Master
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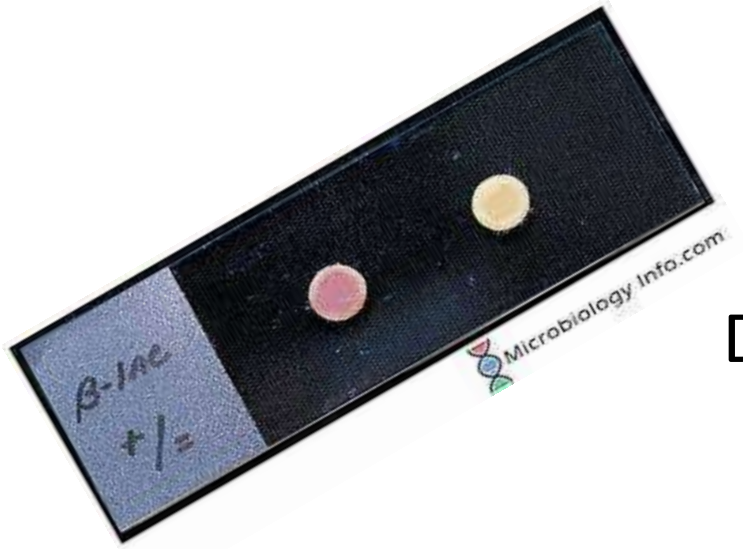
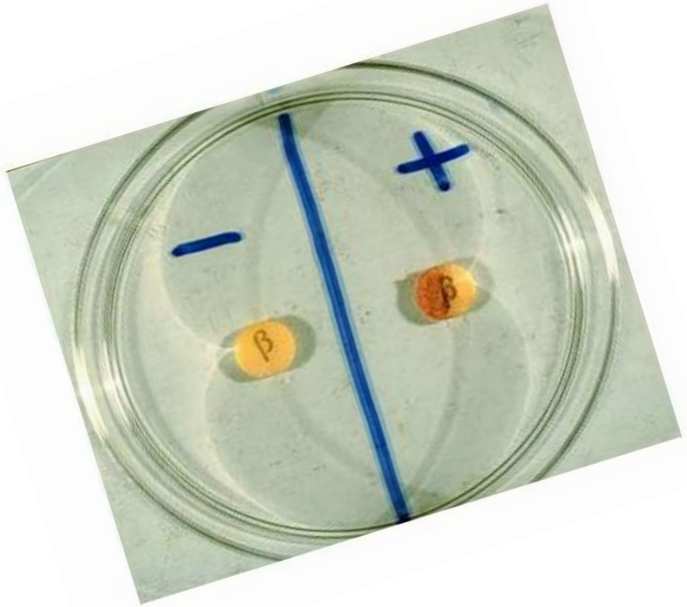
Microbiology

University of Basrah
College of Veterinary Medicine
Department of Microbiology

Beta (β) Lactamase Test



Dr. Tamadher Mohammed



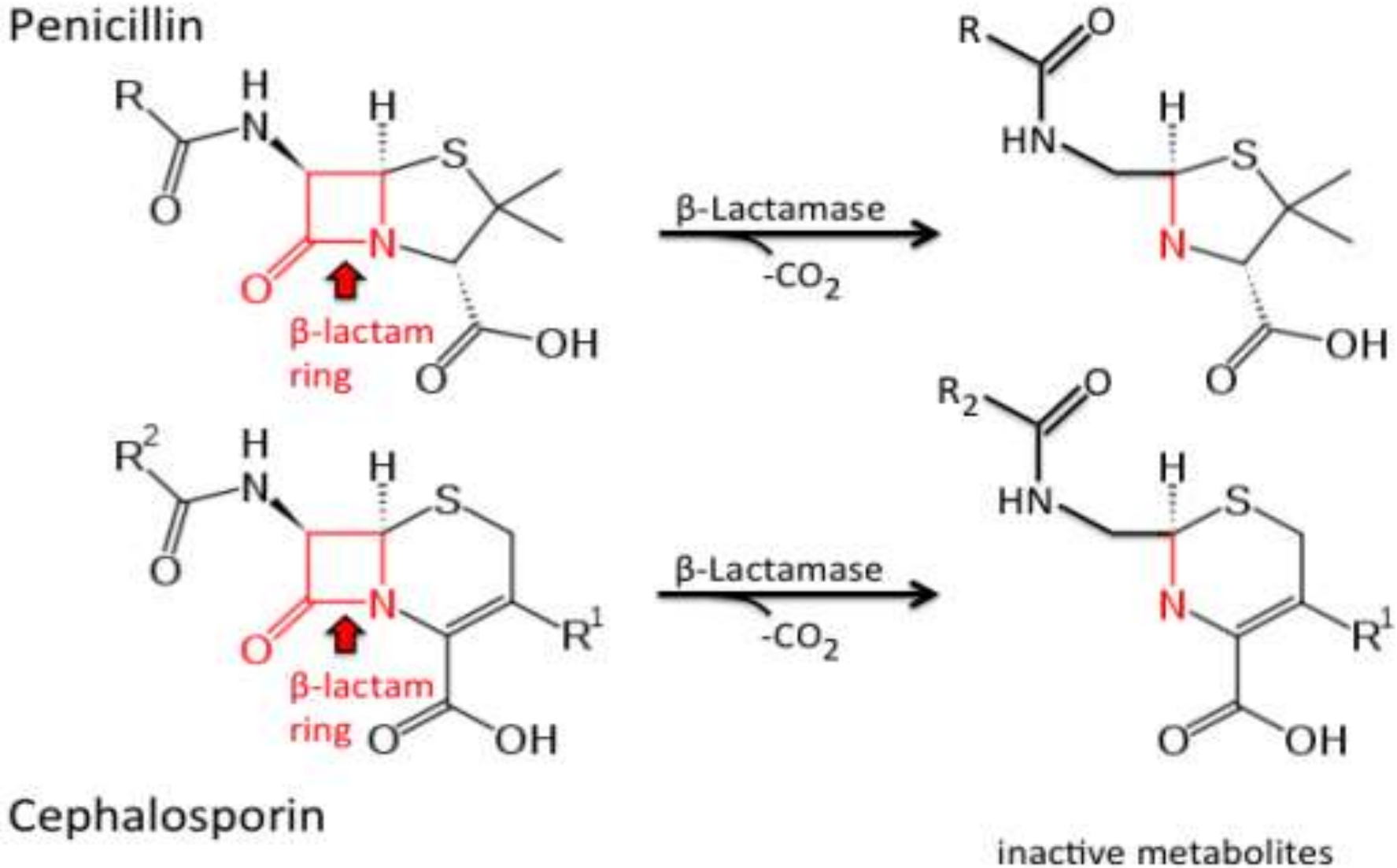
Beta (β) Lactamase

Different bacteria produce an important class of enzymes called beta-lactamases. Beta-lactamase is a plasmid-encoded or chromosomal-encoded enzyme that hydrolyzes the beta-lactam ring of the beta-lactam class of antibiotics resulting inactivation of these drugs. In addition, the beta-lactam gene frequently resides on integron.

These enzymes confer resistance to several of penicillin antibiotics by cleaving the beta-lactam ring of penicillin and cephalosporin antibiotics, resulting the inactivation of these drugs.

They are capable of inactivating “penicillinase-labile-penicillins”, such as amoxicillin, ampicillin, penicillin, carbenicillin, mezlocillin, and piperacillin.

β-lactamases thus play a key role in bacterial resistance to beta-lactam agents, and detection of their presence can provide useful information.


Penicillin
β-lactam ring
β-Lactamase
-CO2
β-lactam ring
β-Lactamase
-CO2
Cephalosporin
inactive metabolites

Various assays are available to detect β-lactamases, such as the iodometric method, the acidometric method, and by the use of chromogenic substrates.

The first beta-lactamase was described in 1940 as a “penicillinase” that is capable of hydrolyzing penicillin in E. coli.
Beta-lactamase enzyme production may be constative or induced by exposure to antimicrobials.
According to the Bush-Medeiros Classification System, beta-lactamase enzymes divide into 4 molecular classes (A-D) based on their amino acid structure.
Beta-lactamase enzymes are detected by rapid beta-lactamase tests. This test gives results earlier than MIC or Disk diffusion test.

chromogenic cephalosporin's test(Nitrocefin test)

One of the most useful tests in clinical laboratories for β- lactamase detection is the chromogenic cephalosporin's test(Nitrocefin test) . The test disk employed consists of a chromogenic cephalosporin which is used as the substrate. Organisms possessing β-lactamases when applied to the disk, exert their effect by opening the β-lactam ring of the substrate.
This process results in a colored product which is conspicuous and hence allows detection. On hydrolysis by the bacterial inoculum, a deep pink color is produced. Lack of color production indicates the absence of β-lactamase.

Objective

To detect the enzyme beta-lactamase, which confers penicillin resistance to various bacterial organisms.

Principle of Nitrocefin test

Nitrocefin disks are impregnated with nitrocefin, a chromogenic cephalosporin.
Bacteria produced a significant amount of β-lactamase enzyme resulting in hydrolyzed amide bond in a β- lactam ring.
Color of the nitrocefin disk changed from yellow to red.
The color change of the disk indicates a positive test, and the colors that remain the same indicate a negative test.

These beta-lactamases are capable of inactivating “penicillinase-labile-penicillins” such as amoxicillin, ampicillin, penicillin, carbenicillin, mezlocillin, and piperacillin.

Requirement of Nitrocefin test

Nitrocefin disk (commercially available, follow manufacture • instructions) stored at 2-8 ℃.
Sterile distilled water •
Glass slide or empty petri dish •
Sterile Pasteur pipette•
Sterile wooden stick and inoculating loop•
The test organism colony was grown overnight (18-24 hrs) on non-• selective media.

The procedure of Nitrocefin test

-Using sterile forceps, dispense the required number of disks onto a clean microscope slide or an empty petri dish.

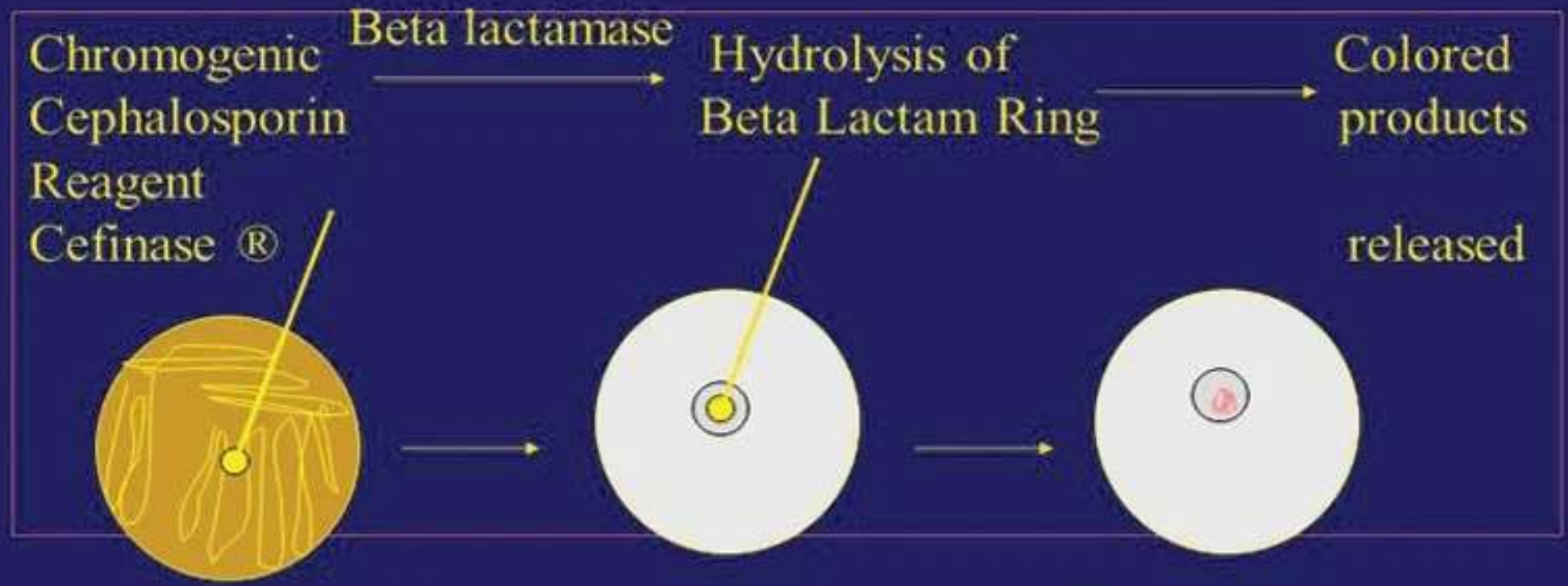
-Before inoculation, allow the Nitrocefin disk to be brought to room temperature.

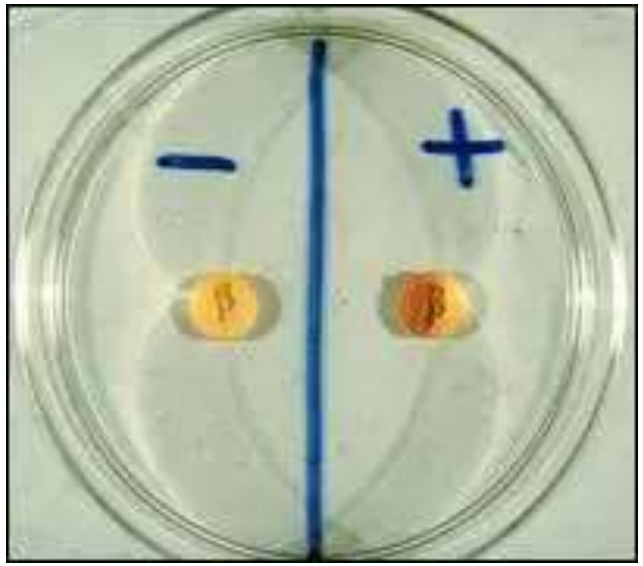
-Moisten each disk with 1 drop of sterile distilled water.

-With a sterile loop or applicator stick, smear several colonies onto the disk surface and also smear the positive control strain colony and negative control strain colony.

-Observe the disk for color change. Positive results usually appear within 15 s to 5 min. If no color change occurs within 5 min, the test is negative. However, positive reactions for some staphylococci may take up to 1 h.

Beta (β)-Lactamase Test





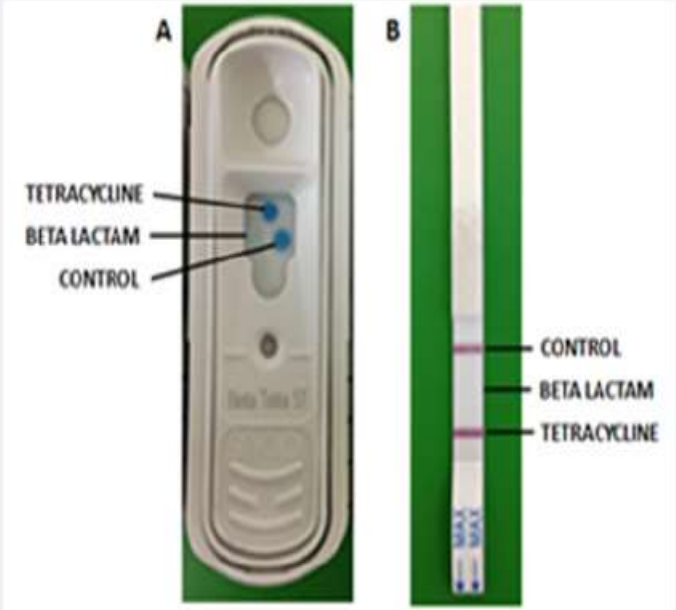


Figure 3: Analysis of a positive beta lactam cow milk sample

Applications of Nitrocefin test

1-The chromogenic cephalosporins test (Nitrocefin) is a biochemical test that is a sensitive method for detecting beta-lactamase-producing strains ;
N. gonorrhoeae,
H. influenza,
Staphylococcus spp,
Enterococcus spp,
Moraxella catarrhalis.

2-It is the most reliable test for detecting beta-lactamase-producing Enterococcus spp.

3-Easy to perform and gives results earlier than other methods.

4-The chromogenic method is quicker and more convenient than the acidimetric and iodometric methods.

5-The sensitivity and efficiency of the chromogenic method are high due to the detection of both penicillinase and cephalosporinase enzymes produced by isolates.

6-Nitrocefin test is only reliable test for detecting β -lactamase production by Enterococcus spp.

7-The chromogenic cephalosporin compound nitrocefin test was also used for detecting β-lactamase produced by Achromobacter.

8-Nitrocefin has a wide susceptibility and sensitivity to the commercially available beta-lactam.

Limitations of the Nitrocefin test

Moisten disk is critical to the development of the color, if the disk begins to dry out, it may be necessary to rehydrate the disk with a small amount of water.

The indistinct and weak reaction was obtained for strains grown on blood agar plates.

Beta-lactamase detection with the nitrocefin disk should not entirely replace conventional susceptibility test methods, as other factors also influence the results of such tests, and on occasion, intrinsic resistance to beta-lactam antimicrobials has not been correlated with the production of beta-lactamase.

Do not over-saturate the tip, as it could dilute the reagent.

Detection of beta-lactamase activity in staphylococci may take up to one hour.

Induction of the enzyme may also be required. This can be done by testing growth from the zone margin around an oxacillin disk.

A negative result does not rule out resistance due to other mechanisms.

Nitrocefin disk method cannot be used to test the members of Enterobacteriaceae, Pseudomonas species, or other aerobic, gram-negative bacilli because the results may not be predictive of susceptibility to the beta-lactams most often used for therapy.

The Nitrocefin disk cannot be used for organisms where penicillin resistance is not due to beta-lactamase production, such as Streptococcus pneumoniae and Streptococci.

Acidimetric method of Beta (β) Lactamase Test

Principle of Acidimetric method

In acidimetric test, penicillin-phenol red substrate reacts with beta-lactamase enzyme resulting in penicilloic acid produced. The color of the disk or solution (in tube test) changes from violet or red to yellow due to the pH decreases. Color change into yellow indicates a positive test or no change in color indicates a negative test.

Requirement of Acidimetric method

For disk or strip test

Acidimetric disk (commercially available, following manufacturer instruction) stored at 2-8 ℃.

Sterile distilled water

Glass slide or empty petri dish

Sterile Pasteur pipettes

Sterile wooden stick and inoculating loop

The test organism colony was grown overnight (18-24 hrs) on non-selective media.

For tube test

0.5% Phenol red solution (add 0.5 g of phenol red to small amount of water, dissolved properly dissolved heat may be needed to dissolve dye and make up volume 100ml then store at 25°C. Shelf life is 6 months.)

Crystalline potassium penicillin G (vial containing 20 million U). Store as indicated by the manufacturer.

1 N NaOH (add 4 g of NaOH crystals to 100 ml of water). Caution: This will cause heat production. Store at 25°C.

Sterile 1- and 10-ml pipettes and pipette bulb

Sterile polystyrene capped tubes (12 by 75 mm)

Sterile wooden applicator sticks or inoculating loops.

Preparation of penicillin-phenol red substrate reagent

Add 2 ml of the 5% phenol red solution to 16.6 ml of sterile distilled water.
Add the phenol red-water solution (18.6 ml) to the vial of crystalline benzylpenicillin G.
Remove the solution from the vial and place it in a sterile container.
Add 1 N NaOH dropwise to this acidic solution until it develops a violet color (pH 8.5).
Dispense in 0.1-ml aliquots into sterile tubes and freeze at – 20°C or lower in a non-frost-free freezer.

Procedures (for disk test) of Acidimetric method

Using sterile forceps, dispense the required number of disks onto a clean microscope slide or an empty petri dish, and the remaining unused disk immediately place into the freezer.
Before inoculation, allow the nitrocefin disk to be brought to room temperature.

Moisten each disk with 1 drop of sterile distilled water.
With a sterile loop or applicator stick, smear several colonies onto the disk surface and also smear the positive control strain colony and negative control strain colony.

Observe the disk for color change. Positive results usually appear within 10 min.

If no color change occurs within 10 min, the test is negative. However, positive reactions for some staphylococci may take up to 1 h. When dry, the color remains for up to 24 h.

Procedure for tube method of Acidimetric method

Remove the desired number of reagent tubes from the freezer and allow them to thaw at room temperature (one tube per organism).

With a sterile loop or applicator stick, add four or five colonies to the test solution to make an opaque, milky suspension.

Observe for color change. A positive reaction will occur in less than 15 min. If no color change occurs within 15 min, the test is negative. A color change after 15 min usually indicates deterioration of the substrate not related to the presence of beta-lactamase and should not be considered positive. Since positive reactions for some staphylococci may take up to 1 h, results that turn positive after 15 min may not be reliable for these bacteria.

Results and interpretation of Acidimetric method

Positive: Violet or red color changes to yellow.

Negative: no change in color occurs.


Organ
Suspe
en'ase
ositiw
s'ase
ative

Applications of Acidimetric method

Acidometric method is more effective than iodometric method for detection of coagulase positive Staphylococcal β-lactamase.

Rapid acidimetric method used for performing a beta-lactamase test on Haemophilus spp, Neisseria gonorrhea, and staphylococcus spp.

The test is easy to perform and interpret.

Limitations of Acidimetric method

This test applies only to aerobic bacteria.

This test don’t differentiate between acylase and β-lactamase activity.

Only detect penicillinase, not cephalosporinase enzyme.

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Iodometric method of Beta (β) Lactamase Test

Principle of Iodometric method

This method is based on the fact that β -lactamase can hydrolyze penicillin G and release a reducing product (penicilloic acid), which reduces iodine and prevent it from combining with starch. Discoloration of dark blue iodine starch complex indicates positive results.

Requirement of Iodometric method

Penicillin: (6,000 µg/ml) dissolved in phosphate buffer (pH 6.0, 0.05 to 1 M) Store at 2 to 8°C. Shelf life is 24 h.

Starch reagent: Add 1 g of soluble starch to 100 ml of distilled water and heat in a boiling water bath until starch dissolves. Store at 2 to 8°C. The shelf life is 1 week.

Iodine reagents: Dissolve 2.03 g of iodine and 53.2 g of potassium iodide in a small volume of distilled water and make the final volume to 100 ml. Store at 2 to 8°C in a dark bottle; replace if precipitate is apparent. Shelf life is 2 months.

Empty sterile microdilution tray or small test tube

Sterile 1 ml pipettes and pipette bulb

Sterile wooden applicator sticks or inoculating loops

Procedure of Iodometric method

-Dispense 0.1 ml of the penicillin solution into a well of a microdilution tray (or a small test tube).

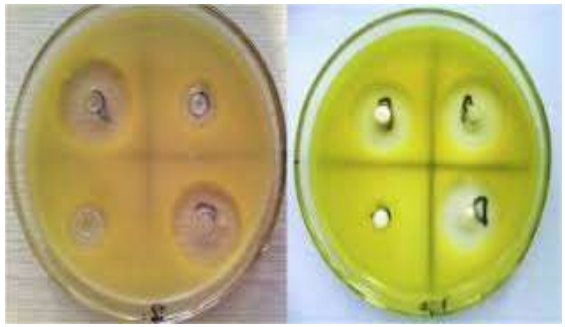
-Add the test organism to make an opaque, milky suspension.

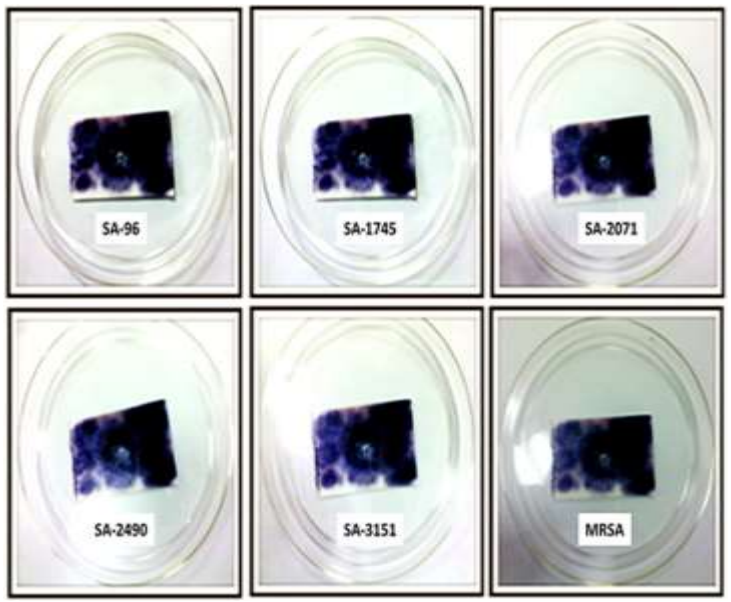
-Add 2 drops of the starch solution and mix.

-Let sit at room temperature (approximately 25°C) for 30 to 60 min.

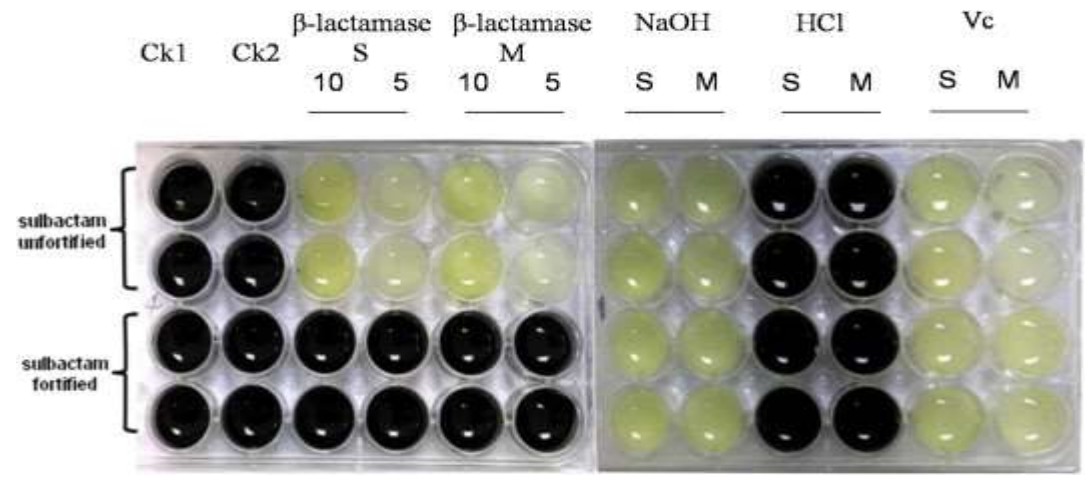
-Add 1 drop of the iodine reagent.

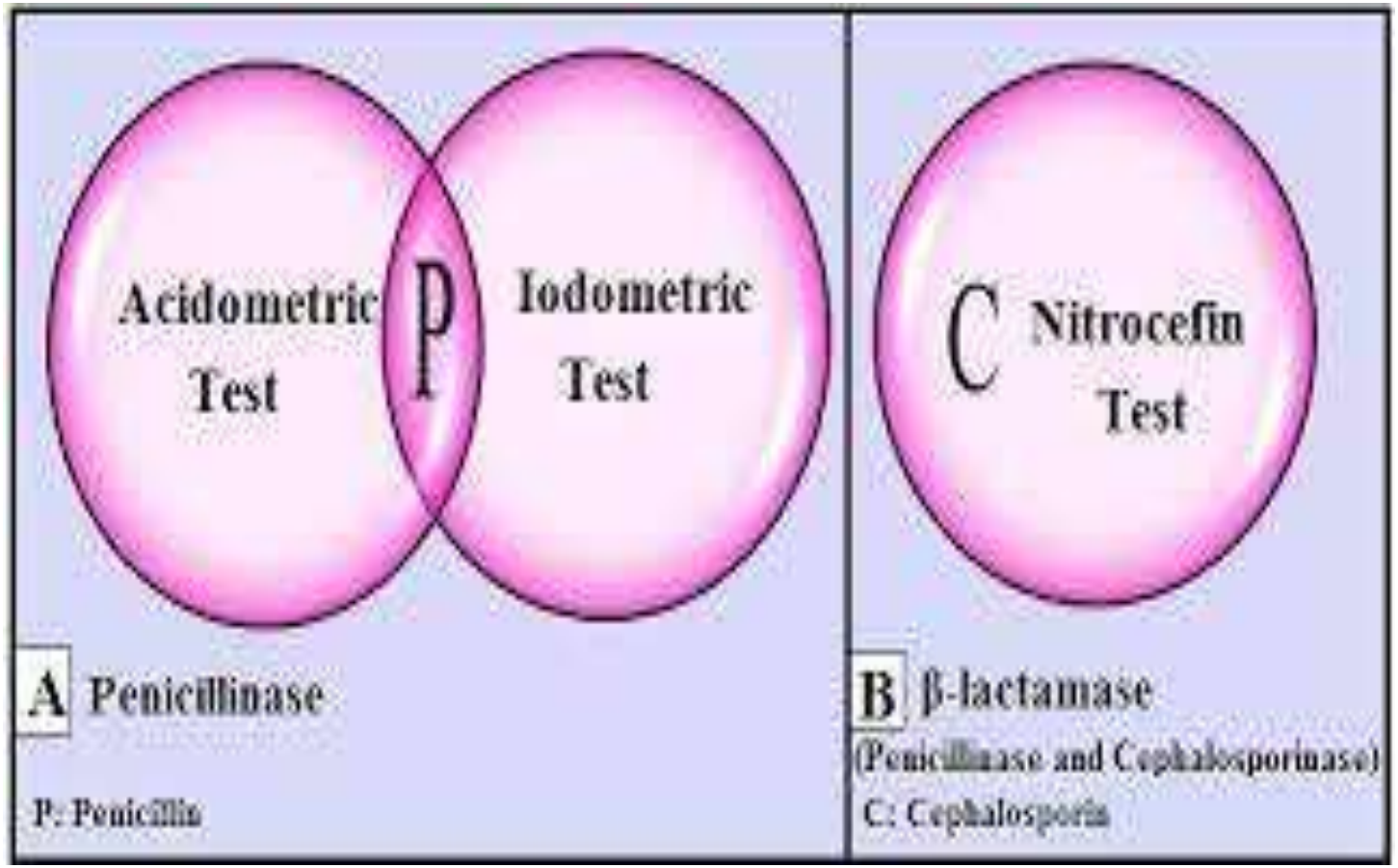
Shake or stir the mixture for 1 min.




SA-96
SA-1745
SA-2071
SA-2490
SA-3151
MRSA


-H2O
β-lactamase


Ck1
Ck2
β-lactamase S
10
5
β-lactamase M
10
5
NaOH
S
M
HCl
S
M
Vc
S
M
sulbactam unfortified
sulbactam fortified


Acidometric Test
P
Iodometric Test
A Penicillinase
P: Penicillin
C Nitrocefin Test
B β-lactamase
(Penicillinase and Cephalosporinase)
C: Cephalosporin