PRIMER

Diphtheria

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Abstract | Diphtheria is a potentially fatal infection mostly caused by toxigenic *Corynebacterium diphtheriae* strains and occasionally by toxigenic *C. ulcerans* and *C. pseudotuberculosis* strains. Diphtheria is generally an acute respiratory infection, characterized by the formation of a pseudomembrane in the throat, but cutaneous infections are possible. Systemic effects, such as myocarditis and neuropathy, which are associated with increased fatality risk, are due to diphtheria toxin, an exotoxin produced by the pathogen that inhibits protein synthesis and causes cell death. Clinical diagnosis is confirmed by the isolation and identification of the causative *Corynebacterium* spp., usually by bacterial culture followed by enzymatic and toxin detection tests. Diphtheria can be treated with the timely administration of diphtheria antitoxin and antimicrobial therapy. Although effective vaccines are available, this disease has the potential to re-emerge in countries where the recommended vaccination programmes are not sustained, and increasing proportions of adults are becoming susceptible to diphtheria. Thousands of diphtheria cases are still reported annually from several countries in Asia and Africa, along with many outbreaks. Changes in the epidemiology of diphtheria have been reported worldwide. The prevalence of toxigenic *Corynebacterium* spp. highlights the need for proper clinical and epidemiological investigations to quickly identify and treat affected individuals, along with public health measures to prevent and contain the spread of this disease.

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Diphtheria is an infectious disease caused by toxigenic bacteria of the *Corynebacterium* genus, mostly *Corynebacterium diphtheriae* and rarely other closely related species, namely *C. ulcerans* and *C. pseudotuberculosis*. The Corynebacteriaceae family comprises >100 species of aerobic, Gram-positive rods that exhibit a club-shaped morphology. Three species, *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*, are known to produce diphtheria toxin (DT), which is encoded by a gene (*tox*) that is carried by a prophage and acquired by horizontal gene transfer. The *tox*-encoding bacteriophage integrates into specific sites of the *C. diphtheriae* chromosome by site-specific recombination. This bacterium was perpetuated by its eponymous name, the Klebs–Löffler bacillus, since it was first identified by Klebs in 1883 and successfully cultured by Löffler in 1884 (FIG. 1). The non-toxigenic strains of *C. diphtheriae* (NTCD) and *Corynebacterium* spp. other than *C. diphtheriae* are considered potential emerging pathogens as they are also capable of causing severe disease, which is not vaccine-preventable[1,2]. Based on colony morphology and biochemical reactions, *C. diphtheriae* is divided into four biovars or biotypes: gravis, mitis, intermedius and belfanti. Among these, belfanti is unusually described as toxigenic[3,4], whereas the other biotypes are named according to the disease severity they are usually associated with: serious, mild and intermediate for the gravis, mitis and intermedius biotypes, respectively.

Diphtheria predominantly affects children of <15 years of age, and several investigations have shown that unimmunized and immunocompromised populations are particularly vulnerable to the disease[5]. Diphtheria was a major cause of childhood morbidity and mortality during the pre-vaccine era. Resurgence of this contagious disease has occurred owing to disruptions in healthcare systems and vaccination programmes. Several major outbreaks of diphtheria have been recorded during 1921–2018, in almost all global regions including the United States, Europe, Asia, the Newly Independent States of the former Soviet Union (NIS), Haiti, Venezuela and Yemen[6–10]. Depending on the anatomical site affected, diphtheria can be classified into respiratory (involving the anterior nasal, pharyngeal and laryngeal cavities and the tonsils), cutaneous (including the genital area) and ocular. The typical advanced symptoms of acute diphtheria include the presence of a thick, grey layer (called the pseudomembrane) on the throat and/or tonsils, enlarged lymph node glands in the neck (bull neck) and, in severe cases, myocarditis and inflammation of the nerves.

Diphtheria has a high case-fatality rate (5–17%) among the unvaccinated population, even in individuals

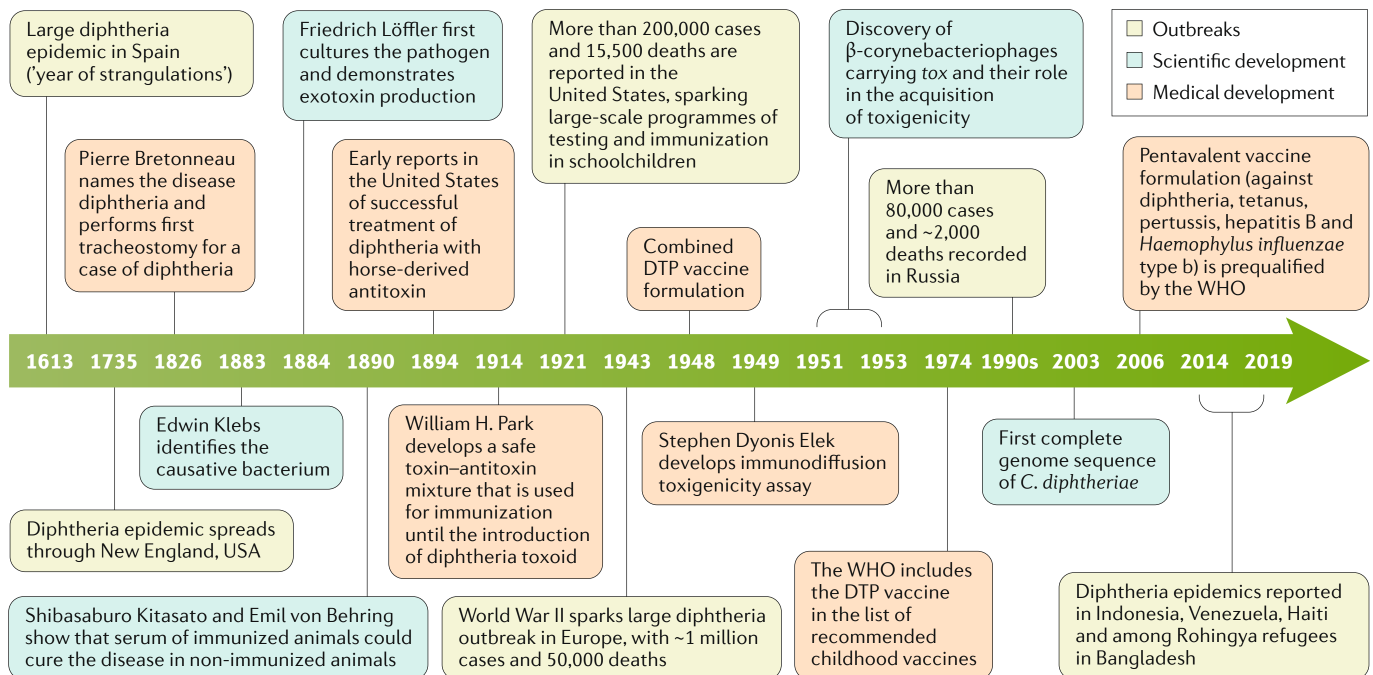

Fig. 1 | **Milestones in the history of diphtheria.** Important milestones in the history of diphtheria, highlighting major disease outbreaks and scientific and medical developments. Crucial scientific discoveries include the discovery of *tox* gene-bearing β-corynebacteriophages[99] and the complete sequencing of the *Corynebacterium diphtheriae* genome[234]. DTP, diphtheria, tetanus and pertussis.

receiving proper care and treatment[11,12]. A trivalent vaccine for diphtheria, tetanus and pertussis (DTP) is available that can protect against diphtheria during childhood. Booster doses of toxoid (inactivated toxin) are recommended for adults[5,13]. Several immunological and molecular tools are now available for the timely detection of the disease. Administration of diphtheria antitoxin (DAT) and antibiotic therapy are effective in patients with typical disease symptoms.

This Primer describes the key aspects of epidemiology, mechanisms and pathophysiology, clinical manifestations, diagnosis, prevention and management of diphtheria in light of the best current evidence.

Epidemiology

Disease epidemiology and outbreaks

The global incidence of diphtheria has declined substantially owing to extensive vaccination coverage; nevertheless, the disease remains endemic in many countries, although accurate reports on the incidence in these countries are limited. Implementation of the DTP vaccine programme has reduced childhood diphtheria in several countries. For example, during the first 13 years of the mass vaccination period (1919–1931), the reported cases of diphtheria were reduced by 82.4% in the Netherlands[14]. Timely vaccination has a substantial public health effect. Studies on the timeliness of the receipt of scheduled vaccine doses in urban Australian indigenous children showed that 72% of children received their first dose of DTP vaccine (at 2 months of age) on time, but only 59% of them received the third dose (DTP-3, at 6 months of age) on time[15]. In endemic regions, the incidence of diphtheria in a hospital setting remains high (27.3%)[16] and the overall case fatality rate ranges from 20 to 31%[17].

During 1982–1994, many countries, such as Jordan[18], Sweden[19,20], the NIS[21] and Thailand[22], reported large numbers of diphtheria cases. In addition, owing to the re-emergence of diphtheria to epidemic levels in Russia and the NIS, the WHO Regional Office for Europe requested the establishment of a European Laboratory Working Group on Diphtheria[23]. This working group was formed in July 1993, with the main objectives of creating a network of laboratories for microbiological surveillance, standardizing laboratory diagnostic methods in epidemic areas and understanding the molecular epidemiology and characteristics of epidemic strains at that time[24]. Later, the network was expanded to the Diphtheria Surveillance Network (now European Diphtheria Surveillance Network), integrating epidemiological and microbiological aspects of diphtheria, and including other infections caused by potentially toxigenic corynebacteria[25].

Large epidemics of diphtheria occurred in Russia in the 1990s; of note, women were more likely to be affected than men[26]. Spread of diphtheria to almost all of the NIS and to neighbouring countries such as Finland, Sweden and Norway was found to be due to imported cases[27]. The 1991–1996 outbreaks in the NIS were mainly due to the lack of active immunization programmes in preceding years as a result of the disruption of the former Soviet Union. However, the epidemic situation of diphtheria in north-western and central regions of Russia during 2001–2002 persisted with high morbidity (40–47%) and mortality (14–19%) primarily among children of 3–6 years of age[28]. For unknown reasons,

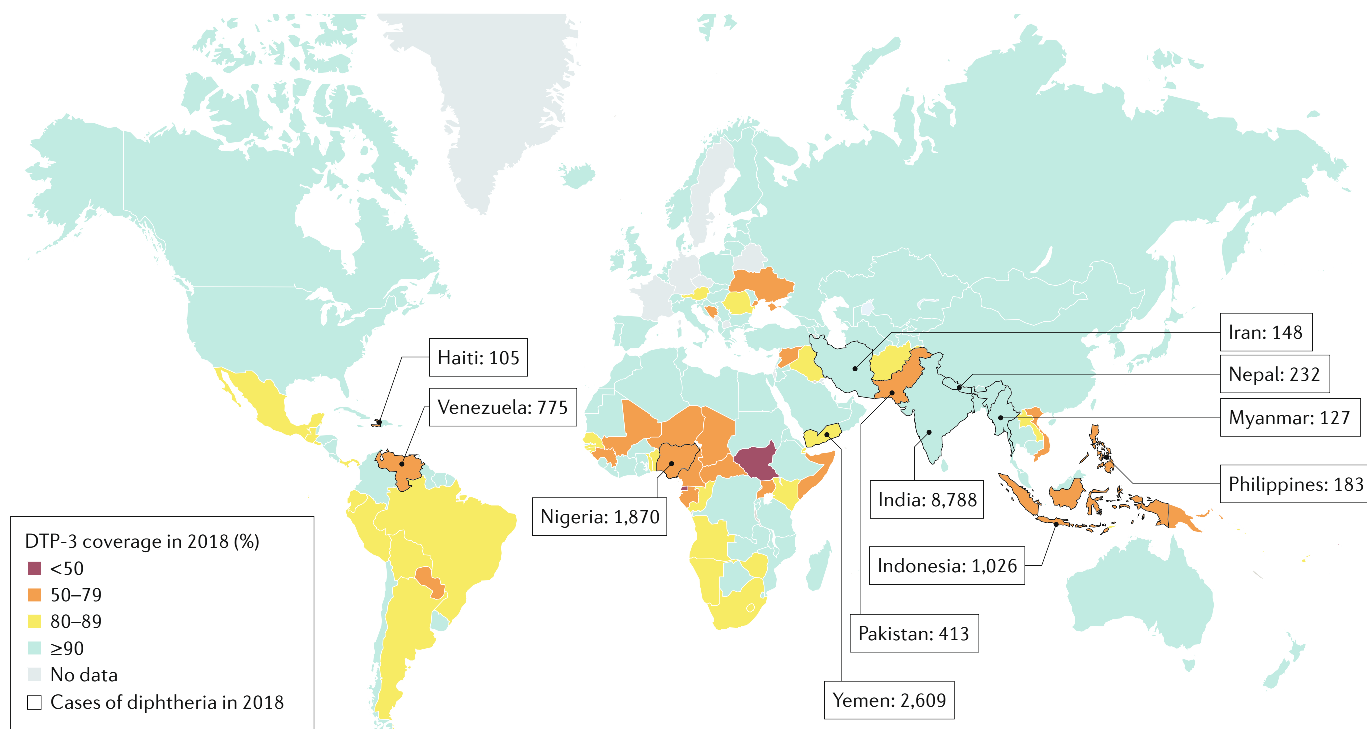

Fig. 2 | **Global DTP vaccine coverage and number of cases of diphtheria.** The map shows the coverage of the third dose of the vaccine for diphtheria, tetanus and pertussis (DTP-3) in 2018. The number of cases of diphtheria reported in the same year is shown for countries with >100 reported cases. DTP, diphtheria, tetanus and pertussis. Data from WHO Reported estimates of DTP-3 coverage and Diphtheria reported cases.

a high proportion (~67%) of fully vaccinated individuals were also affected[18].

In 2018, the WHO recorded 16,611 reported cases (FIG. 2). Generally, diphtheria is under-reported from many regions, including Asian, African and Eastern Mediterranean countries[5]. Outbreaks of respiratory diphtheria have been reported in several countries, including Nigeria in 2011 (REF.[29]) and India during 2010–2016 (REF.[30]). Refugee resettlement centres (for example, in the Rohingya refugee population in Bangladesh)[31] are highly vulnerable, with increased numbers of diphtheria cases and asymptomatic carriers, owing to poor availability of public health services[32,33]. It was estimated that ~76% of refugees had no long-term protection against diphtheria[34]. During 2015–2018, diphtheria outbreaks occurred in Haiti, Venezuela and Yemen owing to socioeconomic crisis or war that resulted in poor access to healthcare and vaccination[7–9,35,36]. The carrier state of diphtheria is important in endemic countries. A study conducted in 1989 in India following a confirmed case of diphtheria identified carriage of *C. diphtheriae* in ~20% of the children tested, and 65% of the organisms were toxigenic[37]. However, in non-endemic countries with optimal vaccine coverage, the carrier status of diphtheria is much lower, except among individuals with low immunization condition[10,20] or people who misuse alcohol[38].

Emerging pathogens. The emergence of respiratory, cutaneous and invasive infections by *C. diphtheriae*-related pathogenic *Corynebacterium* spp., *C. ulcerans* and *C. pseudotuberculosis*, complicates the diagnosis and management of infections. Humans are the only known natural host for *C. diphtheriae*, whereas disease associated with *C. ulcerans* and *C. pseudotuberculosis* infections is of zoonotic origin (from domestic and wild mammals). In the United Kingdom, *C. ulcerans* infection has become more common than *C. diphtheriae* infections, owing to transmission from animals, travel to endemic countries and also lack of vaccination[1]. In Europe and in some developed countries, *C. ulcerans* has been increasingly reported in cases with typical clinical symptoms of diphtheria[13,39,40], including from immunized individuals[41].

The clinical relevance of NTCD has been emphasized by many reported cases[42], and the epidemic nature of invasive NTCD has been documented in several countries with high vaccination coverage[43–47]. NTCD were found to be associated with severe clinical symptoms, such as myocarditis, polyneuritis and bacteraemia[48–50]. Predisposing factors such as alcoholism, hepatic cirrhosis and dental caries facilitate the invasive infections caused by NTCD[51]. In Australia, 70% of the healthy individuals carrying NTCD had a history of travel to tropical nations[52].

Molecular epidemiology

As described in previous sections, the epidemiology of diphtheria is constantly changing owing to varying prevalence of *Corynebacterium* spp. in different regions, travel and migration, variations in socioeconomic status and vaccination rates and other factors. High-resolution molecular typing of strains is vital to identify disease

transmissions and outbreaks. Using genomic information, several typing approaches have been developed over the years to compare the pathogens and to detect the epidemiological links. This section describes several genotyping approaches that have been used for *C. diphtheriae* and other species.

Molecular methods. Molecular typing of *C. diphtheriae* isolates provides baseline information regarding the spread of strains and biotypes during sporadic and outbreak situations[53]. Identification of similarities and differences between bacterial strains and isolates enables their division into types, and identification techniques detect phenotypic or genetic differences among isolates. Phenotypic methods, although globally accepted to characterize the members of specific bacterial species, have several downsides, including false-negative results due to variations in biological expression, interpretation of results, gain or loss of certain genes and so forth. By contrast, DNA-based molecular typing helps in the comprehensive phylogenetic analysis of isolates, revealing their origin, patterns of local and global spread over the years and evolution in response to stress from antimicrobials and the host's immune system. Such information will enable accurate outbreak tracking within certain regions and across continents, and will also help in planning interventions to prevent and treat the disease.

The traditional molecular methods used for typing of various bacteria, including *C. diphtheriae*, are ribotyping (based on restriction patterns of ribosomal RNA genes), pulsed-field gel electrophoresis and multilocus enzyme electrophoresis. In 2004, the ribotype nomenclature of *C. diphtheriae* strains was established: 86 ribotypes were identified by the restriction patterns using *Bst*EII digestion of the DNA. Many ribotypes were allocated a geographical name based on the location of the initial isolate; however, some followed an arbitrary nomenclature[54]. Although widely used, these techniques are time-consuming and require specialized equipment and technical expertise, and, therefore, cannot be performed in all laboratories. By contrast, PCR-based methods are faster and simpler, although they frequently lack adequate discriminatory power and reproducibility and their standardization is a challenge[55]. A multilocus sequence typing (MLST) scheme for *C. diphtheriae* based on allelic determination of seven housekeeping genes has been developed[4], which provides digital, unambiguous and portable results. MLST diversity has grown continuously, with >600 types currently categorized. However, there is no correlation between MLST and biotype test results[56]. Each different sequence existing within an isolate is classified as a distinct allele, and for each isolate, the combination of alleles at the loci analysed defines the sequence type (ST) number. MLST discrimination of 150 isolates from 18 countries and spanning 50 years was consistent with previous ribotyping data, and clonal complexes (clusters of isolates that share at least six out of seven alleles) associated with disease outbreaks were distinctly identified[4].

Two in silico-based approaches have been investigated to enable more-precise characterization of the molecular genetics and epidemiology of diphtheria[57,58], both based on repetitive DNA sequences, namely, variable number tandem repeats (VNTR) and clustered regularly interspaced short palindromic repeats (CRISPR) loci. A study in Poland demonstrated that some of the VNTR loci in *C. diphtheriae* have discriminatory power, although preliminary results were not compared with those from other typing methods[57]. Spoligotyping is a genotyping technique to identify and subtype *C. diphtheriae* isolates at a phylogeographical level, and CRISPR-based spoligotyping had a high level of discrimination for an epidemic clone in Russia and Belarus[58]. In particular, 156 isolates of the epidemic clone (classical ribotypes, Sankt-Petersburg and Rossija) were subdivided into 45 spoligotypes. However, further studies uncovered the limitations of CRISPR-based typing: the three selected CRISPR loci are not present simultaneously in all isolates, and most strains have unique spacers in the leader sequence, which indicates that they evolved independently after diverging from a common ancestor[59]. Hence, CRISPR-based typing might not necessarily provide information on evolutionary relationships between different strains, but it might offer a high level of discrimination to study local diphtheria epidemiology.

In molecular epidemiological technologies, whole genome sequencing (WGS) of the bacterial genome has been demonstrated to be an effective high-resolution typing method to investigate outbreaks caused by *Corynebacterium* spp. As a part of surveillance technologies, WGS data evaluated by core genome MLST or single-nucleotide polymorphism-based methods have shown substantial advances over other techniques[60].

Outbreak-associated clones. Molecular epidemiological investigations suggest the existence of outbreak-associated clones with multiple genotypes circulating around the world. This approach uses various genetic markers to recognize an outbreak, match case isolates involved in the outbreak independently and with known strains and discriminate between outbreak and non-outbreak isolates. This section describes large studies conducted with outbreak-associated *C. diphtheriae*. In several investigations, ribotyping and MLST data show an overall dominance of certain clones in a specific geographical area. Existence of unique clones in these investigations demonstrates that the genome of *C. diphtheriae* is constantly changing in many regions.

Outbreak analysis of >1000 diphtherial cases, mostly with cutaneous lesions from Seattle during 1972–1982, indicated involvement of the intermedius, mitis and gravis biovars, and molecular analysis using restriction fragment length polymorphism with three different probes revealed that the intermedius and gravis biotypes were of clonal origin[61]. Outbreak-associated strains of *C. diphtheriae* during the 1990s in Russia and the NIS exhibited considerable genetic diversity in ribotyping, multilocus enzyme electrophoresis and PCR single-strand conformation polymorphism analysis of *tox* and its regulatory element diphtheria toxin repressor (encoded by *dtxR*)[62,63]. In diphtheria-endemic countries, shifts in the strains of *C. diphtheriae* are typified by changes in the predominance of certain biotypes and ribotypes. The Russian epidemics between the 1950s

and 1960s are first represented by *C. diphtheriae* strains of the gravis biotype, ribotype M11, followed by the mitis biotype, closely related ribotypes M1 and M1v[64]. In the early 1990s, the ribotype provisionally designated D11 was documented amongst strains isolated in the United Kingdom, Russia, Germany, Romania, Italy and Sweden, whereas ribotype D75 has only been reported in the United Kingdom[65]. In Russia, ribotypes G1 and G4 were predominantly found between 1991 and 1997 (REF.[66]). In outbreak-affected areas within the NIS, the *C. diphtheriae* gravis biotype was predominant during 1996–2000 with ribotype Sankt-Peterburg. During 2001–2005, this was replaced by the mitis biotype and ribotype Rossija[67].

The MLST STs of *C. diphtheriae* isolates seem to be country specific, suggesting that the same isolates have been prevailing for many years[68]. Different STs of outbreak-related *C. diphtheriae* were reported from Belarus (ST-8, in the 1990s)[69], Algeria (ST-116, between 1992 and 2005)[70], Thailand (ST-243, in 2012)[71], the United Kingdom (ST-10, between 2007 and 2013)[72], South Africa (ST-378, in 2015)[73] and Malaysia (ST-453, between 1981 and 2016)[74]. A diphtheria outbreak during 2015 in South Africa indicated the prevalence of another ST (ST-395), which has been spreading within the country for >30 years[73].

WGS analysis results have shown close genetic relatedness among toxigenic *C. diphtheriae* isolated from infected wounds of refugees from Northeast Africa and Syria in Europe[75]; circulation of genetically related strains in Malaysia[68]; novel lineages in South Africa[73]; several NTCD outbreak clusters with ST-8, originating from Hamburg and Berlin, Germany[76]; relevance of pilins, adhesion factors and iron utilization in infections caused by NTCD[77]; and the presence of different genetic backgrounds of DT-mediated pathogenicity in *C. diphtheriae*, *C. pseudotuberculosis* and *C. ulcerans*[78].

Mechanisms/pathophysiology

Colonization

Corynebacterium spp. are part of the nasal commensal microbiota and can inhibit the nasal colonization of other opportunistic pathogens, like *Staphylococcus aureus*, and decrease their virulence[79,80]. However, there is scant information regarding the nasal microbial biodiversity and the colonization of *Corynebacterium* spp. in people living in diphtheria-endemic and non-endemic countries. Although immunization protects against clinical diphtheria, it does not prevent carriage of *Corynebacterium* spp., including NTCD that do not express DT. Of note, an increase in the levels of anti-DT antibodies not only eliminates toxigenic strains from the nasal microbial community but may also reduce the diversity of *C. diphtheriae*[81,82].

Virulence and infection

Adhesion and virulence. Adhesion in *C. diphtheriae* is mediated mainly by pili (or fimbriae) that are covalently attached to the bacterial cell wall and contribute to colonization in the host. This pathogen harbours three pilus gene clusters that encode nine pilus proteins, SpaA–SpaI, known as sortase-mediated pilus proteins (or pilins)[83]. Each Spa protein contains the LPXTG motif, which is cleaved by sortase enzymes and has an important role in colonization, virulence and infection by Gram-positive pathogens. Isogenic mutants of a toxigenic *C. diphtheriae* strain, which lack genes encoding SpaA-type pilus proteins (SpaA, SpaB and SpaC) (Δ*spaABC*), showed delayed killing of *Caenorhabditis elegans*, thereby demonstrating the importance of pili in the pathogenicity. The minor pilins SpaB and SpaC act as specific adhesins that facilitate corynebacterial adherence to host pharyngeal cells, which is greatly diminished when these proteins are deficient[84]. The host cell receptors targeted by SpaB and SpaC have not been identified, nor the specific effects of the SpaH-type pilus proteins (SpaH, SpaI and SpaG) on the clinical outcome. Lipoarabinomannan-like lipoglycan and cell surface proteins DIP1281 and DIP1621 are also identified as adhesive factors in *C. diphtheriae*[85–87]. Low iron availability was shown to modify the cell adhesion and expression of surface carbohydrate moieties, which may affect the progression of *C. diphtheriae* infection[88].

C. diphtheriae virulence factors other than the DT (see below) are potentially important in causing human disease. Adhesins, haemagglutinins and surface-exposed non-fimbrial proteins play an important part in the internalization of the pathogen into the host cell[89]. In addition to adhesion and invasiveness, *C. diphtheriae* haemagglutinin (protein DIP0733) might contribute to the cytotoxicity and apoptosis of epithelial cells, as evidenced by cell vacuolization, nuclear fragmentation and the formation of apoptotic bodies, both in vitro and during the early stages of diphtheria and *C. diphtheriae* invasive infection[89,90]. Genome-wide analysis showed the importance of pilins, adhesion factors and iron utilization in infections caused by non-toxigenic, invasive *C. diphtheriae* strains, mostly belonging to ST-32 (REF.[77]). Endocarditis-associated *C. diphtheriae* strains display an aggregative adherence pattern on endothelial cells from the human umbilical vein[91]. This attribute may contribute to more-efficient adherence, internalization, microtubule stability and phosphorylation of proteins in the host cell.

Molecular mechanisms promoting the internalization of *C. diphtheriae* remain unclear. Several putative virulence factors, such as the Shiga-like toxin ribosome-binding protein (encoded by *rbp*), venom serine proteinase (encoded by *vsp2*) and, in the prophage sequence, a factor similar to the RhuM virulence factor of *Salmonella enterica*, have been reported in *C. ulcerans*[92–94]. Several other virulence factors encoded by *cpp*, *pld*, *cwlH*, *nanH*, *rpfI*, *tspA* and *vsp1* were found to be associated with the pathogenesis of *C. ulcerans*[95]. In invertebrate animal model systems, the C-terminal coiled-coil domain of this pathogen was crucial for interaction with epithelial cells[96]. Similarly, a tellurite resistance protein was associated with survival in human epithelial cells and lethality of *C. elegans*[97].

Initiation of infection and mechanism of action of DT. The ADP-ribosylating bacterial toxins are a family of toxins that catalyse the hydrolysis of nicotinamide adenine dinucleotide (NAD) and transfer the ADP-ribose

moiety onto eukaryotic protein targets. ADP-ribosylating bacterial toxins can be classified into four groups on the basis of their domain organization and the nature of their target. DT is an elongation factor 2 (EF-2) ADP-ribosylating toxin[98].

DT is the major known virulence factor of *C. diphtheriae*; the structural gene encoding DT (*tox*) is carried in the genome of a family of corynebacteriophages, and *C. diphtheriae* and other closely related species acquire *tox* by lysogenic integration of the β-prophage genome into their chromosome, thereby becoming toxigenic[99]. The most virulent strains may carry two or three copies of *tox* inserted into the genome as there are different, functionally equivalent bacterial attachment sites (*attB*) for integration of the β-prophage into the chromosome of *C. diphtheriae*[100], and each *att*B site is located within an Arg-tRNA$_2$ gene that is present at two different chromosomal locations[101]. Even though *tox* is of bacteriophage origin, the regulation of toxin production is under bacterial control, since the diphtheria toxin repressor gene (*dtxR*) is on the bacterial chromosome, and toxin production depends upon the expression of *tox* and bacterial iron metabolism. NTCD and other close species represent a potential reservoir for the emergence of toxigenic strains if they possess functional *dtxR* genes[102,103].

DT is an exotoxin: it is secreted across the cytoplasmic membrane without cell lysis. The mature extracellular toxin is a 58-kDa polypeptide comprising 535 amino acid residues. DT is endocytosed into the cells via the binding of its B-subunit to the proheparin-binding epidermal growth factor-like growth factor (HB-EGF) (FIG. 3); the ADP-ribosylation activity of DT prevents protein synthesis in the host cell and results in cell death.

Diphtheria toxin regulation. DtxR is a divalent metal-activated transcription repressor: DtxR regulates siderophore-mediated iron uptake and the expression of haem oxygenase and DT[104,105]. The conserved DtxR binding sequence has been located near the promoters of *tox* and siderophore (*sid*) genes[106]. Siderophores solubilize and bind iron and transport it into the bacterial cell, across specific membrane receptors. The functional activity of the DtxR is controlled by iron (although Ni^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+} and Zn^{2+} may also function as activators), which serves as an essential co-repressor necessary for activation of target DNA binding by DtxR. Binding of the divalent Fe^{2+} cation generates an allosteric change in the conformation of DtxR, which facilitates the activated repressor to bind to the *tox* operator[107]. The regulation of *tox* by DtxR has been detected in response to changing iron concentrations. DtxR is inhibited by low

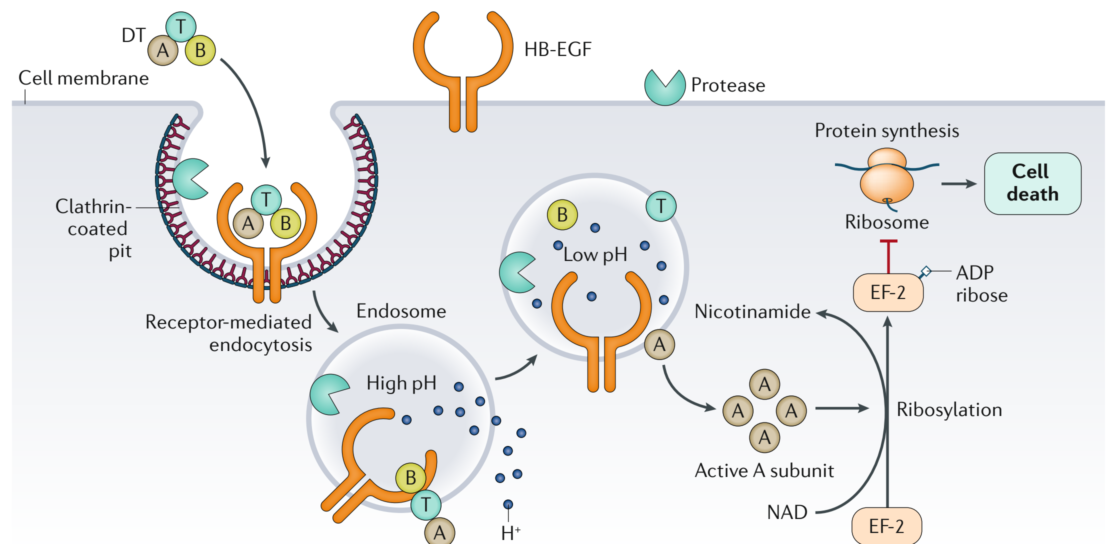

Fig. 3 | **Mechanism of action of DT.** Diphtheria toxin (DT) is composed of two subunits bound by a disulfide bond between cysteine residues: the amino-terminal A-subunit contains the catalytic (C) domain (not shown), and the carboxyl-terminal B-subunit contains a membrane-inserting translocation (T) domain and a receptor-binding (R) domain (not shown). DT secreted from the bacterial cell binds to the proheparin-binding epidermal growth factor-like growth factor (HB-EGF), which acts as a DT receptor. DT enters the cell via receptor-mediated endocytosis through clathrin-coated pits: the R domain recognizes the HB-EGF receptor on human epithelial cells, leading to endocytosis of the entire receptor–toxin complex. Endosome-associated proteases partially cleave the bond between the DT subunits, and exposure of the DT to the acidic conditions triggers a conformational change that enables the T domain to insert into the endosome membrane and the subsequent translocation of the A-subunit across the endosomal membrane into the cytosol. The T domain is thought to be primarily responsible for membrane insertion, although the C and R domains have also been shown to be associated with membranes[262]. In the cytosol, the C domain catalyses the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD) onto the elongation factor 2 (EF-2). EF-2 is a member of the GTP-binding translation elongation factor family. This protein is an essential factor for the cell protein synthesis and enables the transfer of the peptidyl tRNA–mRNA complex from the ribosome to the peptidyl site during protein synthesis. DT targets a post-transcriptionally modified histidine diphthamide on EF-2 (REF.[263]). ADP-ribosylation of this unique diphthamide residue prevents EF-2 translocation activity, resulting in inhibition of protein production and host cell death[264]. Adapted from REF.[265], Springer Nature Limited.

concentrations of iron, which result in increased DT production; thus, iron signalling controls the direct expression of DT through the DtxR[108]. In silico analysis has proved that DtxR may provide a molecular bond for the Fe^{+2}-induced Fenton effect (the generation of reactive oxygen species, which is harmful for bacteria) and protect bacterial DNA from oxidative damage[109]. In a genetic disruption analysis, *dtxR* was shown to have a strong influence on the expression of many genes[110], and the genetic inactivation of *dtxR* results in constitutive DT expression, even under a high iron environment[111]. In addition, adherence, colonization and biofilm formation are the direct response to iron-limited conditions[88]. Most *Corynebacterium* spp. harbour functional *dtxR*[112,113], including many NTCD[102]. If lysogenized by a bacteriophage, these non-toxigenic strains possibly produce DT, and hence signify a potential reservoir for toxigenic *C. diphtheria*.

Systemic infections

Absorption and dissemination of DT through blood circulation from the respiratory tract can lead to systemic effects that are most commonly manifested as damage to the myocardium and peripheral nerves, leading to complications such as myocarditis, peripheral neuropathy and polyneuropathy[114]. DT can be lethal to humans at doses below 0.1 μg/kg of body weight[115]. The HB-EGF precursor acts as the receptor for DT on the plasma membrane of human cells, especially in cerebral neurons and cardiac myocytes[116–118]. DT-induced cytotoxicity is variable, as it involves DNA cleavage and cytolysis (by inhibiting eukaryotic translation elongation factor 2 (EF-2) via ADP-ribosylation)[119] and the depolymerization of actin filaments. DT-induced degradation of actin filaments is important as the toxin is known to cause severe, often lethal, cardiomyopathy associated with diphtheria[120].

During the acute phase of diphtheria, myocarditis has been associated with 60–70% of deaths[121]. DT induces myocardial dysfunction as well as bradyarrhythmias, tachyarrhythmias and complete heart block[122]. Conduction system disturbances are signs of severe myocardial damage due to acute inflammation of sinoatrial and atrioventricular nodes that can be fatal, despite ventricular pacing[122]. Post-diphtheria complications such as cardiomyopathy and neuropathy may result from autoimmune inhibition of epidermal growth factor receptor (EGFR) function, due to antigenic epitope similarities between the DT B-subunit and the extracellular domain of the EGFR, and this inhibition could damage host tissues expressing the EGFR[123]. Post-diphtheritic neuropathy manifests with palatal palsy, limb weakness and requirement of mechanical ventilation in the majority of paediatric cases. Hypoxic neurological injury was observed in some cases[124]. In animal model studies, complete loss of nerve fibres was observed as a long-term effect[125].

Toxigenic *C. diphtheriae* usually causes non-invasive localized infection of the higher respiratory tract with peripheral tissue damage due to the diffusion of DT[126]. However, recently there has been an increase in the incidence of invasive infections caused by non-toxigenic strains. In vitro studies proved that the invasiveness is due to a zipper-like mechanism mediated by receptors on the cell membrane, which may contribute to persistent infections despite antimicrobial therapy[127]. However, host cell receptors and invasion-associated proteins of the pathogen remain unknown. Conversion of blood fibrinogen to fibrin for binding of bacteria was also proposed as one of the factors for pseudomembrane formation in non-toxigenic and toxigenic strains[128].

Other pathogenic Corynebacterium *spp*

***Non-toxigenic* tox*-bearing* C. diphtheriae.** The non-toxigenic *tox*-bearing *C. diphtheriae* (NTTB) strains are genotypically *tox*-positive, but do not express DT owing to nucleotide mutations or deletions. Reported changes that lead to the loss of ADP-ribosyltransferase activity include the loss of 149 C-terminal amino acid residues, a single C–T transition that generated a TAA termination signal and a single G–A transition that substituted glycine-52 with glutamic acid[129]. Other reported changes include a nonsense mutation within the *tox* sequence encoding the B-subunit or *tox* promoter region[130] and deletion of a nucleotide that shifted the open reading frame and formed a stop codon[131]. NTTB and toxigenic *C. diphtheriae* strains existed in Belarus, both during and after the epidemic in the 1990s (REF.[69]). NTTB strains were also widely detected in Russia during 1994–2002 (REF.[131]). These strains belonged to the mitis biotype and ribotype Moskva. Because NTTB strains do not carry a functional *tox* gene, they are considered clinically unimportant. However, they may act as a reservoir for the emergence of toxigenic strains if they acquire new functional *tox* genes by lysogenic conversion[132–134]. The potential risk of such NTTB strains expressing DT has been demonstrated in several investigations[102,135].

The pathogenesis of NTTB infections remains unknown. Skin lesions or dental caries with NTTB are the most probable portals of entry for the pathogen[49,136]. Biofilm formation and pilus proteins (SpaD and SpaH) are thought to be the possible virulence factors in NTTB[48,137]. Several host factors may contribute to the clinical outcome. Bacteraemia caused by invasive NTTB strains is closely associated with homelessness, abuse of alcohol and injection drugs and diabetes mellitus[50].

C. ulcerans. Until the mid 1980s, it was thought that the DT was only produced by *C. diphtheriae* isolates that harbour *tox*-encoding corynebacteriophages[138]. However, cutaneous diphtheria caused by toxigenic *C. ulcerans* has been reported in both humans and animals[139,140], and *C. ulcerans* has been increasingly identified as an emerging zoonotic agent of diphtheria from symptomless pet animals. DT-producing *C. ulcerans* is lysogenized with a *tox*-carrying bacteriophage that has distinct origins from *C. diphtheriae*[141]. The genome of *C. ulcerans* shows a higher similarity with the genome of *C. pseudotuberculosis* than that of *C. diphtheriae*[141]. DT-encoding *tox* of clinically isolated *C. ulcerans* presented changes in the translocation and receptor-binding domains compared with DT sequences of *C. diphtheriae tox*[142]. Production of DT in vitro by *C. ulcerans* was also

lower than in toxigenic *C. diphtheriae*. The *tox* gene and DT from *C. diphtheriae* and *C. ulcerans* diverge from each other in about 5% of their base pair and amino acid composition[142], and five different *tox* and DT sequence groups have been identified in *C. ulcerans*[143]. *C. ulcerans* harbouring *tox* but not secreting DT in vitro has also been reported[144]. In the sequence analysis of toxigenic and non-toxigenic *Corynebacterium* species, four distinct, species-specific clades could be classified, corresponding to *C. diphtheriae*, *C. pseudotuberculosis*, *C. ulcerans* and non-toxigenic *tox*-bearing *C. ulcerans*. The average amino acid homology was >99% for DT and DtxR within these four groups, but lower between each group[78]. This analysis also showed that in most *C. diphtheriae*, *tox* genes were identified within the known prophages, whereas in *C. ulcerans*, diverse *tox*-including mobile genetic elements could be distinguished: either in altered prophages or in an alternative pathogenicity island. In addition to DT, expression of phospholipase D; binding properties to human fibrinogen, fibronectin and type I collagen biotinylated proteins; and virulence to *C. elegans* are considered virulence factors of *C. ulcerans*[145]. Increasing virulence in *C. ulcerans* has been attributed to several factors, including the RhuM prophage[93] and acquisition of other exogenous factors[94].

C. pseudotuberculosis. In the case of *C. pseudotuberculosis* infection, animals are the main source of human infection, such as lymphadenitis[146]. *C. pseudotuberculosis* is considered one of the species that can cause diphtheria as it can be lysogenized by the *C. diphtheriae* bacteriophage[147,148]. Genomic analysis of *C. pseudotuberculosis* isolated from a patient with necrotizing lymphadenitis showed the presence of several putative virulence factors, including Spa proteins, phospholipase D, corynebacterial protease CP40, serine proteases, neuraminidase H and nitric oxide reductase[149].

Diagnosis, screening and prevention

Clinical presentation

The time between *C. diphtheriae* infection and symptom development may range from 1 to 10 days (typically 2–5 days)[5]. People infected with *C. diphtheriae*, even if they are asymptomatic, are infective for up to 4 weeks[150]. Transmission of the disease occurs through direct contact with skin lesions or direct contact or inhalation of airborne oral or respiratory discharges. Infection can also be dispersed by contact with contaminated objects. Lack of vaccination, a compromised immune system, a history of atopic dermatitis (eczema), congested and/or unsanitary living conditions and travel to areas where the disease is endemic are the pragmatic risk factors for diphtheria[151]. Early diagnosis of diphtheria is based on the typical clinical symptoms (FIG. 4) as this helps to initiate the presumptive treatment quickly. Clinical diagnosis of diphtheria usually relies on the presence of pseudo-membranous pharyngitis. The other typical symptoms of acute diphtheria include enlarged lymph node glands in the neck (bull neck), myocarditis and inflammation of the nerves.

Diphtheria is confirmed by isolation of *Corynebacterium* spp. followed by toxigenicity testing. If the cultures of samples from a patient with suspected diphtheria are negative because antibiotic therapy had been started before the samples were collected, a presumptive diagnosis of diphtheria can be made if *C. diphtheriae* is isolated from close contacts of the patient, the patient has a minimal anti-DT antibody titre (<0.1 IU) in serum samples obtained before the administration of DAT (although this parameter is not a key diagnostic indicator) and a direct PCR test of clinical swab samples is positive for diphtheria *tox* genes. Differential diagnoses include acute epiglottitis, oral syphilis, viral pharyngitis, *Borellia vincentii* infection (also known as Vincent angina or trench mouth), oral candidiasis, infectious mononucleosis and streptococcal pharyngitis. Concurrent diphtheria and infectious mononucleosis with exudative pharyngitis are difficult to distinguish, so accurate diagnosis is essential[152], either by culture test for *Corynebacterium* spp. from throat and/or nasal swabs or by reliable molecular methods. Indirect laryngoscopy is recommended in cases with membrane formation. In patients with pharyngitis and a pharyngeal membrane, diphtheria should be suspected. DT penetrates into Schwann cells and inhibits the synthesis of myelin proteolipid and basic proteins[153], leading to diphtheritic polyneuropathy[154,155]. The period between diphtheria and the development of diphtheritic polyneuropathy varies from 10 days to 3 months[155]. The initial symptoms of diphtheritic polyneuropathy are paresis of the soft palate and paraesthesia in the distal parts of the extremities, including respiratory muscle pareses. Common symptoms are hyporeflexia or areflexia and hypotonia, sensory symptoms (paraesthesia, hypaesthesia and hyperaesthesia), facial palsy, nerve palsy, diaphragmatic palsy and loss of vasomotor tone. In a typical diphtheritic polyneuropathy, dysfunction of the cranial nerves might present faster than muscular dystrophy (also known as the cranial stage)[156]. In most cases, diphtheritic polyneuropathy has to be differentiated from Guillain–Barré syndrome, which is common in children. The typical features of diphtheritic polyneuropathy are a high prevalence of bulbar palsy, gradual development of the neuropathy (>4 weeks) and synchronized participation of other organ systems[157].

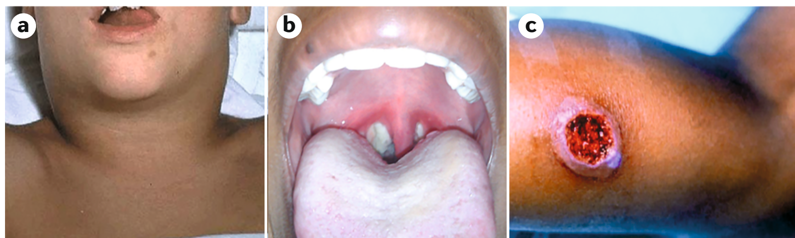

Fig. 4 | **Clinical presentations of diphtheria. a** | Characteristic bull neck caused by enlarged lymph nodes. **b** | Thick pseudomembrane in the posterior pharynx. The pseudomembrane is a layer of bacteria and debris from necrosis of the surrounding tissues due to diphtheria toxin. **c** | Cutaneous lesion caused by *Corynebacterium diphtheriae*.

Microbiological diagnosis

The clinical diagnosis of diphtheria must be confirmed by the isolation and identification of one of the three causative *Corynebacterium* spp. (as diphtheria

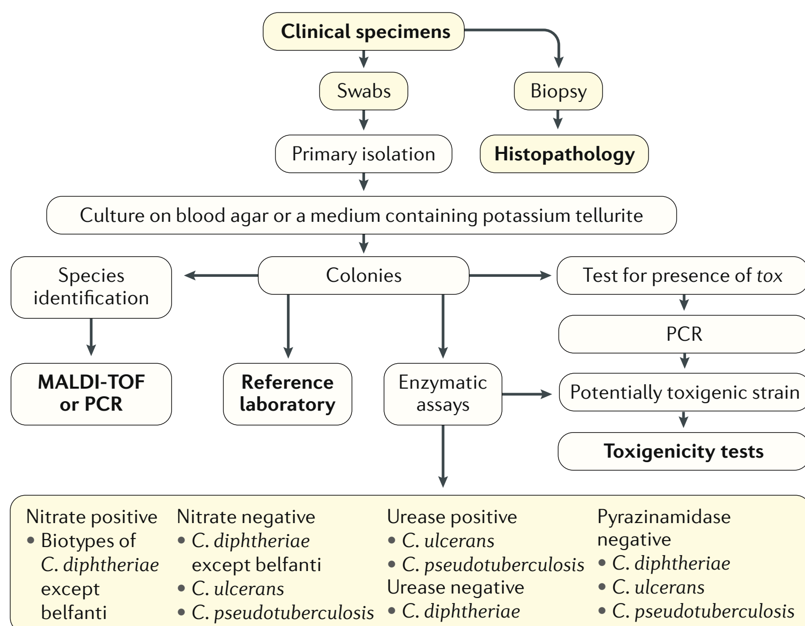

Fig. 5 | **Diagnostic algorithm.** Flow chart showing the methods used in the isolation and identification of *Corynebacterium* spp. MALDI-TOF, matrix-assisted laser desorption/ionization time of flight mass spectrometry.

is a notifiable disease) (FIG. 5). Members of the genus *Corynebacterium* are Gram-positive, non-motile rods, often with a clubbed end, are aerobic or facultatively anaerobic and convert carbohydrates to lactic acid. Of the >100 species in this genus, only a few, *C. diphtheriae, C. pseudotuberculosis* and C. *ulcerans*, are toxigenic and clinically important[158]. Even though all biotypes of toxigenic *C. diphtheriae* are virulent, in some findings, strains belonging to the gravis biotype were found to produce larger amounts of DT than strains of the mitis biotype[159].

Culture and species identification. Bacteria culture of clinical samples is the gold standard for the isolation and identification of *Corynebacterium* spp. Swab samples should be collected from the suspected sites of infection, such as the nasopharyngeal cavity, throat, wounds or skin lesions. If a pseudomembrane is present, swabs should be taken from beneath the membrane, or a piece of the membrane can be collected instead. It is essential to collect the samples regardless of whether antibiotic therapy has been started. If delays in the processing of the clinical samples are expected, specimens should be maintained in Amies transportation medium and can be supplemented with charcoal to preserve the viability of the bacteria.

Sheep or horse blood agar or a medium containing potassium tellurite, such as Hoyle's tellurite agar, is used for primary isolation. This medium is not highly selective for *C. diphtheriae*, as the other bacterial species may also grow. Typical *C. diphtheriae* colonies are grey to black, whereas *Streptococcus* spp. grow as tiny black or brownish colonies. On blood agar, corynebacteria grow as convex, greyish, translucent colonies with a granular appearance, mostly with opaque centres. *C. ulcerans* and *C. pseudotuberculosis* colonies may exhibit β-haemolysis. Bacteria grown in Löffler's medium, which contains coagulated serum with phosphate, accumulate volutin granules (a form of intracellular polyphosphate storage). When stained with polychrome methylene blue (Albert stain), the granules appear violet (metachromatic stain), whereas the rest of the bacterial cell appears blue. Colonies of *Corynebacterium* spp. on tellurite medium appear dark grey or black owing to the intracellular reduction of tellurite to tellurium after 48 h of growth at 37 °C. Using smears made from corynebacterial colonies grown in tellurite medium for immunofluorescence-based toxigenicity tests is not recommended, owing to morphological changes caused by potassium tellurite[160].

Colonies isolated from primary culture plates are identified by enzymatic tests and tested for toxin production (see following section). Enzymatic tests include nitrate, urease, catalase, cystinase and pyrazinamidase tests (to detect the presence of nitrate reductase and the other enzymes), which permit the presumptive identification of the potentially toxigenic *Corynebacterium* spp. within 4 h (REF.[161]). Kits including combinations of such enzyme assays are commercially available.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) can be used to identify the specific *Corynebacterium* sp. directly from a colony isolated from the blood agar plates in about 30 min. The accuracy of the MALDI-TOF system for the identification of *C. diphtheriae*, *C. pseudotuberculosis* and *C. ulcerans* is very high (97–100%)[162]. After MALDI-TOF confirmation of the isolation of a potentially toxigenic colony, bacterial colonies may be used for PCR to identify *tox* and/or for toxin assays. However, in diphtheria-endemic countries, conventional biochemical tests are still widely used.

Toxigenicity tests. The Elek test works on the principle of antigen and antibody immunoprecipitation. In this assay, a known toxigenic strain (positive control), a non-toxigenic strain (negative control) and the sample strains are inoculated onto Elek agar medium with a paper strip containing DAT (500 IU/ml) placed onto the agar surface[163]. In the modified Elek test, the test and control strains are inoculated with a disc containing DAT (10 IU/disc) placed in the centre[164]. After 24–48 h at 37 °C, a clear precipitin line develops at the junction where the toxin produced by the strain and the antibody from the strip or disc meet (FIG. 6). In vitro Vero cell assays and an in vivo rabbit skin test have also been used in the detection or neutralization of DT, but these tests are not recommended for routine use[165].

PCR has been considered a sensitive and specific method for the identification of a specific *Corynebacterium* sp. or to test the clinical samples from suspected diphtheria cases for the presence of *tox*. Although the 16S-ribosomal RNA gene-based identification is widely in use, design of species-specific PCR primers can be difficult, especially when the homologous genes have high similarity. Compared with the 16S-ribosomal RNA gene, sequencing the gene encoding the RNA

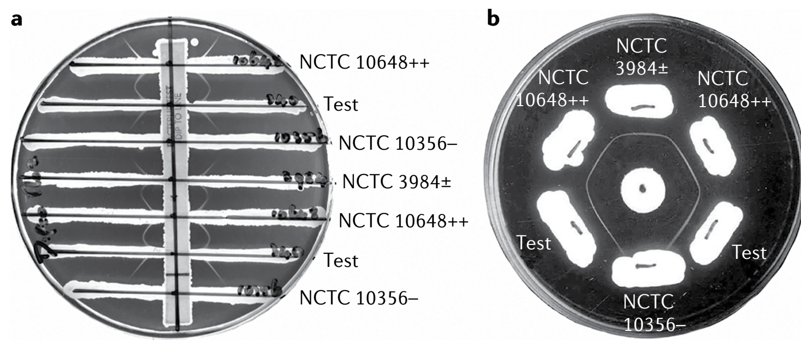

Fig. 6 | **Elek tests for in vitro detection of diphtheria toxin.** The Elek test, also known as the immunodiffusion (immunoprecipitation) technique, is an in vitro virulence test for the detection of toxigenic strains of *Corynebacterium* spp. **a** | In a conventional Elek test, the bacterial colonies grown from culture of clinical samples are spread on a dish perpendicular to a filter paper strip containing diphtheria antitoxin (DAT). If the bacteria produce diphtheria toxin (DT), diffused DAT from the filter paper develops a precipitin line at the zone of equivalence. Appearance of the precipitin line indicates that the tested sample (test) produced DT that reacted with the DAT. **b** | Modified Elek test of the same samples shown in panel **a**, in which the bacteria are grown around a DAT disc (10 IU/disc) that was placed at the centre of the plate. For clear results, the optimum distance between the inoculum and the DAT disc should be 9 mm. National Collection of Type Cultures (NCTC) 3984 (weak positive, ±) and NCTC 10648 (strong positive, ++) are the positive control strains, and NCTC 10356 (negative, –) is the negative control.

polymerase β-subunit (*rpoB*) was found to be useful in identifying the *Corynebacterium* sp.[166]. The *rpoB* sequence has a higher degree of polymorphism than the 16S rDNA sequence[166]. A real-time PCR assay testing for a combination of *tox* and *rpoB* genes was reported for the rapid identification of toxigenic and non-toxigenic strains as well as to differentiate *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*[167,168]. Of note, whereas a *tox*-negative result is final and additional toxigenicity assays are not required, the presence of *tox* does not specify the expression of DT. Hence, the Elek test must be performed on all *tox*-positive isolates from patients with suspected diphtheria[169]; however, patients with *tox*-positive results can be considered for further preventive treatment action, without waiting for the Elek test results. If the clinical laboratories are not equipped for further biochemical or toxigenicity tests, the pure cultures should be submitted to the regional referral centres, in slanted Dorset egg medium or other common agars or on plates at ambient temperature.

The co-agglutination test, passive haemagglutination test, reversed passive latex agglutination assay and bead-based serology assays detecting the expression of glutathione S-transferase fusion proteins are useful for the detection of DT in serum samples and/or pure cultures of toxigenic *C. diphtheriae*[170–173]. Of note, serum samples must always be collected prior to the administration of DAT. However, these techniques have been replaced in many laboratories by a rapid enzyme immunoassay that can detect DT directly from the suspected colonies of corynebacteria[174]. In this enzyme immunoassay, equine polyclonal antitoxin is the capture antibody and an alkaline phosphatase-labelled monoclonal antibody is the detection antibody. The assay is rapid (within 3 h), sensitive (0.1 ng DT/ml) and specific for the detection of fragment A of the DT molecule[174]. Several other diagnostic methods were also developed for serological surveillance studies, including enzyme-linked immunosorbent assay-based detection and quantification of anti-DT antibodies[175], counter-immunoelectrophoresis[176–178] and immunofluorescence assay[179]. Serum anti-DT antibody levels <0.01 IU/ml indicate that an individual is susceptible to diphtheria, levels between 0.01 and 0.09 IU/ml indicate the recommended minimum protective level (basic immunity) and levels ≥0.10 IU/ml are above the protective threshold observed in individuals who have been vaccinated[5].

Histology and imaging

Histopathological imaging analysis of the sites of infection might be required for disease detection, diagnosis and prognosis prediction. This analysis is also important to understand the causal reasons for a specific diagnosis and to provide a more comprehensive view of the disease to complement other clinical investigations. In the case of diphtheria, histological examinations are not performed routinely. Pseudomembrane formation can also be caused by *Streptococcus* spp. and *Staphylococcus* spp. infections, and, therefore, histology might be required to identify the causative pathogen. In a typical disease state, haematoxylin and eosin stain of the pharyngeal pseudomembrane might display necrosis of the epithelium with fibrinosuppurative exudate. Gram-positive *C. diphtheriae* cells can also be detected after Brown–Hopps staining[114].

Myocardium and peripheral nerves are the most susceptible parts during the acute stage of diphtheria[180]. In the histology, the affected myocardium might show extensive areas of hyaline degeneration and necrosis with inflammation in the interstitial spaces. In these areas, infiltrates of mononuclear cells with eosinophilic cytoplasm can be seen. Fluorescent staining of tissue sections with anti-DT antibody demonstrates the presence of DT within myocardial fibres, but not in the areas of advanced necrosis. Ultrastructural variations within the affected myofibres (such as enlarged mitochondria with excess lipid droplets, loss of matrix and disorganization of the cristae, with or without dense osmophilic material) can be identified by electron microscopy[114]. The damaged myofibrils are seen as dislocated scattered foci, with empty, structureless, pale spaces. Clumped chromatin granules can be found near the nuclear membranes[120]. Echocardiography is advantageous in assessing the ejection fraction and identifying indications of ventricular systolic dysfunction, aortic incompetence and acute mitral valve regurgitation[122,181]. Electrocardiography is important to monitor patients with diphtheria-associated myocarditis and detect alterations in the ST-segment (elevation or depression) and T wave, which could be a sign of myocardial infarction, sinus tachycardia, multiple atrial ectopic beats, or prolonged PR or QT intervals, which could lead to arrhythmias, among others[182,183]. CT scans may reveal aortic annular and interventricular septal abscesses and thickening of the pericardium[184,185]. On X-ray examination, patients with diphtheria may present cardiomegaly and bronchopneumonia with thickened pulmonary markings and/or inflammatory infiltrates[186].

Diphtheritic polyneuropathy is due to toxic myelopathy with paranodal demyelination, especially in large myelinated neural fibres. The affected nerves display degeneration of myelin sheaths and axon cylinders. Ranvier nodes appear widened during the early stages of infection, as the paranodal myelin is affected first, followed by demyelination at the later stages[187]. In the most severe cases, axonal degeneration is common, caused by compression made by the folded myelin and invagination of Schwann cell cytoplasm[188]. Muscle biopsies during the acute stages of diphtheritic polyneuropathy show scattered, angulated fibres, predominately of type 2B fibres or cores of type 1 fibres. Histology of intramuscular vessels reveals vasculitis (inflammation of blood vessels) with lymphoid cells[189]. Multiple emboli and diphtheritic neuropathy can be detected using MRI of the brain[157], and CT scans can reveal gyriform enhancement of cerebral lesions[190].

Biomarkers

In acute diphtheria cases, cardiac complications are common, which develop in 10–25% of patients[191] and can result in fatality. In patients with diphtheritic myocarditis, the total leukocyte count and serum glutamic oxalo-acetic transaminase levels are useful biomarkers of prognosis and fatal outcome, as high counts or levels are found to be associated with increased risk[191,192]. Creatine phosphokinase in muscle/brain (CPK-MB) and cardiac troponins levels might be useful outcome predictors, as they are strongly associated with cardiac mortality[183]. Increased systemic levels of IL-6 and tumour necrosis factor (TNF) were noticed during diphtheritic endocarditis[91].

Vaccination

Diphtheria control is mainly based on immunization of the population through vaccination and prevention of the disease in close contacts of patients with confirmed diphtheria by prompt initiation of antibiotic treatment (chemoprophylaxis) followed by vaccination (the three-dose protocol for individuals who had never received the vaccine or a single booster dose for previously immunized contacts). DTP (also known as DPT in some countries) is a combination vaccine against diphtheria, tetanus and pertussis. The vaccine components include diphtheria and tetanus toxoids (inactivated toxins adsorbed onto an adjuvant (aluminium hydroxide or aluminium phosphate)) and killed whole cells of *Bordetella pertussis*. In the DTaP vaccine, the pertussis component is acellular. DTP is also combined with other vaccine antigens, such as hepatitis B virus surface antigen and *Haemophilus influenzae* type b (Hib) conjugates in pentavalent vaccines, and also with inactivated polio vaccine in hexavalent vaccines. A cycle of three doses (via intramuscular injection) of this vaccine is recommended (DTP-3): the first dose should be administered within 6 weeks of age, followed by the other doses at least 4 weeks apart. The third dose has to be completed by 6 months of age. To reduce the number of injections, the DTP vaccine is administered along with other vaccines, such as the Hib vaccine and the hepatitis B vaccine (HepB), scheduled at the same time. Several WHO prequalified vaccines are available in many combinations. Vaccines containing diphtheria toxoid should be stored under refrigerated conditions (2–8 °C) and frozen vaccine should not be used[193]. In 2012, the CDC (Centers for Disease Control and Prevention) recommended DTP vaccination for individuals of ≥65 years of age[194]. The vaccine adverse event reporting system did not find any unfavourable events against tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccine in this population[195]. DTP is safe for pregnant women, and especially useful in protecting the young infant against tetanus and pertussis. DTP vaccination during pregnancy also increases immunity and the duration of protection in mothers who had not received the recommended booster doses[5].

Based on the WHO and UNICEF estimates, global DTP-3 coverage increased during 2007–2010 (from 79 to 84%) and remained stable from 2010 to 2017 (84–85%)[196]. The 2018 global DTP-3 coverage was ~86%, and >80% of the countries reached >80% coverage (FIG. 2). This level of coverage seems adequate to maintain the herd immunity, which for diphtheria has a coverage threshold of 85% (REF.[197]), and lower the risk of outbreaks[198]; however, it is still not fully protective. Generally, there is a long delay in DTP vaccination, especially among newborn babies in low-income and middle-income countries[199]. Societal and cultural issues, poverty, false perceptions about the safety and credibility of vaccines and difficulty for parents to comply with the vaccination schedule are possible reasons for this delay[200]. Completion of vaccination has been significantly correlated with knowledge of mothers on immunization. Parents' forgetfulness about their child's immunization status, many siblings in the family, mother's unemployment and premature birth were significantly associated with a delay in receiving the vaccine[15]. Children whose mothers had poor school-level education are most likely not to receive the DTP vaccination or to discontinue it than children whose mothers had many years of schooling[201,202]. With support from the Global Alliance for Vaccines and Immunization, a combined DTwP-HepB-Hib (pentavalent) vaccine was introduced in Kenya in 2001, and this vaccine has now been used by 72 other countries[203], targeting about 80 million children for immunization[204]. Anti-DT antibody levels decrease with ageing owing to changes within the immune system and/or insufficient vaccination earlier in life[205]. Hence, booster vaccinations during adulthood are recommended to maintain the herd immunity. Reduced antigen-content tetanus, diphtheria and acellular pertussis vaccine is recommended in many countries for boosting immunity in adolescents and adults[206]. A factor that correlates well with the low rate of serological immunity among adults is the time delay in vaccination during their childhood, that is, a delay up to 3 years[207]. Several studies on susceptibility to diphtheria in adults also showed that deficiency of seroprotection was more common in women than in men[207–210]. This difference might be due to gender-specific immune responses subsequent to vaccination[210]. Diphtheria vaccination prevents toxin-related symptoms, but does not prevent colonization of invasive NTCD and other non-toxigenic

Corynebacterium spp. in the host that could cause substantial health risks to unvaccinated individuals and does not provide protection against asymptomatic carriage of *C. diphtheriae*[211].

Management

DAT and antibiotic therapy

The mainstay of treatment is the prompt administration of DAT, which contains antibodies obtained from the serum of horses that have been immunized against DT and, therefore, neutralizes the circulating DT and also reduces disease progression. Some acute cases may require intubation or a tracheotomy[212]. DAT is not effective against DT that has already adhered to body tissues and, therefore, should be administered as soon as possible after the presumptive clinical diagnosis, even before the bacteriological confirmation[213], preferably via intravenous infusion, especially in severe cases[193]. DAT sometimes can induce anaphylaxis in susceptible individuals, for example, in patients with asthma, allergic rhinitis or urticaria who had received a previous dose of serum of horse origin. Hypersensitivity reaction should be prick tested using diluted DAT to test for the development of a skin reaction, such as erythema and itch, within 15–20 min. Recent use of an antihistamine may interfere with the DAT intradermal test and, therefore, histamine should be tested in these patients similar to the DAT prick test. If the DAT intradermal test does not result in a skin reaction, the patient should receive intravenous DAT at increasing doses at regular intervals, to build immune tolerance to DAT[214].

Recommended antibiotics for the treatment of diphtheria include oral penicillin V or erythromycin, or parenteral benzylpenicillin or erythromycin if the patient is unable to swallow[193]. For penicillin-sensitive patients, azithromycin is an alternative. As diphtheria is highly contagious, patients should be kept in an isolation ward. A minimum period of 2 weeks of both antibiotic treatment and culture tests is advocated to ensure elimination of the pathogen. Short-term antibiotic treatment with erythromycin was found to be related to recurrence of the infection[215]. Elimination of the pathogen must be confirmed by negative cultures of two consecutive nasopharyngeal and throat swab samples collected at least 24 h apart and 24 h after the completion of antimicrobial therapy. A further 10 days of antibiotic treatment are needed if the bacteria persist, as confirmed by culture methods. Owing to the infection in the throat, patients might experience pain and difficulty in swallowing solid foods; hence, patients should be provided with liquid nutrition and soft foods until they can swallow comfortably. Long bed rest and avoiding physical exertion might be required during recovery, especially in the case of diphtheritic myocarditis.

Additional considerations

DT causes depletion of myocardial carnitine, which leads to reduced oxidation of long-chain free fatty acids[216]. Carnitine supplementation restores fatty acid oxidation to normal levels, improves ventricular function and reduces mortality associated with diphtheritic myocarditis[212,216–220]. There are also reports on diphtheritic myocarditis treated with a temporary cardiac pacemaker[217]. Erythromycin or penicillin in combination with streptomycin was proposed for early treatment of diphtheritic endocarditis until antibiotic susceptibility testing is completed[221].

There is no specific treatment for diphtheritic polyneuropathy, and the management is conservative. Circulatory collapse is most likely to occur within 4–7 weeks of diphtheritic polyneuropathy, and requires haemodynamic monitoring and vasopressors (which induce vasoconstriction and thereby increase the blood pressure) for 3–10 days. Endotracheal intubation, ventilation support and nasogastric feeding may ensure better survival[222].

C. diphtheriae isolates have often shown either resistance to penicillin[223] or reduced susceptibility to penicillin, cefotaxime, tetracycline and chloramphenicol[70]. Antimicrobial resistance seems to be more frequent in NTCD and *Corynebacterium* spp. other than *C. diphtheriae*[52,224,225]. In vitro studies suggest that reduced susceptibility to penicillin and erythromycin induces biofilm formation and cell surface hydrophobicity; such phenotypes of *C. diphtheriae* may decrease the effect of antimicrobial therapy[226].

Quality of life

A community-based study has shown that diphtheria was one of the infectious diseases identified that may affect mental and physical health-associated quality of life in adults[227]. Based on the severity of diphtheria, the duration of hospital stay may vary from 1 to 3 months[228]. Myocarditis develops in 10–25% of diphtheria patients during the acute phase of illness or after several weeks[191]. The long-term effects of myocarditis include hyaline and granular degeneration of muscle fibres (myolysis), and replacement of lost muscle with fibrotic tissue may cause permanent cardiac damage[114]. Valvular damage may lead to heart failure, and valvular replacement might be required[229]. Diphtheritic polyneuropathy is established as one of the most severe complications of diphtheria: DT is adsorbed on Schwann cells (which provide the myelin sheath for the nerve cells), resulting in the inhibition of myelin synthesis, which in turn can lead to many neurological complications. Depending on the disease severity, diphtheritic polyneuropathy may present in 20–100% of respiratory diphtheria cases[114,230]. Some symptoms of diphtheritic polyneuropathy may have a short-term or long-term effect in both children and adults. Recovery from diphtheritic polyneuropathy may begin 2–4 months after the onset of symptoms[230,231]. The quality of life during and after infections and the long-term effects of neuropathy have yet to be evaluated in detail. Patients diagnosed with diphtheria should be followed up for 3–6 months for the possible development of neurological complications[157].

In addition to effects on individuals' health and quality of life, diphtheria has an economic burden as well (BOX 1). Unfavourable outcomes of diphtheria are mainly related to the lack of knowledge about the disease and late treatment. Practices such as administration of unrecommended antibiotics[35] and improper storage and delivery of the vaccine[232] still exist in many countries.

Box 1 | Economic burden of diphtheria

Evaluation of diphtheria, tetanus and acellular pertussis (DTaP) vaccination in the United States has shown that without a vaccination programme 276,750 diphtheria cases would have occurred, with 27,675 deaths, in a cohort of 4.1 million children. From the economic perspective, these cases would cost approximately US$18,772.4 million[258]. The CDC (Centers for Disease Control and Prevention) contract price under the Vaccines for Children programme is about $18.0 per dose[259]. Reports from the Expanded Program on Immunization Costing (EPIC) project revealed that an average cost per child receiving a combination of diphtheria, tetanus and pertussis (DTP) in six African countries ranged between $27 and $139 (REF.[260]). In some countries, the cost seems high because of the involvement of several factors, such as the status of the hospital, outreach service facility, management, manpower, vaccine delivery to far-off places, extra working days and per-capita gross domestic product. In low-income countries, the cost is about $3.95 per child covered by the three doses of the vaccine for diphtheria, tetanus and pertussis (DTP-3)[261].

Successful treatment depends on the timely administration of DAT with conjunct antibiotic therapy. However, DAT may cause acute or delayed hypersensitivity reactions and, more rarely, acute anaphylactic shock[161]. These adverse effects require immediate treatment and, hence, antihistamines and treatment for anaphylactic shock (including adrenaline) must be kept ready.

Outlook

Strengthening of surveillance and diagnosis

Surveillance of diphtheria is important not only to monitor the disease burden but also to define transmission patterns, detect outbreaks and develop suitable management policies, including vaccination[233]. Diphtheria continues to be one of the main infectious diseases in countries where implementation of the full course of the DTP vaccination programme is not effective, and poses a public health threat in developed countries owing to the emergence of NTCD and species other than *C. diphtheriae*. The NTCD gravis biotype has been isolated with increasing frequency in association with endocarditis and pharyngitis[43]. WGS of *C. diphtheriae* exposed horizontal gene transfer of other virulence factors, such as adhesins, fimbrial proteins and iron-uptake systems[234], thereby increasing the pathogenicity. High-throughput DNA sequencing and bioinformatic approaches might address several unresolved questions on the microbiology and pathogenicity of this organism. Molecular epidemiology of diphtheria could benefit from the application of more precise molecular genetics techniques. WGS (or at least core genome sequencing) analysis could provide a wide range of possible applications, from global tracing of large clonal clusters to fine-tuned strain discrimination. In addition, a multicentre evaluation of recently developed low-cost and discriminatory VNTR and CRISPR methods is warranted to establish whether and in what capacity they could support regional surveillance. Strengthening the microbiological and epidemiological surveillance should be prioritized along with improved strategies for early diagnosis, prevention and treatment measures. National surveillance should ensure early detection and reporting systems for diphtheria outbreaks with reliable identification of toxigenic *C. diphtheriae*, and strengthening of laboratory capacity is essential, with proper external quality assessments. Countries should establish a database for timely reporting of diphtheria cases, such as the existing European Diphtheria Surveillance Network.

Diphtheria vaccines

The Global Vaccine Action Plan 2011–2020, endorsed by the World Health Assembly in 2012, recommends that each country should reach DTP-3 coverage of ≥90% in their routine immunization schedule by 2020 (REF.[235]). The timeliness and completion of childhood DTP vaccination are still a big challenge in many countries. One of the main reasons for low vaccination coverage in many developing countries is the limited awareness about the need for completing the DTP vaccine doses[236]. This aspect should be strengthened through national programmes by governments and non-governmental organizations. Vaccination should also be prioritized for susceptible groups of adults (for example, refugees and asylum seekers) and during outbreak situations.

Several research challenges still persist in advancing vaccine efficacy, including the lack of an international standard for the diphtheria toxoid to be used in the flocculation test (a test with a combination of antigen and antibody that is used to measure the levels of anti-DT antibodies) and blood concentrations of heavy metals, which may lead to reduced levels of antibodies induced by the vaccine[237]. A study conducted in Bangladesh has shown a strong antibody response to diphtheria vaccination with simultaneous administration of vitamin A during DTP immunization[238]. Because elderly people are at increased risk as the antibody concentrations are frequently low (<0.1 IU/ml), it is important to consider vaccination in this group[239]. In addition, fully vaccinated individuals could still acquire the infection with typical diphtheria symptoms; the reason for infection in properly immunized patients who could experience severe clinical outcomes is not well known[240]. In these patients, the role of non-toxigenic *Corynebacterium* spp. that are not influenced by vaccination status cannot be ruled out.

DAT treatment

Although DAT is listed by the WHO as an essential medicine, global access to DAT for both therapeutic and diagnostic purposes seems increasingly arduous, owing to limited production and low market demand, which are due to the decline in the incidence of diphtheria and the intensified use of the vaccine. Currently, DAT is manufactured in few countries, leading to short supply and logistical problems[241]. Hence, there is an urgent need for a safe, cost-effective, quality substitute for DAT. Anti-DT antibody made from unused human blood stocks was tested as a substitute to horse-derived DAT[242]. This practice not only requires administration of large volumes of pooled sera to neutralize the DT but also exposes the patients to blood-borne pathogens.

In the past, peptides that bind to the HB-EGF precursor (thereby preventing DT from binding and being internalized), neutralizing murine monoclonal antibodies against DT A-subunit and human monoclonal antibodies from antibody-secreting plasma cells in the circulation of immunized humans have been established and tested effectively in vitro and in vivo[243–246]; however, these products were not carried forward for further development or past the preclinical stage. A recently discovered human monoclonal IgG1 anti-DT antibody (antibody number S315), which is specific and neutralizes the toxin, showed promise for therapeutic use[243,247].

Several small-molecule compounds exhibit protection against DT by inhibiting the transport of the toxin at various stages in the endocytic pathway[248]. The compound 4-bromobenzaldehyde *N*-(2,6-dimethylphenyl) semicarbazone (EGA) has been shown to protect human cells from intoxication with DT and other bacterial toxins by inhibiting the pH-dependent translocation of the DT catalytic A-subunit across cell membranes, as it interferes with the uptake into the host cell cytosol[249]. These results suggest that EGA may act as a potential candidate for the treatment and/or prevention of diphtheria. The specific mode of action for how other compounds prevent the transport of DT into the cell requires further investigation.

Antibiotic resistance

Antimicrobial resistance in toxigenic *C. diphtheriae* has not been a major problem in the treatment of diphtheria, except sporadic reports from a few countries. However, the other species, *C. ulcerans* and *C. pseudotuberculosis*, are reportedly resistant to many drugs, including penicillin[224,250,251]. Multidrug-resistant *C. pseudotuberculosis* and *C. ulcerans* were reported to cause nosocomial infections[252]. Resistance to daptomycin among non-*C. diphtheriae* isolates and penicillin-resistant and cephalosporin-resistant cutaneous *C. diphtheriae* are increasingly detected in clinical cases[225,253,254]. The genetic characteristics of area-specific *C. diphtheriae* variants are influenced by several factors, including antibiotic pressure, as unique trends in resistance prevail in certain geographical regions, for example, resistance to tetracycline in Indonesia[255], erythromycin in Vietnam and rifampin in France[136,256]. Studies on the mechanisms of antimicrobial resistance have to be intensified; for example, a study showed that the mycomembrane of *Corynebacterium* spp., which is rich in mycolic acids (long fatty acids), helps to protect the bacteria from the antibiotic pressure[257], but its potential as a drug target has not yet been evaluated.

Diphtheria is a life-threatening disease that requires early detection, rapid treatment and intensive care interventions in very severe cases. Diphtheria must be recognized as another important vaccine-preventable disease of the modern age. Prevention via immunization programmes along with booster doses must be prioritized. The manufacturing of DTP vaccine and DAT should be expedited, and a global stockpile should be created and readily available to all regions of the world. The antigen content of the vaccines, including the booster vaccines, and molecular characterization of *C. diphtheriae* must be constantly evaluated. Diphtheria is very much still present in both the developed and the developing world, and increased clinical awareness needs to be addressed urgently.

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Author contributions

Introduction (N.C.S. and T.R.); Epidemiology (N.C.S., A.E., I.M. and B.D.); Mechanisms/pathophysiology (T.R., B.D. and A.E.); Diagnosis, screening and prevention (N.C.S., A.E., I.M. and A.M.); Management (N.C.S. and A.E.); Quality of life (T.R., A.E. and A.M.); Outlook (T.R. and A.E.); Overview of the Primer (T.R.).

Competing interests

All authors declare no competing interests.

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