University of Basra
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Microbiology Department

laboratory diagnosis of veterinary importance agents from genera

Clostridium

and Citrate Utilization Test

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clostridium species

- Categorized into three major groups based on toxin activity

Neurotoxic clostridium

C. tetani

C. botulinum

Enterotoxemic and Enteropathogenic clostridia

C. perfringens type A-E

C. difficile

Histotoxic clostridia

localized lesion in liver and muscle

C. chauvoei

C. septicum

C.novyi type A

C. perfringens type A

C. sordelli

C. haemolyticum

C. novyi type B

GENERAL CHARACTERISTICS

- **Gram-positive rods** (at least early in growth), **in singles, pairs, or chains.**
- Most are **obligate anaerobes**, but some are **microaerophiles**.
- **Produces endospores**, but not aerobically; spore shape and position are variable, but usually distend the cell
- **Most are catalase-negative and oxidase negative.**
- Most are isolated from soil, sewage, or marine sediments.
- Key pathogens are ***C. tetani*** (tetanus), ***C. botulinum*** (botulism), ***C. perfringens*** (food poisoning and gas gangrene), and ***C. difficile*** (pseudomembranous colitis).

GENERAL CHARACTERISTICS

- Endospore's **size, shape and location** is used for differentiation
- **Mostly motile** (except *C. perfringens*)
- Required enriched media for growth
- They are **toxigenic** (has the ability to intoxicate a person)
- They are **non-capsulated** except *C. perfringens*
- **Liquefy gelatin** (gelatin liquefaction +ve)
- **Fermentative**

Laboratory diagnosis

Clostridium spp.

Samples:

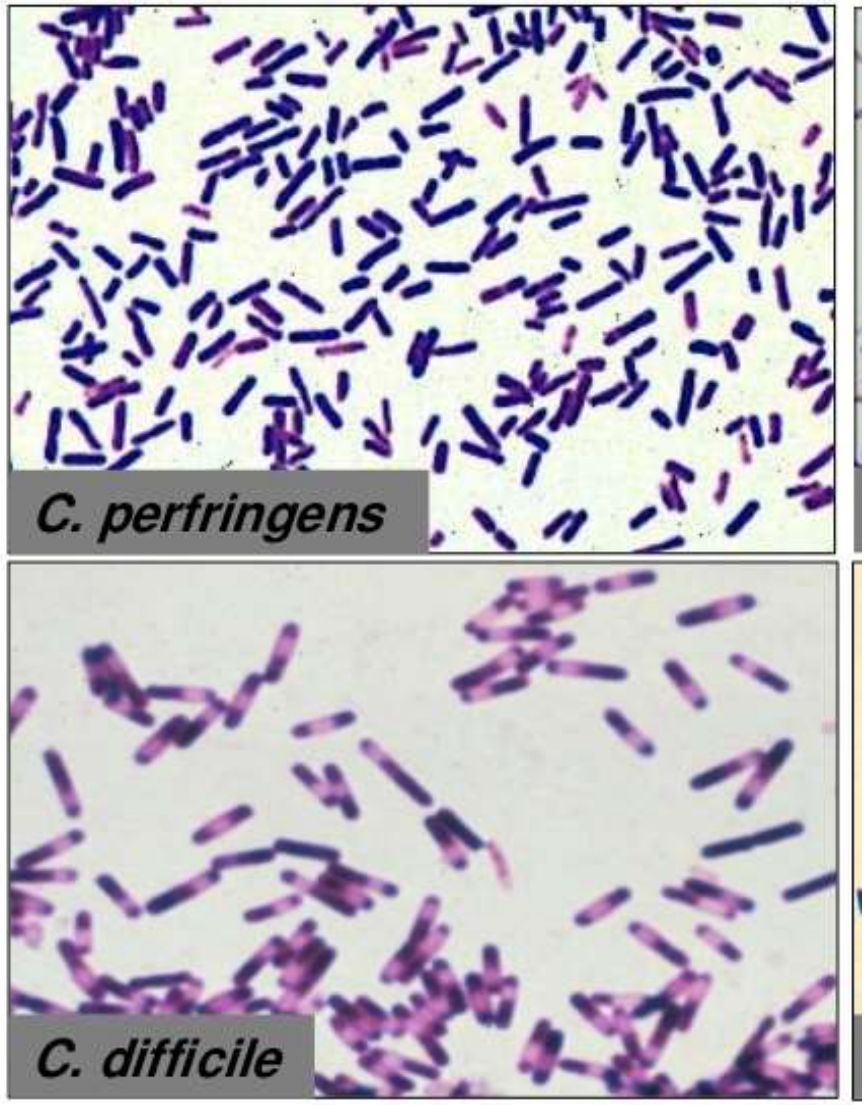
- ***C. perfringens:*** wound swabs, necrotic tissues, muscle fragments, pus, stool and food (food poisoning)
- ***C. difficile:*** stool samples (diarrhoea)
- ***C. botulinum:*** food, faeces or intestinal contents

Clostridium spp.

Microscopic features:

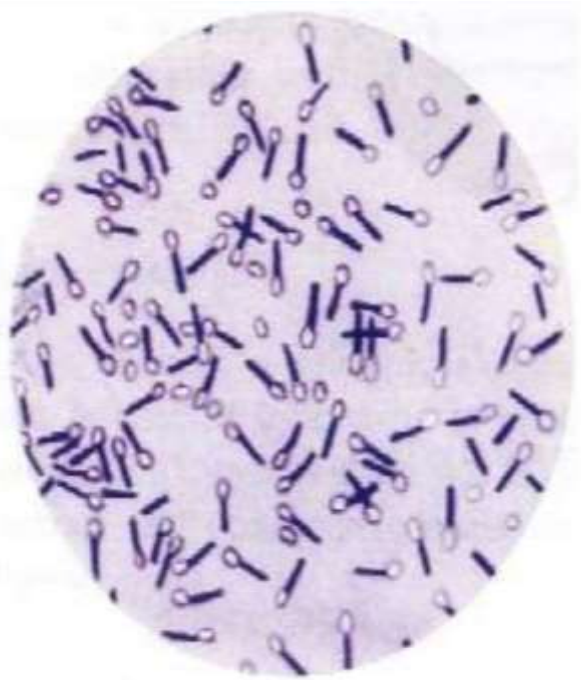
1. ***C. perfringens:*** Gram +ve, pleomorphic rods, oval, sub-terminal spore and non-motile.
2. ***C. tetani:*** Gram +ve, long thin rods, terminal spores, (drumstick appearance) and motile.
3. ***C. botulinum:*** Gram +ve, pleomorphic rods, oval, sub-terminal spore and motile.
4. ***C. difficile:*** Gram +ve, long thin rods, large oval sub-terminal spores and motile.

- **Microscopic features**



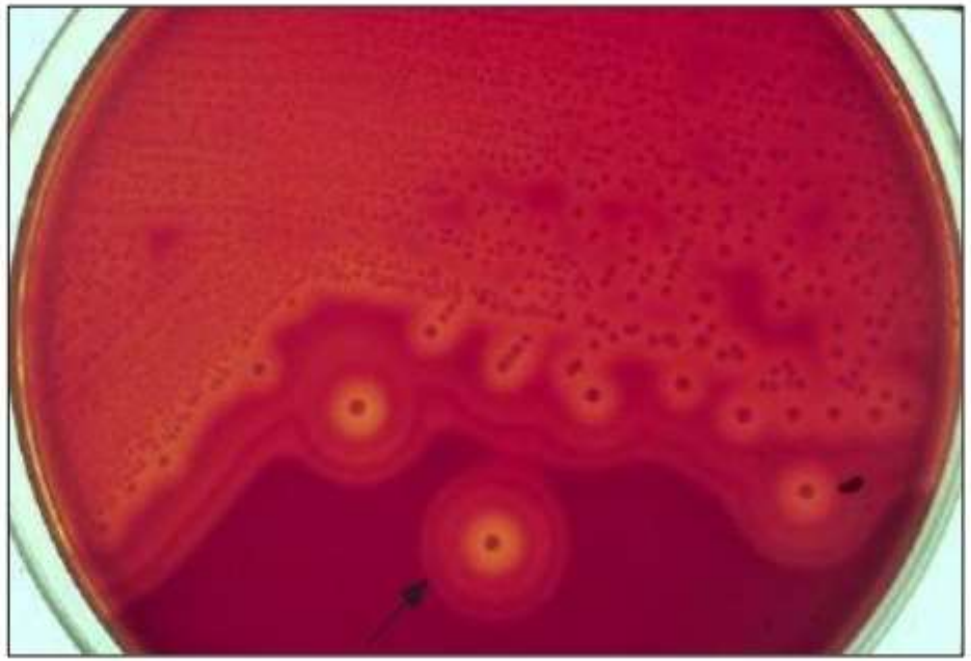
Clostridium tetani- agent of tetanus

Calf with tetanus following castration. Note the rigid limbs due to muscle spasm

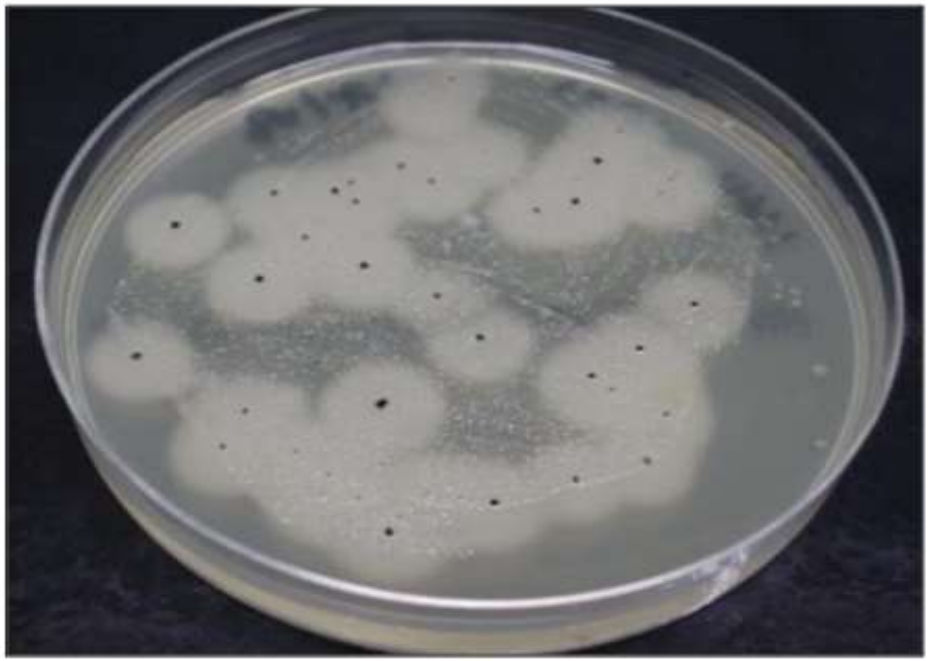


C. perfringens (culture)

- Samples are cultured on **blood agar plates (BAP)** anaerobically (i.e. using jar), **Tryptose sulphite cycloserine agar (TSCA)**, **Robertson's Cooked Meat broth** & **Thioglycolate broth media**.



Double zone of clear beta-hemolysis around a colony is clearly seen (BAP)



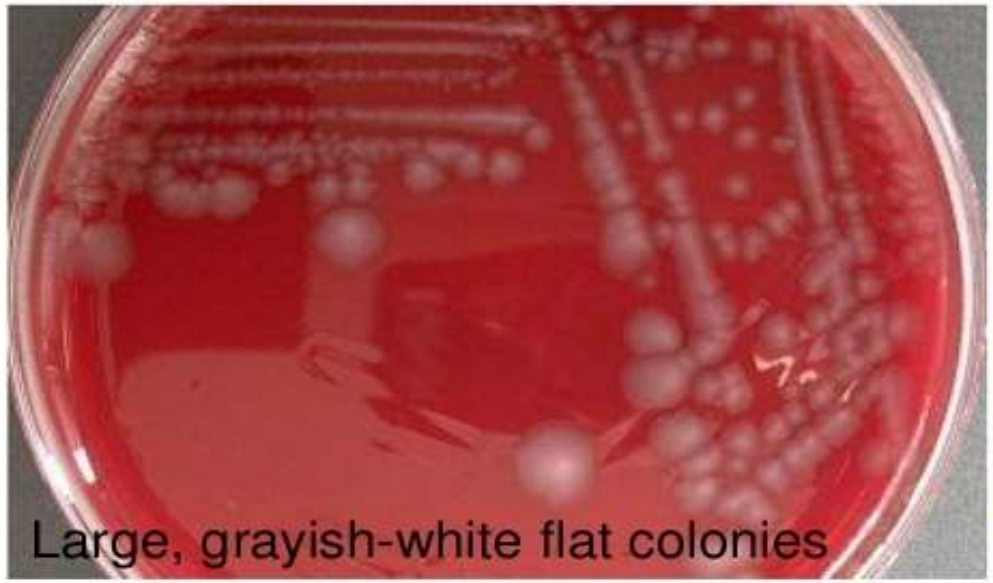
TSCA (C. perfringens)

C. botulinum (culture)

- On blood agar ***C. botulinum*** produces large semi-transparent colonies with a wavy outline.
- Most strains are beta-hemolytic.

C. difficile (culture)

- **Blood agar (anaerobically):** large, flat colonies with barnyard smell.
- **Cycloserine Cefoxitin Fructose Agar (CCFA)/ selective:** 4 mm colonies appear as yellow and ground class-like and have filamentous edge, the odor associated with colonies is very distinct and typically like elephant or horse manure.



Blood agar

CCFA

C. tetani (culture)

- On blood agar it produces a fine film of growth.
- Swarming due to its motility.
- On fresh BAP it is hemolytic (alpha first followed by beta hemolysis).

Anaerobic culture methods

Robertson Cooked Meat Broth

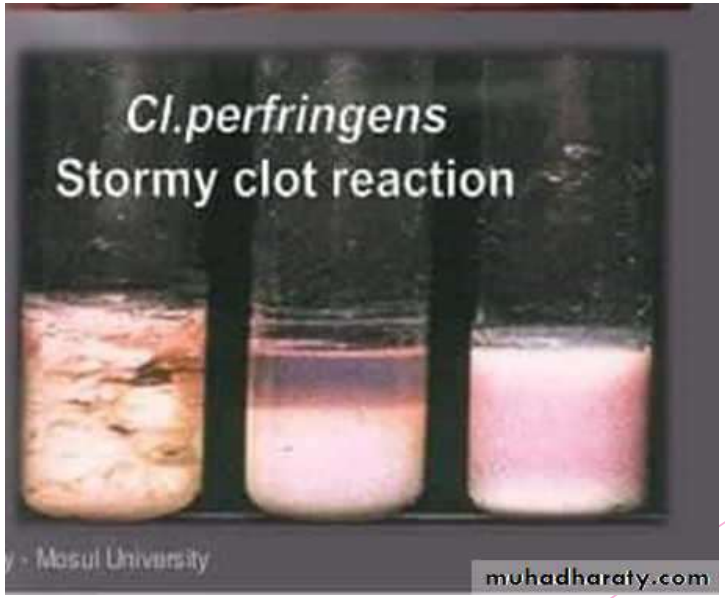
4- Mechanical exclusion of O_2 (anaerobic incubator).

Thioglycolate broth

Biochemical tests:

Litmus Milk test

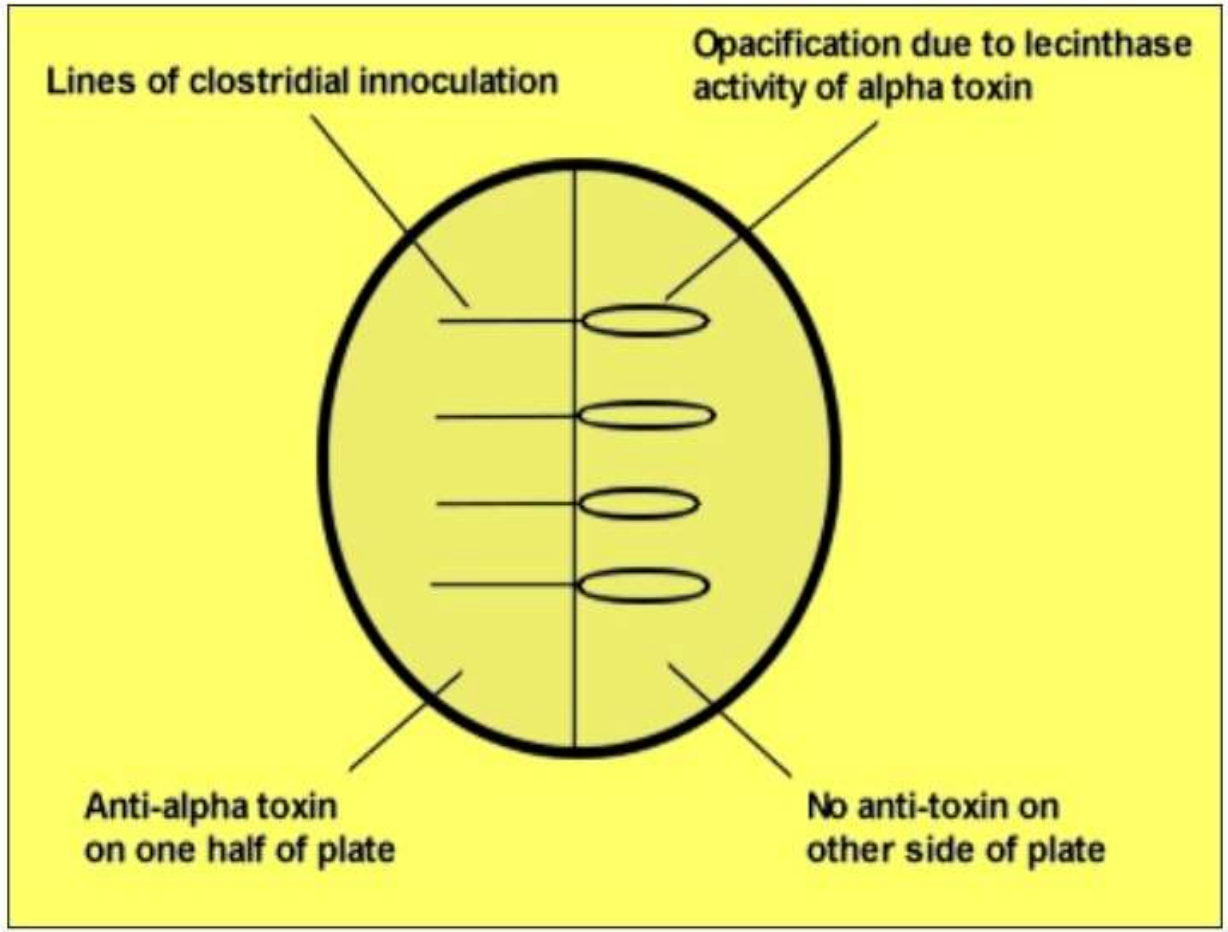
- stormy fermentation In anaerobically-grown Litmus Milk cultures, enzymes of C. perfringens will attack the proteins and carbohydrates of the milk producing a "stormy fermentation" with acid production (the indicator, litmus turns pink), clotting of milk proteins, and gas formation.



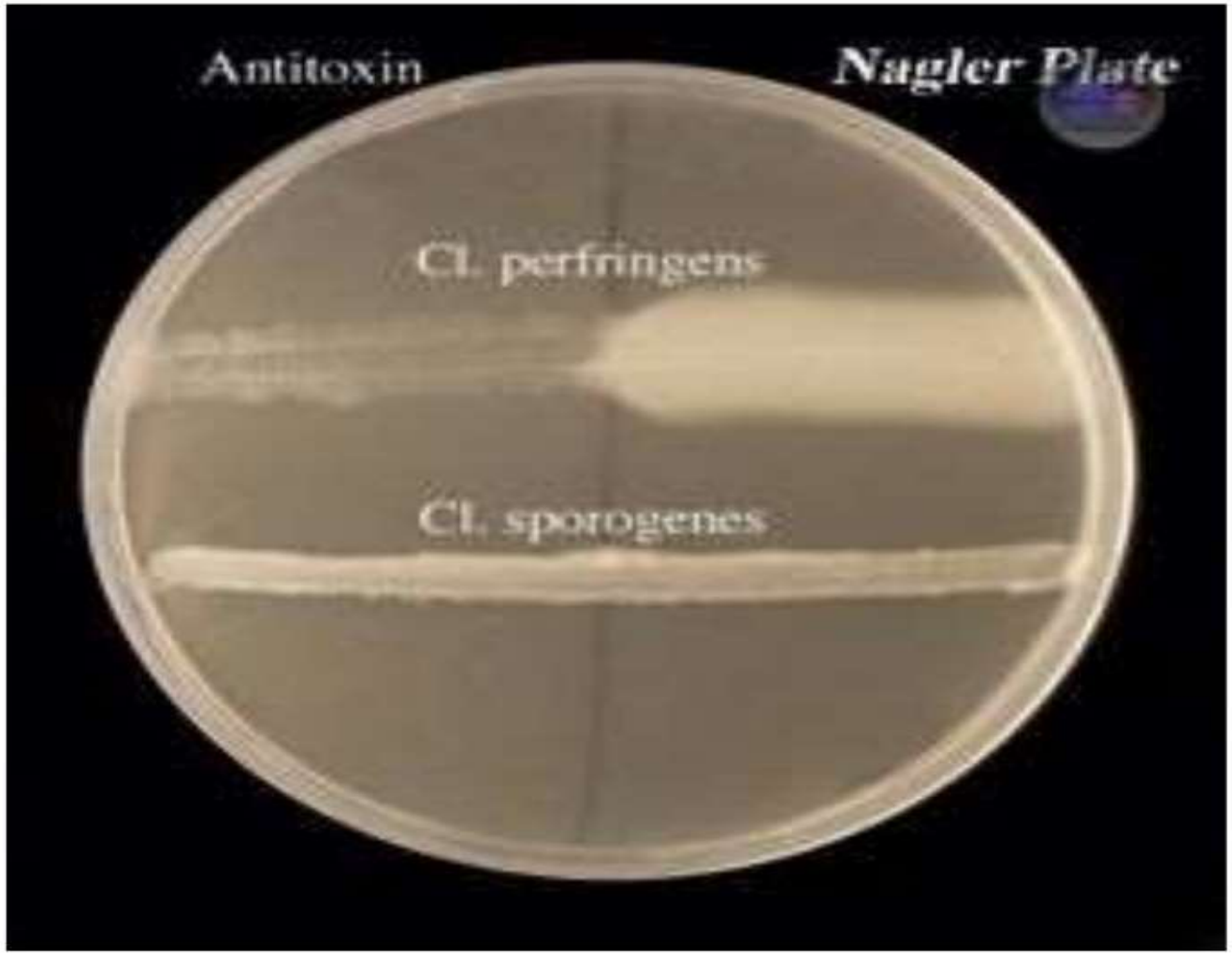
NAGLER TEST (PROCEDURE)

1. Label an egg yolk media plate and mark the plate into two halves.
2. Inoculate 60 µL of *Clostridium perfringens* type A antitoxin in half of the plate, spread over the surface of agar using a spreader and allow to absorb and dry.
3. Mark the side of the plate in which the antitoxin is inoculated.
4. **Streak the test organism in a straight line from the toxin free agar half of the plate to toxin containing side. Repeat the same procedure with control strains on the same plate.**
5. Incubate **anaerobically** at 35-37 °C for 24-48 hrs.
6. Examine the plate for an opalescent halo around the inoculum and inhibition by antitoxin.

- **<u>Nagler reaction:</u>** *C. perfringens* is **Nagler positive** due to lecithinase activity of alpha exotoxin.



Nagler reaction: C. perfringens phospholipase causes turbidity aro colonies on egg-yolk



Laboratory diagnosis

- Direct smears from the mucosa or contents of the small intestine of recently dead animals which contain substantial numbers of large Gram-positive rods are consistent with enterotoxaemia.

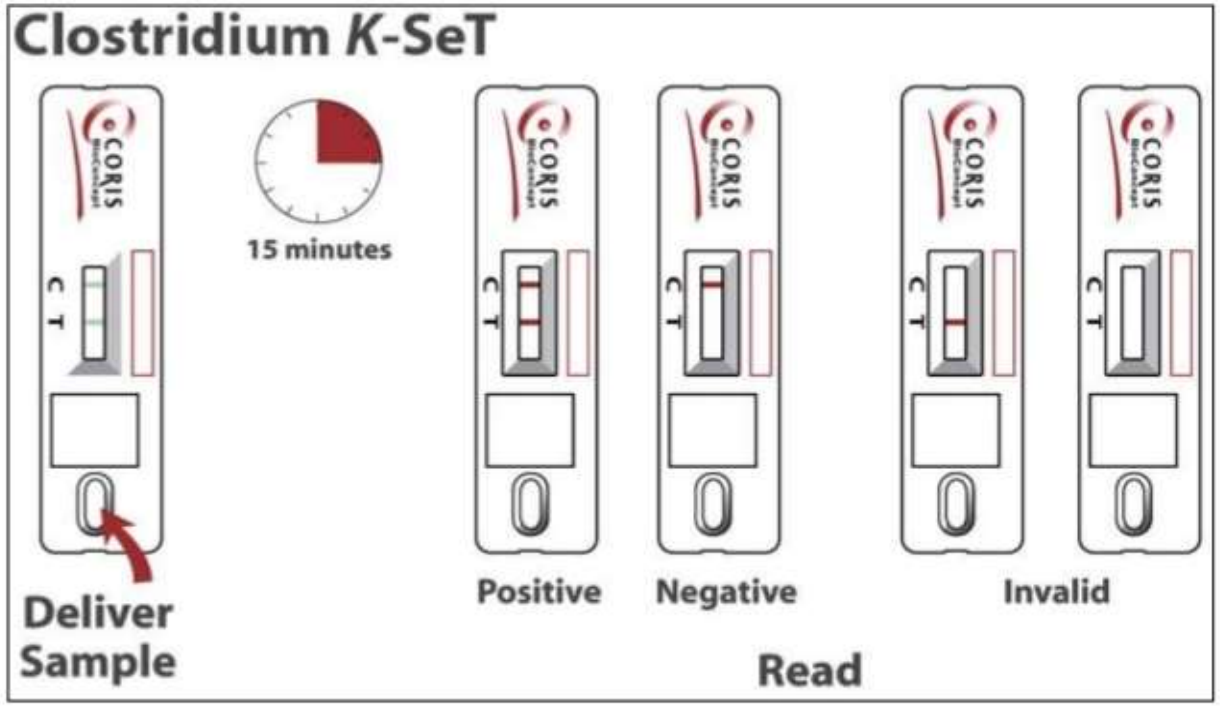
- Toxin neutralization tests using mouse and guinea-pig inoculation can definitively identify the toxins of C. perfringens present in the contents of recently dead animals.
- ELISA can be used for demonstrating toxin in intestinal contents and are of comparable sensitivity to in vivo assays.

C. *perfringens*

1) Gram staining of content of small intestine→ large no. of G + rod
2) Culture → rapid growth property
   → stormy reaction
   → double zone of hemolysis
   → **Nagler reaction**
3) Animal inoculation for demonstration of toxins in vivo from specimens or culture
4) PCR

OTHER TECHNIQUES

- ***Clostridium difficile*** rapid test:



- **Toxin analysis (PCR & ELIZA):** *C. perfringens & C. difficile*

SIMPLE DIFFERENTIATION CHART

Clostridium Species	Spore	Motility	Lecithin C	Lipase hydrolysis	Proteolytic Activity	Ferment Lactose
C.tetani	**Round Terminal (drum stick)**	Motile	-Ve	-Ve	-Ve	-Ve
C.perfringens **(A-E types)**	Oval, sub - terminal	**Non-motile**	**+Ve**	-Ve	-Ve	**+Ve**
C.botulinum **(Type A, B, F)**	Oval, sub-terminal	Motile	-Ve	**+Ve**	**+Ve**	-Ve
C.botulinum **(Type C, D, E)**	Oval, sub-terminal	Motile	-Ve	**+Ve**	-Ve	-Ve
C.difficile	Oval, sub-terminal	Motile	-Ve	-Ve	-Ve	-Ve

- Demonstration of histotoxic clostridia in tissues
  - PCR-based techniques
  - Fluorescent antibody techniques
- ELISA can be used for toxin detection.

Note: They are often derived from the normal flora.

oxygen sensitive, isolation takes several days or longer

Citrate Utilization Test

- This test is among a suite of IMViC Tests (Indole, Methyl-Red, Vogues-Proskauer, and Citrate) that are used to differentiate among the Gram-Negative bacilli in the family Enterobacteriaceae.

- Significance Of Citrate Test
- Use test to identify genera within the bacterial family Enterobacteriaceae that are able to utilize sodium citrate as a sole source of carbon.

Principle Of Citrate Test

- Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. The medium contains **citrate** as the sole **carbon source** and **inorganic ammonium salts (NH4H2PO4)** as the sole source of **nitrogen**..
- Bacteria that can grow on this medium produce an enzyme, citrate-permease,When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity.
- The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6.

Materials

- Simmons Citrate agar which contains :

1. sodium citrate (carbon source) .
2. ammonium salts (nitrogen source).
3. Bromthymol blue indicator.

- Inoculating needle .

Method

1. Streak one organism over the surface of the agar slant, then stab the butt.
2. Incubate the tube at37°C for 48 hours .

Result

- Examine for growth (+) .
- Growth on the medium is accompanied by a rise in pH to change the medium from its initial green color to deep blue.

Result

- Some intestinal bacteria are (+)citrate as: *Klebsiella* .

  Citrobacter.

  Enterbacter aerogenes .

- while others is(-) citrate as: *E. coli*