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ESKAPE bacteria characterization reveals the presence of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* outbreaks in COVID-19/VAP patients

Miguel Ángel Loyola-Cruz MSc a,b, Emilio Mariano Durán-Manuel MSc a, Clemente Cruz-Cruz MSc a, Laura Margarita Márquez-Valdelamar MSc a, Juan Carlos Bravata-Alcántara MSc a, Iliana Alejandro Cortés-Ortiz MSc a, Mónica Alethia Curenó-Díaz MPH a, Gabriela Ibáñez-Cervantes PhD a, Verónica Fernández-Sánchez PhD a, Graciela Castro-Escarpulli PhD b,*, Juan Manuel Bello-López PhD a,**

a Hospital Juárez de México, Mexico City, Mexico
b Laboratorio de Investigación Clínica y Ambiental, Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico
c Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico

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**ABSTRACT**

*Introduction:* A decrease of detection of outbreaks by multidrug-resistant bacteria in critical areas has been reduced due to COVID-19 pandemic. Therefore, molecular epidemiological surveillance should be a primary tool to reveal associations not evident by classical epidemiology. The aim of this work was to demonstrate the presence of hidden outbreaks in the first wave of the COVID-19 pandemic and to associate their possible origin.

*Methods:* A population of 96 COVID-19 patients was included in the study (April to June 2020) from Hospital Juárez de México. Genetic identification and antimicrobial susceptibility testing of VAP causative agents isolated from COVID-19 patients was performed. Resistance phenotypes were confirmed by PCR. Clonal association of isolates was performed by analysis of intergenic regions obtained. Finally, the association of clonal cases of VAP patients was performed by timelines.

*Results:* ESKAPE and non-ESKAPE bacteria were identified as causative agents of VAP. ESKAPE bacteria were classified as MDR and XDR. Only *A. baumannii* and *P. aeruginosa* were identified as clonally distributed in 13 COVID-19/VAP patients. Time analysis showed that cross-transmission existed between patients and care areas.

*Conclusions:* *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were involved in outbreaks non-detected in COVID-19/VAP patients in the first wave of COVID-19 pandemic.

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The COVID-19 pandemic has accumulated a high number of deaths, where it has been estimated that approximately 80% of patients admitted to intensive care units (ICU) require mechanical ventilation, which is a risk factor for the development of ventilator-associated pneumonia (VAP). Patient-associated comorbidities are another risk factor that increases the likelihood of acquiring VAP, complicating the clinical condition of COVID-19 patients and directly impacting the mortality. Among the causative agents of VAP in COVID-19 patients are those belonging to the ESKAPE group. This acronym is composed of bacteria such as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*, among others. These
bacteria have been recognised as pathogens in critically ill and immuno-compromised individuals, and may be associated with resistance to multiple antibiotics.\textsuperscript{5,12,13} The hospital environment play an important role in the development of Healthcare-Associated Infections (HAI) including VAP.\textsuperscript{14,15} This is because these microorganisms have been isolated from inert surfaces, medical devices, and healthcare personnel, suggesting that the nosocomial environment is an important reservoir of pathogens.\textsuperscript{16} The spread of bacteria in ICUs often occurs through cross-transmission events mediated by healthcare personnel.\textsuperscript{17,18} This phenomenon has been demonstrated in previous studies by using molecular Biology assays and case associations, where it was observed that 20\%-40\% of pathogens identified in patients and medical devices were genetically related.\textsuperscript{19,20} These results allowed speculation that the common source of contamination could be the hands of healthcare personnel who previously handled medical devices or patients with active infections. The use of other molecular tools, which have advantages over other techniques, have allowed the identification of cross-transmission in ICUs of COVID-19 patients. The analysis of intergenic regions, demonstrated the clonal dispersion of \textit{A. baumannii} on inert surfaces, healthcare personnel, and on the skin of COVID-19 patients.\textsuperscript{16} In contrast, the application of ERIC-PCR identified that mechanical ventilators of COVID-19 patients play an important role as reservoirs of pathogens and could be involved in the occurrence of undetected outbreaks.\textsuperscript{21,22}

This is why it has been suggested that a molecular approach in the COVID-19 pandemic is necessary for the containment of outbreaks associated by antibiotic-resistant bacteria.\textsuperscript{16} Since \textit{A. baumannii} has been recognised as the main causative agent of VAP in \textit{Hospital Juarez de Mexico} (HJM), it is necessary to implement epidemiological surveillance at this pathogen. This will be done through genetic identification of VAP causative agents and identification of clones by analysis of intergenic regions of \textit{A. baumannii} and other pathogens circulating in ICUs of COVID-19 patients. Implications on the identification of ESKEAPE pathogen clones causing VAP in COVID-19 patients and the use of adequate disinfection process are analysed and discussed.

**MATERIAL AND METHODS**

**Ethical considerations**

The institutional Committee of Research, Ethics, and Biosafety from HJM approved the protocol under the registration number HJM 002/211 in accordance with the Regulation of the General Health Law on Research for Health (http://www.conbioetica.mexico.salud.gob.mx/descargas/pdf/normatividad/normatinaclonal/10_NAL_Reglamento_de_Investigacion.pdf).

**Description of the Hospital Juarez de Mexico in the COVID-19 era**

Derived from the pandemic by the SARS-CoV-2 virus, the HJM was converted to care COVID-19 patients. For this purpose, a total of 160 beds were distributed in seven areas, and multidisciplinary 15 health workers medical staff (morning, evening and night shifts), was involved in the care of each critical COVID-19 patient. Empirical treatments for VAP were ceftriaxone (1-2 g per 24 h) and due to the national shortage of colistin, the final treatments were meropenem (1 g per 24 h), imipenem (500 mg per 6-8 h), and piperacillin/tazobactam (4 g per 6 h). Finally, as an HAI control measures, towels impregnated with \textit{H}\textsubscript{2}O\textsubscript{2} (Oxivir Tb, CA) were used as disinfection method.

**Study population of COVID-19 patients with VAP**

A population consisting of 96 COVID-19 patients was included in the study during the period between April to June 2020 (first wave).

These patients met the inclusion criteria for suspected VAP (fever acquired after 48 h of intubation, purulent pulmonary secretions, leukocytosis, and infiltrates on chest X-ray).\textsuperscript{23-25} In aseptic conditions, COVID-19 patients were sampled through tracheal aspirates and were transported at 4°C to the research laboratory for their microbiological culture. Additionally, demographic data was obtained from medical records in order to describe COVID-19 patient population.

**Isolation of ESKEAPE bacteria from COVID-19 patients with VAP**

Prior to bacteriological isolations, tracheal aspirates were handled under a biosafety level 2 cabinet according to laboratory biosafety with the operator wearing a coverall protective gown. Tracheal aspirates were cultured on selective MacConkey and Mannitol Salt agar (Becton Dickinson & Co., Franklin Lakes, NJ) and were incubated aerobically at 37°C for 24-48 h. Subsequently, bacterial strains were purified in LB agar, were growth in LB-broth, then frozen in glycerol (50\%) and stored at −70°C for future experiments.

**DNA extraction**

For molecular biology assays, total DNA from strains was isolated and purified by using the DNeasy Blood & Tissue Kit (QIAGEN, Venlo, The Netherlands). Integrity of genomic and DNA was visualized on horizontal 0.8\% agarose gels and were used as templates in PCR assays.

**Genetic identification of ESKEAPE bacteria**

Amplification reactions were performed in a T100 Thermal cycler (Bio-Rad, Germany) Polymerase chain reactions of the 16S rRNA gene were performed with universal primers 27F and 1492R by using the conditions recommended by DeSantis et al., (2007)\textsuperscript{26} (Table 1). Amplicons were analysed on horizontal 1.5\% agarose gels by using 1 × Tris-Borate-EDTA buffer (TBE). PCR products were purified and sequenced by the Biology Institute of Universidad Nacional Autónoma de México (UNAM) by using a DNA Analyzer 3730xI (Applied Biosystems, Forrest City, CA). Nucleotide sequences were compared with the nucleotide sequence database (GenBank) by means of the Blast algorithm (http://blast.ncbi.nlm.nih.gov), using parameters of coverage (>80\%) and identity (90\%).

**MDR, XDR, and PDR classification of ESKEAPE bacteria isolated from COVID-19 patients**

The classification of MDR (multidrug-resistant), XDR (extensively drug-resistant) and PDR (pandrug-resistant) of ESKEAPE bacteria isolated from COVID-19 patients, were through of susceptibility/resistance assays, according to “Consenso latinoamericano para definir, categorizar y notificar patógenos multiresistentes, con resistencia extendida o panresistentes”\textsuperscript{27,28} and CLSI (2021),\textsuperscript{29} respectively. Pseudomonas aeruginosa ATCC 27853, E. coli ATCC 25922, and S. aureus ATCC 25923 were used as controls. Results were inferred as susceptible, intermediate, or resistant by measuring the diameter of the inhibition zone. The frequency of antibiotic resistance was calculated and represented in percentages (%) and MDR, XDR, and PDR classification was done.

**Carbapenemase production and their relationship with their genotype in ESKEAPE bacteria**

**Carbapenemases detection by mCIM assay**

ESKEAPE bacteria were subjected to the modified carbapenem inactivation method (mCIM) according to the method recommended by Pierce et al., (2017)\textsuperscript{29} In brief, two 1-µL loopfuls of ESKEAPE bacteria

\textit{P. aeruginosa} ATCC 27853, S. aureus ATCC 25923, and E. coli ATCC 25922 were used as controls. Results were inferred as susceptible, intermediate, or resistant by measuring the diameter of the inhibition zone. The frequency of antibiotic resistance was calculated and represented in percentages (%) and MDR, XDR, and PDR classification was done.
from an overnight blood agar plate were emulsified in 2 mL trypticase soy broth. Subsequently, a 10-μg MEM disk (BD, Brea, CA) was immersed in each suspension and incubated at 37°C for 4 h. Furthermore, a Mueller-Hinton (MH) was massive plated with E. coli ATCC 25922 (MEM6) suspension adjusted to 0.5 McFarland nephelometer. Finally, MEM disks were removed from the bacterial suspension and were deposited on MH plate with indicator MEMS strain. MH plates were incubated at 37°C for 18-24 h and the zones of inhibition were measured as for the routine disk diffusion method. Klebsiella pneumoniae blaNDM-1 was used as positive control.

Molecular screening to confirm carbapenemase production in ESKAPE bacteria

The genetic resistance background in ESKAPE bacteria (only carbapenemase-producer) that confer resistance to β-lactams (previously detected by mCIM assay), RT-PCR assays were performed to detect metallo β-lactamasmes and serine β-lactamasmes genes, by using CRE ELITE MGB (Torino, Italy) kit according to the manufacturer's manual. Isolates with positive PCR in first step were subjected to second PCR assays in order to detect specific carbapenemase genes (blaOXA, blaVIM, blaPER, blaIMP, blaNDM, blaIMV, blaVIM).13,15

Resistance to β-lactams (blaOXA) in Acinetobacter baumannii population

Once detected the phenotype of resistance to β-lactams in A. baumannii strains, a member of blaOXA subfamily (blaOXA-23) in these population (n = 14) was detected by end-point PCR. Amplicons were purified, sequenced, and analysed by nucleotide alignment by using UGENE (42.0). Primers used for this purpose are shown in Table 1.

Molecular screening to detect mcr gene in ESKAPE bacteria

To confirm the absence of colistin resistance in ESKAPE bacteria, RT-PCR assays were performed to detect mcr-1 and mcr-2 genes, using COLISTIN-R ELITE MGB (Torino, Italy) kit according to Girlich et al. (2019).13

Assembly of AdeABC operon and regulators genes AdeRS in A. baumannii

Full operon AdeABC encoding efflux pumps and their regulator genes AdeRS were amplified by end-point PCR in A. baumannii strains. Detection was performed under amplification strategy of conserved genes as follows: first PCR reaction was performed to amplify the AdeA gene encoding a protein forming a dimeric complex that anchors in the periplasmic region of the cell. Once a positive amplification to first molecular target was performed, a second reaction to amplify adeB gene (encoding an intermembrane protein) was carried out. Finally, a third reaction was performed to amplify the AdeC gene (encoding an extramembrane protein). Additionally, AdeK and AdeS genes encoding a regulator protein and activating protein kinase respectively were amplified.16 Sequences of primers used for AdeABCRS operon amplification are shown in Table 1.

Relation of Efflux pump AdeABC and MDR in A. baumannii

The possible relation between the MDR phenotype and the presence of detected efflux pumps was analysed as follows. Strains were tested for resistance before and after exposure to the efflux pump inhibitor with Phe-Arg-β-naphthylamide (PABN; Sigma, St. Louis, MO). This inhibitor was supplemented in the culture medium at a final concentration of 20 μg/mL. Significant inhibition was defined as a 4-fold or greater increase to the halo of inhibition in the presence of PABN and the antibiotic tested.

Molecular typing of ESKAPE members by ERIC-PCR

ESKAPE bacteria was subjected to molecular typing by ERIC-PCR, by using the primers ERIC1R and ERIC2 (Table 1). The total reaction volume was 50 μL and consisted of molecular Biology grade water, 1 × PCR buffer, 20 mM MgCl2, 25 mM deoxyribonucleotides phosphate, 100 μM of each primer, 3 units of Taq DNA polymerase (Thermo Scientific, Foster City, CA, USA) and 300 ng of template genomic DNA. Cycling conditions were as follows: pre-denaturation at 95°C for 7 s, denaturation at 90°C for 30 s, annealing at 58°C for 1 min, and extension at 65°C for 8 min, with a final extension at 68°C for 16 min at the end for 30 cycles. Genetic profiles were run in 1 × TBE buffer, pH 8.3, and separated in horizontal electrophoresis in 1.5% agarose gels, visualized, photographed under UV illumination, and analysed by intra-gel pattern comparison by using ImageLab 5.2.1. To confirm the reproducibility of ERIC-PCR assays three times was done. Tenover criteria was used in order to established the clonal relationship between isolates with the same genus and species.46 Finally, graphical relationship was performed through distance matrix by using a linear semilogarithmic method. The dendrograms were constructed using the UPGMA algorithm, with the Dice similarity index.

### Table 1

| Primer        | Molecular target | Sequence (5'→3') | Size (bp) | Reference |
|---------------|-----------------|-----------------|----------|-----------|
| 27F           | 16S rRNA        | AGAGTTTGATCCTGTCGAG | 1492     | 26        |
| 1492R         | oxa-23-F        | TACGCGACCGGCGGTTGTC | 1065     | 34        |
| adeA-F        | adeA            | TCTTCGTAACGATCTTCGTC | 236      | 16        |
| adeB-F        | adeB            | GCCATTGATTACCCAGCAGC | 541      |           |
| adeC-F        | adeC            | TTACGCGACCGGCGGTTGTC | 560      |           |
| AdeR-F        | adeR            | GCGTCAATGAAAGCAAG | 447      |           |
| AdeR-F        | adeS            | ACTACGATATTGGCGACATT | 544      |           |
| AdeS-R        | adeS            | TTACGCGACCGGCGGTTGTC | 544      |           |
| ERIC1R        | Intergenic consensus | ATGTAAGTCCTGCGGGATCTA | Variable | 35        |
| ERIC2         |                 | AAGTAAAGTGAATCCCCAGC | 139      | 31        |
| MultiIMP_for  | blaIMP          | TTACGCGACCGGCGGTTGTC | 390      |           |
| MultiIMP_rev  | blavIM          | GATGCCAGAATTAAGGCCCAYCT |           |           |
| MultiVIM_for  | blavIM          | GATGCCAGAATTAAGGCCCAYCT |           |           |
| NDM-Fn        | blavNDM         | GGTTGGGCGATCTGTTTTC | 621      | 32        |
| NDM-Rn        |                 | CGGAAATGGCATTACACAGCT |           |           |
Genomic similarity were confirmed with a bootstrap test of 1,000 repetitions using the Past4 program (Version 4.09).

**RESULTS**

**Description of population of COVID-19 patients with VAP**

During the period from April to June 2020 (first wave), patients with suspected COVID-19 were admitted to HJM. According to the CO-RADS system, these patients were classified according to their degree of severity as reported by Prokop et al., (2020). The study population was categorised as critical and therefore underwent mechanical endotracheal intubation. COVID-19/VAP patients were cared for in 6 different areas named COVID-19 AREAS (A, B, C, D, E, F, G). Table 2 shows the distribution of COVID-19/VAP patients by sex and age.

**ESKAPE and non-ESKAPE bacteria isolated from COVID-19/VAP patients**

Bacteriological analysis by classical microbiology revealed that only 56.3% (n = 54) of the tracheal aspirates showed bacterial growth with presumptive clinical value. Polymicrobial and monomicrobial cultures were identified in 42.6% (n = 23) and 57.4% (n = 31), respectively. Patients in the adult (36-64 years old) and elderly (>64 years old) age groups were those who developed polymicrobial infections. Monomicrobial infections were heterogeneously identified in all four age groups. A total of 60 and 23 isolates taxonomically classified as Gram-negative and Gram-positive were identified, respectively. Sequence analysis showed that 63.85% (n = 53) were isolates belonging to the ESKAPE group (Fig 1). Finally, other genera and species implicated in HAI, as well as bacteria considered commensal and environmental, were identified.

**High frequency of MDR and XDR ESKAPE bacteria**

The results showed that A. baumannii (n = 14/26.4%), P. aeruginosa (n = 5/9.4%) and K. pneumoniae (n = 5/9.4%) strains were classified as MDR. XDR phenotype was identified in P. aeruginosa in 3 isolates (5.6%). Resistant phenotypes for Enterobacterales group (except K. pneumoniae) were classified according to Magiorakos et al., (2012) (Table 3).

**Table 2**

| Patient classification | Male | Female |
|------------------------|------|--------|
| Pediatric<sup>a</sup>  | 0/0  | 2.9/1  |
| Young adults<sup>b</sup> | 3.2/2 | 5.9/2  |
| Adults<sup>c</sup>     | 69.3/43 | 55.9/19 |
| Elderly<sup>d</sup>    | 27.5/17 | 35.3/12 |
| **Total**              | 100/62 | 100/34 |

<sup>a</sup>0-18 years old.<br><sup>b</sup>18-35 years old.<br><sup>c</sup>36-64 years old.<br><sup>d</sup>≥64 years old.

Table 2. Distribution of COVID-19/VAP patients by sex, age groups admitted to Hospital Juárez de México.

![Fig1](https://example.com/fig1.png)

**Fig 1.** Frequency of ESKAPE and non-ESKAPE bacteria isolated. A. Gram negative population, B. Gram positive population and C. ESKAPE bacteria population.

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MDR/XDR phenotype/genotype and their relationship in carbapenemases production

ESKAPE bacteria (30.2%/n = 16) and non-ESKAPE bacteria (1.8%/n = 1) were capable to hydrolyse carbapenems. Acinetobacter baumannii (n = 9) was the most prevalent carbapenemase-producer and 6 strains of P. aeruginosa were also carbapenemase producers. Finally, Escherichia coli (n = 1) and E. cloacae (n = 1), were capable to hydrolyse meropenem. The results of antimicrobial resistance genotype are shown in Table 4.

Table 3
Frequency of antimicrobial resistant obtained by CLSI in ESKAPE bacteria isolated of COVID-19/VAP patients from Hospital Juárez de México

| Antibiotic | Acinetobacter baumannii (n = 14) | Pseudomonas aeruginosa (n = 19) | Enterobacteriales (n = 20) |
|------------|----------------------------------|----------------------------------|---------------------------|
| AMC        | ND*                              | ND                              | ND                        |
| AN         | 0 (0)                            | 13 (68.4)                       | 5 (26.3)                  |
| CAZ        | 0 (0)                            | 12 (63.1)                       | 0 (0)                     |
| COL        | 14 (100)                         | 14 (100)                        | 0 (0)                     |
| CRO        | 0 (0)                            | ND                              | ND                        |
| CTX        | 0 (0)                            | ND                              | ND                        |
| CZA        | ND                              | 12 (63.1)                       | 7 (36.8)                  |
| ETP        | ND                              | ND                              | 18 (9)                    |
| FEP        | 0 (0)                            | 12 (63.2)                       | 5 (26.3)                  |
| GM         | 0 (0)                            | 14 (73.7)                       | 2 (10.5)                  |
| IPM        | 1 (7.1)                          | 9 (47.4)                        | 10 (52.6)                 |
| LVX        | 0 (0)                            | 9 (47.4)                        | 2 (10.5)                  |
| MEM        | 1 (7.2)                          | 10 (52.6)                       | 9 (47.4)                  |
| SAM        | 3 (21.4)                         | 7 (50)                          | ND                        |
| STX        | 0 (0)                            | ND                              | ND                        |
| TET        | 3 (21.4)                         | ND                              | ND                        |
| TGC        | 14 (100)                         | ND                              | ND                        |
| TZP        | 0 (0)                            | 14 (100)                        | ND                        |

*Sensitive.
Intermediate.
Resistant.

ND: Nondeterminated.
Abreviations: AMC, Amoxicillin/Clavulanic; AN, Amikacin; CAZ, Ceftazidime; COL, Colistin; CRO, Ceftriaxone; CTX, Cefotaxime; CZA, Ceftazidime/Avibactam; ETP, Ertapenem; FEP, Cefepime; GM, Gentamicin; IPM, Imipenem; LVX, Levofloxacin; MEM, Meropenem; SAM, Ampicillin/Sulbactam; STX, Trimethopim/Sulfamethoxazole; TET, Tetracycline; TG, Tigecycline; TZZ, Piperacillin/Tazobactam.

Table 4
Genetic background of antimicrobial resistance in ESKAPE and non-ESKAPE bacteria isolated from COVID-19/VAP patients from Hospital Juárez de México

| Strain | Carbapenem resistance | mClm | Metallo β-lactamases | Serine β-lactamases | Colistin resistance |
|--------|-----------------------|------|----------------------|---------------------|---------------------|
|        |                       | NDM  | IMP, VIM             | KPC**/OXA-48**      | OXA-23**            | mcr-1/2            |
| AB-10  | +                     | -    | -                    | -                   | -                   | -                  |
| AB-20  | +                     | -    | -                    | -                   | -                   | -                  |
| AB-28  | +                     | -    | -                    | -                   | -                   | -                  |
| AB-66  | +                     | -    | -                    | -                   | -                   | -                  |
| AB-70  | +                     | +    | -                    | -                   | -                   | -                  |
| AB-73  | +                     | -    | -                    | -                   | -                   | -                  |
| AB-84  | +                     | +    | *blaVIM**            | -                   | -                   | -                  |
| AB-86  | +                     | -    | -                    | -                   | -                   | -                  |
| AB-97  | +                     | +    | -                    | -                   | -                   | -                  |
| AB-102 | +                     | +    | -                    | -                   | -                   | -                  |
| AB-104 | +                     | +    | *blaVIM**            | -                   | -                   | -                  |
| AB-106 | +                     | +    | -                    | -                   | -                   | -                  |
| AB-108 | +                     | -    | -                    | -                   | -                   | -                  |
| AB-112 | +                     | -    | -                    | -                   | -                   | -                  |
| PA-5(2) | +                   | +    | *blaVIM**            | -                   | ND*                 | -                  |
| PA-9   | +                     | +    | *blaVIM**            | -                   | ND                  | -                  |
| PA-11  | +                     | +    | *blaVIM**            | -                   | ND                  | -                  |
| PA-27  | +                     | +    | *blaVIM**            | -                   | ND                  | -                  |
| PA-78  | +                     | +    | *blaVIM**            | -                   | ND                  | -                  |
| PA-81(2) | +                  | +    | *blaVIM**            | -                   | ND                  | -                  |
| ECL-77(2) | +                    | +    | *blaVIM**            | -                   | ND                  | -                  |
| EC-23(2) | +                    | +    | *blaVIM**            | -                   | ND                  | -                  |

*Acinetobacter baumannii.
**Pseudomonas aeruginosa.
***Enterobacter cloacae.
**Escherichia coli.
*According to CLSI (2021).
**Modified carbapenem inactivation method.
**Real time-PCR.
End point-PCR.
Non-determinated.
Acinetobacter baumannii of COVID-19/VAP patients carrying full AdeABC operon

Full operon AdeABC and their regulator genes AdeRS were amplified in all A. baumannii MDR strains. Additionally, to genetic detection of the efflux pump, assays of inhibition of this pump were performed to confirm their functionality, through MDR phenotype reversion. The results showed there was no association of efflux pump inhibition and antibiotic resistance.

Molecular typing of ESKAPE members by ERIC-PCR

Profiles of the intergenic products revealed sizes of amplicons from ESKAPE ranged from slightly more than ≈100 bp to about ≈3000 bp. Intergenic region diversity of A. baumannii showed that the 14 isolates were grouped in seven unique clones, where 11 of them identified 4 clonal groups. A broad genomic diversity of P. aeruginosa strains was detected. This is due to the fact that out of the total of 19 strains, 17 were not grouped, and one clonal group was detected (Fig 2). Finally, Klebsiella pneumoniae and E. cloacae isolates, a broad genomic diversity was observed (Fig 3).

Acinetobacter baumannii clonal dispersion investigation

Four clonal clusters of A. baumannii were detected in the last month of the quarter analysed, according to the chronology of their appearance, the origin of these clonal clusters started in the COVID-19 “A and B” areas. No patients survived. Figure 4 shows the timeline for A. baumannii clonal detection events by date and COVID-19 area (A, B, and C). The Table 5 summarises the most relevant characteristics of the COVID-19/VAP patients involved in the detection of the four A. baumannii clonal groups.

DISCUSSION

Research on the new SARS-CoV-2 virus has focused on the study of its pathogenesis, evolution, treatment, vaccines, among others. However, the study of the bacterial agents that complicate the clinical status of COVID-19 patients, as well as the genetic basis of antimicrobial resistance and its clonal dispersion in critical areas has been poorly explored. It is known that VAP-causing pathogens prolong and complicate their hospital stay, so their identification and containment through prospective epidemiological surveillance should be a priority. This study showed that the pathogens that cause VAP in COVID-19 patients form part of the nosocomial reservoir in non-COVID-19 and pandemic periods. This is because pathogens found in ICU patients (ESKAPE and non-ESKAPE group) have also been shown to be identified on inanimate surfaces, medical personnel and devices used in respiratory therapy, such as mechanical ventilators. Statistics from this work showed that the male patient population had the highest prevalence of COVID-19 (Table 2). It has been shown that ACE2 receptor levels are significantly higher in this sex, a factor of susceptibility to SARS-CoV-2 infection and consequently fatal outcomes. Conversely, 88.54% of the population studied was over 41 years, where age has been recognised as a predisposing factor, mainly in those who presented the disease during the first wave of the pandemic (Table 2). Other susceptibility factors include impaired immune response, which leads to impaired control of viral replication and prolonged inflammatory response. All these complications together trigger acute respiratory distress syndrome (ARDS), where mechanical ventilation (MV) plays an important role in supporting respiratory support for the maintenance of these patients. ARDS is a well-known risk factor for VAP and is related to the incidence in patients with MV. The percentage of bacterial co-infections in our study was 56.25%, frequencies of 17.2% to 89% have been reported elsewhere in the...
We speculate that these frequencies may vary depending on factors, such as adherence to correct clinical practices in patient management, handwashing technique, and adequate disinfection methods. As a result of these problems, the need and interest in epidemiological surveillance for the containment of both SARS-CoV-2 and HAI-causing bacteria arose at the HJM. The ESKAPE group was the microorganisms mainly responsible for HAI, including VAP. These bacteria have been previously reported in patients with VAP and SARS-CoV-2 infection. In contrast, 44% of the study population had clinical features suggestive of VAP but failed to isolate the causative agent. We speculate that the microbiological culture bias is due to the fact that only ESKAPE bacteria were targeted. This may have influenced the detection of other microorganisms related to this nosocomial infection. Previous studies report the presence of pathogens that can cause VAP in COVID-19 patients that could be undetected by microbiological analysis. Co-infections of COVID-19 patients with yeast Candida spp., and other bacteria not investigated in this study have been reported. The COVID-19 pandemic has had several consequences, including a possible increase in antimicrobial resistance (AMR), where the irrational use of antibiotics has been described as the main cause. Antibiotic resistance and its persistence are associated with the risk of therapeutic failure and resilient infections. We detected isolates with resistance to tested antimicrobials, mainly β-lactams and other agents used in treatment.

Fig 3. Genomic diversity by dendrogram construction of ESKAPE bacteria isolated from COVID-19/VAP patients of the Hospital Juárez de México by ERIC-PCR.

Fig 4. Timeline of detection of clonal groups of MDR Acinetobacter baumannii isolated from COVID-19/VAP patients of the Hospital Juárez de Mexico.

*Areas for COVID-19 patients
Acute respiratory distress syndrome.

Resistance profiles similar to those of this work have been observed in other studies.11,12 The background of the presence of one of the main mechanisms of antimicrobial resistance in A. baumannii13,14 is a genetic and phenotypic search for efflux pumps in this pathogen was performed, although PCR tests demonstrated the presence of the complete AdeABC operon, pump inhibition tests did not demonstrate its activity. We speculate that the multidrug resistance of the A. baumannii isolates in this work is not conferred by the AdeABC efflux pump. Future works of resistome analysis of A. baumannii will be aimed for searching possible mutations in the AdeABC operon and antimicrobial resistance mechanisms in order to relate phenotypes and genotypes in the isolates found. The high multidrug resistance in A. baumannii isolates, compared to the other members of the ES KAPE group, shows that this pathogen continues to be a problem in our hospital and the main microorganism implicated in cases of VAP caused by MDR bacteria. As reported by Sosa-Hernandez et al.,15 (2019), the incidence of A. baumannii as a causative agent of VAP was higher compared to the other pathogens of the ES KAPE group.12 Derived from this finding, the positive impact of prospective epidemiological surveillance through implementation of a comprehensive quality improvement plan (CQIP) during the following year was demonstrated.16 The classical epidemiological approach of case association limits the identification of outbreaks that might go unnoticed. To our knowledge, the available literature is only limited to the reporting of isolates without an analysis of whether these isolates have a common ancestor. Therefore, classical Epidemiology must resort to alternative molecular tools to understand the origin of adverse events in critical areas. Through the analysis of intergenic regions, clonal dispersion of A. baumannii and P. aeruginosa was demonstrated (Fig. 2 and 3). The timeline analysis of VAP cases in COVID-19 patients showed a direct association between the dispersion of clones detected and the area where patients were hospitalized (Fig. 4 and Table 5). We supposed that healthcare workers may be the main vehicle of transmission of these pathogens. Several reports support that, poor clinical practices lead to the spread of microorganisms in hospitals, and this has an impact on the incidence of HAI and in turn on mortality, especially in critically ill patients.17,20,62,63 It is not known exactly how the pandemic has affected antimicrobial resistance as outbreak detection and control measures were suspended due to the health emergency.21,22 However, some studies suggest that there was an increase in AMR during the pandemic.64,65 This paper demonstrates that molecular tools go beyond the traditional scheme of analysis for enriching the causal chain by detecting hidden outbreaks and revealing associations that are not so obvious to classical Epidemiology. The current epidemiological transition positions molecular Epidemiology as an indispensable tool in the pandemic and post-pandemic era in the bacteriological field.

**Table 5** Characteristics of COVID-19/VAP patients involved in clonal dispersion groups of MDR A. baumannii strains from Hospital Juarez de Mexico

| Sex | Age | ICU admission reason | Clonal group | Date of first CR-AB isolate collection | CR-AB isolate source | Hospital Stay (days) | Patient outcomes |
|-----|-----|----------------------|--------------|----------------------------------------|----------------------|---------------------|-----------------|
| M   | 43  | SDRA                 | 1            | 21/05/20                               | Tracheal aspirate    | 25                  | Deceased         |
| M   | 55  | SDRA                 | 2            | 25/05/20                               | Tracheal aspirate    | 12                  | Deceased         |
| M   | 57  | SDRA                 | 3            | 01/06/20                               | Tracheal aspirate    | 22                  | Deceased         |
| F   | 55  | SDRA                 | 3            | 12/06/20                               | Tracheal aspirate    | 19                  | Deceased         |
| F   | 33  | SDRA                 | 4            | 26/05/20                               | Tracheal aspirate    | 28                  | Deceased         |
| M   | 38  | SDRA                 | 5            | 26/05/20                               | Tracheal aspirate    | 20                  | Deceased         |
| M   | 35  | SDRA                 | 6            | 10/06/20                               | Tracheal aspirate    | 23                  | Deceased         |
| M   | 74  | SDRA                 | 7            | 22/06/20                               | Tracheal aspirate    | 14                  | Deceased         |
| F   | 65  | SDRA                 | 8            | 25/06/20                               | Tracheal aspirate    | 9                   | Deceased         |
| F   | 63  | SDRA                 | 9            | 15/06/20                               | Tracheal aspirate    | 21                  | Deceased         |
| F   | 46  | SDRA                 | 10           | 23/06/20                               | Tracheal aspirate    | 26                  | Deceased         |

*Acute respiratory distress syndrome.

1Carbapenem-resistant Acinetobacter baumannii.

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