CHAPTER 9

Isolation and Identification of *Bacillus* sp. and *Clostridium* sp.

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Inroduction

Bacillus (Latin "stick") is a genus of Gram-positive, rod-shaped bacteria, a member of the phylum Bacillota, with 266 named species. The term is also used to describe the shape (rod) of other so-shaped bacteria; and the plural Bacilli is the name of the class of bacteria to which this genus belongs. Bacillus species can be either obligate aerobes which are dependent on oxygen, or facultative anaerobes which can survive in the absence of oxygen. Cultured Bacillus species test positive for the enzyme catalase if oxygen has been used or is present. Bacillus can reduce themselves to oval endospores and can remain in this dormant state for years. The endospore of one species from Morocco is reported to have survived being heated to 420 °C. Endospore formation is usually triggered by a lack of nutrients: the bacterium divides within its cell wall, and one side then engulfs the other. They are not true spores (i.e., not an offspring). Endospore formation originally defined the genus, but not all such species are closely related, and many species have been moved to other genera of the Bacillota. Only one endospore is formed per cell. The spores are resistant to heat, cold, radiation, desiccation, and disinfectants. Bacillus anthracis needs oxygen to sporulate; this constraint has important consequences for epidemiology and control. In vivo, B. anthracis produces a polypeptide (polyglutamic acid) capsule that kills it from phagocytosis.

The genera Bacillus and Clostridium constitute the family Bacillaceae. Species are identified by using morphologic and biochemical criteria. Because the spores of many Bacillus species are resistant to heat, radiation, disinfectants, and desiccation, they are difficult to eliminate from medical and pharmaceutical materials and are a frequent cause of contamination. Not only are they resistant to heat, radiation, etc., but they are also resistant to chemicals such as antibiotics. This resistance allows them to survive for many years and especially in a controlled environment. Bacillus species are well known in the food industries as troublesome spoilage organisms. Ubiquitous in nature, Bacillus includes symbiotic (sometimes referred to as endophytes) as well as independent species. Two parasitic pathogenic species are medically significant: B. anthracis causes anthrax; and B. cereus causes food poisoning.

Clostridium is a genus of anaerobic, Gram-positive bacteria. Species of Clostridium inhabit soils and the intestinal tract of animals, including humans. This genus includes several significant human pathogens, including the causative agents of botulism and tetanus. It also formerly included an important cause of diarrhea, Clostridioides difficile, which was reclassified into the Clostridioides genus in 2016.

Clostridium species are readily found inhabiting soils and intestinal tracts. *Clostridium* species are also a normal inhabitant of the healthy lower reproductive tract of females.

The main species responsible for disease in humans are:

1. *Clostridium botulinum* can produce botulinum toxin in food or wounds and can cause botulism. This same toxin is known as Botox and is used in cosmetic surgery to paralyze facial muscles to reduce the signs of aging; it also has numerous other therapeutic uses.
2. *Clostridium perfringens* causes a wide range of symptoms, from food poisoning to cellulitis, fasciitis, necrotic enteritis and gas gangrene.
3. *Clostridium tetani* causes tetanus.

Several more pathogens were previously described in *Clostridium*, but have been moved to other genera with additional research

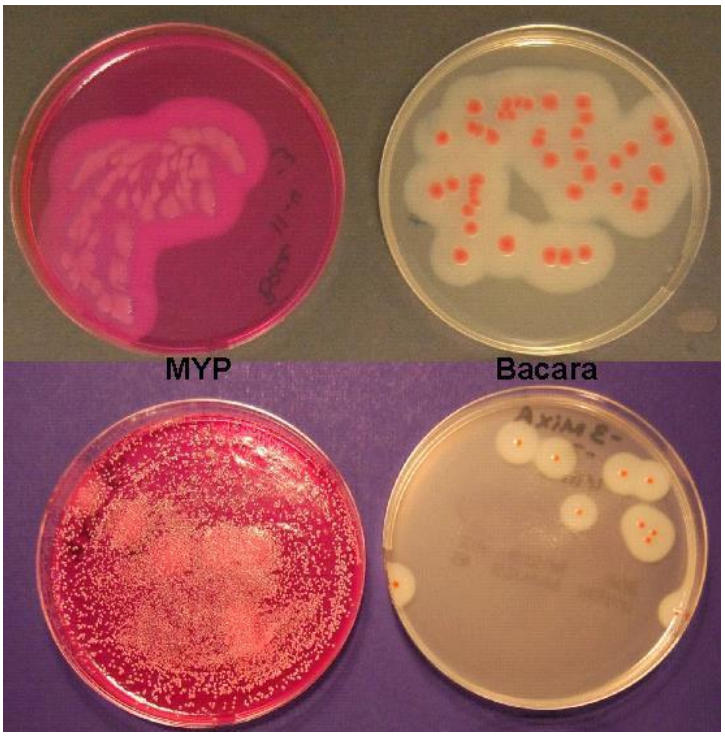
4. *Clostridium difficile*, now placed in *Clostridioides*.
5. *Clostridium histolyticum*, now placed in *Hathewaya*.
6. *Clostridium sordellii*, now placed in *Paeniclostridium*, can cause a fatal infection in exceptionally rare cases after medical abortions.

Bacillus cereus

Bacillus cereus is an aerobic spore-forming bacterium that is commonly found in soil, on vegetables, and in many raw and processed foods. B. cereus food poisoning may occur when foods are prepared and held without adequate refrigeration for several hours before serving, with B. cereus reaching >106 cells/g. Foods incriminated in past outbreaks include cooked meat and vegetables, boiled or fried rice, vanilla sauce, custards, soups, and raw vegetable sprouts. Two types of illness have been attributed to the consumption of foods contaminated with B. cereus. The first and better known is characterized by abdominal pain and non-bloody diarrhea; it has an incubation period of 4-16 h following ingestion with symptoms that last for 12-24 h. The second,

which is characterized by an acute attack of nausea and vomiting, occurs within 1-5 h after consumption of contaminated food; diarrhea is not a common feature in this type of illness1. The MYP agar has been the standard media for plating B. cereus, but it has little selectivity so background flora is not inhibited and can mask the presence of B. cereus. Bacara is a chromogenic selective and differential agar that promotes the growth and identification of B. cereus, but inhibits

the growth of background flora. The chromogenic agar has been suggested for the enumeration of B. cereus group as a substitute for MYP1,2. Typical colonies will grow as pink-orange uniform colonies surrounded by a zone of precipitation. The identification would include all species from the *B. cereus group: B. cereus, B. thuringiensis, B. anthracis,B. mycoides*, and *B. weihenstephanensis*. Biochemical testing will be necessary to delineate to the species level.



Colonies of B. cereus grown on MYP are pink and lecithinase positive, but other bacteria are not inhibited and can interfere with isolation of B. cereus. Colonies of B. cereus grown on Bacara are pink-orange and are lecithinase positive, but other organisms are inhibited.

B. cereus isolates can be identified by 1) produce large Gram-positive rods with spores that do not swell the sporangium; 2) produce lecithinase and do not ferment mannitol on MYP agar; 3) grow and produce acid from glucose anaerobically; 4) reduce nitrate to nitrite (a few strains may be negative); 5) produce acetylmethylcarbinol (VP-positive); 6) decompose L-tyrosine; and 7) grow in the presence of 0.001% lysozyme.

Bacillus anthracis

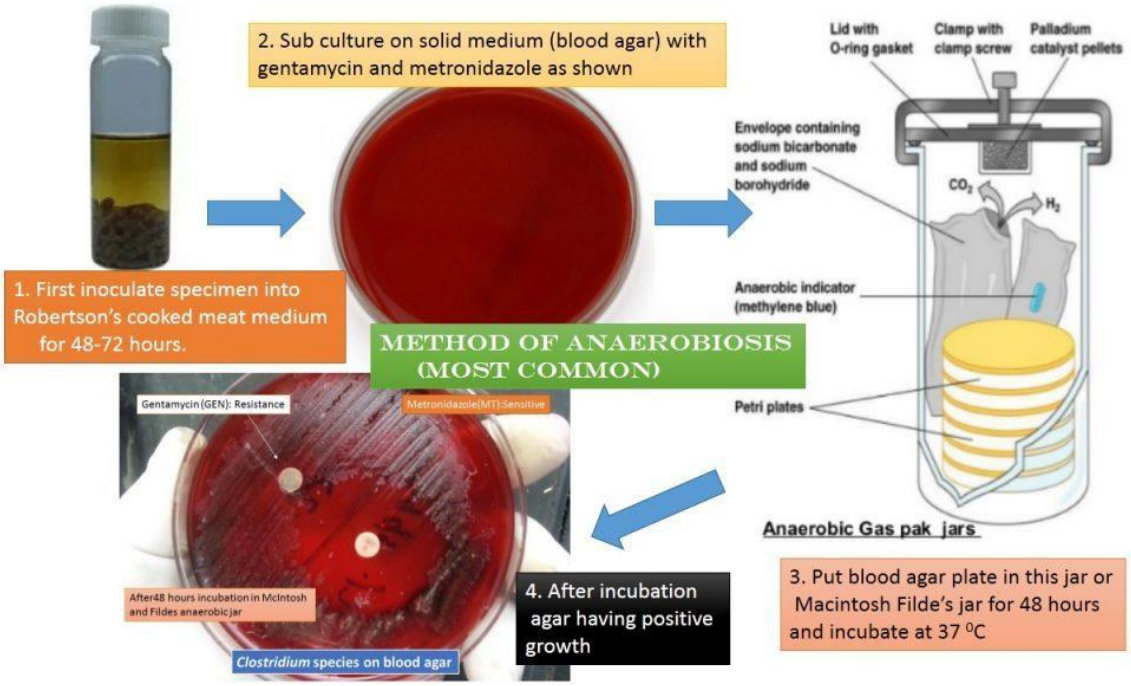
Anthrax is primarily a disease of herbivores. Bacillus anthracis has long been considered a potential agent for biological warfare or bioterrorism. Source: close contact with infected animals

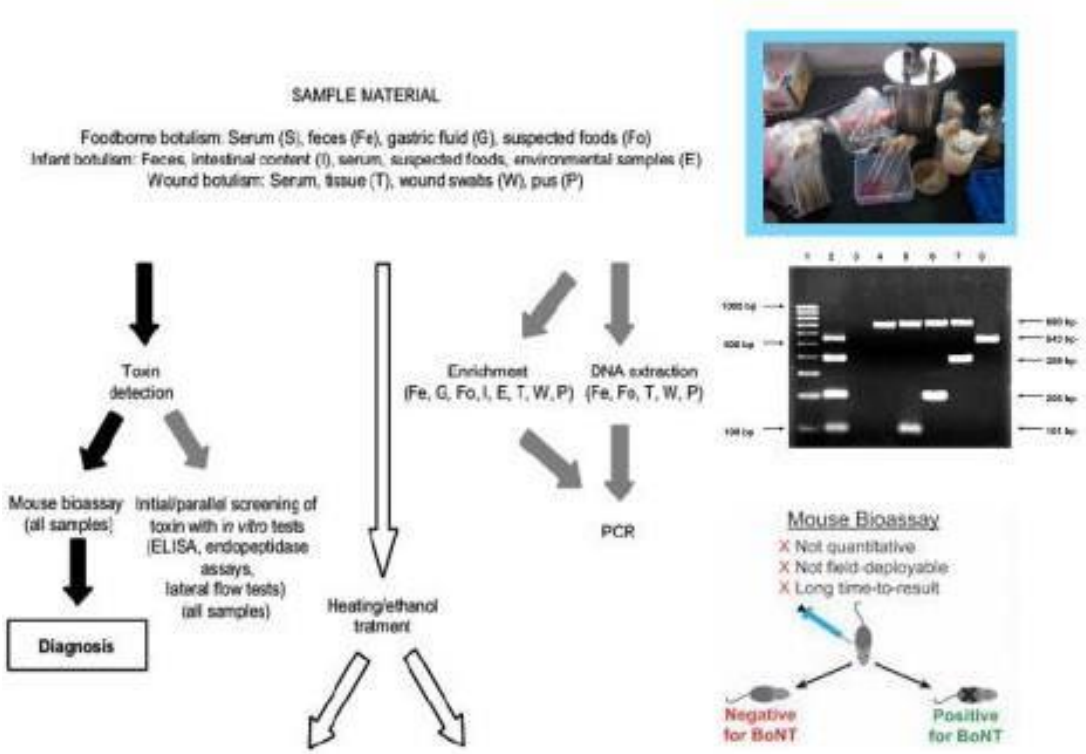
or their carcasses after death. It also causes Meningitis. The symptoms of oropharyngeal infections are fever, toxemia, inflammatory lesions in the oral cavity and oropharynx, cervical lymph node enlargement, and edema, and there is a high case-fatality rate.

Clostridium botulinum

Clostridium botulinum is an anaerobic, rod-shaped sporeforming bacterium that produces a protein with characteristic neurotoxicity. Under certain conditions, these organisms may grow in foods producing toxin(s). Botulism, a severe form of food poisoning results when the toxin-containing foods are ingested. Although this food illness is rare, its mortality rate is high; the 962 recorded botulism outbreaks in the United States from 1899 to 1990 involved 2320 cases and 1036 deaths. In outbreaks in which the toxin type was determined, 384 were caused by type A, 106 by type B, 105 by type E, and 3 by type F. In two outbreaks, the foods implicated contained both types A and B toxins. Due to a limited number of reports, type C and D toxins have been questioned as the causative agent of human botulism. It is suspected that these toxins are not readily absorbed in the human intestine. However, all types except F and G, which have not been as studied thoroughly, are important causes of animal botulism. *C. botulinum* is widely distributed in soils and in sediments of oceans and lakes. The finding of type E in aquatic environments by many investigators correlates with cases of type E botulism that were traced to contaminated fish or other seafoods. Types A and B are most commonly encountered in foods associated with soil contamination. In the United States, home-canned vegetables are most commonly contaminated with types A and B, but in Europe, meat products have also been important vehicles of foodborne illness caused by these types.

The mouse bioassay is a functional assay that detects biologically active toxin. The assay requires a three part approach: toxin screening, toxin titer, and finally toxin neutralization using monovalent antitoxins. The process requires two days of analysis at each step. Recently, rapid, alternative, in-vitro procedures have been developed for the detection of types A, B, E, and F botulinal toxin producing organisms and their toxins. The toxins generated in culture media can be detected using ELISA techniques such as the DIG-ELISA and the amp-ELISA. Biologically active and non-active toxins are detected since the assay detects the toxin antigen. The ELISA assays require one day of analysis. The toxin genes of viable organisms can be detected using the polymerase chain reaction technique and require one days of analysis after overnight incubation of botulinal spores or vegetative cells. In-vitro assays that are positive are confirmed using the mouse bioassay.





Confirmation of Clostridium botulinum toxin by PCR assay and mouse bioassay