *E. coli*
Rectal products	10^3	10^2	
Oral mucosal, gingival, cutaneous, nasal and ear products	10^2	10^1	Absence of *Staph. aureus* and *Ps. aeruginosa*
Vaginal products	10^2	10^1	Absence of *Staph. aureus*, *Ps. aeruginosa* and *Candida albicans*
Inhalation products (excluding nebulized liquids)	10^2	10^1	Absence of *Staph. aureus*, *Ps. aeruginosa* and bile-tolerant Gram-negative bacteria