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Clostridium clostridioforme and *Atopobium minutum* Clinical Isolates with VanB-Type Resistance in France▽

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Acquired vancomycin resistance in Gram-positive anaerobes has been reported only in Australia and Canada from rare *vanB*-positive stool samples in the absence of vancomycin-resistant enterococci (VRE). We report the emergence of VanB-type resistance in *Clostridium clostridioforme* and *Atopobium minutum* involved in human infections in France.

CASE REPORTS

Case 1. A 59-year-old man was admitted to the emergency room of the Central Hospital of Nancy in France for acute abdominal pain with vomiting. The patient had a past medical history that included aortobifemoral bypass surgery complicated by an abdominal wall eventration treated by placement of a prosthetic patch in 2000 and a cystoprostatectomy in 2008. Abdominal and pelvic computerized tomography (CT) indicated hydropneumoperitoneum. Exploratory laparotomy visualized stercoral peritonitis adjacent to a perforated loop of the small intestine. The treatment included peritoneal lavage, lateral ileostomy, adhesiolysis of the small intestine, and antibiotic therapy, including ceftriaxone and metronidazole. The patient left the intensive care unit (ICU) 9 days after surgery. Bacterial culture of a peritoneal fluid sample yielded *Bacteroides fragilis*, *Clostridium clostridioforme*, *Enterococcus durans*, *Enterococcus faecium*, and *Escherichia coli*. Assays for antibiotic susceptibility showed that *C. clostridioforme* was resistant to vancomycin (MIC > 256 μg/ml) but remained susceptible to teicoplanin; in contrast, *E. faecium* and *E. durans* were susceptible to glycopeptides.

Case 2. A 45-year-old man was admitted to the Saint Joseph Hospital in Paris to cure a deep pressure sore with tissue necrosis. The patient had a long history of spina bifida which led to a chronic sacral bedsore. CT visualized profound bony destruction, and the patient was treated by surgical debridement combined with ceftriaxone and minocycline, leading to clinical improvement. Bacterial culture of deep infected tissue yielded *Atopobium minutum*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Prevotella oralis*, and *Staphylococcus aureus*. Interestingly, *A. minutum* was found to be resistant to vancomycin and susceptible to teicoplanin.

A. minutum, formerly designated *Lactobacillus minutus*, is a Gram-positive, non-spore-forming, nonmotile, and strictly anaerobic bacillus that has been classified in the family *Coriobacteriaceae* (4). *Atopobium* spp. are members of the human commensal microbiota which have been reported only rarely in oral infections, abdominal wounds, blood, and pelvic abscesses, and in most instances, these bacteria were found associated with other microorganisms (15).

Microbiological data. *C. clostridioforme* strain CIP (Collection of Institut Pasteur) 110249 was identified by API 20A and Rapid ID 32 A strips (bioMérieux, Marcy l'Etoile, France). Identification of CIP 110250, an obligatory anaerobic, Gram-positive, non-spore-forming rod-shaped bacterium, could not be achieved biochemically. Definitive identification of the organisms was performed by sequencing a 1,483-bp PCR fragment from 16S rRNA using universal primers B27F (5′-AGAGTTTGATCCTGGCTCAG) and U1492R (5′-GGTTACCTTGTTACGACTT) (18). A sequence of 1,327 bp from the CIP 110250 PCR fragment was identical to the corresponding portion of the 16S rRNA gene of *A. minutum* type strain DSM 20586^T (GenBank accession number FN178468.2). A sequence of 1,351 bp from the CIP 110249 PCR product was closely related to the 16S rRNA gene from type strain *C. clostridioforme* ATCC 25537 (DSM 933) (99.7% identity) (GenBank accession number M59089 and DNA sequence revisited in this study).

C. clostridioforme CIP 110249 and *A. minutum* CIP 110250 were grown under anaerobic conditions at 37°C on prereduced brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI) and BHI agar supplemented with 5% horse blood. The MICs of vancomycin were determined by the Etest procedure (AB Biodisk, Solna, Sweden) and by 2-fold serial dilution in blood agar according to CLSI guidelines. Both strains were highly resistant to vancomycin (MIC > 256 μg/ml and MIC = 64 μg/ml for *C. clostridioforme* CIP 110249 and *A. minutum* CIP 110250, respectively) but susceptible to teicoplanin (MIC = 1 μg/ml), suggesting VanB-type resistance.

The *C. clostridioforme* group includes *Clostridium bolteae*, *C. clostridioforme*, and *Clostridium hathewayi* (9), and more recently, *Clostridium aldenense* and *Clostridium citroniae* have been added (19). All these species have been reported from human clinical infections, most often associated with other bacteria from peritoneal fluid in patients with peritonitis. *C. clostridioforme* bacteria have been also isolated from osteomy-

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elitis, blood, liver abscess, and diabetic foot infection (9). Taking into account the role of vancomycin in treatment, the emergence of resistance to this antibiotic is of clinical significance.

The *vanB* gene was detected by PCR using primers p624 (5′-CGTGTGCTGCAGGATACTAC) and p951 (5′-CCGCT GGGCATTTGAATAC) deduced from flanking sequences of *vanB* in Tn*1549* (GenBank accession number AF192329). The 1,843-bp PCR fragment from *C. clostridioforme* CIP 110249 was identical to *vanB2* of the pheromone-responsive plasmid pMG2200 (21), whereas that from *A. minutum* CIP 110250 differed by two nonsynonymous point mutations from *vanB2* of *Eggerthella lenta* MLG043 (16). Heterogeneity in *vanB* genes has led to the description of three subtypes, *vanB1*, *-2*, and *-3* (6). The *vanB2* determinant is part of conjugative transposon Tn*1549* (34 kb) and closely related elements, such as Tn*5382*, and is the most widespread *vanB* subtype (3, 10). Therefore, we searched for the presence of several genes of Tn*1549* in CIP 110249 and CIP 110250. PCR analyses were performed using the following primers: f16F (5′-GTGAAGTCGGAACGGTT GTT) and f16R (5′-CTCCAAACGCTCAAACATGA) for *orf16*, f7F (5′-TTTGTCACAGGCAGATTTGG) and f7R (5′-CGATCTCACCATCTGCCATA) for *orf7*, iF (5′-CGCTTCG GATGAAAGAAAAG) and iR (5′-CGTCGTCCAGAACGT CACTA) for the *int* gene, and xF (5′-GATGAGCCATACCC CGATAC) and xR (5′-CGATCTCACCATCTGCCATA) for *vanX*. PCR products of the expected size were obtained for each gene in both strains (data not shown), confirming the presence of Tn*1549*-type elements. The presence of circular intermediates of Tn*1549* was screened for by amplification and sequencing of the 250-bp PCR product overlapping the jointed ends using a nested PCR with primers VB2 and VBR2 in a first step and internal primers VB1 and VBR3 in a second step, as described previously (13). Circular intermediates were detected in *A. minutum* CIP 110250 and in *C. clostridioforme* CIP 110249. These results indicated that the Tn*1549*-related elements are able to govern the transposition in both microorganisms. Tn*1549* was shown to transfer passively in enterococci by conjugation of plasmids or by the exchange of large chromosomal elements ranging from 90 to 250 kb in size (5). We have demonstrated both *in vitro* and *in vivo* that Tn*1549*-like elements can transfer from *Clostridium symbiosum* to *Enterococcus* spp. (13). In order to test the ability of CIP 110249 and CIP 110250 to transfer vancomycin resistance, conjugation experiments were carried out *in vitro* with *E. faecium* 64/3 as the recipient, as described previously (13). Despite four repeated experiments, attempts to transfer vancomycin resistance were unsuccessful. In fact, we reported the capacity of Tn*1549* to be mobilized by a heterologous transfer system when plasmids or integrative and conjugative elements (ICEs) are present in the bacterial host (17). This accounts for the observation that all the *vanB*-containing anaerobe strains are not able to transfer vancomycin resistance *in vitro*.

Glycopeptide resistance in enterococci is due to operons which lead to the synthesis of peptidoglycan precursors ending in D-Ala-D-Lac (VanA, VanB, VanD, and VanM) or D-Ala-D-Ser (VanC, VanE, VanG, VanL, and VanN), which have low affinity for glycopeptides. In addition, the elimination of the pentapeptide precursors produced by the ligase of the host ending in D-Ala-D-Ala is required (1, 20). VanA and VanB are the most common types and are responsible for more than 95% of the vancomycin-resistant enterococcus (VRE) isolates. The origin of the *van* genes remains hypothetical; however, recent studies indicated that *vanA* might originate from soil microorganisms (12), whereas *vanB* might arise from gene transfer from human intestinal microbiota (2). Anaerobes that could represent the reservoir of *vanB*, including *C. bolteae*, *C. hathewayi*, *Clostridium innocuum*-like, *Clostridium lavalense*, *C. symbiosum*, *E. lenta*, and *Ruminococcus lactaris*-like, have been identified from stool samples positive for *vanB* in the absence of VRE (7, 8, 11, 14, 16). However, these rare strains are limited to Australia and Canada. We report here the description of two *vanB* anaerobes isolated from clinical samples in France. Interestingly, the presence of VRE was not detected from stool specimens in our patients. Several studies have demonstrated a lack of correlation between the fecal detection of *vanB* by PCR or real-time PCR and the carriage of VRE. This result was attributed to the presence of *vanB*-containing anaerobic bacilli (11). Although the *vanB* reservoir has probably spread worldwide, until now, Australian and Canadian authors were the only ones to successfully identify different *vanB*-carrying anaerobes, probably due to the difficulty in cultivating certain strains. The isolation of two *vanB*-carrying anaerobes from infection sites in humans exemplifies the risk of dissemination of VanB-type resistance from the intestinal microbiota, which constitutes a reservoir for antibiotic resistance.

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA genes from isolates CIP 110250, CIP 110249, and ATCC 25537 were deposited in GenBank under accession numbers JF313107, JF313108, and JF313109, respectively. The nucleotide sequences of the *vanB* ligase genes from isolates CIP 110249 and CIP 110250 were deposited in GenBank under accession numbers JF313105 and JF313106, respectively.

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