Nosocomial, Multidrug-Resistant *Klebsiella pneumoniae* Strains Isolated from Mexico City Produce Robust Biofilms on Abiotic Surfaces but Not on Human Lung Cells

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Background: *Klebsiella pneumoniae* (*Kpn*) strains are a leading cause of hospital-acquired infections, including ventilator-associated pneumonia. Resistance to antibiotics, biofilm formation, and the production of certain fimbriae play an important role in the pathogenesis.
Aim: We investigated the genetic relatedness, antibiotic resistance, virulence potential, and ability to form biofilms of *Kpn* strains isolated from hospital-acquired infections (*n* = 76). Strains were isolated at three major hospitals serving the largest metropolitan urban area in Mexico City, Mexico.
Results: Enterobacterial repetitive intergenic consensus (ERIC)–PCR demonstrated that clonal groups predominate in each hospital. Selected strains chosen from clonal groups (*n* = 47) were multidrug resistant (MDR, 83%), although the majority (∼70%) were susceptible to carbapenems. All strains produced robust biofilms on abiotic surfaces, and ∼90% harbored adhesin genes *fimH*, *mrkA*, and *ecpA*. The ultrastructure of biofilms was further studied by high-resolution confocal microscopy. The average height of *Kpn* biofilms on abiotic surfaces was ∼40 μm. We then assessed formation of biofilms on human lung cells, as a surrogate of lung infection. While *Kpn* strains formed robust biofilms on abiotic surfaces, studies on lung cells revealed attachment to human cells but scarce formation of biofilms. Gene expression studies revealed a differential temporal expression of an adhesin (*ecpA*) and a capsule (*galF*) gene when biofilms were formed on different substrates.
Conclusions: *Kpn* strains isolated from nosocomial infections in Mexico City are MDR, although the majority are still susceptible to carbapenems and form more robust biofilms on polystyrene in comparison to those formed on human cells.

Keywords: *K. pneumoniae*, ERIC-PCR, multidrug resistance, biofilms, abiotic surfaces, gene expression

Introduction

KLEBSIELLA PNEUMONIAE (*KPN*) IS a Gram-negative bacterium ubiquitously found in different environments, including the human body, where it commonly resides in the gastrointestinal tract, skin, and nasopharynx.[1,2] When favorable conditions are met, *Kpn* strains are opportunistic pathogens of humans and animals causing a wide range of hospital-acquired infections, particularly among immunocompromised individuals, including pneumonia, cystitis, pyelonephritis, osteomyelitis, meningitis, bacteremia, and septicemia.[1]

In the United States, there were ∼800,000 hospital-acquired infections in 2011 leading to U.S. ∼$6 billion in medical expenses.[3] In the same study, *Kpn* was the third most commonly isolated pathogen.[3] In developing countries, such as Mexico, hospital-acquired infections display double the prevalence observed in industrialized countries; thus, an estimated ∼21% of hospitalized patients in Mexico will develop a hospital-acquired infection. Hospital-acquired infections caused by *Kpn* are commonly chronic and difficult to treat due to important virulence traits, including the production of a thick capsule, antibiotic resistance, and biofilm formation.[2,4]

Kpn strains isolated from hospital-acquired infections often display multidrug-resistant (MDR) phenotypes caused by the presence of extended-spectrum β-lactamases or carbapenemases, making it difficult to choose appropriate antibiotics

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for the treatment.[2] A recent article reports a *Kpn* strain already resistant to ceftazidime–avibactam, the first antimicrobial approved by the U.S. FDA for the treatment of carbapenem-resistant *Kpn* strains.[5] A survey by Fernández-Canigia and Dowzicky found that *Kpn* strains isolated from 12 Latin American countries were resistant to cephalosporins, including ceftriaxone (97.8%), ceftazidime (81.1%), cefepime (58.8%), and also to levofloxacin (56.4%), amoxicillin/clavulanic acid (56.3%), and piperacillin/tazobactam (44.7%).[6] A very similar trend of resistance to antibiotics by *Kpn* strains has been reported by different groups for strains isolated in Mexico.[7–11] Carbapenems such as imipenem and meropenem, however, are still active against *Kpn* strains isolated in Latin America (>90%)[6] and in Mexico.[7–11] On the contrary, strains isolated from outbreaks in different hospitals in Mexico have not only demonstrated resistance to cephalosporins and quinolones, but also to carbapenems.[12,13]

Kpn strains have the ability to produce biofilms on abiotic surfaces, which is directly associated to hospital-acquired infections, given that structures form, and persist, on indwelling devices.[14,15] Biofilm structures protect *Kpn* from commonly used disinfectants, antibiotics, and the attack of host immune responses near the site of contact between the human body and the indwelling devices.[16,17] *Kpn* biofilms formed on abiotic surfaces have been associated with production of type 1 and type 3 fimbriae[4,18,19] and the *Escherichia coli* common pili (Ecp).[20] The capsule, a key virulence factor for pathogenesis, appears to be involved in early stages of biofilm formation as *Kpn* strains with mutations affecting the production of capsule, had a decreased biomass when compared with the wild-type strain.[21,22] Regulation of biofilms has been linked, in part, to a c-di-GMP-dependent MrkH circuit, which upregulates the production of type 3 fimbriae.[23,24] Supporting the existence of a tightly regulated mechanism of biofilm formation, studies have demonstrated that biomass varies when *Kpn* forms biofilms on different abiotic surfaces, including polystyrene, polypropylene, and catheters.[23]

In this study, we isolated *Kpn* strains from hospital-acquired infections in three major hospitals of Mexico City, including hospitals attending only children and two hospitals attending all age groups. Strains were mainly isolated from bacteremia cases (53%), followed by ventilator-associated pneumonia (VAP; 17%) and urinary tract infection (UTI; 10%). We investigated the genetic relatedness, antibiotic resistance, virulence potential, and ability to form biofilms of these strains. The clonality was evaluated by enterobacterial repetitive intergenic consensus (ERIC)-PCR. Antibiotic resistance profile and virulence traits were sought. Since biofilms produced in indwelling medical devices have been implicated as a main source of *Kpn* in hospital-acquired infections, the biofilm phenotype, ultrastructure of biofilms produced by selected strains, and temporal expression of genes involved in biofilm formation were also studied.

Materials and Methods

Isolation and identification of K. pneumoniae strains and bacterial culture media utilized

We collected 76 *Kpn* strains, in the year of 2013, from hospital-acquired infections (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/mdr) at three different hospitals in Mexico City: National

Medical Center "20 de Noviembre" (NMC20N), National Medical Center "La Raza" (NMCR), and the National Institute of Pediatrics (INP). Hospital-acquired infections were determined in each hospital following international standards and criteria published by the Centers for Disease Control and Prevention (CDC).[3] Strains were isolated from specimens obtained for routine testing of nosocomial pathogens at the mentioned hospitals, so neither the Institutional Review Board (IRB) approval nor informed consent were required from adult patients, or parents or legal guardians of children. Strains were identified (one strain from each specimen obtained from patients with documented nosocomial infection) at the genus and species level by a conventional method using EnteroPluri-Test system (Liofilchem, Italy) followed by a molecular identification method that amplified a 108 bp-specific fragment of the *rpo*B gene using primers listed in Table 1. PCR conditions were previously described.[25] Reference, genome-sequenced, strain RYC492 was also utilized in this study.[26] Bacterial culture media utilized throughout the study included the following: Luria-Bertani (LB) (DIBICO, Mexico), Trypticase soy agar (TSA) (Becton-Dickinson, NJ), and Mueller-Hinton agar plate (Becton-Dickinson).

Cell line culture

Human lung Calu-3 cells (ATCC HTB-55) were cultured in minimal essential medium (MEM 1×) (Gibco by Life Technologies, NY) with 10% of heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). Flasks were incubated at 37°C in a 5% CO_2 atmosphere. Cells were detached with 0.25% trypsin and resuspended in 10 ml of MEM 1×.

DNA extraction

All cultures were grown on LB agar plates at 37°C for 18 hr. Genomic DNA of each strain was obtained with the InstaGene Matrix Kit (Bio-Rad, Singapore) as per the instructions provided by the manufacturer. Once purified, genomic DNA was quantified using an AQ-07 AmpliQuant Nucleic Acid Photometer (AmpliQuant) and stored at −80°C until used.

ERIC-typing

DNA fingerprints analysis of *Kpn* isolates by ERIC-PCR was done as previously described[27] using primers listed in Table 1. Band profiles were used to build a binary matrix which was utilized to generate a dendrogram using Jaccard similarity index and the PAST software (version 3.13).

Antimicrobial susceptibility testing

The Kirby–Bauer disc diffusion method was used to investigate susceptibility to antibiotics, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[28] The following antibiotic discs were used (all purchased from Becton-Dickinson): gentamicin (GM) 10 μg, amikacin (AN) 30 μg, piperacillin+tazobactam (TZP) 100/10 μg, imipenem (IPM) 10 μg, meropenem (MEM) 10 μg, ertapenem (ETP) 10 μg, cefazolin (CZ) 30 μg, ceftriaxone (CRO) 30 μg, ceftazidime (CAZ) 30 μg, cefepime (FEP) 30 μg, cefoxitin (FOX) 30 μg, ciprofloxacin (CP) 5 μg, trimethoprim–sulfamethoxazole (SXT) 1.25/23.75 μg, aztreonam (ATM) 30 μg, ampicillin (AMP) 10 μg, piperacillin (PIP) 100 μg, ampicillin/sulbactam (SAM)

TABLE 1. PRIMERS USED IN THIS STUDY

Primer	Sequence (5′–3′)	Target	Expected size (bp)	Reference
ERIC 1	ATGTAAGCTCCTGGGGATTCAC	ERIC sequences	Variable	Versalovic et al.[27]
ERIC 2	AAGTAAGTGACTGGGGTGAGC			
rpoF	CAACGGTGTGGTTACTGACG	rpoB	108	Chander et al.[25]
rpoR	TCTACGAAGTGGCCGTTTTC			
fimHF	CGCCTGGTCCTTTGCCTGCA	fimH	817	Cruz-Córdova et al.[7]
fimHR	CTGCACGTTGCCGGCGGTAA			
mrkAF	GTTAACGGCGGCCAGGGCAGCGA	mrkA	382	Cruz-Córdova et al.[7]
mrkAR	AGGTGAAACGCGCGCCATCA			
ecpAF	AATGGTTCACCGGGACATCATGTCC	ecpA	759	Cruz-Córdova et al.[7]
ecpAR	AAGGATGAAATATCGCCGACATCC			
qcfaF	GATGATGGAGAAAGTGAAACCAT	Ecp	216	This work
qcfaR	CGTCTTATCACCAACACCTTAAC			
qgalFF	CAAAGGCAATTCCAAAGGAG	galF	156	This work
qgalFR	GCTCGTAGGAGGTGTCGAAG			
qrpoDF	TAAGGAGCAAGGCTATCTGACC	rpoD	117	Tan et al.[23]
qrpoDR	ACCTGAATACCCATGTCGTTG			

10/10 μg, amoxicillin+clavulanic acid (AMC) 20/10 μg, tetracycline (TE) 30 μg, and nitrofurantoin (F/M) 300 μg. Isolates were regarded as susceptible, intermediate, or resistant using breakpoints set by the CLSI. Strains were further classified as MDR if they were nonsusceptible to ≥1 agent in ≥3 antimicrobial categories and extensively drug resistant (XDR) if strains were nonsusceptible to ≥1 agent in all but ≤2 categories.[29] Kpn ATCC 700603 and E. coli ATCC 25922 were used as a control.

Quantification of biofilm biomass

To measure biofilm biomass, we used the classic method with Crystal Violet (0.5% w/v) that has been widely utilized to investigate biofilms of human pathogens, including Kpn.[20,30,31] Isolates were cultured on TSA plates overnight at 37°C. These cultures were utilized to make bacterial suspensions adjusted at 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/ml) and $\sim 3 \times 10^5$ CFU/ml were inoculated into a 24-well polystyrene plate (Corning Costar, NY) containing trypticase soy broth (Becton-Dickinson) and incubated at 37°C for 24 hr. Planktonic bacteria were removed and biofilms attached to the bottom of each well were washed with sterile distilled water. Biofilms were fixed with 1 μl of glyceraldehyde 2.5% (v/v) and incubated for 3 min at room temperature. Fixed biofilms were washed three times with sterile distilled water and stained with 1 ml of Crystal Violet 0.5% for 10 min at room temperature. After two washes with distilled water, Crystal Violet was solubilized with 2 ml of ethanol–acetone (80/20, v/v) and the absorbance for each well was measured at 570 nm in a spectrophotometer Optimus 10000 xs (Spectronic 20D Genesys). The isolates were classified, based on the optical density of the assay, as high ($OD_{570nm} \geq 0.501$), moderate ($OD_{570mn} = 0.101–0.500$), or low biofilm producers ($OD_{570nm} \leq 0.100$). Pseudomonas aeruginosa PAO1, a high biofilm former, was used as a positive control and E. coli K12 was used as a negative control.[31,32] Each strain was tested at least five times.

Confocal microscopy analysis of K. pneumoniae biofilms

(a) Biofilms formed on abiotic surfaces. Bacterial suspensions were adjusted at 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/ml) and $\sim 1.5 \times 10^6$ CFU were inoculated into a Chamber Slide™ (eight-well glass slide) (Lab-Tek, NY) containing LB broth and biofilms were incubated at 37°C for 24 hr.

(b) Biofilms formed on human lung Calu-3 cells. Polarized, confluent (100%), monolayers of human lung Calu-3 cells grown on an eight-well glass slide (Lab-Tek) were fixed 15 min with 2% paraformaldehyde (PFA) at room temperature, thoroughly washed with phosphate-buffered saline (PBS) to eliminate residual PFA, and MEM containing 5% of heat-inactivated FBS was added. Immobilized human lung cells were infected with $\sim 1.5 \times 10^6$ CFU of Kpn and biofilms were incubated for 24 hr at 37°C.

Whether produced on an abiotic surface or on human lung cells, culture medium was removed and each well was washed twice with PBS and fixed with 2% PFA for 15 min at room temperature. Then, each well was washed three times with PBS and blocked with 2% bovine serum albumin for 1 hr. Biofilms were stained with rabbit polyclonal anti-Klebsiella antibodies (1.1 μg/ml) (Thermo Fisher Scientific, IL) for 1 hr at room temperature followed by a goat anti-rabbit secondary antibody conjugated to Alexa Fluor 555 (Molecular Probes). Bacterial DNA was stained with TO-PRO3 (Molecular Probes). Stained biofilms were finally washed three times with PBS, mounted with ProLong Diamond Antifade Mountant (Molecular Probes) and analyzed with an Olympus FV1000 confocal microscope. Confocal images were analyzed with ImageJ version 1.49k (National Institutes of Health) and The Imaris software (Bitplane, CT).

Molecular detection of adhesin-encoding genes

Conventional PCR was used to detect genes encoding for type 1, type 3, or Ecp adhesins, that is, genes fimH, mrkA, or ecpA, respectively. PCR primers are listed in Table 1. The reaction was carried out with 5 μl of 10× PCR buffer, 1.8 μl of MgCl₂ (50 mM), 7.5 μl dNTPs (2 mM), 1 μl of primer F (100 μM), 1 μl of primer R (100 μM), 0.5 μl of Taq polymerase (5 U/μL), 2 μl of DNA (80–100 ng), and enough molecular-grade water to adjust to a total of 50 μl. Reactions were performed under the following conditions: initial

denaturing at 92°C for 5 min and 35 cycles of 92°C for 60 sec, 56°C for 45 sec, and 72°C for 45 sec, with a final extension at 72°C for 5 min.

Phenotypic detection of production of siderophores

The Chrome Azurol S (CAS) assay was used to determine the production of siderophores. Approximately 1.5×10^5 CFU of each strain was inoculated into CAS medium and incubated at 37°C for 24 hr. The presence of a yellow or orange halo around the colony in the media was indicative of siderophores production.[33] *Aeromonas hydrophila* ATCC 7966[T] and *Aeromonas caviae* Sch3 were used as a control for siderophores production.[34]

Gene expression studies

We performed gene expression assays using biofilms harvested from six-well plates cultured on an abiotic surface (polystyrene, *i.e.*, plastic), immobilized Calu-3 cells (using 2% PFA), or on live Calu-3 cells. Plates were infected with $\sim 3.5 \times 10^7$ CFU of *Kpn* RYC492. Planktonic cells and biofilms were obtained at 2, 4, 8, and 24 hr postincubation at 37°C in 5% of CO_2 atmosphere. RNA was purified from planktonic cells and biofilms using the RNeasy® Mini Kit (Qiagen, MD) according to the manufacturer's instructions. To eliminate DNA contamination, RNA preps were treated with TURBO DNA-free™ (Ambion, Life Technologies) and 50 ng of purified, DNA-free, RNA was reverse transcribed into cDNA with iScript™ Reverse Transcription system (Bio-Rad). qPCRs with iQ™ SYBR® Green Supermix (Bio-Rad) were used to measure the relative expression of *cfa* (*ecpA* homologous gene in *Kpn*) and *galF* with primers listed in Table 1. Recommended efficiency of reactions (>90% to <110%)[35] using these primers was confirmed. Reactions were carried out with 300 nM primers and 2 μl of cDNA in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) under the following conditions: 1 cycle at 95°C for 3 min and 40 cycles of 95°C for 15 sec, 57°C for 30 sec, and 72°C for 30 sec. Melting curves were generated by a cycle of 95°C for 1 min, 55°C for 1 min, and 80 cycles starting at 55°C with 0.5°C increments.[36,37] Average CT values were normalized to the *rpoD* gene, and the fold differences were calculated using the comparative C_T method ($2^{-\Delta\Delta CT}$)[38] with the Bio-Rad CFX manager software, version 3.0 (Bio-Rad).

Results

Genetic relatedness of K. pneumoniae *strains isolated from nosocomial infections across three major hospitals in Mexico City*

Kpn strains (*n* = 76) were isolated in three different hospitals, mainly from hospital-acquired infections, including bacteremia (*n* = 40), VAP (*n* = 13), UTI (*n* = 8), wound infection (WI; *n* = 7), meningitis (*n* = 4), upper respiratory infection (*n* = 3), and diarrhea (*n* = 1) (Supplementary Table S1). Genetic relatedness was investigated by ERIC-PCR analysis and demonstrated 47 unique patterns (Fig. 1 and Supplementary Fig. S1). Strains were positioned in two clusters. The smallest cluster included four *Kpn* strains isolated from the same hospital (NMCR), three of which were isolated from UTI and one isolated from VAP. The second cluster contained *Kpn* strains

isolated across the three hospitals. Regardless of where the hospital-acquired infection strains were isolated from (*e.g.*, bacteremia, UTI, etc.), genetic relatedness was observed in strains isolated from the same hospital (Fig. 1). For example, *Kpn* strains isolated at the INP from hospital-acquired infections in children formed a clonal group within the second cluster, separated from strains infecting adults isolated from NMCLR.

Antibiotic resistance among K. pneumoniae *strains*

Since antibiotic resistance of *Kpn* strains isolated from hospital-acquired infections has been increasingly reported,[6,14] antibiotic susceptibility was evaluated in 47 representative *Kpn* strains belonging to most clonal groups identified in Fig. 1. Our studies revealed that >60% of strains were resistant to ampicillin (AMP), piperacillin (PIP), cefazolin (CFZ), piperacillin/tazobactam (TZP), ceftriaxone (CRO), nitrofurantoin (NIT), ampicillin/sulbactam (SAM), amoxicillin/clavulanic acid (AMC), and ceftazidime (CAZ), whereas <30% of strains were resistant to imipenem (IPM), ertapenem (ETP), and cefoxitin (FOX) (Fig. 2 and Supplementary Fig. S2). Based on antibiotic resistance profiles, strains were grouped using the Jaccard similarity index and most strains (*n* = 38, 80%) were classified as MDR, whereas one *Kpn* strain showed resistance to all antibiotics (XDR) tested in this study (Supplementary Fig. S2). Only 17% of strains (*n* = 8) had a wild-type phenotype, that is, they were resistant to less than three categories of antibiotics. Therefore, in this study, we found that ∼83% of *Kpn* strains tested displayed resistance to multiple antibiotics.

K. pneumoniae *biofilms*

Kpn strains were isolated from hospital-acquired infections, therefore strains may possess virulence factors allowing them to survive in the hospital environment and cause infection. Given that biofilm-related infections account for ∼50% of all nosocomial infections,[3] we investigated the ability of strains to form biofilms. Our studies demonstrate that all strains were able to produce biofilms on abiotic surfaces, that is, polystyrene. Most of them produced high biofilm biomass, in comparison to the control strain, and therefore were classified as high biofilm producers (72% [*n* = 34]) or moderate producers (28% [*n* = 13]) (Table 2).

Kpn's ability to form biofilms has been attributed to the production of three main fimbriae named type 1, type 3, and the Ecp.[2,20] To assess whether the isolated *Kpn* strains carry genes involved in the production of these fimbriae, genes *fimH*, *mrkA*, and *ecpA* were screened by PCR as a marker for type 1, type 3, or Ecp, respectively. Genes were carried by >89% of strains (Table 2). Using the χ^2 test, there was no statistically significant difference in the carriage of any of the above genes when high biofilm producers were compared with moderate biofilm producers (not shown).

Ultrastructure of biofilms formed by K. pneumoniae *strains on abiotic surfaces*

Despite *Kpn* strains being recognized for their ability to form biofilms, the ultrastructure of *Kpn* biofilms has not been thoroughly investigated. We selected strains producing
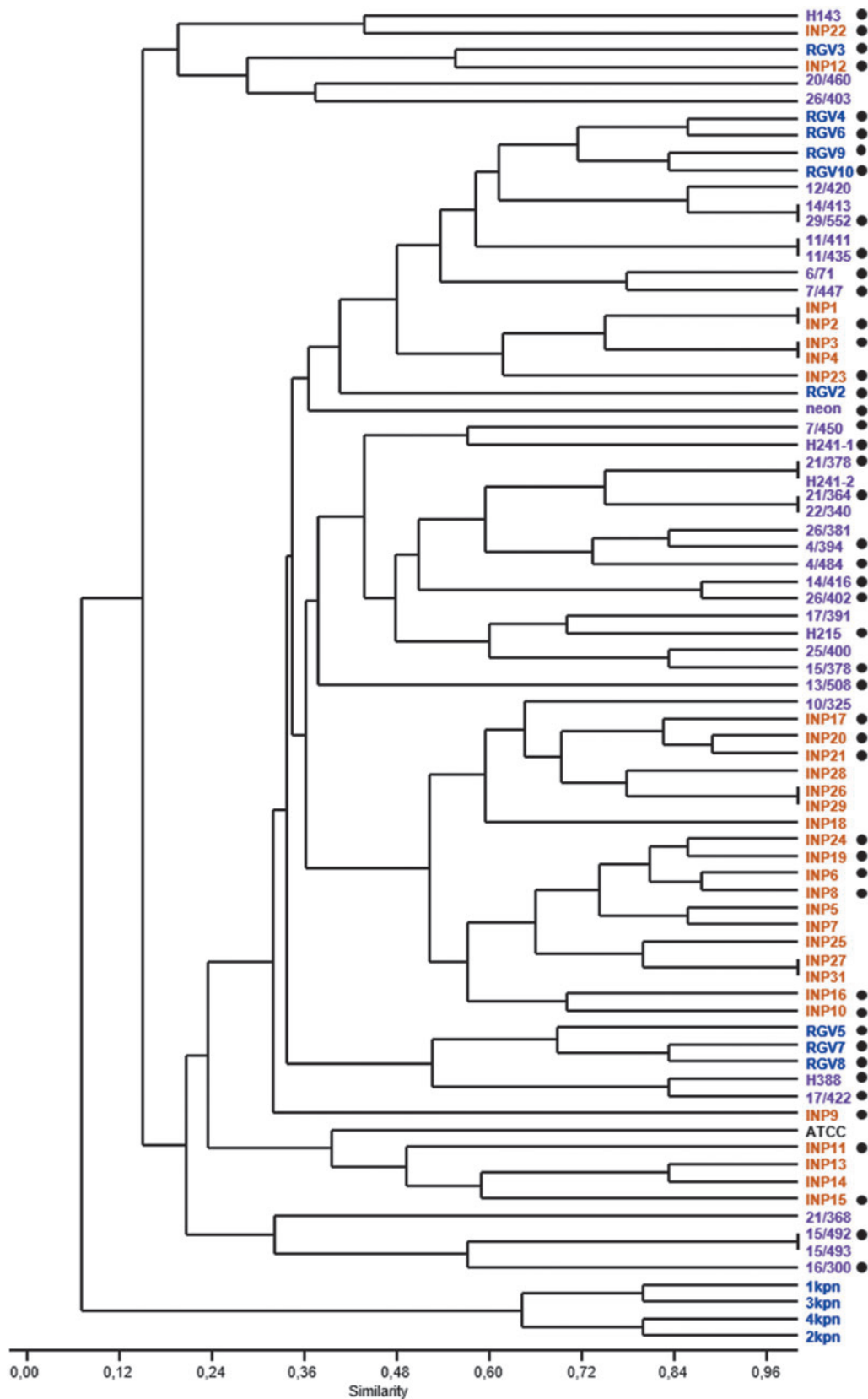
FIG. 1. Genetic relatedness of *Klebsiella pneumoniae* strains isolated from cases of hospital-acquired infection in Mexico City. *K. pneumoniae* strains isolated from National Medical Center ''20 de Noviembre'' (NMC20N, *purple*), National Medical Center ''La Raza'' (NMCLR, *blue*), National Institute of Pediatrics (INP, *orange*), were analyzed by ERIC-PCR. A matrix that considered the presence, or absence, of PCR products was entered into the PAST software, and a dendrogram was generated. The Jaccard similarity index is shown. A *black dot* at right indicates *K. pneumoniae* strains representative from all divergent strains and hospital or isolation that were chosen for further analysis. ERIC, enterobacterial repetitive intergenic consensus.
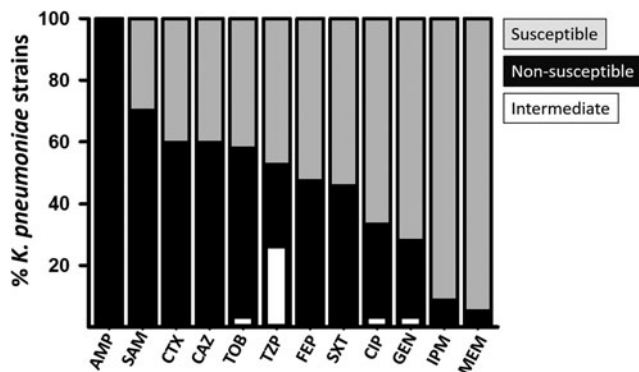
FIG. 2. Antibiotic susceptibility of *K. pneumoniae* strains. Selected *K. pneumoniae* strains ($n = 47$) were tested for susceptibility to antibiotics as specified in the Materials and Methods section and classified as resistant, intermediate resistance (both grouped within the nonsusceptible group), and susceptible based on criteria established by the CLSI. Antibiotics used were the following: ampicillin (AMP), ampicillin/sulbactam (SAM), cefotaxime (CTX), ceftazidime (CAZ), tobramycin (TOB), piperacillin+tazobactam (TZP), cefepime (FEP), trimethoprim-sulfamethoxazole (SXT), ciprofloxacin (CIP), gentamicin (GEN), imipenem (IPM), and meropenem (MEM). CLSI, Clinical and Laboratory Standards Institute.

high biofilm biomass as detected by Crystal Violet, including reference strain *Kpn* RYC492. As shown in Fig. 3, biofilms made by strain RYC492 produced structures that initiated concentrically and then matured toward the top, resembling domes. Biofilm structures were built up from specific points of initiation, that is, not all attached bacteria produced biofilm structures (Fig. 3). A few, nonbiofilm-forming, *Kpn* bacteria adhered to the polystyrene substratum and were detected with anticapsule antibodies; therefore, they were producing capsule. In contrast, capsule staining was only observed on cells surrounding the biofilm structure, but not in those present in the core. Similar observations were made when biofilms of the other three *Kpn* strains were visualized by confocal microscopy (Fig. 4, left panels). The size of *Kpn* biofilms ranged from ~20 μm to as high as 60 μm (Figs. 3 and 4).

TABLE 2. BIOFILM PHENOTYPE, DETECTION OF GENE ENCODING FOR ADHESINS, *CAS* GENES, AND SIDEROPHORE PRODUCTION IN *KLEBSIELLA PNEUMONIAE* STRAINS

	Biofilm phenotype		
Gene encoding fimbriae or adhesion	*High producer* (n=34) n (%)	*Medium producer* (n=13) n (%)	*Total* (n=47) n (%)
fimH	30 (88.2)	12 (92.3)	42 (89.3)
mrkA	31 (91.7)	13 (100)	44 (93.6)
ecpA	33 (97.1)	13 (100)	46 (97.9)
None	1 (2.1)	0 (0)	1 (2.1)
cas genes	3 (6.4)	1 (2.1)	4 (8.5)
Production of siderophores	27 (79.4)	7 (53.8)	34 (72.3)

Comparison of K. pneumoniae *biofilms formed on plastic versus those formed on human cells*

Nosocomial, biofilm-forming, *Kpn* strains ultimately produce human disease by colonizing human epithelia (*i.e.*, the lungs). Strains adhere to *in vitro* cultures of human cells using fimbriae and the Ecp pili.[20] We, therefore, investigated the biofilm phenotype on polarized cultures of human lung Calu-3 cells. The same four strains utilized to inoculate abiotic surfaces, that is, polystyrene plates, were tested. Since *Kpn* might detach *in vitro* cultured cells within 24 hr of incubation, our experiments immobilized Calu-3 cell cultures to assure the cell substrate remained attached for *Kpn* to form biofilms.

In contrast to those large biofilm structures formed by *Kpn* strains on abiotic surfaces, biofilms on human lung Calu-3 cells were barely detected 24 hr postinoculation (Fig. 4, compare left vs. right panels). Therefore, *Kpn* forms robust biofilms on abiotic surfaces, but these structures are not produced on human cells within 24 hr.

Gene expression of K. pneumoniae *planktonic cells and biofilms*

To gain insights into a potential regulatory mechanism controlling the formation of robust biofilms on abiotic surfaces, we evaluated gene expression of planktonic cells and biofilms. We selected the capsule gene, *galF*, and the gene encoding the Ecp fimbriae (*ecp*A). Overall transcription of the *galF* gene was significantly upregulated when on abiotic surfaces compared with immobilized lung cells, whereas transcripts of the *ecpA* gene were significantly upregulated when *Kpn* was inoculated onto immobilized lung cells. Upregulated expression of these genes was generally more prominent 8 hr postinoculation than after 24 hr of incubation (Fig. 5). We then studied transcription in living cultures of lung cells. Consistent with Fig. 5 experiments, in this model, the *ecpA* gene was highly upregulated in comparison to *galF* (Fig. 6). Upregulated transcription of *ecpA* at 4 hr postinoculation was similar in planktonic cells in comparison to transcription in biofilms (Fig. 6A), whereas *galF* transcription was significantly higher in planktonic cells 4 hr postinoculation (Fig. 6B).

Production of siderophores

Another important virulence factor of *Kpn* is the capability to acquire iron from the host. At least 12 distinct iron uptake systems can be identified in *Kpn* NTUH-K2044, including siderophores. We detected siderophore production phenotypically by CAS assay in 34 out of 47 strains (72.3%) (Table 2). The nature of these siderophores was not investigated further.

Discussion

We demonstrated in this study that *Kpn* strains isolated from hospital-acquired infections, from Mexico City hospitals, were resistant to first-line antibiotics, although the majority was still susceptible to carbapenems. Genetic studies found that *Kpn* strains isolated from each hospital were genetically related, indicating a common source of contamination. As expected, given that strains were isolated from nosocomial infections, biofilms were formed by all
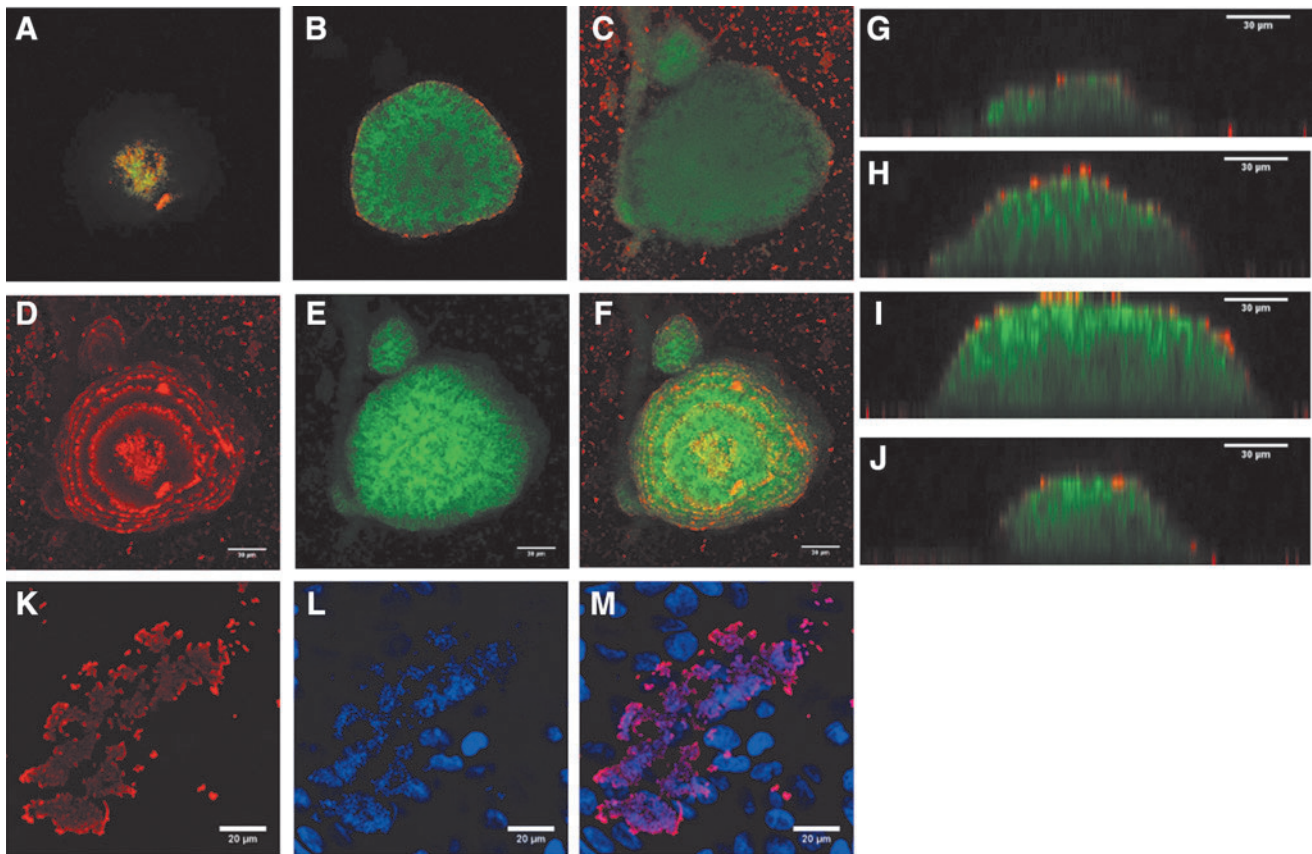
FIG. 3. Ultrastructure of *K. pneumoniae* biofilms. *K. pneumoniae* strain RYC492 was incubated for 24 hr on abiotic surfaces (**A–J**) or on immobilized human lung cells (**K–M**). Bacteria were stained with an anti-*K. pneumoniae* antibody followed by an Alexa 555 conjugated secondary antibody and bacterial DNA was stained with TOPRO3, which was pseudocolored in *green* (**A–J**) or *blue* (**L, M**) for better visualization. Confocal microscopy XY images showing optical sections (**A**) top, (**B**) middle, and (**C**) bottom. XY projections of (**D**) capsule stain, (**E**) DNA stain, or (**F**) merged images. (**G–J**) Optical sections of YZ images collected across the biofilm structure. XY projection of (**K**) capsule stain, (**L**) DNA stain, or (**M**) merged images.

Kpn strains with the majority (74%) classified as high biofilm producers on abiotic surfaces. *Kpn* biofilms growing on abiotic surfaces, which mimic conditions found in hospital environments, grew as high as $\sim 60\,\mu m$.

Biofilm structures were not formed when strains colonized human lung Calu-3 cells during the same period, that is, 24 hr, and under the same culture conditions. Limited formation of biofilms within the first 16 hr postinoculation has also been described on human bronchial epithelial cells cultivated in a flow chamber.[39] Altogether, these pieces of evidence provide further explanation for the high burden of hospital-acquired infections caused by *Kpn*, despite the low prevalence of community-acquired infections attributed to these bacteria. *Kpn* biofilms produced on abiotic surfaces (average $\sim 40\,\mu m$) were larger or comparable to biofilms produced by other human pathogens, including *Streptococcus pneumoniae* ($\sim 10\,\mu m$), and *Vibrio cholerae* ($\sim 30\,\mu m$), respectively.[40,41]

In 2011, *Kpn* strains were the third most important cause of hospital-acquired infections in the United States, accounting for 9.9% of all cases; just below two other important nosocomial pathogens: *Clostridium difficile* (12.1%) and *Staphylococcus aureus* (10.7%).[3] In Mexico, the prev-

alence of hospital-acquired infections produced by *Kpn* was similar to that reported in the United States, with 8.5% of cases attributed to *Kpn* (www.epidemiologia.salud.gob.mx).

Kpn can be involved in a variety of biofilm-related hospital-acquired infections, including pneumonia, surgical-site infections, bacteremia, and UTI.[3] Accordingly, most strains utilized in this study were isolated from biofilm-related diseases and were MDR (83%). Similar antibiotic resistance findings have been published by studies conducted in different settings in Mexico.[7–11,42,43] The situation in other Latin American countries appears to be similar. For example, a recent survey by Fernández-Canigia and Dowzicky found that *Kpn* strains isolated from 12 Latin American countries were resistant to cephalosporins, including ceftriaxone (97.8%), ceftazidime (81.1%), cefepime (58.8%), and to levofloxacin (56.4%), amoxicillin/clavulanic acid (56.3%), and piperacillin/tazobactam (44.7%).[6]

Resistance to most commonly prescribed antibiotics and new generations of cephalosporins is worrisome. Carbapenems such as imipenem and meropenem, however, are still active against *Kpn* strains isolated in Latin America (>90%)[6] and in Mexico (this study and Refs.[7–11,42,43]). It is expected, however, that antibiotic resistance rates will be
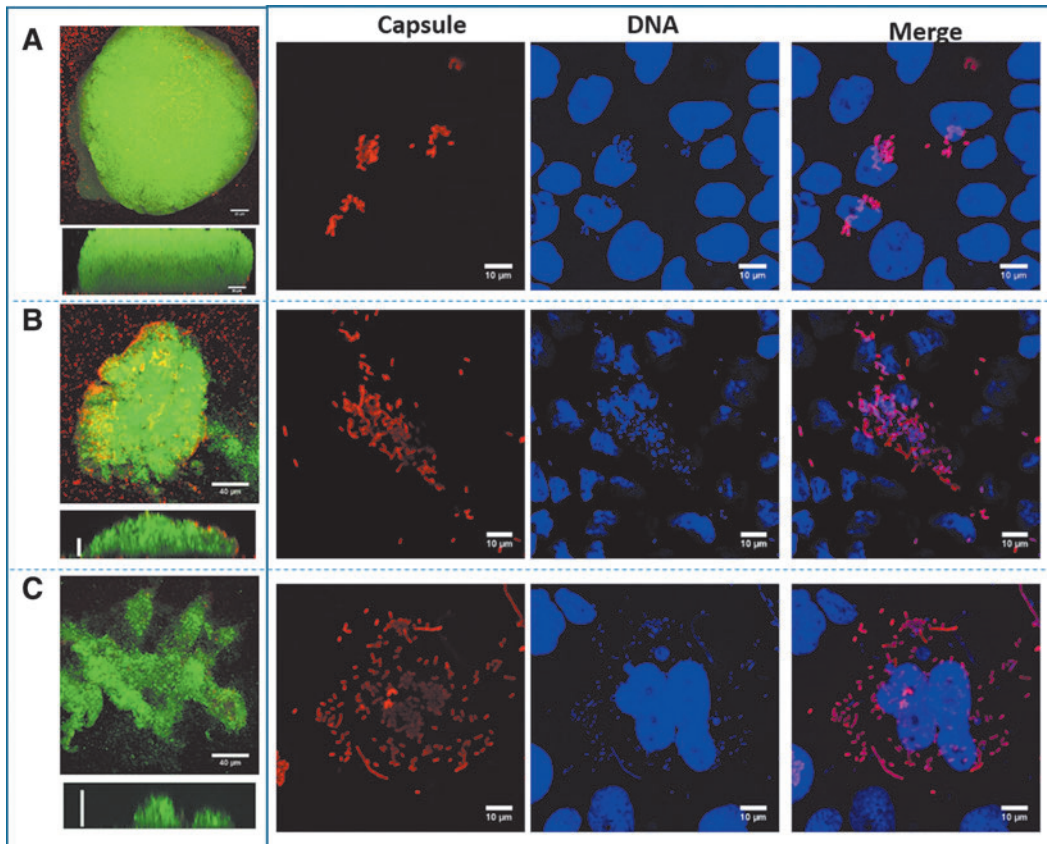
FIG. 4. *K. pneumoniae* forms robust biofilms on abiotic surfaces, but flat structures on lung cells. *K. pneumoniae* strain INP15 **(A)**, RGV2 **(B)**, or 4/484 **(C)** was incubated for 24 hr on an abiotic surface (*left panels*) and on immobilized human lung cells (*right panel*). Bacteria were stained with an anti-*K. pneumoniae* antibody followed by an Alexa 555 conjugated secondary antibody and DNA was stained with TOPRO3, which was pseudocolored in *green* (*left panels*) or *blue* (*right panels*) for better visualization. *Left panels* show an XY projection and underneath a YZ optical middle section from each strain. *Right panels* show capsule stain, DNA stain, and merged.
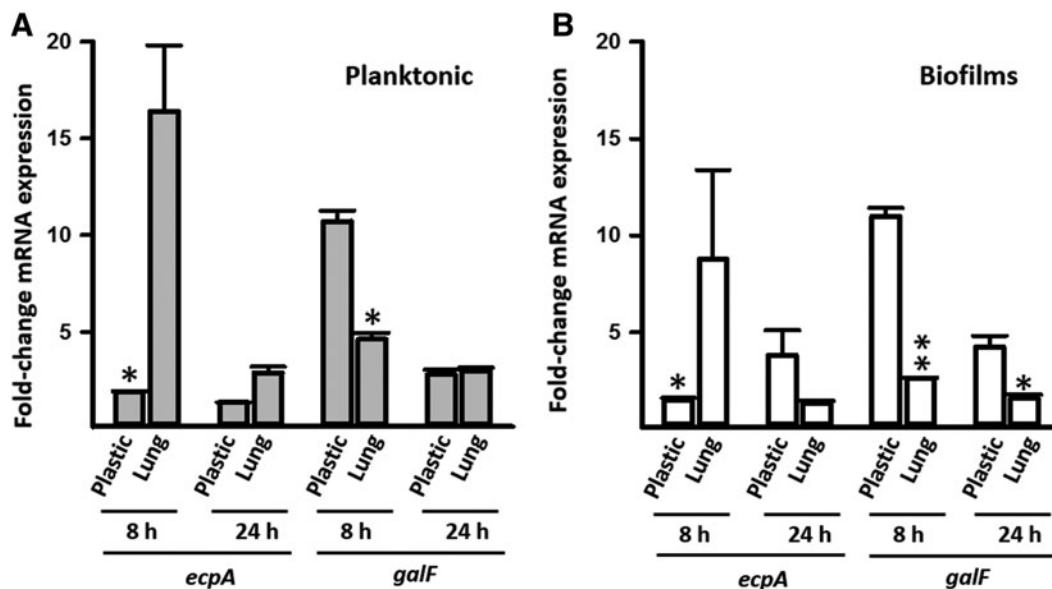
FIG. 5. Transcription of *cfa* and *galF* gene of *K. pneumoniae* planktonic cells and biofilms. *K. pneumoniae* RYC492 was inoculated on polystyrene plates (plastic), or on immobilized human Calu-3 cells (lung) followed by incubation at 37°C for 8 or 24 hr. **(A)** Planktonic cells or **(B)** biofilms were harvested, RNA extracted, and used as a template in qRT-PCRs targeting the *ecpA* or *galF* gene. Average Ct values were normalized to the *rpoD* gene, and the fold differences were calculated using the comparative C_T method ($2^{-\Delta\Delta CT}$).[38] Graphs show fold changes in mRNA expression of 8 and 24-hr planktonic cells and biofilms, relative to the inoculum. Error bars represent the standard errors of the mean calculated using data from at least two independent experiments. Gene expression on plastic was compared with that on immobilized lung cells using two-tailed Student *t* test, *$p \leq 0.05$, **$p < 0.006$.
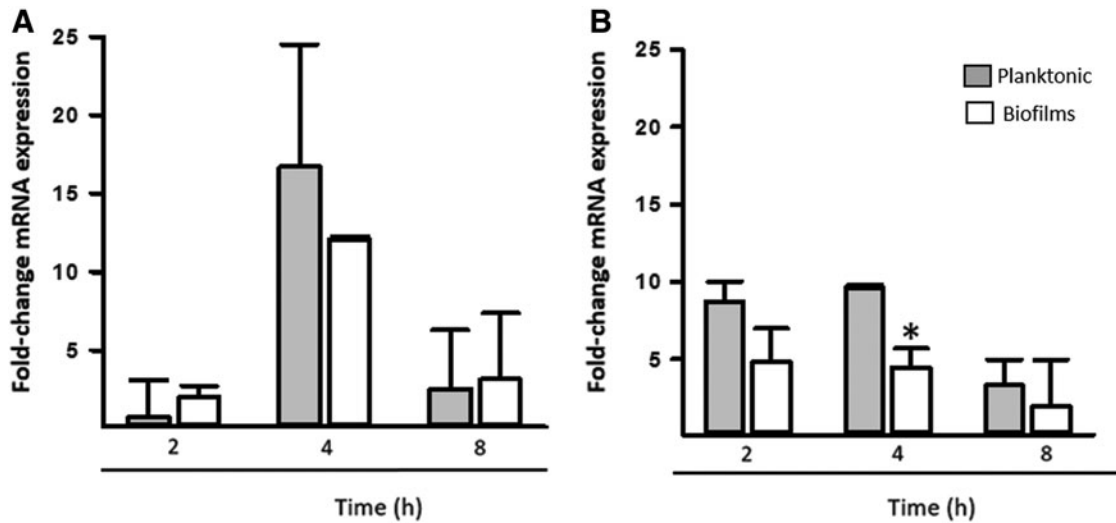
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FIG. 6. Transcription of *cfa* and *galF* gene of *K. pneumoniae* inoculated on living cultures of human lung cells. A monolayer of human Calu-3 cells was infected with *K. pneumoniae* strain RYC492 and incubated for 2, 4, and 8 hr. Planktonic cells (*gray boxes*) and biofilms (*white boxes*) were harvested, RNA extracted, and used as the template in qRT-PCRs targeting the **(A)** ecpA or **(B)** *galF* gene. Average Ct values were normalized to the *rpoD* gene, and the fold differences were calculated using the comparative C_T method ($2^{-\Delta\Delta CT}$).[38] Graphs show fold changes in mRNA expression of biofilms and planktonic cells relative to inoculum. Error bars represent the standard errors of the mean calculated using data from at least two independent experiments. Two-tailed Student *t* test compared gene expression in biofilms versus planktonic cells, at each time point, $*p \le 0.05$.

maintained at steady levels now that a new law, which requires certified prescriptions for purchasing antibiotics, has been recently implemented in Mexico.[44]

Besides antibiotic resistance, strains evaluated in this study produced biofilms, with 72% being classified as high biofilm producers. A study by Cruz-Córdoba *et al.* assessed biofilms of *Kpn* strains isolated from nosocomial infections in children and demonstrated a similar prevalence.[7] Other studies have reported ∼80% of prevalence for strains forming biofilms.[17,20] The variance in prevalence observed in other studies may be due to differences in the methodology utilized or selection bias. For example, while the current study, and that one by Cruz-Córdoba *et al.*, only included nosocomial isolates, those published by other groups evaluated strains isolated from different sources, including community-acquired infections.[17,20] We hypothesize that strains isolated from nosocomial infection may have an increased ability to form biofilms.

Kpn produces different fimbriae implicated in the production of biofilms. These fimbriae include type 1 and type 3, encoded by the genes *fimH* and *mrkA*, respectively.[2] Another adhesin known as the *E. coli* common pilus, or Ecp (encoded by *ecpA*), has recently been shown to be involved in adhesion of *Kpn* to host cells and biofilm formation.[20] This survey demonstrated that ∼89%, ∼94%, and ∼98% of hospital-acquired *Kpn* isolates carried the *fimH*, *mrkA*, and *ecpA* genes, respectively. Prevalence of these genes is in agreement with other epidemiological studies. In our survey, however, we did not target the *fimA* gene, which has been reported to produce another type 1 fimbriae. As expected, given that most strains carried the three genes, no associations could be detected between the presence of individual genes and the biofilm phenotype (not shown).

Biofilms were more robust when *Kpn* strains grew on abiotic surfaces in comparison to those attached bacteria

observed on cultures of human lung cells. It has also been demonstrated that *Kpn* strains attached better to human lung cells than to intestinal cells,[7] further supporting that colonization of the substrate is a tightly regulated process. Moreover, biomass was different when *Kpn* formed biofilms on different abiotic surfaces, including polystyrene, polypropylene, and catheters.[23] Formation of biofilms was regulated by a c-di-GMP-dependent MrkH circuit, which upregulated the production of type 3 fimbriae.[23,24]

Mutagenesis analysis has suggested that the capsule is required for the formation of *Kpn* biofilms.[21,22] Mutants deficient in capsule production were unable to attach to either glass or polyvinyl chloride substrates, and microscopy studies also revealed that the capsule was required for appropriate initial coverage of the substrate.[21] The demonstrated need for the capsule is consistent with the upregulated expression of the capsule gene, *galF*, observed in gene expression studies presented in this work. Both planktonic *Kpn* and biofilms upregulated transcription of *galF* at early stages of biofilm formation on abiotic surfaces, whereas only a modest increase in transcription was observed when *Kpn* was inoculated onto cultures of human lung cells. In contrast, the *ecpA* gene was only upregulated when *Kpn* infected human lung cells, but not during formation of biofilms on polystyrene. These observations support the main role of Ecp for colonization of human HeLa cells, recently demonstrated by Alcantar-Curiel *et al.* The authors demonstrated that Ecp is produced by all strains when colonizing human cells, but its production when forming biofilms was less clear, that is, only 8% of strains produced the fimbriae on abiotic surfaces.[20]

The specific mechanism by which *Kpn* strains regulate production of robust biofilms on abiotic surfaces, versus those "flat structures" of attached bacteria formed on polarized cultures of human lung cells, warrants further investigation. Several lines of evidences suggest the existence

of an efficient regulatory mechanism controlling formation of robust biofilms on abiotic substrates, different to that one allowing bacteria to colonize human cells. To gain further insights into this regulatory mechanism, more sophisticated studies are underway in our laboratories.

In conclusion, *Kpn* strains isolated from hospital-acquired infections from different hospitals located in Mexico City were resistant to most commonly prescribed antibiotics, but a high proportion of isolates remained susceptible to carbapenems. All strains produced biofilms and those structures formed by selected strains were robust on abiotic surfaces when compared with those attached bacteria observed on human lung cells.

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Disclosure Statement

No competing financial interests exist.

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