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Toxigenic diphtheria: A review of the current knowledge

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Abstract

Toxigenic diphtheria is the most feared and vaccine-preventable disease of global importance. It is one of the major health problems in developing countries and causes high morbidity and mortality. Three distinct species (*Corynebacterium ulcerans, C. pseudotuberculosis,* and *C. diphtheriae*) of the renowned genus *Corynebacterium* are responsible for this pathological condition. The review deals with the current advance knowledge concerning the etiology, transmission, distribution, pathogenesis, and control of the three pathogens. These pathogenic bacteria produce highly potent exotoxins: the major virulence factors. *C. diphtheriae* causes respiratory and cutaneous diphtheria most commonly in small children. *C. pseudotuberculosis* and *C. ulcerans* are the etiological agents of caseous lymphadenitis and zoonotic diphtheria, respectively. These species share particular features like specific cell wall composition (peptidoglycan, arabinogalactan and mycolic acid) and high G+C contents. These microorganisms are of considerable importance regarding the medical, veterinary, and biotechnological point of view. The toxins produced by toxigenic strains are highly potent and disseminate quickly, once disseminated then they could be highly lethal. Thus diphtheria toxoid and diagnosis followed by prompt treatment is the only way to decrease the rate of morbidity as well as mortality.

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Introduction

Corynebacterium is a well-known genus that belongs to the supra-generic group of *Actinomycetes*, termed as CMNR that comprised the genera *Corynebacterium*, *Mycobacterium*, *Nocardia,* and *Rhodococcus*. These are gram-positive bacteria and have great medical, veterinary, and biological importance (Soares *et al.*, 2013). The three *Corynebacterium* species, namely, *Corynebacterium ulcerans*, *C. pseudotuberculosis,* and *C. diphtheriae* (Both *et al.*, 2014; Taylor *et al.*, 2010) are capable of producing highly potent exotoxins and responsible for different infectious diseases both in humans and animals (Torres Lde *et al.*, 2013). These species were originally categorized based on their morphology and biochemical characteristics (Riegel *et al.*, 1995). According to some investigators, these three species cannot be distinguished precisely from one another and they share some general characteristics such as fermentation of sugar, hydrolysis of cystine and production of diphtheria toxin (Maximescu *et al.*, 1974).

C. diphtheriae causes an acute and contagious infection known as diphtheria in humans (Engler *et al.*, 2002). Diphtheria can spread from person to person and the most severe presentation of diphtheria is a respiratory disease with a swollen 'bull neck' and strongly adherent pseudo-membrane, which obstructs the airways (Wagner *et al.*, 2010). While *C. ulcerans* and *C. pseudotuberculosis* are responsible for zoonotic "diphtheria like disease" and "lymphadenitis", respectively (Kaufmann *et al.*, 2002; Moussa *et al.*, 2016).

C. ulcerans is the agent of multiple infectious conditions of animals including mastitis in cattle (Dias *et al.*, 2011), and *C. pseudotuberculosis* can cause caseous lymphadenitis in sheep, goats, and buffaloes (Selim *et al.*, 2016). The diseases are characterized by high morbidity and mortality and cause severe economic losses due to expensive medication, reduction in the milk production and lowering of work activity of the affected animals (Selim, 2001b). The bacteria produce diphtheria toxin (DT) which is one of the major virulence factors. These pathogens acquire the toxin-producing ability after infection with the ß-corynebacteriophage encoding the diphtheria tox gene (Seto *et al.*, 2008).

This DT producing group is of major concern in many countries of the world. *C. diphtheriae* is very important from the medical point of view, while *C. ulcerans* and *C. pseudotuberculosis* have both medical and veterinary importance. This article was aimed to review fragmented published research and discuss the pathogens. To the best of our knowledge, there is no such review dealing with these three *Corynebacterium* species.

Hence, in this review, we discussed the current status concerning the etiology, transmission, distribution, pathogenesis, virulence factors, clinical features, treatment, and control of the diseases caused by these pathogens, with a core focus on diphtheria, which is the major health problem in developing countries.

Materials and methods

Search strategy

This review article was designed by collecting previously published research data. Google Scholar, Science Direct, and Pub Med databases were searched for published research articles. Different search indicators such as "*Corynebacterium* species", "diphtheria", "diphtheria toxin", "Phospholipase D", "diphtheria vaccines and drugs", "pathogenesis", "epidemiology of diphtheria", and "control measures of diphtheria" were used for searching articles.

Inclusion criteria

Research articles describing (i) current knowledge of the Diphtheria (ii) containing full information regarding disease description, causative agent, prevalence, transmission, pathogenesis, virulence factors, mode of action, and control of the disease (iii) original research articles (iv) and published in the English language were included. While articles with

uncertain results, inadequate data, and language other than English were excluded.

Table 1. General characteristics.

Pathogen	Importance	Transmission	Host(s)	Biotypes	Toxin(s)	Vaccine	Reference
C. diphtheriae	Causes respiratory/ cutaneous Diphtheria	Through respiratory droplets/ direct contact	Mostly humans, rarely animals	Four biotypes	Diphtheria Toxin (DT)	Available	(Hadfield *et al.*, 2000; Holmes, 2000; Sangal and Hoskisson, 2016; Singh *et al.*, 2013)
C. ulcerans	Classical diphtheria and mastitis	From domestic animals	Mostly animals, rarely humans	-	Diphtheria Toxin Phospholipase D (PLD)	Available	(Barret, 1986; Kaufmann *et al.*, 2002; Sekizuka *et al.*, 2012)
C. pseudotuberculosis	Causes CLA and OSD	From contact with infected animals	Mostly animals, rarely humans	Two biotypes	Diphtheria Toxin Phospholipase D (PLD)	Available	(Almeida *et al.*, 2017; Paton *et al.*, 2003; Soares *et al.*, 2013)

Table 2. Biochemical properties of *Corynebacterium* spp.

Corynebacterium spp.	Hemolysis	DNase	Pirazinamidase	Nitrate reduction	Glucose	Maltose	Catalase	CAMP reaction	References
C. diphtheriae									
Biotype *belfanti*	-	+	-	-	+	+	+	-	(de Mattos Guaraldi *et al.*, 2014; Dorella *et al.*, 2006)
Biotype *gravis*	-	+	-	+	+	+	+	-	
Biotype *intermedius*	-	+	-	+	+	+	+	-	
Biotype *mitis*	+/-	+	-	+	+	+	+	-	
C. ulcerans	+	+	-	+/-	+	+	+	Rev	(Torres Lde *et al.*, 2013)
C. pseudotuberculosis									(de Mattos Guaraldi *et al.*, 2014; Dorella *et al.*, 2006)
Biotype *ovis*	+	-	-	-	+	+	+	Rev	
Biotype *equi*	+	-	-	+	+	+	+	Rev	

Key Rev= Reverse CAMP test

Data presentation

Based on published literature illustrations and tables were formulated. Table 1 was designed to describe the general characteristics of the pathogens and Table 2 for summarizing the biochemical properties of *Corynebacterium* species. Similarly, Fig. 1 was designed to illustrate the chemical structure of diphtheria toxin and Fig. 2 for describing the cellular entry of the toxin. Inkscape (0.92) Draw Freely software (www.inkscape.org) was used for drawing the fiures.

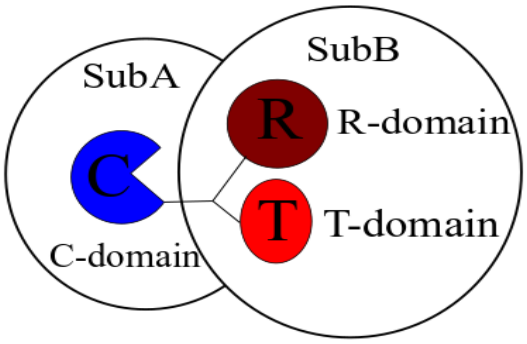

Fig. 1. Structure of diphtheria toxin (DT).

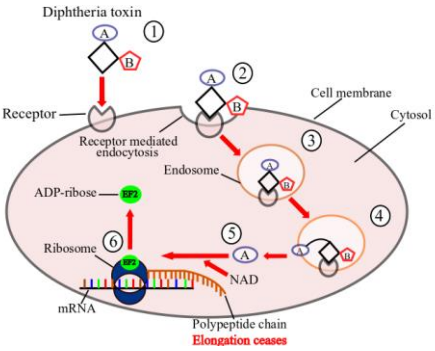

Fig. 2. Cellular entry of diphtheria toxin (1) Binding of DT to Receptor. (2) Receptor mediated endocytosis to enternalize DT. (3) Endosome formation. (4) Cleavage of Enzymatic A subunit. (5) T-domain passes Enzymatic A subunit through endosome membrane into the cytosol. (6) Ribosylation of EF2 by Enzymatic A subunit inhibits protein synthesis that results in cell death.

Results and discussion

Corynebacterium diphtheria (Diphtheria)

Description of the pathogen

C. diphtheriae is the causative agent of Diphtheria: an acute, extremely transmissible disease (Engler *et al.*, 2002) of the upper respiratory tract, and infrequently the skin (Both *et al.*, 2014), commonly affect young children (Singh *et al.*, 2013) with high morbidity and mortality (Table 1) (Guiso, 2015). Diphtheria was first described by Bretonneau in 1826, visualized by a bacteriologist Edwin Klebs in a stained specimen from pseudo-membrane in 1883, while Friedrich Loeffler isolated the bacteria in 1884 (Alcano, 1997; Holmes, 2000). *C. diphtheriae* is an aerobic, non-capsulated, non-spore forming, gram-positive bacillus (Maheriya *et al.*, 2014), which is transformed by a bacteriophage carrying the toxic gene (Kolybo *et al.*, 2013). The bacterium produces a diphtheria toxin (DT): a potent polypeptide exotoxin, that has the ability to act on various types of tissues particularly myocardium, nervous system, kidneys and suprarenal glands (Dias *et al.*, 2011). In the case of circulatory failure, death can occur (Meera and Rajarao, 2014), however, the severity of the disease depends upon the toxigenicity of the affecting strain and host immune response (Singh *et al.*, 2013).

C. diphtheriae has four biotypes included *mitis*, *intermedius*, *belfanti* and *gravis* (Sangal and Hoskisson, 2016). These all are different in colony morphology and growth characteristics. All of these have the toxin-producing ability except biotype *belfanti* (Efstratiou and George, 1999) but the most severe disease is often associated with the *gravis* biotype (Kolybo *et al.*, 2013).

Transmission

C. diphtheriae is a highly communicable microorganism. Transmission of *C. diphtheriae* occurs either through direct contact or sneezing, or a cough (Hadfield *et al.*, 2000), or droplet infection from the case or carrier (Singh *et al.*, 2013). Infrequently the disease may occur from skin injuries or particles soiled with discharge from wounds from infected persons (CDC, 2015). Although recently sexually transmitted cases also have been reported (Berger *et al.*, 2012).

Poor or no personal cleanliness and crowding have added to the spread of diphtheria (Mattos-Guaraldi *et al.*, 2003). Besides all these nutritional factors and low rates of immunizations also playing an important role in diphtheria transmission (Sharma *et al.*, 2013).

Prevalence

Previously diphtheria was widespread and occurred cyclically every ten-twelve years (Guiso, 2015). After the introduction of routine vaccination epidemiology of diphtheria changed in many countries (Divino-Goes *et al.*, 2007) and in the 1980s, the frequency of diphtheria decreased to an extent that the disease was near to elimination in many countries of the world (Sadoh and Sadoh, 2011). Regardless of high immunization against diphtheria, the disease is still prevalent in several areas including Africa, India, Bangladesh, Vietnam, the tropics, and some parts of South America, including Brazil (Mattos-Guaraldi *et al.*, 2003). Though diphtheria is still a serious problem in various areas of the world (Efstratiou *et al.*, 2000).

In the 1990s, a major epidemic of diphtheria emerged in Russian Federation (Mattos-Guaraldi *et al.*, 2003) and by 1993 and 1994 subsequently transmitted to all of the Newly Independent States (Mikhailovich *et al.*, 1995) and Baltic states (Mattos-Guaraldi *et al.*, 2003). About 64% to 82% of the cases was ≥ 15 years old. At the start of 1999, diphtheria had affected ≥ 157,000 individuals and caused 5000 deaths. People of 40-49 years of age were more susceptible and accounted for almost half of all expiries in some countries (Mattos-Guaraldi *et al.*, 2003). Between 1990 and 1995 about 794 cases of diphtheria were reported in Belarus out of which 25 were fatal. However, in 1996 the morbidity was stabilized as a consequence of mass immunization and in 2005 the disease declined to 0.11 per 100,000 population (Kolodkina *et al.*, 2006).

In the United Kingdom (UK) from 1986 to 1994 majority of the toxigenic strains isolated were introduced from the India subcontinent, Somalia, Pakistan, Africa, and the tropics. In Brazil, over-all 27,134 cases of diphtheria were described during 1980 and 1999, in which half of the infections were described in the Northeast areas and 26% (715) infections were in the Southeast area. Only 9% (2409) of affected individuals were described during the

1990s. Though diphtheria is taught to be decreasing in Brazil but still the disease is widespread in most states (Mattos-Guaraldi *et al.*, 2003).

In Turkey, the mortality due to diphtheria declined from 1.78/million infected individuals in 1970 to 0.26/million individuals in 1984. After the implantation of the Expanded Program of Immunization in 1985, a total of 427 cases were described between 1985 and 2004, with 124 cases occurring during 1993-1996 (Karakus *et al.*, 2007). The overall rate of diphtheria cases has been declining in Nigeria, the documented cases were 5,039 in 1989, 3,995 in 2000, 2,468 in 2001, 790 in 2002 and 312 in 2006 (Sadoh and Sadoh, 2011). The occurrence of diphtheria into religious societies of Netherlands, those who rejecting vaccination maximized the risk of spread of the microorganism because more than 60% of conventional reformed individuals had no defensive diphtheria antibody level (Mattos-Guaraldi *et al.*, 2003).

India showed a great decline in the incidence of diphtheria as the Expanded Program of Immunization and Universal Immunization Program was introduced in 1978 and 1985 respectively. In 1987, there were 12,952 cases while in 1999 only 2,725 cases of diphtheria were reported (Maheriya *et al.*, 2014).

In Pakistan DEWS had reported 45 cases of diphtheria from 1st January to September 30th, 2014, in which 30 (67%) were from Punjab, 11 (24%) Khyber Pakhtunkhwa (KP), 3 (7%) Sindh and 1 (2%) Azad Jammu and Kashmir (AJK). The National Institute of Health Islamabad (NIH) had received a total of 30 samples of humans during this period, among which 3 revealed positive (NIH, 2015). In 2018, 427 cases were being reported with a mortality rate of 3% in general population of Bannu division and internally displaced persons (IDPs) of KP (Ali *et al.*, 2018).

Pathogenesis

Virulence factors

C. diphtheriae is locally invasive (Meera and Rajarao, 2014) and various proteinaceous elements are involved to disseminate, colonize and, aid in the establishment of pathogenesis. The pilin protein form pili on microbial surfaces (Ton-That and Schneewind, 2003) and a soluble protein, the diphtheria toxin, are the renowned virulence factors that cause the infection (Meera and Rajarao, 2014).

Pili

Adherence to the host tissues with the help of pili has a prominent role in the progression of the disease by a number of bacterial pathogens (Mandlik *et al.*, 2007). An essential aspect of diphtheria pathogenesis that is needed to be further studied is the capability of *C. diphtheriae* to inhabit and effectively compete in the nasopharyngeal niche of the susceptible host (Broadway *et al.*, 2013). Several factors contribute to *C. diphtheriae* colonization on the epithelial tissues of the host including pili (Colombo *et al.*, 2001; Mandlik *et al.*, 2008b). In the late 1970s, electron microscope revealed the presence of pili on the *C. diphtheriae* cell surfaces (Yanagawa and Honda, 1976), though, in gram-positive bacteria, the molecular nature and how the pilus assembly take place were investigated only at the end of the century (Ton-That and Schneewind, 2003). The nucleotide sequence of *Corynebacteria* genome (Cerdeño-Tárraga *et al.*, 2003) elicited that these pathogens possess three distinct pilus gene clusters that composed of closely related genes (Ton-That and Schneewind, 2003), encoding for pilin subunits, sorting signals and five sortases viz Srt-A, Srt-B, Srt-C, Srt-D, and Srt-E. Another sortase termed as the housekeeping sortase Srt-F is located somewhere else on the chromosome (Ton-That and Schneewind, 2003). *C. diphtheriae* assembles 3 different kinds of heterotrimeric pili including SpaA, SpaD, and SpaH type (*S*ortase-mediated *P*ilus *A*ssembly (Spa)) (Broadway *et al.*, 2013; Mandlik *et al.*, 2007). The three pili own same structures (Broadway *et al.*, 2013) and comprised of three pilin subunits (Ton-That and Schneewind, 2003); one major pilin protein subunit (SpaA) while two minor pilin subunits (SpaB and SpaC) (Gaspar and Ton-That, 2006). For instance, in SpaA form of pilus, the main pilin subunit SpaA contributes in the formation of the pilus shaft, the minor SpaC protein is found at the tip and the SpaB is distributed in the shaft as well as the base of the pilus (Broadway *et al.*, 2013). This

SpaA type pilus of *C. diphtheriae* is encoded by the gene cluster (*spaA-srtA-spaB-spaC*) (Mandlik *et al.*, 2008a).

SpaA is crucial to forming the structure of the pilus, though SpaB and SpaC are dispensable (Ton-That *et al.*, 2004).

Adherence to host cells

Pili play a fundamental role in the adherence of the pathogen to the host and ultimately form colonies on susceptible tissues by several pathogens. Recent studies have revealed that the three types of pili possessed by the human pathogen *C. diphtheriae* (Mandlik *et al.*, 2007), the pilus type SpaA is mainly responsible to bind with the pharyngeal epithelial tissues of the host (Broadway *et al.*, 2013). The SpaA pili mediated binding to pharyngeal epithelial cells is accredited to the minor subunits SpaB and SpaC (Mandlik *et al.*, 2007), that not just contribute to the pilus formation but most interestingly, is associated to the cell wall of the bacteria in monomeric and heterodimeric forms as well (Chang *et al.*, 2011). The remaining two; SpaD and SpaH pili show specificity to adhere to the lungs and laryngeal epithelium (Mandlik *et al.*, 2007).

Diphtheria toxin

Diphtheria toxin (DT) is an AB-toxin (Shapira and Benhar, 2010), a protein molecule consisted of 535 amino acids (Jamal *et al.*, 2017) that have two functionally active subunits; subunit-A with a molecular weight 21 kD (Enzymatically Active) and subunit-B with a molecular weight 39 kD (Receptor Binding) (Labyntsev *et al.*, 2014; Ren *et al.*, 1999). The x-ray crystallography has shown that DT consists of three domains; the amino-terminal C-domain that ribosylate ADP with the EF2; the T-domain specified to be inserted into the cell envelopes at acidic pH, to form channels, and to translocate the C-domain via endosomal membranes into the cell cytoplasm; and the carboxyl-terminal R-domain that binds DT to the HB-EGF that acts as a receptor for diphtheria toxin on vulnerable cells (Holmes, 2000) (Fig. 1).

C-domain includes the fragment A (SubA-Subunit-A), T-domain and R-domain together constitute the fragment B (SubB-Subunit B) (Fig. 1) (Kolybo *et al.*, 2013). The Heparin-Binding Epidermal Growth Factor precursor (HB-EGF precursor) a protein and acts as a receptor for DT. Vulnerable cells have a large quantity of DT receptors on their cell membranes. Diphtheria Toxin is a protein consisted of 535 amino acids residues and is a pro-enzyme that must be processed to trigger its dormant NAD: EF2-ADPR-transferase action (Holmes, 2000).

DT mechanism of action

The intoxication of eukaryotic cells involves the attachment of the toxin to the cellular receptor HB-EGF. The fragment B of DT is responsible for interaction with a receptor on the cell surface and translocation of the fragment A across the membrane of endosome into the cell cytoplasm (Srivastava and Luqman, 2015). The two domains of the subunit B, namely, R- domain and T-domain have different roles in toxin activity. R-domain is responsible for initiating endocytosis of the toxin receptor complex as it binds with the receptor HB-EGF while T-domain facilitates C-domain translocation across the lipid bilayer. As soon as it is formed endosomal low pH/acidic condition induces conformational changes that result in diphtheria toxin T-domain interaction with the endosomal membrane (Liu and Li, 2009; Rodnin *et al.*, 2017). This permits T-domain to translocate C-domain into the cell cytosol. After C-domain translocation, it restores the ability to inactivate the eukaryotic Elongation Factor 2 (eEF2). Subunit-A possesses ADP-ribosyl transferase activity and specifically inactivates eEF2. Accumulation of a large number of inactivated eEF2 leads to the inhabitation of cellular protein biosynthesis and cell death (Fig. 2) (Kolybo *et al.,* 2013). Nicotinamide Adenine Dinucleotide (NAD) is essential to inhibit protein formation. DT acts enzymatically by relocating the Adenosine diphosphate ribose (ADPR) moiety from NAD to EF2, thus deactivating EF2 and constraining chain formation during the course of protein synthesis. The site at which ADP-ribosylation

is mediated by Subunit-A is a post-transitionally modified histidine residue, called Diphthamide.

Kinetic study of NAD: EF2-ADPR transferase reaction reveals a progressive mechanism that involves the binding of DT to NAD and then it interacts with EF2. DT is an extremely potent toxin with a minimal lethal dose of less than 0.1g/kg of body weight (Efstratiou *et al.*, 1998).

Role of Iron

C. diphtheriae is capable to get control over host conditions, partially by producing siderophores or other mechanisms to take up iron that enables the pathogen to express virulence factors like toxins and enzymes (de Oliveira Moreira *et al.*, 2003). Iron has an important role in the toxinogenesis of *C. diphtheriae*. A study has shown that the production of DT is greatest in iron-limiting environments while severely repressed under high-iron environments (Holmes, 2000).

Iron represses the expression of diphtheria toxin while transcription of the *tox* gene is controlled by the diphtheria toxin repressor protein, DtxR, in association with iron. DtxR is a global iron-dependent repressor in *C. diphtheriae* that controls the expression of at least 50 genes (Allen and Schmitt, 2009). Structurally, DtxR has one amino-terminal domain contains the DNA binding site and the second domain for dimerization and metal binding (Hantke, 2001). In the absence of divalent transition metal ions, apo-DtxR exists as an inactive monomer that is in weak equilibrium with a dimeric form (Tao *et al.*, 1995). Whereas Fe^{++} is the physiological activator of DtxR *in vivo*, Fe^{++}, Ni^{++}, Co^{++}, Cd^{++}, and Mn^{++} have been shown to activate the aporepressor *in vitro* (White *et al.*, 1998).

Clinical manifestations

Sometimes, it seems difficult to diagnose diphtheria clinically, as it is tangled with other diseases like Vincent's angina, glandular fever or severe streptococcal sore throat (Efstratiou *et al.*, 2000). Respiratory diphtheria is often confined to the upper respiratory system: nasal, pharyngeal, tonsillar, and laryngeal. Initial symptoms include a sore throat, low fever, and most importantly the formation of the membrane occurs at the site where bacteria form colonies (Kolybo *et al.*, 2013).

In children, the common site of infection is the upper respiratory mucosa whereas, in adults with the buccal mucosa, mucosal lesions, lower and upper lips, soft and hard plates and tongue are included (Hadfield *et al.*, 2000). Serosanguinous or seropurulent nasal discharge is the characteristic of nasal diphtheria, which is frequently related to obvious white patches on the mucosal membrane of the septum. Erosion of the external nares and upper lips also mediated by nasal discharge (Hadfield *et al.*, 2000).

Faucial diphtheria includes the posterior structures of the mouth and proximal pharynx. For clinical diphtheria, it is the most common site of infection. Usually, a pseudo-membrane formation occurs on the uvula, soft palate, either or both tonsillar pillars, oropharynx, and nasopharynx. Primarily, pseudo-membrane is whitish but later develops dirty gray in color. With the progression of disease patches of green and black necrosis may also appear. Edema and pseudo-membrane formation cause obstruction of the respiratory tree, which results in cyanosis or suffocation and eventually leads to death. Lymphadenitis occurs when the lymph nodes especially in the cervical area become enlarge and swell up and may seem blackish red and be hemorrhagic. Respiratory embracement, severe adenitis, and soft tissue edema result in a "bull neck" appearance. When the concentration of toxin increases, it is absorbed in the blood and readily distributed by the circulatory system, as a result, the toxic effects spread out beyond the local area. Diphtheria toxin can affect all the organs/tissues of the body and is not specific in their action, but myocardium (causing myocarditis) and peripheral nerves are commonly affected (causing neuritis). Myocarditis may lead to cardiac failure while neuritis to paralysis (Hadfield *et al.*, 2000).

Laboratory findings and diagnosis

The microbiological diagnosis of diphtheria is very crucial. The first step to clinically diagnose diphtheria is to take a swab from the throat or nasopharynx of the suspected case. The specimen must be transported to the test center instantly because of the quick inoculation of special culture media is essential (Efstratiou *et al.*, 2000). Screening tests for presumptive identification of a tellurite comprising medium are used. Clinical samples are preferably cultured onto the blood and selected tellurite media; Hoyle's tellurite is recommended. As the growth of normal oral flora is inhibited by tellurite medium, whereas, *C. diphtheriae* decreases the tellurite salt-producing characteristics of black colonies (Efstratiou *et al.*, 2000).

Recommended screening test, for the distinction of possibly toxinogenic species of *Corynebacteria*, is the existence of cystinase (either Tinsdale or Pizu media) and the nonexistence of pyarinamidase. Bio-typing, pathogenic strains are biochemically identified by the use of simple tests, commercial kits such as "API CORYNE" and "Rosco Diagnostica" tests are readily available. The use of bio-typing is limited in epidemiologic studies because discrimination is poor. The Elek immunoprecipitation test can also be used to diagnose diphtheria (Efstratiou *et al.*, 2000).

Polymerase Chain Reaction (PCR): Recently use of PCR for recognition of the diphtheria toxin has been introduced. Other methods for the detection of toxigenicity include enzyme immunoassay (EIA). It is a simple, quick, accurate and precise phenotypic technique for the uncovering of toxigenicity. The EIA technique practices equine polyclonal antitoxin as the capture of antibody and an alkaline phosphatase labeled monoclonal as the detecting antibody, precise to subunit A of the DT molecule. It takes ~ 3 hours to give results (Efstratiou *et al.*, 2000).

Medical management

Diphtheria antitoxin, which is primarily produced in horses, is the mainstay of medical management. The horse sera are used without waiting for laboratory confirmation to neutralize the unbound toxin in the blood and prevent the progression of the disease (Maheriya *et al.*, 2014).

Antimicrobials such as penicillin, erythromycin, clindamycin, and metronidazole are used for the treatment of disease as *Corynebacterium* is susceptible to these antibiotics.

Therapy should be last for 14 days. Control and prevention of diphtheria are primarily based on vaccination. During the pre-vaccine era, the disease was most predominant with yearly stated cases of 125,000 and 10,000 expiries each year due to diphtheria in the United States of America (Sadoh and Sadoh, 2011). Protection against diphtheria is possible either by active immunization or recovery from clinical/subclinical infection with a toxinogenic strain of *C. diphtheriae* (Holmes, 2000).

At present four combination vaccines are used to prevent Diphtheria, Tetanus, and Pertussis: DTaP, Tdap, DT, and Td. The two (DTaP & DT) are prescribed for children younger than 7 years of age, and two (Tdap & Td) are given to older children and adults. Diphtheria, pertussis, and tetanus (DPT) vaccine can prevent Diphtheria, but its protection does not last forever (Sharma *et al.*, 2013).

Corynebacterium ulcerans
(Diphtheria like-disease)
Description of the pathogen

C. ulcerans was primarily recovered from infected human throats by Gilbert and Stewart in 1926 (Gilbert and Stewart, 1926). *C. ulcerans* was assigned as a distinct species of the genus *Corynebacterium* in 1995 (Riegel *et al.*, 1995) and it is responsible for multiple infectious conditions of animals including mastitis in cattle (Dias *et al.*, 2011). Human infections are rare and cause zoonotic diphtheria affecting the throat and causes diphtheria like symptoms (Kaufmann *et al.*, 2002).

C. ulcerans is a commensal pathogen having the capability of producing diphtheria toxin (Sekizuka *et al.*, 2012) when lysogenized by phage virus (Maximescu *et al.*, 1974; Trost *et al.*, 2011). It is a gram-positive, non-motile, non-spore forming and facultative anaerobic pleomorphic bacterium. They

form grey-white colonies on 5% sheep blood agar (Riegel *et al.*, 1995).

Transmission

Primarily *C. ulcerans* is regarded as the pathogen of animals and can cause numerous complex conditions in animals.

However, the bacterium has the capacity to cause diphtheria of zoonotic nature in humans. *C. ulcerans* is contagious and able to transmit from animals to human by close contact or through untreated/unpasteurized milk and other derivatives (Barret, 1986; Bostock *et al.*, 1984; Henrisksen, 1955). *C. ulcerans* infections in numerous mammals including cattle, sheep, goats, and dogs, etc. have been described worldwide. Consequently, the risk of spread amongst small animals and humans has become a cause for concern. But it is worth noting that still there is no confirmation of person to person transmission of *C. ulcerans* (Dias *et al.*, 2011).

Prevalence

C. ulcerans has a low incidence rate of infection, hence little is known about the disease spectrum. The bacterium was described in 1926 since then sporadic cases of infection occurred throughout the world. In the United Kingdom, thirty-three cases of individuals infected with *C. ulcerans* were documented from 1986-1999 in which 24 out of 33 were isolated between 1993-1999 (Kaufmann *et al.*, 2002). There is an obvious increase in the rate and severity of *C. ulcerans* infections in the UK (De Zoysa *et al.*, 2005). In Denmark, only two cases of classical diphtheria were stated in 1956-1989 (Nielsen *et al.*, 1991). A lethal case of necrotizing sinusitis caused by *C. ulcerans* was described in a formerly healthy agriculturalist in Germany (Wellinghausen *et al.*, 2002) and similarly, diphtheria like disease and two cases of severe pneumonia complicated by diffuse pseudo-membrane on the bronchi were reported in Japan, recently (Hatanaka *et al.*, 2003; Yasuda *et al.*, 2018). *C. ulcerans* has also been isolated from a 71 years old woman in Switzerland in 2002 (Kaufmann *et al.*, 2002). In Brazil, a single lethal case of humans affected by *C. ulcerans* was demonstrated in 2008 and 2010 subsequently. *C. ulcerans* infections are also reported in North and South America, but there is no evidence of reporting classical diphtheria in Asia, the Middle East, Africa and Oceania till now (Dias *et al.*, 2011).

Virulence factors

Two distinct types and highly potent exotoxins are produced by *C. ulcerans* (De Carpentier *et al.*, 1992); a diphtheria toxin (DT) that is identical to *C. diphtheriae* toxin in ADP-ribosylating activity and their synthesis is regulated by iron and Phospholipase D (PLD) toxin which is shared with the *C. pseudotuberculosis* (Table 1) (Wong and Groman, 1984). *C. ulcerans* harbors the lysogenic ß-corynebacteriophage virus that encodes for the diphtheria *tox* gene and enables the bacterium to produce the DT exotoxin (Selim *et al.*, 2016). DT produced by *C. ulcerans* is a 62 KiloDalton (kDa) highly toxic protein (Wong and Groman, 1984) that constrains biosynthesis of protein by inactivation of elongation-factor 2, while PLD is a dermonecrotic toxin (von Hunolstein *et al.*, 2003) biochemically affects phospholipase D, sphingomylinase, and cellular permeability (De Carpentier *et al.*, 1992).

Clinical manifestations

C. ulcerans exotoxins may lead to several clinical manifestations depend upon the presence and production of the toxin produced. The disease spectrum caused by *C. ulcerans* is not yet fully understood probably because of the low incidence of infection (Wellinghausen *et al.*, 2002). The toxigenic strains are reported to cause severe respiratory diphtheria like illness and skin lesions in humans (Tiwari *et al.*, 2008). Infrequent manifestations of *C. ulcerans* infection comprise pharyngitis, necrosis, mucosal ulceration, necrotizing, granulomatous inflammation and pneumonia (Wellinghausen *et al.*, 2002).

Laboratory findings and diagnosis

Clinical features, laboratory findings, treatment and preventive measures of zoonotic diphtheria are similar to that of respiratory diphtheria affected by *C. diphtheriae*. Culturing of *C. ulcerans* on growth media such as Tinsdal or Tellurite agar is the gold standard for diagnosis of zoonotic diphtheria. Moreover, the modified Elek test is used to investigate toxin production, PCR based genotypic tests are used for the recognition of *tox* gene and recently a multiplex PCR has been

recommended for the diagnosis of *C. ulcerans* (de Mattos Guaraldi *et al.*, 2014).

Medical management

Antibiotics against diphtheria are used to eradicate the pathogens and minimize the production of the exotoxin by them as well as to decrease the risk of spread of these microbes.

The antimicrobial treatment recommended for *C. ulcerans* is penicillin G, erythromycin (de Mattos Guaraldi *et al.*, 2014), amoxicillin, benzylpenicillin, ceftriaxone, ciprofloxacin, vancomycin, linezolid, and tetracycline, and also susceptible to clindamycin (Meinel *et al.*, 2015). In addition administration of diphtheria antitoxin is also recommended, diphtheria antitoxin is the mainstay for treatment that neutralizes the unbound toxin in the blood and prevents progression of the disease (Maheriya *et al.*, 2014). Diphtheria toxoid vaccine is used to minimize infections triggered by *C. ulcerans*, though, the efficacy of the diphtheria toxoid vaccine against the classical diphtheria is still unclear. The toxoid vaccine has a defensive effect against *C. ulcerans* mediated diphtheria and this vaccination simply inhibits the effect of DT and does not obstruct colonization caused due to toxigenic *Corynebacteria* (de Mattos Guaraldi *et al.*, 2014).

Corynebacterium pseudotuberculosis
(Caseous lymphadenitis)

Description of the pathogen

C. pseudotuberculosis was first observed by Nocard in 1888 while Priesz thoroughly investigated this microorganism in 1894 (Dorella *et al.*, 2006). *C. pseudotuberculosis* is an anaerobic intracellular, gram-positive, facultative pleomorphic bacterium that belongs to the genus *Corynebacterium* (Soares *et al.*, 2013). This is a non-motile, non-sporulating as well as non-capsulated bacterium (Dorella *et al.*, 2006). This is very essential livestock pathogen and the causal agent of infectious and chronic ailment commonly known as Caseous lymphadenitis (CLA) in sheep, goats and other small ruminants (Soares *et al.*, 2013) while acute oedematous skin disease in buffaloes (OSD) (Moussa *et al.*, 2016). *C. pseudotuberculosis* is also responsible for a variety of diseases including hepatitis, pneumonia, mastitis, arthritis as well as subcutaneous abscesses in a broad spectrum of hosts (de Mattos Guaraldi *et al.*, 2014). Human infections caused by this facultative pathogen are a rare and manifest abscess in the liver and internal lymph node (Moussa *et al.*, 2016). Human lymphadenitis caused by *C. pseudotuberculosis* was reported in 1966 for the first time (Peel *et al.*, 1997).

C. pseudotuberculosis has been classified into two biovars based on morphological and biochemical features (Table 2): on the ability to convert nitrate into nitrite; one is nitrate negative called the biovar *ovis* (recovered from small ruminant) the causative agent of Caseous lymphadenitis and another is nitrate positive the biovar *equi* (recovered from horse and bovines) the agent of ulcerative lymphadenitis (Almeida *et al.*, 2017). The bacterium is able to produce two kinds of exotoxins; the major virulence factors, one is the exotoxin phospholipase D (PLD) and the other is diphtheria toxin (DT) (Selim *et al.*, 2016).

Transmission

C. pseudotuberculosis has the potential to persist for a long period of time in the environment possibly contributes to its capability to spread within a herd or flock (Augustine and Renshaw, 1986; Yeruham *et al.*, 2004). The pathogen is mainly transmitted among sheep/goats through superficial wound contamination (resulting from ear tagging, castration as well as shearing) or through other animal's body injuries resulting from traumatic events. Occasionally, infected sheep cough the pathogen on skin wounds of other sheep, establishing alternative means of transmission (Paton *et al.*, 1995; Williamson, 2001). In cattle and buffaloes, there is confirmation of the mechanical spread of this pathogen by houseflies and other Diptera, however, the usual mechanisms of infection with *C. pseudotuberculosis* are not well known (Selim, 2001a; Yeruham *et al.*, 1996; Yeruham *et al.*, 2004). To humans, the disease can be spread through the ingestion of raw milk from infected animals (Cetinkaya *et al.*, 2002).

Prevalence

C. pseudotuberculosis is a sporadic zoonosis (Bonmarin *et al.*, 2009), and less than 30 infections

have been described in the literature since 1966 (Lopez *et al.*, 1966). The reported infections are mostly associated with domestic animals. In the literature one eosinophilic pneumonia (Keslin *et al.*, 1979) and one eye infection (Liu *et al.*, 2005), all other human infections due to *C. pseudotuberculosis* have had lymphadenitis (Join-Lambert *et al.*, 2006; Peel *et al.*, 1997).

The 22 reported cases in Australia did not produce diphtheria toxin (Peel *et al.*, 1997), and the data isn't accessible in the other detailed cases.

Pathogenesis

The mechanism of caseous lymphadenitis caused by *C. pseudotuberculosis* is poorly understood, though, the importance of Phospholipase D is known. Normally, the infection initiates due to a break in the skin, bacteria reproduce at the infection site, the inflammatory reaction is initiated, produces a primary abscess, and the infection propagates through the lymph to infect lymph nodes, which also become abscessed (Morris *et al.*, 1997). *C. pseudotuberculosis* PLD mostly occurs in the discharged fraction; and degrading sphingomyelin and lysophosphatidylcholine. PLD in its purified form is dermonecrotic and deadly when injected into animals (Massenburg *et al.*, 1994). Though, in culture, PLD secreted by *C. pseudotuberculosis* can't cause hemolysis. It appears to be plausible that *C. pseudotuberculosis* PLD action induces host enzymes that are responsible for cytolysis, though how precisely the exotoxin applies its toxic impacts are unknown (Morris *et al.*, 1997).

Virulence factors

C. pseudotuberculosis has peculiar characteristics of producing two types of exotoxins: these virulence factors have a crucial role in the attachment, colonization, spread inside the host and escape from the host system allow this facultative pathogen to penetrate and survive inside the host.

Phospholipase D is the key virulence factor expressed by *C. pseudotuberculosis* (Soares *et al.*, 2013). PLD has the capacity to hydrolyze and degrade the ester linkages in the sphingomyelin (Morris *et al.*, 1997) in the mammalian endothelial plasma membranes thus inducing the vascular permeability and allow the pathogen to spread from the early site to the secondary sites of infection (de Mattos Guaraldi *et al.*, 2014; Dorella *et al.*, 2006; Soares *et al.*, 2013). Several environmental factors such as cell density and heat shock regulate the expression of PLD (McKean *et al.*, 2007).

C. pseudotuberculosis is one of the members of the diphtheria group and can produce diphtheria toxin (DT) (Kraeva *et al.*, 2007). DT toxin is a highly toxic protein comprised of one polypeptide chain (62 Kilodalton MW). It comprises of A and B fragments, which are essential to intoxicate cells in the culture or animals. Enzymatic activity is accomplished by fragment A; active site of the toxin while fragment B facilitates the attachment of toxin with the host cell receptors (Seto *et al.*, 2008).

Clinical manifestations

CLA causes major financial losses especially in sheep and goats, the disease causes reduced wool and milk production, reproductive ailments, early culling, carcass condemnation and infrequently death (Cetinkaya *et al.*, 2002). CLA clinically manifests three dissimilar disease forms; (a) external abscesses in the superficial lymph nodes and in the subcutaneous tissues, (b) ulcerative lymphangitis of the limbs and (c) visceral CLA characterized by abscesses in the internal structures such as the kidney, lungs, spleen, and liver (Soares *et al.*, 2013).

It also causes a disease in buffalo termed as an oedematous skin disease in which redness and swelling occur at the infection site. The swelling may spread to drainage of lymph nodes and may involve the entire hind and forelimbs (Selim *et al.*, 2016).

Laboratory findings and diagnosis

CLA is mainly diagnosed by the clinical symptoms of the disease present and isolation of the pathogen from the site of infection. Cultured microorganisms are identified as a *C. pseudotuberculosis* by various biochemical tests, however, it is difficult because of

general variability in the features of the bacterium (Cetinkaya *et al.*, 2002).

A number of serological tests such as ELISA, complement fixation tests, and the hemolysis inhibition tests have been developed for the clinical documentation of CLA (Cetinkaya *et al.*, 2002), however, most have been testified to lack either sensitivity or specificity.

ELISA test for the detection of gamma interferon has been recently developed and seems to be highly sensitive than usual antibody ELISA. PCR tests can also be employed to identify *C. pseudotuberculosis* bacteria isolated from the site of infections (Dorella *et al.*, 2006).

Medical management

To control caseous lymphadenitis with antibiotics is not an easy job because the pathogens are capsulated and thus remain protected inside the abscesses. *C. pseudotuberculosis* is susceptible to a variety of antibiotics including ampicillin, chloramphenicol, lincomycin, gentamycin, tetracycline, penicillin G, and sulfamethoxazole-trimethoprim. While some of the strains show resistance to the antibiotics streptomycin (Paton *et al.*, 2003).

Commercially available vaccines for caseous lymphadenitis are usually used in combination with other vaccines for pathogens like *Clostridium tetani, Clostridium perfringens, and Clostridium septicum,* etc.(Paton *et al.*, 2003), (Piontkowski and Shivvers, 1998), (Stanford *et al.*, 1998), (Williamson, 2001). One vaccine is a toxoid vaccine; this is the formalin-inactivated vaccine derived from the Phospholipase D (PLD) (Moussa *et al.*, 2016) and the other one is a live attenuated vaccine that has been approved for practice in Brazil since 2000 (Dorella *et al.*, 2006).

Conclusion

From this review, it is concluded that diphtheria is a highly infectious and fatal disease though vaccine-preventable. *Corynebacterium* strains other than *diphtheriae* are emerging quickly worldwide and causing high morbidity and mortality both in humans and animals as well. There is an obvious increase in the rate and severity of *C. ulcerans* infections in the UK and other developed countries. The toxins produced by toxigenic strains are highly potent and disseminate quickly, once disseminated then they could be highly lethal. Thus diphtheria toxoid and diagnosis followed by prompt treatment is the only way to decrease the rate of morbidity as well as mortality.

Conflict of interest

All the authors have read and approved the manuscript and they have declared that there is no conflict of interest.

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