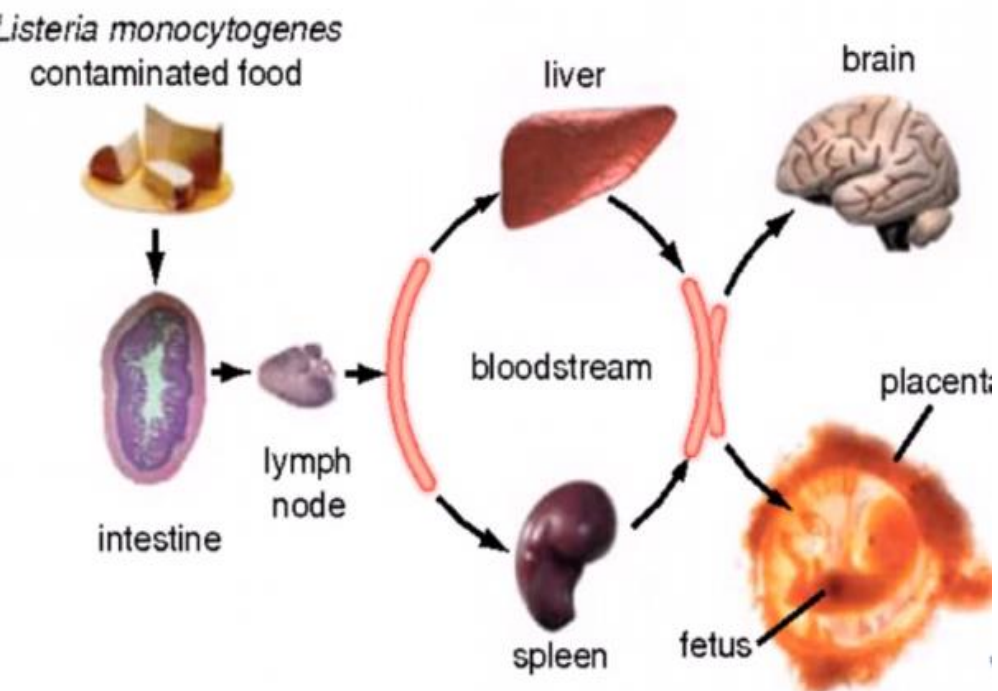


Enumeration of *Listeria monocytogenes* and *Staphylococcus aureus*

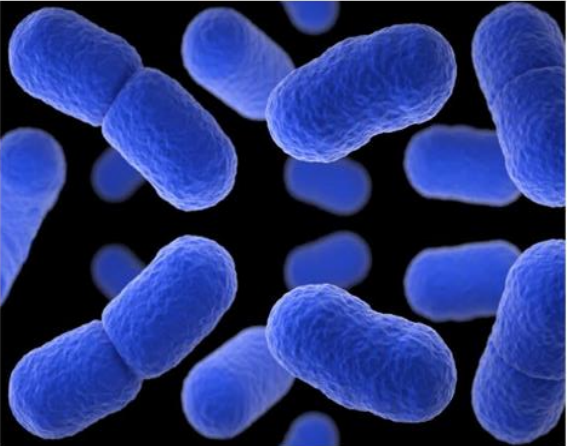
- ✓ ***Listeria monocytogenes*: Intracellular pathogen, affects healthy as well as immuno-suppressed populations.**
- ✓ **Cause gastroenteritis and fever.**
- ✓ **Mortality rate: 20–30%.**
- ✓ **Three forms of disease gastrointestinal form, systemic listeriosis, and abortion and neonatal listeriosis.**
- ✓ **Post-processing contamination from plant environments is most common source of *L. monocytogenes* contamination in pasteurized dairy products.**



Listeria Monocytogenes Infections Treatment Market



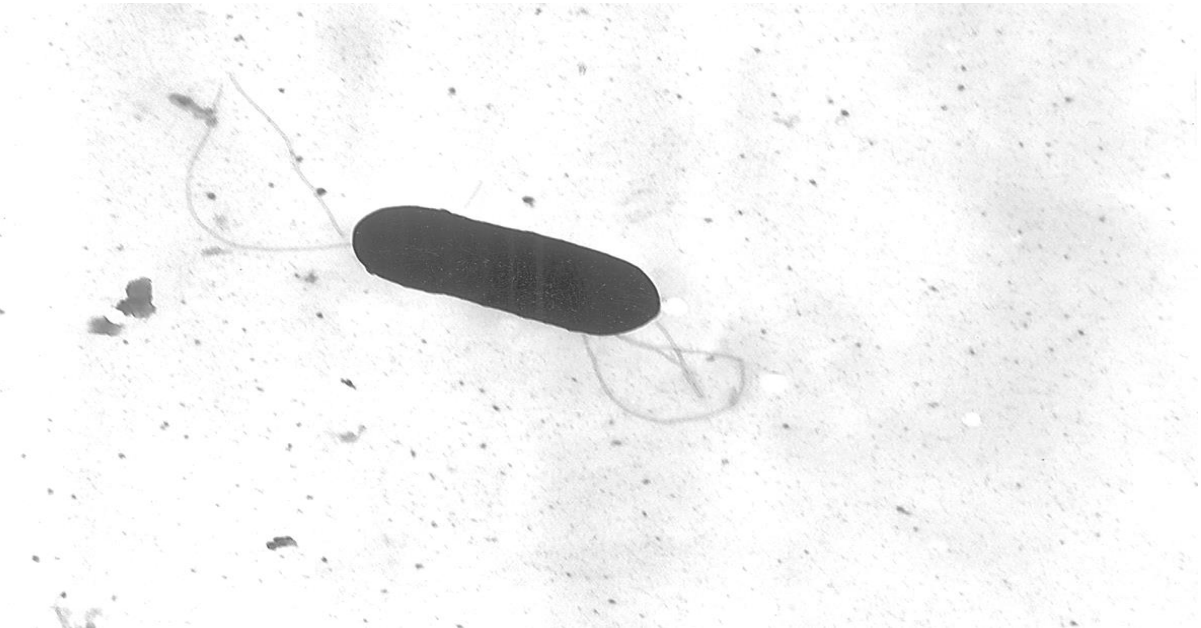
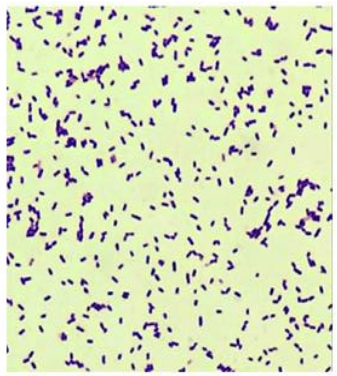
- ✓ Gram-positive, non-sporing rods about 0.5 μm \times 0.5-2.0 μm , produce catalase and positive in methyl red and Voges-Proskauer reactions.
- ✓ Indole, oxidase, H_2S , urease, nitratase and gelatinase negative.
- ✓ Non-capsular facultative anaerobic *Listeria* spp. grows well at water activity of >0.95 .
- ✓ Grow well at 10% salt concentration but survive at 25.5%.
- ✓ *L. monocytogenes* and *L. ivanoviare* are β -haemolytic and pathogenic



Beta-hemolytic colonies



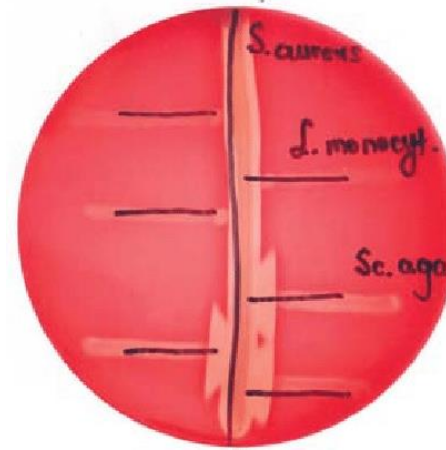
Gram positive coccobacilli



Species	β -Hemolysis ^a	Mannitol	Rhamnose	Xylose	Virulence ^b
<i>L. monocytogenes</i>	+	-	+	-	+
<i>L. ivanovi</i> ^c	+	-	-	+	+

➤ **CAMP test:** Confirm pathogenic species.

- Test is performed by streaking β -haemolytic *Staphylococcus aureus* and *Rhodococcus equi* culture in parallel and diametrically opposite each other on sheep blood agar plate.
- *Listeria* cultures are streaked at right angles to two other cultures.
- After 48 h of incubation at 35-37°C, β -haemolysis by *L. monocytogenes* and *L. seeligeri* is enhanced near *S. aureus* streak, *L. ivanovii* becomes enhanced near *R. equi* streak.
- Other *Listeria* species remain non-haemolytic.



CAMP:

S. aureus

- *L. monocytogenes*
- *Sc. agalactiae*

Isolation and Enumeration Principle of *Listeria* Species from Dairy Products

Direct isolation of organism from infected and contaminated material is often unsuccessful. Reliability of isolation method for *Listeria* intended for food analysis would depend on ability to cope with low-level contamination (<10² cells per ml).

Enumeration of *Listeria* spp. is based on principle that organism show tolerance to selective agent

Ability to hydrolyse esculin by enzyme β -glucosidase

Organism show weak β -haemolysis

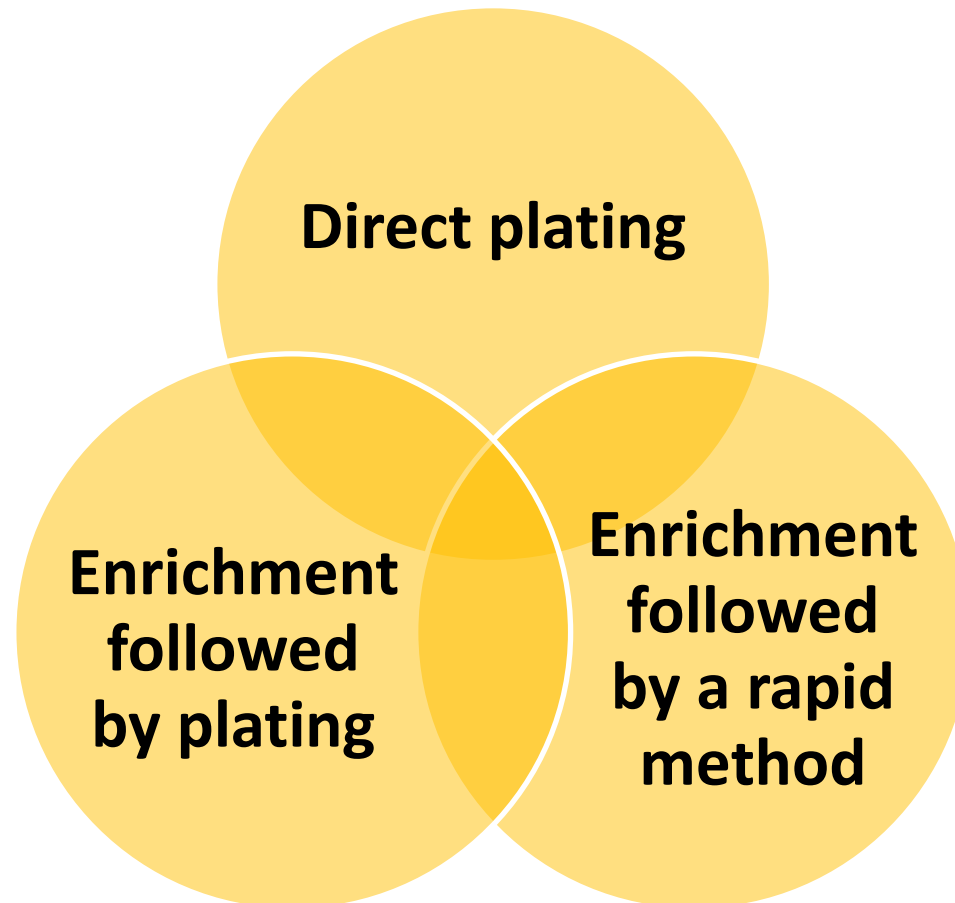
Ability to produce phosphatidylinositol-specific phospholipase-C (PI-PLC)

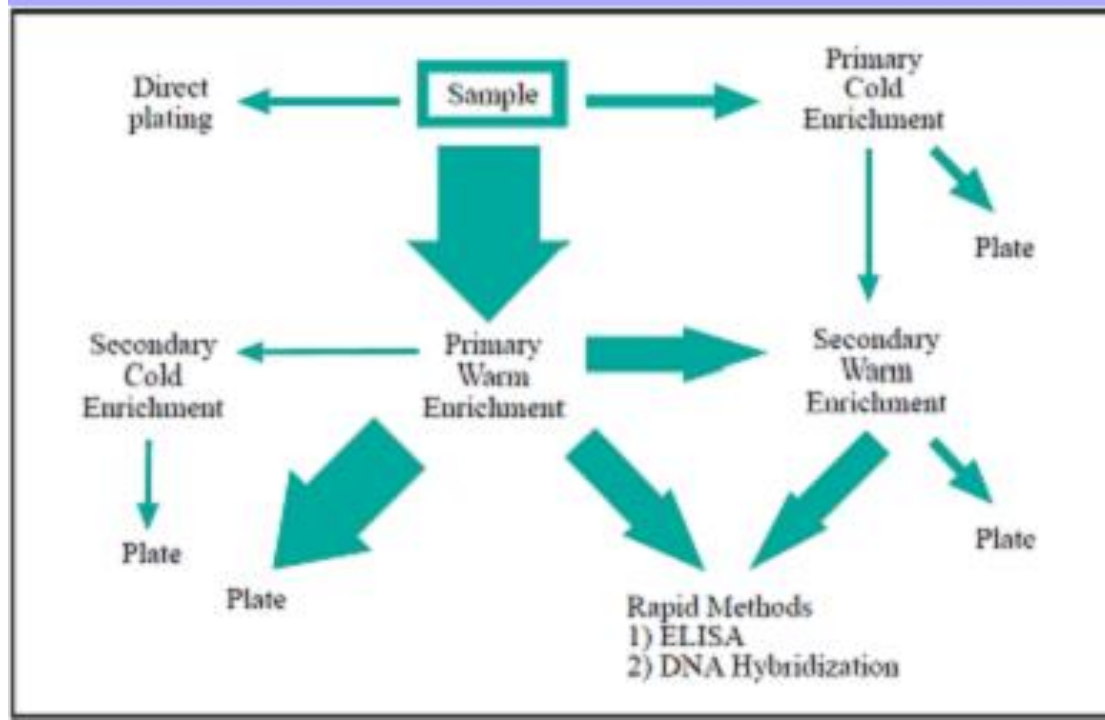
Ability to ferment rhamnose

Enumeration principle

Conventional Method for Isolation of *Listeria* spp. From Dairy Products

First advocated by Gray et al. (1948) in which sample material was inoculated into tryptose broth without selective agents and held at 4°C for long periods. This excellent isolation technique; however need prolonged incubation (up to several months or even a year) which is serious disadvantage.





Scheme for isolation and detection methods of *Listeria*. The size of arrows denotes an estimated popularity of various methods

1. First and least used is direct plating of food suspension onto selective solid agar medium such as LiCl-phenylethanol-moxalactam (LPM), PALCAM containing Polymyxin B, Acriflavin. Lithium chloride, Ceftazidime, phenylethanol, moxalactam medium with or without esculin and modified Oxford (MX) agar.
2. Direct plating quantify populations of listeriae cells in food directly.
3. Direct plating is rather insensitive and can detect only 100 CFU of *Listeria* per gram food.

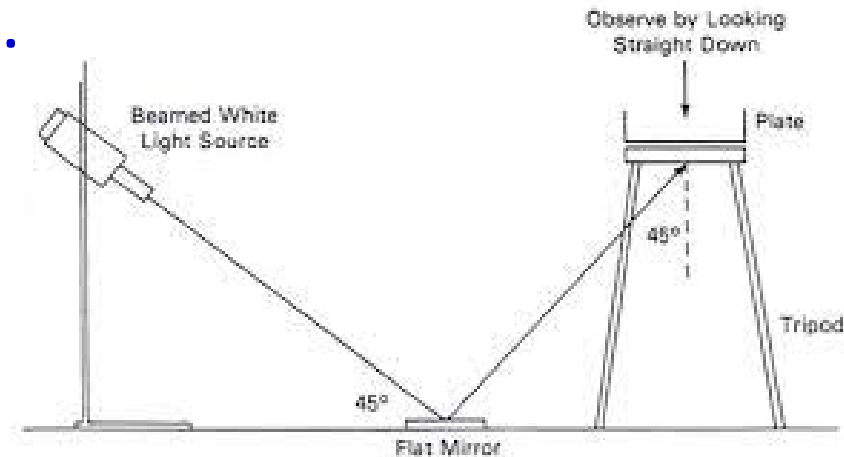
4. Approach for isolating and detecting *Listeria* spp. uses one/two warm enrichment steps in nutritious broth, followed by plating onto selective agar.
5. Warm enrichment is based on accelerated growth of listeriae at increased incubation temperature (30-37°C), with addition of selective compounds to inhibit growth of competitive microflora.
6. Simplified warm enrichment procedure in combination with chromogenic and fluorogenic substrates, DNA and immuno-technology reduce isolation and identifications times.

Antimicrobial Agent	mg/L	Organism inhibited
Lithium chloride	0.5	Gram positive bacteria
Acriflavin	5-25	gram-positives, including <i>Lactobacillus bulgaricus</i> and <i>Streptococcus thermophilus</i>
Nalidixic acid	20-40	gram-negatives except <i>Pseudomonas</i> and <i>Proteus</i>
Moxalactam, phenylethanol	20	Gram-negative bacteria
Ceftazidime	50	Gram-negative bacteria
Cycloheximide	50	Fungi
Fosfomycin	100	Inhibitory to <i>Staphylococcus</i> and <i>Bacillus</i>

Example of antimicrobial agents that are commonly used in *Listeria* selective broth and agar

Procedures for isolation of *Listeria* spp. from dairy products:

- **FDA method** employs single enrichment step using FDA enrichment broth at 30°C.
- After 24 and 48 h, culture is streaked onto selective LiCl-phenylethanol-moxalactam (LPM) and Oxford/ PALCAM agars.
- Plates are then incubated at 30/35°C (LPM/Oxford/PALCAM) for 24-48 h.
- Suspected plates are examined by using beamed white light striking the plate bottom at 45° angle. Technique is known as **Henry's Light technique** and shows *Listeria* colonies as sparkling blue or white.
- In PALCAM agar, which contains esculin/ ferric iron indicator system, *Listeria* colonies show up as black with black halo under ordinary viewing and appears as light green colonies.
- Five or more typical colonies are picked and transferred to trypticase soy agar and incubated at 30°C for 24-48 h.



- **USDA method** is a two-stage enrichment procedure involving primary enrichment in *Listeria* enrichment broth I (LEB I), secondary enrichment in Fraser broth and subsequent plating on modified Oxford agar (MXA).
- Typical *Listeria* colonies exhibit black halos from esculin hydrolysis.
- **ISO/ AFNOR** method uses 1/2 Fraser broth (with half amount of selective agents) as first enrichment step and complete Fraser broth as a second enrichment step. Both steps are followed by selective plating on PALCAM and/ or Oxford agars.
- Typical colonies on Oxford agar show black due to esculin hydrolysis.
- PALCAM agar contains two indicator systems: esculin/ ferric iron and D-mannitol with phenol red.
- On PALCAM agar, *Listeria* colonies are green with black haloes against a pink-purple background.

Rapid enumeration of *Listeria* spp. using chromogenic media:

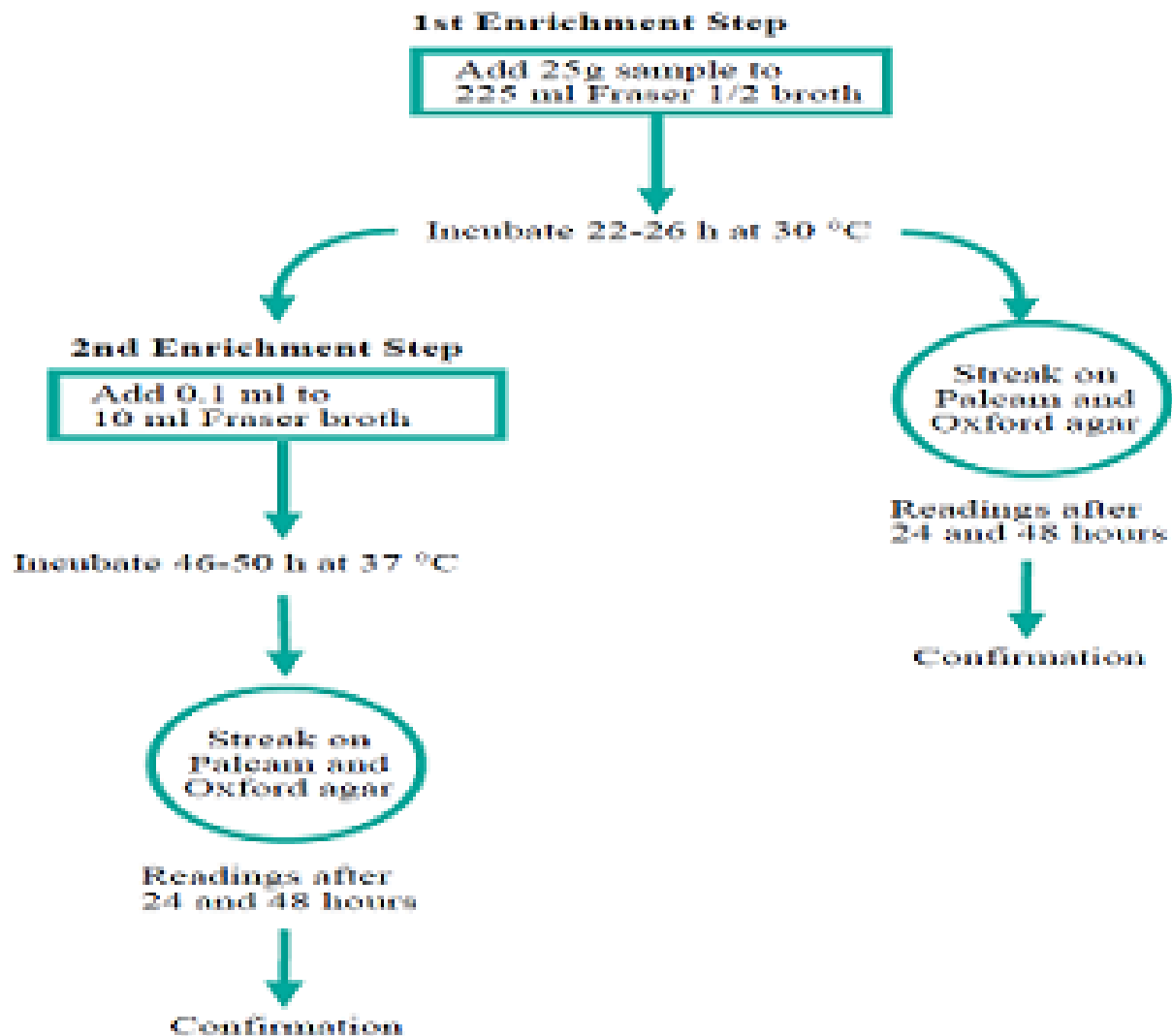
- Chromogenic and fluorogenic media are microbiological growth media that contain enzyme substrates linked to chromogen (color reaction), fluorogen (light reaction) or a combination of both.
- Enzyme substrates, e.g. **5-Bromo-4-chloro 3-indolyl- β -D-glucoside**, **5-Bromo-4-chloro-3-indoxyl myo-inositol-1-phosphate, ammonium salt**, **4-Methylumbelliferyl myo-inositol-1-phosphate N-methyl-morpholine salt** are commonly used chromogenic and fluorogenic substrate for detection of *L. monocytogenes*.



CHROMagar™
Listeria

CHROMagar™
Identification
Listeria

Target population are characterized, colour change in medium and/ or fluorescence under long wave UV light.



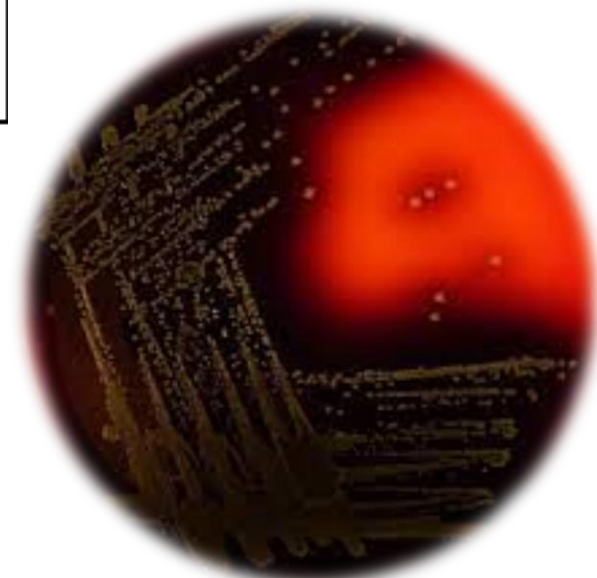
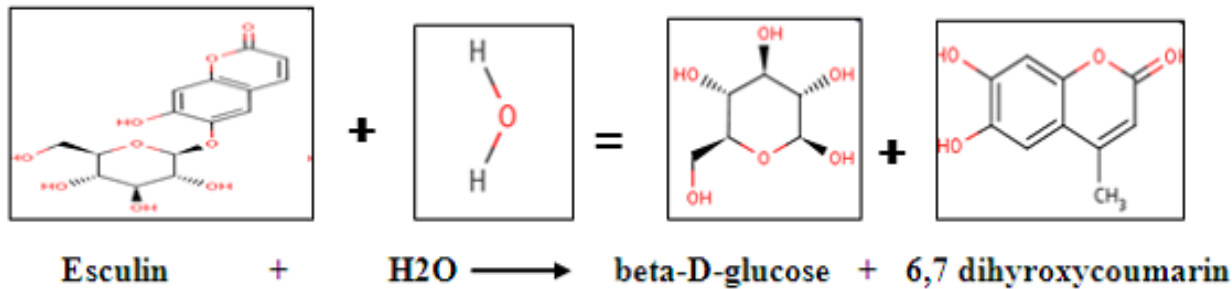
Conventional enrichment method for the isolation and detection of *Listeria* according to the NF ISO 11290-1/ AFNOR standard

PALCAM agar base

Enumeration principle and interpretation:

- **Differentiation on PALCAM Agar Base is based on esculin hydrolysis and mannitol fermentation.**
- **Hydrolyzes esculin, which appears as blackening in medium.**
- **Blackening by esculin-hydrolyzing bacteria results from formation of 6,7-dihydroxycoumarin, which reacts with ferric ions that are present in medium as ferric ammonium citrate.**
- **Mannitol and pH indicator (phenol red) added to differentiate mannitol-fermenting strains of possible contaminants (enterococci and staphylococci).**
- **Do not ferment mannitol, demonstrated by color change in colony and/or surrounding medium from red or grey to yellow based on the production of acidic end products.**

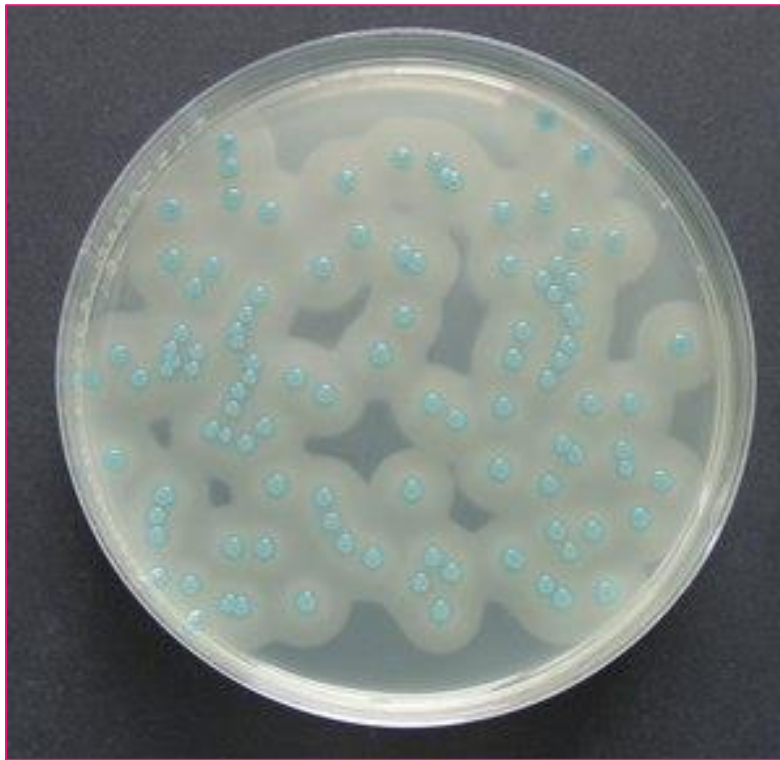
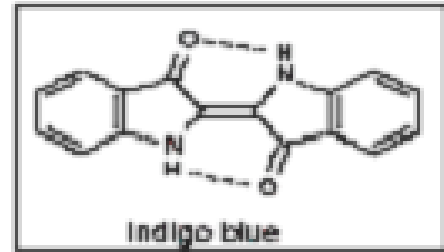
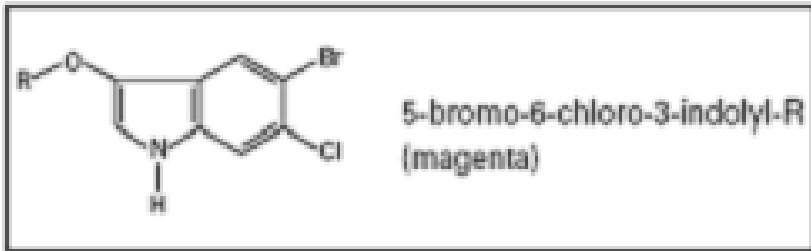
- Polymyxin B, acriflavin, ceftazidime, and lithium chloride are selective agents that suppress Gram-negative and Gram-positive bacteria.
- Reaction catalysed by beta-glucosidase results in formation of black color compound for detection of *Listeria* sp.
- *Listeria* spp. are presumptively indicated by **grey-green colonies with black precipitate** following incubation for 24-48 h at 35°C on PALCAM Agar Base.



Chromocult listeria selective agar base

Enumeration principle and interpretation:

- Addition of 5-bromo-4-chloro-3-indolyl- β -Dglucopyranoside which differentiate between β -D-glucosidase positive and negative bacteria.
- *Listeria* is β -D-glucosidase-positive and grows on medium in form of blue-green colonies.
- To detect *L. monocytogenes*, L- β -phosphatidylinositol is added to medium.
- *L. monocytogenes* has enzyme phosphatidylinositol phospholipase C (PI-PLC) described as a virulence factor.
- Phospholipase activity results in formation of opaque haloes around *L. monocytogenes* colonies.
- *L. ivanovii* shows phospholipase C activity.
- Beta-glucosidase forms indigo blue color by hydrolysing chromogenic substrate.
- Colonies which appear **blue-green with an opaque halo** on the medium are *L. monocytogenes* colonies.



Listeria monocytogenes

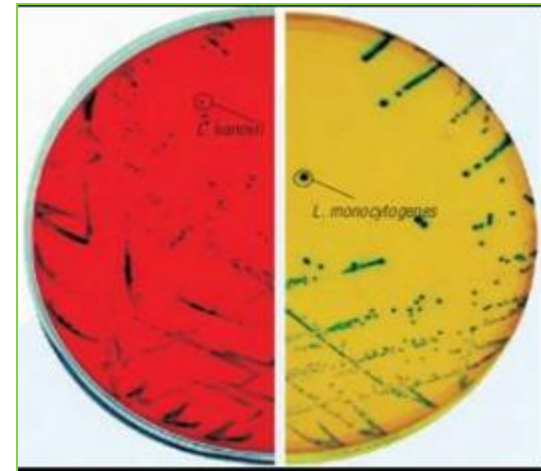


Listeria innocua

HiCrome Listeria Agar Base, Modified

Enumeration principle and interpretation:

- Medium is based on specific beta-glucosidase activity detection and rhamnose fermentation.
- Colonies of *L. ivanovii* appear blue without yellow halo (rhamnose -ve) while, colonies of *L. monocytogenes* and *L. innocua* are blue with yellow halo (rhamnose +ve).
- Peptone, yeast extract and meat extract provide nitrogenous substances, vitamin B complex and other essential growth nutrients.
- Rhamnose is fermentable carbohydrate with phenol red as an indicator.
- Sodium chloride maintains osmotic equilibrium.
- Lithium chloride and Hi-Crome Listeria Selective Supplement inhibit growth of Gram positive bacteria, Gram negative bacteria, yeasts and molds.



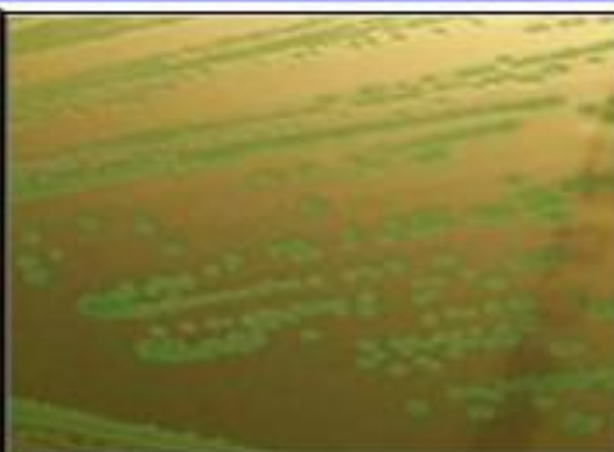
ALOA Chromogenic Agar

Enumeration principle and interpretation:

- To minimise growth of contaminating organisms, lithium chloride and balanced antimicrobial mixture is employed.
- Incorporation of chromogenic substrate X-glucoside for detection of β -glucosidase demonstrates presence of *Listeria* spp., whilst, detection of specific phospholipase C enzyme produced by pathogenic *Listeria* spp. including *L. monocytogenes* is also achieved.
- *Listeria* spp. produce blue/green colonies, with pathogenic species producing similar coloured colonies surrounded by characteristic opaque halo after 24 h at 37 C.
- Non-*Listeria* spp. produces white colonies.



Typical colonies of *L. monocytogenes*



Typical colonies of *L. innocua*

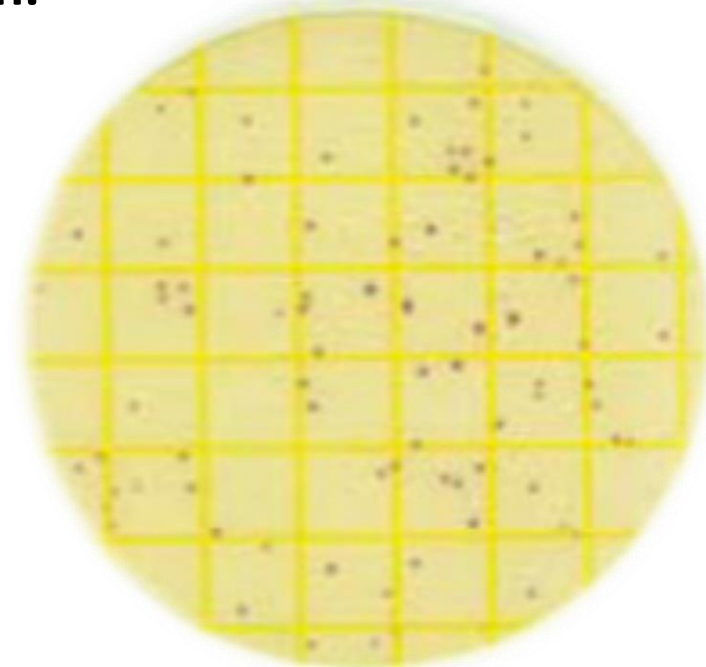


Typical colonies of *L. ivanovii*

3M™ Petrifilm™ Environmental *Listeria* Plates

Enumeration principle and interpretation:

- Enumeration of *Listeria* in environmental samples.
- Petrifilm Environmental Listeria Plate detects *Listeria* sp. as **red-violet colonies**: *L. monocytogenes*, *L. innocua* and *L. welshimeri*, but does not differentiate these organisms from one another.
- A repair broth (5 mL of buffered peptone water) is added to collected environmental sample and allowed at room temperature for 1 h.
- Three ml of sample is pipetted onto the 3M Petrifilm Environmental Listeria Plate and plate incubated for 26-30 h.



Prepare a 10^{-1} dilution of sample



Homogenise by stomaching (food, swabs) or mix by vortexing (swabs)



Prepare further dilutions if required in peptone saline diluent



Surface spread 0.5 ml of 10^{-1} dilution on two *Listeria* chromogenic plates. If high counts are expected, also inoculate 50 μ l of a 10^{-1} and 10^{-3} dilution onto *Listeria* chromogenic agar media using a spiral plater



Incubate *Listeria* chromogenic agar plates at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for up to 48 hours in aerobic conditions



Examine at 24 ± 3 hours and after a further 24 ± 3 hours.



Sub-culture 5 presumptive colonies onto horse blood agar



Incubate at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 24 ± 3 hours



Calculate the counts of *Listeria* species (and *L. monocytogenes* if present) per gram or ml

Enumeration procedure

Enumeration of
Staphylococcus aureus

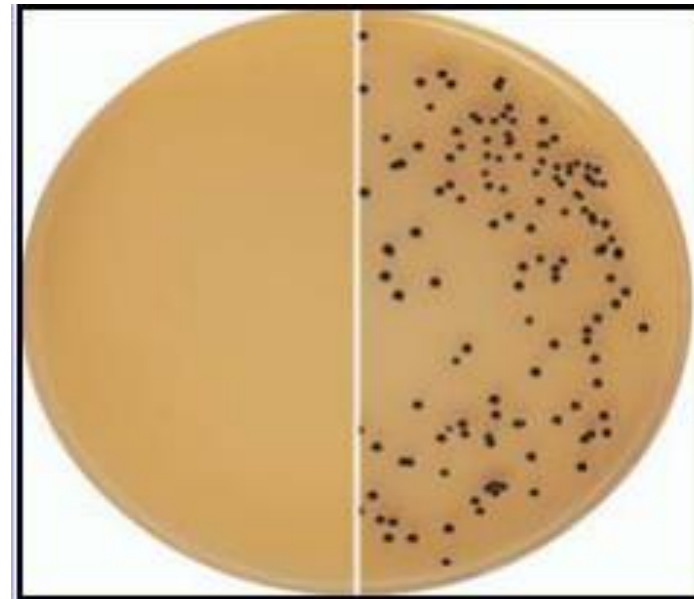
- ✓ ***Staphylococcus aureus*: Highly vulnerable to destruction by heat treatment and sanitizing agents.**
- ✓ **Presence of this bacterium or its enterotoxins in processed foods or on food processing equipment is indication of poor sanitation.**
- ✓ **Staphylococcal Food Poisoning.**
- ✓ **Colonial morphology on Baird-Parker agar, lysostaphin sensitivity, coagulase and thermo-nuclease production, and glucose and mannitol fermentation has been conducted.**
- ✓ **Contains lithium chloride and tellurite to inhibit other microbial flora, whereas; pyruvate and glycine selectively stimulate growth of staphylococci.**
- ✓ **Two characteristic features when grown in this opaque medium (opaque, because of its egg-yolk content): **characteristic zones and rings are formed as a result of lipolysis and proteolysis and reduction of tellurite to tellurium produces a black colouration.****

Suspend 58 g in 0.95 litre, autoclave (15 min at 121°C). Cool to 45-50°C, mix in 50 ml egg yolk tellurite emulsion and 50 mg sulphamethazine/ litre. Pour plates. pH: 6.8 ±0.2 at 27°C. Plates are opalescent and yellowish-brown in colour.

Typical Colony characteristics

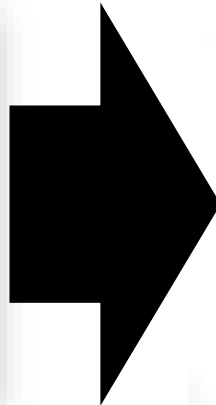
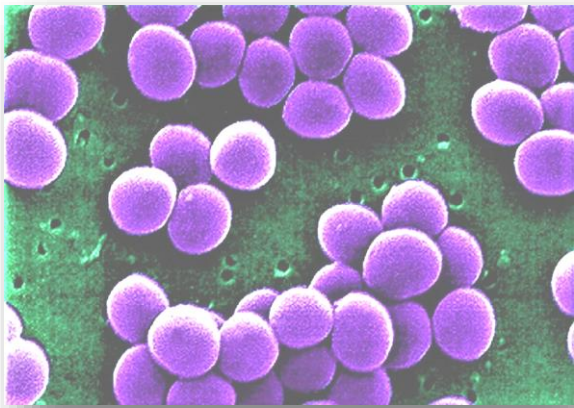
- ✓ *S. aureus* colonies are convex, irregular shape, black, shiny 1-5 mm in diameter with narrow, white edge surrounded by clear zone 2-5 mm wide. Opaque rings within clear zones only appear after 48 h of incubation. Opaque zone develop around colonies after 24 h.
- ✓ *S. epidermis*: Very small, brown to neither black, non clear zones.
- ✓ *Micrococcus*: Dark brown, dull, clear zones sometimes appear after 48 h.
- ✓ *Bacillus* species are white; yeasts have no clear zones.

S. aureus on Baird-Parkar agar

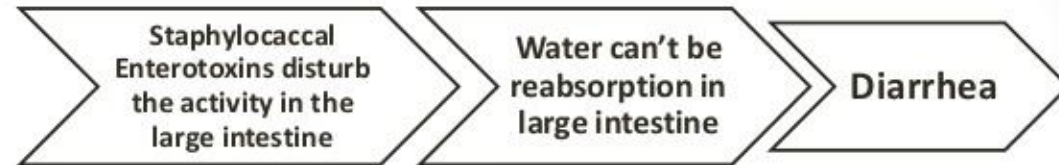


Enumeration principle and interpretation:

- Facultative anaerobic, Gram-positive coccus, which appears as grape-like clusters and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates.
- Enterotoxin that causes *S. aureus* gastroenteritis.
- Colonies are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowned plates, gray to jet-black, frequently with light-coloured (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone.



Mechanism of Staphylococcal Enterotoxins - Diarrhea



Direct plate counts

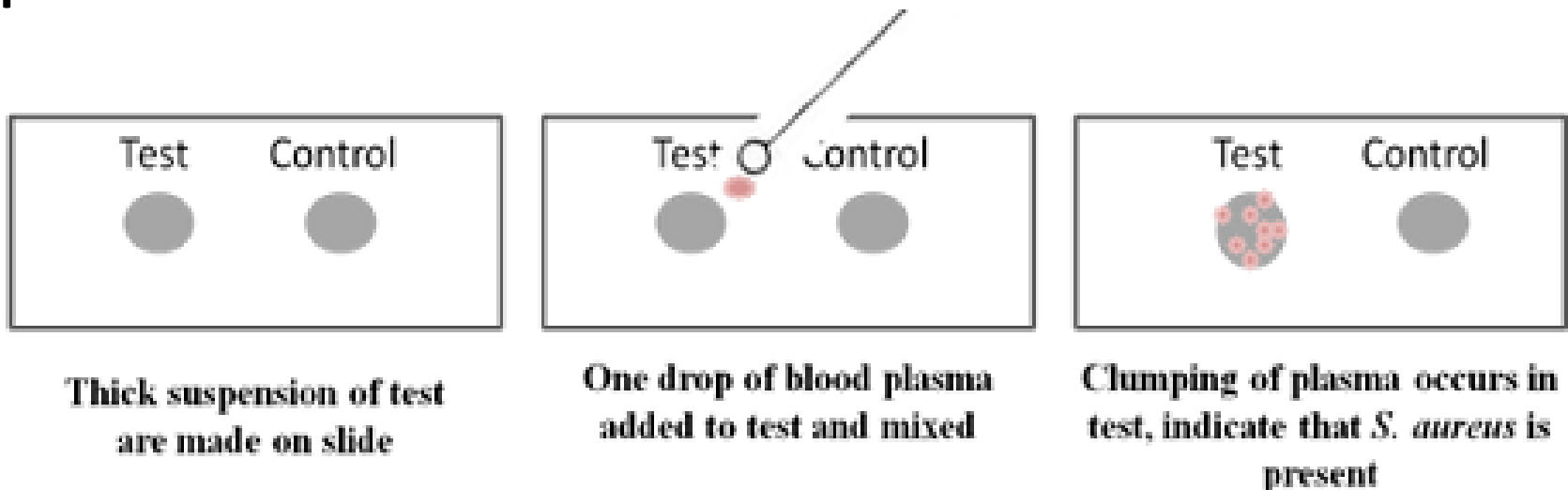
- **Aseptically transfer one ml sample suspension to 3 plates of Baird-Parker agar, distributing 1 ml of inoculum equitably to 3 plates (e.g., 0.4 ml, 0.3 ml, and 0.3 ml).**
- **Spread inoculum over surface of agar plate, using sterile bent glass streaking rod.**
- **Retain plates in upright position until inoculum is absorbed by agar.**
- **If inoculum is not readily absorbed, place plates upright in incubator for about 1 h.**
- **Invert plates and incubate 45-48 h at 35°C.**
- **Select plates containing 20-200 colonies.**
- **Select > 1 colony of each type counted and test for coagulase production.**

Coagulase Test (Clotting of dilute mammalian blood):

- Produce coagulase, which can clot plasma into gel in tube or agglutinate cocci on slide.
- Test is useful in differentiating *S. aureus* from other coagulase-negative staphylococci.
- Two types of coagulase: **free coagulase and bound coagulase.**
- Free coagulase is an enzyme that is secreted extracellularly, bound coagulase is a cell wall associated protein.
- Free coagulase is detected in tube coagulase test and bound coagulase is detected in slide coagulase test.
- Slide coagulase test may be used to screen isolates of *S. aureus* and tube coagulase may be used for confirmation.
- There are seven antigenic types of free coagulase, only one antigenic type of bound coagulase exists.
- Free coagulase is heat labile while bound coagulase is heat stable.

Coagulase Test (Procedure):

- Dense suspensions of Staphylococci from culture are made on two ends of clean glass slide.
- One should be labelled as “test” and other as “control”.
- Control suspension serves to rule out false positivity due to auto-agglutination.
- Test suspension is treated with drop of citrated plasma and mixed well.
- Agglutination or clumping of cocci within 5-10 secs is taken as positive.



DNase Test:

- Colonies producing DNase hydrolyse DNA content of this medium located in their immediate vicinity.
- If medium is then flooded and acidified with 1 N HCl (acid kills the viable cells), DNA precipitates out (turbidity) and clear zones appear around DNase-positive colonies.
- Some authors recommend instead flooding medium with toluidine blue solution or use of DNase test agars containing toluidine blue or methyl green.
- Dyes form coloured complexes with DNA.
- Presumptive pathogenic *S. aureus* shows colour changes on DNA hydrolysis with pink zone or almost colourless on media containing toluidine blue and methyl green respectively.
- Pathogenic *S. aureus* metabolize mannitol to form acid.

DNase Test (Procedure):

- Streak pure culture to be tested onto surface of test agar.
- Incubate under optimal conditions (24 h at 35°C).
- Check plates for mannitol fermentation, and flooded surface of plates with 1 N hydrochloric acid.
- Before addition of 1 N HCl into medium, look for yellow surrounded by yellow zone by mannitol positive *S. aureus*.
- After addition of 1 N HCl into medium HCl appears yellow, surrounded by yellow zone before addition of 1 N HCl, it appears well defined, pink or clear zones for coagulase positive *S. aureus* otherwise turbid culture medium.



+ Clear zone around inoculation line

- No clearing, green colored agar throughout