CORYNEBACTERIA

The genus Corynebacterium consists of a diverse group of bacteria including animal and plant pathogens, as well as saprophytes. Some corynebacteria are part of the normal flora of humans, finding a suitable niche in virtually every anatomic site, especially the skin and nares. Corynebacteria are Gram-positive, aerobic, nonmotile, rod-shaped bacteria classified as Actinobacteria. Corynebacteria are related phylogenetically to mycobacteria and actinomycetes. “Diphtheroids” or “coryneform” bacteria are recognized as causing opportunistic disease under specific circumstances, such as in patients who are immunocompromised, have prosthetic devices, or have been in hospitals/nursing homes for long-term periods of time.

The best known and most widely studied species is Corynebacterium diphtheriae, the causal agent of the disease diphtheria.\(^1\) C. pseudodiphteriae has been associated mainly with respiratory disease and less commonly with endocarditis, prostheses or wound infections or colonizations. Most of the respiratory disease occurred in immunosuppressed hosts. Other organisms such as C. ulcerans, C. pseudotuberculosis and C. xerosis may also be able to cause infection of the nasopharynx and skin.

CORYNEBACTERIUM DIPHTHERIAE

The pathogenicity of Corynebacterium diphtheriae includes two distinct phenomena:
1. **Invasion** of the local tissues of the throat, which requires colonization and subsequent bacterial proliferation. Little is known about the adherence mechanisms of C. diphtheriae, but the bacteria produce several types of pili. The diphtheria toxin, as well, may be involved in colonization of the throat.

2. **Toxigenesis**: bacterial production of the toxin. The diphtheria toxin causes the death of eucaryotic cells and tissues by inhibition protein synthesis in the cells. Although the toxin is responsible for the lethal symptoms of the disease, the virulence of C. diphtheriae cannot be attributed to toxigenicity alone, since a distinct invasive phase apparently precedes toxigenesis. However, it has not been ruled out that the diphtheria toxin plays an essential role in the colonization process due to short-range effects at the colonization site.

**The role of B-phage.**

Only those strains of Corynebacterium diphtheriae that are lysogenized by a specific Beta phage produce diphtheria toxin. A phage lytic cycle is not necessary for toxin production or release. The phage contains the structural gene for the toxin molecule.

The zoonotic agents Corynebacterium pseudotuberculosis (the cause of caseous lymphadenitis primarily in sheep and goats), transmitted to humans by contact with diseased animals and C. ulcerans, historically thought to cause disease in humans after contact with...
contaminated milk or farm animals, but more recently linked to transmission between humans and their companion pets such as cats and dogs, are also able to produce diphtheria toxin and cause diphtheria-like disease in humans. It is therefore recommended that those in frequent contact with animals (veterinarians, animal care technologists, or farm workers) ensure that they maintain adequate vaccine coverage against diphtheria.

Coryneform bacteria are increasingly being recognized as causing opportunistic disease under specific circumstances, such as in patients who are immunocompromised, have prosthetic devices, or have been in hospitals/nursing homes for long-term periods of time. The most significant pathogen of this group remains *Corynebacterium diphtheriae*, the primary cause of diphtheria, a disease which has essentially disappeared from developed countries after implementation of universal vaccination that targets the primary virulence factor, the diphtheria toxin (DT).

**CORYNEBACTERIA - GRAM STAIN**

Corynebacteria are Gram-positive, catalase positive, non-spore-forming, non-motile, rod-shaped bacteria that are straight or slightly curved, with clubbed ends (from the Greek *koryne*, club).

![Corynebacterium diphtheriae - Gram stain.](image)

Corynebacteria are pleomorphic rods that occur in angular arrangements. They undergo snapping movements just after cell division, which brings them into characteristic forms resembling „Chinese-letters“ or „palisades“. This is due to the incomplete separation of the daughter cells after binary fission. Their size falls between 2-6 micrometers in length and 0.5 micrometers in diameter. Corynebacteria do not form spores or branch as do the actinomycetes.
**Corynebacterium pseudodiphteriae** are pleiomorphic, Gram-positive rods, irregularly shaped, they are arranged as single cells, in pairs, in V forms, in palisades, or in clusters with Chinese-letter appearance.

**CORYNEBACTERIA – ALBERT STAIN (METACHROMATIC GRANULES)**

Metachromatic granules are irregularly sized granules found in the protoplasm of numerous bacteria. It stains a different color from that of the dye used. Granules are usually present in Corynebacteria, representing stored phosphate regions. They are composed of complex polyphosphate, lipid, and nucleoprotein molecules (volutin) and serve as an intracellular phosphate reserve. Called also Babès-Ernst body or granule.

**Abert’s stain Procedure**

1. Prepare a smear on clean grease free slide.
2. Air dry and heat fix the smear.
3. Treat the smear with Albert’s stain and allow it to react for about 7 mins.
4. Drain of the excess stain do not water wash the slide with water.
5. Flood the smear with Albert’s iodine for 2 minutes.
6. Wash the slide with water, air dry and observe under oil immersion lens.

Corynebacterium diphtheriae appears green coloured rod shaped bacteria with bluish black metachromatic granules at the poles.

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*Fig. 2 Corynebacterium pseudodiphtheriae - Gram stain.*

*Fig. 3 Corynebacterium diphteriae - Albert stain.*
CORYNEBACTERIA – CULTIVATION ON BLOOD AGAR

The causative agent of diphtheria is an aerobe or a facultative aerobe. The optimal temperature for growth is 37° C and the organism does not grow at temperatures below 15°C and above 40°C. The pH of medium is 7.2-7.6. Corynebacterium diphtheriae usually grows on media with blood with a weak beta-hemolysis (C. diphtheriae biotype mitis and gravis) or is nonhemolytic (biotype intermedius).

![Corynebacterium diphtheriae - Blood agar culture.](image)

Three strains of Corynebacterium diphtheriae are recognized, gravis, intermedius and mitis. All strains produce the identical toxin and are capable of colonizing the throat. The differences in virulence between the three strains can be explained by their differing abilities to produce the toxin in rate and quantity, and by their differing growth rates. The gravis strain has a generation time (in vitro) of 60 minutes; the intermedius strain has a generation time of about 100 minutes; and the mitis stain has a generation time of about 180 minutes. The faster growing strains typically produce a larger colony on most growth media. In the throat (in vivo), a faster growth rate may allow the organism to deplete the local iron supply more rapidly in the invaded tissues, thereby allowing earlier or greater production of the diphtheria toxin. Also, if the kinetics of toxin production follow the kinetics of bacterial growth, the faster growing variety would achieve an effective level of toxin before the slow growing varieties.

CORYNEBACTERIA – CULTIVATION ON TELLURITE AGAR AND TINSDALE AGAR

Tellurite Blood Agar – in which 0.4% tellurite inhibits other bacteria, diphtheria bacilli reduce tellurite to metallic tellurium which is incorporated in the colonies giving them a grey or black colour (Fig. 73).

Tinsdale agar (TIN) is used for the primary isolation and identification of Corynebacterium diphtheriae. The medium differentiates between C. diphtheriae and diphtheroids found in the upper respiratory tract. It contains L-cysteine and sodium thiosulfate that are H₂S indicators.
Potassium tellurite is the selective agent (inhibits most of the upper respiratory tract normal flora) that turns the media brown-black as a result from the reduction of potassium tellurite to metallic tellurite. This differentiation is based on the ability of *C. diphtheriae* to produce black (or brown) colonies, surrounded by a brown/black halo (Fig. 74). The dark halo is due to the production of $\text{H}_2\text{S}$ from cystine, interacting with the tellurite salt (cystinase activity).

**Fig. 5 C. diphteriae - Tellurite agar, strain gravis.**

**Fig. 6 C. diphteriae – Tinsdale agar. Small, brownish-black colonies surrounded by brown halo.**

**ELEK TEST**

The **Elek culture plate precipitin test** is routinely used for the detection of exotoxin from toxigenic strains of *Corynebacterium diphtheriae*. The test for toxigenicity, which detects the potent exotoxin, a phage-encoded protein, is the most important test and should be done without delay on any suspect isolate that is found by routine screening or while investigating a possible case of diphtheria. The toxigenic species *C. diphtheriae* acquire this characteristic when infected by the family of $\beta$-phages or other families of corynephages. The Elek test was
first described in 1949 and replaced the in vivo virulence test in guinea pigs, a test that was used by many countries at that time.

The Elek test principle

A filter paper strip impregnated with diphtheria antitoxin is buried just beneath the surface of a special agar plate before the agar hardens. Strains to be tested, known positive and negative toxigenic strains are streaked on the agar's surface in a line across the plate, and at a right angle to the antitoxin paper strip. After 24 hours of incubation at 37° C, plates are for the presence of fine precipitin lines at a 45-degree angle to the streaks (Fig. 75). The presence of precipitin lines indicated that the strain produced toxin that react with the antitoxin.

Diphteria toxin – mode of action

Diphtheria toxin (DT) is an extracellular protein of Corynebacterium diphtheriae that inhibits protein synthesis and kills susceptible cells. Diphtheria toxin is a single polypeptide chain consisting of two subunits linked by disulfide bridges, known as an A-B toxin. Binding to the cell surface of the B subunit (the less stable of the two subunits) allows the A subunit (the more stable part of the protein) to penetrate the host cell. The diphtheria toxin catalyzes the transfer of NAD⁺ to a diphthamide residue in eukaryotic elongation factor-2 (eEF2), inactivating this protein. It does so by ADP-ribosylating the unusual amino acid diphthamide. In this way, it acts as a RNA translational inhibitor. The acceptor is diphthamide, a unique modification of a histidine residue in the elongation factor found in archaeabacteria and all eukaryotes, but not in eubacteria.

The catalysed reaction is as follows:

\[ \text{NAD}^+ + \text{peptide diphthamide} \rightleftharpoons \text{nicotinamide} + \text{peptide N-(ADP-D-ribosyl)diphthamide} \]
Diphtheria toxin has also been associated with the development of myocarditis. Myocarditis secondary to diphtheria toxin is considered one of the biggest risks to non-immunized children.

Diphtheria toxin is extraordinarily potent. The lethal dose for humans is about 0.1 μg of toxin per kilogram of bodyweight. A massive release of toxin into the body will likely cause lethal necrosis of the heart and liver.

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**Fig. 8** Diphtheria toxin - mode of action.

Diphtheria toxin is extraordinarily potent. The lethal dose for humans is about 0.1 μg of toxin per kilogram of bodyweight. A massive release of toxin into the body will likely cause lethal necrosis of the heart and liver. The toxin is labile, prolonged storage, incubation at 37° C for 4-6 weeks, treatment with 0.2-0.4% formalin, acid pH converts it to toxoid.

**Schick skin test**

In 1913, Schick designed a skin test as a means of determining susceptibility or immunity to diphtheria in humans. Diphtheria toxin will cause an inflammatory reaction when very small amounts are injected intracutaneously. The Schick Test involves injecting a very small dose of the toxin under the skin of the forearm and evaluating the injection site after 48 hours. A positive test (inflammatory reaction) indicates susceptibility (nonimmunity). A negative test (no reaction) indicates immunity (antibody neutralizes toxin).


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BORDETELLA

The *Bordetellae* are small, Gram-negative, aerobic coccobacilli. The genus *Bordetella* contains the species *B. pertussis* and *B. parapertussis*, which cause pertussis in humans. Other members of the genus are *B. bronchiseptica*, causing respiratory disease in various animals and occasionally in humans, and *B. avium* as well as *B. hinzii*, which cause respiratory disease in poultry and are very rarely found in humans. Closely related organism, *B. parapertussis* can cause a milder form of bronchitis. *B. parapertussis* causes similar, but often milder type of cough with posttussive vomiting. *B. bronchiseptica* is the causative agent of respiratory diseases in cats and swine, but can cause broncho-pulmonary symptoms in severely immunosuppressed individuals.

BORDETELLA PERTUSIS

*Bordetella pertussis* is the only organism of major clinical significance within this genus; it causes whooping cough in infants and young children. There does not appear to be a zoonotic reservoir for *B. pertussis* - **humans are its only host.** *B. pertussis* is an extremely small, strictly aerobic, Gram-negative, non-motile coccobacillus (short rod). Compared to other *Bordetella* species, *B. pertussis* does not grow on common laboratory media and can be distinguished from *B. parapertussis* in that *B. pertussis* is oxidase positive but urease negative, while *B. parapertussis* is oxidase negative and urease positive. *B. bronchiseptica* is positive for both enzymes.

**Whooping cough (pertussis)**

Whooping cough (pertussis) is a highly contagious respiratory tract infection. In many people, it's marked by a severe hacking cough followed by a high-pitched intake of breath that sounds like "whoop."

Before the vaccine was developed, whooping cough was considered a childhood disease. Now whooping cough primarily affects children too young to have completed the full course of vaccinations and teenagers and adults whose immunity has faded.

After an incubation period of 1 to 2 weeks, whooping cough begins with the catarrhal phase. The subsequent paroxysmal phase, lasting 2 to 4 weeks, is characterized by severe and spasmodic cough episodes.

At the end of the catarrhal phase, a leukocytosis with an absolute and relative lymphocytosis frequently begins, reaching its peak at the height of the paroxysmal stage. At this time, the total blood leukocyte levels may resemble those of leukemia (≥ 100,000/mm³), with 60 to 80 percent being lymphocytes.

The convalescent phase, lasting 1 to 3 weeks, is characterized by a continuous decline of the cough.

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2 *B. -* Bordetella
**B. pertussis - toxins**

**Pertussis toxin**
Pertussis toxin is an oligopeptide AB-type exotoxin that is the major cause of pertussis. Following binding of the B component to host cells, the A subunit is inserted through the membrane and released into the cytoplasm in a mechanism of direct entry. The A subunit gains enzymatic activity, the membrane-bound regulatory protein G$i$ that normally inhibits the eukaryotic adenylate cyclase. The G$i$ protein is inactivated and cannot perform its normal function to inhibit adenylate cyclase (G$i$ protein is a G protein subunit that inhibits the production of cAMP from ATP). The conversion of ATP to cyclic AMP cannot be stopped and intracellular levels of cAMP increase. This results leads to increased mucus secretion and interferes with many cellular functions (disruption of cellular functions, decrease phagocytic activities such as chemotaxis, engulfment, the oxidative burst, and bactericidal killing). Pertussis toxin causes T cell lymphocytosis, hypoglycemia, increased IgE\(^3\) synthesis, and increased histamine and endotoxin sensitivity.

**Adenylate cyclase toxin**
This exotoxin penetrates the host cells, is activated by calmodulin and catalyzes the conversion of ATP to cAMP. Like pertussigen, it also inhibits phagocyte and NK cell functions. However, in contrast with pertussigen, the cAMP increase caused by this toxin is short-lived.

**Tracheal cytotoxin**
This is a peptidoglycan-like molecule (monomer) which binds to ciliated epithelial cells, thus interfering with ciliary movement. In higher concentrations, it causes ciliated epithelial cell extrusion and destruction. The destruction of these cells contributes to pertussis.

**Dermonecrotic (heat-labile) toxin**
Dermonecrotic toxin is a very strong vaso-constrictor and causes ischemia and extravasation of leukocytes and, in association with tracheal cytotoxin, causes necrosis of the tracheal tissue.

**Filamentous haemagglutinins (agglutinogens)**
These are not exotoxins but are filament-associated lipo-oligo-saccharides which are implicated in the binding of the organism to ciliated epithelial cells. Antibodies against these molecules are protective, probably by preventing bacterial attachment.

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**PERTUSSIS - SAMPLING**

Laboratory diagnosis of pertussis is highly important. Culture has remained the gold standard of diagnosis. The specimens should be taken from the posterior nasopharynx by intranasal aspiration or by a swab (Fig. 77). Calcium alginate swabs are better than dacron, rayon or cotton wool swabs. The specimens should be plated immediately onto selective media, and

\(^3\) IgE – Immunoglobulin E
the charcoal supplemented with 10% horse blood and 40 mg/l cephalaxin is currently the medium of choice.

![Fig. 9 B. pertussis - sampling.](image1)

The cough plate is the best means of early diagnosis. The exposed plate is held 4-5 inches from the patient's mouth during several expulsive coughs (Fig. 76).

**BORDETELLA PERTUSSIS – GRAM STAIN**

*B. pertussis* is an extremely small, strictly aerobic, Gram-negative, non-motile cocobacillus, that is encapsulated and does not produce spores. It is arranged singly, in pairs or in small groups (Fig. 78) and is not easily distinguished from *Haemophilus* species. *B. pertussis* and *B. parapertussis* are nonmotile.

![Fig. 10 B. pertussis - Gram stain.](image2)

**BORDETELLA PERTUSSIS – CULTIVATION**

*(BORDET GENGOU BLOOD AGAR, CHARCOAL HORSE BLOOD AGAR)*

*Bordetella pertussis* is a slow-growing organism that requires specialized conditions for growth. It is the most fastidious species within the genus. The culture positivity is often highest during the first 2 weeks of illness, and cultures are seldom positive if cough has lasted more than 4 weeks.

*Bordet Gengou Agar* is a solid medium recommended for use in qualitative procedures for
the isolation of *Bordetella pertussis* and *Bordetella parapertussis*. Also used for the “cough plate” method in case of whooping cough. Bordet Gengou Blood Agar contains potato infusion and glycerol to supply the nutrients necessary to support the growth of *B. pertussis*. Defibrinated animal blood supplies additional nutrients and enables the detection of hemolytic reactions, which aid in the identification of *B. pertussis*.

![Fig. 11 Bordetella pertussis - Bordet Gengou Blood Agar.](image)

*Bordetella pertussis* produces small, domed, glistening colonies that resemble bisected pearls (Fig. 79). The colonies are usually surrounded by a zone of hemolysis; however, some strains of *B. pertussis* are not hemolytic. *B. parapertussis* grows faster than *B. pertussis* and is oxidase negative.

**Charcoal horse blood agar** with cefalexin, beef extract, peptone, and nicotinic acid provide essential nutrients for the growth of *Bordetella* spp. *Bordetella pertussis* in particular, requires the addition of charcoal and horse blood (10%) to neutralize the growth-inhibiting effects. Plates are incubated in air without elevated carbon dioxide at 35°C for a minimum of 7 days before being reported as negative (most isolates are detected in 3 to 4 days).

![Fig. 12 Bordetella pertussis on Charcoal horse blood agar with cefalexin.](image)

Colonies are small, shiny and round (Fig. 80). With age they become whitish grey. Repeated subculture of *B. pertussis* leads to loss of fastidiousness and laboratory adaptation to a variety of media.
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