Increase in detection of *Corynebacterium diphtheriae* in Canada: 2006–2019

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Abstract

Background: Increasingly, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) has been used to provide rapid, inexpensive and precise identification of bacteria, including *Corynebacterium* species. Only three *Corynebacterium* species are able to produce diphtheria toxin (DT), and strains recovered may be either toxin-producing or non-toxin-producing. It appears the more precise bacterial identification provided by MALDI-TOF systems has led to an increase in requests submitted to the National Microbiology Laboratory (NML) for toxin testing.

Objective: To describe the number of isolates identified as *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*, submitted to the NML between January 2006 and July 30, 2019, including their geographic area, source, and whether they produce DT.

Methods: Referrals to the NML of human or animal isolates that were identified as any of those three *Corynebacterium* species were studied with respect to province, source and toxigenicity. Species identification was confirmed and then specimens were tested by polymerase chain reaction for the presence of *tox* genes and, if positive, for expression of DT by the modified Elek method. Analysis was descriptive.

Results: Over the study period, 639 isolates were identified as *C. diphtheriae*, 22 isolates as *C. ulcerans*; no isolates were identified as *C. pseudotuberculosis*. There was an increase in *C. diphtheriae* referrals for DT testing: from eight per year in 2006 to an average of 15 per month in 2019, or a 1,200% increase over the 13.6-year period. The referrals were primarily from western Canada (n=609/639; 95%). Most (638/639, 99%) were human isolates and most were obtained from cutaneous sites. Of those isolates, 87/639 (13.6%) were found to be toxigenic and 552/639 (86.4%) non-toxigenic. Among *C. ulcerans* referrals, 17/22 (77%) were from humans and five (23%) were from animals, with 10/22 (45%) being toxigenic.

Conclusion: There has been a marked increase in referrals to the NML for DT testing of *Corynebacterium* species. This could be due to the enhanced ability to identify these bacteria using MALDI-TOF systems. Ongoing monitoring will help to assess whether the increase is due solely to increased precision of diagnosis or whether these are emerging cutaneous pathogens.

Suggested citation: Bernard KA, Pacheco AL, Burdz T, Wiebe D. Increase in detection of *Corynebacterium diphtheriae* in Canada: 2006–2019. Can Commun Dis Rep 2019;45(11):296-301. https://doi.org/10.14745/ccdr.v45i11a04

Keywords: *Corynebacterium diphtheriae*, diphtheria-toxin-producing species, increase in recovery, MALDI-TOF

Introduction

Classic *Corynebacterium diphtheriae* (*C. diphtheriae*) infection—causing sore throat, fever and respiratory symptoms—is rare in Canada (1–3). Since vaccination against diphtheria toxin (DT) is now part of the routine immunization schedule, there are fewer than four cases of this notifiable disease annually (4,5).

In the past, *Corynebacterium* species isolated from non-respiratory clinical specimens were understudied and underreported. These bacteria were nearly always considered as contaminants, and were neither identified (i.e. genus and species) nor tested for antimicrobial susceptibility, even if recovered in pure culture from sterile body fluids or deep

wound abscesses (3). As well, unambiguous identification of strains to genus and species could be difficult and expensive to perform solely using phenotypic test methods (1,3).

It is now more widely appreciated that non-respiratory *C. diphtheriae* can cause skin and wound infections. Cutaneous diphtheria often presents as well-demarcated, sometimes foul-smelling ulcers or as nodules that are slow healing and highly contagious. People who are particularly at risk include those with co-morbidities such as HIV, hepatitis B or C, diabetes, a history of recurrent ulcers, alcohol abuse, a history of intravenous drug use, living in poorer-socioeconomic conditions, homeless shelters or refugee camps, or who travelled to countries where these pathogens are endemic (6–11). Treatment for cutaneous diphtheria often includes the use of the same systemic antibiotics as those used for respiratory disease, plus the implementation of isolation precautions (6–10) to prevent bacteria from lesions serving as a reservoir for further spread of infection (11,12). However, in some instances, difficult-to-treat multidrug resistant strains of *C. diphtheriae* have been detected from cases of cutaneous diphtheria (13). Occasionally, sepsis and death may result. The role of prior vaccination with diphtheria toxoid in preventing cutaneous infections is not well understood. The use of antitoxin is generally not recommended, unless signs of systemic toxicity are present (7).

Identification of the genus and species of *Corynebacterium* species has now been simplified by the widespread use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) instruments and more precise molecular methods. Proteins are extracted from bacteria and the resulting fingerprints are then compared to entries housed in the system's data library. High confidence identification of species can be obtained within a few minutes, at a cost of less than $1.00 Canadian per sample (14,15). The MALDI-TOF procedure has made it possible to routinely identify the diphtheria-toxin-producing species: *C. diphtheriae*; *C. ulcerans*; and *C. pseudotuberculosis*. Infections involving *C. ulcerans* and *C. pseudotuberculosis* are rare in humans and are acquired through infected animals. The *C. ulcerans* infections in humans have been linked to contact with diseased pets, such as dogs or cats, whereas *C. pseudotuberculosis* occurs in sheep and goats, so veterinarians or animal handlers are at increased risk for acquisition (3).

In Canada, the use of MALDI-TOF instruments began about 2012. These instruments are now widely distributed across the country—in all provincial public health laboratories, in many private/public hospital institutions and in veterinary laboratories specializing in infectious diseases of animals. The clear benefits for routine use of MALDI-TOF systems are the significantly reduced costs for characterizing bacteria and the greatly improved speed at which pathogens such as *C. diphtheriae* can be identified. These benefits result in prompt implementation of appropriate treatment options. However, MALDI-TOF systems can only identify these bacteria and cannot determine whether or not any of these *Corynebacterium* species actually produce DT. Over the past few years, the National Microbiology Laboratory (NML) began to receive an increasing number of requests for DT testing from public health laboratories and veterinary laboratories across the country. The objective of this study was to describe the number and features of the *C. diphtheriae*, *C.ulcerans* and *C. pseudotuberculosis* samples submitted to the NML between January 2006 and July 30, 2019, including the percentage that produced DT.

Methods

Record review

The NML's Special Bacteriology Unit (SBU) laboratory records were reviewed from 2006 to July 30, 2019 for test requests for DT detection. Information reviewed here included province, site of infection if known, the species of DT-producing *Corynebacterium* involved and whether or not the strain was toxigenic. Referrals received from January to July 2019 were further analyzed with respect to the source of the strain, as these data were typical of referrals in other years. Information regarding vaccination history and underlying patient history was generally scant or not provided.

Overview of specimen types received

Since 2011, Cadham Provincial Laboratory (Manitoba's public health laboratory) only forwards strains to the NML that have been found to be positive for *tox* gene targets by polymerase chain reaction (PCR) (*D. Alexander, personal communication, July 24, 2019*). Other provincial centres forward all strains that may require testing for DT to the NML, as toxin analyses are not offered in their own laboratory settings. The NML receives pure cultures of bacterial isolates that have been recovered at provincial public health laboratories or by their clientele, which the provincial public health laboratories then forward to the NML for toxin testing. Occasionally strains are received from veterinary laboratories.

Confirmation of *Corynebacterium diphtheriae* isolates

From 2006 to approximately 2018, SBU strains had been tested phenotypically using an API CORYNE (bioMérieux, France) panel to provide species-level identification and to assist with categorizing the isolate with respect to one of the four *C. diphtheriae* biovars (i.e. biotypes: gravis; mitis; belfanti; and intermedius) (1,3). The API CORYNE panel unambiguously identified *C. diphtheriae* for strains previously characterized by the gold standard 16S rRNA gene sequencing method; molecular methods are otherwise used at the NML to corroborate species level identification as required. The NML ceased characterization of strains to biovar as of May 2019, except upon request. *Corynebacterium diphtheriae* identification

was validated for use by the NML for the Bruker Microflex and Biotyper library, where this method also provides unambiguous identification of species (data not shown).

Identification of *Corynebacterium ulcerans* and *C. pseudotuberculosis* isolates

These species are readily discernable from all others in the genus *Corynebacterium*, including *C. diphtheriae*, but are otherwise not easily differentiated from each other by commonly-used laboratory methods (1). Phenotypic results for these species, using conventional sugars or API CORYNE panels, are very similar (1,2). These species have 99.4% similarity to each other by 16S rRNA gene sequencing, which is too close to provide definitive discrimination from each other, and can produce similar scores to each other using MALDI-TOF systems (data not shown) (1). Therefore, precise identification of these species is corroborated at the NML by use of partial *rpo*B gene sequencing (16).

Detection of diphtheria *tox* gene targets and expression of diphtheria toxin

The SBU had used conventional PCR approaches to detect *tox* gene targets (13), but switched to a real time PCR-based method in 2018. Real-time PCR detects the *tox* gene and a region of the *rpo*B gene specific for *C. diphtheriae* using a Quant Studio platform (Applied Biosystems) (17,18). The toxin-producing strain, *C. diphtheriae* ATCC 19409 (NCTC 3984), and the non-toxigenic strain, *C. xerosis* ATCC 373^T, were used as positive and negative controls, respectively. Expression of DT was detected by performing the modified Elek test only for strains where *tox* genes had been detected by PCR (19,20). Elek-positive results may be reported at 48 hours, or as soon as expression is visually detected, with negative results being reportable after 48 hours. Isolates that are found to be positive for *tox* gene by PCR but negative for DT expression by Elek are reported as non-toxigenic (20); these occur rarely in Canada, accounting for 8% or fewer of all *tox* gene-positive strains (21).

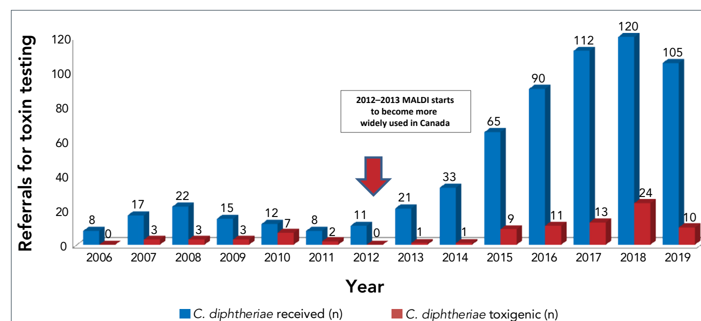
Results

Over the study period there were a total of 639 isolates identified as *C. diphtheriae*, where 638/639 (99%) were from human disease and 1/639 (1%) was from a horse. Twenty-two isolates (n=17; 77% from humans and n=5; 23% from animals) were identified as *C. ulcerans* and no isolates were identified as *C. pseudotuberculosis*.

Corynebacterium diphtheriae

Between 2006 and 2012, an average of fourteen isolates of *C. diphtheriae* was referred annually. Between 2013 and 2015 (which coincided with the increasing use of MALDI-TOF), the number of annual referrals increased to an average of 40. Between 2016 and 2018, the number of annual referrals increased to 115. Between January and July 2019, an average of 15–16 referrals per month had been received at the NML: extrapolated, this would be approximately 185 by the end of the year. Comparing the pre-MALDI-TOF era for toxin testing of eight strains (in 2006) to an estimated 185 referrals projected for 2019, this represented a 1,200% or 12-fold increase over the review period (**Figure 1**).

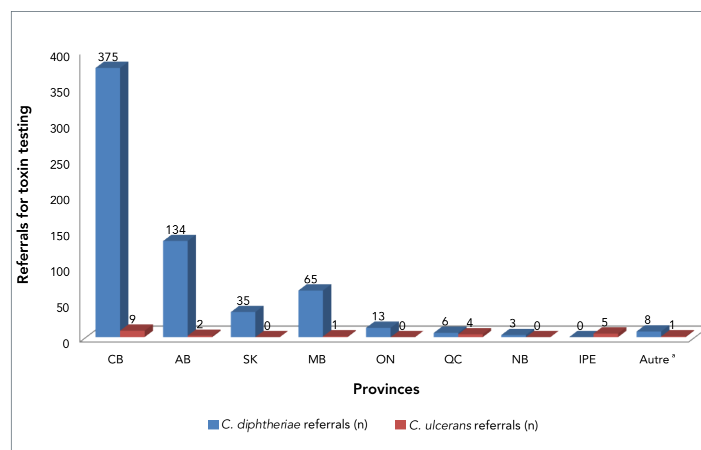
Figure 1: *Corynebacterium diphtheriae* referrals for toxin testing by year, subset by number of toxigenic strains



Abbreviations: *C. diphtheriae*, *Corynebacterium diphtheriae*; MALDI, matrix-assisted laser desorption/ionization spectrometry

Strains identified as *C. diphtheriae* were derived primarily from western Canada (n=609/639; 95%), that is, from British Columbia, Alberta, Saskatchewan and Manitoba, with small numbers from Ontario, Quebec and New Brunswick and from outside of Canada (**Figure 2**).

Figure 2: *Corynebacterium diphtheriae* and *C. ulcerans* referrals, by province



Abbreviations: AB, Alberta; BC, British Columbia; *C.*, *Corynebacterium*; MB, Manitoba; NB, New Brunswick; ON, Ontario; PEI, Prince Edward Island; QC, Quebec; SK, Saskatchewan
ª Other, sources outside of Canada

Referrals of *C. diphtheriae* subdivided by province were as follows (% of n=631): British Columbia, 375 (59%); Alberta, 134 (21%); Saskatchewan, 35 (5%); Manitoba, 65 (10%); Ontario, 13 (2%); Quebec, six (0.9%); and New Brunswick, three (0.4%). No *C. diphtheriae* referrals were received from Nova Scotia, Prince Edward Island or Newfoundland, and eight were received

from outside of Canada. Referrals of *C. ulcerans* by province were as follows (% of n=21): British Columbia, nine (43%); Alberta, two (9%); Manitoba, one (5%); Quebec, four (19%); and Prince Edward Island, five (24%, all from animals). No strains were referred from Saskatchewan, Ontario, New Brunswick, Nova Scotia or Newfoundland, and one from outside of Canada.

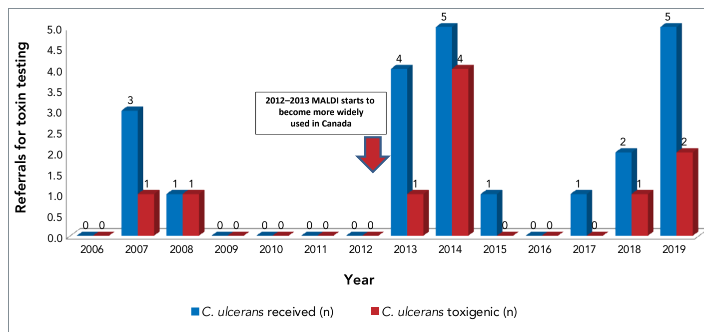
The majority of the 105 *C. diphtheriae* strains from 2019 were obtained from skin infections. There were 89 (85%) specimens from cutaneous sites that included the abdomen and abscesses/wounds from the ankle, arm, elbow, foot, finger, head/scalp, hip, leg, knee, shin, thigh, thumb, tibia and toe; 16 specimens (15%) from non-cutaneous sites that included blood culture (n=1), uterine (n=1), ear (n=2), sinus, sinus washing, nasal wound and nose (n=5), throat (n=4), sputum (n=1) and unknown sites (n=2).

In 2019, there were 10 toxigenic strains recovered from nose or cutaneous sites. These referrals were from Alberta (n=8), British Columbia (n=1) and Manitoba (n=1). No patient was symptomatic of classic, respiratory-type diphtheria.

Corynebacterium ulcerans

A small number of referrals (n=22, **Figure 3**) were identified as *C. ulcerans*. Seventeen (77%) samples were obtained from human cases, with sources described as pus or from cutaneous sources (arm, elbow, foot, leg or toe wounds). Five animal-derived specimens were also received from mink (n=1, lung), dog (n=1, ear), cat (n=1) and horse (n=2, abscess, skin). Such testing had also increased by approximately 35% between 2006–2012 and 2013–2019 (Figure 3). Critically, although numbers were small, we found that 10/22 (45%) of *C. ulcerans* strains produced DT.

Figure 3: *Corynebacterium ulcerans* referrals for toxin testing by year, subset by number of toxigenic strains



Abbreviations: *C. ulcerans*, *Corynebacterium ulcerans*; MALDI, matrix-assisted laser desorption/ionization spectrometry
Notes: Five *C. ulcerans* strains shown from 2019 were received from a veterinary laboratory. The remaining 17 samples were obtained from human sources

Discussion

The NML has documented a 1,200% or 12-fold increase in referrals of *Corynebacterium* specimens for DT testing in less than 15 years. These are predominately non-toxigenic *C. diphtheriae* strains from cutaneous sources in humans recovered in pure culture or co-recovered in association with other Gram-positive bacteria (6). Although the overall numbers were small, a 35% increase of referrals involving *C. ulcerans* was found, that included strains from humans and animals. Similar increases of both *C. diphtheriae* and *C. ulcerans* have been reported in other countries including the United States (7–11). This increase is likely due in part to the increasingly widespread use of MALDI-TOF systems and precise molecular identification methods. It may also reflect that *C. diphtheriae* is an emerging pathogen for non-respiratory infections.

Clinical implications

Canadian clinicians should be aware that identification of *C. diphtheriae* and *C. ulcerans* from certain patient and specimen types is becoming increasingly common. Strains should be tested for toxin production and, if toxigenic, a public health response, including testing/monitoring of contacts of patients, may be indicated in addition to the usual treatment approaches (5).

Antibiotic sensitivity testing of these species has only rarely been requested but may be useful. In a recent article, we described antimicrobial susceptibility testing (AST) results based on an ancillary study of 195 strains of *C. diphtheriae* and 20 strains of *C. ulcerans* (22). Although these species are usually susceptible to first line antibiotics (22), the occasional strain of *C. diphtheriae* has been found to be penicillin or multidrug resistant (13,23); in some instances, azithromycin may be indicated (10). Of note, the Canadian notifiable disease definition for diphtheria includes *C. diphtheriae* as well as both *C. ulcerans* and *C. pseudotuberculosis* and, if identified in a symptomatic patient, would be classified as a confirmed case (5). Cutaneous diphtheria would also be classified as a confirmed case if a toxigenic strain was recovered from a wound/cutaneous site (5).

Limitations

There are some inherent limitations to the results shown here. It is not mandatory for laboratories to send these taxa to the NML for further characterization. For example, Cadham Provincial Laboratory conducted their own DT testing by PCR on more than 600 strains of *C. diphtheriae* in the first six months of 2019—all were found to be negative for the presence of *tox* genes (*D Alexander, personal communication, July 24, 2019*). These were not referred to the NML so the data presented here from the NML is an underestimate.

Identification of *C. diphtheriae* or *C. ulcerans* from a variety of clinical specimens, especially cutaneous sites, requires that local laboratories are able to select for/recognize these agents from among flora found in the biomes of infected cutaneous or respiratory sites. An algorithm may assist in the process of determining putative *C. diphtheriae* or *C. ulcerans* strains (1). Furthermore, it is difficult to conjecture if the lack of strains received for DT testing from central and eastern Canada reflects that no actual cases exist, or they occur more rarely than in the

west. It may reflect the use of different guidance documents or different laboratory practices.

Future research

Ideally, the next steps could include both clinical and laboratory research, which would include assessment of cutaneous diphtheria as an emerging infection and collection of more complete clinical data on treatment versus outcome. Further laboratory research on these agents includes consensus-based development of a national and internationally-based typing scheme for characterization of such strains at whole genome sequence level, for use *in silico* tracking of strains and for better characterization of virulence factors.

Conclusion

In less than 15 years, there has been a marked increase in requests for DT testing of *C. diphtheriae* and *C. ulcerans* in Canada. This increase could be due to an enhanced ability to identify these agents using MALDI-TOF systems. Ongoing monitoring of these bacterial strains will help to assess whether the increase in NML referrals is due solely to increased precision of diagnosis or whether these are emerging cutaneous pathogens.

Authors' statement

KAB wrote the manuscript and reviewed the data
ALP, TB and DW performed all technical assays and reviewed the manuscript

Conflict of interest

None.

Acknowledgements

We gratefully acknowledge the following centres for sending study strains to the National Microbiology Laboratory:
Dr. L Hoang, BC Centre for Disease Control, Vancouver, British Columbia; Dr. G Tyrell and S Gee, Provincial Laboratory, Edmonton, Alberta; R Kitzel, R Romanow Provincial Laboratory, Regina, Saskatchewan; Dr. P Van Caeseele and Dr. D Alexander, Cadham Laboratory, Winnipeg, Manitoba; Dr. J Kus, Public Health Ontario, Toronto, Ontario (ON); Dr. H Dick, Ideex Laboratories, Richmond Hill, ON; Dr. MC Domingo, Laboratoire de Santé Publique du Québec, Sainte-Anne-de-Bellevue, Quebec; Staff, the Moncton Hospital, Moncton, New Brunswick (NB); Staff, Hôpital Georges-Dumont, Moncton, NB; M Saab, Atlantic Veterinary Hospital, Charlottetown, Prince Edward Island. We thank current and former students A Vachon, D Kielich and J Ung for technical services provided.

Funding

Public Health Agency of Canada A Base funding was used for this study.

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