Antibiotic resistance mechanisms in *Acinetobacter* spp. strains isolated from patients in a paediatric hospital in Mexico

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ABSTRACT

Objectives: The aim of this study was to identify *Acinetobacter* spp. strains from paediatric patients, to determine their genetic relationship, to detect antibiotic resistance genes and to evaluate the role of efflux pumps in antibiotic resistance.
Methods: A total of 54 non-duplicate, non-consecutive *Acinetobacter* spp. isolates were collected from paediatric patients. Their genetic relationship, antibiotic resistance profile, efflux pump activity, antibiotic resistance genes and plasmid profile were determined.
Results: The isolates were identified as 24 *Acinetobacter haemolyticus*, 24 *Acinetobacter calcoaceticus–baumannii* (Acb) complex and 1 strain each of *Acinetobacter junii*, *Acinetobacter radioresistens*, *Acinetobacter indicus*, *Acinetobacter lwoffii*, *Acinetobacter ursingii* and *Acinetobacter venetianus*. The 24 *A. haemolyticus* were considered genetically unrelated. One strain was resistant to carbapenems, two to cephalosporins, two to ciprofloxacin and sixteen to aminoglycosides. The antibiotic resistance genes $bla_{OXA-214}$ (29%), $bla_{OXA-215}$ (4%), $bla_{OXA-264}$ (8%), $bla_{OXA-265}$ (29%), bla_{NDM-1} (4%), $aac(6')-Ig$ (38%) and the novel variants $bla_{OXA-575}$ (13%), $bla_{TEM-229}$ (75%), $aac(6')-Iga$ (4%), $aac(6')-Igb$ (13%) and $aac(6')-Igc$ (42%) were detected. Among 24 Acb complex, 5 were multidrug-resistant, carbapenem-resistant strains carrying bla_{OXA-51} and bla_{OXA-23}; they were genetically related and had the same plasmid profile. Other species were susceptible. In some strains of *A. haemolyticus* and Acb complex, the role of RND efflux pumps was evidenced by a decrease in the MICs for cefotaxime, amikacin and ciprofloxacin in the presence of an efflux pump inhibitor.
Conclusions: This study identified isolates of *A. haemolyticus* carrying new β-lactamase variants and shows for the first time the contribution of efflux pumps to antibiotic resistance in this species.

1. Introduction

Antibiotic-resistant bacteria currently pose a high risk in the infant population and are a major challenge in paediatric hospitals. Treatment of these patients is more complicated because the selection of antibiotics to treat infections is more limited than for adults [1,2]. One of the main hospital pathogens worldwide is carbapenem-resistant *Acinetobacter baumannii,* which is considered by the World Health Organization (WHO) as one of the critical priority pathogens. However, other members of this bacterial genus have become clinically important, especially in immunocompromised patients who suffer burns, trauma or who are in intensive care units (ICUs) [3].

There are extensive studies about the mechanisms of resistance in *A. baumannii,* including extended-spectrum β-lactamases (ESBLs), carbapenemases, efflux pumps and decreased

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permeability; however, these studies are scarce for other members of this genus [4]. Studies on the mechanisms of resistance in species such as *Acinetobacter johnsonii*, *Acinetobacter calcoaceticus*, *Acinetobacter haemolyticus*, *Acinetobacter radioresistens*, *Acinetobacter lwoffii* and *Acinetobacter bereziniae* have reported the presence of some chromosomal OXA-type β-lactamases, ESBLs, metallo-β-lactamases (MBLs) and aminoglycoside-modifying enzymes (AMEs), but it is still necessary to study other mechanisms involved [5]. In this context, we studied a collection of *Acinetobacter* spp. strains isolated from paediatric patients by analysing different mechanisms of resistance and the contribution of efflux pumps to the antibiotic-resistant phenotype.

2. Materials and methods

2.1. Bacterial isolation and typing

A collection of 54 *Acinetobacter* spp. isolates was obtained from samples collected during routine diagnosis of paediatric patients at Hospital para el Niño Poblano (Puebla, Mexico) from April 2010 to July 2017. The strains were selected according to the following criteria: (i) non-duplicate samples and not isolated consecutively; (ii) belonging to different patients; and (iii) showing resistance to at least one antibiotic tested.

All isolates were identified by the VITEK®2 system (bioMérieux), the sequence of the *rpoB* gene, and detection of $bla_{OXA-51-like}$ and $bla_{OXA-214-like}$ genes, which can support the presumptive species identification [6]. Multiple sequence alignment for molecular typing was performed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). A dendrogram was constructed using MEGA v.7 software [7].

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the agar disk diffusion method for a total of 16 antibiotics, including piperacillin, ampicillin/sulbactam, piperacillin/tazobactam (TZP), ticarcillin/clavulanic acid, ceftazidime, cefepime, cefotaxime, ceftriaxone, imipenem, meropenem, gentamicin, amikacin, tetracycline, ciprofloxacin, levofloxacin and trimethoprim/sulfamethoxazole (SXT) (BBL™ Sensi-Disc™). Minimum inhibitory concentrations (MICs) were determined by the agar microdilution method for cefotaxime, ceftriaxone, imipenem, meropenem, gentamicin, amikacin and ciprofloxacin. Both methods are described in the Clinical and Laboratory Standards Institute (CLSI) guidelines [8].

2.3. Effect of efflux pump inhibitor (EPI) on the minimum inhibitory concentrations of cefotaxime, meropenem, amikacin and ciprofloxacin

The EPI phenylalanine-arginine β-naphthylamide (PAβN) (Sigma-Aldrich) was used to determine the contribution of the resistance–nodulation–cell division (RND) efflux pump family to antibiotic resistance. Strains were selected according to their resistance phenotype. MICs of cefotaxime, meropenem, amikacin and ciprofloxacin were determined by the agar dilution method in the presence and absence of PAβN (25 mg/L). A decrease of ≥2-fold in the MIC in the presence of PAβN was considered as positive for the role of RND-type efflux pumps in resistance to these antibiotics. *Pseudomonas aeruginosa* PAO1 (negative control) and *A. haemolyticus* AN54 (positive control) were used as control strains [9,10].

2.4. Pulsed-field gel electrophoresis (PFGE)

The clonal relationship of *A. haemolyticus* and carbapenem-resistant *A. calcoaceticus–baumannii* (Acb) complex isolates was determinate by PFGE using the restriction enzyme *Apa*I [11], but with a modification in the running time of 21 h. *Acinetobacter haemolyticus* 11616 and *A. baumannii* Ab23 were used as the respective reference strains. Photographic images of the gels were saved digitally with MiniBIS Pro-DNR Bio-Imaging Systems and analysed with GelQuant Express Analysis Software. Comparison and analysis of the band patterns were performed with NTSYS pc 2.2. software using the Dice coefficient/UPGMA to determine strain relationships based on the criteria established by Tenover et al. [12].

2.5. Detection of antimicrobial resistance genes

Genes conferring resistance to β-lactams (bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, bla_{OXA-58}, $bla_{OXA-214}$, bla_{CTX-M}, bla_{SHV}, bla_{TEM}, bla_{GES}, bla_{PER}, bla_{BEL}, bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SPM} and bla_{NDM}) and aminoglycosides [*aac(6´)-I*] were identified by PCR and Sanger sequencing (Supplementary Table S1). Determination of new variants was carried out by translation to amino acids and subsequent comparison with those reported in GenBank.

2.6. Extraction of plasmids from carbapenem-resistant Acinetobacter calcoaceticus–baumannii complex isolates

Plasmid extraction was performed using QuickPrep methodology [13]. *Escherichia coli* NCTC 50192 harbouring four plasmids (154, 66, 38 and 7 kb) was used as a control strain.

3. Results

3.1. Clinical isolates

A total of 54 *Acinetobacter* spp. were isolated from paediatric patients. One sample per patient (35 female and 19 male) was obtained, mainly admitted to the internal medicine ward and emergency unit. The age of the patients ranged between 3 days and 17 years. The most frequent sources of isolation were peritoneal dialysis fluid and wounds (Table 1).

Table 1
Percentage of strains by hospital ward and site of isolation from paediatric patients (*n* = 54).

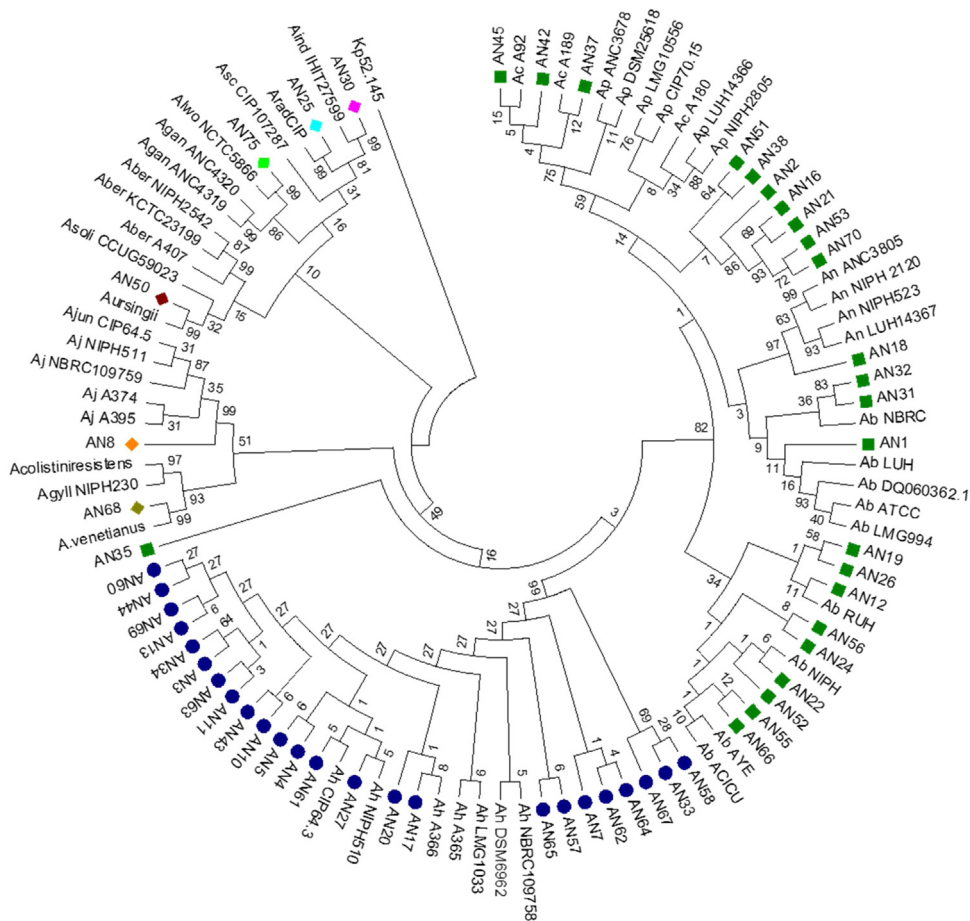| Hospital ward	No. (%) of isolates	Isolation site	No. (%) of isolates
Internal medicine	26 (48.1)	Peritoneal dialysis fluid	35 (64.8)
Infectiology	1 (1.9)	Ulcer	1 (1.9)
Emergency unit	16 (29.6)	Abdominal abscess	2 (3.7)
Surgical	3 (5.6)	Vulvar exudate	1 (1.9)
Haematology	1 (1.9)	Biopsy	5 (9.3)
Oncology	1 (1.9)	Wound	10 (18.5)
ICU	1 (1.9)		
Burn unit	5 (9.3)		

ICU, intensive care unit.

Fig. 1. Dendrogram representing the phylogenetic relationship of the 54 typed *Acinetobacter* spp. strains derived from the partial sequence of the *rpoB* gene. Green squares represent members of the *Acinetobacter calcoaceticus–baumannii* complex, blue circles represent *Acinetobacter haemolyticus* and coloured rhombuses represent different *Acinetobacter* spp. Bootstrap consensus tree by the neighbour-joining method inferred from 100 replicates conducted in MEGA7 software. Reference strains: Ab, *A. baumannii* (NIPH 1734, KJ956460.1; RUH 134, HQ123410.2; ACICU, NC 010611.1; AYE, CU459141.1; NBRC 109757, LC102671.1; LUH 4722, HQ123415.1; ATCC 17904, HQ123408.1; LMG994, EF611385.1; DQ060362.1); Aber, *A. bereziniae* (A407, KJ789097.1; KCTC 23199, LC102674.1; NIPH 2542, FJ754460.2); Ac, *A. calcoaceticus* (A180, KJ788881.1; A189, KJ788890.1; A92, KJ788793.1); *A. colistiniresistens* (NIPH 1861, KY458050.1); Agan, *A. gandensis* (ANC 4319, KJ569690.1; ANC 4320, KJ569691.1); Agyll, *A. gyllenbergii* (NIPH 230, NZ KI530704.1); Ah, *A. haemolyticus* (A365, KJ789055.1; NBRC 109758, LC102681.1; LMG1033, EF611392.1; A366, KJ789056.1; NIPH 510, EU477110.2; CIP 64.3, NZ KB849803.1; DSM6962, EF611391.1); Aind, *A. indicus* (IHIT27599, KU507500.1); Aj, *A. junii* (NIPH 511, EU477110.2; CIP 64.5, DQ207486.1; NBRC 109759, LC102684.1; A395, KJ789085.1; A374, KJ789064.1); Alwo, *A. lwoffii* (NCTC 5866, NZ KB851227.1); An, *A. nosocomialis* (ANC 3805, HQ123402.1; NIPH 2120, KJ956466.1; NIPH 523, EU477118.2; LUH 14367, HQ123406.1); Ap, *A. pittii* (LUH 14366, HQ123387.1; NIPH 2805, HQ123381.1; LMG 10556, HQ123380.1; CIP 70.15, DQ207479.1; DSM 25618, LC102689.1; ANC 3678, HQ123388.1); Arad, *A. radioresistens* (CIP 103788, NZ KB849747.1); Asc, *A. schindleri* (CIP 107287, NZ KB849574.1); Asoli, *A. soli* (CCUG 59023, HQ148175.1); Aurs, *A. ursingii* (NIPH3649, EU742582.1); *A. venetianus:* (NIPH 2310, EU496381.2); Kp52.145, *Klebsiella pneumoniae* (Kp52.145, FO834906.1).

The 54 *Acinetobacter* spp. corresponded to 24 *A. haemolyticus*, 24 Acb complex and 1 strain each of *Acinetobacter junii*, *A. radioresistens*, *Acinetobacter indicus*, *A. lwoffii*, *Acinetobacter ursingii* and *Acinetobacter venetianus* (Fig. 1).

Of the 54 *Acinetobacter* spp. strains, 23 were isolated with other micro-organisms such as *Klebsiella pneumoniae*, *E. coli*, *P. aeruginosa*, *Enterococcus faecium* and *Staphylococcus* spp., among others. The remaining 31 strains were obtained in pure culture, of which 15 were *A. haemolyticus*, 13 Acb complex and 3 other *Acinetobacter* spp.

3.2. Antibiotic resistance profiles of Acinetobacter spp. Strains

In the susceptibility profile by species, we observed that all *A. haemolyticus* strains showed resistance to β-lactams between 4–13% towards piperacillin, ticarcillin, TZP, ceftazidime, cefepime, cefotaxime and ceftriaxone, including carbapenems (imipenem and meropenem). These strains also showed resistance to aminoglycosides, mainly to amikacin (67%), as well as to tetracycline (4%), ciprofloxacin (8%) and SXT (21%) (Fig. 2).

In contrast to *A. haemolyticus*, the 24 Acb complex strains had a notable increase in the percentage of resistance towards different families of antibiotics. They showed 21–46% resistance towards β-lactams, including cephalosporins (mainly cefotaxime and ceftriaxone) and carbapenems. Moreover, 29% of the strains showed resistance to amikacin, 25% to tetracycline, 29% to fluoroquinolones (ciprofloxacin and levofloxacin) and 33% to SXT (Fig. 3). The other six *Acinetobacter* spp. were susceptible to the antibiotics tested; only 17% of the strains showed resistance to levofloxacin (Fig. 4).

3.3. Clonal relationship of the Acinetobacter haemolyticus and carbapenem-resistant Acinetobacter calcoaceticus–baumannii strains

PFGE results for the group of *A. haemolyticus* strains showed 20 different PFGE pulsotypes, therefore the isolates were non-related. Strains AN33, AN34, AN58 and AN69 were non-typeable under the conditions tested (Fig. 5).

The clonal relationship of five carbapenem-resistant Acb complex strains was determined due to the phenotype they presented and the fact that were isolated from the burns unit, which is an independent building of the hospital. Four strains were
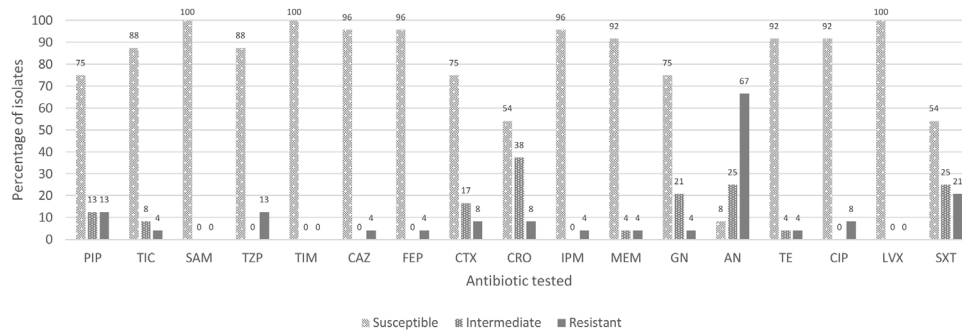
Fig. 2. Percentages of antibiotic susceptibility in *Acinetobacter haemolyticus* isolates (*n* = 24) determined by the Kirby–Bauer method [8]. PIP, piperacillin; TIC, ticarcillin; SAM, ampicillin/sulbactam; TZP, piperacillin/tazobactam; TIM, ticarcillin/clavulanic acid; CAZ, ceftazidime; FEP, cefepime; CTX, cefotaxime; CRO, ceftriaxone; IPM, imipenem; MEM, meropenem; GN, gentamicin; AN, amikacin; TE, tetracycline; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulfamethoxazole.

Fig. 3. Percentages of antibiotic susceptibility in *Acinetobacter calcoaceticus–baumannii* complex isolates (*n* = 24) determined by the Kirby–Bauer method [8]. PIP, piperacillin; TIC, ticarcillin; SAM, ampicillin/sulbactam; TZP, piperacillin/tazobactam; TIM, ticarcillin/clavulanic acid; CAZ, ceftazidime; FEP, cefepime; CTX, cefotaxime; CRO, ceftriaxone; IPM, imipenem; MEM, meropenem; GN, gentamicin; AN, amikacin; TE, tetracycline; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulfamethoxazole.
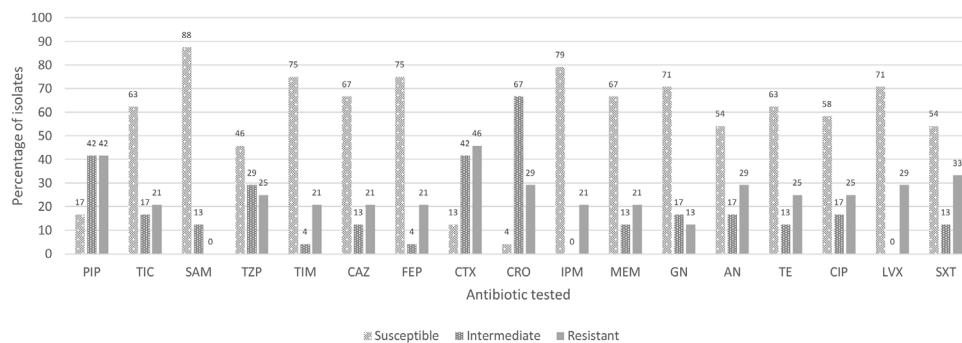

Fig. 4. Percentages of antibiotic susceptibility in *Acinetobacter* spp. isolates (*n* = 6) determined by the Kirby–Bauer method [8]. PIP, piperacillin; TIC, ticarcillin; SAM, ampicillin/sulbactam; TZP, piperacillin/tazobactam; TIM, ticarcillin/clavulanic acid; CAZ, ceftazidime; FEP, cefepime; CTX, cefotaxime; CRO, ceftriaxone; IPM, imipenem; MEM, meropenem; GN, gentamicin; AN, amikacin; TE, tetracycline; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulfamethoxazole.
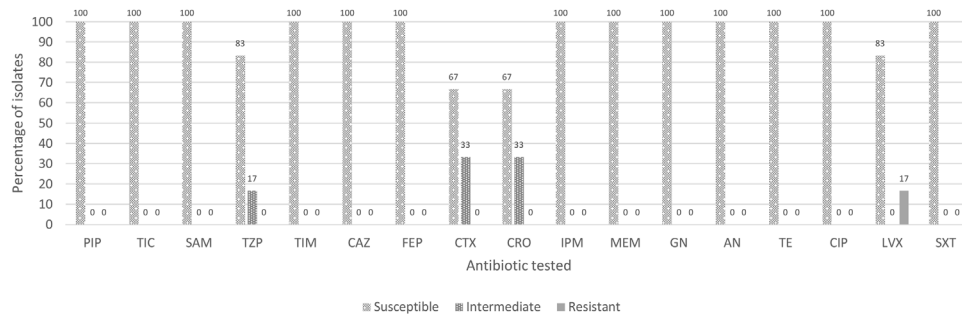
genetically related, which suggests that the same strain was circulating in the burn unit (Fig. 6); subsequently no more strains were received with these characteristics.

3.4. Resistance genes harboured by Acinetobacter haemolyticus *isolates*

Although 96% of *A. haemolyticus* were susceptible to carbapenems, 17% showed intermediate resistance to cefotaxime; these isolates carried genes encoding OXA- and TEM-type β-lactamases. Besides, our data showed that of 24 *A. haemolyticus* isolates, 29% (*n* = 7) carried *bla*$_{OXA-214}$, 4% (*n* = 1) carried *bla*$_{OXA-215}$, 8% (*n* = 2) carried *bla*$_{OXA-264}$, 29% (*n* = 7) carried *bla*$_{OXA-265}$ and 13% (*n* = 3)

carried *bla*$_{OXA-575}$. The latter is a novel variant of the *bla*$_{OXA-214}$ family and was deposited in the GenBank database (accession no. MG821355.1). Interestingly, only one of the three strains (AN3) carrying the new variant showed high MICs for cephalosporins. Moreover, three strains that carried *bla*$_{OXA-265}$ had the insertion sequence IS*Aba125* at nucleotide 106 and one strain presented a premature stop codon at amino acid position 253. In these strains, no changes in the MICs of the β-lactams relative to other strains with the entire gene were detected (Table 2).

On the other hand, 75% (*n* = 18) of the strains harboured *bla*$_{TEM-229}$, which is a novel variant (GenBank accession no. MG821356.1), 4% (*n* = 1) presented deletion of 78 amino acids at position 209 of the gene and 21% (*n* = 5) did not carry this β-lactamase. We also
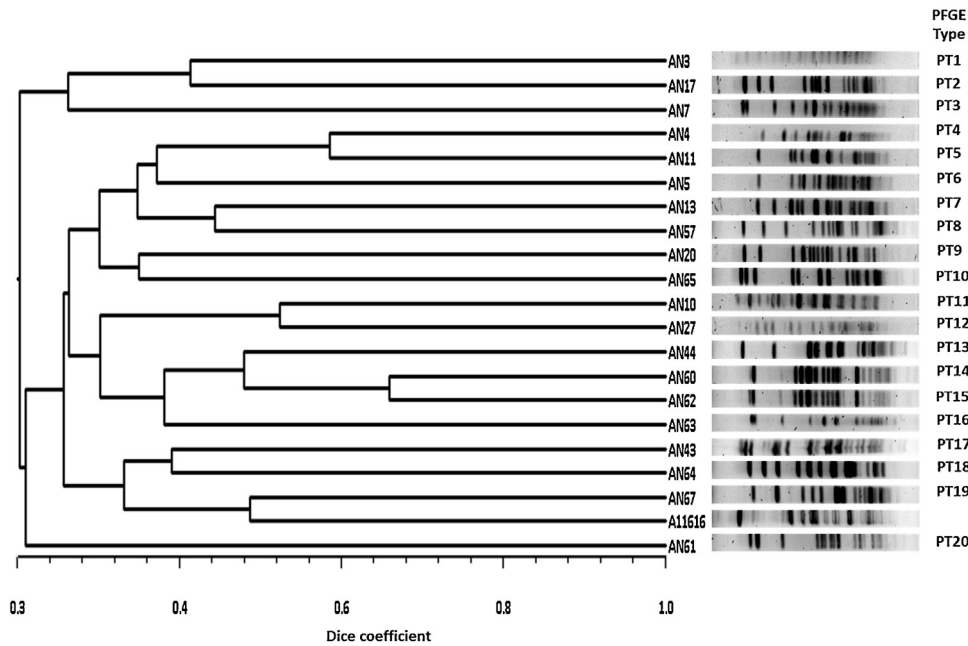
Fig. 5. Dendrogram of 24 *Acinetobacter haemolyticus* isolates. Isolates showing a Dice coefficient of <80% were considered as genetically unrelated. A11616, *A. haemolyticus* control strain; PT, pulsotype. Strains AN33, AN34, AN59 and AN69 were non-typeable.
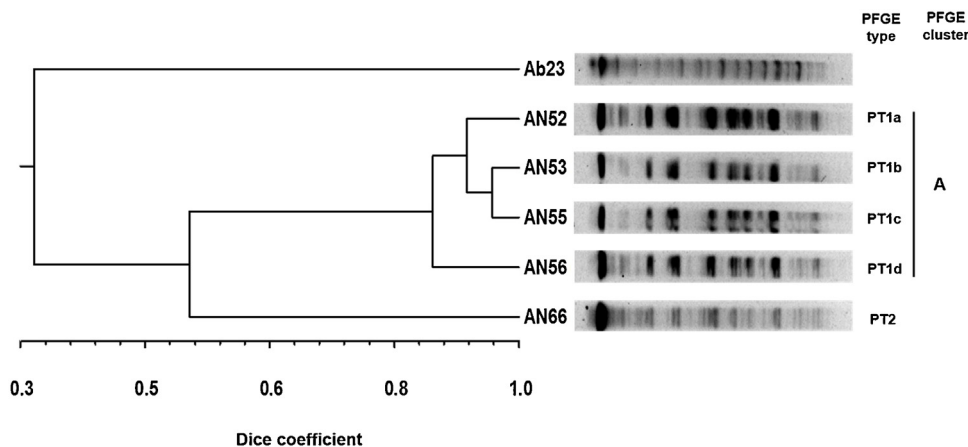

Fig. 6. Dendrogram of five carbapenem-resistant *Acinetobacter calcoaceticus–baumannii* complex isolates. Four isolates showing a Dice coefficient of ≥80% were considered as genetically related (PFGE cluster A). Ab23, *A. baumannii* control strain; PFGE, pulsed-field gel electrophoresis; PT, pulsotype.

detected a strain (AN57) resistant to cephalosporins and carbapenems that carried $bla_{OXA-265}$, bla_{NDM-1} and $bla_{TEM-229}$ (Table 2).

In the case of the strains with a resistant phenotype to aminoglycosides, MICs showed that all isolates were intermediate or resistant to gentamicin and amikacin. Fourteen strains carried *N*-acetyltransferase genes, among which it was found that 38% (*n* = 9) carried *aac(6′)-Ig*, with three new variants including *aac(6′)-Iga* (4%; *n* = 1), *aac(6′)-Igb* (13%; *n* = 3) and *aac(6′)-Igc* (42%; *n* = 10). These variants were deposited in GenBank with accession nos. MK468739, MK468740 and MK468738, respectively (Table 2). In Supplementary Fig. S1 we show the diversity and relationships among allelic variants of the AAC(6′)-I AME family.

3.5. Resistance genes harboured by Acinetobacter calcoaceticus–baumannii complex isolates

From the 24 strains belonging to the Acb complex, 5 isolates were selected as they showed resistance to different families of

antibiotics and resistance to carbapenems. They were searched for diverse resistance genes such as ESBLs, MBLs and the AAC(6′)-I AME family, of which the five strains carried bla_{OXA-51} and bla_{OXA-23} only (Table 3).

3.6. Contribution of efflux pumps to minimum inhibitory concentrations of cefotaxime, meropenem, amikacin and ciprofloxacin in resistant Acinetobacter haemolyticus and Acinetobacter calcoaceticus–baumannii complex strains

To determine the role of efflux pumps in the β-lactam-resistant phenotype, six *A. haemolyticus* strains that were resistant or intermediate to cefotaxime were selected. We tested the MIC of cefotaxime in the absence and presence of the EPI PAβN (25 mg/L), observing that three *A. haemolyticus* strains showed a 2-fold decrease in cefotaxime MIC, one strain showed a decrease in the order of 4-fold and a one strain showed a decreased of 8-fold. On the

Table 2
General characteristics of *Acinetobacter haemolyticus* isolates obtained in this study (*n* = 24).

Strain	Hospital ward	Isolate site	Isolation date (d/m/y)	PFGE type	CTX (CTX + EPI[a])	AN (AN + EPI[a])	CIP (CIP + EPI[a])	CRO	IPM	MEM	GN	β-Lactamase		AME[b]
AN3	Emergency	PD fluid	05/04/2010	PT1	512 (256)	>512 (>512)	>64 (64)	>128	0.25	0.5	>512	OXA-575	TEM-229	AAC(6')-Igc*
AN4	Infectiology	Ulcer	26/11/2010	PT4	8	64 (16)	8 (8)	2	1	1	4	OXA-214	TEM-229	AAC(6')-Iga≠
AN5	IM	PD fluid	02/09/2011	PT6	8	64 (32)	<0.5	4	1	2	4	OXA-265	TEM-229	AAC(6')-Igc*
AN7	IM	PD fluid	02/11/2011	PT3	8	32 (32)	<0.5	2	0.25	0.5	4	OXA-575	TEM-229	AAC(6')-Igc*
AN10	IM	PD fluid	16/03/2012	PT11	8	64 (32)	<0.5	2	0.5	0.5	4	OXA-264	TEM-229	AAC(6')-Igc*
AN11	IM	PD fluid	29/03/2012	PT5	16 (8)	64 (32)	<0.5	8	1	2	6	OXA-265	TEM-229	AAC(6')-Igb□
AN13	Surgery	Abdominal abscess	17/10/2012	PT7	8	32 (32)	<0.5	4	0.5	1	8	OXA-265	TEM-229	AAC(6')-Igb□
AN17	Emergency	PD fluid	10/03/2013	PT2	4	16	<0.5	2	0.25	0.25	4	Truncated by ISAba125	TEM-229	AAC(6')-Ig
AN20	Emergency	PD fluid	13/06/2013	PT9	8	64 (32)	<0.5	2	0.25	0.25	4	Truncated by ISAba125	TEM-229	AAC(6')-Igc*
AN27	Emergency	Wound	24/02/2014	PT12	32 (4)	64 (16)	<0.5	8	0.25	2	6	OXA-265	NC	AAC(6')-Igb□
AN33	IM	PD fluid	12/08/2014	NT	8	128 (64)	0.5	6	0.5	1	8	OXA-214	TEM-229	AAC(6')-Ig
AN34	Emergency	PD fluid	22/08/2014	NT	4	128 (32)	<0.5	2	0.5	0.5	6	OXA-214	TEM-229	AAC(6')-Igc*
AN43	IM	PD fluid	24/04/2015	PT17	8	64 (16)	<0.5	2	0.5	0.5	6	Stop codon at AA 253	TEM-229	NC
AN44	Emergency	PD fluid	11/08/2015	PT13	16 (4)	128 (64)	<0.5	4	0.5	2	4	OXA-265	TEM-229	AAC(6')-Igc*
AN57	IM	PD fluid	28/03/2016	PT8	>512 (>512)	16	<0.5	>128	128	>128	4	OXA-265 and NDM-1	TEM-229	AAC(6')-Ig
AN58	IM	PD fluid	02/04/2016	NT	8	64 (64)	<0.5	4	1	0.5	8	OXA-214	TEM-229	AAC(6')-Ig
AN60	Emergency	PD fluid	10/04/2016	PT14	8	32 (32)	<0.5	2	1	0.25	4	OXA-215	TEM-229	AAC(6')-Igc*
AN61	IM	PD fluid	26/01/2016	PT20	4	32 (32)	<0.5	2	0.5	0.25	4	OXA-264	TEM-229	AAC(6')-Igc*
AN62	IM	PD fluid	02/05/2016	PT15	8	64 (16)	<0.5	2	0.5	0.5	4	OXA-214	TEM-229	AAC(6')-Ig
AN63	Emergency	PD fluid	03/05/2016	PT16	8	32 (16)	<0.5	4	1	0.5	4	OXA-575	209 Δ 78 AA	AAC(6')-Igc*
AN64	IM	PD fluid	23/05/2016	PT18	8	64 (32)	<0.5	2	0.5	0.25	4	Truncated by ISAba125	NC	AAC(6')-Ig
AN65	IM	PD fluid	29/05/2016	PT10	8	128 (64)	<0.5	2	1	1	8	OXA-214	NC	AAC(6')-Ig
AN67	IM	PD fluid	03/09/2016	PT19	8	64 (64)	<0.5	4	1	2	8	OXA-214	NC	AAC(6')-Ig
AN69	Emergency	PD fluid	18/11/2016	NT	16 (8)	32 (32)	<0.5	8	1	4	4	OXA-265	NC	AAC(6')-Ig

PFGE, pulsed-field gel electrophoresis; MIC, minimum inhibitory concentration; CTX, cefotaxime; EPI, efflux pump inhibitor; AN, amikacin; CIP, ciprofloxacin; CRO, ceftriaxone; IPM, imipenem; MEM, meropenem; GN, gentamicin; AME, aminoglycoside-modifying enzyme; PD, peritoneal dialysis; PT, pulsotype; IM, internal medicine; NC, not carried; NT, non-typeable; AA, amino acid.
Interpretative breakpoints according to the Clinical and Laboratory Standards Institute (CLSI) [8].
[a] The EPI was phenylalanine-arginine β-naphthylamide (25 mg/L).
[b] AAC(6')-I AME variants: accession nos.: *MK468738; ≠MK468739; and □MK468740.

Table 3
Characteristics of carbapenem-resistant *Acinetobacter calcoaceticus–baumannii* complex isolates (*n* = 5).

Strain	Hospital ward	Isolate site	Isolation date (d/m/y)	PFGE type	MIC (µg/mL)								Genotype
					CTX (CTX + EPI[a])	MEM (MEM + EPI[a])	AN (AN + EPI[a])	CIP (CIP + EPI[a])	CAZ	CRO	IPM	GN	
AN52	Burn unit	Biopsy	02/01/2016	PT1a	1024 (512)	32 (32)	128 (64)	256 (256)	>128	>512	64	32	bla_{OXA-51}, bla_{OXA-23}
AN53	Burn unit	Biopsy	03/02/2016	PT1b	1024 (512)	32 (32)	64 (16)	256 (256)	>128	>512	64	32	bla_{OXA-51}, bla_{OXA-23}
AN55	Burn unit	Biopsy	02/03/2016	PT1c	1024 (512)	32 (32)	64 (16)	256 (128)	>128	>512	64	32	bla_{OXA-51}, bla_{OXA-23}
AN56	Burn unit	Biopsy	29/03/2016	PT1d	1024 (512)	32 (32)	64 (16)	256 (128)	>128	128	64	32	bla_{OXA-51}, bla_{OXA-23}
AN66	Burn unit	Biopsy	01/06/2016	PT2	1024 (512)	32 (32)	>128 (16)	256 (256)	>128	>512	64	>128	bla_{OXA-51}, bla_{OXA-23}

PFGE, pulsed-field gel electrophoresis; MIC, minimum inhibitory concentration; CTX, cefotaxime; EPI, efflux pump inhibitor; MEM, meropenem; AN, amikacin; CIP, ciprofloxacin; CAZ, ceftazidime; CRO, ceftriaxone; IPM, imipenem; GN, gentamicin; PT, pulsotype.
Interpretative breakpoints according to the Clinical and Laboratory Standards Institute (CLSI) [8].
[a] The EPI was phenylalanine-arginine β-naphthylamide (25 mg/L).

other hand, one *A. haemolyticus* strain (AN57), which carried $bla_{OXA-265}$ and bla_{NDM-1}, did not show MIC variation.

The variation of the MIC for amikacin in the presence or absence of PAβN was evaluated in 22 *A. haemolyticus* strains. The results showed that in 5 strains the MIC decreased 4-fold, whilst 9 strains showed a decrease of 2-fold and in 8 strains no decrease of the MIC was observed. It is important to highlight that only one strain did not carry the *aac(6')-I* gene. One of two ciprofloxacin-resistant strains showed an MIC decrease of <2-fold in the presence of PAβN (Table 2).

Fig. 7. QuickPrep plasmid extraction of carbapenem-resistant *Acinetobacter calcoaceticus–baumannii* complex strains. Lane 1, AN52; lane 2, AN53; lane 3, AN55; lane 4, AN56; lane 5, AN66; and lane 6, *Escherichia coli* NCTC 50192 with four plasmids (154, 66, 38 and 7 kb).

In the case of five Acb complex strains in which *aac(6')-I* was not detected, we observed that one strain had a 2-fold decrease in the MIC for amikacin, three strains had a 4-fold decrease and one strain had an 8-fold decrease. MICs for ciprofloxacin in the presence of PAβN showed that only two strains showed a 2-fold decrease in the MIC for ciprofloxacin. For cefotaxime, the five resistant strains showed a decrease of 2-fold in the MIC. The five strains did not confirm the participation of the efflux pump for meropenem (Table 3).

3.7. Plasmid profile of carbapenem-resistant Acinetobacter calcoaceticus–baumannii complex strains

Knowing that the five carbapenem-resistant Acb complex strains carried the same resistance genes, that they came from the same hospital ward and they had a close clonal relationship by PFGE, we performed plasmid extraction to investigate whether they carried the same plasmid profile. We found that they had four identical plasmids (Fig. 7).

4. Discussion

Infections caused by *Acinetobacter* spp. have been increasing in recent years, becoming a public-health problem worldwide owing to an increase in resistance to antibiotics in hospitals [14]. Mexico is not the exception, where several reports indicate that *A. baumannii* is the most frequently reported micro-organism causing infections in immunosuppressed patients admitted in ICUs [15,16]. In this work, we report the presence of species considered environmental 'emerging' in paediatric patients. Our findings show that the predominant non-*baumannii Acinetobacter* spp. was *A. haemolyticus* (44%), however there are reports that show contrary results, where isolates of this species have been described at a smaller proportion in other countries [17,18]. Other species such as *A. venetianus*, *A. junii*, *A. indicus*, *A. lwoffii*, *A. radioresistens* and *A. ursingii* were also reported, but these cannot be identified with routine techniques so that they are frequently misidentified in certain cases; therefore, this is the first report in a Mexican hospital where mainly species out of the Acb complex were isolated.

In this study, the isolates of *Acinetobacter* spp. came mainly from peritoneal dialysis fluid (65%) and from the internal medicine ward (48%), in contrast to the report by Morfín-Otero in 2013 where the primary isolation site was secretions (>50%), followed
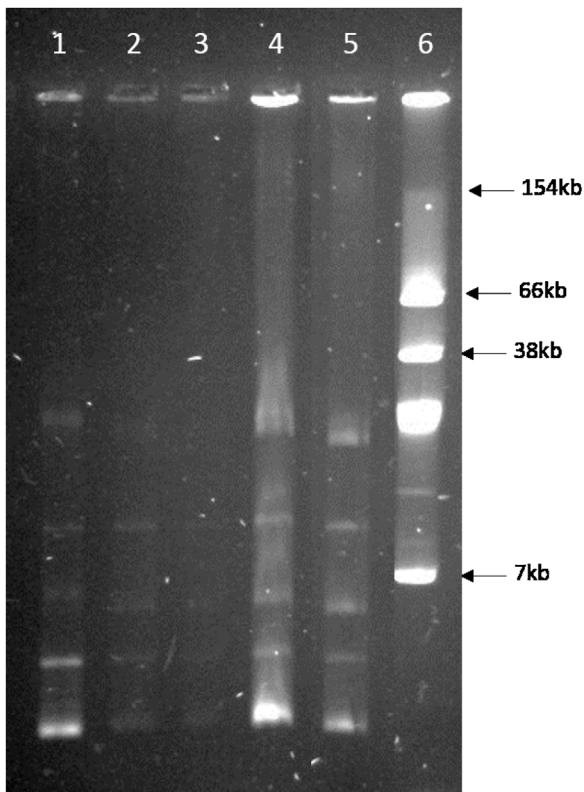
by vascular catheter and respiratory sources; the area with the highest number of isolates was the ICU in the paediatric ward [19].

It is important to highlight that some strains of *A. haemolyticus* and other non-*baumannii Acinetobacter* spp. were isolated from pure culture, and although the pathogenicity of these species is still a matter of study, there is a possibility that these emerging environmental species may be causing an infectious process, mainly in immunocompromised patients, those undergoing invasive procedures or with broad-spectrum antibiotic treatment. In addition is the fact that the *Acinetobacter* genus is known to be intrinsically resistant to desiccation and to have a metabolic dynamism that allows them to adapt to different environments, together with being important reservoirs of resistance genes causing the ineffectiveness of treatments. Therefore, they must be isolated and typed to carry out epidemiological control of these species in hospitals [14,20].

Although most strains of *A. haemolyticus* studied here were mainly susceptible to β-lactam antibiotics, the presence of intrinsic β-lactamases is the most prevalent mechanism of resistance to β-lactam antibiotics. These micro-organisms are potential reservoirs and dispensers of resistance determinants, particularly in hospital environments; our isolates were carriers of β-lactamases from the $bla_{OXA-214}$ family. This gene has been previously located in the chromosome of these species [21]. It is important to highlight that in this work we found a new variant $bla_{OXA-575}$, with 97.85% identity to $bla_{OXA-214}$; although only one of the strains harboured this new variant and it resulted in a high MIC for cephalosporins, these results suggest that other mechanisms of resistance could be involved. On the other hand, variants of this family of OXA-214 that are truncated by IS*Aba125* were also found; this insertion sequence has been reported forming a transposon named Tn*125* that mobilises the New Delhi metallo-β-lactamase 1 gene (bla_{NDM-1}) in different genera and bacterial species [22].

Besides, a strain of *A. haemolyticus* carrying NDM-1 was found. This enzyme has emerged around the world, conferring a wide range of resistance to β-lactams, including carbapenems, and whose early detection is extremely important [23]. Likewise, the strains studied carried the novel variant $bla_{TEM-229}$, which derives from $bla_{TEM-116}$ (99.65% identity), that has been reported in other non-*baumannii Acinetobacter* spp. and other bacterial genera such as *Citrobacter freundii*, *Providencia stuartii* and *Shigella flexneri* [24–27].

Regarding aminoglycoside resistance, it is known that strains of *A. haemolyticus* carry chromosomal genes encoding AAC(6')-I AMEs [28,29]. In our isolates, we found three new variants of *N*-acetyltransferase. On the order hand, one strain did not carry these enzymes, suggesting that other mechanisms could be acting in aminoglycoside resistance, and this study showed the participation of efflux pumps in the resistance to amikacin in several strains.

In this work, strains typified as members of the Acb complex showed higher resistance to antibiotics than the other species; however, only five Acb complex strains from the burn unit, which is an independent module of the hospital, turned out to be multidrug-resistant and carbapenem-resistant. These strains carried bla_{OXA-51} and bla_{OXA-23}; they are frequently found in the chromosome and plasmids, and overexpression of these genes by insertion sequences is the most frequent mechanism of resistance to carbapenems [30,31]. Our isolates did not carry other resistance genes such as ESBLs or MBLs, in contrast to other studies [32,33]. The presence of these strains in the burn unit occurred in a short period and no more strains with the same phenotypic, genotypic and plasmid characteristics were identified, which could suggest a local spread in that area.

In *A. baumannii* strains, other mechanisms involved in resistance to antibiotics have been studied. One of them is efflux pumps, mainly those of the RND system, specifically AdeABC, AdeFGH and AdeIJK, whose overexpression has been related to resistance to different antimicrobials such as aminoglycosides, β-lactams, fluoroquinolones, chloramphenicol, erythromycin and tigecycline [34]. However, experimental studies have not been carried out on non-*baumannii Acinetobacter* spp.

Our results showed that in *A. haemolyticus* and Acb complex strains carrying OXA-type β-lactamases there was a decrease of 2-, 4- or even 8-fold in the MIC for cefotaxime in the presence of the EPI PAβN. These results suggest that efflux pumps also participate in resistance to this antibiotic in different species. Also, we observed the participation of efflux pumps in resistance to amikacin, even when the strains carried AAC(6')-I. Previous works affirm that there is simultaneous active participation of efflux pumps together with resistance genes in strains of *A. baumannii* [35,36]. Therefore, to our knowledge, this is the first study that evidences efflux pump-mediated resistance to antibiotics in clinical strains of *A. haemolyticus*. Interestingly, in the *A. haemolyticus* strain that carried bla_{NDM-1} and the Acb complex strains that carried bla_{OXA-23}, there was no decrease in the MICs for meropenem in the presence of the EPI, suggesting that the activity of the enzymes they carry is enough to maintain resistance to carbapenems [37].

On the other hand, only two carbapenem-resistant Acb complex strains and one *A. haemolyticus* presented a decrease in the MIC to ciprofloxacin in the presence of PAβN, which probably suggests the participation of efflux systems. However, it is known that the mechanism of resistance to quinolones is mostly conferred by mutations in the quinolone resistance-determining region (QRDR) in the *gyrA* and *parC* genes in strains of *A. baumannii* and members of the Acb complex [38,39]. However, this mechanism was not studied in our collection, but it is interesting to note that *A. haemolyticus* AN3 showed resistance to ciprofloxacin, but the contribution of efflux pumps was not evidenced, so chromosomal mutations could be contributing.

In addition, our results showed that strains of *A. haemolyticus* also use their RND efflux systems to extrude antimicrobials, which will allow them to survive under antibiotic selection pressure in hospital environments.

5. Conclusions

In this hospital, we found an important number *A. haemolyticus* and other environmental species in clinical samples, unlike other hospitals where *A. baumannii* is the principal isolated bacterium. In this study, we found *A. haemolyticus* with new variants of β-lactamases and acetylases, in addition to other mechanisms of resistance such as efflux pumps causing bacterial adaptation to the hospital environment, complicating its treatment and eradication.

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Conflict of interest

None declared.

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Ethical approval

The protocol for this study was carefully reviewed and approved by the Hospital Ethical Committee [no. HNP/ENS/177/2016]. In this work, samples collected during routine diagnosis were used for bacterial isolation and did not constitute additional risks to patients. Patient information was considered anonymous.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2020.08.014.

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