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Introduction

Diphtheria is caused by *Corynebacterium diphtheria* (*C. diphtheria*), a non-sporulating, non-encapsulated, non-motile, pleomorphic gram-positive bacillus. Diphtheria is an epidemic disease that remains as a threat to health in the developing world **(Dass *and* Deepika, 2008).**

Isolation of *C. diphtheriae* by bacteriological culture is essential for confirming diphtheria After *C. diphtheriae* has been isolated, the biotype (substrain) should be determined. The four biotypes are intermedius, belfanti, mitis, and gravis **(Roush *et al.,* 2008)**. The most severe disease is associated with the gravis biotype, but any strain may produce toxin **(Atkinson *et al.,* 2007).**

The incidence of vaccine-preventable infections in the context of systemic diseases is not quantified **(Hanslik *et al.,* 2009),** so that vaccination programs have made a major contribution to the elimination of many vaccine-preventable diseases and significantly reduced the incidence of others **(Sandra *et al.,* 2007).**

In addition to the bacterial exotoxin, cell-wall components such as the O- and K-antigens are important in the pathogenesis of the disease. The heat-stable O-antigen is common to all corynebacteria, whereas the variable, heat-labile K-antigen permits differentiation between individual strains. Also, while the K-antigen is important for mucosal attachment, invasiveness is facilitated by the cord factor, a toxic glycolipid **(WHO, 2006)**.

In countries where diphtheria is still endemic, preschool and school-age children are most commonly affected **(WHO, 2006).** Homelessness is associated with numerous behavioral, social, and environmental risks that expose persons to many communicable infections, which may spread among the homeless and lead to outbreaks that can become serious public health concerns. The prevalence of these transmissible diseases among the homeless varies greatly according to living conditions (**Badiaga *et al.,* 2008).**

Routine childhood immunization with diphtheria toxoid is the key to controlling diphtheria while the role of routine adult reimmunization is less established; mass immunization will remain an important control measure for widespread diphtheria outbreaks **(Vitek, 2006).**

The designation of the Klebs-Loeffler bacillus as the cause of diphtheria in the early 1890s and the subsequent development of the antitoxin treatment in the years immediately following were at the time and continue to be viewed as triumphs of scientific medicine **(Condran, 2008).**

The diphtheria, tetanus, and pertussis vaccines confer protection by inducing neutralizing antibodies to the conserved bacterial toxins that are the major virulence factors **(Telford, 2008).**

Immunity against diphtheria is obtained by the induction of a neutralizing Th2- dominant (mainly immunoglobulin G1 [IgG1]) humoral immune response against DTx. The conventional vaccine consists of the alum-adsorbed, formaldehyde-treated toxin **(Miyaji *et al,* 2001).**

The pathophysiology of the sex-difference in local reactions (induration, tenderness, erythema, pruritus) following vaccination is clearly multifactorial with hypersensitivity reaction (type III, Arthus reaction-antigen/antibody immune complex formation), route of administration and hormonal factors being suggested **(Cook, 2009).**

Aim of the work:

Different immunological methods are to be used to evaluate the immunogenicity of diphtheria toxin (DT) purified by various techniques thus; it would be possible to delineate the best purification technique that yields a better immunogen to be used as a vaccine. Evaluation of the immunogenicity of the vaccine is by assessment of its ability to induce profound humoral and cellular immune responses.

Chapter 1

Biology of the organism and pathogenesis of infection

Gram-positive organisms are the most common bacterial pathogens that cause diseases in humans **(Metzger *et al.,* 2009).** *C. diphtheriae* is a slender gram positive bacillus, usually with one end being wider, thus giving the often-described club-shaped appearance **(Wharton and Vitek, 2004**), they have a thick cell wall that causes it to appear purple under the microscope **(Murray *et al.,* 1998)**. The organisms are resistant to environmental changes, such as freezing and drying. The term diphtheria is said to be derived from the Greek word for leather or "tanned skin" even though the disease was not so named until many years later **(English, 1985).**

History of the disease

Historical descriptions of diphtheria-like illness (throat membrane, neck swelling, frequent suffocation) appear in ancient literature. The earliest historical recording is that of Hippocrates in the 5th century BC. Also Egyptian writing from circa 1550 BC describes a throat condition associated with cyanosis and temporary paralysis usually affecting children **(Zink *et al.,* 2001)**.

In 1883, Klebs first described the characteristic organism in stained preparations of diphtheritic membranes, and Loffler reported the successful growth of these organisms in culture a year later **(Loffler, 1884).** He suggested that the damage at distant sites might be caused by a toxic substance produced by the pathogen and transported throughout the body **(Andrewes *et al.,* 1923; Holmes, 2000).**

In 1888, Roux and Yersin demonstrated that culture filtrates of the *diphtheria bacillus* contained a potent heat-labile toxic protein called DT, which caused death of experimental animals with a similar pattern of tissue damage to that which occurred when virulent organisms were injected including myocarditis, focal necrosis in organs, such as the adrenal glands, kidneys and liver and polyneuritis **(Michel *et al.,* 1972; Holmes, 2000; Wharton and Vitek, 2004; Hawgood, 2008).**

1n 1890, Von Behring and Kitasato demonstrated that susceptible animals could be immunized by injection with graded doses of *diphtheria bacilli*, and the serum from such immunized animals protected other susceptible animals from the toxic effects of DT **(Holmes, 2000**), Behring named this preparation antitoxin. For his discovery, he received the Nobel Prize in 1901 **(Wharton and Vitek, 2004)**

In 1913, Schick introduced a simple test which was based on the response to intradermal injection of a small dose of DT to distinguish between individuals who are immune or not. Susceptible individuals (Schick positive) develop prolonged erythrema and induration at the site of toxin injection, but immune individuals (Schick negative) do not **(Holmes, 2000).**

The Schick test was used both to determine the prevalence of immunity to diphtheria among persons of different ages and to assess the efficacy of different vaccine regimens in eliciting protective antitoxic immunity. During the last few decades, direct measurement of anti-DT antibodies in serum has replaced the Schick test as the basis for assessing immunity against diphtheria in individuals or population **(Holmes, 2000).**

In 1957, two groups reported that low concentrations of DT were lethal for many cell lines **(Lennox and Kaplan, 1957; Placido Sousa and Evans, 1957).** In 1959, Strauss and Hendee reported that the first effect of DT on Hella cells was cessation of protein synthesis at approximately 90 min **(Strauss and Hendee, 1959).**

Description of the disease

Diphtheria is a severe and sometimes fatal disease caused by toxin-producing strains of *C. diphtheriae* **(Danilova *et al.*, 2006; Bitragunta *et al.*, 2008).** Diphtheria is an acute communicable upper respiratory illness that was a major cause of childhood mortality in prevaccine era **(George, 2005; Vitek, 2006).** *C. diphtheriae* is well known as an agent of localized respiratory tract disease potentially complicated by systemic effects of exotoxin **(Zasada *et al.*, 2005).** The organisms do not actively invade deep tissue or the blood, but multiply locally, producing DT. These results in necrosis of the mucosal cells and production of a thick, grey pseudomembrane containing fibrin, epithelial cells, bacteria and neutrophils. Diffusion of toxin into the circulation causes toxic neurological and myocardial complications **(George, 2005; Oram and Holmes, 2006).** Symptoms and evidence of peripheral neuritis do not surface until 10 days to three months after the onset of the disease. The delayed onset of symptoms of neuritis may be a model for damage to the nerves in the ear causing hearing loss **(Schubert *et al.*, 2001)**.

The incubation period, in which bacteria are present, plus the period from the onset of the disease until admission is 2 to 4 days **(Danilova *et al.*, 2006).** Symptoms of diphtheria are initially nonspecific and mild; throughout the course of disease, fever does not usually exceed 38.5°C (101.3°F). Other early symptoms in children include diminished activity and some irritability **(Wharton and Vitek, 2004).** It is convenient to classify diphtheria into a number of manifestations, depending on the site of disease into**:** anterior nasal diphtheria, pharyngeal, tonsillar diphtheria, laryngeal diphtheria and cutaneous (skin) diphtheria **(Atkinson *et al.*, 2007).**

Death can result from suffocation caused by occlusion of the air passage that toxin induces tissue necrosis and an inflammatory reaction resulting in tough, adherent membranes. The membrane islets tend to spread outside the tonsils and to be confluent in most cases. The

membrane size usually corresponds to the spread of local edema **(Danilova *et al.,* 2006)**. The overall case-fatality rate for diphtheria is 5%–10%, with higher death rates (up to 20%) among persons younger than 5 and older than 40 years of age **(Atkinson *et al.,* 2007).**.

Epidemiology and mode of transmission

In the past, many diphtheria outbreaks have occurred all over the world and many people, especially infants, have died of the disease **(Nakajima *et al.,* 2008).** *C. diphtheriae* appears to have a phylogeographical structure mainly represented by area-specific variants whose circulation is under strong influence of human host factors, including health control measures, first of all, vaccination, and social economic conditions **(Mokrousov, 2009)**. Diphtheria was a major threat in the pre-vaccine era until widespread vaccination was implemented **(Danilova *et al.,* 2005).** Pharyngeal or cutaneous diphtheria caused by toxigenic *C. diphtheriae* and *C. ulcerans* remains a serious health problem in many regions of the world **(Sharma *et al.,* 2007).** Humans are the only natural host for *C. diphtheriae*, although the organism has also been isolated from the environment of persons infected with *C. diphtheriae* **(Wharton and Vitek, 2004**). Infections of the airway in children may present to the anesthetist as an emergency in several locations: the Emergency Department, the Operating Department or on Intensive Care. In all of these locations, relevant and up to date knowledge of presentations, diagnoses, potential complications and clinical management will help the anesthetist and the surgical team, not only with the performance of their interventions, but also in buying time before these are undertaken, avoiding complications and altering the eventual outcome for the child **(Jenkins and Saunders, 2009)**, transmission is person-to-person, primarily by intimate respiratory and physical contact. Laryngeal diphtheria may occur at any age but is particularly prone to occur in children younger than 4 years that are associated with greater morbidity and mortality as a result of airway obstruction and to more toxin absorption from the extensive membrane **(Wharton and Vitek, 2004**).

Cutaneous lesions appear to be important in transmission in warm climates **(Belsey *et al.,* 1969).** Also as a sexually transmitted disease, bacterial culture from swabs taken from the genital ulcer, grew organisms morphologically and biochemically characteristic of *C. diphtheriae* **(Vetrichevvel *et al.,* 2008)**. Under conditions of poor hygiene, it is supposed that the predominant route for bacterial contamination and penetration into the bloodstream are various skin lesions such as cutaneous ulcers, bullous pemphigoid, scabies and open fractures **(Gruner *et al.,* 1994; Zasada *et al.,* 2005).**

Diphtheria is still endemic in Eastern Europe and other regions of the world although it has virtually disappeared in developed countries following mass immunization in the 1940s **(Zasada *et al.,* 2005).** It may now be prudent to re-vaccinate the sector of the Egyptian community that has been found to have inadequate immunity against diphtheria **(Redwan and El-awady, 2005).**

Diagnosis

Diagnostic tests used to confirm infection include isolation of *C. diphtheriae* on culture and toxigenicity testing **(Roush *et al.,* 2008).** Diagnosis of diphtheria is based on clinical symptoms and signs and on the detection of *C. diphtheriae*. The complications and mortality of diphtheria are inversely related to the promptness of diagnosis and treatment. Administration of anti-diphtheria antitoxin during the early stage of the disease is often crucial to preventing complications and death **(Danilova *et al.,* 2006).** A throat swab for culture should be obtained under direct visualization, preferably from the edge or beneath the edge of the membrane. Swabs should be inoculated promptly onto tellurite containing media and onto blood agar **(Efstrtiou *et al.,* 2000; George, 2005).** Suspicious colonies are identified by a black or grey appearance on this medium **(George, 2005).**

Because not all *C. diphtheriae* recovered on culture are toxigenic, testing for toxin production must be carried out by the Elek immunoprecipitation test or by cutaneous testing in guinea pigs **(Engler *et al.,* 1997; Efstrtiou *et al.,* 2000).** The diagnosis is also supported by low levels of diphtheria antibodies in serum.

In addition, the DT gene can be detected by polymerase chain reaction (PCR) **(Pollen, 1991; Hauser *et al.,* 1993; Aravena-Romaán *et al.,* 1995; Mikailovich *et al.,* 1995)** which can be performed directly on clinical specimens **(Nakao and Popovic, 1997).** The Control Disease Center (CDC) diphtheria laboratory developed a PCR test for the A and B subunits of the DT gene in 1994 **(Mikailovich *et al.,* 1995; Nakao and Popovic, 1997)**. Clinical specimens submitted to the CDC laboratory included throat swabs or biopsies, pieces of membrane, and pure cultures. Specific PCR assay that targets the DT repressor protein DtxR gene is documented as a procedure for differentiating *C. diphtheriae* from corynebacterium –like colonies **(Pimenta *et al.,* 2008)**, also real-time PCR is a rapid tool to confirm the presence of the DT gene (tox) in an isolate or specimen **(Cassiday *et al.,* 2008).**

Virulence

The metal ion-activated DtxR is responsible for the regulation of virulence and other genes in *C. diphtheria* **(D'Aquino *et al.,* 2009)** which in high-iron conditions, toxin production is repressed due to the activity of the DtxR, a global iron-dependent regulator that controls the iron