

Resistome and a Novel *bla*_{NDM-1}-Harboring Plasmid of an *Acinetobacter haemolyticus* Strain from a Children's Hospital in Puebla, Mexico

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Acinetobacter calcoaceticus-baumannii complex isolates have been frequently associated with hospital and community infections, with *A. baumannii* being the most common. Other *Acinetobacter* spp. not belonging to this complex also cause infections in hospital settings, and the incidence has increased over the past few years. Some species of the *Acinetobacter* genus possess a great diversity of antibiotic resistance mechanisms, such as efflux pumps, porins, and resistance genes that can be acquired and disseminated by mobilizable genetic elements. By means of whole-genome sequencing, we describe in the clinical *Acinetobacter haemolyticus* strain AN54 different mechanisms of resistance that involve *bla*_{OXA-265}, *bla*_{NDM-1}, *aphA6*, *aac(6')*-I_g, and a resistance-nodulation-cell division-type efflux pump. This strain carries six plasmids, of which the plasmid pAhaeAN54e contains *bla*_{NDM-1} in a Tn125-like transposon that is truncated at the 3' end. This strain also has an insertion sequence IS91 and seven genes encoding hypothetical proteins. The pAhaeAN54e plasmid is nontypable and different from other plasmids carrying *bla*_{NDM-1} that have been reported in Mexico and other countries. The presence of these kinds of plasmids in an opportunistic pathogen such as *A. haemolyticus* highlights the role that these plasmids play in the dissemination of antibiotic resistance genes, especially against carbapenems, in Mexican hospitals.

Keywords: *A. haemolyticus*, plasmid, NDM-1, antibiotic resistance

Introduction

THE GENUS *ACINETOBACTER* INCLUDES a group of bacteria that can be isolated from a wide variety of environmental sources, including soil and water. However, some of them have become important nosocomial pathogens, such as those included within the *Acinetobacter calcoaceticus-baumannii* complex. Members of this genus have been associated with severe nosocomial and community infections with high mortality rates. Nevertheless, isolates of other non-*baumannii* spp. have gained medical relevance because

of their increased frequency in recent years, for example, *Acinetobacter haemolyticus*, *Acinetobacter lwoffii*, *Acinetobacter ursingii*, *Acinetobacter parvus*, and *Acinetobacter junii*.^{1–3}

Acinetobacter spp. have developed resistance to multiple classes of antimicrobial agents, including broad-spectrum cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides. This resistance is due to multiple mechanisms, such as resistance-nodulation-cell division (RND)-type efflux pumps, CarO porin, and resistance genes. In addition, the ability of *Acinetobacter* spp. to acquire mobilizable

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elements that carry antibiotic resistance genes increases the resistance dissemination.^{4–6} In particular, *bla*_{NDM-1}, which encodes the New Delhi Metallo- β -lactamase-1 (NDM-1), hydrolyzes a broad spectrum of β -lactam antibiotics, including carbapenems, and is among the most worrisome resistance determinants that have spread around the world, severely complicating the treatment of nosocomial infections.^{7–10} In Latin America, its presence has been reported in Enterobacteriaceae isolates such as *Klebsiella pneumoniae*, *Escherichia coli*, and *Providencia rettgeri*,^{11–13} and in non-fermentative bacilli, such as *A. baumannii*, *Acinetobacter pittii*, *Acinetobacter bereziniae*, and *A. haemolyticus*.^{14–17} In this work, we report the presence of diverse antibiotic resistance genes in a strain of *A. haemolyticus* obtained from a Mexican pediatric patient and the characterization of the complete plasmid carrying *bla*_{NDM-1} isolated from this strain.

Materials and Methods

Bacterial isolation

Acinetobacter haemolyticus AN54 was recovered from peritoneal dialysis fluid culture from a 12-year-old male patient who had been admitted to the hospital for end-stage renal disease in February 2016. The patient was previously treated with ceftriaxone (CRO) and trimethoprim-sulfamethoxazole (SXT). The isolate was identified with the VITEK 2 system (bioMérieux) and molecular typing by sequencing of the *rpoB* gene.^{18,19}

Antimicrobial susceptibility testing

An antimicrobial susceptibility test was performed by using the agar disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines.²⁰ The following antimicrobials were tested: piperacillin (PIP), ticarcillin (TIC), ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin-clavulanic acid (TIM), ceftazidime (CAZ), cefepime (FEP), cefotaxime (CTX), CRO, imipenem (IPM), meropenem (MEM), gentamicin, amikacin (AN), tetracycline, ciprofloxacin, levofloxacin, and SXT. The minimal inhibitory concentration (MIC) for CTX, CAZ, FEP, MEM, IPM, and AN was determined by the agar dilution method.²⁰ Metallo- β -lactamase (MBL) detection was performed by IPM and MEM disks supplemented with 10 μ L of 0.5 M ethylenediaminetetraacetic acid.²¹

Test of the activity of the efflux pump in antibiotic resistance

The activity of the efflux pump was evaluated by using phenylalanine-arginine β -naphthylamide (Sigma-Aldrich) as an efflux pump inhibitor (EPI). The test was performed as follows: MICs for AN, CTX, and MEM were determined by the agar dilution method in the presence and absence of EPI (25 mg/L). A twofold or greater decrease in MIC in the presence of EPI was considered indicative of a role of RND-type efflux pumps in the resistance to the antibiotics tested. Test veracity was checked by using the strain *Acinetobacter haemolyticus* HNP11 as a positive control. To evaluate the effect of EPI on bacterial growth, all bacteria were cultured in Mueller–Hinton broth with and without EPI (25 mg/L).²²

Whole-genome sequencing and data analysis

A high-quality draft genome sequence from isolate AN54 was obtained by using an Illumina MiSeq platform (2 \times 300 paired-end reads) (IBT-UNAM) and one SMRT cell of PacBio RS II system (Yale Center for Genome Analysis). With data obtained from both platforms, a hybrid assembly was performed with the Unicycler assembler version 0.4.1.²³ and SPAdes version 3.11.1.²⁴ ResFinder 2.1²⁵ was used to identify and determine the location of antibiotic resistance genes. MAUVE version 20150226,²⁶ CLC Sequence Viewer 8.0 and BLAST were used to align and compare sequences. EASYFIG 2.2.2 was used to draw figures.²⁷

Pulsed-field gel electrophoresis and Southern blot

The S1 nuclease-pulsed-field gel electrophoresis (PFGE) method was carried out to determine the plasmids number by using the *Escherichia coli* strain NCTC 50192 as a reference. To detect the resistance gene in the plasmid, the PFGE gel was transferred to a nylon membrane (Hybond-N; GE Healthcare Life Sciences), and hybridization was performed with the Dig-High Prime DNA Labeling and Detection Starter Kit II (Roche).

Conjugation assays

Conjugation assays from AN54 to recipient strains *Escherichia coli* C600 (rifampicin resistant) and *Escherichia coli* DH5 α (nalidixic acid resistant) were performed. Mueller–Hinton agar plates (BD Bioxon) supplemented with rifampicin (100 μ g/mL) or nalidixic acid (32 μ g/mL) containing AN (32 μ g/mL) and MEM (8 μ g/mL) were used for the selection of transconjugant strains.

Nucleotide sequence accession numbers. Draft genome and plasmid sequences of the AN54 strain were deposited in the GenBank database, with accession numbers CP041224.1 to CP041229.1.

Results

In this work, we report the identification of an *Acinetobacter haemolyticus* (AN54) strain resistant to carbapenems, which was isolated from peritoneal dialysis fluid. This strain was initially identified as *Acinetobacter* spp. by a VITEK 2 System and subsequently reclassified as *A. haemolyticus* by analysis of the *rpoB* gene. The AN54 strain exhibited resistance to AN, and to the broad-spectrum β -lactams antibiotics, except for TIM. The *bla*_{NDM-1} and *bla*_{OXA-265} were previously detected by PCR and sequencing. A twofold decrease in the MIC for AN and no change in the MICs for CTX and MEM in the presence of EPI were observed. In addition, the phenotypic test to detect MBL production was positive (Table 1).

Plasmid DNA extraction revealed that the AN54 isolate carries six different bands. Southern blot hybridization indicated that *bla*_{NDM-1} was present in one of the plasmids (Fig. 1).

Whole-genome sequencing analysis revealed that AN54 possess a chromosome with a size of \sim 3.61 Mbp, and we obtained the complete sequence of four plasmids named

TABLE 1. ANTIMICROBIAL SUSCEPTIBILITIES TEST AND GENOTYPE OF *ACINETOBACTER HAEMOLYTICUS* AN54

Strain	Anatomical site	Resistance phenotype	MIC ($\mu\text{g}/\text{mL}$)						Genotype ^a	Efflux pumps genes ^a
			CAZ	FEP	IPM	CTX + EPI	MEM + EPI	AN + EPI		
AN54	Peritoneal dialysis fluid	PIP, TIC, SAM, TZP, CAZ, FEP, CTX, CRO, IPM, MEM, AN	>128	>128	>128	>128 (>128)	>128 (>128)	64 (32)	<i>bla</i> _{NDM-1} , <i>bla</i> _{OXA-265} , <i>aphA6</i> , <i>aac</i> -(6')-Ig	<i>adeA</i> , <i>adeB</i> , <i>adeC</i> , <i>adeI</i> , <i>adeK</i> , <i>adeS</i> , <i>adeR</i> , <i>macA</i> , <i>macB</i>

Control strain *Acinetobacter haemolyticus* HNP11 MIC values: CTX: 16 $\mu\text{g}/\text{mL}$, CTX + EPI: 8 $\mu\text{g}/\text{mL}$, AN: 64 $\mu\text{g}/\text{mL}$, AN + EPI: 32 $\mu\text{g}/\text{mL}$.

^aObtained by PCR and whole-genome sequencing analysis.

^bRegulators of AdeABC efflux pump.

AN, amikacin; CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; EPI, efflux pumps inhibitor (phenylalanine-arginine- β -naphthylamide) (PA β N) 25 mg/L; FEP, cefepime; IPM, imipenem; MEM, meropenem; MIC, minimal inhibitory concentration; PIP, piperacillin; SAM, ampicillin/sulbactam; TIC, ticarcillin; TZP, piperacillin/tazobactam.

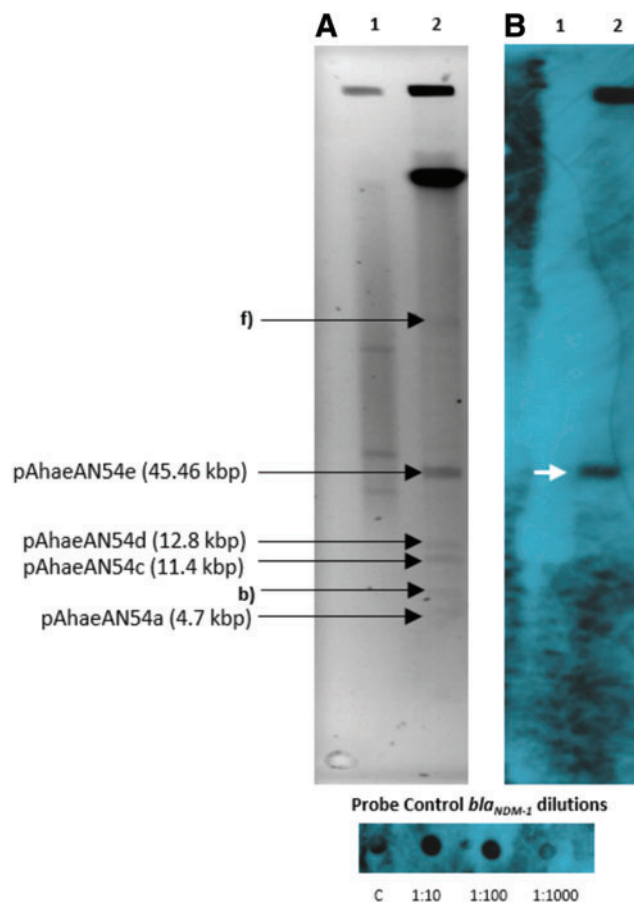


FIG. 1. (A) PFGE-S1 gel showing the plasmids of *Acinetobacter haemolyticus* AN54 strain, Line 1: Control strain *Escherichia coli* NCTC 50192, Line 2: AN54. (B) Hybridization autoradiography with the *bla*_{NDM-1} probe (DIG-High Prime DNA Labeling and Detection Starter Kit II, Roche). The white arrow indicates that *bla*_{NDM-1} was detected in the plasmid pAhaeAN54e. The plasmid sizes were determined by WGS. PFGE, pulsed-field gel electrophoresis. Color images are available online.

pAhaeAN54a (4.7 kbp), pAhaeAN54c (11.4 kbp), and pAhaeAN54d (12.8 kbp) that do not carry antimicrobial resistance genes, and pAhaeAN54e (45.46 kbp) that carries *bla*_{NDM-1} and *aphA6*. In the chromosome, we detected the presence of *bla*_{OXA-265} and the aminoglycoside-modifying enzyme-encoding gene *aac*-(6')-Ig. In addition, genes for efflux pumps and their regulators that mediate multidrug resistance were found; *adeA*, *adeB*, *adeC*, *adeI*, *adeK*, *adeR*, *adeS*, *macA*, and *macB*, as well as heavy metal resistance genes *czcA* and *arsH*.

The *bla*_{NDM-1} was located in the 45.46 kbp plasmid, which we named pAhaeAN54e. This plasmid has a 41% guanine-cytosine content and 53 open reading frames. Sixteen of these genes are related to plasmid maintenance and transfer functions (plasmid backbone) and include the plasmid-partitioning genes *parA* and *parB*, and transfer genes *traA*, *traC*, and *traD*, as well as some genes of the type IV secretion system (T4SS). Twelve genes of this plasmid are located within the composite transposon carrying *bla*_{NDM-1}, and the remaining genes encode 25 hypothetical

proteins. Conjugation assays were performed to test the transferability of this plasmid; however, no transconjugants were obtained under the tested conditions.

Plasmid pAhaeAN54e has 99% nucleotide identity, with 100% coverage, to plasmids pNDM-BJ02 (accession no. JQ060896.1) and pNDM-BJ01 (accession no. JQ001791.1) of *A. lwoffii*.²⁸ Figure 2 shows the detected differences, especially the insertion of seven genes encoding hypothetical proteins along the T4SS gene cluster, near the *parA* and *traA* genes, and a deletion of a hypothetical protein upstream of *traC*. The pAhaeAN54e plasmid also has a truncated composite transposon that is similar to transposon Tn125. Both these transposons consist of the IS*Aba14* insertion sequence and the *aphA6*, *bla*_{NDM-1}, *ble*, and *trpF* genes, but in contrast to Tn125, the truncated transposon of strain AN54 has only one copy of the insertion sequence IS*Aba125*. The structure of the truncated transposon is similar to that found in plasmid pNDM-BJ02 and to the partially sequenced plasmids pABC7926 (accession no. JQ080305.2) and pNDM-69122 (accession no. LN611576.1) of *A. haemolyticus* (Fig. 3).^{29,30}

Plasmid pAhaeAN54e contains an IS*91* family transposase, which is 330 bp larger than the *insE* transposase gene carried on the pNDM-BJ02 and pNDM-BJ01 plasmids in *A. lwoffii*. In addition, pAhaeAN54e has an insertion of two genes encoding hypothetical proteins downstream of IS*91* and one more that is adjacent to the recombinase gene.

Interestingly, plasmid pAhaeAN54e has 99% identity with more than 73% of coverage with the *P. rettgeri* plasmid p06-1619-NDM (accession no. KX832928.1) reported in Mexico (Fig. 4).³¹ These plasmids share 33 genes, including *traA*, *traC*, *traD*, and genes from the type IV secretion system. Plasmid p06-1619-NDM also has a Tn125-like element.

Discussion

A. baumannii is one of the principal etiological agents causing nosocomial infections in Mexico and the rest of the world.³²⁻³⁴ In Mexican hospitals, there are a few reports of carbapenem-resistant *Acinetobacter* isolates that are not included in the *A. calcoaceticus-baumannii* complex,¹⁷ possibly because the molecular typing of these species is not routinely implemented in Mexican hospitals. In this work, we studied the resistome of an *A. haemolyticus* strain resistant to carbapenems. The strain carries different resistance mechanisms, such as *bla*_{OXA-265}, which is a member of the *bla*_{OXA-214}-like family on the *A. haemolyticus* chromosome.³⁵ The strain also harbors aminoglycoside-modifying enzyme genes, such as *aac(6')*-I_g, which is responsible for AN resistance and belongs to the AAC(6')-I aminoglycoside N-acetyltransferase family reported in *Acinetobacter* spp.,^{36,37} and *aphA6*, which confers resistance to AN and is associated with the presence of *bla*_{NDM-1} in a plasmid from this strain, as has been reported in another study.³⁸

Another antibiotic resistance mechanism studied in *A. baumannii* is the use of efflux pumps, mainly the RND family of efflux pumps, such as the AdeABC and AdeIJK systems, which are located on the bacterial chromosome. This family exhibits a wide substrate range that includes dyes, biocides, detergents, and antiseptics; however, its presence has been little studied in non-*baumannii* spp.³⁹ The

overexpression of this system was shown to be responsible for decreasing susceptibility to a broad spectrum of antimicrobials, such as aminoglycosides, tetracyclines, erythromycin, chloramphenicol, trimethoprim, fluoroquinolones, some β -lactams, and ethidium bromide, and has recently also been associated with tigecycline.⁴⁰ The genes that encode the AdeABC efflux pump are organized in an operon (*adeABC*). There are two regulatory genes, *adeS* and *adeR*, and their products are closely related to proteins of the two-component regulatory system. These genes regulate efflux pump expression in response to cellular environment stimuli (antibiotics). This type of expression is called inductive.⁴¹

AdeIJK encoded by the *adeIJK* operon is the second RND efflux system described in *A. baumannii*,⁴² which contributes to the resistance to β -lactams, such as TIC, cephalosporins, aztreonam, fluoroquinolones, tetracyclines, tigecycline, lincosamides, rifampicin, and chloramphenicol; however, aminoglycosides are not substrates for this pump.⁴³ The *Acinetobacter haemolyticus* AN54 strain contains homologues of the regulator proteins AdeS and AdeR and the efflux pump components AdeA, AdeB and AdeC with the *Acinetobacter baumannii* AYE strain, these proteins share an amino acid identity of 72%, 85%, 86%, 92% and 77% respectively, to the corresponding proteins. In addition, homologues of AdeI and AdeK proteins with 82% and 89% identity with *Acinetobacter baumannii* AYE were also found. However, the AdeJ subunit in strain AN54 was not located.

In this work, EPI was used to evaluate the role of the efflux pump in antibiotic resistance. The results obtained indicate that the efflux pump only participates in conferring resistance to AN, as shown by a twofold decrease in the MIC (from 64 to 32 $\mu\text{g}/\text{mL}$). The strain did not show a decrease in the MICs of CTX and MEM, possibly due to the presence of *bla*_{NDM-1}.⁶

The presence of *bla*_{NDM-1} in a non-*baumannii* spp. of environmental origin such as *A. haemolyticus* is medically and epidemiologically relevant. This is especially true considering the lack of successful treatment for patients with some underlying diseases and the ease of *bla*_{NDM-1} dissemination through complex recombination events mediated by insertion sequences, transposons, and plasmids, as reported in previous works.^{31,44,45}

The *Acinetobacter haemolyticus* AN54 strain harbors *bla*_{NDM-1} within a Tn125-like transposon in a plasmid highly similar to pNDM-BJ02 of *A. lwoffii*, which has been previously reported in China.²⁸ The backbone of the plasmids carrying *bla*_{NDM-1} between *Acinetobacter* spp. is relatively conserved; however, one difference found in our plasmid is the presence of IS*91*. This gene is designated as ISCR, and one of its functions is the mobilization of additional sequences upstream of the transposase gene.^{46,47} The second difference is the insertion of seven genes encoding hypothetical proteins in the putative conjugation region; the function of these proteins is yet unknown. Currently, there are no methodologies to characterize this kind of replicon, due to the absence of a typical Rep protein, such as the one presented by Enterobacteriaceae plasmids, indicating that these plasmids possess an uncharacterized replication system; therefore, additional studies are needed. No transconjugants were obtained under the tested conditions. However, we cannot exclude other alternative mechanisms of plasmid

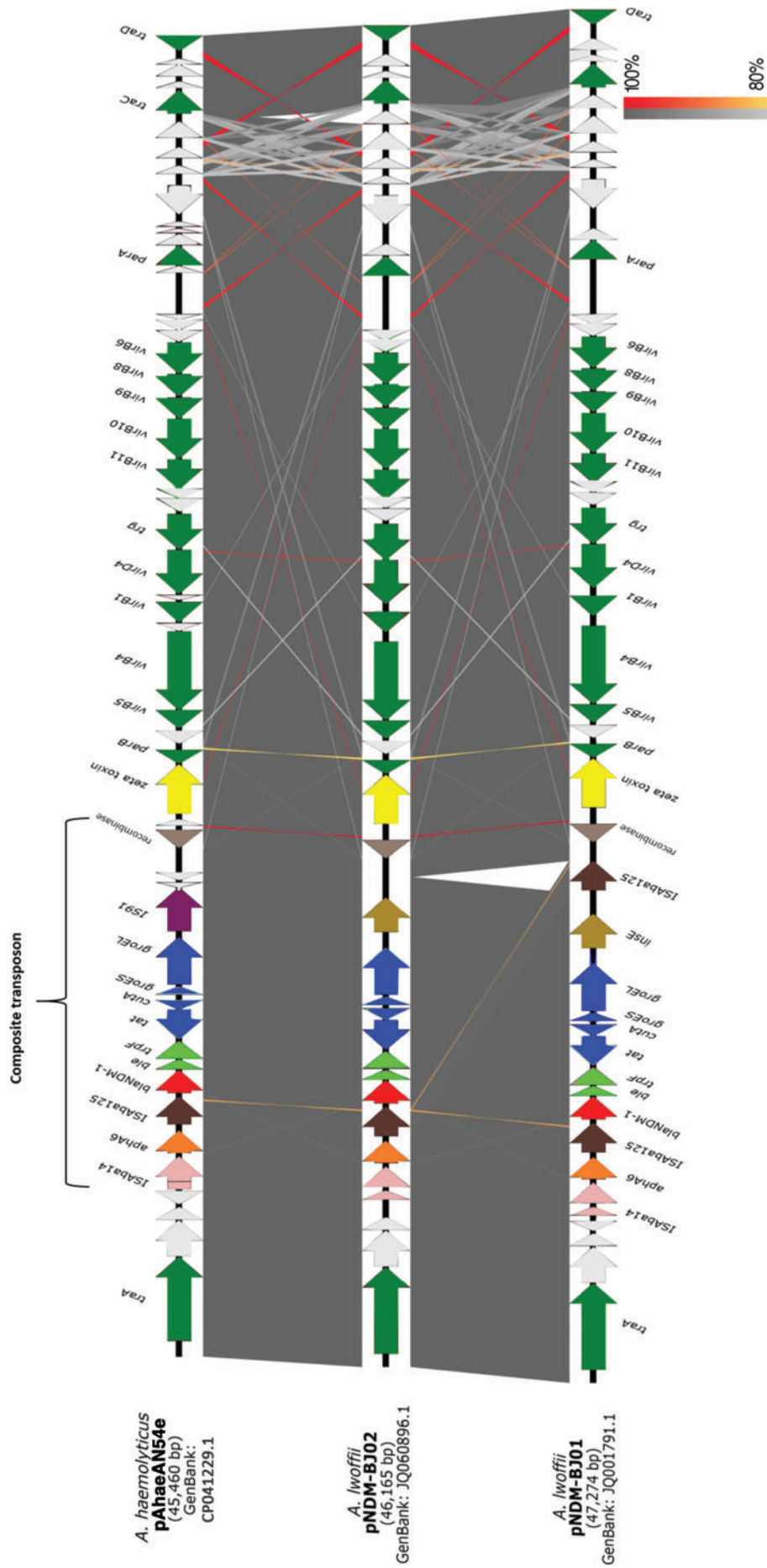


FIG. 2. The schematic representation and alignment of plasmid pAhaeAN54e of *Acinetobacter haemolyticus* and plasmids pNDM-BJ02 and pNDM-BJ01 of *Acinetobacter Iwoffii* are divided into two sections: the composite transposon in which *ISAbal25*, *blaNDM-1*, and *IS91* are inserted and the putative conjugation machinery. The known genes are marked in *green*, and putative proteins are marked in *white*. The *gray bars* indicate 99% identity, and the inverted regions are indicated in *red*. Color images are available online.

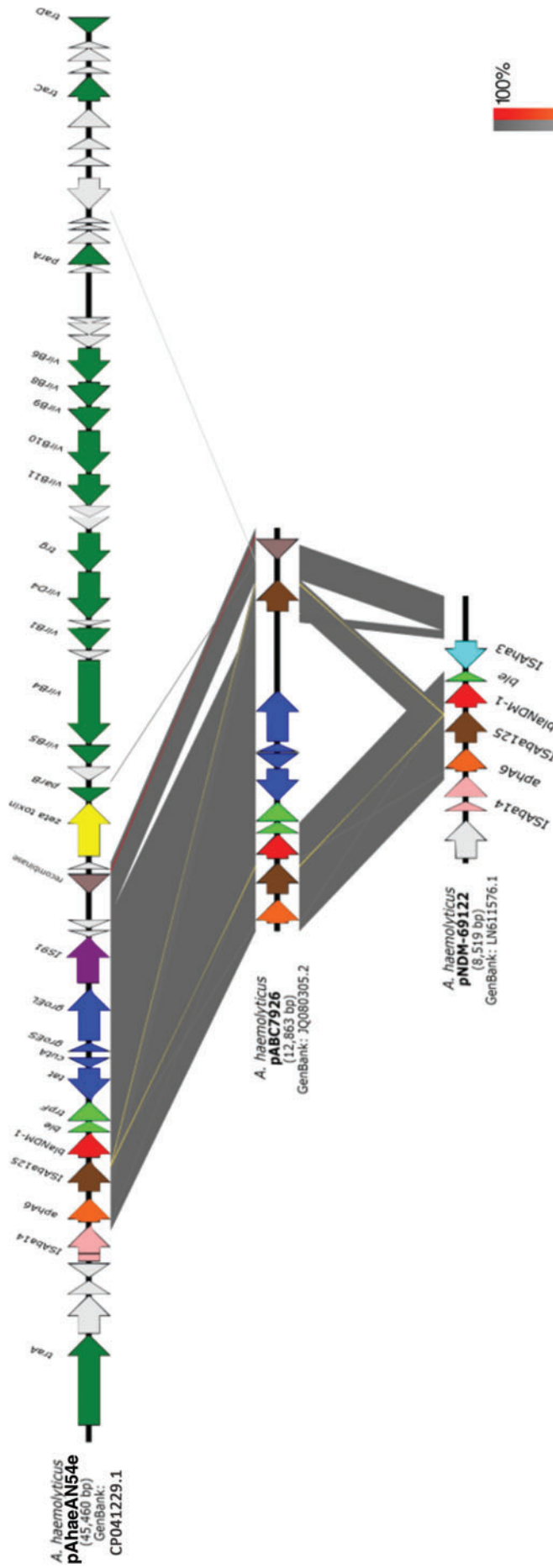


FIG. 3. Comparison between the plasmid pAhaeAN54e and the partially sequenced plasmids pABC7926 and pNDM69122 of *Acinetobacter haemolyticus*. Color images are available online.

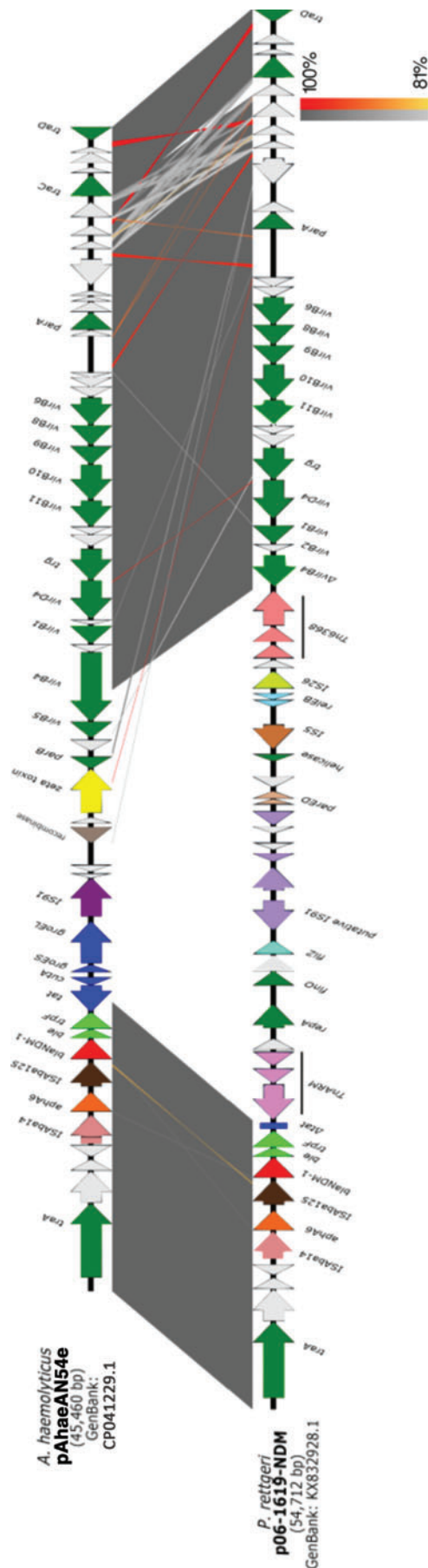


FIG. 4. Comparison between plasmids pAhaeAN54e of *Acinetobacter haemolyticus* and p06-1619-NDM of *Providencia rettgeri*, both of which are isolated from México. Color images are available online.

transference, as reported in other studies of *Acinetobacter* spp. carrying bla_{NDM-1} .^{14,15,38,48}

Recently, Duran-Bedolla *et al.*¹⁷ partially reported the genetic context of bla_{NDM-1} in strains of *Acinetobacter* spp., including the *Acinetobacter haemolyticus* 10256 strain isolated in a different geographical area of Mexico. The plasmid reported in our work showed specific differences in comparison with the plasmid of *Acinetobacter haemolyticus* 10256 (55 kbp plasmid); these included size (45.4 kbp), absence of ISAb125 sequence in the 3' end, presence of genes encoding hypothetical proteins along the structure of the plasmid, and IS91 instead of ISCR27. These results suggest that the genetic context of bla_{NDM-1} in *A. haemolyticus* is partially conserved.^{17,29,30}

Our study shows that plasmid pAhaeAN54e has a high similarity with plasmid p06-1619-NDM of *P. rettgeri* and, as noted by Marquez-Ortiz *et al.*,³¹ the similarities among pNDM-BJ01-like plasmids and plasmids of *P. rettgeri* may be the result of recombination events that lead to a chimeric plasmid that can be transmitted and replicated among Enterobacteriaceae and *Acinetobacter* isolates.⁴⁹

Concluding Remarks

In this study, we report a multidrug-resistant clinical *A. haemolyticus* strain isolated from a pediatric patient carrying bla_{NDM-1} in a novel variant of pNDM-BJ01-like plasmids. This plasmid could constitute a dissemination mechanism of antimicrobial resistance genes among diverse bacterial species. The findings reported in this work contribute to providing information about the diverse mechanisms of resistance that can coexist in *A. haemolyticus* strains.

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Ethical Statement

Acinetobacter haemolyticus AN54 isolate was collected during routine sampling, and patient data were anonymized. The protocol for this study was approved by the Ethical Committee of Hospital number: HNP/ENS/177/2016.

Disclosure Statement

No competing financial interests exist.

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