

Multiplex polymerase chain reaction to identify and determine the toxigenicity of *Corynebacterium* spp with zoonotic potential and an overview of human and animal infections

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Corynebacterium diphtheriae, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* constitute a group of potentially toxigenic microorganisms that are related to different infectious processes in animal and human hosts. Currently, there is a lack of information on the prevalence of disease caused by these pathogens, which is partially due to a reduction in the frequency of routine laboratory testing. In this study, a multiplex polymerase chain reaction (mPCR) assay that can simultaneously identify and determine the toxigenicity of these corynebacterial species with zoonotic potential was developed. This assay uses five primer pairs targeting the following genes: rpoB (*Corynebacterium* spp), 16S rRNA (*C. ulcerans* and *C. pseudotuberculosis*), pld (*C. pseudotuberculosis*), dtxR (*C. diphtheriae*) and tox [diphtheria toxin (DT)]. In addition to describing this assay, we review the literature regarding the diseases caused by these pathogens. Of the 213 coryneform strains tested, the mPCR results for all toxigenic and non-toxigenic strains of *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* were in 100% agreement with the results of standard biochemical tests and PCR-DT. As an alternative to conventional methods, due to its advantages of specificity and speed, the mPCR assay used in this study may successfully be applied for the diagnosis of human and/or animal diseases caused by potentially toxigenic corynebacterial species.

Key words: mPCR - *C. diphtheriae* - *C. ulcerans* - *C. pseudotuberculosis* - diphtheria toxin

Corynebacterium species constitute a group of microorganisms related to different infectious processes involving both animal and human hosts (Seto et al. 2008). The gene for diphtheria toxin (DT) is present in bacteriophages capable of infecting *Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* (Wong & Groman 1984, Pappenheimer 1993).

C. diphtheriae is the primary causative agent of diphtheria, a toxæmic disease whose prevention depends on the implementation of effective immunisation programs using toxoid molecules (Hadfield et al. 2000). In many developing countries, diphtheria continues to have a high case fatality rate due to the inadequate nationwide coverage of immunisation programs (Mattos-Guaraldi et al.

2003, Saikya et al. 2010). The potential for *C. diphtheriae* to spread epidemically and the fact that some strains have recently been isolated from domestic animals indicate that there is a risk for the zoonotic circulation of the tox gene although the spread of toxigenic *C. diphtheriae* among humans is considered as under control (Hall et al. 2010, Leggett et al. 2010).

Recently, cases of diphtheria (Aaron et al. 2006), mostly in European and North American countries (CDC 2000, von Hunolstein et al. 2003, DeWinter et al. 2005, Bonmarin et al. 2009, Wagner et al. 2011), caused by *C. ulcerans* have been reported and some infections were reported to be related to animal transmission (Hogg et al. 2009, Wagner et al. 2011). However, there have been few reported cases of zoonotic diphtheria due to *C. ulcerans* in developing countries (Dias et al. 2011), especially in countries with large livestock populations and/or deficient child immunisation programs. Other types of human infections caused by *C. ulcerans* have been described (Taylor et al. 2002, Mattos-Guaraldi et al. 2008). Moreover, infection by toxigenic *C. ulcerans* has also reported in animals, including cattle, pigs and small pets, such as cats and dogs (De Zoysa et al. 2005, Lartigue et al. 2005, Katsukawa et al. 2009, 2012, Schuegger et al. 2009, Sykes et al. 2010). In Brazil, a survey performed in

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an animal shelter for abandoned dogs found an asymptomatic dog colonised by a non-toxigenic *C. ulcerans* strain (Dias et al. 2010).

C. pseudotuberculosis is the aetiological agent of caseous lymphadenitis (CLA) in small ruminants, such as sheep and goats. This infection sometimes presents as pneumonia, hepatitis, pericarditis, mastitis, arthritis or subcutaneous abscesses. This pathogen is also associated with lymphadenitis in horses and with ulcerative lymphangitis in cattle (Foley et al. 2004, Perkins et al. 2004, Baird & Fontaine 2007, Sharpe et al. 2010). Two biotypes of *C. pseudotuberculosis*, which are differentiated based on nitrate-reducing ability, have been reported: nitrate-negative strains are referred to as serotype I (biotype *ovis*) and nitrate-positive strains are classified as serotype II (biotype *equi*). Isolates from sheep and goats are usually nitrate negative, whereas strains from horses are typically nitrate positive; isolates from cattle are variable (Tejedor-Junco et al. 2008, Wagner et al. 2011). Although *C. pseudotuberculosis* is distributed worldwide, it has the most serious economic impact in Oceania, Africa and South America, including Argentina and Brazil (Estevao et al. 2007, Komala et al. 2008, Stefanska et al. 2008, Tarello & Theneyan 2008, Seyffert et al. 2010). Once established, CLA is difficult to eradicate because drug therapy is not effective and the clinical detection of infected animals is of limited efficiency (Dorella et al. 2006). Early microbiological diagnosis and long-term antimicrobial treatment are important for a successful outcome in horses with *C. pseudotuberculosis* infection (Pratt et al. 2005). Similar to diphtheria in humans (Kombarova et al. 2001, Saikya et al. 2010), *C. pseudotuberculosis* can cause clonally expanding epidemics in animals. The increase in the number of infections could be the result of a reporting bias, environmental factors that facilitate infection or host factors, such as greater herd susceptibility (Foley et al. 2004).

C. pseudotuberculosis can be spread among animals by fly vectors, such as *Musca domestica* and *Hippobosca equina*, as previously observed in the United States of America (USA), Israel and Egypt. This pathogen can be found in up to 20% of flies in the vicinity of diseased animals (Yeruham et al. 1996, Braverman et al. 1999, Selim 2001, Spier et al. 2004). Oedematous skin disease (OSD) is an endemic disease of buffaloes in Egypt. *C. pseudotuberculosis* serotype II (biotype *equi*; nitrate positive) is the primary cause of OSD. The pathogenesis of these strains is related to the secretion of toxigenic factor(s) and phospholipase D (PLD) and the lipid contents of their cell walls (Selim 2001). The appearance of OSD outbreaks during the summer months was found to be related to the fact that the summer months are the breeding season for *H. equina*, the primary vector of the causative agent. The control of OSD presents several problems because there is insufficient knowledge about the epidemiology and pathogenesis of this disease (Syame et al. 2008). The role of DT in CLA, OSD and other *C. pseudotuberculosis* diseases in animals has not been adequately studied.

Few studies have demonstrated the isolation of the causal agent of CLA and OSD from humans worldwide. Although rare, human infections caused by *C. pseudotuberculosis* are frequently similar to those observed in sheep and goats (CLA); these infections usually require the excision of infected lymph nodes accompanied by supplementary antimicrobial treatment, but do not involve toxæmic manifestations. *C. pseudotuberculosis* is usually acquired after close contact with an infected animal and no underlying diseases or predisposing conditions have been identified in infected patients (Peel et al. 1997, Romero-Perez et al. 2004, Join-Lambert et al. 2006, Hemond et al. 2009). Only one patient with toxæmic symptoms, an injecting drug user with endocarditis, has been reported. This patient had no history of animal contact and no possible source of the *C. pseudotuberculosis* infection was identified (Wagner et al. 2011). Additional studies are necessary to investigate the correlation between the prevalence of *C. ulcerans* and *C. pseudotuberculosis* infections in humans and the prevalence in local cattle populations, most notably in developing countries, as previously performed for bovine tuberculosis infection in animal and human populations in Ethiopia (Shitaye et al. 2007).

There has been an increase in the incidence of disease caused by non-toxigenic *C. diphtheriae*. Non-toxigenic strains generally cause persistent sore throats and severe pharyngitis/tonsillitis and invasive diseases, such as endocarditis, septic arthritis, splenic abscesses and osteomyelitis, are not uncommon (Galazka 2000, Dzipova et al. 2005, Hirata Jr et al. 2008, Farfour et al. 2012). In non-industrialised countries there is an overall lack of information on the prevalence of colonisation by and diseases caused by non-toxigenic *C. diphtheriae* in the population due to a reduction in the frequency of routine screening for this organism. Because of non-toxigenic *C. diphtheriae* infection may not be recognised by healthcare professionals and non-toxigenic *C. diphtheriae* is considered an emerging pathogen of increasing significance worldwide there is an urgent need for increased clinical awareness, especially for immunocompromised patients, in whom complications can arise (Gomes et al. 2009, Edwards et al. 2011, Mattos-Guaraldi et al. 2011).

The data indicate that there is still a lack of information on the prevalence of disease caused by potentially toxigenic corynebacteria. Therefore, to better understand the mechanisms of carriage/transmission, determine the incidence rates and analyse the variation among non-toxigenic and toxigenic strains, it is imperative to use assays that can rapidly and accurately detect these three pathogens (Connor et al. 2007, Estevao et al. 2007, Kraeva et al. 2007, Baird & Malone 2010, Komiya et al. 2010, Konrad et al. 2010, Sharpe et al. 2010). In the present study, a multiplex polymerase chain reaction (mPCR) assay was developed to identify *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* and differentiate between toxigenic and non-toxigenic strains. This assay will serve as a complementary method for the timely laboratory diagnosis of infected animals and/or humans and for epidemiological studies.

MATERIALS AND METHODS

Bacterial strains and identification procedures - This study was carried out using 213 strains of *Corynebacterium* and coryneform bacteria (Supplementary data). Microorganisms from international reference laboratories [the National Culture Type Collection (NCTC) (UK), the American Type Culture Collection (ATCC) (USA) and the Centers for Disease Control and Prevention (CDC) (USA)] were used as controls: non-toxicogenic *C. diphtheriae* biotype *mitis* (ATCC 27010) and *C. pseudotuberculosis* (FRC41, genome sequence deposited in GenBank), toxicogenic *C. ulcerans* (CDC KC279) and *C. diphtheriae* biotypes *mitis* (ATCC 27012 and CDC E8392), *intermedius* (CDC D7920) and *gravis* (NCTC 13129), *C. jeikeium* (ATCC 43734), *C. minutissimum* (ATCC 23348), *C. striatum* (CDC F378), *Rhodococcus equi* (ATCC 33701 and ATCC 10146), *Nocardia asteroides* (ATCC 7772) and *Nocardia brasiliensis* (ATCC 7771).

The identification of corynebacteria was based on the results of both standard biochemical tests (Efstratiou & George 1999, Funke & Bernard 2007) and a commercial kit: the API Coryne System (bioMérieux, La-Balme-les-Grottes, France) (Soto et al. 1994). A DNase test was also performed to differentiate *C. diphtheriae* and *C. ulcerans* from *C. pseudotuberculosis* and other *Corynebacterium* spp (Pimenta et al. 2008b). A reverse CAMP reaction was performed using *Staphylococcus aureus* ATCC 25923. A nitrate test was used to differentiate the *C. pseudotuberculosis* biovars *equi* and *ovis*. The conventional phenotypic tests used to identify the potentially toxicogenic species are listed in Table I.

Stock cultures were maintained as suspensions in 10% skim milk containing 25% glycerol at -20°C. The microorganisms were cultured on Columbia agar base (CAB) (BBL, Sparks, USA) containing 5% sheep blood for 24-48 h at 37°C for all the phenotypic and genotypic procedures described in this study. For DNA extraction, microorganisms were grown on CAB.

Toxicogenicity tests - All *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* strains were evaluated for the presence of the *tox* gene by PCR using the DT primer set (PCR-DT), which targets fragment A of diphtheria toxin. The primer pair (forward, ATCCACTTTTAGTGCGA-GAACCTTCGTCA and reverse, GAAAACCTTTCTTCGTACCACGGGACTAA) was able to amplify a 248 bp fragment from both the control and clinical strains (Pallen et al. 1994, Efstratiou et al. 1998).

mPCR - The conditions for amplification using this mPCR assay were based on reactions described previously (Pacheco et al. 2007, Pimenta et al. 2008a, b). The nucleotide primers used for mPCR are listed in Table I. In this study, primers targeting the 16S rRNA sequences (816 bp) of both *C. pseudotuberculosis* and *C. ulcerans* strains were used along with a reverse primer for the amplification of a *pld* sequence specific for *C. pseudotuberculosis* (203 bp) to discriminate *C. pseudotuberculosis* from *C. ulcerans*. In addition, a primer pair, Dipht 4 (Nakao et al. 1996), that amplifies a region linking the A and B fragments of the DT gene was used to generate an

TABLE I
Phenotypic tests used for differentiation of potentially toxicogenic *Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*

Microorganisms	Phenotypic properties ^a												
	Haemolysis ^b	Cistinasase (H ₂ S) ^c	Dnase	Urease	Nitrate reductase	CAMP Reaction ^d	Glucose	Maltose	Sucrose	Glycogen	Starch	Pyrazinamidase	Catalase
<i>C. diphtheriae</i> ^e													
biotype <i>gravis</i>	-	+	+	-	+	-	+	+	-/+	+	+	-	+
biotype <i>mitis</i>	+/-	+	+	-	+	-	+	+	-/+	-	-	-	+
biotype <i>intermedius</i>	-	+	+	-	+	-	+	+	-/+	-	-	-	+
biotype <i>belfanti</i>	-	+	+	-	-	-	+	+	-/+	-	-	-	+
<i>C. ulcerans</i> ^e	+	+	+	+	-/+	Reverse	+	+	-/+	+/-	+	-	+
<i>C. pseudotuberculosis</i> ^e													
biovar <i>ovis</i>	+	+	-	+	-	Reverse	+	+	-/+	-/+	+	-	+
biovar <i>equi</i>	+	+	-	+	+	Reverse	+	+	-/+	-/+	+	-	+

a: phenotypic tests accordingly with Efstratiou and George (1999), Funke and Bernard (2007) and Pimenta et al. (2008b); b: Columbia agar base with 5% sheep blood; c: H₂S production on Tinsdale medium when isolates are stabbed into the surface; d: phospholipase production leading to inhibition of the beta-haemolysis of *Staphylococcus aureus*, characterising the reverse CAMP reaction; e: identification may be also performed by the API-Coryne System version 3.0; +: positive; -: negative.

amplicon of 303 bp, which is between the size of the amplicons for *rpoB* (446 bp) and *dtxR* (258 bp) of *C. diphtheriae* strains. The amplification of the *rpoB* gene was used both as an internal control and to confirm that the isolate was a *Corynebacterium* sp. when no other amplicons were observed after the amplification reaction.

Microbial DNA was extracted by boiling a suspension containing a loopful of freshly grown bacteria on CAB in 500 µL of sterile deionised MilliQ water for 10 min. The suspension was centrifuged at 13,000 rpm and 1 µL of the supernatant was used in the final multiplex reaction described in Table II, with primers diluted to 2 mM. The amplification was performed in a MyCycler thermal cycler (BioRad Laboratories).

The amplified products were observed after electrophoresis on 1% agarose gels and SYBR green staining.

RESULTS

Identification of C. diphtheriae, C. ulcerans and C. pseudotuberculosis isolates - The microorganisms were identified by both biochemical assays and mPCR. All sucrose-fermenting and non-sucrose-fermenting *C. diphtheriae* and *C. ulcerans* strains exhibited DNase activity, whereas 93 of the *C. pseudotuberculosis* strains belonging to biotypes *ovis* and *equi* were negative for the expression of DNase. The urease production assay showed positive results for *C. ulcerans* and *C. pseudotuberculosis*; both species also inhibited the beta-haemolysis of *S. aureus* ATCC 25923, indicating a positive reverse CAMP reaction.

The API profiles identified all microorganisms included in this study to the species level, with percentages greater than 98%. Only *Corynebacterium* strains that were identified with API at percentages higher than 98%, except for five strains belonging to the *C. amycolatum* complex, were used in this investigation. Using these confirmed strains and conventional biochemical tests was important for the accuracy of the mPCR assay.

The mPCR was identified all *C. pseudotuberculosis* strains independently of the biotype (*equi* or *ovis*), yielding at least three amplicons: 816 bp, corresponding to *16S rRNA*, 446 bp, corresponding to *rpoB*, and 203 bp, corresponding to *pld*. *C. ulcerans* had only two amplicons, which corresponded to *16S rRNA* and *rpoB*. The *C. ulcerans* strains did not produce amplicons for the *pld* gene, although PLD was expressed by all strains, confirming the specificity of the primer (*pld* R) used in this study for *C. pseudotuberculosis*. The mPCR also identified all sucrose-fermenting and non-sucrose-fermenting *C. diphtheriae* strains independently of the biotype (*gravis*, *mitis*, *intermedius* and *belfanti*) or site of colonisation (respiratory tract, skin, bone and blood), yielding amplicons for at least two genes, *rpoB* (446 bp) and *dtxR* (258 bp).

In this investigation, all isolates of *C. diphtheriae*, *C. pseudotuberculosis* and *C. ulcerans* yielded the amplicons targeted in the mPCR assay, giving the amplification pattern presented in Figure. No isolates identified as the species above yielded unequivocally different amplification patterns using the mPCR conditions described in this study. The additional amplicon observed in DT gene-

TABLE II

Deoxyoligonucleotide primers and amplification steps for simultaneous characterization of *Corynebacterium diphtheriae*, *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* by the multiplex polymerase chain reaction used in this study

Components ^a	Volume (µL)	Amplification reaction
Accuprime buffer (Invitrogen)	1.0	
<i>16SF</i> -ACCGCACTTTAGTGTGTGTG	0.3	
<i>16SR</i> -TCTCTACGCCGATCTTGAT	0.3	
C2700 F (<i>rpoB</i>) CGTATGAACATCGGCCAGGT	0.2	
C3130 R (<i>rpoB</i>) TCCATTTGCCGAAGCGCTG	0.2	1: initial denaturation (95°C - 3 min)
<i>pld</i> F-ATAAGCGTAAGCAGGGAGCA	0.2	
<i>pld</i> R-ATCAGCGGTGATTGTCTTCC	0.2	2: 35 cycles (95°C - 1 min, 55°C - 40s, 68°C - 1 min: 30s)
<i>dtxR</i> 1F-GGGACTACAACGCAACAAGAA	0.2	
<i>dtxR</i> 1R-CAACGGTTTGGCTAACTGTA	0.2	3: final extension (68°C - 5 min)
<i>diph</i> 4F-GAACAGGCGAAAGCGTTAAGC	0.2	
<i>diph</i> 4R-TGCCGTTTGATGAAATTCTTC	0.2	
Accuprime Taq polymerase	0.5	
H ₂ O milliQ deionized	5.3	
DNA extracted	1.0	
Total volume	10	

^a: AccuPrime buffer (Invitrogen Co) contains all components necessary for optimal amplification reactions. Primers were used in concentration of 2mM.

bearing strains (*dipht* 4- 303 bp) was correlated with amplification by the DT primer set (PCR-DT), which targets fragment A of DT (248 bp). The primers targeting fragment A of DT gave a product with a molecular mass similar to the *dtxR* amplicon (258 bp) and therefore, these primers not included in this mPCR assay.

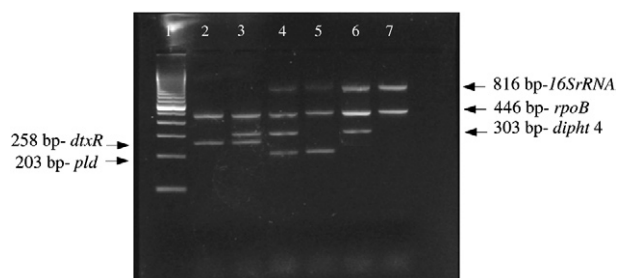
All tested *Corynebacterium* spp that were not associated with *C. diphtheriae*, *C. pseudotuberculosis* or *C. ulcerans*, except for *Corynebacterium pseudodiphtheriticum* strains, only yielded amplicons for *rpoB*. The *rpoB* sequence of *C. pseudodiphtheriticum* could only be amplified using the cycling conditions described in this study after replacing C3130 R (*rpoB*) TCCATTTCGC-CGAAGCGCTG with the reverse primer 5'-TCCATCT-CACCGAAGCGCTC-3'.

The Gram-positive bacteria *Nocardia* sp, *R. equi* and *Oerskovia* sp. included in this investigation did not yield amplicons in this mPCR assay using the cycling conditions described.

DISCUSSION

The prevalence of *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* infections is greater than previously recognised by the medical and veterinary communities in many regions worldwide due to the difficulty in accurately identifying these pathogens and their ability to produce DT.

The laboratory identification of *Corynebacterium* organisms is usually complex and expensive, requiring the use of conventional and/or miniaturised biochemical methods (Funke & Bernard 2007). The molecular typing methods used for the characterisation of *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* are important tools for the rapid detection and identification of bacterial clones that harbour the *tox* gene and these methods have several advantages over the traditional methods used to determine the toxigenicity of corynebacterial isolates (Hauser et al. 1993, Pallen et al. 1994, Nakao & Popovic 1997, Cetinkaya et al. 2002, Mothershed et al. 2002, Sing et al. 2003, Spier et al. 2004, Pacheco et al. 2007, Pimenta et al. 2008a, b).



Amplification profile by multiplex polymerase chain reaction of *Corynebacterium diphtheriae*, *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans*. Lane 1: molecular weight (100-bp DNA ladder); 2: *C. diphtheriae* ATCC 27010 (tox⁺); 3: *C. diphtheriae* ATCC 27012 (tox⁺); 4: *C. pseudotuberculosis* biovar *equi* E31 (tox⁺); 5: *C. pseudotuberculosis* biovar *ovis* E40 (tox⁺); 6: *C. ulcerans* CDC KC279 (tox⁺); 7: *C. ulcerans* BR-AD22 (tox⁺); +: positive; -: negative.

In this study, a species-specific mPCR assay for the identification of potentially toxigenic human and animal isolates of *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* was developed. The taxonomic position of most of the isolates could also be determined using this mPCR assay, which includes an *rpoB*-targeting genus-specific primer pair for the identification of *Corynebacterium* sp. (Khamis et al. 2004). All *Corynebacterium* strains used in this study, except for *C. pseudodiphtheriticum* isolates, yielded amplicons with the *rpoB* primers, as previously observed (Pacheco et al. 2007).

The mPCR method using primers for the detection of the *dtxR* gene was also able to identify *C. diphtheriae* isolates independent of DT production, including isolates of the biotypes *gravis*, *mitis*, *intermedius* and *belfanti*, as previously demonstrated (Pimenta et al. 2008a).

C. ulcerans and *C. pseudotuberculosis* yielded amplicons with the 16S rRNA primers, whereas only *C. pseudotuberculosis*, regardless of the biovar (*ovis* or *equi*), yielded amplicons for *pld*, as previously described (Pacheco et al. 2007). No diverse amplicons were observed for *C. diphtheriae* after amplification with primers targeting 16S rRNA or *pld*.

To detect toxin production by the three species included in this study, primers targeting *Dipht* 4 (Nakao et al. 1996), a sequence between the A and B fragments of DT, were chosen due to the adaptation of the primer sequences to the cycling conditions used for mPCR and the molecular mass of the generated amplicon, which allowed for an easier interpretation of the mPCR results after electrophoresis with agarose gels.

The mPCR assay yielded reliable results when performed by at least two different technicians who used the same strains at different times. For clinical purposes, *C. pseudotuberculosis*, *C. ulcerans* and toxigenic and non-toxigenic *C. diphtheriae* control strains should be included in the assay. The inclusion of these strains was shown to be essential to monitor the amplification profiles of the isolates. No non-specific amplicons were observed for other *Corynebacterium* spp with mPCR in this study. Amplicons were not observed for the coryneform strains tested.

The advantages of this mPCR assay over conventional biochemical procedures are its rapidity, ease of performance, the large number of strains that can be simultaneously tested and the easy interpretation of the mPCR results. This novel species-specific mPCR system may facilitate routine laboratory diagnosis and/or epidemiological research on this group of potentially zoonotic and toxigenic corynebacterial pathogens: *C. pseudotuberculosis*, *C. ulcerans* and *C. diphtheriae*.

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