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► To cite this version:

Romain Lotte, Laurène Lotte, Philippe Bouvet, Nicolas Degand, Antonin Bal, et al.. 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. *Anaerobe*, 2016, 42, pp.44 - 49. 10.1016/j.anaerobe.2016.08.001 . pasteur-01787653

HAL Id: pasteur-01787653

<https://pasteur.hal.science/pasteur-01787653>

Submitted on 31 Jul 2018

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

5 Authors name: Romain Lotte^{1,2,3,#}, Laurène Lotte^{1,2}, Philippe Bouvet⁴, Nicolas Degand¹,
6 Michel Carles^{2,5}, Regis Bernard de Dompierre⁶, Michel Popoff⁴ and Raymond Ruimy^{1,2,3}

7 ¹*Laboratoire de bactériologie, Centre Hospitalier Universitaire de Nice, Nice, France*

8 ²*Université de Nice Sophia-Antipolis, Faculté de Médecine, Nice, France*

9 ³*INSERM U1065(C3M), “Bacterial toxins in host pathogen interactions”, C3M, Bâtiment
10 universitaire Archimed, Nice, France.*

11 ⁴*Centre de Reference des Bactéries Anaérobies et du Botulisme, Institut Pasteur, France*

12 ⁵*Service d'anesthésie réanimation, Centre Hospitalier Universitaire de Nice, Nice, France*

13 ⁶*Service de traumatologie, Centre Hospitalier Universitaire de Nice, Nice, France*

14 **#Corresponding author : Dr Lotte Romain**

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16 Laboratoire de Bactériologie

17 Centre Hospitalier Universitaire de Nice, Hôpital de l'Archet II,

18 151 route de Saint Antoine de Ginestière CS 23079 06202 Nice Cedex 3, France

19 Tel: 33 (0) 4 92 03 62 14

20 Fax: 33 (0) 4 92 03 59 52

21 E-mail: lotte.r@chu-nice.fr

22 Key words: *Clostridium*, Osteitis, Emerging pathogen

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

28 In august 2014, a 37 years-old woman was admitted to the surgical intensive care unit of our
29 tertiary care center, because of an open Cauchoux III fracture of left tibia and fibula with skin
30 damages after a motorcycle injury. On admission, the patient was intubated and ventilated.
31 Body temperature was 36°C. Heart rate and blood pressure were normal. Ionogram, blood
32 cells count and C-reactive protein (CRP) were all within normal range whereas serum
33 creatinine phosphokinase (CPK) level was increased to 697 UI/L secondary to muscular lysis.
34 She underwent an urgent surgery consisting in a tibial-fibular osteosynthesis performed by a
35 multidisciplinary surgical critical care team involving orthopedic, vascular and plastic
36 surgeon. In early 2015, she presented a subcutaneous collection located close to orthopedic
37 screws. This process has grown chronically and was well tolerated during 4 months. On 15
38 June 2015, she was readmitted because of an acute purulent discharge from this collection that
39 started 4 days before. Her body temperature was normal. Laboratory investigations revealed
40 inflammation markers such as slightly elevated CRP (7.5 mg/L) and moderate neutrophil
41 polynucleosis (7.9 G/L). The orthopedic treatment consisted in the removal of two screws and
42 washing. Five bacteriological specimens (screws and collection) were sampled as
43 recommended by the Société de Pathologie Infectieuse de Langue Française (SPILF) and sent
44 to our microbiology laboratory for analysis. All samples grew with *Enterobacter cloacae* (on
45 all media seeded: 5 out of 5) displaying wild type antibiotic susceptibility pattern to beta-
46 lactams. On the basis of these results and according to the recommendations of SPILF for
47 osteo-articular infections on osteosynthesis material, a six-weeks antibiotic course of Intra-

48 Venous (IV) cefepime (2g t.i.d) associated with ciprofloxacin IV (400 mg b.i.d) was initiated.

49 One week later, a new intervention was performed to remove the intramedullary nail materiel.

50 Four surgical samples (3 soft tissues and 1 bone) were sent to our laboratory. Bacteriological

51 analysis retrieved *E. cloacae* displaying cephalosporinase hyperproduction phenotype from all

52 biological samples (4 out of 4). At the end of July 2015, 4 weeks after the beginning of

53 antibioticotherapy, she underwent a surgical revision with medial fibula transport and an Ilizarov

54 external fixator was set because of tibial pseudarthrosis. A total of 3 surgical specimens were

55 sampled (2 soft tissues and one bone). Cultures on Columbia agar plates supplemented with 5

56 % sheep blood (Oxoid, Dardilly, France) incubated both under anaerobic and aerobic

57 atmosphere, and cultures on chocolate agar plates (Oxoid, Dardilly, France) incubated under

58 5% CO₂ remained sterile after 96 hours. Culture was positive on Rosenow (Biorad, Marnes-

59 la-Coquette, France) enriched liquid medium after 2 weeks of incubation. Gram staining

60 showed large spore-forming Gram-variable bacilli. Subculture performed on blood agar plates

61 under anaerobic conditions grew with medium size mucoid colonies surrounded by a single

62 zone of beta-hemolysis (two out of three samples). MALDI-TOF mass spectrometry (MS)

63 performed directly on the colonies identified *Clostridium indolis* (Log score value of 2.19

64 matching *Clostridium indolis* reference strain DSM755T; MALDI Biotype v2.3). This

65 identification was confirmed by the National Reference Center of Anaerobic Bacteria and

66 Botulism (Institut Pasteur, Paris, France) by 16S rDNA gene sequencing using forward

67 AAGGAGGTGATCCAGCCGCA and reverse primers AGAGTTGATCATGGCTCAG,

68 (99,6% of identity with the sequence of *Clostridium indolis* type strain DSM755T, GenBank

69 accession number Y18184) (Strain 570.15, see *figure* for phylogenetic analysis) [1–5].

70 Antimicrobial susceptibility testing was performed on the isolated strain using E-test strips

71 (bioMérieux, Marcy-l'Etoile, France), with a 1 McFarland suspension, on Brucella agar plate

72 supplemented with vitamin K (1 mg/L) and 5% sheep blood that were incubated under

73 anaerobic atmosphere as recommended by the CASFM 2013. The Minimal Inhibitory
74 Concentrations (MICs) were read following the CASFM 2013 interpretative standard for
75 anaerobes (see table1). The antibiotic treatment was changed to oral metronidazole (500 mg
76 t.i.d) for a total duration of four weeks. Biological markers of inflammation returned normal
77 at 4 weeks (CRP 4.1 mg/L and normal neutrophils count 2.6 G/L). In February 2016, the
78 patient reported no painful phenomenon and clinical examination showed clean scars and
79 body temperature was normal. CRP was negative. Radiological control did not show signs of
80 osteitis.

81 *Clostridium* species are spore forming Gram-positive or Gram-variable anaerobic bacilli that
82 are ubiquitous in the environment. Some species are part of human digestive microbiota.
83 *Clostridium* species like *Clostridium difficile*, *Clostridium perfringens* and *Clostridium tetani*
84 are well known for their involvement in human infections mostly related to the production of
85 specific toxins. *Clostridium* species can also be associated with post-traumatic infections [6]
86 and only few species among the genus *Clostridium* are involved in osteo-articular infections
87 [7–11]. Interestingly, *C. indolis*, which belongs to the *C. saccharolyticum* group along with
88 *Clostridium sphenoides*, *Clostridium celerecrescens*, *Clostridium methoxybenzovorans* and
89 *Desulfotomaculum guttoideum*, has never been reported in any human infection so far.
90 *C. indolis* was isolated only once from a blood culture of a patient presenting without sepsis
91 syndrome [12]. Furthermore, the clinical significance of *C. saccharolyticum* species group in
92 human is not well established. Only 4 cases of infections due to *C. sphenoides* were reported
93 (2 gastro-intestinal infections, one bacteremia and one osteomyelitis) [13–16]. Three cases of
94 *C. celerecrescens* infections were also described including an osteomyelitis, an abscess
95 secondary to an open fracture and a post traumatic wound infection [7,8,17] (see table 2 for
96 details). Here we report the first case of osteitis related to *C. indolis* identified by MALDI-
97 TOF MS using Biotype database. The recent spread of new identification tools in routine

such as MALDI-TOF MS allows an accurate identification of bacterial species that were previously underestimated. Nevertheless, there is only one reference spectrum of *C. indolis*, *C. sphenoides* and *C. celerecrescens* included in the Bruker database (Biolyper 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 will be soon included in the upcoming databases. In this study, we further attempted to identify the patient strain (Strain 570.15) using Rapid ID 32A strips (BioMérieux, Marcy-l'Etoile, France) that is still widely used in routine microbiological diagnosis. The test misidentified the strain as *C. 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 [17]. 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* species group in the table 3. Concerning the clinical presentation, the patient was successfully treated by metronidazole, which is active on *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 to *E. cloacae* in monobacterial culture, and only four weeks after an appropriate antibiotic treatment with cefepime and ciprofloxacin targeting *E. cloacae*, *C. indolis* was isolated in culture. The strains showed high resistance level to cefepime and ciprofloxacin with MICs values 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% [18,19]. This data suggests that, in our case, the resistance of the strain to quinolones was probably acquired under

123 antibiotic pressure. This could be explained by the pre-existence of *C. indolis* in the wound
124 and selection of resistant mutants after the first course of antibiotics. Indeed, even if we did
125 not detect *C. indolis* in the first samples as co-pathogen, it seems very likely that it has been
126 overgrown by *E. cloacae*. In addition, *Clostridium* species are environmental organisms,
127 considered as soil saprophytes, or are part of normal human intestinal flora [20]. Almost all
128 anaerobic osteomyelitis occur by direct extension from an adjacent focus of infection and are
129 rarely due to bacteremia [21]. These data along with the absence of other positive
130 bacteriological samples and/or digestive symptoms, suggest that this post-traumatic chronic
131 osteitis occurred after a telluric contamination. Furthermore, the prolonged contact between
132 the open wound and the ground after the accident can easily explain this mode of infection.

133 In conclusion, we described here the first case of osteitis due to *Clostridium indolis* in an
134 immunocompetent patient successfully treated by a multidisciplinary medico-surgical
135 approach while this bacterium was previously considered as non-pathogenic. Data concerning
136 *C. saccharolyticum* species group are still lacking in the literature. Hopefully, the spread of
137 new identification tools in bacterial routine identification such as MALDI-TOF MS and 16S
138 rDNA sequencing would help its diagnosis and provide a better understanding of its clinical
139 significance in human infections.

140 **Acknowledgments**

141 We would like to thank the department of Bacteriology (Nice teaching hospital) for the
142 technical support and Dr Antonin Bal for the help in the collection of clinical data.
143 This work has been supported by FRM (Grant#FDM20-150632804 to Romain Lotte).

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