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Porin expression in clinical isolates of *Klebsiella pneumoniae*: a comparison of SDS-PAGE and MALDI-TOF/MS and limitations of whole genome sequencing analysis

Cristina Elías-López[1,2*], Montserrat Muñoz-Rosa[1,3], Julia Guzmán-Puche[1,2,3], Elena Pérez-Nadales[1,2,4], Eduardo Chicano-Galvez[1,5] and Luis Martínez-Martínez[1,2,3,4]

Abstract

Background The permeability of the outer membrane barrier modulates the susceptibility of microorganisms to antimicrobial agents. Loss or structural alterations of porins contribute to decreased antibiotic concentration of multiple antimicrobial agents. Precise de□nition of porin pro□les is of critical importance to understand the role of porins in antimicrobial resistance. The objectives of this study are to compare the expression patterns of major outer membrane proteins (OMP) of clinical isolates of *Klebsiella pneumoniae* obtained with Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry (MALDI-TOF/MS), with those obtained with sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and to correlate porin expression patterns with the sequences of porins genes de□ned with whole genome sequencing (WGS).

Methods The OMP pro□les of 26 clinical isolates of *K. pneumoniae* and of strain ATCC 13883 (wild-type) and ATCC 700603 (producing SHV-18) have been determined using both SDS-PAGE and MALDI-TOF/MS. SDS-PAGE was performed using both homemade and commercial gels, and protein bands were identi□ed by liquid chromatography coupled to mass spectrometry. A rapid extraction method was used to analyse OMPs by MALDI-TOF/MS. The sequences of porin genes were obtained by WGS and mutations were de□ned by BLAST.

Results Same results were obtained for all strains either using SDS-PAGE or MALDI-TOF/MS. SDS-PAGE showed protein bands of ~ 35, ~36, and ~ 37 kDa, identi□ed as OmpA, OmpK36 and OmpK35, respectively. By MALDI-TOF/MS, peaks at ~ 35,700 (OmpA), ~ 37,000 (OmpK35), and ~ 38,000 (OmpK36) m/z were detected. *ompK35* was intact in nine wild-type isolates and was truncated in 13 isolates, but OmpK35 was not observed in 3 isolates without mutations in *ompK35*. One point mutation was detected in another isolate and multiple mutations were detected in the remaining isolate. *ompK36* was truncated in two isolates lacking this protein and presented one point mutation ($n = 1$) or multiple mutations in the remaining isolates.

*Correspondence:
Cristina Elías-López
cristina.elias@imibic.org

Full list of author information is available at the end of the article

Conclusion MALDI-TOF/MS was reliable for porin detection, but because of the complex regulation of porin genes, WGS cannot always anticipate protein expression, as observed with SDS-PAGE and MALDI-TOF/MS.

Keywords *Klebsiella pneumoniae*, Porins, MALDI-TOF/MS, Multidrug resistance

Introduction

Klebsiella pneumoniae is one of the most relevant microorganisms in the ESKAPE group and can cause a wide range of di cult to treat infections due to its potential multiresistance to antimicrobial agents [1–3].

e outer membrane (OM) of Gram-negative bacteria acts as a selective barrier that, by combining a highly hydrophobic lipid bilayer with pore-forming proteins (porins), contributes to exclude substances based on their size and/or charge [4, 5]. Some porins allow non-speci c passage of low molecular weight solutes, while others are speci c di usion channels for certain substrates (i.e. LamB for maltose derivatives and maltodextrins, or PhoE for phosphate) [6, 7]. e major non-speci c porins of *K. pneumoniae* are OmpK35 and OmpK36, orthologs of OmpF and OmpC in *Escherichia coli*. Besides, *K. pneumoniae* usually expresses OmpA and other porins, such as OmpK26 and OmpK37, which could be relevant in the absence of OmpK35 and OmpK36 [8, 9].

e permeability of the OM barrier modulates the susceptibility of microorganisms to antimicrobial agents [10, 11]. Loss or structural alterations of porins contribute to decreased antibiotic concentration of multiple antimicrobial agents (i.e. cephalosporins, monobactams, carbapenems, uoroquinolones…) in the periplasm. is cooperates synergistically with the expression of speci c (i.e. β-lactamases, type II topoisomerase mutations) or non-speci c (active e ux pumps) mechanisms to increase resistance to antimicrobial agents [12].

Porins are subject to complex regulatory mechanisms and their expression can be conditioned by environmental factors such as osmolarity, pH, temperature or the presence of toxins or antimicrobials [13, 14]. Expression of OmpK35 is downregulated in high osmolarity media, while OmpK36 production is favoured in these media [15].

On the other hand, whole genome sequencing (WGS) currently allows recognition of genes coding for resistance markers and virulence factors, and provides relevant epidemiological information, such as sequence type (ST) and clonal relationship of multiresistant organisms.

e bioinformatic analysis of WGS data can be explored for inferring a relationship between mutations in porin genes and decreased susceptibility/resistance to antibiotics used in clinical practice [16–18]. Porin loss can be predicted from stop codons in the encoding genes, but regulatory post-transcriptional modulating porin expression can be di cult to identify. For this reason, the ultimate analysis of the role of porins in resistance may require an actual evaluation of porin expression. Traditionally, OMP expression has been studied using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). However, this is a time-consuming methodology and is not usually implemented in clinical microbiology laboratories. New methodologies are being established to detect these proteins, including soft ionization techniques such as Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry (MALDI-TOF/MS) or liquid chromatography coupled to mass spectrometry (LC-MS/MS), which is nowadays commonly used in clinical laboratories for routine identi cation of microorganisms [19–21].

e objectives of this study are (i) to compare the expression patterns of major OMP of clinical isolates of *Klebsiella pneumoniae* obtained with MALDI-TOF/MS, using a rapid extraction method, with those obtained with SDS-PAGE, and (ii) to correlate porin expression patterns with the sequences of porins genes de ned with WGS.

Materials and methods

Bacterial isolates

Twenty-six *K. pneumoniae* clinical isolates with di erent antimicrobial susceptibility pro les [wild-type (WT), n=9; extended-spectrum β-lactamase producers (ESBL), n=3; carbapenemase producers (KPC, n=7; OXA-48, n=7)] and two ATCC strains (ATCC 13883 and ATCC 700603) were tested. Bacterial isolates were identi ed by MALDI-TOF/MS (Biotyper®, Bruker, Germany) and con rmed by SpeciesFinder and KmerFinder (Center for Genomic Epidemiology). e production of ESBL was detected by disk di usion, using discs (Bio-Rad, USA) of ceftazidime, cefepime and cefotaxime alone or combined with clavulanic acid, according to Clinical and Laboratory Standards Institute (CLSI) guidelines [22]. Carbapenemases were detected using an immunoassay (NG-Test CARBA 5, Biotech, France); the presence of genes encoding the corresponding enzymes was con rmed by WGS (see below) and bioinformatic analysis with Res nder (Center for Genomic Epidemiology).

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) of multiple agents were determined by broth microdilution, using commercial panels (Sensititre® DKMGN and EUMDROXFF, ermoFisher Scienti c, USA) according to manufacturer's instructions. MICs were interpreted according to the European Committee on Antimicrobial

Susceptibility Testing (EUCAST) breakpoints (v_13.0) [23].

SDS-PAGE

Bacteria were grown in nutrient broth (BD-Difco, USA) and cation-adjusted Muller-Hinton broth (Sigma-Aldrich, Germany), representative of low- and high-osmolarity growth media, respectively. Osmolarity of the media was measured with Osmo-Station OM-6060 (Arkray, Japan).

OMPs for SDS-PAGE analysis were extracted as previously described [24, 25] (Supplementary Fig. 1A). Brie y, bacteria were grown overnight at 35±2 °C in broth and subsequently harvested at late logarithmic phase. ey were washed with sodium phosphate bu er (10 mM, pH 7.2) and resuspended in the same bu er. Cells were disrupted by sonication (Omni International, USA). After a rst centrifugation (2300 x g, 10 min, 4ºC) the supernatant was retained and centrifuged at 16200 x g, 45 min, 4ºC. e pellet was incubated at room temperature in sodium phosphate bu er containing 2% sodium lauroyl sarcosinate (Sigma-Aldrich). e insoluble fraction was recovered by centrifugation (16200 x g, 45 min, 4ºC), washed and resuspended with Trizma base bu er (0.0625 M, pH 6.8) (Sigma-Aldrich).

Protein concentration was measured by spectrophotometry (Denovix DS-11, DeNovix Inc, USA). Reduced Laemmli bu er obtained by mixing 50 µl of 2-mercaptoethanol and 950 µl of Laemmli 2x (Bio-Rad) was used as loading bu er. e proteins/loading bu er mixture was boiled for 5 min before electrophoretic analysis. OMPs were rst determined by SDS-PAGE using 1 mm thick homemade gels containing 12% of acrylamide and different concentrations of bisacrylamide (0.23-0.38-0.6%) [25]. Protein bands were visualized with Coomassie brilliant blue R-250 (Sigma-Aldrich) staining. In a second assay, commercial stain-free gels with 12% polyacrylamide (Mini-PROTEAN® TGX Stain-Free, Bio-Rad) were used, and proteins were visualized with the Gel Doc EZ Imager system and the Image Lab software (Bio-Rad).

For the 9 Kp-WT isolates and for both ATCC, protein bands from bacterial growth in nutrient broth were excised from the gel and identi ed by liquid chromatography (EvosepOne nanoLC, Evosep, Denmark) and mass spectrometry (TIMSTOF-Flex, Bruker). Brie y, gel bands were destained with 10 mM ammonium bicarbonate, reduced with dithiothreitol (20 mM, 25 mM ammonium bicarbonate) and alquilated with iodoacetamide (40 mM, 25 mM ammonium bicarbonate). Later, gel bands were washed with sequential steps containing incremental concentrations of acetonitrile. After that, gel bands were incubated with MS-grade trypsin 12.5 ng/µl in 25 mM ammonium bicarbonate (Promega, USA) overnight. e reaction was stopped using 10% tri uoroacetic acid (TFA) during 10 min and additional washes were applied to samples. Finally, obtained peptides were loaded onto Evotips (Evosep, Denmark). All samples were analyzed by LC-MS/MS using the EvosepOne- TIMSTOF-Flex Clinical Proteomics Platform. e samples identi cation was carried out using MSFragger through its graphical interface Fragpipe. Subsequently, the results of the MSFragger searches were ltered using the TransProteomicsPipeline tool using its ltering options to obtain those proteins with 2 or more peptides that had not been identi ed in the decoy database associated with the fasta le used.

MALDI-TOF/MS

For MALDI-TOF/MS analysis, OMPs were extracted using a rapid outer membrane protein (ROMP) protocol [26] (Supplementary Fig. 1B). Strains were grown in broth and incubated overnight. A bacterial suspension (1 McFarland) was prepared, the pellet obtained after centrifugation (8000 x g, 5 min) was resuspended and incubated at room temperature with HEPES bu er (10 mM, pH 7.3) (Nzytech, Portugal) and 2% of sodium lauroyl sarcosinate (Sigma-Aldrich) during 30 min. After another centrifugation (8000 x g, 5 min) step, the pellet was resuspended with HEPES (10 mM, pH 7.3).

Following this step, OMPs were diluted (1/10, v/v) with a 2,5-dihydroxybenzoic acid solution (20 mg/ml, 50% acetonitrile, 2.5% TFA). Later, one microliter of this solution was applied to the plate and air dried. Subsequently, samples were analysed with a MALDI Biotyper® Sirius (Bruker) benchtop mass spectrometer using FlexControl software (Bruker, v3.4) with a modi ed acquisition method from LP_66KDa.par standard method. Protein Standard II (Bruker) was used for calibration. Mass spectra were analysed using FlexAnalysis software (Bruker, v3.4).

WGS

Bacterial DNA from clinical isolates was extracted using an automatic MagCore® HF16 Plus System with the MagCore® Genomic DNA Bacterial Kit 502 (RBC Bioscience, Taiwan). DNA was sequenced using the Illumina platform. Libraries were generated using a Nextera DNA XT Sample Preparation Kit (Illumina, USA) and sequenced using an Illumina Novaseq6000 sequencer system with 2×150-bp paired-end reads. e quality of the raw data was veri ed by FASTQC, the quality of the sequences was corrected with TRIMGALORE software and the short reads were assembled de novo into contigs using Unicycler. e quality of assembly was reviewed with SQUAT.

For ATCC 700603 the genome with GenBank identi er NZ_CP014696.2 was used. ATCC 13883 *K. pneumoniae* (GenBank ID: NZ_KN046818.1) was used as the reference genome for de ning mutations in the *ompA*,

ompK26, *ompK35*, *ompK36*, *ompK37*, *phoE* and *lamB* genes, using BLAST. Additionally, in those isolates where no mutation or point mutation in the *ompK35* gene was found and no band/peak was observed, mutations in porin regulatory genes such as *marR*, *marA*, *marB*, *envZ*, *ompR*, *mzrA*, *ramA*, *ramR and soxR* were searched for.

Prediction of a signal peptide was established with SignaIP 6.0.

Results

e obtained MIC values against the evaluated isolates corresponded to the expected phenotypes and genotypes. MICs, relevant β-lactamases and STs data are presented in Table 1.

Osmolarities of nutrient broth and cation-adjusted Muller-Hinton broth were 39 and 315 mOms, respectively. e best separation of OMPs with homemade SDS-PAGE gels was observed with gels containing 12% acrylamide and 0.23% bisacrylamide. e same OM proles were obtained with commercial precast gels and homemade gels (data not shown).

Protein analysis by SDS-PAGE gels showed bands with sizes of ~35, ~36 and ~37 kDa. By LC-MS/MS analysis, the ~35 kDa band was identi ed as OmpA, the ~36 kDa band as OmpK36 and the ~37 kDa band as OmpK35. By mass spectrometry MALDI-TOF, peaks at ~35,700, ~37,000, and ~38,000 m/z were detected and had previously been reported as OmpA, OmpK35 and OmpK36, respectively [27].

For all isolates studied, the same results for OmpA, OmpK35 and OmpK36 were obtained using either SDS-PAGE or MALDI-TOF/MS. For both methodologies, two OMP bands/peaks were observed for all Kp-WT strains and ATCC 13883 when protein extraction was performed from bacteria growing in cation-adjusted Muller-Hinton broth, however, three bands were observed when extraction was performed from nutrient broth; representative results of the osmoregulation process for CHURS-170744 strain are presented in Fig. 1 (SDS-PAGE) and Fig. 2A (MALDI-TOF/MS).

In strains RHURS-0115, RHURS-0132 and CHURS-000694 only one band/peak corresponding to OmpA was detected, regardless of the broth used. In the remaining non- wild-type isolates, two bands/peaks corresponding to OmpA and OmpK36 were observed.

In addition, on SDS-PAGE gels, a ~48 kDa band corresponding to the LamB protein was found in all strains for both broths, but by MALDI-TOF/MS a peak presumably corresponding to LamB (~45300 m/z) was only detected in 14 of the 28 isolates (50%) when the organisms were grown in cation-adjusted Muller-Hinton broth (but not when grown in nutrient broth). In all Kp-KPC strains studied by MALDI-TOF/MS, a peak at ~28,550 m/z was observed, regardless of the broth used in the extraction, consistent with the expected peak for KPC carbapenemase expression (Fig. 2B).

Mutations of OMP genes found by WGS and expression of the corresponding proteins (SDS-PAGE and MALDI-TOF/MS) are presented in Table 2. A signal peptide of 21 amino acids was predicted in 5 genes (*ompA*, *ompK35*, *ompK36*, *ompK37* and *phoE*). e signal peptide of *ompK26* and *lamB* had 20 and 25 amino acids, respectively. No mutations were found in *lamB* and *phoE* of clinical isolates, but point mutations were identi ed in the *ompA*, *ompK26* and *ompK37* genes (Supplementary Table 1).

e *ompK35* gene presented a great sequence variation. In the Kp-WT and Kp-ESBL strains there was no mutation in *ompK35*. In all 7 Kp-KPC isolates, a single nucleotide insertion (G) produced a premature stop codon (TGA) at position 67 of the mature protein, resulting in a truncated protein, corresponding to a small fragment of 19 amino acid following the signal peptide. In 6 of the 7 Kp-OXA-48 isolates, a single nucleotide (T) deletion caused a premature stop codon (TAG) at position 15 of the protein sequence, which truncates the protein within the signal peptide region; a point mutation (T119S) was found in the remaining isolate of Kp-OXA-48 (CHURS-009-IS), but *ompK35* was also not expressed.

e *ompK36* sequences in the Kp-WT isolates had multiple mutations compared to strain ATCC 13883. In isolate RHURS-0115 (SHV-12 producer), a point mutation was observed in the region coding for the leading peptide (E2K), but no others changes were noted. Two strains of Kp-OXA-48 had a premature stop codon in *ompK36* at di erent positions: in strain CHURS-000694, a 4-nucleotide deletion (ACTT) truncates the protein at position 171, while in strain RHURS-0132, a 2-nucleotide deletion (TA) at position 65 of the protein sequence was observed. In addition, four of the seven Kp-KPC strains (CHURS-170942, CHURS-180130, CHURS-175691 and RHURS-0308), all of them corresponding to ST512, had alterations in the conserved PEFGGD motif of the L3 region of OmpK36, including a GD duplication at position 115–116 [28, 29].

Considering strain ATCC 13883 as a reference, the S82G mutation in the *marR* gene was found in clinical isolates RHURS-0113, RHURS-0114, and RHURS-0115 and CHURS-009-IS, which do not express OmpK35, although mutations were not found in the *ompK35* gene of those isolates. However, this mutation was also found in Kp-WT isolates expressing OmpK35. Additionally, CHURS-009-IS also had the E85K mutation in *marR*. e *envZ* gene was found truncated in isolate RHURS-0113 and the *ramR* gene in isolate CHURS-0009-IS.

Table 1 MICs (mg/L) of different antimicrobials against the 28 isolates

ID_Isolate	Relevant β-lactamase	ST	AZT	CTX	CAZ	CZA	FEP	ETP	IMI	IMR	MER	MEV	CIP	GEN
ATCC 13883	SHV-1	3	≤1	≤0,5	≤0,5	≤0,25	≤1	≤0,12	≤1	1	≤0,12	≤0,06	≤0,06	≤0,5
CHURS-5-1534	SHV-11	268	≤1	≤0,5	≤0,5	≤0,25	≤1	≤0,12	≤1	0.12	≤0,12	≤0,06	≤0,06	≤0,5
CHURS-9-1075	SHV-11	17	≤1	≤0,5	≤0,5	≤0,25	≤1	≤0,12	≤1	0.5	≤0,12	≤0,06	≤0,06	≤0,5
CHURS-170652	SHV-26	34	≤1	≤0,5	≤0,5	≤0,25	≤1	≤0,12	≤1	0.12	≤0,12	≤0,06	≤0,06	≤0,5
CHURS-170744	SHV-11	1628	≤1	≤0,5	≤0,5	≤0,25	≤1	≤0,12	≤1	0.5	≤0,12	≤0,06	≤0,06	≤0,5
CHURS-170943	SHV-26	34	≤1	≤0,5	≤0,5	≤0,25	≤1	≤0,12	≤1	0.12	≤0,12	≤0,06	≤0,06	≤0,5
CHURS-171503	SHV-187	6428	≤1	≤0,5	≤0,5	≤0,25	≤1	≤0,12	≤1	0.12	≤0,12	≤0,06	≤0,06	≤0,5
CHURS-171703	SHV-11	1628	≤1	≤0,5	≤0,5	≤0,25	≤1	≤0,12	≤1	0.25	≤0,12	≤0,06	≤0,06	≤0,5
CHURS-174873	SHV-108	6429	≤1	≤0,5	≤0,5	≤0,25	≤1	≤0,12	≤1	0.25	≤0,12	≤0,06	≤0,06	≤0,5
CHURS-175802	SHV-11	1825	≤1	≤0,5	≤0,5	≤0,25	≤1	≤0,12	≤1	0,12	≤0,12	≤0,06	≤0,06	≤0,5
ATCC 700603	SHV-18	489	32	8	16	1	≤1	≤0,12	≤1	0,12	≤0,12	≤0,06	0,25	8
RHURS-0113	SHV-12	38	>32	2	16	0.5	≤1	≤0,12	≤1	0,12	≤0,12	≤0,06	1	>8
RHURS-0114	SHV-12	38	>32	8	>16	0.5	≤1	≤0,12	≤1	0,12	≤0,12	≤0,06	2	>8
RHURS-0115	SHV-12	38	>32	>8	>16	2	>16	2	≤1	0,12	≤0,12	≤0,06	2	>8
RHURS-0039	KPC-2	258	>32	>8	>16	1	16	>2	8	0,25	>16	≤0,06	>2	1
RHURS-0308	KPC-3	512	>32	>8	>16	2	>16	>2	>8	0,12	>16	1	>2	2
CHURS-000952	KPC-3	512	>32	>8	>16	1	>16	>2	>8	0,25	>16	≤0,06	>2	4
CHURS-170942	KPC-3	512	>32	>8	>16	2	>16	>2	>8	0,12	>16	0,5	>2	1
CHURS-175691	KPC-3	512	>32	>8	>16	2	>16	>2	>8	0,25	>16	0,5	>2	2
CHURS-180130	KPC-3	512	>32	>8	>16	2	>16	>2	>8	0,25	>16	0,25	>2	1
CHURS-184677	KPC-3	512	>32	>8	>16	1	16	>2	8	0,12	>16	≤0,06	>2	4
RHURS-0132	OXA-48	15	>32	>8	>16	0.5	32	1	≤1	1	1	0.5	>2	≤0,5
CHURS-009-IS	OXA-48	11	>32	>8	>16	0.5	16	0.5	≤1	0.25	≤0,12	≤0,06	>2	>8
CHURS-000694	OXA-48	15	>32	>8	>16	1	>16	>2	>8	>8	>16	>16	>2	≤0,5
CHURS-000958	OXA-48	15	>32	>8	>16	1	>16	>2	>8	8	>16	>16	>2	≤0,5
CHURS-001262	OXA-48	15	≤1	4	≤0,5	0.5	≤1	>2	≤1	0.5	1	1	>2	≤0,5
CHURS-181073	OXA-48	15	>32	>8	>16	0.5	16	>2	≤1	0,5	0,5	0,25	>2	≤0,5
CHURS-183019	OXA-48	15	>32	>8	>16	≤0,25	16	>2	≤1	1	2	1	>2	1

Aztreonam (AZT), cefotaxime (CTX), ceftazidime (CAZ), ceftazidime/avibactam (CZA), cefepime (FEP), ertapenem (ETP), imipenem (IMI), imipenem/relebactam (IMR), meropenem (MER), meropenem/vaborbactam (MEV), ciprofloxacin (CIP) and gentamicin (GEN)

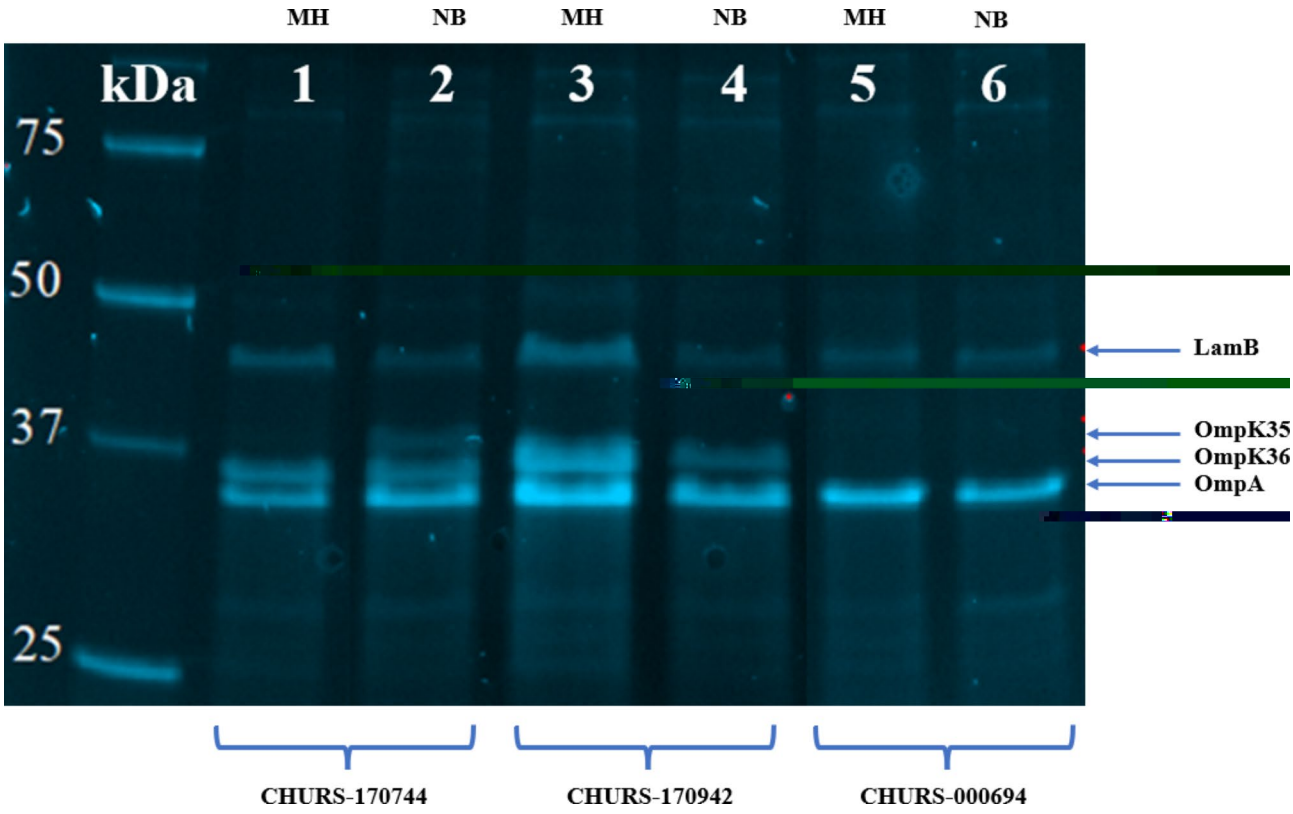

Fig. 1 Analysis of OMPs by SDS-PAGE with precast gels. Lane 1: CHURS-170744 (Kp-WT) in cation adjusted Mueller-Hinton broth (MH), lane 2: CHURS-170744 in nutrient broth (NB), lane 3: CHURS-170942 (Kp-KPC) in MH, lane 4: CHURS-170942 in NB, lane 5: CHURS-000694 (Kp-OXA-48) in MH and lane 6: CHURS-000694 in NB

Discussion

High level resistance to β-lactams in *K. pneumoniae* usually involves both the production of e cient β-lactamases and the loss or structural alteration of porins OmpK35 and OmpK36, with a double mutation in OmpK35-OmpK36 being the cause of most clinically relevant increases in the MICs of cephalosporins and carbapenems. Loss of OmpK35 and OmpK36 also contributes to a moderate (2-4-fold) increase in the MICs of uoroquinolones [15, 30, 31].

For OMPs analysis, we performed the commonly used SDS-PAGE method, comparing homemade and precast gels in terms of reliability, cost and execution time. In the homemade gels, we used a xed concentration of acrylamide (12%) and di erent concentrations of bisacrylamide, because the proximity of the molecular masses of OmpA, OmpK35 and OmpK36 requires very controlled and precise electrophoresis conditions. As previously described [25, 32], porins from di erent clinical isolates can run on gels with di erent apparent molecular masses, and for some isolates, resolution of the two porins requires changing the bisacrylamide concentration. e best resolution of the three bands in the Kp-WT strains cultured in nutrient broth was obtained when the lowest bisacrylamide concentration (0.23%) was used. Although the same results were obtained with homemade and commercial gels for all the samples analysed, better separation was observed with commercial gels, and with these gels, time was saved in gel preparation, electrophoresis and staining.

In this study, bacterial growth was performed with two di erent broths to test osmoregulation processes: cation-adjusted Muller-Hinton broth (high osmolarity) and nutrient broth (low osmolarity). In both types of gels and with MALDI-TOF/MS, Kp-WT isolates expressed OmpK35 when grown in nutrient broth but not in cation-adjusted Muller-Hinton broth. When the gel bands were analyzed by LC-MS/MS, the protein with the apparent size of 35 kDa corresponded to OmpK36 while that running at 36 kDa corresponded to OmpK35 [9, 32]. is is in contrast to the interpretation of the results based on the mobility of the two major *K. pneumoniae* porins in polyacrylamide gels (SDS-PAGE and, additionally, Western-blot), which, traditionally, have been suggesting that the OMP with the apparent highest molecular weight is OmpK36 and that with the lowest molecular weight is OmpK35. is study indicates that using new spectrometric techniques and protein sequence databases would allow for a more accurate and reliable identi cation of these proteins.

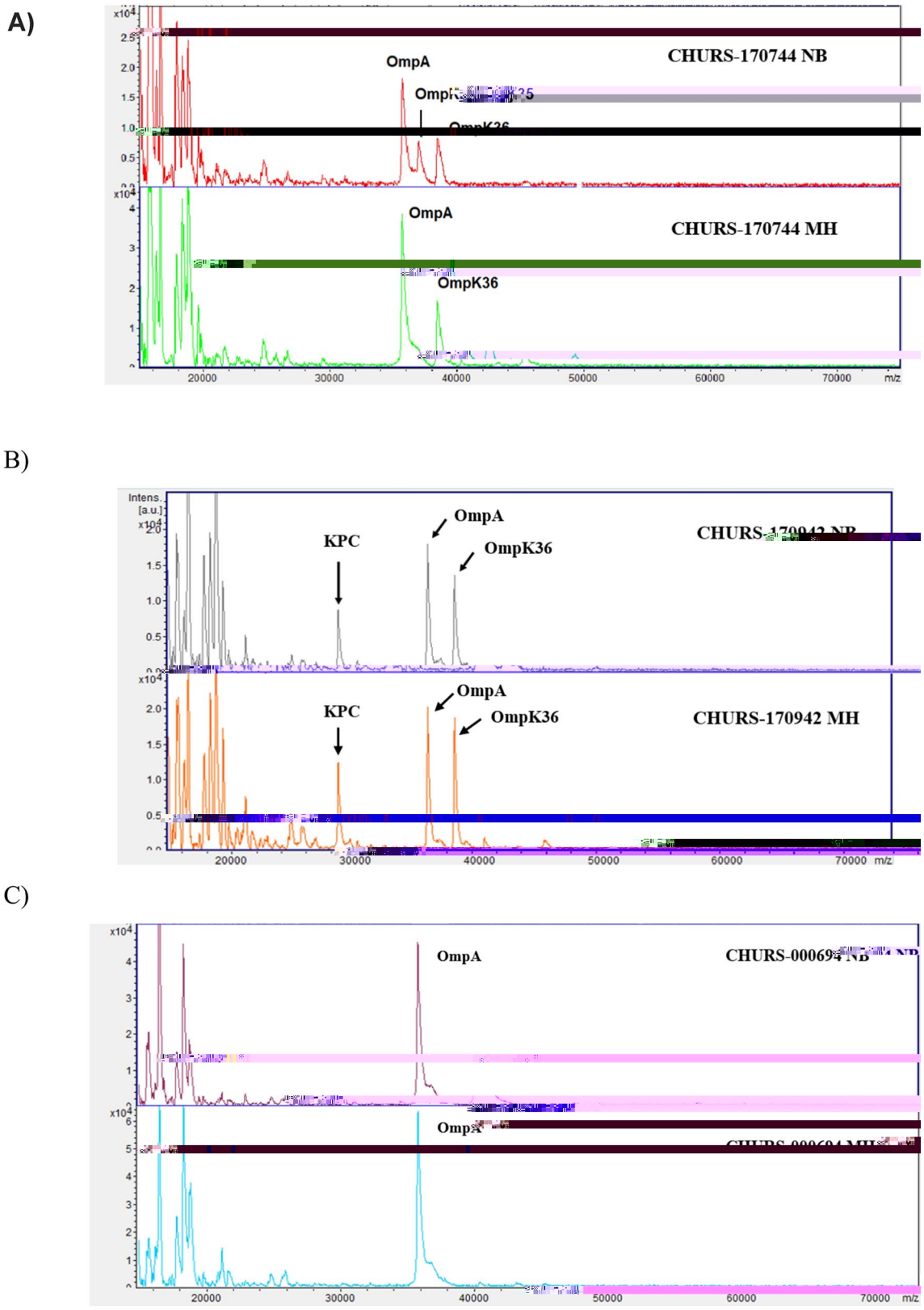

Fig. 2 Analysis of OMPs by MALDI-TOF/MS. The X-axis represents the mass/charge in Daltons (m/z) and the Y-axis represents the relative intensity. (**A**) CHURS-170744 (Kp-WT) in cation adjusted Mueller-Hinton broth (MH) and nutrient broth (NB); (**B**) CHURS-170942 (Kp-KPC), where the KPC and the OmpK36 porin peaks are observed in both media. (**C**) CHURS-000694, where none of the two major porins are observed

Table 2 Expression of porins grown in nutrient broth, mutations in the corresponding genes and molecular weight

ID_Isolate	OmpA				OmpK35				OmpK36			
	Protein expression	WGS	MW WGS (kDa)	MW MALDI-TOF/MS (kDa)	Protein expression	WGS	MW WGS (kDa)	MW MALDI-TOF/MS (kDa)	Protein expression	WGS	MW WGS (kDa)	MW MALDI-TOF/MS (kDa)
ATCC 13883	+	Reference	35.96	35.75	+	Reference	37.23	37.03	+	Reference	38.69	38.55
CHURS-5-1534	+	NM	35.96	35.65	+	NM	37.23	36.94	+	NM	38.69	38.45
CHURS-9-1075	+	NM	35.96	35.77	+	NM	37.23	37.04	+	M	37.97	37.77
CHURS-170652	+	NM	35.96	35.76	+	NM	37.23	37.04	+	M	38.12	37.93
CHURS-170744	+	NM	35.96	35.77	+	NM	37.23	37.07	+	M	37.96	37.79
CHURS-170943	+	NM	35.96	35.77	+	NM	37.23	37.03	+	M	38.12	37.93
CHURS-171503	+	NM	35.96	35.75	+	NM	37.23	37.04	+	M	37.94	37.77
CHURS-171703	+	NM	35.96	35.76	+	NM	37.23	37.05	+	M	37.96	37.78
CHURS-174873	+	NM	35.96	35.75	+	NM	37.23	37.06	+	M	37.89	37.70
CHURS-175802	+	NM	35.96	35.77	+	NM	37.23	37.02	+	NM	38.69	38.58
ATCC 700603	+	M	35.96	35.75	-	M	37.25	X	+	M	37.54	37.36
RHURS-0113	+	NM	35.96	35.76	-	NM	37.23	X	+	M	37.98	37.81
RHURS-0114	+	NM	35.96	35.76	-	NM	37.23	X	+	M	37.98	37.79
RHURS-0115	+	NM	35.96	35.76	-	NM	37.23	X	-	P	37.98	X
RHURS-0039	+	P	36.13	35.97	-	Stop-codon-67	8.01	X	+	M	37.96	37.80
RHURS-0308	+	NM	35.96	35.76	-	Stop-codon-67	8.01	X	+	M	38.13	37.95
CHURS-000952	+	NM	35.96	35.84	-	Stop-codon-67	8.01	X	+	M	37.96	37.85
CHURS-170942	+	NM	35.96	35.76	-	Stop-codon-67	8.01	X	+	M	38.13	37.94
CHURS-175691	+	NM	35.96	35.76	-	Stop-codon-67	8.01	X	+	M	38.13	37.95
CHURS-180130	+	NM	35.96	35.75	-	Stop-codon-67	8.01	X	+	M	38.13	37.96
CHURS-184677	+	NM	35.96	35.76	-	Stop-codon-67	8.01	X	+	M	37.96	37.77
RHURS-0132	+	NM	35.96	35.89	-	Stop-codon-15	1.57	X	-	Stop-codon-65	7.27	X
CHURS-009-IS	+	NM	35.96	35.87	-	P	37.45	X	+	M	37.96	37.90
CHURS-000694	+	NM	35.96	35.85	-	Stop-codon-15	1.57	X	-	Stop-codon-171	18.65	X
CHURS-000958	+	NM	35.96	35.83	-	Stop-codon-15	1.57	X	+	M	37.96	37.84
CHURS-001262	+	NM	35.96	35.84	-	Stop-codon-15	1.57	X	+	M	37.96	37.89
CHURS-181073	+	NM	35.96	35.83	-	Stop-codon-15	1.57	X	+	M	37.96	37.85
CHURS-183019	+	NM	35.96	35.84	-	Stop-codon-15	1.57	X	+	M	37.96	37.83

Presence (+) / absence (-) of band or peak. NM (no wt mutation). M (multiple mutations). P (point mutation). Stop-codon-x (position of the protein with a stop codon). X (no peaks)

Porin expression in *K. pneumoniae* and other clinically relevant organisms has traditionally been evaluated by SDS-PAGE, but this is a laborious and time-consuming methodology. For this reason, alternative techniques are being sought. MALDI-TOF/MS has been introduced in clinical laboratories in recent years for primary identi cation of bacteria and fungi, but other applications have been developed in the study of antimicrobial resistance. Several studies have validated MALDI-TOF/MS as a reliable approach for detection of carbapenemases [20, 33], and other investigators have already identi ed OMPs in *K. pneumoniae* using MALDI-TOF/MS. In the studies by Cai JC et al. [27] and Pinto NA et al. [34] OmpA, OmpK35, and OmpK36 corresponded to peaks of ~36,000, ~37,000, and ~38,500 m/z. In our study, we have obtained similar m/z values for the same proteins, with the advantage of performing a rapid protein extraction assay by optimizing the ROMP method [26], allowing us to obtain protein extracts for MALDI-TOF/MS in only 1.5 h. All strains tested showed the same results in SDS-PAGE and MALDI-TOF/MS, so we can consider that the mass spectrometry technique is a fast, reliable and easy to use procedure to detect porin loss in *K. pneumoniae* isolates. Furthermore, protein extraction of all Kp-KPC isolates studied by ROMP and subsequent analysis by MALDI-TOF/MS showed a peak at ~28,550 m/z, corresponding to the KPC enzyme [35, 36].

In general, WGS provides an accurate genetic description of the mutations present in the porin genes, although it does not always correlate with the results obtained by SDS-PAGE and MALDI-TOF/MS.

ESBL-producing *K. pneumoniae* strains express OmpK36 alone more frequently than strains lacking these enzymes, which generally produce both OmpK35 and OmpK36 [15]. Interestingly, although the OmpK35 band was not observed in the studied Kp-ESBL strains, we did not identify any mutation in the *ompK35* sequence, indicating that additional regulation leading to altered gene expression has occurred [37, 38]. RHURS-0115 strain, unlike the other ESBL strains, did not show an OmpK36 band/peak by proteomic studies, but it had a single mutation located in the signal peptide coding region, which may be related to the observed OmpK36 loss. Additional studies are warranted to evaluate this possibility.

Numerous mutations of the OmpK35 protein have already been reported, especially in isolates carrying KPC enzymes. A very common mutation in OmpK35 in isolated with ST512 and 258 [39, 40] was found in all Kp-KPC isolates in our study, which is in agreement with the SDS-PAGE and MALDI-TOF/MS results, where only one band/peak belonging to OmpK36 was observed. In four of the seven Kp-KPC strains, L3 loop modi cations in OmpK36, including a duplication of amino acids G115-D116, located in the highly conserved motif PEFGGD were observed. L3 is not exposed on the surface, but folds towards into the barrel, forming a constriction zone in the center of the channel that determines the size exclusion limit and ionic selectivity of the pore. e G115-D116 mutation contributes to resistance to meropenem, imipenem, ertapenem and cephalosporins [40–43].

In all ST15 Kp-OXA-48 isolates, a premature stop codon was identi ed in the porin gene *ompK35*, but the contribution of this alteration to clinically relevant resistance is doubtful if the organisms still express OmpK36, as this porin still facilitates permeation of drugs throughout the outer membrane.

e *mar* locus is an operon with two divergent transcriptional units, *marC* and *marRAB* separated by the operator *marO*. Under normal conditions, MarR represses the *marRAB* operon by binding to two sequences within the *marO* operator. Expression of *marA* induces a decrease in OmpF in *E. coli*. is negative regulation is carried out by transcription of the untranslated antisense RNA *micF* located upstream of the gene encoding the OmpC porin [44–46]. In isolates RHURS-0113 (Kp-ESBL), RHURS-0114 (Kp-ESBL), RHURS-0115 (Kp-ESBL) and CHURS-009-IS (Kp-OXA-48) the S82G mutation was found in the *marR* gene, but this mutation was also found in all Kp-WT isolates (expressing OmpK35) evaluated, indicating that this mutation should not be the cause of the loss of OmpK35. Isolate CHURS-0009-IS had an additional mutation (E85K) in *marR* and also had a truncated *ramR* gene. e *envZ* gene was found truncated in isolate RHURS-0113, but further studies are needed to determine the relationship of these mutations with the OmpK35 disruption. Because of the complex regulation of OMP genes, the simple analysis of their sequences, which are easily obtained by WGS, cannot always anticipate the expression of the corresponding proteins. Accordingly, the combined study of porin expression and analysis of porin genes is recommended for a reliable evaluation of the role of porins in antimicrobial resistance.

As a limitation of this study, we have only considered a single species, *K. pneumoniae*, and a rather limited number of isolates (those with the most frequent phenotypes identi ed in clinical practice). Porin analysis in *K. pneumoniae* producing plasmid-mediated AmpC showing resistance to carbapenems seems of particular interest. Additional research on other gram-negative bacteria is also warranted.

Abbreviations

CLSI	Clinical and Laboratory Standards Institute
ESBL	Extended-spectrum β-lactamase producers
EUCAST	European Committee on Antimicrobial Susceptibility Testing
LC-MS/MS	Liquid chromatography coupled to mass spectrometry

MALDI-TOF/MS	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry
MIC	Minimal inhibitory concentrations
OM	Outer membrane
ROMP	Rapid outer membrane protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
ST	Sequence type
TFA	Tri□uoroacetic acid
WGS	Whole genome sequencing
WT	Wild-type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12941-024-00761-9.

Supplementary Material 1

Supplementary Material 2

Author contributions

CEL: acquisition of data, data analysis and interpretation, writing and editing. MMR, JGP, EPN and ECG: data analysis and interpretation. EPN and ECG: review and editing, LMM: funding acquisition, conceptualization of the study, data analysis and interpretation, writing and □nal approval of the version to be submitted.

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Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (https://proteomecentral.proteomexchange.org) via the PRIDE (47) partner repository with the dataset identi□er PXD053879.

Declarations

Ethics approval and consent to participate

Human Ethics and Consent to Participate declarations: not applicable.

Competing interests

The authors declare no competing interests.

Transparency declarations

All authors: none to declare.

Author details

[1]Reina Sofía University Hospital, Maimonides Biomedical Research Institute of Cordoba, University of Cordoba (IMIBIC/HURS/UCO), Cordoba, Spain
[2]CIBER de Enfermedades Infecciosas (CIBERINFEC), Instituto de Salud Carlos III (ISCIII), Madrid, Spain
[3]Unit of Microbiology, Reina Sofía University Hospital, Cordoba, Spain
[4]Department of Agricultural Chemistry, Soil sciences and Microbiology, University of Cordoba, Cordoba, Spain
[5]IMIBIC Mass Spectrometry and Molecular Imaging Unit (IMSMI), Reina So□a University Hospital, Maimonides Biomedical Research Institute of Cordoba, University of Cordoba (IMIBIC/HURS/UCO), Cordoba, Spain

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