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ORIGINAL ARTICLE

Characteristics of Multidrug-Resistant Corynebacterium spp. Isolated from Blood Cultures of Hospitalized Patients in Japan

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SUMMARY: Corynebacterium is a genus consisting of Gram-positive, rod-shaped bacteria, that is widely distributed in nature. We report the epidemiological characterization of Corynebacterium spp. isolated from blood specimens at the Kurume University Hospital, between June 2008 and November 2011. Twenty-two strains that were likely Corynebacterium spp. were isolated from 22 hospitalized patients, of which 12 (54.5%) were identified as Corynebacterium striatum. Minimum inhibitory concentration tests were performed after biochemical and genotypic identifications. Biofilm production was detected using a 96-well microplate assay. The dissemination of C. striatum was investigated using pulsed-field gel electrophoresis (PFGE). All strains showed the tendency to be resistant to multiple drugs except vancomycin. Fourteen (82.4%) strains, including 9 C. striatum strains were capable of producing biofilms. Four distinct PFGE patterns were detected among C. striatum strains; 6 of which were identified as dominant pattern A (defined in this study) and had high biofilm production ability. During the 3-year monitoring period, these strains might have repeatedly infected the patients or could have readily colonized the hospital environments. C. striatum appeared to be a potential risk factor for bloodstream infections in hospitalized patients. More surveillance and enhanced control strategies are necessary to decrease Corynebacterium spp. infections in hospitals.

INTRODUCTION

Corynebacterium spp. consists of Gram-positive rods with 88 valid, published species, including 3 species in press or published since the Bergey’s Manual chapter was compiled (1). Corynebacterium spp. is widely disseminated in the environment and can colonize the skin and mucous membranes of humans as part of the normal microbiota (2). Because of these characteristics, as well as challenges in its identification, Corynebacterium spp. were traditionally regarded as contaminants in clinical microbiological laboratories. However, Corynebacterium spp. has been increasingly recognized as an important cause of significant human infections including respiratory tract infections (3), surgical site infections (4), prosthetic joint infections (5), endocarditis (6), osteomyelitis (7), and other infections. Recently, Corynebacterium spp. was also reported to cause several nosocomial outbreaks (8–10), and its pathogenic potential has been proven, particularly in the setting of immunosuppression and medical devices (11–14). Drug-resistant Corynebacterium spp. strains were suggested to be an emerging opportunistic nosocomial pathogen in long-term hospitalized patients and can be the origin of major outbreaks (15,16). Furthermore, Corynebacterium striatum was reported as an emerging pathogen in both immunocompetent and immunocompromised hosts (17). For these reasons, it was deemed a priority to establish more reliable methods to identify Corynebacterium clinical isolates at the species level (2,10,18,19). There is still little information regarding bloodstream infections caused by Corynebacterium spp., or the characteristics of devices related to infections caused by Corynebacterium spp. biofilms (20,21). In our study, we performed an investigation of Corynebacterium spp. isolated from blood specimens in a university-affiliated hospital in Japan.

MATERIALS AND METHODS

Isolates: The study was performed in Kurume University Hospital, which is affiliated with Kurume University located in Kurume city, Japan. There are 541 doctors and 1,097 nurses working in this hospital with 1,025 beds and 24 wards. A total of 22 isolates that were likely Corynebacterium spp. recovered from blood specimens of 22 admitted patients (man: 16; woman: 6), were investigated between June 2008 and November 2011. Corynebacterium isolates were only included based on the criteria that they had been identified at least twice from independent blood specimens. When multiple Corynebacterium isolates were obtained...
from the same patient, only the first isolate was included. The clinical records of all patients, from whom suspected Corynebacterium spp. had been recovered, were reviewed.

**Identification:** Identification was performed based on colony morphology and pigmentation, Gram staining, motility, and catalase reaction. Gram-stained smears and cultures of good quality specimens obtained as recently as possible, as well as all isolates, were analyzed to initially identify with the clinical laboratory routine workflow using the MicroScan system (Siemens, Sacramento, CA, USA). The identifications were further confirmed by API Coryne (BioMerieux, Etoile, France), partial rpoB (19) and 16S rRNA genes sequencing as described previously (22). The rpoB-based identification at the species level required ≥95% sequence identity with >2% separation between species, as described previously (23). On the other hand, 16S rRNA-based identification at the species level required ≥99% identity and >0.8% separation between species according to CLSI guidelines (24). The amplified sequences were compared to those available in the GenBank database using the basic local alignment search tool (BLAST) program.

**In vitro antimicrobial susceptibility:** Minimal inhibitory concentration (MIC) tests were performed to investigate in vitro antimicrobial susceptibility of all Corynebacterium spp. strains using 12 antimicrobial agents. These included penicillin, ampicillin/sulbactam, amoxicillin/clavulanate, cefditoren, ceftriaxone, cefepime, meropenem, levofloxacin, clindamycin, erythromycin, sulfamethoxazole-trimethoprim (SXT), and vancomycin (VAN). Susceptibility was investigated using a commercial micro-dilution method (MicroScan AST panel; Beckman Coulter, Brea, CA, USA) according to CLSI guidelines (25).

**Microtiter biofilm assay (MBA):** Biofilm formation in Corynebacterium spp. was assessed using 96-well microplates as previously described (26). All strains were tested in quadruplicate for each experiment, and the results were reported as the average ± standard deviation (SD) of 3 independent experiments. An OD$_{950}$ value ≥ 0.5 was used as the cut-off value for this study, which indicated the capability to form biofilms.

**Observation of the biofilms:** Biofilms of CK-7 and CK-21 strains were grown in brain heart infusion broth to mid-log phase and were then diluted to an optical density of 0.1 (OD$_{950}$). A 1-ml aliquot of the cell suspension, approximately 1 × 10$^{9}$ CFU/ml, was used to inoculate into the bottles with glass cover-slips, which were subsequently incubated for 24, 48, and 72 h at 37°C. During this incubation, bacteria were expected to tightly adhere to the glass cover slip. Biofilm architecture was studied using inverted confocal laser scanning microscopy (Olympus, Tokyo, Japan). Biofilms were rinsed with phosphate buffered saline and then stained with Live/Dead BacLight from Invitrogen (Carlsbad, CA, USA). To ensure reproducibility, each experiment was performed in triplicate. Images were analyzed using FV10-ASW Viewer ver. 1.7b (Olympus) (26).

**Pulsed-field gel electrophoresis (PFGE):** PFGE was performed for all Corynebacterium striatum strains. Chromosomal digestions with Smal (Takara Bio, Shiga, Japan) and PaeI (New England BioLabs, Tokyo, Japan) were performed to determine genetic relatedness using a CHEF Mapper (Bio-Rad, Hercules, CA, USA) as described previously (27), and the interpretation of PFGE patterns was based on the criteria described by Tenover et al. (28).

## RESULTS

**Patient characteristics and isolate identification:** Patients were recruited from 8 different wards. The average age of the patients was 57.3 years (range, 1–93). All patients had serious underlying diseases such as tumors, heart failure, and strokes. They had been hospitalized for prolonged periods (mean, 90.8 days; range, 4–373 days) and had central or peripheral vein catheters. Nineteen patients had been exposed to previous antibiotic treatments, and 7 patients died during the study.

Twenty-two isolates were identified as suspected Corynebacterium strains by standard phenotypic methods. The results of genotypic identification were as follows: 12 isolates (54.5%) of C. striatum, 2 isolates (9.1%) of Corynebacterium afermentans, 1 Corynebac-

| Strain | CK-1 | CK-2 | CK-5 | CK-7 | CK-8 | CK-11 | CK-12 | CK-13 | CK-14 | CK-15 | CK-19 | CK-21 |
|--------|------|------|------|------|------|-------|-------|-------|-------|-------|-------|-------|
| PEN    | >4   | 1)   | >4   | >4   | >4   | >4    | >4    | >4    | >4    | >4    | >4    | >4 |
| SAM    | >4   | 4)   | >4   | >4   | >4   | >4    | >4    | >4    | >4    | >4    | >4    | >4 |
| AMC    | >1   | ≤1   | >4   | >4   | >4   | >4    | >4    | >4    | >4    | >4    | >4    | >4 |
| CDN    | >1   | >1   | >1   | >1   | >1   | >1    | >1    | >1    | >1    | >1    | >1    | >1 |
| CRO    | >8   | 8    | >8   | >8   | >8   | >8    | >8    | >8    | >8    | >8    | >8    | >8 |
| FEP    | >2   | >2   | >2   | >2   | >2   | >2    | >2    | >2    | >2    | >2    | >2    | >2 |
| MEM    | >2   | 0.5) | >2   | >2   | >2   | >2    | >2    | >2    | >2    | >2    | >2    | >2 |
| LVX    | >8   | >8   | >8   | >8   | >8   | >8    | >8    | >8    | >8    | >8    | >8    | >8 |
| CLI    | >1   | >1   | >1   | >1   | >1   | >1    | >1    | >1    | >1    | >1    | >1    | >1 |
| ERY    | >1   | >1   | >1   | >1   | >1   | >1    | >1    | >1    | >1    | >1    | >1    | >1 |
| SXT(1) | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5  | ≤0.5  | ≤0.5  | ≤0.5  | ≤0.5  | ≤0.5  | ≤0.5 |
| VAN(2) | 0.5  | 0.5  | 0.5  | 0.5  | 0.5  | 1     | 0.5   | 0.5   | 0.5   | 0.5   | 0.5   | 0.5 |

1) The susceptibility according to the breakpoints of CLSI guidelines. PEN, penicillin; SAM, ampicillin/sulbactam; AMC, amoxicillin/clavulanate; CDN, cefditoren; CRO, ceftriaxone; FEP, cefepime; MEM, meropenem; LVX, levofloxacin; CLI, clindamycin; ERY, erythromycin; SXT, sulfamethoxazole-trimethoprim; VAN, vancomycin.
Corynebacterium amycolatum, and 1 Corynebacterium jeikeium. Five isolates were identified as non-Corynebacterium and were Zimmermannella alba, Actinomyces hordeoovulneris, Staphylococcus caprae, Gordonia terrae, and Janibacter limosus, based on 16S rRNA analysis. The misidentification rate of MicroScan and the API system was 22.7% and 27.3%, respectively.

**In vitro antimicrobial susceptibility:** In total, 17 isolates of Corynebacterium spp. were tested for antimicrobial susceptibility. VAN (100%, 17/17) was active against all Corynebacterium spp. strains, and SXT (82.4%, 14/17) was active against the majority of strains. All isolates seemed to be resistant to at least 2 types of antibiotics. Only 1 strain C. striatum (CK-2) showed higher susceptibility to more antibiotics compared to other isolates (Table 1).

**Characterization of biofilm formation:** The biofilm formation abilities of Corynebacterium spp. isolates were variable. Fourteen strains (82.4%) were able to form biofilms, of which 9 (64.3%) strains were identified as C. striatum strains (data not shown). Biofilm architectures were visualized at 20 × (original magnification; Fig. 1). The CK-7 strain produced mature biofilms that consisted of a large cluster of bacterial cells after 72 h of culture. The CK-21 strain presented different cellular morphologies, which suggested very low ability to produce biofilms, even after 72 h of incubation. The data showed congruent results with MBA results.

**PFGE and epidemiological investigation of C. striatum:** Molecular typing by PFGE revealed 4 distinct PFGE patterns in C. striatum isolates. Six strains presented PFGE pattern A (defined in this study), which was predominant. Four strains presented pattern D, and only 1 strain showed pattern B and C. Similar results were obtained using 2 different endonuclease restriction enzymes (Fig. 2).

The epidemiological characteristics of the C. striatum isolates are presented in Table 2. Six pattern A strains were isolated from the Cardiovascular Surgery, Cardiovascular Internal Medicine, Emergency, Hematology, and Urology wards. All of these strains had increased ability to produce biofilms. Two pattern D strains were isolated from patients admitted to the Emergency ward, and CK-15 was recovered 5 months later when CK-13 was isolated. There was no direct evidence suggesting that the patient deaths were associated with Corynebacterium spp.

**DISCUSSION**

Recently, Corynebacterium spp. have been increasingly recognized as causes of significant human infections (14,29–31). Many reports have mentioned that it remains a priority to identify Corynebacterium spp. at the species level in clinics (1,19,23). According to our results, we agree with the previously proposed opinion that partial rpoB sequencing is a simple and efficient method for the identification of Corynebacteria (23). However, multiple molecular tests are recommended in conjunction with routine tests for definitive identification in some ambiguous cases.

The antibiogram might contribute to exposure to multiple-antibiotics and consequently extended hospital stays since all strains exhibited multidrug-resistance in this study. VAN and SXT seemed to be still active against most strains, however, the limited therapeutic options might affect management of patients, and increase reliance on VAN (32). The selective pressure exerted by prior antimicrobial treatments might favor the overgrowth of multidrug-resistant Corynebacterium spp. as a secondary colonizer in immunocompromised hosts.

C. striatum has previously been considered a saprophyte on the skin and nasal mucosa, but it has also been reported to be responsible for various types of infections. It is also a potentially pathogenic microorganism with the ability to produce outbreaks of nosocomial infections. The isolation of C. striatum from sterile sites is a rare event and has been reported mainly in patients with indwelling devices or immunosuppression (14,29,31,33,34). In this study, C. striatum was isolated from blood specimens of patients with under-
Biofilm Producing *Corynebacterium striatum*

Fig. 2. Pulsed field gel electrophoresis profiles of *Corynebacterium striatum*. Digestion patterns of chromosomal DNA with *Smil* (A) and *PacI* (B) to determine genetic relatedness and the interpretation of PFGE patterns based on the criteria described by Tenover et al. (28). Lane M contains a molecular size marker. Four distinct PFGE profiles were investigated, and defined as pattern A, B, C, and D (*Smil*), and pattern a, b, c, and d (*PacI*). For example, patterns that are closely or possibly related to the outbreak pattern A are considered subtypes of A and are designed as subtypes A1 and A2.

Table 2. Summary of epidemiological analysis of *Corynebacterium striatum*

| Strain No. | Ward                  | Admitted date | Sampling date | Outcome | PFGE pattern | Biofilm formation (OD<sub>590</sub>) |
|------------|-----------------------|---------------|---------------|---------|--------------|-------------------------------------|
| CK-1       | Cardiovascular Surgery| March 19, 2008| June 26, 2008 | Died    | A            | 1.3                                 |
| CK-7       | Cardiovascular Internal Medicine | August 21, 2009 | August 27, 2009 | Recovered | A            | 1.99                                |
| CK-11      | Emergency             | May 20, 2010  | June 8, 2010  | Died    | A            | 1.08                                |
| CK-12      | Hematology            | August 4, 2010| August 29, 2010| Recovered | A            | 1.7                                 |
| CK-14      | Urology               | October 6, 2010| December 27, 2010| Recovered | A            | 2.26                                |
| CK-19      | Cardiovascular Surgery| April 6, 2011 | April 27, 2011| Died    | A            | 2.1                                 |
| CK-8       | Cardiovascular Surgery| October 6, 2009| October 22, 2009| Recovered | D            | 0.72                                |
| CK-13      | Emergency             | August 27, 2010| August 30, 2010| Died    | D            | 0.81                                |
| CK-15      | Emergency             | January 8, 2011| November 19, 2011| Recovered | D            | 0                                   |
| CK-21      | Hematology            | June 13, 2011 | July 10, 2011 | Died    | D            | 0                                   |
| CK-2       | Emergency             | February 17, 2009| March 2, 2009 | Died    | B            | 1.56                                |
| CK-5       | Cardiovascular Internal Medicine | June 19, 2009 | June 24, 2009 | Recovered | C            | 0                                   |

Lying diseases and with central or peripheral vein catheters. Only 4 distinct PFGE patterns were investigated during a 3-year monitoring period, which suggested that these strains possibly repeatedly infected the patients or efficiently colonized the hospital environments. Two pattern A and pattern D strains were isolated from the Cardiovascular Surgery and Emergency wards, respectively. It was difficult to classify these infections as nosocomial outbreaks, since very large intervals existed between the sampling dates of these isolates. Recently, Renom et al. reported that multidrug-resistant *C. striatum* contributes to high morbidity and mortality (41%) in nosocomial respiratory infection patients (35). Since most patients received long-term hospitalizations in this study, a high level of concern remains regarding the continuous monitoring of *C. striatum* disseminating in the hospital environment.

Biofilm-related infections are considered a major cause of morbidity and mortality in hospitals (36). *Corynebacterium* is known as a biofilm producing pathogen and is responsible for reduced susceptibility to antibiotics (37,38). To the best of our knowledge,
this is the first report of variable biofilm formation by different *C. striatum* strains isolated from blood specimens. Strains might escape from killing by producing robust biofilms, which could be reported to allow microorganisms to exchange genetic material and to become persistent colonizers and/or to gain greater resistance to antibiotics (39). These findings might explain why pattern A strains can survive in hospital patients for months or, even years. However, our presumption is still controversial, because it cannot explain why 2 pattern D strains that do not produce biofilms also showed signs of dissemination over a period of 5 months. This study also had many limitations, for example, small sample size and a limited study period. We also did not investigate the catheters from patients and did not reveal the association between biofilm formation and pathogenicity in *C. striatum* or the mechanisms of antimicrobial resistance.

In conclusion, evidence highlighting the clinical importance of *Corynebacterium* spp. continues to accumulate. In addition, *C. striatum* as an emerging pathogen, might be found responsible for bloodstream infections. It thus appears important to perform active surveillance focused on *Corynebacterium* spp. colonization. This might foster the implementation of enhanced control strategies to decrease *Corynebacterium*-related infections in hospitals.

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Conflict of interest None to declare.

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