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Identification of NDM-1 in a Putatively Novel *Acinetobacter* Species ("NB14") Closely Related to *Acinetobacter pittii*

Paula Espinal,[a] Noraida Mosqueda,[a] Murat Telli,[b] Tanny van der Reijden,[c] Dora Rolo,[a] Dietmar Fernández-Orth,[a] Lenie Dijkshoorn,[c] Ignasi Roca,[a] Jordi Vila[a]

ISGlobal, Barcelona Centre for International Health Research, Hospital Clínic, Universitat de Barcelona, Barcelona, Spain[a]; Department of Clinical Microbiology, School of Medicine, Adnan Menderes University, Aydin, Turkey[b]; Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands[c]

In this study, we describe the molecular characterization of a plasmid-located bla_{NDM-1} harbored by an *Acinetobacter* clinical isolate recovered from a patient in Turkey that putatively constitutes a novel *Acinetobacter* species, as shown by its distinct ARDRA (amplified 16S ribosomal DNA restriction analysis) profile and molecular sequencing techniques. bla_{NDM-1} was carried by a conjugative plasmid widespread among non-*baumannii Acinetobacter* isolates, suggesting its potential for dissemination before reaching more clinically relevant *Acinetobacter* species.

Acinetobacter spp. belonging to the *Acinetobacter baumannii* group are a major cause of nosocomial intensive care unit (ICU) infections, with *A. baumannii* being the species with the highest clinical relevance, followed by *Acinetobacter pittii* and *Acinetobacter nosocomialis* (1, 2).

The implementation of molecular identification methods and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has led to increasing reports of infections caused by non-*baumannii Acinetobacter* isolates and to the identification of putative novel *Acinetobacter* species (3, 4). The increase of carbapenem resistance in *Acinetobacter* spp. is also of concern, and we are witnessing the worldwide dissemination of NDM enzymes among different *Acinetobacter* spp. (5). Here, we report the molecular characterization of bla_{NDM-1} from a putatively novel *Acinetobacter* species isolated in Turkey.

An *Acinetobacter* species isolate (JVAP01) was recovered from a six-year-old female patient admitted to the Adnan Menderes University Hospital, Turkey, in August 2009 with a urinary tract infection. Empirical treatment with cefuroxime axetil (75 mg/kg/day) and nitrofurantoin (1 mg/kg/day) was initiated and sustained upon availability of microbiological data, since remission of the patient's symptoms was observed. Therapy was continued for 1 week, and upon recovery, she was discharged from the hospital. The patient had no history of travel or admission to other hospitals in Turkey.

JVAP01 was initially reported as belonging to the *Acinetobacter calcoaceticus-A. baumannii* complex by the Phoenix system (Becton Dickinson, USA) but identified as *A. pittii* (97%) and as *A. calcoaceticus* (99%) by *recA* and 16S-23S rRNA gene internal transcribed spacer (ITS) sequencing, respectively (6). Multilocus sequence typing (MLST) performed according to the Pasteur MLST scheme for *A. baumannii* (http://pubmlst.org/abaumannii/) identified a novel *recA* allele (*recA106*), and JVAP01 was assigned a novel sequence type (ST606).

Amplified 16S ribosomal DNA restriction analysis (ARDRA) (7) showed a 2-5-1-1-3-17 ARDRA pattern for CfoI, AluI, MboI, RsaI, MspI, and BfaI, respectively, matching the band pattern of several strains from the *Acinetobacter* reference collection of Leiden University. This collection of strains apparently constituted a novel *Acinetobacter* species, preliminarily termed "NB14," and the strains showed high similarity in amplified fragment length polymorphism (AFLP) fingerprint analysis (data not shown).

A comparative sequence analysis of the RNA polymerase β-subunit gene (*rpoB*) of JVAP01 was performed using the Bionumerics 5.1 software with default parameters as previously described (8, 9). The *rpoB* sequences of *Acinetobacter* spp. were used to create a neighbor-joining dendrogram for phylogenetic clustering with the Jukes-Cantor algorithm. As shown in Fig. 1, strains from each species of the *A. calcoaceticus-A. baumannii* complex formed respective clusters, supported by high bootstrap values, with strains belonging to NB14 forming a distinct branch that was closest to both *A. pittii* and *A. calcoaceticus* (Fig. 1). The bootstrap value for linkage of *A. pittii* to NB14 was 98%.

The *rpoB* interspecies similarity values for the species of the *A. calcoaceticus-A. baumannii* complex ranged between 89.6 and 98% (data not shown), while the intraspecies similarity of NB14 strains ranged between 99.1 and 100%, and this group was closest to *A. pittii* (97 to 98%).

Antimicrobial susceptibility testing performed by Etest (AB-bioMérieux, Sweden) and interpreted according to EUCAST guidelines (version 4.0, 2014) showed that JVAP01 was resistant to β-lactams and kanamycin and susceptible to amikacin, gentamicin, ciprofloxacin, and colistin (Table 1). JVAP01 also yielded positive results in the imipenem-EDTA synergy test and the modified Hodge test (MHT), suggesting metallo-β-lactamase (MBL) production. PCR screening for class B and class D β-lactamase genes (10) was negative except for bla_{NDM-1}, as confirmed by DNA se-

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Address correspondence to Ignasi Roca, Ignasi.roca@isglobal.org.
P.E. and N.M. contributed equally to this work.

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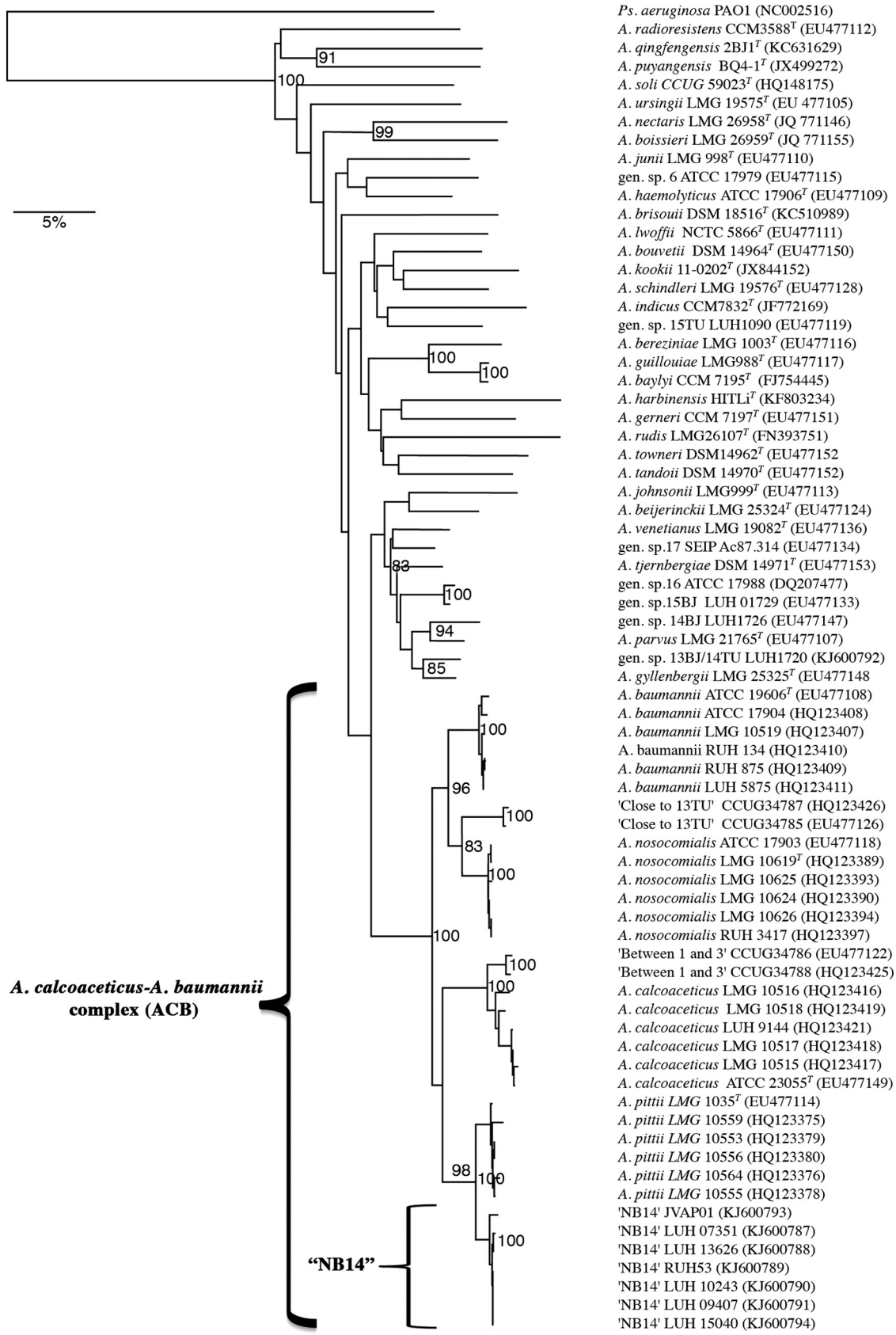

FIG 1 Cluster analysis of the *rpoB* sequences from *Acinetobacter* spp. A rooted neighbor-joining tree based on the partial sequence of *rpoB* (nucleotide positions 2915 to 3775) of 70 strains of the *A. calcoaceticus-A. baumannii* complex, including those of NB14 and representatives of different species of the genus *Acinetobacter*, is shown. *P. aeruginosa* PAO1 was used as the outgroup. Bootstrap percentages based on 1,000 simulations are shown. GenBank accession numbers are given in parentheses. The bar indicates 5% sequence divergence.

TABLE 1 Antimicrobial susceptibility testing by Etest for the indicated strains

Antimicrobial(s)	MIC (mg/liter)[a]			
	NB14 JVAP01	*A. baumannii* ATCC 17978	*A. baumannii* ATCC 17978-C	*A. baumannii* ATCC 17978-T
Cefepime	>256	2	>256	>256
Ceftazidime	>256	3	>256	>256
Cefotaxime	>256	6	>256	>256
Ciprofloxacin	0.38	0.5	0.5	0.38
Amikacin	3	0.38	1	1.5
Gentamicin	0.125	0.19	0.25	0.5
Kanamycin	24	0.5	8	12
Piperacillin-tazobactam	128	0.32	96	32
Imipenem	>32	0.25	>32	>32
Meropenem	>32	0.38	>32	>32
Tigecycline	1	0.5	0.25	1
Colistin	0.38	0.125	0.38	0.38
Rifampin	2	>32	32	32

[a] C, transconjugant strain; T, transformed strain.

quencing. Of note, JVAP01 showed reduced susceptibility to amikacin despite carrying a full-length *aphA6* gene, thus suggesting reduced expression of *aphA6*, as previously noted (11, 12).

S1 nuclease pulse-field gel electrophoresis and Southern blot analysis (10) located both bla_{NDM-1} and *aphA6* on a plasmid (pNDM-JVAP01) of approximately 50 kb that could not be assigned to any plasmid replicon type (13). pNDM-JVAP01 was successfully transferred to *A. baumannii* ATCC 17978 by both biparental mating and electroporation, but not to *E. coli* MC1061 (12). *A. baumannii* ATCC 17978 transconjugants and transformants acquired both bla_{NDM-1} and *aphA6* as well as resistance to carbapenems and reduced susceptibility to kanamycin and amikacin, in agreement with MICs of the parental strain (Table 1). pNDM-JVAP01 was unstable in JVAP01, as it was spontaneously lost after 5 successive passages on blood agar plates in the absence of antibiotic pressure.

Genomic DNA from JVAP01 was used to sequence the pNDM-JVAP01 plasmid (paired-end 150-bp reads) in an Illumina MiSeq system, revealing the presence of a putative composite transposon Tn*125* bracketing the bla_{NDM-1}, ble_{MBL}, *trpF*, *dsbC*, *cutA1*, *groES*, *groEL*, and IS*CR21* array of genes (14). Overall, pNDM-JVAP01 is a molecule of 47,268 bp with a GC content of 40.8% also containing genes for a type IV secretion system presumably involved in plasmid conjugation and a Z toxin of unknown function. Interestingly, pNDM-JVAP01 displays 99.98% identity at the nucleotide level with pNDM-BJ01 (NC_019268) isolated from *Acinetobacter lwoffii* and more than 96% identity with NDM-harboring plasmids recovered from *A. lwoffii*, *Acinetobacter bereziniae*, and *A. pittii* (KJ547696, KF702385, and KJ003839, respectively), with only minor differences in the plasmid backbone sequences. The partial sequences of additional plasmids containing bla_{NDM-1} and recovered from *A. haemolyticus* (JQ080305) and *A. junii* (KJ018154) also show >99% identity with the nucleotide sequence of pNDM-JVAP01.

The appearance of several tentative novel species within the genus *Acinetobacter* makes accurate identification in routine diagnostic laboratories difficult, since phenotypic identification can only be considered presumptive. ITS and *recA* sequencing identification methods in this study provided inconclusive results, reflecting the limitation of methods that rely on the availability of curated databases (6). ARDRA analysis upon the inclusion of BfaI, however, highlighted the genetic relatedness between JVAP01 and *Acinetobacter* isolates tentatively belonging to a novel *Acinetobacter* species (NB14). Cluster analysis of *rpoB* sequences showed that JVAP01 formed a distinct monophyletic group that also included additional NB14 strains and that was closer to both *A. pittii* and *A. calcoaceticus* within the *A. calcoaceticus-A. baumannii* complex, providing further evidence that JVAP01 and NB14 strains represent a novel presumptive *Acinetobacter* species.

Genetic analysis of the JVAP01 strain revealed the common transposon Tn*125* structure carrying bla_{NDM-1}. Tn*125* has been described as being inserted both in the chromosome and on plasmids of *A. baumannii*; however, in non-*baumannii Acinetobacter* species, Tn*125* has been located exclusively on plasmids (5, 11, 12, 15–17). In JVAP01, Tn*125* was integrated within a transferable plasmid of approximately 50 kb, highly similar to bla_{NDM}-carrying plasmids recovered from non-*baumannii Acinetobacter* isolates and containing a type IV secretion system (5, 11, 15–17), suggesting the widespread nature of this plasmid and a role in bla_{NDM-1} transfer among different *Acinetobacter* species. This study reports the first identification of bla_{NDM-1} in a putatively novel *Acinetobacter* species which has been informally named NB14 and which is closely related to *A. pittii*, which has been recognized as the potential resistant reservoir for the dissemination of NDM-1, being found in food of animal origin and sewage and causing human infections and outbreaks in hospital units (18).

Nucleotide sequence accession numbers. The *rpoB* sequences of NB14 strains and the pNDM-JVAP01 sequence were submitted to GenBank with accession numbers KJ600793 (JVAP01), KJ600787 (LUH 07351), KJ600788 (LUH 13626), KJ600789 (RUH 53), KJ600790 (LUH 10243), KJ600791 (LUH 09407), KJ600794 (LUH 15040), and KM923969.1 (pNDM-JVAP01).

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We have no conflicts of interests to declare.

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