Corynebacterium: Molecular Typing and Pathogenesis of *Corynebacterium diphtheriae* and Zoonotic Diphtheria Toxin-Producing *Corynebacterium* Species

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1.1 Introduction

Molecular typing techniques have been successfully applied for determining virulence potential, origin, and routes of diphtheria and atypical invasive infections, confirming endemicity, outbreaks, and trace cross-transmission caused by *Corynebacterium diphtheriae* and zoonotic toxin-producing *Corynebacterium* species.

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1.2 Clinical Significance and Epidemiology of Diphtheria Toxin-Producing *Corynebacterium* spp.

Corynebacterium diphtheriae is a major etiologic agent of classic respiratory diphtheria, including local pharyngeal symptoms and systemic manifestations, mainly caused by the action of diphtheria toxin (DT). Symptoms typically begin 2 to 5 days after infection. *C. diphtheriae* usually localizes in the upper respiratory tract, ulcerates the mucosa, and induces the formation of an inflammatory pseudomembrane. Systemic toxicity increases as the pseudomembrane spreads from the tonsillopharyngeal area. A form of malignant diphtheria is associated with extensive "membranous pharyngitis" plus massive swelling of the tonsils, uvula, cervical lymph nodes, submandibular region, and anterior neck (the so-called bull neck of toxic diphtheria). Acute disease of the respiratory tract usually involves one or more of the following: tonsillar zones, larynx, soft palate, uvula, and nasal cavities or, less commonly, in the stomach or lungs. The exceedingly potent DT is absorbed into the circulation, and lesions may also occur in vital organs, including the heart (myocarditis), nervous system, and kidneys, potentially resulting in death [1].

Diphtheria toxin is an extracellular protein that inhibits protein synthesis and ultimately exerts death of susceptible eukaryotic cells. DT was the first member to be identified of a group of bacterial protein toxins that act by ADP ribosylation of a target protein. DT contains a toxic A subunit (active toxin with enzymatic activity) and the receptor binding B subunit. The B subunit (fragment) facilitates translocation of the A subunit from the phagosome to the cytosol, followed by separation, allowing full activity of the A subunit on its target protein elongation factor-2 (EF-2). EF-2 transfers polypeptidyl-transfer RNA from acceptor to donor sites on the ribosome of the host cell. The A subunit catalyzes the transfer of adenine, ribose, and phosphate from NAD to EF-2 (ADP ribosylation), inactivating EF-2 and turning off inhibiting protein synthesis. DT causes local destruction at the site of membrane formation and may be also absorbed into the bloodstream and distributed, resulting in systemic complications including demyelinating neuritis and myocarditis. The *tox* gene that encodes DT is present in β and ω corynephages, and DT is only produced by *C. diphtheriae* isolates that harbor *tox*+ corynephages. Although the *tox* gene is part of the phage genome, the regulation of DT expression is under bacterial control, as the corresponding iron-sensing regulator DtxR is encoded by a gene on the *C. diphtheriae* chromosome [2–4].

Cutaneous diphtheria is the most common nonrespiratory clinical manifestation of infection due to *C. diphtheriae* strains. The disease is characterized by the presence of shallow skin ulcers, usually chronic, which can occur anywhere on the body, mostly on the legs, feet, and hands. This type of diphtheria may cause pain, redness, and swelling similar to other bacterial cutaneous infections. Cutaneous diphtheria is likely to be diagnosed less quickly than respiratory infection due to the nonspecific clinical appearance and often coinfections with pathogens, mostly *Staphylococcus aureus* and *Streptococcus pyogenes*. Cutaneous diphtheria frequently occurs in warm tropical climates and is normally associated with colonization of preexisting

skin lesions, including surgical wounds, burns, impetigo, psoriasis, leishmaniotic ulcers, and insect bites [5, 6].

In the United Kingdom, cases of travel-related cutaneous diphtheria were reported, including patients with high diphtheria vaccination coverage and from tropical countries. The authors emphasized that with increasing travel to and from diphtheria-endemic countries, more cases may occur. These lesions are an important reservoir of infection and can cause respiratory and cutaneous infections in contacts as well as outbreaks. In several outbreaks, secondary transmission has been higher in contacts of patients with cutaneous infection than in those with respiratory tract infection. Cutaneous diphtheria may also cause greater environmental contamination, through dust and fomites. The potential for secondary transmission leads to a large number of contacts requiring follow-up, especially children at school [6]. Therefore, awareness of clinicians and microbiologists of the importance of obtaining swab specimens from any chronic nonhealing skin lesions in patients who have traveled to or a disease-endemic area is necessary, especially in tropical countries. Wound swab samples from these patients should be examined for diphtheria toxin-producing *Corynebacterium* species. Early diagnoses and reporting are crucial to trigger effective public health control measures (skin ulcers, which can occur anywhere on the body and are usually chronic) [5–8].

In many countries, diphtheria is considered an infrequent disease once there are treatment and diphtheria toxoid-containing vaccines to prevent it. Since 1990, diphtheria reemerged in the Russian Federation and spread to all Newly Independent States (NIS) and Baltic states. Awareness of characteristics of the largest diphtheria epidemic in the last decades that seized several European countries should be used to help predict the spread of future epidemics. The epidemic demonstrated conclusively the potential susceptibility of adults to diphtheria in the vaccine era. Important characteristics included, among several other factors, the emergence of distinct epidemic clonal group, a progressive spread of disease from urban centers to rural areas. However, epidemic diphtheria outbreaks remain poorly understood and continues to challenge industrialized and developing countries. Higher risk of acquiring *C. diphtheriae* infections and potentially life-threatening complications may be possible related to inadequately immunized or unimmunized conditions of persons and travelling from/to countries with endemic diphtheria [5, 9–12].

Although immunization is one of the most successful and cost-effective health interventions known, there are still many regions of the world with low vaccine coverage. Diphtheria caused by *C. diphtheriae* is still endemic worldwide, mostly among developing countries, including Nigeria, Venezuela, India, and Brazil. Diphtheria resurgence or epidemic outbreaks remain an important cause of morbidity and mortality that may occur in places where vaccination programs are not maintained or there is a proportion of adults susceptible to disease due to decline in antibody levels provided by vaccine, especially in low socioeconomic and health conditions areas (Fig. 1.1) [14–21]. Previous investigations reported an epidemic outbreak in Dhule, a predominantly tribal and rural district in Northern Maharashtra, India, with diphtheria cases mostly observed among adolescents (10–15 years), despite poor immunization coverage (below 50%) [17]. A diphtheria outbreak was
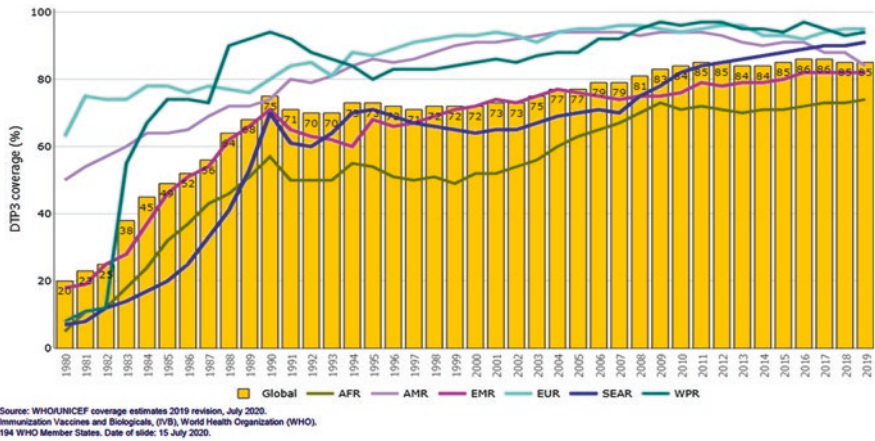
Fig. 1.1 Diphtheria global immunization 1980–2019. Global coverage from three doses of a diphtheria toxoid-containing vaccine (DTP) at 85% in 2019 [13]

also verified in villages of three municipalities of Maranhão, a northern state from Brazil. Most cases occurred in partially or completely immunized patients, including pharyngitis without pseudomembrane formation [22].

Clinical features of diphtheria in partially vaccinated patients may still be similar to those that were observed in the pre-vaccine era. However, mass vaccination has also altered clinical features of some diphtheria cases, independent of immunization status and age of individuals. Therefore, health professionals should be aware of the possibility of atypical cases of DT-producing *C. diphtheriae* infections, including pharyngitis without pseudomembrane formation. Cases of coincidental respiratory diphtheria with infectious mononucleosis were also reported [5, 17, 22, 23].

C. diphtheriae has been increasingly reported not only as the etiological agent of diphtheria but also as the causative agent of atypical invasive infections. *C. diphtheriae* was originally characterized as an extracellular pathogen with local growth pharynx mucosa. During the last decades, cases of atypical and/or invasive infections caused by both non-DT-producing and DT-producing *C. diphtheriae* strains have been reported, such as pneumonia, arthritis, endocarditis, bacteremia, and catheter-related infections, including cancer patients, leading patients to death in varied opportunities independent of age and sex. Septicemia, renal failure, and/or arthritis are frequently reported in patients with *C. diphtheriae* endocarditis [24–30].

C. diphtheriae is usually transmitted by respiratory droplets, direct contact, and fomites when individuals are at home or during occupational activities, especially in laboratory and hospital environments. In 1941, during the pre-vaccine era, the occurrence and persistence of diphtheria bacilli in floor dust of hospital wards and the resultant contamination of the air were verified. DT-producing *C. diphtheriae* strains were found capable to survive in dust and clothing for an extended period of time [31]. Once considered a strictly human pathogen, *C. diphtheriae* strains have been found to be able to infect animals, including cows, horses, and cats [32–35].

Changes of varied aspects in the epidemiology of diphtheria pathogens have been occurring worldwide. Starting in the middle of the 1980s, DT-producing and non-DT-producing *Corynebacterium ulcerans* have been increasingly identified as the etiologic agent of diphtheria of zoonotic nature and extrapharyngeal infections in varied industrialized and developing countries, including immunized individuals in the American continent [8, 12, 36–40]. Similar to *C. diphtheriae* strains, *C. ulcerans* strains were found to produce clinical syndromes of the lower respiratory tract, such as pneumonia and pulmonary granulomatous nodules, independent of DT production [36, 41–44]. At first, zoonotic diphtheria cases were mainly restricted to rural populations and associated with contact with dairy cattle and consumption of unpasteurized dairy products. Lately, *C. ulcerans* has been increasingly isolated as emerging zoonotic agent from companion animals such as cats and dogs. Therefore, there is a potentially large reservoir of infection with little knowledge about the risks of zoonotic transmission, since *C. ulcerans* strains were already found among animals from farms, domestic and natural settings [8, 37–39, 45].

Since the epidemic in European countries during the 1990s, the number of diphtheria cases due to *C. ulcerans* was found to exceed the number of reported cases related to *C. diphtheriae* [45]. Although *C. ulcerans* have been increasingly recognized in several industrialized countries as an emerging zoonotic pathogen, its capacity to cause disease in humans, including among the inhabitants of urban centers, is still often neglected [38]. Detection of *C. ulcerans* strains in Canada, during the period of 2006–2019, showed that 77% of the isolates were from humans and mostly obtained from cutaneous sites and 23% were from animals – mink (lung), dog (ear), and cat and horse (abscess and skin) – comprising 45% DT-producing strains [39]. In Brazil, a case of concurrent zoonotic diphtheria by *C. ulcerans* and infectious mononucleosis (IM) was first reported in the literature [46]. Moreover, a case of fatal pulmonary disease caused by a unusual penicillin and clindamycin resistant non-DT-producing *C. ulcerans* disseminated from primary nonhealing lesions on lower legs was documented. Both legs of the elderly patient with *C. ulcerans*-invasive infection presented skin ulcers covered by yellowish membranes [36]. Previous studies also reported the isolation of *C. ulcerans* strains from nares and/or skin wounds of asymptomatic dogs (companion dogs or kept in animal shelter), from Duque de Caxias and Niterói cities, located at the metropolitan area of Rio de Janeiro [37, 47].

Similar to *C. ulcerans*, *Corynebacterium pseudotuberculosis* is also a zoonotic etiologic agent and also considered a public health concern. *C. pseudotuberculosis* is a diphtheria toxin-producing pathogen of medical, veterinary, and biotechnological interest that mainly affects small ruminants, causing caseous lymphadenitis (CLA), throughout the world and generates significant economic losses. Sheep and goats are the most common animals infected within the broad spectrum of hosts in which *C. pseudotuberculosis* causes clinical disease. This zoonotic pathogen may also infect bovines, pigs, and equines. Therefore, contamination of meat and milk by *C. pseudotuberculosis* may possibly occur, putting children and adult consumers at risk. However, human infections due to *C. pseudotuberculosis* remain apparently

rare and have been mostly reported among those with close contact to animals, including farm workers and travelers to rural areas [48–50].

In many countries, routine procedures for identification of DT-producing *Corynebacterium* spp. are uncommonly undertaken by diagnostic laboratories due to a low prevalence of diphtheria cases alongside the difficulties of diagnosis through conventional biochemical tests and the ever-increasing need for cost-effectiveness. This fact contributes to justify the low number of reported cases of human infections by zoonotic *C. pseudotuberculosis* strains over the years [49].

In a previous investigation of zoonotic potential of *C. pseudotuberculosis*, a summary data from all 33 cases of human infections reported over a period of 42 years (from 1966 to 2008) showed that a main group of non-DT-producing *C. pseudotuberculosis* strain-infected patients presented a characteristic of lymphadenopathy. Profiles of most of these patients included adult males, 21–40 years old, and previously exposed to raw milk or meat, farm animals (mostly sheep), and/or rural areas. Only two cases involved clinical presentations other than the characteristic lymphadenopathy: from the United States, a 28-year-old (male) veterinary student who worked with equines, diagnosed with eosinophilic pneumonia, and from China, a 63-year-old (male) with ocular infection post-retinal reattachment intervention. Until the present moment, only one case of human infection due to DT-producing *C. pseudotuberculosis* was reported. The zoonotic pathogen was isolated in the United Kingdom from the aortic root vegetation of an intravenous drug user with endocarditis [50–53].

1.3   Treatment and Prevention of DT-Producing *Corynebacterium* spp. Infections

Antibiotics are needed to kill DT-producing *Corynebacterium* spp., eliminate diphtheria toxin production, limit carriage that may persist even after clinical recovery, and prevent further transmission from asymptomatic carriers and colonization of close contacts. Penicillin and erythromycin have long been the drugs of choice for the eradication of DT-producing strains of *Corynebacterium* spp. The World Health Organization (WHO) has recently added azithromycin as part of the standard antibiotics for these pathogens. However, the increasing problems of resistance to penicillin, oxacillin, erythromycin, and other drugs including rifampicin, tetracycline, and clindamycin are examples of challenges confronting both industrialized and developing countries. Resistance to ß-lactams should also be considered in invasive infections, since failure to eliminate *C. diphtheriae* in cases of endocarditis treated with penicillin has been reported. Data emphasize the need for a continuous survey of antibiotic susceptibility for these pathogens, especially in tropical and developing countries where diphtheria is endemic and invasive infections may occur [20, 22, 28, 46, 54–57].

In cases of classic and zoonotic diphtheria, patients with severe infections should be immediately admitted to a hospital *intensive care unit* and given diphtheria *antitoxin (DAT),* consisting of *antibodies* isolated from the *serum* of horses that have

been challenged with diphtheria toxin. Since antitoxin does not neutralize toxin that is already bound to tissues, delaying its administration increases risk of death. Therefore, the decision to administer diphtheria antitoxin is based on clinical diagnosis, and should not await laboratory diagnosis [20]. Administration of diphtheria vaccine is recommended during convalescence because diphtheria infection does not always confer immunity [53].

In the early 1880s, *C. diphtheriae* was first visualized in stained specimens from pseudomembranes and shown to be the cause of diphtheria isolated by bacteriologists Edwin Klebs and Friedrich Loeffler. In 1890, Emil von Behring isolated the first diphtheria antitoxin from blood samples of an infected horse. A few years later, William H. Park and Anna W. Williams isolated a *C. diphtheriae* strain that produced an unusually large amount of diphtheria toxin, later named the Park-Williams no. 8 (PW8) strain. Since the 1920s, a diphtheria toxoid vaccine has been produced from diphtheria toxin treated with formalin to inactivate the toxicity and to maintain the immunogenicity of the protein. *C. diphtheriae* PW8 is lysogenized by two copies of corynephage ω^{tox+}, suggesting that the enhanced DT synthesis is due to a gene dosage effect of the *tox* gene [3].

Protection against diphtheria is mainly due to the development of neutralizing toxin antibodies. Diphtheria antitoxin production, primarily of IgG type, can be induced by absorption of native toxin during clinical infection or in the carrier state or by immunization with diphtheria toxoid. It is believed that a circulating diphtheria antitoxin level of 0.01 IU/ml, as determined by the neutralization test in animals or in cell culture, provides clinical immunity against disease. The outcome of revaccination of adults depends on several factors, including the immunization schedule, potency, and time since the last dose of toxoid [58, 59]. In developing countries where diphtheria is endemic, the process of maintaining immunity usually operates through natural mechanisms, including frequent skin infections caused by *C. diphtheriae*. Nowadays, adults might become susceptible to diphtheria due to reduced opportunities of subclinical infections. Since diphtheria infection may also occur among previously vaccinated persons, the immunity gap observed among adults should be closed by regular diphtheria boosters [9, 60–62].

1.4 Identification of DT-Producing *Corynebacterium* spp. in Diagnostic Laboratories

Accurate and fast diphtheria laboratory diagnosis is not only a matter of acute patient management but also an important issue in public health due to international notification and management requirements, and there is an urgent need for a reliable, robust, and fast laboratory method for diagnosing DT-producing *Corynebacterium* spp., especially in the light of the continuing loss of laboratory expertise even in national reference laboratories for diphtheria [5, 7, 8, 22, 63].

Phenotypic characterization of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* may be performed by conventional biochemical assays and semiautomatized systems, including API Coryne System (bioMérieux). However, it takes at

least 16 hours after isolation of suspicious colonies from screening plates of catalase-positive irregular Gram-positive bacilli (IGPB) [7, 22, 29, 36, 64].

In many countries, routine procedures for identification of *C. diphtheriae* and *C. ulcerans* are not commonly undertaken by diagnostic laboratories due to a low prevalence of diphtheria cases in recent years alongside the ever-increasing need for cost-effectiveness. Consequently, many diagnostic laboratories have suspended screening for diphtheria etiologic agents, further increasing the potential for missed and delayed diagnoses. Therefore, screening tests remain currently essential for the presumptive identification of these pathogens in clinical microbiology laboratories [5, 8, 22, 28, 65].

The use of DNase screening test provided a substantial improvement in the existing standard identification algorithm of DT-producing *Corynebacterium* spp. of routine diagnostic laboratories. DNase assays have been useful for differentiating DNAse-positive *C. diphtheriae* and *C. ulcerans* from DNAse-negative *C. pseudotuberculosis* and other suspected pathogenic corynebacteria, particularly in the surveillance of cases of diphtheria, asymptomatic carriers, and invasive infections in endemic or epidemic areas with unfavorable economic conditions [64]. The reverse CAMP test is particularly a screening assay effectively used as part of the identification of *C. ulcerans* and *C. pseudotuberculosis* zoonotic pathogens. A reverse CAMP test is based on the inhibition of hemolytic activity of beta-hemolysin from *S. aureus* through the production of phospholipase D by *C. ulcerans* and *C. pseudotuberculosis* [36].

The application of molecular techniques for the identification of bacterial pathogens has been expanded for use in clinical microbiology laboratories. Molecular procedures have been also proposed for the identification of *Corynebacterium* species. Improvements should become widely available for the rapid and precise detection of DT-producing *Corynebacterium* spp., including direct analysis of swabs and other clinical samples, as already done with *C. diphtheriae* and *C. ulcerans* in some laboratories [66–68].

1.5   MALDI-TOF Assays

Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) is one of the most recently established technologies used for species identification based on the protein composition of microbial cells. MALDI-TOF MS has been increasingly applied worldwide in routine analysis of clinical microbiology laboratories due to easy procedure, rapid results (15 minutes), and accurate identification of several bacterial species, including misidentified pathogens in specific clinical specimens. MALDI-TOF MS became a powerful tool that has initiated a revolution in the clinical microbiology laboratory for identification of nosocomial pathogens, including *Corynebacterium* spp. Misidentification of some human-pathogenic clinical isolates commonly occurs when using conventional and commercially available methods in microbiology laboratories. The ability to rapidly identify bacterial species, including rarely described as pathogens in specific

clinical specimens, may help to study the clinical burden resulting from the emergence of these species as human pathogens and MALDI-TOF MS may be considered an alternative to DNA-based methods in clinical laboratories. Due to the difficulties of diagnosis in the laboratory routine through conventional biochemical tests, MALDI-TOF MS also represents an important alternative method for the identification of *C. diphtheriae*, *C. ulcerans*, and *C pseudotuberculosis* strains [39, 46, 69–71].

1.6   DNA-Based Methods

The timely and precise diagnosis of corynebacterial infections, especially those involving DT-producing strains, is indispensable for the patient management and for establishment of surveillance and control strategy of the disease. Consequently, different molecular methods, such as end-point and real-time PCR (polymerase chain reaction), have been used since the 1990s for the characterization of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* [7, 49, 66, 68, 72–78]. Since the investigation of the toxigenic potential of clinical isolates is one of the most critical aspects of diphtheria diagnosis, conventional end-point PCR assays targeting the *tox* gene were the first to be developed. Subsequently, the *tox* gene detection was combined with species identification PCR targets, as *dtx*R (*d*iphtheria *t*oxin *r*epressor gene) from *C. diphtheriae*, in multiplex assays [7, 49, 66, 68, 72, 73]. Since detection of the *tox* gene only provides presumption of toxigenicity, additional phenotypic investigations such as Elek test and Vero cell cytotoxicity assays have been currently used to demonstrate DT production by *Corynebacterium* strains [7, 66, 74].

Multiplex PCR represents a fast, simple, and reliable methodology for identification and differentiation between DT-producing and non-DT-producing strains of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*. In Brazil, an mPCR protocol was developed, and it has been used for clinical diagnosis and epidemiological and virulence research, during the last decade. Direct analyses of swabs and other clinical samples have also been done. Brazilian mPCR allows the detection of *tox* gene from potentially DT-producing *Corynebacterium* spp., in addition to 16S rRNA from both *C. pseudotuberculosis* and *C. ulcerans*, *pld* from *C. pseudotuberculosis*, *dtx*R from *C. diphtheriae*, and *rpo*B from *Corynebacterium* spp. [22, 27, 37, 46, 49].

Real-time PCR instruments are increasingly common in public health laboratories, mostly in industrialized countries. Real-time PCR (qPCR for quantitative PCR) presents some advantages than classical PCR, including faster data collection, low contamination risks, and high sensitivity, especially for pathogen detection in host carriers and clinical samples which often contain components that inhibit PCR [69, 79].

During the last decades, different qPCR assays have been developed for detection of the *tox* gene and/or identification of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* directly from clinical samples. However, similarly to end-point PCR,

currently available qPCR assays allow only the detection of *tox* gene from *Corynebacterium* spp. strains. However, the confirmation of DT expression still requires phenotypic investigations [66, 69, 75, 80, 81].

1.7   rpoB Gene Sequencing Technique

The genus *Corynebacterium* is a heterogeneous group of species comprising human and animal pathogens and environmental bacteria. It is defined on the basis of several phenotypic characters and the results of DNA-DNA relatedness and, more recently, 16S rRNA gene sequencing. The *rpoB* gene, encoding the beta-subunit of RNA polymerase, has emerged as a core gene candidate for phylogenetic analyses and identification of corynebacteria, especially when studying closely related isolates. However, the 16S rRNA gene is not polymorphic enough to ensure reliable phylogenetic studies and needs to be completely sequenced for accurate identification. Previous studies verified that higher proportions (91%) of corynebacterial isolates were positively identified by partial *rpoB* gene determination than by that based on 16S rRNA gene sequences [82–84].

1.8   Diphtheria Toxin-Producing Group Becoming Diverse: A Novel *C. diphtheriae* Complex

C. diphtheriae was historically classified into four biovars – *gravis*, *mitis*, *intermedius*, and *belfanti* – based on biochemical phenotypic testing [4, 85]. Recent investigations documented *C. diphtheriae* to be genetically heterogeneous and that genomics does not support the use of biovars to reliably classify diphtheria bacilli isolates, since *C. diphtheriae* strains within a certain biovar were found to be genetically more distant than between biovars [3, 86, 87].

A switch in populations causing endemic infections from DT-producing to non-DT-producing isolates in the 1990s and the 2000s and other countries with vaccination coverage has been documented as a direct consequence of the large-scale use of diphtheria toxoid. In Brazil, several *C. diphtheriae* isolates were found capable of degrading sucrose, a phenotypic characteristic rarely described in other parts of the world. Results of *rpo*B sequence analysis confirmed all sucrose-fermenting isolates as *C. diphtheriae* species. Sucrose-fermenting and DT-producing *C. diphtheriae* strains were predominantly isolated from human respiratory tract of diphtheria patients. However, cases of endocarditis due to sucrose-fermenting *C. diphtheriae* phenotypes were also observed in South American countries [28, 88, 89].

During the 1980s and 1990s, studies dealing with *C. diphtheriae* biovar belfanti were occasionally reported in literature. *C. diphtheriae* biovar *belfanti* were mostly isolated from human respiratory samples. In Brazil, the case of pulmonary infection in a cancer patient was reported [90]. The *tox* gene, which codes for diphtheria toxin, was infrequently reported in isolates of biovar *belfanti* [7, 90–93].

Taxonomic status of *C. diphtheriae* biovars has been increasingly investigated [91, 93, 94]. Phylogenic analysis described two lineages of non-DT-producing *C. diphtheriae* biovar belfanti obtained from 18 countries, covering a time period from 1957 to 2006 [91]. In France, the number of non-DT-producing *C. diphtheriae* biovar *belfanti* increased between 1977 and 2011, and it is the most frequent biovar recovered in recent years. Non-DT-producing belfanti isolates were mostly isolated from human respiratory samples, including from a woman presenting with rhinitis. However, there were also found *belfanti* phenotypes isolated from blood, skin, and bone lesions. Phylogenic analyses of French non-DT-producing *C. diphtheriae* biovar *belfanti* human isolates were distributed among three distinct lineages. Almost all belfanti isolates belonged to a single clonal complex. A third new lineage was composed of a single clinical isolate (rhinitis) and phylogenetically distant from other two partially studied belfanti lineages from France [91, 94].

Recent investigations by genomic sequencing, biochemical, and chemotaxonomic analyses indicated that *C. diphtheriae* biovar belfanti represents a branch that is clearly demarcated from *C. diphtheriae* biovar *mitis* and *gravis*. Data, including the inability to reduce nitrate, allowed to differentiate biovar *belfanti* from other *C. diphtheriae* strains. Consequently, the name *Corynebacterium belfantii* sp. nov. for the group of nitrate-negative strains, previously considered as *C. diphtheriae* biovar *belfanti*, was recently reported [95].

In a further study, it was proposed that *C. diphtheriae* taxon should be subdivided into two subspecies, *C. diphtheriae* subsp. *diphtheriae* and nitrate-negative *C. diphtheriae* subsp. *lausannense* [93]. However, given that *C. belfantii* was validly published a few months before the taxonomic proposal *C. diphtheriae* subsp. *lausannense* was validated, the latter subspecies was suggested to be a heterotypic synonym of *C. belfantii* [92].

Most recently, a group of clinical isolates previously identified as *C. diphtheriae* biovar *belfanti* strains isolated from human cutaneous or peritoneum infections and from one dog were characterized by genomic sequencing, biochemical analysis, and MALDI-TOF assays as *Corynebacterium rouxii* sp. nov. for the novel group. Phenotyping data revealed an atypical negative or heterogeneous intermediate maltose fermentation reaction for both human and animal isolates. Atypical, maltose-negative, and DT-negative *C. diphtheriae* biovar *belfanti* isolated from domestic cats, including with severe otitis, was also previously described in the United States [33, 92].

C. pseudotuberculosis are classified into biovars *equi* and *ovis* based on the ability to convert nitrate to nitrite, due to genetic characteristic that includes the presence of the nitrate reduction operon: *equi*, nitrate-positive strains, and *ovis*, nitrate-negative strains. Disease caused by *C. pseudotuberculosis* biovars has different clinical manifestations in the susceptible hosts, and biovar identification is important for understanding the epidemiology of infection and consequently for disease control. *C. pseudotuberculosis* biovar *equi* strains are etiologic agents of ulcerative lymphangitis in horses, cows, camels, buffaloes, and occasionally humans. *C. pseudotuberculosis* biovar *ovis* strains are the causative agents of caseous lymphadenitis (CLA) in small ruminants, mostly ovine and caprine herds. CLA

causes important economic losses in ovine and caprine herds by reducing wool, meat, and milk production. Cases of human infections due to non-DT-producing *C. pseudotuberculosis* biovar *ovis* were more frequently described worldwide than cases by biovar *equi*. Lymphadenitis in human hosts due to *C. pseudotuberculosis* was also reported in literature, mostly occurring in those who were visitors or were occupationally exposed to animals in rural areas, especially sheep farms [78, 96, 97].

Only one case of DT-producing *C. pseudotuberculosis* was described in literature. The zoonotic pathogen was isolated from the aortic root vegetation of an intravenous drug user with endocarditis; this patient had no history of animal contact, and no possible source of infection was identified. This isolation occurred in the United Kingdom and biovar was not reported [53].

1.9   Pathogenomics of Potentially DT-Producing *Corynebacterium* spp.

Diphtheria toxin is one of the best investigated bacterial toxins and a leading virulence factor of toxigenic *C. diphtheriae* and *C. ulcerans* strains. Different investigations demonstrated that *tox* genes of *C. diphtheriae* strains showed similar nucleotide sequence identity. Phylogenetic analyses of *C. ulcerans* revealed diverse diphtheria toxin suggesting that *C. ulcerans* tends to acquire mutations more frequently than *C. diphtheriae*. Two possible explanations for this phenomenon are that *C. ulcerans* strains are maintained by various animals and have a phage-independent pathway to acquire the DT-encoding gene, increasing its diversity compared with *C. diphtheriae* [2, 3, 98, 99].

The diphtheria toxin repressor DtxR is known as an iron-dependent regulator that controls the transcription of the diphtheria toxin gene *tox* and a complex gene regulatory network involved in iron homeostasis. Variations of *dtx*R genes and in the regulatory network of DtxR might lead to differences in iron supply of the bacterial cell, thereby influencing the expression of the *tox* gene and the virulence of DT-producing *Corynebacterium* spp. [3, 100, 101]. Therefore, naturally occurring diversity of *tox* genes and variations on the expression of diphtheria toxin due to *dtx*R regulatory activities may exert influence on efficacy of diphtheria toxoid vaccine and diphtheria antitoxin for preventing and treating infections caused by DT-producing *Corynebacterium* spp. pathogens [3, 5, 99, 100].

The occurrence of diphtheria among immunized persons, as well as the increasing frequency of cases of atypical and invasive diseases, caused by non-DT-invasive clones also points the relevance of multiple virulence factors of the potentially DT-producing *Corynebacterium* spp. [26, 30, 37, 102–105]. In previously reported cases of invasive infections, non-DT-producing *C. diphtheriae* and *C. ulcerans* strains were found capable of expressing additional proteins with cytotoxic effects similar to Shiga-like toxins, characterized as ribosome-binding proteins (Rbps). Experimental evidence for the cytotoxic function of Rbps toxins were provided by the interaction of *C. diphtheriae* and *C. ulcerans* wild-type, mutant, and complementation as well as overexpression strains with invertebrate model systems,

Caenorhabditis elegans and *Galleria mellonella*, and on various animal and human macrophage and epithelial cell lines, including Vero cells [28, 36, 105, 106].

The dermonecrotic phospholipase D (PLD) exotoxin may be also produced by both zoonotic pathogens that have been investigated as a prominent virulence factor, especially for *C. pseudotuberculosis*. The *pld* gene encoding the phospholipase D is included among the subset of genes homologous for *C. pseudotuberculosis* and *C. ulcerans* species. Studies with *C. pseudotuberculosis* strains with inactivated PLD have convincingly demonstrated the necessity of PLD for establishment of diseases in animals, including caseous lymphadenitis [8, 46, 98, 107, 108]. However, a case of diphtheria due to *C. ulcerans* strain that is unable to express both PLD and DT activities was recently reported. These data emphasize that virulence mechanisms and pathogenic potential of *C. ulcerans* species may arise independent of PLD and DT production. *C. ulcerans* virulence potential and zoonotic pathogenicity traits need further investigation [36–38, 46, 98, 104, 105, 109].

Basic mechanisms and specific virulence determinants, other than DT, involved in the pathogenic potential of *C. diphtheriae* have been investigated for almost half a century [110]. Since 2003, data from whole-genome sequencing (WGS) of *C. diphtheriae*, including a pangenomic study with Brazilian clinical isolates from cases of classical diphtheria, endocarditis, and pneumonia, involving sucrose-fermenting *C. diphtheriae* strains, exposed horizontal gene transfer of virulence factors, such as adhesins, fimbrial proteins, and iron uptake systems [3, 111].

In an attempt to further investigate mechanisms that promote *C. diphtheriae* survival within different environmental conditions, infection and dissemination through host tissues, several features have been of concern [112–114]. A putative determinant (CDCE8392_813 gene), coding for tellurite (TeO_3^{2-}) –resistance (TeR) was detected, and the influence on virulence attributes of *C. diphtheriae* strains was verified. Tellurium (Te) is a metalloid that exists as a trace component in natural environments. Although TeO_3^{2-} is toxic to most microorganisms, TeR bacteria, including *C. diphtheriae*, exist in nature. The presence of TeR determinants in pathogenic bacteria might provide selective advantages in the natural environment. The *C. diphtheriae* TeR-disrupted mutant strain expressed increased susceptibility to TeO_3^{2-} and reactive oxygen species (hydrogen peroxide) but not to other antimicrobial agents. Moreover, TeR determinants contributed to the survival of *C. diphtheriae* strains by using in vivo and in vitro models of infection [115].

The ability of biofilm formation on varied biotic and abiotic surfaces was also investigated. Low-dose antibiotics was reported to favor biofilm formation by *C. diphtheriae*, similar to observations for other human pathogens. *C. diphtheriae* strains expressed higher cell-surface hydrophobicity and biofilm formation on different abiotic surfaces in the presence of penicillin and erythromycin. Moreover, *C. diphtheriae* was also recognized as a potential cause of catheter-related infections, independent of DT production [29, 89, 102, 115–118].

C. diphtheriae was also found to express the ability to invade and survive within different types of human cells and the capacity to cause invasive bloodstream infections. Systemic complications of *C. diphtheriae* bacteremia are not unusual and include endocarditis, joint infections, and peripheral embolic disease. A

strain-dependent ability to induce osteomyelitis by DT-negative *C. diphtheriae* strains was probed by an in vivo assay using Swiss Webster mice, as first reported for *C. ulcerans* [27, 104].

Mechanisms of interaction with different cell types have been also investigated, such as erythrocytes, macrophages, and endothelial and epithelial cells [26, 27, 30, 102, 115, 119]. The pathogenic role of aggregative-adhering properties in *C. diphtheriae*-invasive disease was investigated. *C. diphtheriae* biovar *mitis* and *gravis*, isolated from cases of endocarditis, expressed aggregative adherence (AA) patterns to human epithelial cells. The predominance of localized (LA) and diffuse adherence (DA) patterns have been reported for *C. diphtheriae* strains mostly isolated from throat and skin lesions [26, 64]. *C. elegans* nematodes have been also applied as an infection model system for *C. diphtheriae* and *C. ulcerans* with invasive phenotypes [102, 115, 120–122].

During years of research, the adhesive properties of *C. diphtheriae* strains have been already defined as multifactorial, relying on specific and general mechanisms. Functions and mechanisms of action of fimbriae; non-fimbrial adhesins – 67-72p (DIP0733) and DIP2093 – trans-sialidase; hydrophobins; and sugar residues are already recognized at different levels, especially how they jointly participate in the adherence to the host cells and in the colonization of these cells during bacterial infection [113, 117, 123–125].

Genomic analysis of *C. diphtheriae* revealed the identification of three distinct pili clusters (spaABC, spaDEF, spaGHI) together with five sortase-encoding genes (srtA–E), which are essential for pilus assembly. Adherence rates are not strictly correlated with pili formation, and the pili repertoire of *C. diphtheriae* strains is highly variable. spaA-type is the pilus mostly detected among *C. diphtheriae* strains. As shown by genome comparisons, it is necessary to investigate various isolates on a molecular level to understand and to predict the colonization process of different *C. diphtheriae* strains [3, 123, 124, 126].

The DIP0733 was initially described as a non-fimbrial 67-72p protein responsible for the adherence of *C. diphtheriae* strains to human erythrocytes. Further studies demonstrated DIP0733 protein as a microbial surface component recognizing adhesive matrix molecule (MSCRAMM). The influence of DIP0733 in *C diphtheriae* interaction with human epithelial cells and macrophages in addition to the ability to induce host cell death, giving a signal for apoptosis in the early stages of infection, was also reported. These findings support the idea that DIP0733 is a multifunctional virulence factor of *C. diphtheriae* that enhances the ability to spread throughout the whole human body via the bloodstream [125, 127, 128].

1.10   Molecular Typing Methods for DT-Producing *Corynebacterium* spp.

Molecular typing methods are expected to be reproducible with high discriminatory power, stable and cost-effective, and easy to perform and interpret. Several typing methods have been developed to investigate epidemiological relationship of strains

in disease outbreaks. Investigation of outbreaks is possible due to phylogenetic analysis of origin of strains and patterns of local and global dissemination over the years, among other features [23, 129, 130].

In the past, epidemiological surveillance of diphtheria was limited, depended largely on phenotypic characterization of strains, first by differentiation into biotypes and subsequently by serotyping and phage and bacteriocin typing. At the request of the World Health Organization Regional Office for Europe, the European Laboratory Working Group on Diphtheria (ELWGD) was formed in July 1993 because of the re-emergence of diphtheria to epidemic levels in the Russian Federation and Newly Independent States. The main objectives were to form a network of laboratories for microbiological surveillance, to standardize laboratory diagnostic methods, and to understand the molecular epidemiology and characteristics of *C. diphtheriae* strains at that time. In 2001, the network was expanded to become a Diphtheria Surveillance Network (DIPNET) (http://www.dipnet.org) concerned with epidemiological and microbiological aspects of diphtheria and other infections caused by other DT-producing *Corynebacterium* species, including Brazilian scientists. The main purpose of DIPNET is to establish a Pan-European network of expertise for the prevention of diphtheria and other related infections across the EU Member States and beyond. Among the specific objectives of DIPNET are to (i) determine the disease prevalence and characteristics of toxigenic and non-toxigenic *C. diphtheriae* and *C. ulcerans* in a variety of populations with emphasis upon higher-risk countries, (ii) expand the DIPNET external quality assurance schemes for laboratory diagnosis to include epidemiological typing and serological immunity, and (iii) develop novel tools for integrated molecular epidemiological characterization so as to gain a clearer understanding of the spread of epidemic clones throughout the WHO European Region [3, 23, 108, 131–136].

Over the years, several molecular typing methods have been applied for *C. diphtheriae*, including ribotyping, pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), multilocus enzyme electrophoresis (MEE), multilocus sequence typing (MLST), and spoligotyping. Some of these genotyping approaches have been also used for epidemiologic investigations of both *C. ulcerans* and *C. pseudotuberculosis* zoonotic pathogens [92, 137–141].

1.10.1 Ribotyping Assays

Ribotyping methods, based on restriction patterns of ribosomal RNA genes, had been previously considered the gold standard procedure for *C. diphtheriae* epidemiological surveillance, due to its high discriminatory power, reproducibility, and optimal typeability [142–144]. At the beginning, two ribotypes were identified by the restriction enzyme BstEII and were named G and M, since they seemed to be related to biovars *gravis* and *mitis*, respectively. However, G ribotypes were found in *C. diphtheriae* biovar *mitis* strains, and M ribotypes were also found in *C. diphtheriae* biovar *gravis* strains. The ten most frequent ribotypes of *C. diphtheriae* strains from Russia during the period of 1984 to 1996 were shown in Fig. 1.2.
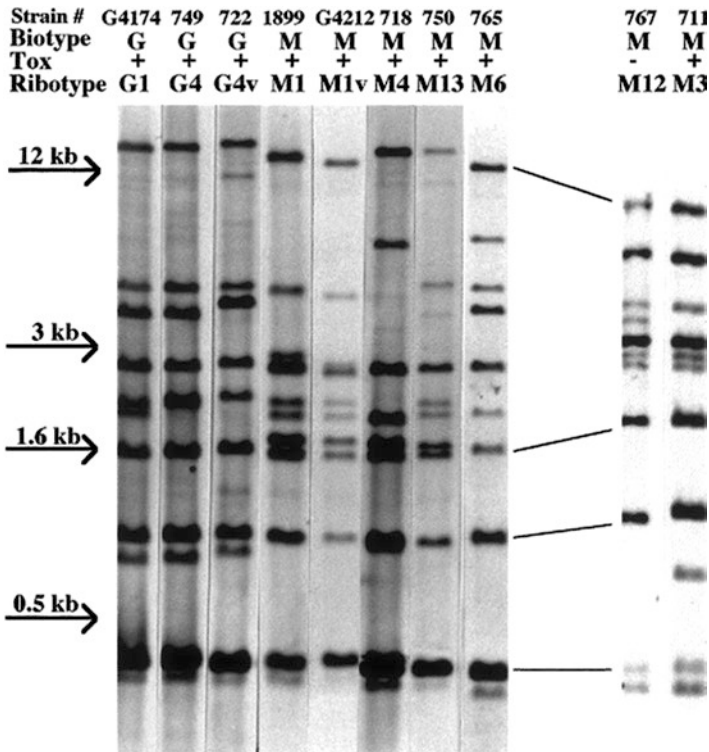
Fig. 1.2 Ribotyping assay based on restriction patterns of ribosomal RNA genes. Predominant riboprofiles identified in *Corynebacterium diphtheriae* strains (*n* = 156) from Russia during the period of 1984 to 1996. Tox, diphtheria toxin-producing. G, *gravis*. M, *mitis*. (Reprinted from Popovic et al. [142])

During an accurate interlaboratory comparative analysis, a revised nomenclature for the designation of ribotypes was proposed. Prefixes "G" and "M" were both replaced by prefix "D" (diphtheria). In 2004, an international ribotyping database was established at the Pasteur Institute and several collaborating laboratories supported by ELWGD/WHO. The ribotype nomenclature was revised and named using the geographical origin to reflect the location where one of the strains was isolated or studied. Eighty-six ribotypes were identified by the restriction patterns using BstEII digestion of the DNA [142, 145].

Ribotyping methods have been also used in studies of *C. ulcerans* zoonotic pathogen. In Japan, DT-producing *C. ulcerans* strains isolated from pharyngeal swabs of two patients attended at a hospital unit were indistinguishable by PFGE analysis and distinguished by ribotyping methods [146]. In 2016, a case of asphyxia death due to pseudomembrane caused by a DT-producing *C. ulcerans* strain, also recovered from the patient's domestic cat, was reported in a Japanese woman. Ribotyping analysis during this case in 2016 detected identical ribotype observed for *C. ulcerans* 0102 strain isolated from the first case in Japan during the year 2001

[57]. In a previous study, 9 different ribotypes, designated U1 to U9, were identified when evaluating 81 *C. ulcerans* strains, 50 of which were clinical isolates from the United Kingdom (90% toxigenic), 7 isolates from domestic cat, and the remaining from different places and sources of origin. The U1 ribotype was the predominant pattern found among human clinical isolates from the United Kingdom (20 isolates) and four isolates from other countries (Germany, France, Ukraine, and Italy). The seven domestic cat isolates also generated ribotypes found among human clinical isolates [147].

Limitations of ribotyping as a genotyping method for *C. ulcerans* were also reported. Genome sequence of *C. ulcerans* FH2016-1 isolated from the first fatal case described above was sequenced and compared with genomes of *C. ulcerans* strains of the first and second cases from Japan, 0102 and 0211, respectively. Although the analyses demonstrated a low variability between genomes, the isolate FH2016-1 was genetically distinct from 0102 and 0211, indicating that conventional ribotyping did not accurately reflect the strain with consequent inaccurate classification [148].

1.10.2 Pulsed-Field Gel Electrophoresis (PFGE) Technique

PFGE technique consists of the separation of DNA fragments with high molecular weight of bacterial genoma, obtained by using restriction enzymes [149]. PFGE technique consists of the separation of DNA fragments with high molecular weight of bacterial genoma, obtained by using restriction enzymes were used, and the *SfiI* restriction endonuclease was chosen, producing 18 to 25 DNA fragments ranging in size from 24 to 290 kb. In comparison with ribotyping, PFGE was not able to distinguish three ribotypes [137]. Thereafter, minor changes in the PFGE protocol were done, and both PFGE and ribotyping showed identical discriminatory ability. In that opportunity, all isolates grouped in one PFGE type were grouped in a ribotype, and vice versa. In addition to the PFGE protocol changes, some explanations seemed plausible for the results found [150]. A protocol using difference in three or more bands was used to distinguish *C. diphtheriae* PFGE types. In a later study, a difference in only two bands to distinguish the PFGE types of a limited number of *C. diphtheriae* strains was used [137, 150].

Nowadays, PFGE typing method has been scarcely used to investigate epidemiological relationship of *C. diphtheriae* strains in disease outbreaks. During the diphtheria outbreak in Northeastern Brazil in 2010, most of the confirmed cases occurred in partially or completely immunized children, including three fatal cases. Molecular analysis demonstrated the spread of predominant PFGE type related strains (Fig. 1.3) [22].

PFGE typing assays have been also used in investigations of origin, transmission, and dissemination of zoonotic *C. ulcerans* strains, especially in Japan. DT-producing *C. ulcerans* strains were isolated from pharyngeal swabs of two patients from the same hospital unit during 2001 and 2002 and were characterized by PFGE and ribotyping. The isolates could not be distinguished by PFGE;
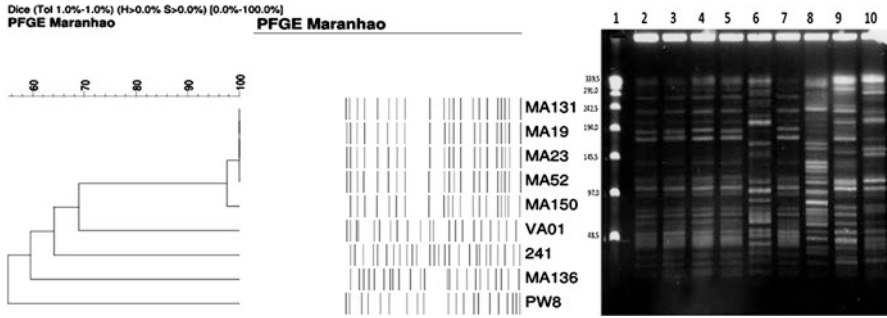
Fig. 1.3 Pulsed-field gel electrophoresis (PFGE) types of *Corynebacterium diphtheriae* biovar *intermediu*s strains isolated from children with diphtheria living in the state of Maranhão, Brazil. Lane 1, λ DNA ladder PFGE marker; lanes 2–5, PFGE type Ia (MA19, MA23, MA52, MA131 strains, respectively); lane 6, PFGE type II (MA136 strain); lane 7, PFGE type Ib (MA150 strain). Other Brazilian DT-producing *C. diphtheriae* strains: lane 8, profile III (sucrose-positive TR241 biovar *mitis* strain); lane 9, profile IV (sucrose-negative VA01 biovar *gravis* strain). Toxoid vaccine producer strain: lane 10, profile V (PW8 strain). (Reprinted from Santos et al. [22])

however, ribotyping showed discriminatory results [146]. In contrast, a study of a fatal case due to a DT-producing C. *ulcerans* strain, PFGE analyses, and ribotyping of C. *ulcerans* strains from the patient and his cat belonged to the same molecular type [57].

1.10.3 Multilocus Enzyme Electrophoresis (MEE)

This electrophoresis technique detects amino acid substitutions capable of altering charge and structural conformation of cellular housekeeping enzymes. Each electromorph or mobility variants of the same enzyme are visualized in a starch gel matrix as bands with different migration rates. Twenty-seven enzymes are tested, and each electromorph is considered a different allele of the specific enzyme. An electromorph profile defines the electrophoretic type (ET) of each bacterial strain. The genetic distance of ET is calculated as a dendrogram generated by the average linkage method of clustering the ETs [151]. Several studies had used MEE methods to estimate *C. diphtheriae* genetic diversity and epidemiological features of endemic and epidemic diseases. Diphtheria epidemic in the 1990s, which initiated in the Russian Federation and dispersed to several European countries, was characterized by the simultaneous presence of different ET that were also detected in *C. diphtheriae* strains isolated during the pre-epidemic period. The majority of *C. diphtheriae* strains with D1 and D4 ribotype patterns (previously named G1 and G4) belonged to the clonal group called ET8 complex [139].

1.10.4 Random Amplification of Polymorphic DNA (RAPD) Assays

The RAPD technique aims to amplify random segments of DNA with single primers of arbitrary nucleotide sequence that may be used to construct genetic maps in a variety of species [152]. The RAPD assay for *C. diphtheriae* was recently standardized by using *C. diphtheriae* strains isolated worldwide. Initially, *C. diphtheriae* strains from Russia (1985–1994) were evaluated, and primers 3 and 4 classified the isolates previously ribotyped into 19 and 24 genotypic profiles, respectively. However, epidemic ribotypes D1 and D4 could not be differentiated by primers 3 and 4. Subsequently, 120 *C. diphtheriae* strains isolated from Russia (1994–1995), Kazakhstan (1996), and the Republic of Georgia (1995–1996) were included and presented RAPD profiles typical of the epidemic ribotypes D1 and D4 [153]. Difficulties in the standardization of RAPD assays for *C. diphtheriae* such as the use of crude DNA and different thermocyclers resulted in poor amplifications, and nonreproducible patterns were also verified [138]. RAPD assays have advantages for being simple, rapid, and inexpensive. However, RAPD assays for *C. diphtheriae* demonstrated low reproducibility in some opportunities [138, 150, 153].

In a previous study, DT-producing and non-DT-producing *C. diphtheriae* strains isolated from cases of infective endocarditis showed different RAPD profiles, demonstrating the invasive properties and circulation of these different clones in Brazil [28]. Analysis performed with purified DNA of several *C. diphtheriae* strains from 26 countries resulted in the differentiation of Eastern European epidemic ribotypes D1 and D4 corresponding to the RAPD profiles Rp1 and Rp4, respectively [143] (Fig. 1.4).
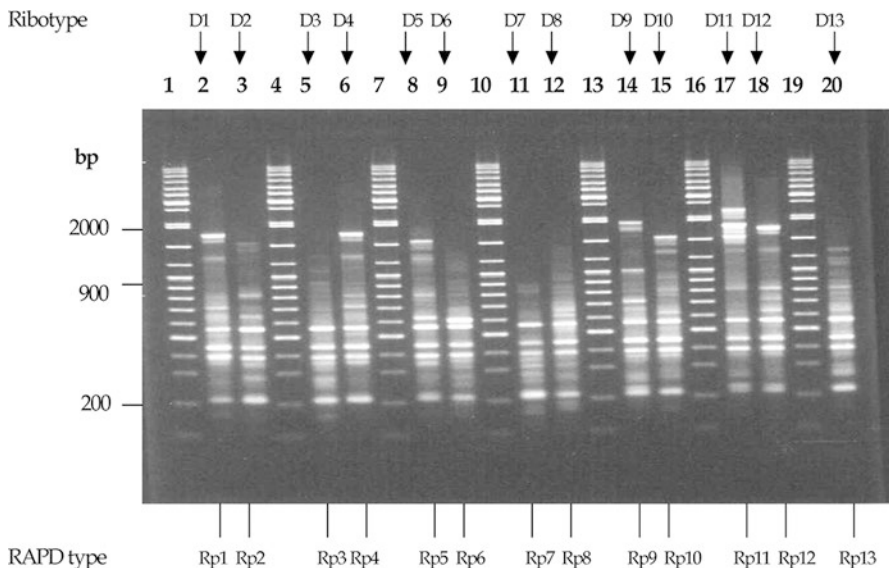

Fig. 1.4 Thirteen RAPD profiles of *C. diphtheriae* isolates illustrating the differentiation of epidemic ribotypes D1 e D4 as Rp1 and Rp4, respectively. (Reprinted from De Zoysa et al. [143])

1.10.5 Amplified Fragment Length Polymorphism (AFLP)

AFLP technique is based on PCR amplification of restriction fragments from total genomic DNA digestion, by using generic primers that do not require prior information of the target DNA sequence. DNA restriction and ligation of oligonucleotide adapters are made to form the binding sites. Subsequently, the selective amplification of the restriction fragments occurs, and finally the fragments are visualized in gel [154]. Evaluation of the AFLP technique was verified during a study conducted with *C. diphtheriae* strains (*n* = 57) presenting nine different ribotypes. A total of ten AFLP profiles were assigned to *C. diphtheriae* tested strains; however, it was not able to discriminate the predominant ribotype during the Eastern European epidemic. AFLP is a PCR fingerprint method easy to perform, rapid, inexpensive, and suitable for most laboratories. Moreover, the AFLP standards are representative of the complete genome [155]. However, AFLP method was less discriminatory than ribotyping in some studies [143, 156].

1.10.6 Multilocus Sequence Typing (MLST) Method

MLST assays have been widely used in different countries for molecular typing of circulating *C. diphtheriae* strains and investigations of epidemic outbreaks. The method aims to group strains related to cloned complex after sequencing and analyzing fragments of seven constitutive genes that encode essential functions for microbial metabolism. *C. diphtheriae* MLST scheme uses fragments of the following housekeeping genes: ATP synthase alpha chain (*atp*A), DNA polymerase III alpha subunit (*dna*E), chaperone protein (*dna*K), elongation factor G (*fus*A), 2-isopropylmalate synthase (*leu*A), 2-oxoglutarate dehydrogenase E1 and E2 components (*odh*A), and DNA-directed RNA polymerase beta chain (*rpo*B) [91, 144].

The PubMLST website hosts a collection of open-access, curated databases that integrate sequence data with phenotype information for many microbial species, including *C. diphtheriae* and *C. ulcerans*. Currently, more than 700 categorized types are deposited (October 2020) in the PubMLST database (http://pubmlst.org/cdiphtheriae/). In 2010, the MLST scheme was proposed for *C. diphtheriae* by using the sequences of the 7 constitutive genes described above, with a total of 150 tested strains (toxigenic isolates *n* = 96) from 18 different countries, during the period of 1957–2006. The results were consistent with previous ribotyping data, which was considered the "gold standard" typing method of *C. diphtheriae* for many years [91].

Although MLST is recognized as a valuable fast and simple PCR-based method used for the tracking the spread of important clones and evolutionary investigation of bacteria, this methodology has some limitations, such as the identification of hypervirulent clones, since the MLST data are based on changes in the core genome, while changes in the accessory genome are responsible for *C. diphtheriae* virulent variants. Each ST can be represented by toxigenic noninvasive, nontoxigenic invasive, and nontoxigenic noninvasive strains [157, 158]. Furthermore, some *C.*

diphtheriae strains with identical ST may differ in up to 290 genes; noncorrelated results between MLST and biotype tests may also occur [158, 159].

The ST-8 clone was responsible for the beginning of the Eastern European epidemic and spread of more than 157,000 registered cases of diphtheria, which resulted in approximately 5,000 deaths. Non-DT-producing profiles of ST-8 strains, previously isolated as toxigenic in Russia, were recently described in Poland. This change was attributed to the environmental pressure exerted by the increase in the number of vaccinated individuals. ST-8 continued to circulate after the epidemic period, as reported realized in Germany, which points to the persistence of ST-8 until today [158, 160, 161]. From 2016 to 2017 in Germany, there was an increase in the circulation of nontoxigenic *C. diphtheriae* characterized mostly by the ST-8. This ST is the most abundant found in the MLST database for *C. diphtheriae* and probably in Europe [160].

Since invasive infections caused by *C. diphtheriae* in vaccinated and non-vaccinated individuals have been reported in Brazil, a genetic relationship of *C. diphtheriae* strains isolated from classic diphtheria and invasive infections in Rio de Janeiro metropolitan area was investigated by using MLST. Four strains presented an atypical sucrose-fermenting ability and corresponded to new STs. Interestingly, a sucrose-fermenting *C. diphtheriae* biovar *mitis* strain, isolated in 1999 patient with endocarditis, formed a clonal complex with a DT-positive *C. diphtheriae* biovar *mitis* strain isolated in Argentina (1995) causing classic diphtheria, suggesting a *C. diphtheriae* biovar *mitis* clonal complex circulation in South America. Moreover, a sucrose-negative strain isolated from a case of endocarditis in 2003 generated an MLST profile that had been previously deposited in the database, ST128, a single locus variant (SLV) of ST-80, the clonal complex that comprises strains currently isolated in different countries including France (ST128) and Canada. These data indicated that *C. diphtheriae* clonal complexes comprising clinical strains related to diphtheria disease may also include invasive phenotypes [9, 28, 91, 94, 111, 162].

An MLST protocol *C. ulcerans* was based on protocol described for *C. diphtheriae.* The website PUBMLST comprises data for both species [91, 163, 164]. MLST methods have been also used in phylogenetic analyses, epidemiology, and zoonotic transmission investigations of *C. pseudotuberculosis* [55, 97, 163, 165].

1.11 In Silico-Based Approaches

Bacterial in silico typing based on repetitive DNA sequences has been also established with the objective of genomic characterization and epidemiological surveillance of diphtheria. Two approaches named CRISPR (clustered regularly interspaced short palindromic repeats) loci and VNTR (variable number tandem repeats) were investigated. In Poland, a study used the complete genome sequence of the NCTC 13129 *C. diphtheriae* strain to identify 75 VNTR loci, of which 14 were selected. Primers were designed, and PCR conditions were optimized to amplify the selected VNTR markers. Fourteen markers were tested and eight were considered

potentially useful. This approach showed discriminatory genotyping ability for the *C. diphtheriae* tested strains (*n* = 28), but the preliminary results were not compared with other genotyping methods [166].

CRISPR-based spoligotyping, defined as a genotyping technique to identify *C. diphtheriae* strains at the phylogeographic level, was also described. According to the authors, this typing methodology presents a high level of discrimination and may be employed to study local epidemiology. One of the limitations is the need for expensive equipment or the use of external services, but the comparison of results between laboratories is easy, and the data generated can be compared in a database. In a study conducted with *C. diphtheriae* of Russia and Belarus epidemic clone, the 156 strains tested were subdivided into 45 spoligotypes. A high level of discrimination in the study of the local epidemiology of diphtheria was observed. However, the three selected CRISPR loci were not present in all *C. diphtheriae* tested strains, and most of them had unique spaces in the leader sequence, indicating that they evolved independently after diverging from a common ancestor [141, 167, 168].

Whole-genome sequencing (WGS) has become an essential tool for molecular epidemiology of infectious disease studies. In recent years, WGS has become the gold standard of high-resolution typing methods, allowing the understanding of the molecular epidemiology and global transmission of pathogens. Genome sequencing remains expensive to be employed in routine genotyping. Nevertheless advances in *C. diphtheriae* genomics concern an increasing number of complete genomes in GenBank may benefit sequence-based genotyping methods as identification of SNPS, tandem repeats (VNTRs), and CRISPR-based spoligotyping. The development of more inexpensive and discriminatory methodologies for use in epidemiological studies will be crucial in our understanding of the molecular epidemiology and carriage of *C. diphtheriae* and *C. ulcerans* [23, 87, 144].

1.12 Conclusions

Molecular typing methods have become essential during the analysis of studies involving epidemiology outbreaks, endemic conditions, recurrent infections, transmission, and virulence potential of *C. diphtheriae* and zoonotic diphtheria toxin-producing *Corynebacterium* species. Application of any typing method depends on the objectives of the study, the level of resolution desired (species vs. strain), and the laboratory conditions and technical expertise available. In studies of complex epidemiological situations and strain-dependent virulence mechanisms, it is recommended not to rely on a single method but to use combinations of methods for strain identification and to interpret results within the context of the epidemiological background and evolution of once acquired pathogenicity features during vaccine era [22, 23, 38, 55, 74, 97, 143, 155].

Epidemiological investigations demonstrated the prevalence of *C. diphtheriae* biovars *gravis* and *mitis* in Eastern Europe and most of Brazilian outbreaks, respectively. Diphtheria cases and deaths caused by *C. diphtheriae* biovar *intermedius* were also documented in previously immunized individuals in India and during the

most recent diphtheria outbreak in Brazil [5, 17, 22, 169, 170]. Nowadays, molecular epidemiological investigations demonstrated the prevalence of different *C. diphtheriae* genotypes in specific geographic regions, including epidemic outbreak: Thailand (ST-243), South Africa (ST 379), and Malaysia (ST453) [171–173].

The occurrence of diphtheria among immunized persons and the increasing frequency of atypical infections caused by non-DT-producing clones indicated that other microbial factors should be used as one of the antigens in the potential vaccine development in the near future. In conclusion, molecular typing methods became a remarkable achievement in wide-ranging research to potentially DT-producing *Corynebacterium* species (*C. diphtheriae* complex), group of extremely dangerous human pathogenic species.

1.13   Summary

Corynebacterium diphtheriae is the leading causing agent of respiratory and cutaneous diphtheria, an acute disease with local and systemic manifestations, which remains as an important cause of morbidity and mortality in different continents. Diphtheria vaccination programs implemented in industrialized and developing countries led to an increasing number of atypical cases of diphtheria in addition to localized and systemic infections, including fully immunized adults. Changes in the clinical epidemiology and virulence features of diphtheria pathogens have been investigated. Cases of infections due to diphtheria toxin (DT)-producing and non-DT-producing *C. diphtheriae* and *Corynebacterium ulcerans*, a zoonotic pathogen, have been increasingly reported. The timely and precise diagnosis of DT-producing *Corynebacterium* strains is indispensable for the patient management and for establishment of surveillance and control strategy of disease. Different molecular methods, such as real-time PCR (polymerase chain reaction) and multiplex PCR, have been used for the characterization of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* and the detection of the gene for DT (*tox*). Recent investigations by genomic sequencing and chemotaxonomic analyses reported DT-producing *C. diphtheriae* subsp. *lausannense* and *Corynebacterium belfantii* sp. nov. in addition to *Corynebacterium rouxii* sp. nov. Genotyping methods have been used as essential epidemiological tools for *C. diphtheriae* and *C. ulcerans* infection prevention and control, including pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), and multilocus sequence typing (MLST) assays. Molecular typing methods are required in studies involving characterization, virulence potential, and susceptibility to antimicrobial agents of *C. diphtheriae* and *C. ulcerans* clinical isolates; origin, routes, and transmission of diphtheria and atypical invasive infections; and endemicity, outbreaks, recurrent infections, and trace cross-transmission caused by non-DT-producing and DT-producing *Corynebacterium* spp.

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