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First isolation of *Clostridium indolis* in a patient with chronic osteitis: a case report and literature review of human infections related to *Clostridium saccharolyticum* group species

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ABSTRACT

Clostridium indolis is an anaerobic spore-forming Gram-positive bacillus belonging to the *Clostridium saccharolyticum* group. Its clinical significance in human remains poorly known. We describe the first case of osteitis related to *C. indolis*, identified by MALDI-TOF mass spectrometry and provide a literature review of human infections related to *C. saccharolyticum* group species.

In August 2014, a 37 years-old woman was admitted to the surgical intensive care unit of our tertiary care center, because of an open Cauchoix III fracture of left tibia and fibula with major skin damages and soft tissue defects after a motorcycle injury. On admission, the patient was intubated and ventilated. Body temperature was 36 °C. Heart rate and blood pressure were normal. Ionogram, blood cell count and C-reactive protein (CRP) were all within normal ranges whereas serum creatinine phosphokinase (CPK) level was increased to 697 UI/L secondary to muscular lysis. She underwent emergency surgery involving orthopedic, vascular and plastic surgical procedures. The treatment consisted in trimming and washing followed by centromedular tibial osteosynthesis, anterior tibial artery bypass, deep peroneal nerve graft and finally soleus muscle flap for covering soft tissue defects. In early 2015, she presented a subcutaneous collection of fluid located close to orthopedic screws which had developed during the previous four months. On 15 June 2015, she was readmitted because of an acute purulent discharge that had started four days earlier. Her body temperature was normal and laboratory investigations revealed inflammation markers such as slightly elevated CRP (7.5 mg/L) and moderate neutrophil polynucleosis (7.9 G/L). The orthopedic treatment consisted in the removal of two screws and washing. Five bacteriological specimens were sampled as recommended [1]. Three samples of the fluid collection and two necrotic bones fragment located next to the proximal and distal part of the screw were sent to our microbiology laboratory for analysis. All samples grew wild-type *Enterobacter cloacae* (on all media seeded: 5 out of 5) displaying natural resistance pattern to beta-lactams. On the basis of these results and according to the recommendations of *Société de Pathologie Infectieuse de Langue Française* (SPILF) [2] for osteoarticular infections on osteosynthesis material, a six-weeks

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antibiotic course of intra-venous (IV) cefepime (2 g t.i.d) and ciprofloxacin IV (400 mg b.i.d) was initiated. One week later, a new intervention was performed to remove the intramedullary nail materiel. Four surgical samples (3 soft tissues and 1 bone) were sent to our laboratory. Bacteriological analysis revealed *E. cloacae* displaying cephalosporinase hyperproduction phenotype from all biological samples (4 out of 4). At the end of July 2015, 4 weeks after the beginning of antibiotic therapy, she underwent a surgical revision with medial fibula transport and an Ilizarov external fixator was set because of tibial pseudarthrosis. A total of 3 surgical specimens were sampled. Two tibial soft tissues interpositions and one tibial necrotic bone fragment were directly placed into a specific transport medium Ultra Turrax tube (Dutscher, Brumath, France) and sent to our laboratory. Cultures on Columbia agar (Oxoid, Dardilly, France) plates supplemented with 5% sheep blood incubated in anaerobic and aerobic atmosphere, and cultures on chocolate agar plates (Oxoid) incubated in 5% CO_2, remained sterile after 96 h. Culture was positive on Rosenow enriched liquid medium (Biorad, Marnes-la-Coquette, France) after 2 weeks of incubation. Gram staining showed large spore-forming Gram-variable bacilli. When subcultured on blood agar plates under anaerobic conditions two out of three samples grew with medium size mucoid colonies surrounded by a single zone of beta-hemolysis. MALDI-TOF mass spectrometry (MS) performed on the colonies by direct transfer onto target identified *Clostridium indolis* Log score value of 2.19 matching *Clostridium indoli*s DSM 755T; MALDI Biotyper v2.3 (Brüker Daltonics, Bremen, Germany). This identification was confirmed by the National Reference Center of Anaerobic Bacteria and Botulism (Institut Pasteur, Paris, France) by *16S rDNA* gene sequencing using forward AAGGAGGTGATCCAGCCGCA and reverse primers AGAGTTTGATCATGGCTCAG, displaying 99.6% of identity with the sequence of *C. indolis* type strain DSM 755T (GenBank accession number Y18184 [3]). Phylogenetic relationship between the isolated strain (Strain 570.15) and the type strain of the species is shown in Fig. 1. The tree was constructed using a neighbor-joining method (Kimura 2 parameter method) and 500 bootstraps with MEGA6 (Molecular Evolutionary Genetics Analysis version 6.0) software as previously described [4]. Values above the

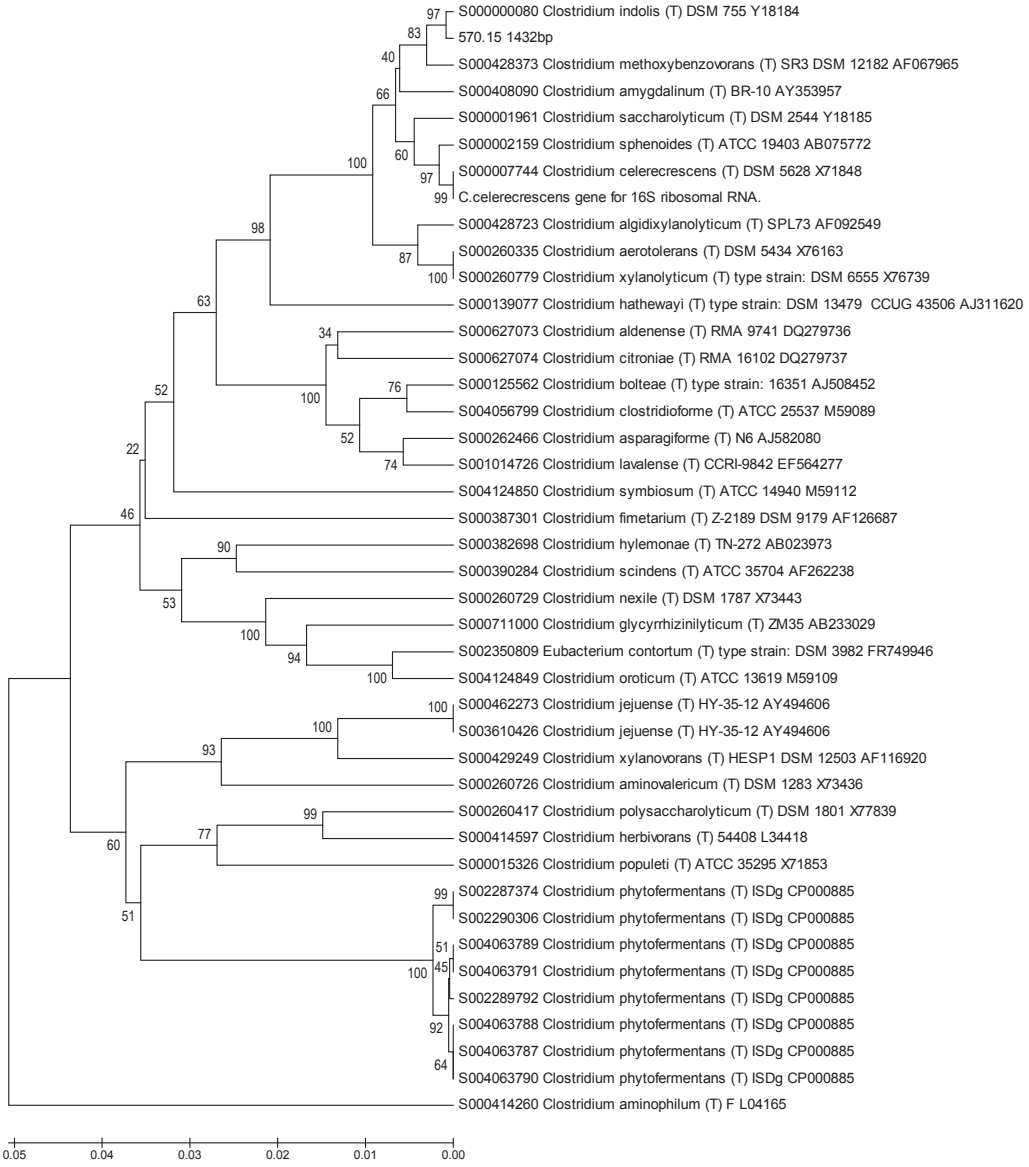

Fig. 1. Evolutionary relationships of strain 570.15. Phylogenetic tree based on 16r DNA sequence analysis as described in the text shows that the strain 570-15 is related to *C. indolis* type strain DSM755T and more distantly related to the clostridia of the *C. saccharolyticum* group.

lines are bootstrap values expressed as percentages. Antimicrobial susceptibility testing was performed on the isolated strain by using E-test strips (bioMérieux, Marcy-l'Etoile, France), with a McFarland 1 suspension in Schaedler broth, seeded on Brucella agar plate supplemented with vitamin K (1 mg/L) and 5% sheep blood that were incubated under anaerobic atmosphere for 48 h at 35–37 °C as recommended by the *Comité de l'Antibiogramme de la Société Française de Microbiologie* (CASFM) 2013 [5]. The Minimal Inhibitory Concentrations (MICs) were read following the CASFM 2013 interpretative standard for anaerobes (Table 1). To date, specific guidelines concerning the antibiotic treatment of chronic osteitis related to anaerobic bacteria do not exist. The antibiotic course was switched to oral metronidazole (500 mg t.i.d) because of its excellent oral bioavailability, good bone diffusion and because the strain was highly susceptible. The treatment was prolonged as recommended by the SPILF for a total duration of four weeks [2], in spite of potential occurrence of various side effects with prolonged metronidazole [6,7]. The patient had no secondary infections and presented no other side effects. Biological markers of inflammation returned normal at four weeks (CRP 4.1 mg/L and normal neutrophil count 2.6 G/L). In February 2016, the patient reported no pain and clinical examination showed clean scars, normal body temperature and CRP was negative. Radiological films did not show any sign of osteitis.

Clostridium species are spore forming Gram-positive or Gram-variable anaerobic bacilli that are ubiquitous in the environment. Some species are part of human gut microbiota. The most common *Clostridium* species in the intestinal microbiota are those from cluster IV such as *Clostridium leptum* and from cluster XIVa (*Clostridium coccoides, Clostridium celerecrescens, Clostridium sphenoides, Clostridium clostridioforme*) [8]. Other *Clostridium* species like *Clostridium difficile*, *Clostridium perfringens* and *Clostridium tetani* are well known for their involvement in human infections mostly related to the production of specific toxins. *Clostridium* species can also be associated with post-traumatic infections [9] and only a few species among the genus *Clostridium* have been reported to be involved in osteo-articular infections [10–14]. Interestingly, *C. indolis*, which belongs to the *Clostridium saccharolyticum* group along with *C. sphenoides, C. celerecrescens, Clostridium methoxybenzovorans and Desulfotomaculum guttoideum*, has never been reported in any human infection so far. *C. indolis* has once been isolated from a blood culture of a patient without sepsis syndrome and was therefore considered of no clinical importance [15]. Furthermore, the clinical significance of *C. saccharolyticum* group species in human is not well established. Only four cases of infections due to *C. sphenoides* were reported of which two were gastro-intestinal infections, one bacteremia and one osteomyelitis [16–19]. Three cases of *C. celerecrescens* infections were also described including an osteomyelitis, an abscess secondary to an open fracture and a post traumatic wound infection [10,11,20] (Table 2). Here we report the first case of osteitis related to *C. indolis* identified by MALDI-TOF MS with direct deposit on target using the Biotyper database. The recent availability of new identification tools in routine such as MALDI-TOF MS allows an accurate and rapid identification of bacterial species that previously were underestimated. In their study, AlMogbel et al. compared the efficiency of two MALDI-TOF MS systems (Bruker MALDI-TOF MS and Vitek2) for the identification of 144 *Clostridium* spp. isolates. Bruker MALDI-TOF-MS reliably identified to the species level 88.8% (128/144) of the isolates and to the genus level 92.3% (133/144), whereas, ViteK2 identified only 77.7% of the isolates. An extraction step with formic acid was used in this study [21]. Grosse-Herrenthey et al. showed that the MALDI-TOF technology using Bruker Microflex LT mass spectrometer allowed the identification of all the clostridial strains tested after preparation of samples with TFA and acetonitrile [22]. Finally Justensen et al. reported 93.9% (62/66) *Clostridium* spp. identified to the species level using Bruker system without pretreatment [23]. It is important to note that there is only one reference spectrum of *C. indolis, C. sphenoides* and *C. celerecrescens* included in the Bruker database (Biotyper v2.3) and there is still no reference for *C. methoxybenzovorans* and *D. guttoideum*. These limits may lead to an underestimation of these species. Hopefully, new reference spectra soon will be included in the upcoming databases. In this study, we further attempted to identify the patient strain *C. indolis* 570.15 using Rapid ID 32A strips (bioMérieux) that are still widely used in routine microbiological diagnosis. The test misidentified the strain as *Clostridium clostridioforme* with an acceptable significance (biochemical API profile: 45362000, 99.4% T = 0.77) except for a positive reaction for indole production. Interestingly, Bouvet et al. reported the misidentification of *C. celerecrescens* as *C. clostridioforme* using RAPID ID 32A strips [20]. This type of mistakes pinpoints the need to integrate new strains to refine biochemical databases. In order to help identifying these phylogenetically related anaerobic species in routine diagnosis, and waiting for enlarged databases, we provide a summary of the main biochemical characteristics of *C. saccharolyticum* group species [20,24–28] (Table 3). Concerning the clinical presentation, the patient was successfully treated by metronidazole, which is active against *C. indolis*, and follow-up consultation at six months after the last surgery reported no physical, biological or radiological signs of infection. Of note, bacteriological samples obtained after the first orthopedic surgery were positive for *E. cloacae* in pure culture, and only four weeks after an appropriate antibiotic treatment with cefepime and ciprofloxacin targeting *E. cloacae*, *C. indolis* was isolated in culture. *C indolis* 570.15 displayed high resistance level to cefepime and ciprofloxacin with MICs of 256 mg/L and 32 mg/L, respectively. Interestingly, the literature review reported high rates of *Clostridium* sp. susceptible to moxifloxacin, from 69% to 79% [29,30]. These data suggest that, in our case, the resistance of the strain to quinolones might have been acquired under antibiotic pressure. Concerning β-lactams, the nitrocefin test resulted negative, showing that the resistance was not mediated by production of beta-lactamase. The resistance could be due to modification of penicillin binding protein (PBP). This could be explained by the pre-existence of *C. indolis* in the wound and selection of resistant strains after the first antibiotic course. Indeed, even if we did not detect *C. indolis* in the first samples as co-pathogen, it seems very likely that it has been overgrown by *E. cloacae*. In addition, *Clostridium* species are environmental organisms, considered as soil saprophytes, or are part of normal human intestinal flora [31]. Almost all cases of anaerobic osteomyelitis occur by direct extension from an adjacent focus of infection and are rarely due to bacteremia [32].

Table 1
Antimicrobial susceptibility testing determined with the E-test method.

Antimicrobial agent	MIC (μg/mL)	Clinical categorization
Amoxicillin	0.75	S
Amoxicillin + clavulanic acid	0.75	S
Cefepime	192	*
Cefotaxime	32	R
Tazocillin	4	S
Erythromycin	2	*
Clindamycin	0.190	S
Tigecyclin	0.016	S
Rifampicin	0.0750	S
Ciprofloxacin	32	*
Levofloxacin	6	*
Moxifloxacin	4	R
Trimethoprim + sulfamethoxazole	32	*
Metronidazole	0.064	S

*No breakpoint available for this molecule.

Table 2
Main features of reported cases of human infections related to *C. saccharolyticum* group species.

Sex/age of patient (years)	*Clostridium* species	Underlying condition(s)	Type of infection	Risk factors	Treatment	Outcome	Positive sample/ associated pathogens	Methods of identification	Author
M/68	*C. sphenoides*	Unknown	Bacteremia	Vehicle injury spleen rupture	Doripenem IV (14 days), metronidazole IV (11 days) and vancomycin IV (7 days)	Recovery	Blood culture/none	*16S rDNA* sequencing	Kelesidis *et al.*, 2011 [19]
F/39	*C. sphenoides*	None	Gastroenteritis	Ate contaminated food 8 h before the onset of symptoms	None	Recovery	Stool/*Bacteroides vulgatus*	Gram staining, colony morphology, API20A, and use of Virginia Polytechnic Institute criteria	Sullivan *et al.*, 1980 [17]
M/13	*C. sphenoides*	None	Right ulnar osteomyelitis	Right wrist injury	Phenethicillin IV (3 months)	Recovery	Bone/none	Chemical activity, physiologic characteristics, and the appearance of the spores	Isenberg *et al.*, 1975 [18]
F/6	*C. sphenoides*	Periodic neuropenia	Peritonitis	None	None	Death	peritoneal sample/none	Gram staining, colony morphology and biochemical features	Felitti *et al.*, 1970 [16]
M/55	*C. celerecrescens*	Peripheral artery occlusive disease, diabetes mellitus and alcoholism	Right tibial osteomyelitis	Open tibial fracture 9 years before the infection	Cefuroxime (1.5 mg t.i.d) and metronidazole 400 mg t.i.d) for a total duration of 6 weeks (3 weeks IV followed by 3 weeks per os)	Relapse the following year	Intraoperative swab of the right tibia and tissue specimens taken from the medullary cavity tissue/none	API rapid 32 A strips misidentified as *Clostridium clostridioforme* (99.8%) MALDI-TOF led to *C.sphenoides* with unacceptable score (1.6). *16S rDNA* sequencing performed for exact species determination	Mischnik *et al.*, 2011 [11]
M/45	*C. celerecrescens*	Heart arythmia and chronic bronchitis	Abscess	Metaphysis interior open fracture of the left thigh bone	Combination of oral sulfamethoxazole (800 mg 6 times a day) trimethoprim (160 mg b.i.d), clindamycin (600 mg b.i.d) and metronidazole (250 mg b.i.d)	Recovery	Drained abscess/non	*16S rDNA* sequencing	Glazunova *et al.*, 2005 [10]
M/20	*C. celerecrescens*	None	Post traumatic wound infection	Open wound with a piece of rusty iron	Oral amoxicillin/clavulanic acid (1 g t.i.d) during 7 days	Recovery	Wound tissue/none	*16S rDNA* sequencing	Bouvet *et al.*, 2012 [20]
F/37	*C. indolis*	None	Post traumatic wound infection	Open wound, prolonged contact with the road	Oral metronidazole 500 mg t.i.d during 4 weeks	Favorable evolution at 6 months follow-up	Bones and tissues biopsy	MALDI-TOF MS and *16S rDNA* sequencing	Our case

Table 3
Enzymatic activity tests and profile numbers (Rapid ID 32A gallery) and production of acid from some sugars for differentiation of species of *C. indolis*, *C. sphenoides*.[a] *C. celerecrescens* and "C. *Clostridioform* group".

RAPID ID 32A	*C. indolis (Strain* 570.15)	*C. indolis* T	*C. sphenoides* CIP 104283T	*C. celerecrescens* CIP14809	*C. clostridioforme*
Characters[b]					
α-Galactosidase	+	+	+	+	+ (−)
β-Galactosidase	+	+	+	+	+ (−)
α-Glucosidase	+	+	+	+	− (+)
β-Glucosidase	+	+	+	+	−
α-Arabinosidase	+	+	+	+	− (+)
β-Glucuronidase	−	−	−	−	− (+)
β-N-Acetyl-glucosaminidase	−	−	−	+	−
Mannose	+	+	−	−	−
Raffinose	+	−	−	−	− (+)
Indole production	+	+	+	+	−
Alkaline phosphatase	±	−	−	−	− (+)
Arginine arylamidase	−	−	−	−	−
Proline arylamidase	−	−	−	−	−
Leucyl glycine arylamidase	−	−	−	−	−
Leucine arylamidase	−	−	−	−	−
Pyroglutamic acid arylamidase	−	−	−	−	−
Rapid ID 32A profile numbers	4536200000	4532200000	5530300000	45120000	00000000 41000000 41000400 45160000

+ = Positive; +w = weakly positive; − = negative; + (−) = positive (rarely negative); − (+) = negative (rarely positive); ± = positive or negative.
[a] Data were obtained from published studies [20,24–27] and from some strains studied in the NRC of Anaerobic Bacteria and Botulism (Institut Pasteur, Paris, France) with 16S rDNA gene sequenced (2 *Clostridium indolis* DSM755 et NCIB9731, 2 *C. clostridioforme).*
[b] The following tests were always found negative: urease, arginine dihydrolase, β-galactosidase-6-phosphate, reduction of nitrates, phenylalanine arylamidase, tyrosine arylamidase, alanine arylamidase, and glycine arylamidase.

These data along with the absence of other positive bacteriological samples and/or digestive symptoms, suggest that this post-traumatic chronic osteitis occurred after a telluric contamination. Furthermore, the prolonged contact between the open wound (with major soft tissue injury) and the ground after the accident can easily explain this mode of infection.

In conclusion, the first case of osteitis caused by *C. indolis* has been described. The patient was immunocompetent and successfully treated by a multidisciplinary medico-surgical approach. The bacterium has previously been considered as non-pathogenic and data concerning *C. saccharolyticum* group species are still missing in the literature. Hopefully, the use of new tools in the bacterial routine identification, such as MALDI-TOF MS and *16S rDNA* sequencing, will provide a better understanding of the clinical significance of clostridia in human infections.

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