Journal of Clinical Microbiology®

EPIDEMIOLOGY

Molecular and Epidemiological Characterization of Toxigenic and Nontoxigenic *Corynebacterium diphtheriae*, *Corynebacterium belfantii*, *Corynebacterium rouxii*, and *Corynebacterium ulcerans* Isolates Identified in Spain from 2014 to 2019

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ABSTRACT This study examines the microbiological and epidemiological characteristics of toxigenic and nontoxigenic *Corynebacterium* isolates submitted to the national reference laboratory in Spain, between 2014 and 2019, in order to describe the current situation and improve our knowledge regarding these emerging pathogens. Epidemiological information was extracted from the Spanish Surveillance System. Microbiological and molecular characterization was carried out using phenotypic methods, multilocus sequence typing (MLST), whole-genome sequencing (WGS), and core genome MLST (cgMLST). Thirty-nine isolates were analyzed. Twenty-one isolates were identified as *Corynebacterium diphtheriae* (6 toxigenic), 14 as *C. belfantii*, 4 as *C. ulcerans* (3 toxigenic), and 1 as *C. rouxii*. One *C. diphtheriae* isolate was identified as nontoxigenic *tox* gene bearing (NTTB). Ages of patients ranged from 1 to 89 years, with 10% (3/30) of nontoxigenic and 22% (2/9) of toxigenic isolates collected from children less than 15 years. Twenty-five of the patients were males (17/30 in nontoxigenic; 8/9 in toxigenic). MLST identified 28 sequence types (STs), of which 7 were described for the first time in Spain. WGS analysis showed that 10 isolates, including 3 toxigenic isolates, harbored a variety of antibiotic resistance genes in addition to the high prevalence of penicillin resistance phenotypically demonstrated. Phylogenetic analysis revealed one cluster of isolates from family members. Risk information was available for toxigenic isolates (9/39); 3 patients reported recent travels to countries of endemicity and 3 had contact with cats/dogs. One unvaccinated child with respiratory diphtheria had a fatal outcome. Including nontoxigenic *Corynebacterium* infections in disease surveillance and using WGS could further improve current surveillance.

KEYWORDS *Corynebacterium*, *Corynebacterium* infections, diphtheria toxin, microbiology, diphtheria, epidemiology

Diphtheria is an acute infectious disease that affects the upper respiratory tract and occasionally the skin. Classical diphtheria is due to the production of a toxin during the infection by strains lysogenized by a bacteriophage (corynephage) harboring

Citation Hoefer A, Pampaka D, Herrera-León S, Peiró S, Varona S, López-Perea N, Masa-Calles J, Herrera-León L. 2021. Molecular and epidemiological characterization of toxigenic and nontoxigenic *Corynebacterium diphtheriae*, *Corynebacterium belfantii*, *Corynebacterium rouxii*, and *Corynebacterium ulcerans* isolates identified in Spain from 2014 to 2019. J Clin Microbiol 59:e02410-20. https://doi.org/10.1128/JCM.02410-20.

Editor Daniel J. Diekema, University of Iowa College of Medicine

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Received 17 September 2020
Returned for modification 29 October 2020
Accepted 21 November 2020

Accepted manuscript posted online 9 December 2020
Published 18 February 2021

the toxin gene. Diphtheria toxin (DT) is produced by toxigenic strains of the human pathogen *Corynebacterium diphtheriae* as well as zoonotic *C. ulcerans* and *C. pseudotuberculosis*. *C. diphtheriae* isolates have traditionally been categorized into the four biovars Gravis, Mitis, Intermedius, and Belfanti (1). Recently, on the basis of genomic sequencing and biochemical and chemotaxonomic analyses, Dazas et al. proposed the name of *C. belfantii* for the strains previously considered *C. diphtheriae* bv. Belfanti (2) Additionally, a novel member of the *diphtheriae* species complex named *C. rouxii* has recently been described (3).

The incidence of diphtheria in Europe is very low, with a notification rate of less than 0.01 cases per 100,000 habitants (4, 5). The reported cases are mostly diagnosed among travelers, refugees, asylum seekers, or immigrants from countries of diphtheria endemicity (4–9). The incidence of diphtheria in Spain is similar to that in the rest of Europe (4). A highly effective toxoid-based vaccine for diphtheria exists that is considered to have saved millions of lives. Vaccination of diphtheria was introduced in Spain in the 1960s as a primary course of three doses in the first year of life. Boosters in the second year of life, childhood, adolescence, and adulthood (≥65 years) were gradually introduced in the national vaccination schedule (10). In 2015, there was a fatal case of respiratory diphtheria in an unvaccinated 6-year-old boy in Catalonia (11).

Spain had surveillance in place only for cases involving respiratory toxigenic *C. diphtheriae* prior to 2014, but since then, all toxigenic diphtheria cases must be reported following the European Union case definition. A confirmed case of diphtheria is defined as any person meeting the laboratory criteria (isolation of toxin-producing *C. diphtheriae*, *C. ulcerans*, or *C. pseudotuberculosis* from a clinical specimen) and at least one of the clinical forms (classic respiratory, mild respiratory, cutaneous, or diphtheria of other sites). As a result of the improving diagnostic capacity in hospitals (e.g., matrix-assisted laser desorption ionization–time of flight mass spectrometry [MALDI-TOF MS]), the reports of suspected cases of *C. diphtheriae* and *C. ulcerans* have increased over time. It has, however, highlighted the lack of knowledge regarding the prevalence and origins of both toxigenic and nontoxigenic corynebacteria circulating in the population. Nontoxigenic corynebacteria have been associated with severe diseases (12, 13). The aim of this study was to review the molecular and epidemiological characteristics of toxigenic and nontoxigenic isolates of corynebacteria, collected from 2014 to 2019 and to improve our knowledge regarding *Corynebacterium* sp. infections in Spain.

MATERIALS AND METHODS

Diphtheria surveillance. The Epidemiology Surveillance Service of each autonomous region in Spain urgently notifies the Health Alert and Emergency Coordination Centre (CCAES) and National Centre for Epidemiology (CNE) for any suspected, probable, or confirmed case of respiratory diphtheria (14). The guidelines of the National Surveillance Network (RENAVE) are shown in Table 1.

Microbiological characterization. Microbiological procedures were carried out in accordance with the WHO manual for the laboratory diagnosis of diphtheria (15). Initially, the clinical sample is plated onto an enriched medium, Mueller-Hinton agar supplemented with 5% sheep blood and potassium tellurite, such as Hoyle's tellurite medium. Potential corynebacterial colonies are small greyish colonies with a granular appearance on blood agar and have a gray to black pinpoint appearance on Hoyle's medium. These colonies are then subcultured to Hoyle's, Mueller-Hinton with 5% sheep blood and Tinsdale, where the colonies appear black with a brown halo (16). Subsequently, the *Corynebacterium* species and subspecies were identified using the API Coryne System adhering to the manufacturer's instructions (bioMérieux, Durham, NC). PCR was used to distinguish between *C. ulcerans*, *C. diphtheriae*, and *C. pseudotuberculosis* as well as to detect the *tox* gene (17, 18). If the *tox* gene was detected, the phenotypic expression of the toxin was confirmed via the modified Elek test performed at the WHO Collaborating Centre for Diphtheria and Streptococcal Infections at Public Health England (15, 19).

Antimicrobial susceptibility. Phenotypic antimicrobial susceptibility testing was performed using an Etest diffusion assay in accordance with EUCAST guidelines (20). To classify the sensitivity of the isolates to penicillin G, we used the breakpoints set by EUCAST. For erythromycin, the breakpoints are currently in preparation, so the corresponding CLSI breakpoints were used instead (21). Resistance genes in the WGS data were identified using ResFinder-3.2 (22).

Multilocus sequence typing. The sequence type (ST) of the isolates received was assessed using the MLST scheme described by Bolt et al. (23). We included modified primers described by Both et al. to be able to amplify *dnaE* and *dnaK* of *C. ulcerans* (24). The assigned STs were obtained by uploading the allelic profiles to the PubMLST database (25).

TABLE 1 Diphtheria surveillance as described in the guidelines of the National Surveillance Network

Site of infection	Definition	Action required[a]
Respiratory diphtheria		
Suspected case	A person who meets the clinical criteria, i.e., upper respiratory tract disease with laryngitis, nasopharyngitis, or tonsillitis and a membrane or pseudomembrane.	Notify the CCAES and the CNE
Probable case	A person who meets the clinical criteria and has an epidemiologic link with a confirmed human or animal case.	
Confirmed case	A person who meets the clinical and laboratory criteria, i.e., isolation of toxigenic *C. diphtheriae*, *C. ulcerans*, or *C. pseudotuberculosis*, in a clinical specimen (Elek test positive). The test must be confirmed at the CNM[b].	
Cutaneous diphtheria		
Confirmed case	A person who meets the clinical criteria, i.e., chronic nonprogressive ulcerative lesion that may appear with a dirty gray membrane and the laboratory criteria, i.e., isolation of toxigenic *C. diphtheriae*, *C. ulcerans*, or *C. pseudotuberculosis*, in a clinical specimen (Elek test positive). The test must be confirmed at the CNM.	Notify CNE
Diphtheria in other localization		
Confirmed case	A person who meets the clinical criteria, i.e., a conjunctiva or mucosal lesion and laboratory criteria, i.e., isolation of toxigenic *C. diphtheriae*, *C. ulcerans*, or *C. pseudotuberculosis*, in a clinical specimen (Elek test positive). The test must be confirmed at the CNM.	Notify CNE

[a]CCAES, Health Alert and Emergency Coordination Centre; CNE, National Centre for Epidemiology.
[b]CNM, National Centre for Microbiology.

Whole-genome sequencing. For DNA extraction, we used isolates from blood agar plates. We extracted genomic DNA by using a modified protocol of the Qiamp DNA minikit (Qiagen, Germany).

Details of the WGS and its analysis can be found in the supplemental material.

The resulting core genome MLST (cgMLST) scheme consisted of 1,441 target loci for *C. diphtheriae/C. belfantii and* 1,209 for *C. ulcerans*. An accessory target scheme with 735 and 961 more loci was defined during the same process (for *C. diphtheriae/C. belfantii* and *C. ulcerans*, respectively).

We performed next-generation-based cgMLST with reference-based alignments after read trimming and assembling by using FastQC, Unicycler v.0.4.6, QUAST v.4.1, and Kmerfinder v.3.1, by the Bioinformatics Unit at the Instituto de Salud Carlos III. We performed *in silico* cgMLST by using the generated cgMLST or extended cgMLST scheme. After typing and assigning allele numbers, we calculated distances for tree building. Alleles with missing values in at least one sample and samples with missing values in more than 10% of distance columns were excluded from the comparison table. Subsequently, minimum spanning trees were generated. A cluster was defined as a group of closely related cgMLST-analyzed isolates differing by ≤5 alleles and subclusters with the same similarity threshold but after extended cgMLST.

We performed a comprehensive analysis of the prophages in all isolates using the PHASTER software (https://phaster.ca/). We also assessed the *C. ulcerans* isolates for the presence of a novel pathogenicity island harboring the DT as described by Dangel et al. (26). All isolates were assessed for the presence of the diphtheria toxin regulator gene (*dtxR*) via alignment using a *dtxR* reference sequence (GenBank accession number KU869770).

Data analysis. The epidemiological data were described and presented based on their toxigenicity. Contact tracing was undertaken with every confirmed case. Secondary cases were identified only from the 2015 fatal case of respiratory toxigenic diphtheria. Ten asymptomatic cases were identified in 2 stages, as follows: 9 cases were identified from contacts of the confirmed case and the 10th case was identified from contacts of cases identified at the 1st stage (11). Contact tracing of a case of cutaneous diphtheria in 2019 did not identify any secondary cases, but one dog and two cats did test positive for *Corynebacterium ulcerans*. The isolates from contacts were excluded from the main analysis. Characterization of risk factors was possible only for patients with toxigenic diphtheria. The analysis was conducted using Stata 16 (StataCorp. 2019). The algorithm eBURST was used to conduct clonal analysis and visualize potential phylogenetic relationships between STs using Bionumerics (AppliedMaths) software (27).

Consent and ethical approval. Samples and data used in this study were collected without patient identifiable data for diagnostic and surveillance purposes; therefore, a consent form was not required.

Data availability. All sequence data have been deposited in the ENA database under accession numbers ERS5375606 to ERS5375642 (BioProject accession number PRJEB41500).

RESULTS

Epidemiological and microbiological characteristics. From 2014 to 2019, *C. diphtheriae*, *C. belfantii*, and *C. ulcerans* were isolated from 39 specimens, and they were also isolated from 10 specimens obtained during contact tracing of the 2015 case. Specimens included throat swabs, aspirates, skin swabs, biopsy, sputum and blood.

The microbiological characteristics of the 39 isolates are shown in Table 2. Thirty

TABLE 2 Microbiological and clinical profiles of nontoxigenic and toxigenic *C. diphtheriae*, *C. belfantii*, and *C. ulcerans* isolates in Spain, 2014 to 2019

Characteristic	Total no. (%) of isolates ($n = 39$)	No. (%) of nontoxigenic isolates ($n = 30$)	No. (%) of toxigenic isolates ($n = 9$)
Type and subtype			
C. diphtheriae	21 (54)	15 (50)	6 (67)
Gravis	7 (18)	7 (23)	0 (0)
Mitis	13 (33)	8 (27)	5 (56)
Undetermined[a]	1 (3)	0 (0)	1 (11)
C. belfantii	14 (36)	14 (47)	0 (0)
C. ulcerans	4 (10)	1 (3)	3 (33)
Antimicrobial resistance[a]			
Penicillin G			
Resistant (>0.125 mg/liter)	29 (76)	23 (77)	6 (75)
Susceptible (≤0.125 mg/liter)	9 (24)	7 (23)	2 (25)
Erythromycin			
Resistant (>2 mg/liter)	0 (0)	0 (0)	0 (0)
Intermediate (1 mg/liter)	0 (0)	0 (0)	0 (0)
Susceptible (≤0.5 mg/liter)	38 (100)	30 (100)	8 (100)
Sample type			
Respiratory	21 (54)	18 (60)	3 (33)
Cutaneous	16 (41)	10 (33)	6 (67)
Other	2 (5)	2 (7)	0 (0)

[a]One patient had undetermined subtype and antimicrobial resistance because of starting the antibiotic treatment prior to the specimen collection, therefore $n = 38$ in antimicrobial resistance.

isolates were nontoxigenic and nine were toxigenic, i.e., from confirmed cases. One patient was administered extensive antibiotic treatment before the sample was collected and the causative agent could therefore not be cultured for further analyses. However, the PCR analysis performed directly from the clinical sample demonstrated the presence of toxigenic *C. diphtheriae*. This finding, in combination with the clinical presentation, indicative of toxigenic respiratory diphtheria, lead to this patient being classified as a confirmed case. One nontoxigenic *tox* gene-bearing (NTTB) isolate was identified.

Overall, ages ranged from 1 to 89 years, and 64% (25/39) of the patients were males. Figure 1 shows the distribution of sex and age group per toxigenicity group. The ratio of male to female was 1.3:1 among the nontoxigenic isolates while in toxigenic isolates it was 8:1.

There were three cases with a respiratory presentation; one *C. diphtheriae* in 2015

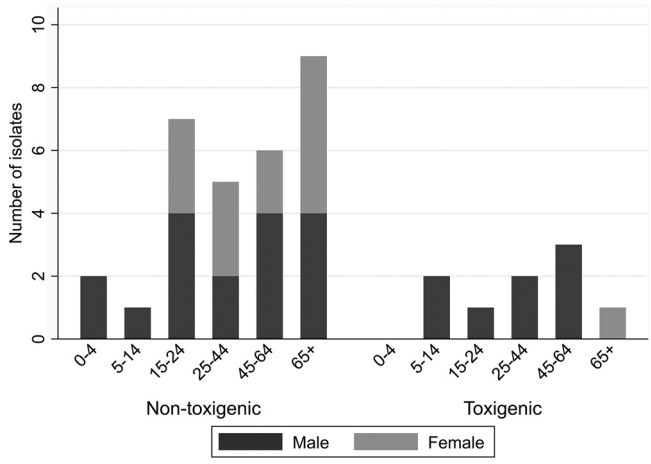

FIG 1 Nontoxigenic and toxigenic *C. diphtheriae*, *C. belfantii*, and *C. ulcerans* isolates in Spain by age and sex groups, 2014 to 2019.

and another in 2018, as well as one *C. ulcerans* in 2019. The 2015 case had a fatal outcome. The other four *C. diphtheriae* cases and the two *C. ulcerans* cases presented cutaneous manifestations. No fatalities were observed among *C. ulcerans* cases. Approximately half of the nontoxigenic patients (16/30) had available clinical information. Among them, five patients had respiratory symptoms, including rhinorrhoea, sinusitis, and pharyngitis. In eight patients, cutaneous symptoms, such as wounds and impetigo, were reported. Other recorded presentations included osteomyelitis and endocarditis (Table 3).

The largest number of isolates was collected in 2017 and 2018, while the lowest number was in 2014 (Fig. 2). None of the 2017 isolates were identified as toxigenic.

A total of 28 sequence types (STs) were identified among the 39 *C. diphtheriae*, *C. belfantii*, and *C. ulcerans* analyzed, of which 21 (75%) appeared only once. The most common sequence type was ST-32 (10%). ST-325 was observed in a toxigenic *C. ulcerans* isolate in 2014 and then in 2016 in a nontoxigenic *C. ulcerans*. ST-297 was observed in 2017 as nontoxigenic *C. diphtheriae* and in 2018 appeared as toxigenic, resulting in respiratory diphtheria. Seven of them, namely, ST-377, ST-482, ST-483, ST-484, ST-511, ST-670, and ST-704, were newly allocated types.

Clonal analysis classified *C. diphtheriae* and *C. belfantii* isolates in 11 clonal complexes designated by eBurst groups and 1 singleton. The five toxigenic *C. diphtheriae* bv. Mitis pertained to five different groups (3, 9, 32, 50, and 51). Of the three toxigenic *C. ulcerans*, two were part of eBurst group 47, and the other was part of eBurst group 48. The nontoxigenic *C. ulcerans* was part of eBurst group 47 (see Fig. S1 in the supplemental material).

Genetic diversity. One of the *C. belfantii* isolates (Cb2017004) had missing values in more than 10% of the distance columns. Consequently, it was excluded from the cgMLST analysis. Finally, 1,091 (*C. diphtheriae/C. belfantii* scheme) and 1,174 alleles (*C. ulcerans* scheme) were used to generate the minimum spanning tree (MST) as 350 and 35 alleles with missing values in at least 1 sample were excluded, respectively. When mapping the results of the cgMLST into an MST, a high degree of diversity among the isolates collected was apparent (Fig. 3A). We identified a single cluster of two nontoxigenic *C. diphtheriae* isolates using a cluster threshold of five allelic differences. These two isolates originated from brothers that travelled from Guinea. As expected, the biovars of *C. diphtheriae* grouped, all toxigenic isolates were dispersed among the *C. diphtheriae* biovar Mitis (Fig. 3B), and *C. belfantii* clustered independently from *C. diphtheriae*. In the case of *C. ulcerans*, the toxigenic isolates did not group separately on the MST from the nontoxigenic isolates (Fig. 3C and D). We generated further MSTs based on the localization of the infection of the *C. diphtheriae* (see Fig. S2 in the supplemental material) that demonstrated no corresponding groupings. To further assess the phylogenetic relationships of the isolates obtained with regard to their epidemiological and clinical background, we generated a neighbor joining tree (Fig. 3B). The isolates exhibited no grouping based on time, origin, toxigenicity, or clinical manifestations.

Based on the molecular characterization of isolate 2017004 (registered as *C. belfantii* with a genome size of approximately 2.4 Mb), we compared our WGS assembly with the reference sequence of *C. rouxii* FRC0190 (GenBank accession number NZ_LR738855.1) (Fig. 4). Alleles with missing values in at least one sample were excluded from the comparison table, but we kept samples with missing values in more than 10% of distance columns. Finally, a 969 cgMLST scheme was used to generate the MST, and 472 alleles with missing values in at least 1 sample were excluded.

***In silico* antimicrobial resistance and virulence factors.** ResFinder analysis identified a total of 38 resistance genes with 2 or more resistance genes in 26% (10/39) of the isolates, of which all were identified in the *C. diphtheriae* isolates. A list of the resistance genes identified has been included in the supplemental material.

All strains were assessed for the presence of the *dtxR* gene required for toxin expression. This analysis demonstrated that of the *C. diphtheriae* isolates analyzed, 100% harbor the regulator gene.

PHASTER prophage analysis identified from one to four prophages in all isolates

TABLE 3 *Corynebacterium* isolates included in the study

Species	Isolate	Isolation yr	Source	Disease	Gender	Age (yrs)	Risk factors	Biovar[a]	Toxin PCR result[a,b]	Elek test result[a,b]	ST	Penicillin G MIC (mg/liter)	Erythromycin MIC (mg/liter)
Corynebacterium diphtheriae	2014018	2014	Cutaneous	Foot ulceration	M	12	Travel (Afghanistan), mixed infection *S. pyogenes*	Mitis	P	P	389	0.38	<0.016
	2015002	2015	Blood	Endocarditis	M	62	Cirrhosis, hepatitis C virus	Gravis	N	NA	32	0.5	<0.016
	2015004	2015	Pharyngeal membrane	Diphtheria	M	7	Unvaccinated	Mitis	P	P	377	0.38	<0.016
	2015005	2015	Pharyngeal swab	Asymptomatic	M	8	Contact	Mitis	P	P	377	0.38	<0.016
	2015006	2015	Pharyngeal swab	Asymptomatic	M	8	Contact	Mitis	P	P	377	0.38	<0.016
	2015007	2015	Pharyngeal swab	Asymptomatic	M		Contact	Mitis	P	P	377	0.38	<0.016
	2015008	2015	Pharyngeal swab	Asymptomatic	F	0	Contact	Mitis	P	P	377	0.38	<0.016
	2015009	2015	Pharyngeal swab	Asymptomatic	M	7	Contact	Mitis	P	P	377	0.38	<0.016
	2015010	2015	Pharyngeal swab	Asymptomatic	M	35	Contact	Mitis	P	P	377	0.38	<0.016
	2015011	2015	Pharyngeal swab	Asymptomatic	M	7	Contact	Mitis	P	P	377	0.38	<0.016
	2015012	2015	Pharyngeal swab	Asymptomatic	F	8	Contact	Mitis	P	P	377	0.38	<0.016
	2015013	2015	Pharyngeal swab	Asymptomatic	F	40	Contact	Mitis	P	P	377	0.38	<0.016
	2015087	2015	Pharyngeal swab	Asymptomatic	M	8	Contact	Mitis	P	P	377	0.38	<0.016
	2016005	2016	Oropharyngeal swab		F	19	Unknown	Gravis	N	NA	32	0.38	<0.016
	2016006	2016	Cutaneous		M	20	Travel (Senegal)	Mitis	P	P	484	0.25	<0.016
	2017001	2017	Oropharyngeal swab		F	22	Unknown	Mitis	N	NA	5	0.5	<0.016
	2017013	2017	Cutaneous	Cutanea	F	25	Unknown	Gravis	N	NA	96	0.38	<0.016
	2017014	2017	Pharyngeal swab	Pharyngitis	M	26	Unknown	Mitis	P	N	212	0.75	<0.016
	2017015	2017	Cutaneous	Infected ulcer	M	41	Travel (Sri Lanka), mixed infection *S. pyogenes*	Mitis	N	NA	422	0.38	<0.016
	2017016	2017	Cutaneous	Impetigo	M	10	Travel (Guinea), mixed infection *S. pyogenes*	Mitis	N	NA	511	0.38	<0.016
	2017017	2017	Cutaneous	Impetigo	M	4		Mitis	N	NA	297	0.75	<0.016

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TABLE 3 (Continued)

Species	Isolate	Isolation yr	Source	Disease	Gender	Age (yrs)	Risk factors	Biovar[a]	Toxin PCR result[a,b]	Elek test result[a,b]	ST	Penicillin G MIC (mg/liter)	Erythromycin MIC (mg/liter)
							Travel (Guinea), mixed infection *S. pyogenes*						
	2017018	2017	Cutaneous	Impetigo	M	1	Travel (Guinea)	Mitis	N	NA	297	0.50	<0.016
	2018042	2018	Cutaneous	Wound infection	M	18	Immigrant, boat trip	Mitis	N	NA	704	0.19	<0.016
	2018043	2018	Cutaneous	Wound infection	M	16	Immigrant, boat trip	Gravis	N	NA	542	0.38	<0.016
	2018046	2018	Blood	Endocarditis	M	21	Unknown	Gravis	N	NA	319	0.39	<0.016
	2018047	2018	Cutaneous	Ankle laceration	F	31	Unknown	Mitis	N	NA	134	0.094	0.032
	2018051	2018	Cutaneous	Pharyngeal membrane, myocarditis	M	53	Unknown	NA	P	NA	297	NA	NA
	2019026	2019	Pharyngeal swab		M	24	Unknown	Gravis	N	NA	32	0.38	<0.016
	2019027	2019	Cutaneous	Leg necrotizing wound	M	40	Travel (Philippines)	Mitis	P	P	458	0.5	0.016
	2019028	2019	Oropharyngeal swab		F	15	Unknown	Gravis	N	NA	32	0.19	<0.016
	2019030	2019	Cutaneous		M	34	Travel (Pakistan)	Mitis	P	P	377	0.25	<0.016
Corynebacterium belfantii	2014013	2014	Expectoration		M	73	Unknown	NA	N	NA	81	0.5	< 0.016
	2014017	2014	Expectoration		F	76	Unknown	NA	N	NA	23	0.5	<0.016
	2015001	2015	Nasopharyngeal swab	Rhinorrhea, epistasis	F	84	Unknown	NA	N	NA	482	0.25	<0.016
	2015167	2015	Cutaneous	Carcinoma in the atrial pavilion, costrosus area in scalp graft	M	79	Mixed infection *S. aureus*	NA	N	NA	106	0.125	<0.016
	2015202	2015	Expectoration	Sinusitis	F	79	Unknown	NA	N	NA	106	0.25	<0.016
	2016001	2016	Expectoration	Asymptomatic	F	54	Cystic fibrosis	NA	N	NA	483	0.125	<0.016
	2016007	2016	Nasal biopsy specimen		F	37	Unknown	NA	N	NA	42	0.25	<0.016
	2017002	2017	Nasopharyngeal swab	Respiratory	M	49	Unknown	NA	N	NA	92	0.19	<0.016
	2017004	2017	Bone biopsy specimen	Osteomyelitis	M	79	Unknown	NA	N	NA	537	0.38	<0.016
	2017008	2016	Expectoration	Respiratory	M	62	Unknown	NA	N	NA	163	0.047	<0.016
	2018032	2018	Expectoration		M	85	Unknown	NA	N	NA	42	0.125	<0.016
	2018045	2018	Expectoration	Respiratory	M	89	Unknown	NA	N	NA	106	0.19	<0.016
	2018048	2018	Expectoration		F	61	Unknown	NA	N	NA	163	0.094	<0.016
	2018076	2018	Nasal swab	Sinusitis	F	53	Unknown	NA	N	NA	670	0.38	<0.016

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TABLE 3 (Continued)

Species	Isolate	Isolation yr	Source	Disease	Gender	Age (yrs)	Risk factors	Biovar[a]	Toxin PCR result[a,b]	Elek test result[a,b]	ST	Penicillin G MIC (mg/liter)	Erythromycin MIC (mg/liter)
Corynebacterium ulcerans	2014016	2014	Cutaneous		M	62	Unknown	NA	P	P	325	0.064	<0.016
	2016002	2016	Bone biopsy specimen		F	74	Unknown	NA	N	NA	325	0.125	<0.016
	2017009	2016	Cutaneous	Chronic vascular ulcers	F	85	Animal contact (cat)	NA	P	P	337	0.012	0.016
	2019012	2019	Pharyngeal swab	Odynophagia, pharyngeal lesions	M	60	Animal contact (cat, dog)		P	P	514	0.75	0.016

[a]NA, not applicable.
[b]N, negative; P, positive.

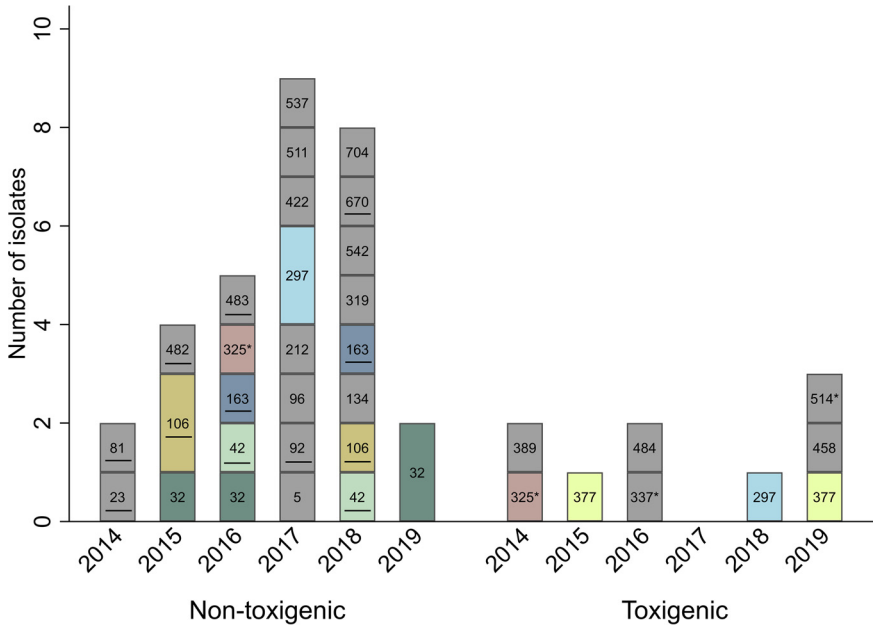

FIG 2 ST profiles of toxigenic and nontoxigenic *C. diphtheriae*, *C. belfantii*, *and C. ulcerans* isolates in Spain, 2014 to 2019. The asterisk (*) indicates the *C. ulcerans* isolates and underlined STs correspond to *C. belfantii* isolates. The STs that were identified once are gray, and those that have been identified in more than one specimen share a color.

which were sequenced in this study (see Table S1 in the supplemental material). Further analysis of the genetic environment of the *C. ulcerans tox* gene did not identify pathogenicity islands.

Risk characterization. Among the patients with toxigenic diphtheria, three had recent travels to African or Asian countries and one was an immigrant from a country where diphtheria is endemic. Two of the patients with toxigenic *C. ulcerans* had reported contact with companion animals. In the latter case, the individual had reported contact with a large number of animals, of which some were stray. Samples were taken from the animals, and one of the dogs and two of the cats were shown to harbor *C. ulcerans*. Interestingly, the cats harbored the same strain, while the dog harbored a different one. Two patients harboring a toxigenic isolate had documented vaccinations against diphtheria, while three reported vaccination but were unable to confirm it. The respiratory diphtheria case from 2015 was not vaccinated. Limited information was available for patients with nontoxigenic isolates, but among those with available data, four had a recent travel history and two were immigrants that had arrived in Spain by boat (Table 3).

DISCUSSION

In this report, we described the microbiological and epidemiological characteristics of *C. diphtheriae*, *C. belfantii*, *C. ulcerans*, and *C. rouxii* isolates submitted to the national reference laboratory (CNM) in Spain over the last 6 years (2014 to 2019). During this period, 39 isolates were submitted, of which 9 were toxigenic. The isolates exhibited a large degree of genetic diversity, belonging to 28 different sequence types, of which 7 were first described in this study. We also identified one NTTB isolate.

In well-vaccinated populations, diphtheria occurs sporadically. The surveillance systems must be sensitive and specific with protocols able to capture any diphtheria circulation in the population. Advances in laboratory techniques, including molecular studies, improve the specificity of surveillance and can help to better describe transmission routes and potential sources of infection. According to the guidelines of the RENAVE, only nine cases were classified as confirmed cases and therefore reported

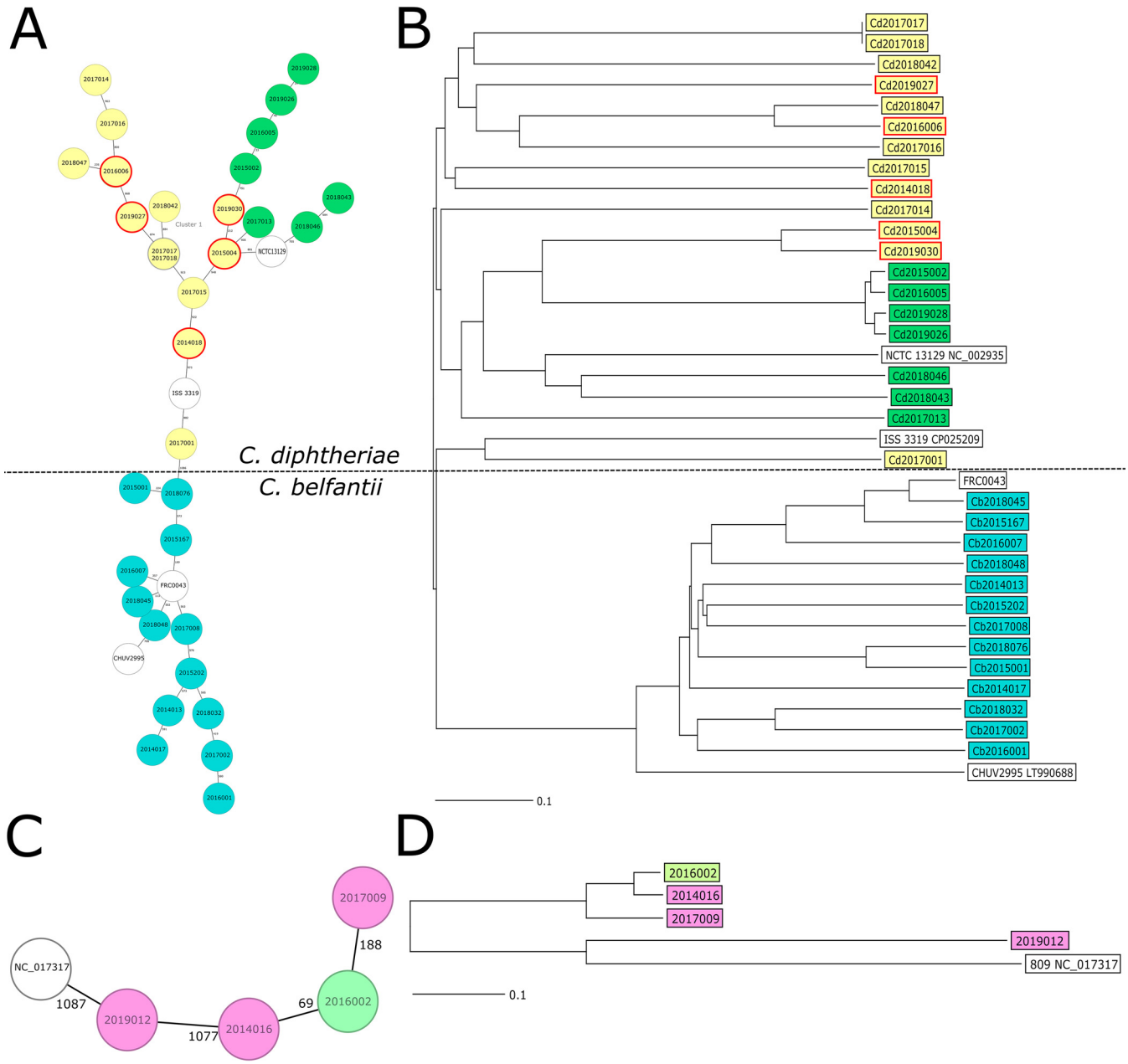

FIG 3 cgMLST analysis. (A) Minimum spanning tree of *C. diphtheriae* and *C. belfantii* isolates generated using a cluster distance threshold of ≤5 alleles. Species and biovars are color coded as follows: *Corynebacterium belfantii* (turquoise), Mitis (yellow), and Gravis (green); and all toxigenic isolates are marked with a solid red circle. The reference genomes used are white. (B) Neighbor joining tree generated from the *C. diphtheriae* cgMLST scheme. Reference genomes used were *C. belfantii* (FRC0043), *C. diphtheriae* bv. Gravis (NCTC 13129) and Mitis (ISS3319), and *C. diphtheriae* subsp. *lausannense* (CHUV2995). Isolates were color-coded according to their subtypes as in A, with red rectangles denoting the toxigenic isolates. (C) Minimum spanning tree of the *C. ulcerans* isolates generated using a cluster distance threshold of ≤5 alleles. Isolates are color-coded according to their toxigenicity; toxigenic isolates (purple), nontoxigenic isolates (green), and the reference genome used NCTC 017317 (white). (D) Neighbor joining tree of *C. ulcerans* isolates consisted of 1,209 target loci and 961 more loci for an accessory target scheme. Toxigenic isolates are denoted with a red rectangle.

to the surveillance network. The updated WHO guidance on surveillance, clinical care, and outbreak response suggests a case-based surveillance with laboratory confirmation of all suspected cases, including a set of definitions and classification of cases (laboratory-confirmed classic respiratory diphtheria, laboratory-confirmed mild respiratory/asymptomatic diphtheria, nonrespiratory confirmed diphtheria, epidemiologically linked, clinically compatible, and discarded case) that more accurately depicts the situations occurring in surveillance practice (28). By adopting the definitions and classification of cases proposed by the WHO, information and

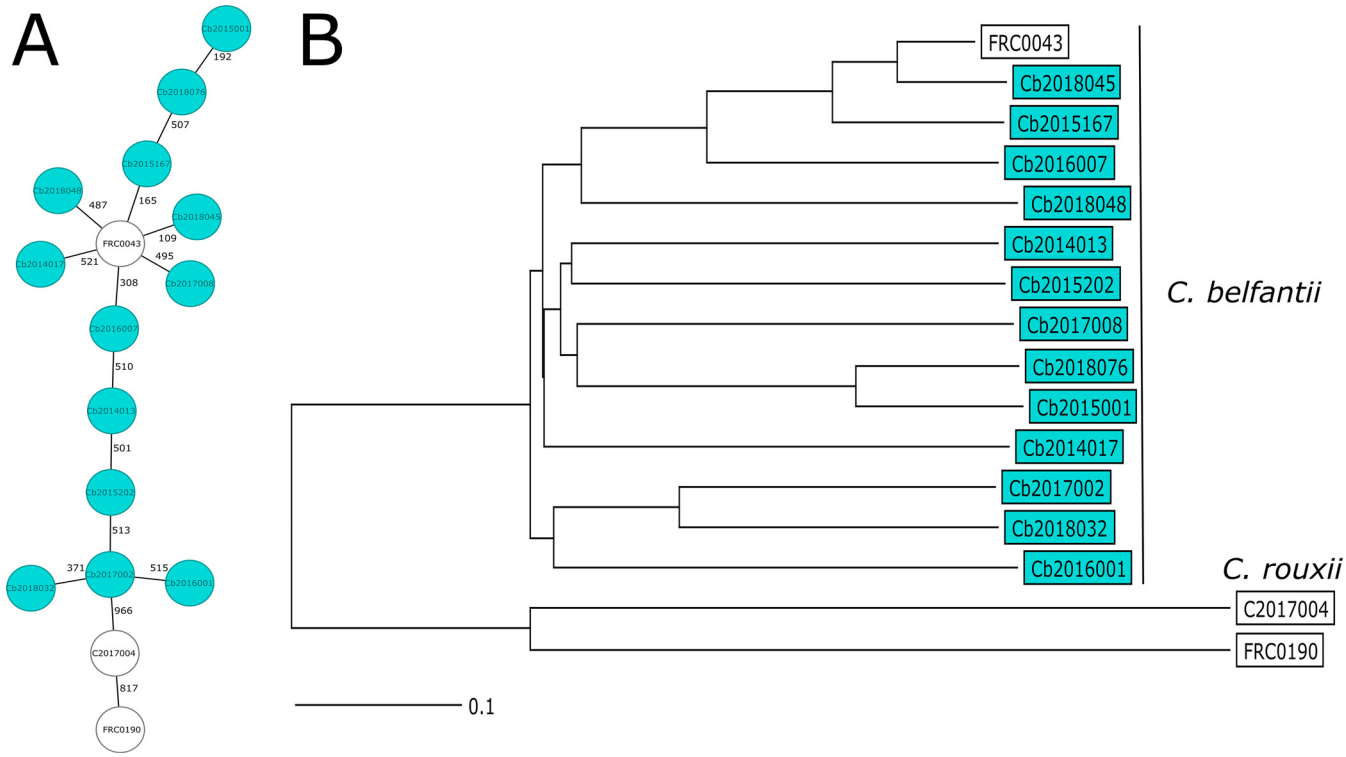

FIG 4 cgMLST analysis of *C. belfantii* and *C. rouxii*. (A) Minimum spanning tree of *C. belfantii* and *C. rouxii* isolates generated using a cluster distance threshold of ≤5 alleles. Reference genomes for *C. belfantii* (FRC0043) and *C. rouxii* (FRC0190) are white. (B) Neighbor joining tree generated from the cgMLST scheme using the reference genomes for *C. belfantii* (FRC0043) and *C. rouxii* (FRC0190) (in white).

knowledge of the clinical, epidemiological, microbiological, and molecular aspects of diphtheria in Spain would be significantly improved.

Prior to 2015, Spain had been diphtheria free for more than 30 years, and the laboratory capacity for the diagnosis of diphtheria in Spain relies on reference laboratories at regional and national levels. As there is no national microbiological surveillance program, it is difficult to estimate how extensive screening is for diphtheria in our country. Nevertheless, the increasing awareness of clinicians due to the fatal case in 2015 and the progressive introduction of MALDI-TOF analysis to routine hospital laboratories in Spain could be related to the increased number of *Corynebacterium* sp. isolates identified in 2017 and 2018. Those isolates are sent to the National Center for Microbiology for confirmation and characterization on a voluntary basis. However, similar to reports from other countries, the majority of isolates were identified as nontoxigenic *Corynebacterium* spp. and are therefore unlikely to be the causative agent (29–31).

Interestingly, the gender distribution of the toxigenic isolates was strongly skewed toward males (8:1), which is not reflected in the literature. Based on the small number of toxigenic isolates, we are hesitant to draw firm conclusions as to why this may be the case in Spain. Perhaps, as the immigrants arriving from countries where diphtheria is endemic are predominantly males, the observed tendency may be a result of that bias.

Less than a quarter (24%) of the isolates showed susceptibility to penicillin G. Resistance to this antibiotic was also observed in Algeria and Brazil, as well as in an outbreak among refugees from Northeast Africa and Syria in Switzerland (4, 26, 27). The interpretation of the EUCAST clinical breakpoints for corynebacteria should, however, be interpreted carefully, as they were developed for species other than *C. diphtheriae*. Moreover, in a recent study, a tentative epidemiological cutoff (TECOFF) of 0.5 mg/liter for penicillin G was suggested, as the wild-type mode for the tested *C. diphtheriae* and *C. ulcerans* isolates was above the EUCAST breakpoints (32). Currently,

there are no EUCAST erythromycin breakpoints for *Corynebacterium* spp. According to CLSI breakpoints and the TECOFF proposed by Marosevi et al., all isolates were susceptible to erythromycin, which is in agreement with other authors, although isolates resistant to erythromycin have been reported in other settings (7, 33). As such, in severe cases, it may improve the patient outcome to administer erythromycin over penicillin as a first-line treatment.

The WGS data analysis using ResFinder identified several other resistance genes *in silico*. Three out of the nine toxigenic isolates as well as seven of the nontoxigenic isolates harbored a variety of resistance genes, but none of them conferred resistance to β-lactams or macrolides.

Similar to results from other authors (9, 26), we identified common prophage insertions in *C. diphtheriae*, *C. belfantii*, and *C. ulcerans* genomes, demonstrating that phage infections commonly occur in Spanish *Corynebacterium* spp. To draw further conclusions regarding the DT-mediated pathogenicity of our isolates, a more exhaustive study of the genetic environment and regulatory components is necessary.

The MLST results showed that there was a large variety in the sequence types of the isolates. According to the PubMLST database, ST-377, ST-482, ST-483, ST-484, ST-511, ST-670, and ST-704 have been first described in Spain. A few STs reoccurred over different years, such as ST-32, which was also the most common sequence type. ST-32 is a nontoxigenic *C. diphtheriae* clone that has been circulating in Europe, with the earliest isolate collected in France in 1984, as documented in PubMLST (25). Since then, it has been described in Germany, Algeria, Romania, and Belgium. A recent genomic study suggested that ST-32 was also endemic in Australia and was suspected to have enhanced virulence, with higher adhesion rates than toxigenic strains of *C. diphtheriae* (34). As the number of isolates pertaining to the other sequence types identified was very small, it is difficult to ascertain their origin in Spain. Noteworthy, ST-377 that was first described in the 2015 respiratory toxigenic diphtheria case reappeared at the end of 2019. However, analyzing the specimens using WGS instead of traditional MLST revealed that the 2019 ST-377 actually differed from the 2015 isolate by 284 different alleles.

With regard to the STs identified in *C. ulcerans*, ST-325 has been previously described in cats, dogs and humans in Germany, France and Belgium (25). Our study identified two isolates pertaining to this ST without epidemiological data available. ST-337 was first described in France in 1998, where it was isolated from a synovial fluid sample. In our study, this ST was identified from a wound exudate in a patient presenting with chronic vascular ulcers who also had a cat. As contact with companion animals is considered a primary risk factor for *C. ulcerans* infection (35), this could explain the origin of this infection; however, to confirm this infection, a sample would have needed to be analyzed. One of the *C. ulcerans* isolates we identified did have corresponding samples taken from contact animals which harbored the same ST-514 found in the human sample, suggesting this as a likely origin of infection. This finding demonstrates the urgent need to collect epidemiological data containing information on companion animals and appropriate samples when toxigenic *C. ulcerans* is identified.

As previously published, our findings support the conclusion that modern, low-cost WGS could replace traditional MLST methods in the surveillance of diphtheria to describe transmission events and sources of infection with the highest possible resolution (36).

The WGS genetic diversity studies demonstrated a profile typical of that expected in a country where the cases are imported. The only cluster we identified was found in closely linked individuals traveling together from a country where diphtheria is endemic. The grouping of *Corynebacterium* species, biovars, toxigenicity, and colonization sites appeared to have no epidemiological, geographical, or chronological link. With more complete clinical, epidemiological, and vaccination information of the

nontoxigenic isolates circulating in Spain, it is highly probable that clusters would be identified.

Ongoing mass movements of travelers, refugees, asylum seekers, or immigrants from countries of diphtheria endemicity to areas of no endemicity and rising numbers of unvaccinated individuals have resulted in an increase in the global incidence of diphtheria (26). Unfortunately, it was not possible to understand the contribution of these factors to corynebacterial infections in Spain, as risk characterization was limited to the patients with confirmed toxigenic diphtheria. Important variables such as country of origin, travel history, contact with animals, and vaccination status were not collected for most of the nontoxigenic isolates, as it is currently not mandatory based on national surveillance guidelines. Of the confirmed cases, traveling to countries of diphtheria endemicity or having an origin from an country with endemicity seemed to be a risk factor. At least two patients with *C. ulcerans* reported contact with animals. In one case, the microbiological investigation identified one dog and two cats positive for *C. ulcerans*. Nontoxigenic *Corynebacterium* spp. are classified as an emerging pathogen, as they are increasingly associated with severe clinical outcomes, such as endocarditis and bacteremia (12, 13). Cases of invasive bacteremia due to nontoxigenic *C. diphtheriae* have been reported in certain at-risk populations (37). Endocarditis was observed in one of the patients with a nontoxigenic isolate in this study; however, it is not clear if this condition was attributed to infection by *C. diphtheriae*. Currently, the potential of these nontoxigenic *Corynebacterium* spp. to cause disease is likely to be underestimated by clinicians. Analysis of the WGS data demonstrated that all of the *C. diphtheriae* harbor the diphtheria toxin regulatory gene, meaning that these isolates are capable of becoming fully toxigenic if they acquire the toxin-bearing β-phage. Disease due to nontoxigenic variants is not vaccine preventable using the toxoid-based vaccine (1).

Although biochemically, isolate 2017004 is almost indistinguishable from *C. belfantii*, our cgWGS analysis suggests that it is likely a member of the *C. rouxii* species, making this the first description of this species in Spain.

This was the first study to describe the microbiological and epidemiological characteristics of *Corynebacterium* isolates identified in Spain. Using modern techniques like WGS and cgMLST, we provided a detailed molecular characterization of the isolates. Introducing WGS in the routine diagnostics for corynebacteria will be an invaluable addition to the surveillance of diphtheria in Spain and will be useful for the harmonization of diagnostics within the European Union and with other countries. To gain insights into the nontoxigenic *Corynebacterium* circulating in Spain, it would be of interest to collect comprehensive epidemiological data from all patients with corynebacterial infections as per the new WHO guidelines.

In conclusion, this study was the first to describe the diphtheria isolates collected in Spain, after the implementation of the new surveillance guidelines in 2014. Nontoxigenic corynebacteria are a concern for public health, as they can cause severe outcomes and have the capacity to become fully toxigenic. Collecting relevant disease and exposure information from patients with nontoxigenic corynebacteria and encouraging their notification could strengthen the current surveillance and improve the epidemiological knowledge regarding these emerging pathogens. Furthermore, the introduction of WGS in describing corynebacteria would be an invaluable addition to the surveillance of diphtheria in Spain, due to its greater discriminative power over traditional techniques.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

ACKNOWLEDGMENTS

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

We thank the clinicians and microbiologists at the National Health System who submitted isolates at the National Reference Laboratory and completed enhanced surveillance forms and the regional Epidemiological Surveillance Services. We are also grateful to the microbiologists at the WHO Collaborating Centre Laboratory for Diphtheria and Streptococcal Infections at Public Health England for performing the Elek tests. Moreover, we thank the head scientific coordinator of EUPHEM Aftab Jasir and the scientific coordinator of the EPIET fellowship program Frantiska Hruba for their guidance and feedback.

L.H.-L. and S.H.-L. proposed the concept of the analyses. L.H.-L., S.H.-L., S.P., and A.H. conducted the laboratory work and the WGS analyses. S.V. conducted the WGS assembly and quality control. D.P. guided by J.M.-C. and N.L.-P. performed the epidemiological analysis. A.H. and D.P. drafted the first version of the manuscript and provided the graphs and figures. N.L.-P., S.H.-L., J.M.-C., and L.H.-L. supervised the work and revised and approved the final manuscript. We alone are responsible for the views presented in the manuscript.

We declare no conflict of interest.

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