Andreas Burkovski *Editor*

Corynebacterium diphtheriae and Related Toxigenic Species

Genomics, Pathogenicity and Applications

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Preface

Diphtheria, the strangling angel of children, has lost its threatening potential with the development of anti toxin, toxoid vaccine and antibiotics in the last century. However, even today several thousand cases per year are reported to the World Health organization and especially the outbreak of diphtheria in the former states of the Soviet Union demonstrated impressively that diphtheria is not completely eradicated.

The outbreak in the 1990s, the development of new molecular biology tools and especially the availability of genome sequence information gave new impetus to research in this field. Several strains of *Corynebacterium diphtheriae*, the etiological agent of diphtheria and the type species of the genus *Corynebacterium* have been sequenced and genome data are available for two closely related pathogenic species, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*.

The book summarizes the latest advances made in understanding physiology and host pathogen interaction of *C. diphtheriae* and its relatives. Topics addressed are genomics of toxigenic corynebacteria, host-pathogen interactions, diagnosis, surveillance and treatment strategies as well as application aspects.

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Chapter 1
Diphtheria and its Etiological Agents

Andreas Burkovski

Abstract Diphtheria, the 'strangling angel of children', plagued mankind for thousands of years. With the discovery of its etiological agent, *Corynebacterium diphtheriae*, it became a paradigm of an infectious disease. According to Koch's postulates *C. diphtheriae* was isolated by Klebs and Loeffler from infected patients, grown in pure culture and used to re-infect guinea pigs as test animals. Loeffler also recognized that the bacterium predominantly colonizes the nasopharyngeal cavity and, based on this observation, postulated that the secretion of a toxin might cause the often fatal damage of distant organs, a hypothesis, which was further supported by Roux and Yersin. While toxin production is the most dangerous aspect of diphtheria infection, the diphtheria toxin has also been the basis for effective diphtheria treatment and control. Already in 1890, von Behring suggested antitoxin application as a means of diphtheria treatment; in 1913 he developed a first vaccine against diphtheria toxin and in 1920 first mass vaccinations started. Mass immunization is still the most efficient means to prevent and control diphtheria, while antibiotics are effective to eradicate the bacteria from infected patients.

Keywords *Corynebacterium diphtheriae* · *Corynebacterium pseudotuberculosis* · *Corynebacterium ulcerans* · Toxoid

1.1 Introduction

Diphtheria of the upper respiratory tract is characterized by pseudomembrane, which renders breathing difficult. Eventually, coughing can remove parts of the pseudomembrane, easing the situation of the patient temporarily, and after several fits of coughing the pseudomembrane might even be removed and healing might be achieved. More often obstruction of airways results in suffocation, agony and death.

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In tropical and subtropical regions respiratory tract diphtheria is outnumbered by cutanous diphtheria, which is characterized by skin lesions located predominantly on feet, lower legs and hands. Typically this infection is persistent for months.

Treatment of diphtheria is unproblematic today since *C. diphtheriae* can be eliminated easily using antibiotics, and the diphtheria toxin can be neutralized by antitoxin application. Nevertheless, mass immunization is the means of choice for diphtheria control. A highly effective toxoid vaccine is available which made diphtheria an extremely rare disease in industrialized countries. However, local outbreaks and even a full scale epidemic have been observed during the last three decades. Several thousand diphtheria cases are reported to the World Health organization each year, showing that diphtheria is not completely eradicated and that reservoirs still exist. Putative carriers are people with insufficient access to medical care including vaccination programs, for example poor parts of the population in developing countries. Consequently, for citizens of industrialized countries a major risk factor for infection with *C. diphtheriae* might be travel to an endemic country; however, animals also seem to play an increasing role in transmission of the infection. Isolation of *C. diphtheriae* strains was reported from domestic cats and horses. Animal reservoirs are even more common for other toxigenic *Corynebacterium* species, which might also be connected to diphtheria-like illness. *Corynebacterium ulcerans* has been primarily recognized as a commensal bacterium in domestic and wild animals; however, an increasing frequency and severity of human infections associated with *C. ulcerans* is observed and *C. ulcerans* strains producing diphtheria toxin are reported with rising frequency from industrialized countries.

1.2 A brief History of Diphtheria

Diphtheria has been well known since Babylonian and Sumerian times and documents describing this disease can be found in the Talmud and in writings by Hippocrates. Its alternative designation ‘strangling angel of children’ refers to the dreadful death by suffocation especially of infants infected by classical diphtheria of the upper respiratory tract. Based on the appraisal of written reports, diphtheria seems to originate from the Middle East and was most likely disseminated from there first to Europe and later to all other continents. Numerous waves of epidemic outbreaks of diphtheria occurred in Europe, often connected to times of war. The cycles include gaps of 100 years and more (English 1985) and outbreaks were reported, e.g. in the sixteenth century in France and Germany, in the seventeenth century in Italy and Spain, and in the eighteenth century in Germany and Sweden. During the eighteenth century, diphtheria also reached the East coast of North America, where in 1799 George Washington became maybe the most prominent victim of this disease. At the end of the eighteenth and the beginning of the nineteenth century, diphtheria became more prevalent and developed into a leading cause of infant mortality. Up to four fifth of children infected with diphtheria died. The reason for this increase in infection numbers and mortality might be the concomitant rise of urban population with beginning industrialization coupled with inferior housing and nutrition conditions of working class people.

The start of industrialization at the end of the nineteenth century brought not only an increase of diphtheria and other diseases connected with poverty, overcrowding and malnutrition, such as tuberculosis, but also the rise of bacteriology. In fact, diphtheria became a paradigm of an infectious disease and provided key evidence for Koch's postulates on the germ theory (Dolman 1973; Levy 1975; English 1985). In 1883 Klebs delivered the first description of *C. diphtheriae* isolated from a patient's swab (Klebs 1883) and based on Klebs' work, Loeffler was the first to grow *C. diphtheriae* in pure culture. In consistence with Koch's postulates, this pure culture was able to evoke diphtheria in guinea pigs used as test animals. Furthermore, Loeffler passed the disease from guinea pig to guinea pig and found that even after more than twenty of these passes the animals still developed diphtheria-like symptoms. In his groundbreaking work, Loeffler described that *C. diphtheriae* colonizes the membranes of the nasopharyngeal cavity but not deeper parts of the body. Based on this observation, he postulated the secretion of a toxin being responsible for the often fatal damage of internal organs connected with upper respiratory tract diphtheria (Loeffler 1884). His hypothesis was supported by Roux and Yersin (1888), who injected bacteria-free filtrates of *C. diphtheriae* cultures in animals and observed that these developed organ damages indistinguishable from those of human diphtheria cases.

While toxin production is the most dangerous aspect of diphtheria infection, leading to severe, often fatal damages of heart, nervous system and muscles (see below), the diphtheria toxin has also been the key to successful treatment, vaccination and control, based on scientific advances made in the late nineteenth century. Already in 1890, von Behring and Kitasato suggested antitoxin application as a means of choice to treat diphtheria (von Behring and Kitasato 1890; for overview, see Grundbacher 1992). In fact, von Behring was also the first who used antitoxin from a horse to successfully treat diphtheria and in 1913 he developed the vaccine against diphtheria toxin. The basis of the vaccine and the vaccination programs starting in the 1920s is the diphtheria toxoid, a formaldehyde-inactivated diphtheria toxin, produced and secreted in vast amounts by strain Park-Williams no. 8 (PW8), originally isolated from a mild case of diphtheria (Park and Williams 1896; Iwaki et al. 2010). Immunization with the inactivated toxin that remains antigenetically intact is extremely effective and changed diphtheria from a former main cause of infant mortality to an extremely rare disease, which will never be seen by most pediatricians or other physicians (English 1985).

Despite this extraordinary success story, diphtheria is not eradicated today. From 2000 to 2008 between three to eleven thousand cases were reported to the World Health Organization per year. Furthermore, especially the epidemic outbreak starting after the breakdown of the former Soviet Union in 1989 showed impressively that diphtheria is still a serious health hazard.

This large scale epidemic started in 1990 with the Russian Federation and Ukraine being centers of the outbreak. 15,211 diphtheria cases were reported for Russia and further 2,987 cases for Ukraine in 1993 (Galazka et al. 1995; Eskola et al. 1998; Popovic et al. 2000), from where the disease spread quickly to neighboring countries. The most characteristic feature of the outbreak was the

infection of adolescents and adults, instead of infants being victims of the disease. In addition to Belarus, Russia and Ukraine, this was especially pronounced in the Baltic States Estonia, Latvia and Lithuania, where diphtheria cases among people from 15 years and older ranged from two thirds to four fifths of total cases (Hardy et al. 1996; Dittmann et al. 2000). Consequently, mass immunization of adults, which was started in 1993, was one crucial step to eradicate the disease and stop the epidemic.

The 1990s epidemic showed impressively that socioeconomic instability, large-scale population movements and absence or break-down of health infrastructure favor the spread of diphtheria. It also indicated, together with constant numbers of case reports from various countries, that *C. diphtheriae* might persist in a population for a long time (for example see Marston et al. 2001). In many cases, carriers of infection are unknown, sometimes these are groups of people with insufficient access to medical care including vaccination programs (John 2008) or drug abusers (Lowe et al. 2011). For inhabitants of industrialized countries, a major risk factor for infection with *C. diphtheriae* might be travel to a country where diphtheria is endemic (Connell et al. 2005; Bonmarin et al. 2009; Wagner et al. 2010).

1.3 Transmission and Clinical Manifestations of Diphtheria

1.3.1 Classical Diphtheria of the Upper Respiratory Tract

Diphtheria is a classical infectious disease which typically spreads from person to person by respiratory droplets produced by an infected person by coughing or sneezing (Fig. 1.1). Establishment of the disease takes 2–5 days and patients are infectious for two to three weeks. Besides people showing diphtheria symptoms, asymptomatic carriers may also contribute to spreading of the disease. Loeffler showed before that about five percent of the school children in Berlin (Germany) were carriers of *C. diphtheriae* without showing diphtheria-like symptoms.

Classical diphtheria is an infection of the upper respiratory tract that causes sore throat and low fever. Symptoms range from mild pharyngitis to suffocation due to airway obstruction by pseudomembrane formation. In severe cases, the air passages might be completely blocked. Typically, infection starts with the colonization of epithelial cells of the upper respiratory tract by *C. diphtheriae*. The bacteria multiply on the surface of the mucous membrane, but do not advance into deeper parts of the body, as already described by Loeffler (1884). Tissues infected show inflammatory symptoms, later edema and necroses develop due to the detrimental action of the diphtheria toxin. The toxin is also responsible for inflammation of deeper lying capillaries which results in fibrin secretion into the damaged epithelium. The released fibrin protein, destroyed epithelial cells and bacteria together form the so-called pseudomembrane, a grayish or yellowish-white coating. Later during infection, the

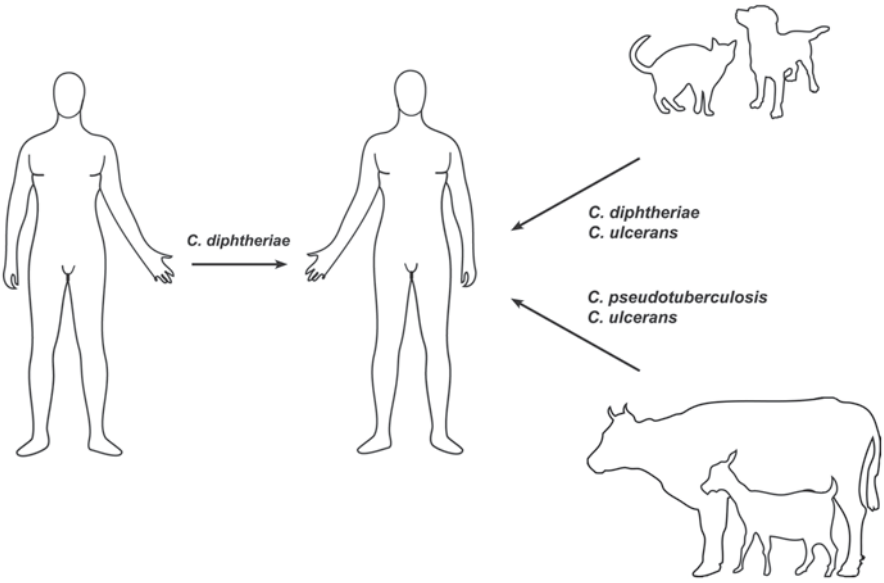

Fig. 1.1 Transmission of toxigenic corynebacteria. Transmission of *Corynebacterium diphtheriae* from person to person occurs by close physical contact. Respiratory diphtheria is mainly disseminated by respiratory droplets; however, smear infections are also possible, especially in case of cutaneous diphtheria. *C. ulcerans* infections occur via close physical contact with pets and farm animals or animal bites. Man-to-man transmission was not reported. *C. pseudotuberculosis* is transferred from infected cattle, especially sheep and goats, to humans by close physical contact

damage of the capillaries due to inflammatory processes causes bleedings, and due to decaying erythrocytes, the color of the pseudomembrane can turn into a dirty brown and the breath of the patients becomes sweetish-putrid, a characteristic indication of diphtheria. With progressive pseudomembrane formation reaching the larynx, a barking cough develops. Furthermore, the voice of the patients becomes affected and hoarseness develops which can even result in complete loss of the voice. Later, trachea and bronchi may be covered by firmly or loosely attached pseudomembranes. At this stage, breathing renders difficult for the patients and their lips turn blue. With increasing dyspnea the patients become restless, their pulse beats become faster and the look of their faces becomes timid and frightened. Sometimes coughing can remove parts of the pseudomembranes at this stage of the disease, easing the situation of the patient temporarily. After several of these fits of coughing, the pseudomembranes might even be completely removed and healing might be achieved in some cases. More often, suffocation results in death agony and, finally, the patients faint and heart failure results in death.

As mentioned above, the colonization of the human host by *C. diphtheriae* remains localized to the upper respiratory tract, although satellite infections may occur in the esophagus, stomach or lower airpassages. The severe complications observed in later stages of infection are the result of the diphtheria toxin which is secreted by *C. diphtheriae* and distributed by the circulatory system to remote parts of the body.

Diphtheria toxin is synthesized depending on the iron concentration in the environment. When iron becomes limiting, as it is the case in the human host, the bacteria start to synthesize the toxin which is then secreted into the extracellular medium as a single polypeptide chain (Pappenheimer 1977; Holmes 2000). In this form the toxin is inactive, can be absorbed into the circulatory system and disseminated to distant parts of the body. When binding to its receptor, the uncleaved precursor of the heparin-binding EGF-like growth factor (Naglich et al. 1992), it can enter the cell by endocytosis. Once inside the endosome, the inactive diphtheria toxin, consisting of an A and B chain, is cleaved, and the A chain is released into the cytoplasm, where it is activated by further cleavage into the active toxin and inactivates its cellular target elongation factor 2 (EF-2) by ADP-ribosylation (Pappenheimer 1977; Lord et al. 1999; Falnes and Sandvig 2000).

Especially myocardium and peripheral motor neurons are affected by diphtheria toxin (Harrison et al. 1972; Murray and Noble 1985; Hadfield et al. 2000; Perles et al. 2000). Up to two thirds of patients show some evidence of myocarditis and 10–25 % of cases develop clinical cardiac dysfunctions. Cardiac symptoms are directly correlated to the extent and severity of local *C. diphtheriae* infection in the patient's upper respiratory tract and often may prove fatal weeks later during convalescence (MacGregor 1995; Hadfield et al. 2000; Perles et al. 2000). Histological changes in the heart differ significantly from patient to patient and may include edema, congestion, infiltration by mononuclear cells and presence of fat drops. The myocardium may show areas of granular degradation, hyaline degradation, necrosis, inflammation and loss of cross striation (Kline and Kaplan 1998; Hadfield et al. 2000; Perles et al. 2000). The heart may be dilated, pale and flabby (Hadfield et al. 2000) and as a result of toxin damage to the cardiac conduction, muscle and nervous system electrical disturbances such as bradyarrhthmia, tachyarrythmia, artioventricular and bundle branch blocks are often found (Perles et al. 2000).

The nervous system is, besides the heart, a main target of the toxin. About three-fourths of patients with severe diphtheria infection develop neurologic complications. First symptoms of neuropathy are paralysis of the soft palate and posterior pharyngeal wall, resulting in regurgitation of swallowed fluids through the nose. Additionally, often a paralysis of the muscles of the eyes and dysfunction of facial, pharyngeal or laryngeal nerves are observed. In later stages, the nerves of trunk, neck, arms and hands might be affected leading to paralysis. Histological investigations to characterize the basis of the neuropathies led to the observation of paranodal and segmental demyelination, resulting in degeneration of myelin sheaths and axons (Baba et al. 1984; Hadfield et al. 2000).

1.3.2 Cutaneous Diphtheria

In tropical and subtropical regions, cutaneous diphtheria is more common and prevails over the respiratory tract form (Höfler 1991). It is still endemic in some African and Asian countries, where climate, overcrowding, poverty, poor hygiene and frequent, slowly healing wounds favor the infection. Cutaneous diphtheria is easily

spread by contact with infected skin, respiratory droplets of a patient infected with respiratory tract diphtheria or exposure to dust or clothing contaminated with *C. diphtheriae* (Höfler 1991).

Common sites for cutaneous diphtheria are feet, lower legs and hands (for example see Hamour et al. 1995; Connell et al. 2005). Due to the various skin lesions that can be colonized by the bacteria and frequently occurring co-infections by different other bacterial pathogens the clinical manifestation of cutaneous diphtheria can be extremely variable.

The infection typically starts with a painful, fluid-filled pustule which breaks down later and progresses as a punched-out ulcer. The diameter of the ulcer might range between a few millimeters to centimeters. During the first weeks of infection lesions are covered by a smeary grayish-brown pseudomembrane, which might change color to a dirty or dark reddish brown over time. Later, the infection becomes anesthetic and the pseudomembrane falls off, leaving a hemorrhagic base with a surrounding of edematous grayish-white, pink or purple-colored tissue (Höfler 1991; Hadfield et al. 2000). Spontaneous healing takes several weeks to months, cases lasting one year have been observed (Höfler 1991). This long persistence might favor dissemination of the disease and might explain the extremely high infection rates of skin lesions with *C. diphtheriae* observed previously. Infection rates of up to 60 % were found in some African and Asian countries (Liebow et al. 1946; Livingood et al. 1946; Bezjak and Farsey 1970a, 1970b; Höfler 1991).

1.3.3 Systemic Infections

C. diphtheriae is not only the etiological agent of classical diphtheria of respiratory tract and skin, but can also cause, although generally rare, systemic infections. Cases of bacteraemia, endocarditis, hepatic and splenic abcesses, meningitis, mycotic aneurysm, osteomyelitis, pneumonia as well as septic arthritis caused by nontoxigenic and toxigenic *C. diphtheriae* were reported (Isaac-Renton et al. 1981; Puliti et al. 2006; Hirata et al. 2008; Honma et al. 2009; Muttaiyah et al. 2011; and references therein). The best documented systemic infections are related to *C. diphtheriae* endocarditis. Endocarditis as a result of *Corynebacterium* infection has been described as aggressive disease often requiring surgical intervention (Mishra et al. 2005). Typically, the left heart of adult males is infected and underlying valvular disease is frequently found. Up to 28 % of patients require valve replacements and more than 40 % die (Belmares et al. 2007).

1.4 Diagnosis, Treatment and Control

The diagnosis of respiratory tract diphtheria is, as in former times, still based on the classical symptoms of this disease: sore throat, formation of a pseudomembrane and the typical sweetish-putrid smell of the patients' breath. With the

identification of *C. diphtheriae* as its etiological agent by Loeffler (1884) and the development of modern biochemistry and molecular biology, the diagnostic toolbox was constantly improved. Besides different screening and identification tests, the classical Elek test (Efstratiou et al. 1998) for toxicity testing is most commonly used (for a overview of tests and quality evaluation, see Neal and Efstratiou 2009). Furthermore, also a number of other toxigenic *Corynebacterium* species (see below) can also be reliably identified today (Schuhegger et al. 2008; Sing et al. 2011).

Before the introduction of antitoxin and antibiotics, physicians were restricted to treatments preventing suffocation such as tracheostomy introduced by Bretonneau in 1825 and intubation introduced by Bouchut in 1859 (English 1985). Despite some success, mortality stayed high, since, besides the severe side-effects of the proposed treatments, the detrimental action of the toxin could not be avoided by these treatments. The situation improved dramatically with the introduction of antitoxin, which is able to neutralize the toxin in the circulatory system. Even more effective for controlling diphtheria is the immunization with diphtheria toxoid, formaldehyde-inactivated diphtheria toxin that remains antigenetically intact. With increasing levels of immunity, the annual incidence of diphtheria dropped to 0.1–0.2 per million (Kwantes 1984; Höfler 1991; Murphy 1996; Vitek 2006; Roush and Murphy 2007). Diphtheria toxoid is widely used as a component of DPT (diphtheria, pertussis, tetanus) vaccine. Immunization typically starts with early childhood; after four doses of the vaccine within the first 2 years immunization against diphtheria is effective up to 97%. Since antibody titers wane over time, a large percentage of adults in the United States and Europe have antitoxin levels below the protective level (Murphy 1996; von Hunolstein et al. 2000). Therefore, booster immunization of adults is recommended.

The discovery of antibiotics was the next hallmark of effective diphtheria treatment. In contrast to other corynebacteria such as *Corynebacterium jeikeium*, which causes severe infections in intensive care units, multi-resistance against antibiotics is not the problem in *C. diphtheriae* and penicillin and erythromycin are first line antibiotics used for its eradication (Begg 1994; Kneen et al. 1998; Pereira et al. 2008; Zasada et al. 2010). In cases of cutaneous diphtheria, additional local application of drugs such as bacitracin or gentian violet is recommended (Höfler 1991). The therapy with antibiotics might become more difficult in future with the emergence of multidrug resistant strains in some countries. While a recent study on antimicrobial resistance found no multidrug resistant strains among isolates circulating in Poland (Zasada et al. 2010), a considerable number of isolates resistant against one or more antibiotics were observed among Brazilian *C. diphtheriae* strains and reservations about the use of penicillin were risen (Pereira et al. 2008).

To avoid complications due to action of diphtheria toxin, e.g. myocarditis, antitoxin is normally given. However, even if properly treated with antibiotics and antitoxin, five to ten percent of cases can end fatally. Therefore, mass immunization of the entire population is the best means to control diphtheria.

1.5 Diphtheria as a Zoonotic Infection

Besides humans, animals seem to play a role as a reservoir of the disease (Fig. 1.1). Isolations of *C. diphtheriae* strains were reported for example from domestic cats (Hall et al. 2010), cows (Corboz et al. 1996) and horses (Henricson et al. 2000; Leggett et al. 2010). The existence of animal reservoirs is even more common, when other toxigenic *Corynebacterium* species are taken into consideration (Bonmarin et al. 2009). *C. diphtheriae* is closely related to two further *Corynebacterium* species, *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* (Riegel et al. 1995). The three species can be lysogenized by similar corynebacteriophages and, if these carry a *tox* gene, all three species produce diphtheria toxin (Groman 1984; Buck et al. 1985; Cianciotto and Groman 1996), making them a potential health threat.

Infections due to *C. pseudotuberculosis*, which causes caseous lymphadenitis in sheep and goat populations worldwide (Dorella et al. 2006; Baird and Fontaine 2007), are rare in humans and classical diphtheria of the upper respiratory tract or skin connected with *C. pseudotuberculosis* have not been observed. However, *C. pseudotuberculosis* may serve as a reservoir of corynebacteriotoxphages. In contrast, during the last decade, the frequency and severity of human infections associated with *C. ulcerans* appear to be increasing in various countries. Cases of respiratory tract diphtheria caused by toxigenic *C. ulcerans* strains were reported from various industrialized countries (Wagner et al. 2001; Hatanaka et al. 2003; De Zoysa et al. 2005; Tiwari et al. 2008) and became even more common than *C. diphtheriae* infections in the United Kingdom (Wagner et al. 2010). Infections with toxigenic *C. ulcerans* can be fatal in unvaccinated patients and usually occur in adults. Besides upper respiratory tract diphtheria, *C. ulcerans* can also cause severe skin and pulmonary infections (Dessau et al. 1995; Nureki et al. 2007; Mattos-Guaraldi et al. 2008). Transmission by person to person contact was not reported up to now. In contrast, close contact with domestic animals (Wagner et al. 2010) and consumption of raw, unpasteurized milk (Bostock et al. 1984; Hart 1984) seem to be risk factors. This observation is in accordance with the identification of *C. ulcerans* strains commensals in various domestic and wild animals (Schuhegger et al. 2008; Dixon 2010; Sykes et al. 2010) , which may serve as a reservoir for zoonotic infections.

1.6 Development and Persistence of *C. diphtheriae* Populations

As described above, there seems to be a shift from *C. diphtheriae* to *C. ulcerans* as etiological agent of diphtheria in at least some Western countries. Interestingly, a shift within *C. diphtheriae* populations has been observed as well. With the introduction of diphtheria toxoid vaccines, not only the number of diphtheria cases but also the number of isolated toxigenic *C. diphtheriae* strains decreased, suggesting

a protection by the vaccine not only against the fatal action of the toxin but, at least partially, against the bacteria itself. Interestingly, anti-parallel to this development, an increasing number of non-toxigenic strains has been isolated (Zuber et al. 1992; Gilbert 1997; Hadfield et al. 2000; Wagner et al. 2011). These non-toxigenic *C. diphtheriae* are persisting over years in different, often poor populations (Romney et al. 2006; Lowe et al. 2011; Shashikala et al. 2011), where they are connected especially to skin infections. Unfortunately, also an increasing number of systemic infections by non-toxigenic strains has also been observed.

Following the outbreak by a unique clonal group of *C. diphtheriae* in Russia in 1990 (Popovic et al. 2000), a rising heterogenicity of circulating strains after the epidemic, emergence of new toxigenic variants, and persistence of invasive non-toxigenic strains were observed (Mokrousov 2009).

All in all, the emergence of new diphtheria-causing corynebacteria such as *C. ulcerans* and the adaptation of *C. diphtheriae* populations to medical treatment are supporting the need of continuous surveillance of *C. diphtheriae* and its relatives and justify, besides the basic scientific interest, experimental efforts to characterize these pathogens.

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