1   **Carriage of a single strain of non-toxigenic *Corynebacterium diphtheriae***

2   **biovar Belfanti *(Corynebacterium belfantii)***

3   **in four patients with cystic fibrosis**

4

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20   **Running title**: *C. diphtheriae* in cystic fibrosis

ABSTRACT

Cystic fibrosis (CF) patients are commonly colonized by bacterial pathogens, which can induce persistent lung inflammation and may contribute to clinical deterioration. Colonization of CF patients and cross-transmission by *Corynebacterium diphtheriae* has not been reported so far. The aim of this article was to investigate the possibility of a cross transmission of *C. diphtheriae* biovar Belfanti between four patients of a CF center. *C. diphtheriae* biovar belfanti (now formally called *C. belfantii*) isolates were collected from four patients in a single CF care center over a 6 years period and analyzed by microbiological methods and whole genome sequencing. Epidemiological links among patients were investigated. Ten isolates were collected from 4 patients. Whole genome sequencing of one isolate from each patient showed that a single strain was shared among them. In addition, one patient had the same strain on two consecutive samplings nine months apart. The strain was non-toxigenic and was susceptible to most antimicrobial agents. Ciprofloxacin resistance was observed in one patient. Transmission of the strain among patients was supported by the occurrence of same-day visits to the CF center. This study demonstrates colonization of CF patients by *C. diphtheriae* biovar Belfanti (*C. belfantii*) and shows persistence and transmission of a unique strain during at least six years in a single CF patient care center.

42   **Abbreviations**

43   CF: cystic fibrosis

44   MLST: multilocus sequence typing

45   MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight

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61   **Accession numbers**

62   The genomic sequence data generated in this work were submitted to the European

63   Nucleotide Archive and are available from the International Nucleotide Sequence Database

64   Collaboration (NCBI/ENA/DDBJ) databases under project accession number PRJEB28372

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INTRODUCTION

A large fraction of the mortality of cystic fibrosis (CF) patients is attributed to infections of the respiratory tract, which can be caused by multiple pathogens, and cross-transmission within CF centers themselves is an important healthcare related issue (1, 2)(3). The genus *Corynebacterium* includes a high number of pathogens, most of them being opportunistic (4). So far, only *C. pseudodiphtheriticum, C. propinquum and C. accolens* were reported from CF patients (5–7). *C. diphtheriae*, the most pathogenic *Corynebacterium* species that causes diphtheria, was not reported in pauci- or asymptomatic CF lung colonization to our knowledge. Typical diphtheria is caused by strains that produce the diphtheria toxin. Although the disease has almost disappeared in countries with high toxoid vaccine coverage, the pathogen still circulates in the human population (8–10). Further, non-toxigenic *C. diphtheriae* strains can be recovered from a variety of infections including respiratory tract infections, skin infections and bacteremia (11, 12). Three biovars of *C. diphtheriae* are distinguished by biochemical characteristics. Whereas biovars Mitis and Gravis can harbor the diphtheria toxin gene, biovar Belfanti isolates were only exceptionally described as toxigenic (13–15). Recently, *C. diphtheriae* biovar Belfanti isolates were recognized as representing a novel species called *C. belfantii* (16). The aim of this study was to investigate potential cross-transmission within a group of four patients with lower respiratory tract colonization by nontoxigenic *C. diphtheriae.* These patients were followed in a single CF center during a period of six years, and our genomic analyses showed that they were colonized by a single *C. diphtheriae* strain.

87   METHODS

88   **Identification of cases**

89   Cases were identified in the regional consultation CF Center of a university hospital

90   between January 2011 and November 2016. During their visits at the center, patients are

91   screened for the presence of opportunistic infectious agents and the evolution of antimicrobial

92   resistance is monitored. The inclusion of cases was performed retrospectively based on at

93   least one sample positive for *C. diphtheriae* upon microbiological screening from sputum or

94   induced sputum. Clinical and laboratory data (sex, age at the time of diagnosis, pulmonary

95   functionality, long-term or sequential antibiotic therapies, symptomatology at the time of

96   diagnosis, respiratory co-infections) were collected for each patient.

97

98   **Epidemiological investigations**

99   The CF center includes three wards (consultation, pulmonary function testing and

100   chest physiotherapy) and an imaging department. The timeline of patients visits to the various

101   wards of the CF center was investigated from their clinical records. Patients undergo chest

102   physiotherapy either before or after their consultation with the physician, and a sputum

103   specimen was systematically collected. Afterwards, they are directed to the pulmonary

104   function testing ward.

105   To investigate infection control measures and detect possible factors that might have

106   favored patient-to-patient transmission, all healthcare workers of the three wards who had

107   worked during the study period were met and interviewed about implementation of standard

108   precautions, material management and patient care organization. Besides, healthcare workers

109   who were in charge of the included patients were screened based on a voluntary basis at the

110   time of the study (June to October 2017) for *C. diphtheriae* colonization in the nasopharynx.

111   For this purpose, swabs were plated onto blood agar medium, on which five fosfomycin disks

112 (50 µg/disk) were then deposited. Colonies growing around the disks after 18-24 h at 37°C

113 were sub-cultivated on Tinsdale medium agar and incubated at 37°C.

114

115 **Bacterial identification and characterization**

116 The isolates were identified as *C. diphtheriae* at the local microbiology laboratory by

117 MALDI-TOF (Bruker) since Sept. 2016, or using API Coryne (bioMerieux) until August

118 2016. Confirmatory analysis and *tox* gene detection were performed at the National Reference

119 Center. The biovar of isolates was determined based on the combination of nitrate reductase

120 (positive in Mitis and Gravis, negative in Belfanti) and glycogen fermentation (positive in

121 Gravis only). Antimicrobial susceptibility was characterized by the disk diffusion method

122 (Bio-Rad, Marnes-la-Coquette, France) and the minimum inhibitory concentration was

123 determined by the E-test method (BioMérieux, Marcy l'Etoile, France). The sensitivity was

124 interpreted using CA-SFM/EUCAST V.1.0 (mars 2017) criteria for *Corynebacterium*

125 (http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFMV1_0_MARS_2017.pdf).

126 Susceptibility was tested for the following antimicrobial agents: vancomycin, kanamycin,

127 gentamycin, penicillin G, oxacillin, amoxicillin, imipenem, cefotaxime, clindamycin,

128 azithromycin, spiramycin, clarithromycin, erythromycin, ciprofloxacin, moxifloxacin,

129 cotrimoxazole, trimethoprim, sulfonamide, pristinamycin, rifampicin, tetracyclin and

130 linezolid. Genomic sequencing was performed using a NextSeq-500 instrument (Illumina, San

131 Diego, USA) with a 2 x 150 nt paired-end protocol and based on Nextera libraries. Contig

132 sequences were assembled using SPAdes v3.9 (http://cab.spbu.ru/software/spades/).

133 Multilocus sequence typing (MLST) was performed from genomic assemblies using the

134 international nomenclature database webpage (https://pubmlst.org/cdiphtheriae/). Read

135 mapping and calling of high quality single nucleotide polymorphisms (SNP) were performed

136 as described (17). Identification to *C. belfantii* was performed by genomic comparison with

137   the type strains of *C. belfantii* and *C. diphtheriae* based on the average nucleotide identity

138   metric calculated with JspeciesWS (18) as in (16).

139

140   **Ethical statement**

141   The work was conducted in accordance with local and national regulation, as well as the

142   Helsinki Declaration, and was approved by the local Ethics committee (Committee for the

143   Protection of Persons EST I, France).

144 **RESULTS**

145 **Patients.** From January 2011 to November 2016, four patients of the CF Center had positive

146 screening respiratory samples for *C. diphtheriae*. This species was identified from these

147 patients because of the presence of abundant, even though not numerically dominant,

148 coryneform gram-positive colonies in their oropharyngeal microbiological flora. The timeline

149 of patients visits to the CF center and detection of *C. diphtheriae* is represented in **Figure 1**.

150 The patient characteristics and medical records are summarized in **Table 1**. Only patients 2

151 and 4 presented with respiratory exacerbation at the time of *C. diphtheriae* detection and

152 received antibiotics, but they did not require hospitalization. None of the four patients had a

153 dermatological disease or chronic wound. Patient 2 was positive for *C. diphtheriae* during at

154 least 15 months.

155

156 **Phenotypic and molecular identification of the isolates.** One isolate from patients 1

157 (February 2011), 3 (January 2016) and 4 (November 2016), and 2 isolates from patient 2

158 collected 9 months apart (April 2015, January 2016), were stored and available for analysis

159 (**Figure 1**). The five isolates were identified as *C. diphtheriae*. None of the isolates was

160 toxigenic and all were of biovar Belfanti. MLST showed that the five isolates belonged to the

161 same sequence type, ST208. Whole-genome sequence variation among the 5 isolates revealed

162 only 62 SNPs among them. The largest SNP distance between two isolates was 58 SNPs,

163 observed between isolates FRC0074 and FRC0381. Hence, the five isolates were very closely

164 related, showing that they belong to a single strain. Phylogenetic analysis based on SNPs

165 (**Figure S1**) revealed three subtypes, comprising respectively (1) the isolate from patient 1

166 (FRC0074); (2) One isolate from patient 2 (isolate FRC0318) and the isolate from patient 3

167 (FRC0381); and (3) The other isolate (FRC0382) from patient 2 and the isolate from patient 4

168 (FRC0455). Within subtypes, only eighteen SNPs separated FRC0318 and FRC0381, and 4

8

169   SNPs separated FRC0382 and FRC0455. Following the recent description of *C. belfantii* (16),

170   the five isolates were re-identified based on their genomic sequence. Their average nucleotide

171   identity with *C. belfantii* type strain (FRC0043[T]) was 99.47%, whereas it was only 94.89%

172   with NCTC11397[T], the type strain of *C. diphtheriae*. Therefore, the five isolates belong to the

173   novel species *C. belfantii*.

174   Antimicrobial susceptibility profiles of the five isolates showed that they were

175   susceptible to all antimicrobial agents, with one remarkable exception: isolate FRC0074 from

176   patient 1 was non-susceptible to ciprofloxacin. Genomic sequence inspection showed that this

177   isolate had a unique mutation, A to G at position 277 of the *gyrA* gene, coding for subunit A

178   of gyrase, the target of ciprofloxacin. This SNP corresponds to a deduced amino-acid change

179   of D to N at protein position 91, which is located within the quinolone-resistance determining

180   region of the gyrase of *Corynebacteria*.

181

182   **Investigations of possible strain transmission risks.** Patient 1 had no recorded contact

183   opportunity with the three other patients at the clinic. In contrast, patients 2 and 3 visited the

184   CF center the same day on eight occasions for consultations, physiotherapy or for pulmonary

185   function tests between January 2014 and November 2016 (**Figure 1**). Further, patient 4 had a

186   consultation and physiotherapy session 30 minutes after patient 2 in April 2016 and then in

187   November 2016, on the day when he had a positive expectoration sample for *C. diphtheriae*.

188   In addition, patient 4 had a pulmonary function test on the same day as patient 2 in September

189   2014 and November 2016 (**Figure 1**). Therefore, several opportunities for cross-transmission

190   within the CF center were identified among patients 2, 3 and 4, even though social interaction

191   between CF patients was not identified.

192   Inspections of hygiene measures was retrospectively conducted by the infection

193   control team between June 2016 and June 2017. Local infection control protocols and

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194   recommendations regarding healthcare staff hygiene (mostly masks, disposable mouthpieces

195   and filters, specific equipment and hand hygiene) and rooms and equipment disinfection were

196   correctly observed. However, it was noted that the salbutamol inhalation chamber was

197   disinfected with low-level disinfectant instead of a mid-level disinfectant. Furthermore, after

198   their physiotherapy session, patients did not always wear masks while undergoing pulmonary

199   function testing.

200   Ten healthcare workers of the CF center who were regularly in contact with the four

201   patients were retrospectively screened for throat colonization by *C. diphtheriae*. No

202   *Corynebacterium* was isolated in any of the samples from the screened workers.

DISCUSSION

We report four cases of CF patients colonization by non-toxigenic *C. diphtheriae* biovar Belfanti (now formally called *C. belfantii*). To our knowledge, *C. diphtheriae* was never described from CF patients. Although non-toxigenic isolates can cause a variety of infections, including bacteremia (11), two patients were not symptomatic, whereas the two other had lung exacerbation at the time of first *C. diphtheriae* detection. Other opportunist pathogens of the *Corynebacterium* genus might be involved in CF lung exacerbations (5, 19). In recent years, *C. diphtheriae* became easier to identify by the use of MALDI-TOF. However, *C. diphtheriae* may still be difficult to detect in the CF lungs, given the polymicrobial colonization occurring in most samples, as was observed during this study (**Table 1**). It is therefore not unlikely that additional cases of colonization might have gone undetected.

Multiple CF patients are typically followed in a given CF center, which creates opportunities for bacterial transmission among patients despite the enforcement of strong infection control measures. A strong suspicion of cross-transmission of *C. diphtheriae* between four patients in our clinic arose given the repeated observation of patients colonized by *C. diphtheriae*. Microbiological investigations fully supported the hypothesis of a single strain. MLST showed that the five isolates belonged to the same sequence type. The MLST genotype of the isolates, ST208, was not reported previously in the *C. diphtheriae* MLST database, suggesting that it is not common. Whole genome sequencing defines the genetic relatedness among *C. diphtheriae* isolates with high precision (20–22). This approach demonstrated that the five isolates belong to the same strain and provided strong support to the hypothesis of cross transmission among patients and/or contamination from a common source. Besides, the data showed that the strain persisted within patient 2 for at least 9 months. Unfortunately, the additional isolates detected from patient 2 and 3 were not stored.

11

227   The SNP variation uncovered by the genomic analysis reflects evolution of the strain

228   since the last common ancestor of the five isolates. Three subtypes were distinguished, two of

229   which comprised isolates from two patients. Subtypes shared by patients may reflect direct

230   transmission between them. Epidemiological investigations support this possibility in one

231   case, as patients 2 and 3 had simultaneous visits to the CF center on several occasions.

232   However, knowledge on subtype diversity within patients would be required to infer

233   transmission chains with confidence (23). In this study, only one isolate was kept and

234   characterized from each sample. Therefore, one cannot exclude that subtypes coexisted within

235   single patients, which would lead to the possibility of other transmission patterns than those

236   suggested by the phylogeny.

237   Biovar Belfanti of *C. diphtheriae* (*C. belfantii*) is commonly isolated from the respiratory

238   tract, generally from the nose or throat and often in association with ozaena (24). In contrast,

239   its isolation from skin infections is extremely rare. Therefore, skin wounds of patients or the

240   personnel is an unlikely reservoir. Transmission by direct respiratory contamination between

241   patients appears as the most likely transmission route. Transmission of *C. striatum* in an

242   intensive care unit and silent transmission of *C. pseudodiphtheriticum* among CF patients

243   were previously reported (5, 25).

244   Evolution of antimicrobial resistance occurs frequently in bacterial isolates that colonize

245   CF lungs (26). Our results showed ciprofloxacin resistance in one isolate, whereas the others

246   were susceptible. As no prescription of quinolone antimicrobials was recorded for this patient,

247   one possibility is that the strain had evolved resistance to ciprofloxacin in another

248   ciprofloxacin-treated patient, in which the strain was not detected.

249   The main limitation of this study is that the infection control investigation was performed

250   retrospectively and that no detailed pattern of transmission could therefore be ascertained.

251   Healthcare workers or the materials used for patient care may have played the role of vector

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252 of *C. diphtheriae* between the patients (27), even though the retrospective screening did not

253 reveal a potential carrier or source of infection. Further, hidden patient-to-patient cross

254 transmission cannot be excluded, as some patients may have been undetected for

255 *C. diphtheriae* carriage. Further studies are needed to better define carriage of *C. diphtheriae*

256 by CF patients and to investigate the possible role of patients, healthcare workers or

257 environmental sources in cross transmission. In addition, the clinical significance of non-

258 toxigenic *C. diphtheriae* will need to be determined in order to define strategies of treatment,

259 prevention and control of contamination of CF patients by this bacterial pathogen.

260

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347 **Table 1.** Summary of the medical records of the four patients with positive *C. diphtheriae* isolates in expectoration samples

Patient	Age/ Gen der	Baseline lung function	Symptoms at the time of positivity	Long-term or recurrent antibiotic therapy	Date of the first positive sample	Sampling	Other bacteria (co-infection)	Treatment following positivity	Current status
1	27/F	Non-invasive ventilation, oxygen requirement FEV 30%	Chronic respiratory failure	Amoxicillin, cotrimoxazole for exacerbations	Feb 2011	Sputum	*S. maltophilia* *H. influenzae* *E. coli*	No treatment	Deceased in 2013
2	25/ M	FEV 106%	Asymptomatic in Apr 2015, Exacerbation in Jul 2015	Inhaled tobramycin for one month on Jan 2014; Ofloxacin, fusidic acid	Apr 2015	Sputum	*S. aureus*	Fusidic acid, ofloxacin	Alive
3	39/ M	FEV 69%	Asymptomatic	amoxicillin/clavulanic acid, ofloxacin, amikacin for exacerbation	Jan 2016	Sputum	*S. aureus* *A. baumannii* *S. maltophilia*	No treatment	Alive
4	23/ M	FEV 75%	Exacerbation in Nov 2016	Piperacillin/tazobactam, teicoplanin, imipenem, cotrimoxazole for exacerbation in 2015 and 2016	Nov 2016	Sputum	*S. aureus* *Achromobacter xylosoxidans*	Amoxicillin/ clavulanic acid, cotrimoxazole	Alive

348 FEV: force expiration volume

349

350

351   **Figure 1. Timeline of events and *C. diphtheriae* detection in four cystic fibrosis patients.**

352   Months correspond to different columns in the grid; inside each month, days (not shown) are

353   distinguished as separate columns. The upper tree recapitulates the phylogenetic relationships

354   among the isolates, which are indicated in red boxes, linked with dotted arrows to their

355   respective patient and isolation time. The numbers of single nucleotide polymorphisms (SNP)

356   per genome inferred to have occurred are given for each branch of the tree.

357

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Patient:	Feb 2011	Jan 2014	Apr 2014	Jul 2014	Sep 2014	Oct 2014	Jan 2015	Apr 2015	Jul 2015	Oct 2015	Nov 2015	Jan 2016	Mar 2016	Apr 2016	Jun 2016	Jul 2016	Sep 2016	Nov 2016
1	+ deceased																	
2																		
3																		
4																		
Month	Feb 2011	Jan 2014	Apr 2014	Jul 2014	Sep 2014	Oct 2014	Jan 2015	Apr 2015	Jul 2015	Oct 2015	Nov 2015	Jan 2016	Mar 2016	Apr 2016	Jun 2016	Jul 2016	Sep 2016	Nov 2016

Legend:
- Imaging
- Pulmonary functional testing
- Physiotherapy
- Consultation
- + Sputum positive for *C. diphtheriae*
- FRC0xxx *C. diphtheriae* isolate