Biofilm formation, interaction and survival within A549 pneumocytes of *Klebsiella pneumoniae* clinical strains: identification of pulsotypes, multidrug-resistance and genes coding for adhesins

Formação de biofilme, interação e sobrevivência dentro dos pneumócitos A549 de cepas clínicas de *Klebsiella pneumoniae*: identificação de pulsótipos, multirresistência a drogas e codificação de genes para adesinas

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ABSTRACT
Klebsiella pneumoniaehas become one of the major causes of hospital-acquired infections over decades due to the spread of virulent clones harboring resistant genes to multiple antimicrobial agents. The aim of this study was to investigate phenotypic and genotypic features of virulence mechanism expressed by K. pneumoniae clinical isolates of different PFGE types, including biofilm formation, interaction with pneumocytes A549 lineage and experimental infection by using C. elegans nematodes. A total of 17 K. pneumoniae strains were isolated from different clinical specimens including blood, urine and respiratory infections. In this present study, 11 strains presented a varied multidrug-resistance profile harboring resistance genes coding for betalactams, aminoglicosydes, fluorquinolones and carbapenemases. PFGE analysis demonstrated the presence of four distinct pulsotypes among K. pneumoniae strains harboring virulence genes for siderophores and fimbiae type 1 and type 3. High adherence and biofilm formation were positively correlated for both polystyrene and glass surfaces in all K. pneumoniae strains analyzed. K. pneumoniae clinical strains showed the ability of adherence, internalization and persistence within human pulmonary epithelial A549 cell line, at different levels. Respiratory infections demonstrated a higher heterogeneity of PFGE types and levels of adherence, intracellular survival and persistence.K. pneumoniae strains were also submitted to Carnohabidits elegans in vivo infection model and data showed that after 24

hr almost 10% of urine-culture isolates worms were dead evidencing virulence profile. Notably, K. pneumoniae strains, presenting virulence genes, was significantly more virulent than those who did not presented any virulence gene after 5 days (survival >60% and >40%).

Keywords: *Klebsiella pneumoniae*, virulence factors, resistance genes, biofilm formation, PFGE, hospital settings.

RESUMO

Klebsiella pneumoniaehas tornou-se uma das principais causas de infecções adquiridas em hospitais durante décadas devido à propagação de clones virulentos que abrigam genes resistentes a múltiplos agentes antimicrobianos. O objetivo deste estudo foi investigar as características fenotípicas e genotípicas do mecanismo de virulência expresso pelos isolados clínicos de K. pneumoniae de diferentes tipos de PFGE, incluindo formação de biofilme, interação com pneumócitos da linhagem A549 e infecção experimental usando C. elegans nematodes. Um total de 17 linhagens de K. pneumoniae foram isoladas de diferentes amostras clínicas, incluindo sangue, urina e infecções respiratórias. Neste estudo, 11 cepas apresentaram um perfil multirresistência variado, abrigando genes de resistência que codificam betalactâmicos, aminoglicosídes, fluorquinolonas e carbapenemases. A análise PFGE demonstrou a presença de quatro pulsótipos distintos entre as cepas de K. pneumoniae que abrigam genes de virulência para sideróforos e fimbiae tipo 1 e tipo 3. Alta aderência e formação de biofilme foram positivamente correlacionados tanto para superfícies de poliestireno quanto de vidro em todas as cepas de K. pneumoniae analisadas. As cepas clínicas de K. pneumoniae mostraram a capacidade de aderência, internalização e persistência dentro da linha celular do epitélio pulmonar humano A549, em diferentes níveis. As infecções respiratórias demonstraram uma maior heterogeneidade dos tipos e níveis de aderência, sobrevivência intracelular e persistência. As cepas de K. pneumoniae também foram submetidas ao modelo de infecção in vivo por Carnohabidits elegans e os dados mostraram que após 24 horas quase 10% dos vermes isolados da cultura da urina estavam mortos, evidenciando o perfil de virulência. Notadamente, as cepas de K. pneumoniae, apresentando genes de virulência, eram significativamente mais virulentas do que aquelas que não apresentavam nenhum gene de virulência após 5 dias (sobrevivência >60% e >40%).

Palavras-chave: *Klebsiella pneumoniae*, fatores de virulência, genes de resistência, formação de biofilme, PFGE, ambiente hospitalar.

1 BACKGROUND

Klebsiella pneumoniae is ubiquitous in the environment, part of the normal intestinal microbiota in humans and capable of colonizing the skin and nasopharynx of healthy individuals. In immunocompromised or debilitated hospitalized patients with severe underlying diseases, *K. pneumoniae* causes urinary tract, respiratory tract and bloodstream infections as well as other less frequent diseases, including osteomyelitis, arthritis, and meningitis [1;2;3]. *K. pneumoniae* is the most frequently reported

opportunistic pathogen among nosocomial and community environmentscausing infections worldwide. [4].

The World Health Organization (WHO) has indicated K. pneumoniae as one of the multidrug resistant (MDR) microorganisms constituting an immediate threat for human health. *K. pneumoniae* invasive infections are associated with high rates of morbidity and mortality due to the high prevalence of resistance to most available antimicrobial agents. Genetic plasticity and high plasmid burden are among the traits that enable *K. pneumoniae* to accumulate and disseminate antimicrobial resistance genes and to occupy different niches. Resistance has a significant impact on clinical outcomedue to a lack of appropriate antimicrobial therapy, resulting in an increase of mortality rates and costs [5;1].

Carbapenemase-producing *Klebsiella pneumoniae* is one of the most urgent healthcare threats and may play an important role in resistance gene exchange and dissemination in both healthcare and non-healthcare settings. Clinically significant carbapenems resistance occurs particularly in Klebsiella spp. and is often mediated by bla_{KPC}carbapenemases genes, one of the most common globally [6].

Treatment of infections is complicated due to the continuous emergence of strains resistant to several antimicrobial agents [7]. *K. pneumoniae* presenting mobile genetic elements is a key factor for its dissemination not only with respect to the possibility of becoming resistant to antibiotics, but also of evolving more virulent phenotypes thanks to genes that may provide a survival benefit to microorganisms [1;8].

Pathogenic*K. pneumoniae*strains have the potential to cause a widevariety of infectious diseases, including urinary tract, respiratory tractand blood infections. Some virulent factors have been described, including hypermucoviscosity-associatedgene specific to K1 capsule serotype (magA,rpmA), adhesins (fimH,mrkD), lipopolysaccharides (wabG,uge,ycfM) and iron acquisition systems (KfuBc) and other virulence factors thatenable them to overcome host defenses, although it is not clear thelinkage of these genes with antibiotic resistance [9;4].

Usually, bacterial strains are categorized as pathogens when are able to colonize, invade and damage the host causing illness. Pathogenic bacteria have several factors related to their virulence, including the capacity to penetrate into the host and spread which are two crucial properties to cause infections [10; 11;12].

Among in vivo models, *Caenorhabditis elegans* have been proposed as a model to study phenotypic and genotypic virulence determinants [13]. *C. elegans*, an ubiquitous

free-living nematode, sharing with humans many biological pathways, has become a widely used model organism for studying host interactions and virulence mechanisms of bacterial infections [14]. It has been reported that free-living nematodes may serve as carriers or vectors of human enteric pathogens and these nematodes have been shown to be resistant to free chlorine and to offer protection to ingested pathogens against chemical sanitizers [15].

Antimicrobial resistance and bacterial virulence are complementary mechanisms that help microorganisms to survive under adverse environments. Hence, while virulence is needed to outgrow host cell defense systems, antibiotic resistance is essential to enable bacteria to overcome medical therapies [16]. The aim of this study was to investigate phenotypic and genotypic features of virulence mechanism expressed by *K. pneumoniae* clinical isolates of different PFGE types, including biofilm formation, interaction with pneumocytes A549 lineage and experimental infection by using *C. elegans* nematodes.

2 MATERIALS AND METHODS

Isolation and identification of *K. pneumoniae* strains. This study was carried out inMicrobiology and Immunology Department of Medical Science College from Rio de Janeiro State University, Rio de Janeiro, Brazil. *K. pneumoniae* isolates were received from the Bacteriology Laboratory of the university hospital during January to December of 2017.

Seventeen isolates were recovered from different clinical specimens: blood (n=5), urine (n=4), tracheal secretion (n=3), sputum (n=2), pleural fluid (n=1), tracheal aspirate (n=1) and oropharynx swab (n=1). All specimens were collected in sterile containers, streaked onto MacConkey agar (Oxoid, UK) and incubated at 37ºC for 24 h. The isolates were identified using MALDI-TOF mass spectrometry.

Antimicrobial resistance profiles. Antimicrobial susceptibility testing was done by the disc diffusion method, and the results were interpreted according to Clinical Laboratory Standards Institute (CLSI) guidelines [17]. The following antimicrobial drugs were tested: cephalothin, cefazolin, cefoxitin, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, cefepime, gentamicin, amikacin, kanamycin, tobramycin, ampicillin, piperacillin/taxobactam, amoxicillin/clavulanic acid, ampicillin/sulbactam, ciprofloxacin, norfloxacin, imipenem, ertapenem, meropenem, aztreonam, chloramphenicol, tetracycline, cotrimoxazole and colistin. Multidrug resistance was

considered when strains were resistant to three or more antimicrobial agents of interest class (beta-lactams, fluorquinolones, aminoglycoside and carbapenems) [18].

Genetic relatedness of MDR *K. pneumoniae* strains by pulsed-field gel electrophoresis (PFGE). Chromosomal DNA preparation and PFGE were performed as previously described [19]. PFGE banding patterns were analyzed by visual comparison among strains and GelJ program, version 2.0 [20]. Similarity coefficient (Dice) was calculated with a band position tolerance of 1.5% and the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method for cluster analysis [21; 22]. Isolates sharing 80% similarity were included in the same pulse type.

2.1 PCR DETECTION OF ANTIMICROBIAL RESISTANCE AND VIRULENCE GENES

DNA preparation. DNA was obtained by thermal lysis as previously described. Briefly, for 1 mL sample: 10 min at 100ºC, then 5 min at -20ºC and then centrifugation for 5 min at 14.000 rpm [23].

Beta-lactam-resistant strains were analyzed for bla_{TEM}, bla_{SHV}, bla_{TOHO}, $bla_{CTX\text{-}M}$ genes [24; 25]. Aminoglycoside-resistant strains were analyzed for *accC2* and *accC3* genes [26]. Fluoroquinolone-resistant strains were investigated for *qnr*A and *qnr*B genes [27].

PCR assays were also used for detection of genescoding for lipopolysaccharide capsule (*K1* and *K2*) [28; 29], type 1 and type 3 adhesins (*fimH* and *mrkD*) [30] and iron uptake system (*KfuBc*) [31].

Biofilm formation on hydrophobic polystyrene surface. Biofilm assays on polystyrene surfaces were performed for all 17 MDR strains[32]. The optical density (OD) of the stained attached bacteria and control wells were read at λ = 570 nm. The cut-off OD (ODc) was defined as the mean OD of the negative control (TSB only). Based on the ODs of the bacterial biofilms, all strains were classified into the following categories: non-adherent (-: OD ≤ ODc), weakly adherent (+: ODc> OD ≤ 2x ODc), moderately adherent (++: 2x ODc> OD ≤ 4x ODc), or strongly adherent (+++:OD>4x ODc). Each assay was performed in triplicate and repeated three times. *S. epidermidis* strain ATCC 35984 was used as a positive control [33].

Biofilm formation on a hydrophilic glass surface. Microorganisms were inoculated in glass tubes (15x100 mm) containing 5 mL of TSB medium and incubated at 37ºC for 48 h. The supernatants containing non-adherent bacterial cells were discarded.

Fresh sterile TSB (5 mL) was added to the test tubes and re-incubated for 48 h. This procedure was repeated twice. Glass-adherent bacteria created a confluent coat of cells on the sides of the tube. Microorganisms were classified as non-adherent (-: absence of adherence), weakly adherent (+: adherent bacteria appeared as a ring at the interface between the medium and the air), moderately adherent (++: bacteria attached on the side of the glass tubes), or strongly adherent (+++: bacteria attached on the side of the glass tubes and at the interface between the medium and the air). *S. epidermidis* strain ATCC 35984 was used as a positive control [34; 33].

Bacterial interaction with human lung carcinoma A549 cell line. Human lung carcinoma A549 cell line were grown Minimum Essential Medium Eagle (MEM—Sigma Chemical Co., MO, USA) supplemented with 10% bovine fetal serum (Gibco BRL, NY, USA). Bacterial viable counts were performed using monolayers of A549 cells grown to about 95% confluence and infected with K. pneumoniae strains (approximately $1x10^8$ bacteria). After incubation periods of 60 min, infected A549 cells were washed three times with mineral salt solution (PBS), lysed with 0.1% (v/v) Triton X-100 in PBSand diluted and plated onto Muller Hinton agar base. The number of bacteria per infected cell was determined by visual inspection and expressed as the mean number of adherent bacteria. To determine the intracellular viable bacteria counts, monolayers were washed six times with PBS and treated with 150 µg mL gentamicin for 1 h. The number of viable bacteria was determined after lysis of the monolayers with 250 µL of 0.1% Triton X-100 in PBS. For each incubation period, the percentage of intracellular bacteria was deduced from the number of A549 cell-associated bacteria. Index values of the viable bacteria related to bacteria associated with A549 cell monolayers were also determined. The bacterial counts (CFU) in supernatants and A549 monolayer lysates were determined at each incubation period. The percentage of cell-associated bacteria was calculated as follows: lysate CFU × (lysate CFU + supernatant CFU)$^{-1}$ × 100 (Moreira et al. 2003). To evaluate bacterial persistence, viable bacteria were recovered from the lysates of monolayers maintained in the presence of gentamicin for 24 h. Briefly, the monolayers were washed three times with PBS at 1 h post-infection and subsequently treated with 150 µg mL^{-1} gentamicin for 24 h. The persistence of intracellular bacteria was determined by viable counts (CFU mL^{-1}) conducted after the monolayers were lysed with 250 µL of 0.1% Triton X-100 in PBS at 24 h post-infection [35; 36].

Nematode killing assay.*K. pneumoniae* strains were presented as food to *Caernohabiditis elegans* nematode instead of *E. coli* strain OP50, an avirulent strain, and

their usual food in the lab.Briefly, *C. elegans* N2 were maintained on plates containing nematode growth medium (NGM) agar for approximately six-seven days at 20ºC and use in infection assays of four MDR*K. pneumoniae*strains isolated from urinary tract infections (UTI). Twenty L3 stage larval worms were infected with 20 µL of each bacterial strain (obtained from an overnight culture) on NGM plates at 20ºC for 24 h. The worms were observed daily following infection and the dead nematodes will be counted and removed every 24 h for seven days. For each strain, approximately 60 nematodes were used[37; 38].

3 RESULTS AND DISCUSSION

During a period of a year, the survey of clinical samples collected from hospitalized patients yielded the isolation of 17 *K. pneumoniae* strains: blood (n=5/ 29.4%), urine (n=4/ 23.5%), tracheal secretion (n=3/ 17.6%), sputum (n=2/ 11.7%), pleural fluid (n=1/ 5.8%), tracheal aspirate (n=1/ 5.8%) and oropharynx swab (n=1/ 5.8%). *K. pneumoniae* strains were identified by MALDI-TOF mass spectrometry with $\geq$ 2 score. PFGE analysis demonstrated the presence of five distinct pulsotypes among *K. pneumoniae* strains, most of them were characterized as MDR: MDS- A'1; MDR- B'1, B'2, C'1, D'1, D'2, D'3, D'4, E'1 and E'2. Data indicated a high and increasing heterogeneity among *K. pneumoniae* profiles mostly MDR in a hospital located at Rio de Janeiro metropolitan area, Brazil.

In recent studies was considered the possibility of contributing factors to the emergence, rise, andspread of antibiotic resistance, including nosocomial areas with a high endemicity of MDR bacteria, lack of new antimicrobial therapeutics, acquisition and transfer of antibiotic resistance genes; immunosuppressed conditions; healthcareexposure; use of indwelling medical devices andinappropriate and excessive antibiotic use[39]. Therefore, many of these risk factors may also havecontributed to the high rates of antibiotic resistance found in ourstudy.

K. pneumoniae have been the second most frequent pathogen related to hospital infections, including respiratory infections, liver abscess, meningitis as well as urinary, bloodstream and wound infections [40; 41; 42]. MDR *K. pneumoniae*is recognized in healthcare settings as a cause of high morbidity and mortality among patients with severe infections. Some MDR *K. pneumoniae* isolates have evolved to become extensively drug-resistant (XDR) isolates that have few therapeutic options, mainly via the spread of high-risk clones and epidemic resistance plasmids [43].

In this present study, 64.7% (n=11) were found to express varied MDR profiles and presence of resistance genes (**Table 1**). All MDR *K. pneumoniae* strains expressed resistance to third and fourth cephalosporins generation, aminoglycosides and fluorquinolones concomitantly, except the B10 strain isolated from tracheal secretion. MDR *K. pneumoniae* strains expressed phenotypic resistance to beta-lactams, aminoglycosides and fluorquinolones, although some of them did not present the correspondent resistance gene.

Extended-spectrum beta-lactamase (ESBLs) tested genes were currently expressed in all MDR *K. pneumoniae* nosocomial isolates: $bla_{CTX\text{-}M}$ (n=9), bla_{TEM} (n=6), bla_{SHV} (n=4). The first ESBL were derived from TEM and SHV beta-lactamases enzymes and are still mainly found in health-care associated infections. CTX-M was first identified in South America and is prevalent in Brazil. It is also the predominant. ESBL type found in other regions of the world and is increasing in frequency, particularly in the context of community-acquired infections. The prevalence and frequency of ESBL types vary from region to region and even between institutions within the same region [44; 45; 46; 47].

Interestingly, among MDR *K.pneumoniae* strains, six clinical isolates from tracheal secretion (n=3), blood (n=2) and urine (n=1) presented phenotypical resistance to carbapenems and bla_{KPC} (n=4) gene (Table 1). Previously studies reported that *K. pneumoniae* carbapenemase-producing is a major bacterial pathogen responsible for hospital outbreaks worldwide mainly via the spread of high-risk clones and epidemic resistance plasmids [6; 8]. In Brazil, a previous study described a gradual increase in antimicrobial resistance in *K. pneumoniae*, including an outbreak of KPC and its spread in a hospital during a nine year period.ESBL prevalence has led to an increase in carbapenem prescriptions, resulting in the emergence of ertapenem-resistant strains [43;48].

Our results indicated the rise of ESBLs endemicity until the development of carbapenems resistance, including the presence of bla_{KPC} genes. A considerable amount of genetic variation was also observed among MDR*K. pneumoniae* isolates.

PFGE analysis demonstrated the presence of four distinct pulsotypes among MDR *K. pneumoniae* strains. These four PFGE types presented resistance to beta-lactams and fluorquinolones groups of antimicrobial agents tested. Moreover, B and C pulsotypes also presented resistance to carbapenems and aminoglycosides respectively. Pulsotypes D and E presented resistance to all four first choice antimicrobial groups. Data indicate a high

and increasing heterogeneity among MDR profiles in presence of resistance genes in the hospital.

Previously studies indicated that acquisition of resistance to antimicrobial agents and virulence traits are necessary for dissemination and survival for human pathogens, including *K. pneumoniae* infections. Capsule serotype (K1 and K2), lipopolysaccharide (LPS), siderophores (*Kfu*) and fimbriae (types 1 and 3) are included among major virulence factors that contribute to pathogenicity of *K. pneumoniae*. Fimbriae type 1 (*fimH* gene) have been described to mediate bacterial adhesion to many types of epithelial cells, including bladder epithelium and are expressed in most of *K. pneumoniae* strains [49]. Investigation of virulence properties of MDR *K. pneumoniae* strains demonstrated the presence of *fimH* gene for all strains tested independent of MDR profiles and presence of resistance genes. Most *K. pneumoniae* strains may express two types of fimbrial adhesins, type 1 and type 3 fimbriae. Type 3 fimbriae are present in practically all *K. pneumoniae* isolates and mediate adhesion to several cell types *in vitro*. High adherence and biofilm formation were positively correlated with bacterial type 3 fimbriae expression and were not dependent upon the strain's origin[50].Aditionally, all MDR *K. pneumoniae* showed *mrkD* gene coding for type 3 fimbriae, except B10, B18 and B19 strains.

The *KfuBc* gene mediates uptake of ferric iron and is associated with capsule formation, hypermucoviscosity, purulent tissue infection. Moreover, is also associated in cases of with invasive infections and most prevalent among hypervirulent strains. The presence of *KfuBc* gene was previously detected from blood, exudates, respiratory secretions and urine samples in a few studies in different countries [51; 49]. In this study, the presence of *KfuBc* gene was observed in two MDR *K. pneumoniae* strainsisolated from urine samples.

PFGE analysis demonstrating the presence of four distinct pulsotypes among MDR *K. pneumoniae* strains showed that indicated the presence of virulence genes for type 1 fimbiae (*fimH*), type 3 fimbrae (*mrkD*) and iron uptake (*Kfubc*) (**Table 1**). All MDR *K. pneumoniae* strains of varied PFGE types presented the *fimH* gene coding for fimbriae type 1. The *mrkD* gene was detected inMDR *K. pneumoniae* strains exhibiting PFGE types B'2, C'1, D'2, D'3, E'1 and E'2. In the meantime the presence of *KfuBc* gene was only observed in PFGE type D'1 (MDR *K. pneumoniae* B18 and B19 clinical strains) isolated from urine samples.

K. pneumoniae was currently found as ethological agent of urinary tract (n=4), respiratory (n=7) and bloodstream (n=5) infections. The ability of biofilm formation by

clinical *K. pneumoniae*on biotic and abiotic surfacesplays a major role in development of nosocomial infections. Previous studies characterized the role of type 1 and type 3 fimbriae in *K. pneumoniae* biofilm formation by use of isogenic mutants. Most clinical *K. pneumoniae* isolates tested expressed both types of fimbrial adhesins (type 1 and 3). Data showed type 3 fimbriae, but not type 1 fimbriae, strongly promoted biofilm formation in *K. pneumoniae*[50].

Analysis of biofilm formation ability of MDS and MDR *K. pneumoniae* strains demonstrated that all strains strongly promoted biofilm formation on polyestirenesurface independent of type 3 fimbriae coding for *mrkD* gene. Moreover, data indicated a multifatorial nature of biofilm formation ability of *K. pneumoniae* since 58.8% (n=10) presented both *fimH* and *mrkD* genes; 11.7% (n=2) presented *fimH* and *kfuBc* genes and 5.8% (n=1) only presented *fimH* gene. Interestingly, 17.6% strains, isolated from sputum (n=1) and urine (n=2), considered strongly adherent to abiotic polyestirene surface, did not harbored any virulence genes, including type 3 fimbriae.

Further analysis investigated the heterogeneity ability of biofilm formation on abiotic surface of *K. pneumoniae* independent of clinical sites, resistance profiles and virulence genes by using hydrophilic glass surfaces. Data demonstrated 52.9% (n=9) strongly adherent, 17.6% (n=3) moderate adherent and 29.4% (n=5) non adherent strains (**Table 1**).

Until the present moment, the process of biofilm formation by*K. pneumoniae* has not been clearly elucidated. Only a few biofilm functions have been identified apart from surface structures such as type 3 pili, type 1 pili and the contribution of quorum-sensing regulatory system.The use of signature-tagged mutagenesis (STM) and surfaces coated with human extracellular matrix showed that proteins involved in transport and/or synthesis of sugars and extracellular components (capsule and fimbriae) were also shown to be involved during biofilm formation by *K. pneumoniae*. Most strains of *K. pneumoniae* are heavily capsulated and the presence of a thick polysaccharide (LPS) capsule maycover bacterial cell surface components[52].

Both capsular polysaccharides and LPS have been shown to mask short bacterial adhesins and to prevent the assembly of functional type 1 fimbriae on *K. pneumoniae* surface. However, the exact role of these surface components remains not well understood during biofilm formation process. Recent studies demonstrated the involvement of several bacterial functions at early and/or late stages of biofilm formation, and highlighted the preponderant and dual role of surface *K. pneumoniae* exopolysaccharides, which

either mask potential bacterial surface structures or rather promote biofilm maturation. A high increase in bacterial density to thedetriment of extracellular matrix in mature biofilm formedby the non-capsulated mutants suggested an increase in bacterial biomassformed by capsule-deficient mutants in the later stages ofbiofilm formation[52].

Iron environmental conditions may influence growth, biofilm formation and enhance virulence of *K. pneumoniae* causing human infections. Previously studies demonstrated that a lower expression of siderophore genes (*kfuBc*) correlated with increased virulence of liver abscess-causing *K. pneumoniae*[53].

Polysaccharide capsule, known as hypercapsule, consists of a mucoviscous exopolysaccharide bacterial coating that is more robust than the typical capsule, may contribute significantly to the pathogenicity of *K. pneumoniae* strains. Studies revealed that acapsular*K. pneumoniae* strains are dramatically less virulent than encapsulated strains in mouse models, based on decreased bacterial loads in the lungs, lower rates of mortality, and an inability of the bacteria to spread systemically. Both classical capsule and hypercapsule are made up of strain-specific capsular polysaccharides termed K antigens (i.e., K1 and K2, up through K78). Lineages of *K. pneumoniae* strains presenting capsular serotype K1 and K2 are more virulent due to their ability to inhibit phagocytosis acting as a protective barrier against the action of antimicrobial agents. Both serotypes are associated with the phenotype of hypermucoviscosity and are often detected in severe cases of pneumonias and liver abscesses [54; 55]. In this opportunity hypervirulent genotypes were not detected in *K. pneumoniae* strains isolated from clinical samples of variety PFGE types, including strains isolated from respiratory tract infections.

Analyses of*K. pneumoniae* isolated from blood nosocomial infections expressed high ability to produce mature biofilm on hydrophilic glass surfaces. All strains presented both *fimH* and *mrkD* genes and were able to produce biofilm on polystyrene and glass surfaces. High ability of biofilm formation production on hydrophobic and hydrophilic surfaces was observed in *K. pneumoniae* PFGE type E'2 harboring two MDR type strains that also expressedcarbapenemases resistant bla_{KpC} gene.

Data from *K. pneumoniae*strains isolated from respiratory tract infections showed different PFGE types and heterogenic properties on resistance, virulence genes and biofilm formation properties. MDR *K. pneumoniae* B'1 pulsotype (B10), isolated from tracheal secretion, were able to produce biofilm on hydrophobic polyestirene surfaces. Although expressing *fimH*gene, did not express ability to produce mature biofilm on hydrophilic glass surfaces.Interestingly, MDS *K. pneumoniae* PFGE type B'2 (B5),

obtained from pleural fluid, did not express ability to produce mature biofilm on hydrophilic glass surfaces and presented any resistance or virulence genes. However, were able to produce biofilm on hydrophobic glass polyestirene surface.

MDR *K. pneumoniae*nosocomial strains, isolated from respiratory clinical sites, were classified as PFGE types D'2 (B13, B15 and B17) andMDS *K. pneumoniae* strain, isolated from tracheal secretion, was classified as D'3 (B11)pulsotype (**Image 1**). All strains presented both *fimH* and *mrkD* genes and were able to produce biofilm on polystyrene and glass surfaces. Interestingly, MDS *K. pneumoniae* (B14) sputum isolate, characterized as PFGE type D'1, was able to produce biofilm in both hydrophilic and hydrophobic surfaces independent of the presence of resistance and virulence genes. Data indicate the involvement of additional virulence mechanisms related to biofilm formation without the involvement of*fimH, mrkD* and *kfuBc* genes by *K. pneumoniae* strains.

Data from *K. pneumoniae* strains isolated from respiratory tract infections showed different PFGE types (**Image 1**) and heterogenic properties on resistance, virulence genes and biofilm formation properties. MDR *K. pneumoniae* B'1 pulsotype (B10), isolated from tracheal secretion, were able to produce biofilm on hydrophobic polyestirene surfaces. Although expressing *fimH*gene, did not express ability to produce mature biofilm on hydrophilic glass surfaces. Interestingly, MDS *K. pneumoniae* PFGE type B'2 (B5), obtained from pleural fluid, did not express ability to produce mature biofilm on hydrophilic glass surfaces and presented any resistance or virulence genes. However, were able to produce biofilm on hydrophobic glass polyestirene surface.

K. pneumoniae urine clinical strains (n=4) presented two different PFGE type (**Image 1**): A'1 (B20 and B23) and D'4 (B18 and B19) and ability of biofilm formation on polystyrene surfaces. Differences were observed between these pulsotypes during production of biofilms on glass surfaces: A'1- negative and D'4- positive. PFGE type A'1 strains were classified as MDS and did not present resistance and virulence genes while D'4 pulsotype strains were classified as MDR, presenting resistance genes coding for ESBLs and fluorquinolones and *fimH* and *kfuBC* genes virulence genes (**Table 1**).

Although virulence genes may be related to invasive infections, including the presence of fimbriae type 1 and type 3, presence of K1 and K2 serotype and iron acquisition ability, virulence mechanisms of *K. pneumoniae* need further investigation.

Molecular differences observed in bacterial adherence to host cells may correlate with maintenance and dissemination of different clones. It is known that sugar residues, particularly those related to capsular composition, including terminal sialic acid, may also

contribute to the expression of hydrophilic characteristics and biofilm formation on glass surface.Molecular diversity observed in bacterial adherence mechanisms during interaction with host cells may influence on maintenance and dissemination of specific cloneswith regard to cells that they efficiently infect and the kind of inflammatory response that they trigger [56; 57].*K. pneumoniae*capsule consists of polysaccharides components and contributes to the mucoid phenotype, which may help the bacteria evade phagocytosis and impede bacterial clearance from the host. Several capsular polysaccharides associated characteristics have been shown to correlate with the occurrence of invasive infections and a muco-polysaccharide outside the capsule was described as hypervirulent expressing a hypermucoviscosity phenotype[58]. The composition of capsular polysaccharides from *K. pneumoniae* can be determined by enzyme treatment and lectin binding assay, and the presence of terminal sialic acid and fucose in capsular polysaccharides is accessible by fucosidase after removal of terminal sialic acid by neuraminidase [59].Previously studies reported that sialic acid terminal moieties are expressed mainly on the surface of highly glass-adherent bacterial strains, including *Corynebacterium diphtheriae* strains[60].

Presently,*K. pneumoniae* clinical strains, from different PFGE types, showed the ability of adherence, internalization and persistence within human pulmonary epithelial A549 cell line,at different levels (**Table 2**). *K. pneumoniae* strains isolated from blood clinical samples presented *fimH* and *mrkD* virulence genes with similar levels of adherence, invasion and persistence among A549 cell line and were classified in three different PFGE types: C'1 (B7 and B9), D'3 (B12) and E'2 (B1 and B2). Despite the MDR *K. pneumoniae* strains,classified as C'1 pulsetype, present different ability of biofilm formation (B7- negative and B9- positive) on glass surfaces, both strains harbored: B7- adherence ability of 33.2%, intracellular survival of 0.44% and persistence of 0.16%; B9 – adherence of 35.7%, followed by 0.37% of intracellular survival and 0.1% persistence strains 24h post infection. PFGE type E'2 (B1 and B2) resistant to bla_{KPC} gene and strongly adherent to glass on biofilm formation, demonstrated the higher levels of adherence ability: 36.8% and 35.7% respectively among blood strains isolates. Both strains also demonstrated similar results related to intracellular survival (B1- 0.25% and B2-0.3%) and persistence (B1- 0.08% and B2- 0.1%). Interestingly, MDS *K. pneumoniae* B12 strain, classified as D'3 pulsetype presented high level of glass biofilm formation and lower levels of adherence (33.9%), intracellular survival (0.3%) and persistence (0.09%) when compared to the others blood isolates (**Table 2**).

Respiratory infections demonstrated a higher heterogeneity of PFGE types and levels of adherence, intracellular survival and persistence. MDR *K. pneumoniae* B10 strain (tracheal secretion) was classified as B'1 pulsetype and presented: adherence capacity of 26.2%, followed by 0.53% of intracellular survival and 0.19% of persistence ability. Interestingly, PFGE type B'2 presented two different *K. pneumoniae* strains (B5 and B17) with no ability on glass biofilm formation. MDS B5 strain, isolated from pleural fluid, presented no resistance and virulence genes while MDR B17 strain harbored bla_{KPC}carbapenemases gene, *fimH* and*mrkD* virulence genes. Both strains presented lower adherence levels (B5- 14.3% and B17- 17%), however, B5 MDS *K. pneumoniae* strain was capable to intracellular survive (0.58%) and persist (0.29%) in higher levels when compared to B17 MDR *K. pneumoniae* strain: 0.28% of intracellular survival and 0.07% of persistence. PFGE type D'1 (B14) and D'3 (B11) isolated from sputum and tracheal secretion respectively, presented ability of biofilm formation despite the presence of virulence genes. Both MDS *K. pneumoniae* strains presented different levels of adherence (B11- 36.2% and B14- 20.3%), intracellular survival (B11- 0.58% and B14- 0.6%) and persistence (B11- 0.1% and B14- 0.38%) 24h post-infection. MDR *K. pneumoniae* strains B13 (oropharynx swab) and B15 (sputum) harboring *fimH* and *mrkD* virulence genes with ability of biofilm formation on glass surface, were classified as D'2 pulsetype and presented lower levels of adherence (16% and 19.4% respectively) followed by intracellular invasion (B13- 0.48% and B15- 0.7%) and persistence (B13- 0.09% and B15- 0.07%) 24h post-infection. MDR *K. pneumoniae* strain B4, isolated from tracheal secretion, was classified as PFGE type E'1 and harbored both resistance (bla_{KPC}) and virulence genes (*fimH* and *mrkD*), was capable of adhere (31.7%), intracellular survive (0.4%) and persist (0.1%) 24h post-infection (**Table 2**).

A total of four *K. pneumoniae* strains were isolated from urine samples and classified in two different pulsetypes: A'1 and D'4. MDS *K. pneumoniae* strains (B20 and B23) with no resistance or virulence genes, presented higher ability to adherence (B20- 34.2% and B23- 36.6%), intracellular survival (B20 and B23 with 0.46%) and persistence(B20- 0.2% and B23- 0.26%) in A549 human lung cell line when compared to different pulsetypes. MDR *K. pneumoniae* strains (B18 and B19), classified as PFGE type D'4 and carrying virulence genes (*fimH* and *mrkD*) were able to adhere (B18- 31% and B19- 32.8%), intracellular survive (B18- 0.5% and B19- 0.47%) and persist (B18- 0.23% and B19- 0.25%) 24h post-infection (**Table 2**).

*K. pneumoniae*strains are able to adhere and replicate at extracellular sites and cellular internalization, despite is less well defined, is considered a virulence mechanism because it enables the bacteria to avoid host defenses and antimicrobial drugs. Data indicates that *K. pneumoniae* ability to adhere and entry into lung epithelial cells represents a host defense mechanism related to a persistent infection in sites where the bacteria are protected from antibiotics and immune system. However, bacterial entry into epithelial cells is not necessarily associated with invasive infection [61].

Once *K. pneumoniae* interacted with epithelial cells, some microorganisms entered into the cells and did not proliferate but rather exhibited decline viability over the 24 h after cell entry. These observations suggested that *K. pneumoniae* entry into human lung epithelial cells might not represent a virulence mechanism. Internalization of the bacterial pathogens did not promote subsequent invasive infection but rather represented a mechanism to contain infection [62; 63; 64; 65].Although internalization of bacterial cells represents a mechanism to control infection, in our experiments, most of the bacterial cells remained in the extracellular environment.

Several Gram-negative human pathogens, including *Pseudomonas aeruginosa*, *Salmonella enterica*, and extraintestinal pathogenic *E. coli*, have been shown to kill *C. elegans* when presented to the nematodes as a source of nutrient [66; 67; 68]. In this study, we showed that *K. pneumoniae* can also kill *C. elegans*.To evaluate this behavior, we used four MDR *K. pneumoniae* isolated from urinary tract infection. PFGE analysis of four *K. pneumoniae* strains has indicated different pulsotypes (A'1 and D'4) and the presence of virulence genes. To this aim, an infection assay was performed by measuring the survival of *C. elegans* fed on pure cultures of these strains; *E. coli* strains OP50 (standard *C. elegans* laboratory food) were used as controls. Infection data (**Graphic 1**) showed that after 24 hr almost 10% of urine-culture isolates worms were dead evidencing that both pulsotypes (A'1 and D'4) presented the same virulence profile. Notably, D'4 pulsotype*K. pneumoniae* strains, presenting virulence genes, was significantly more virulent than A'1 pulsotype, which did not presented any virulence gene after 5 days(survival >60% and >40%).

Recent data suggest a relationship between *K. pneumoniae* virulence and drug resistance [69; 70]. Expression of efflux pumps in *K. pneumoniae* as a mechanism of antibiotic resistance may be associated with expression of virulence factors required to resist to innate immune defense mechanisms in nematode models [69]. The impact of carbapenemase expression on virulence is weak. A recent study suggests that virulence

factors such as K1 and K2 capsular polysaccharides and ferric iron uptake system gene (*KfuBC*) were absent in KPC-producing isolates and these strains present low virulence in an *in vivo* model [71].However, we found the presence of the genes encoding adhesins, including type 1 and type 3 fimbriae in all the strains that demonstrate a virulence potential of the resistant strains.

Some other virulence factors are now well established in *K. pneumoniae* strains such as K antigen - a capsular polysaccharide, which can be classified into 77 serological K antigen types. K1 serotype was the predominant serotype causing pyogenic liver abscess while K2 serotype is among the most common capsule types isolated from patients with UTI, pneumonia or bacteremia. It can be assumed that K2 is the predominant serotype of human clinical isolates worldwide [72; 4]. This present study demonstrates the absence of these serotypes limiting the effect of this important virulence factor [71].

4 CONCLUSION

All *K. pneumoniae* strains clinical isolates expressed ability of biofilm formation independent of multidrug-resistance profile. Moreover, biofilm formation at higher levels was expressed by *K. pneumoniae* strain isolated from patients presenting blood, urine and lower respiratory tract infections. Data emphasized that capsular component, scialic acid, seems to be involved in biofilm formation in both glass and polystirene surfaces. However, the expression of hypercapsular genes did not contributes to biofilm formation on both hydrophilic and hydrophobic surfaces. Biofilm formation ability was found independent of MDR profile. Most of *K. pneumoniae* strains presenting *fimH* gene associated to *mrkD* or *KfuBc* genes were verified as highly biofilm formation indicating a multifatorial nature of this process.

K. pneumoniae clinical isolates with ability of biofilm formation were also found able to express virulence mechanism related to adherence, intracellular survival and persistence within epithelial cells, including pneumocytes during host-pathogen interaction.

Further studies remain necessary to investigate the expression of phenotypes, specific cell surface components and metabolic pathways involved in *K. pneumoniae* interaction with varied biotic and abiotic components.

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The authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence their work. All authors declare that they have no competing interests.

REFERENCES

1- Fasciana, T.; Gentile, B.; Aquilina, M.; Ciammaruconi, A.; Mascarella, C.; Anselmo, A.; Fortunato, A.; Fillo, S.; Petralito, G.; Lista, F.; Giammanco, A.Co-existance of virulence factors and antibiotic resistance in new *Klebsiella pneumoniae* clones emerging in south of Italy. **BMC Infectious Diseases**, v. 19, 2019.

2- Ferrari, C.; Corbella, M.; Gaiarsa, S.; Comandatore, F.; Scaltriti, E.; Bandi, C.; Cambieri, P.; Matone, P.; Sassera, D. Multiple *Klebsiella pneumoniae* KPC clones contribute to an Extended Hospital outbreak. **Frontiers in Microbiology**, v. 29, n. 10, p. 2767, 2019.

3- Melot, B.; Colot, J.; Guerrier, G.Bacteremiccommunityacquired infections due to *Klebsiella pneumoniae*: clinical and microbiological presentation in New Caledonia, 2008–2013. **International Journal of Infectious Disease**, v. 41, p. 29–31, 2015

4- Podschun, R.; Ullmann, U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. **Clinical Microbiology Review**, v. 11, p. 589-603, 1998.

5- Longo, L. G. A.; Sousa, V. S.; Kraychete, G. B.; Justo-da-Silva, L. H.; Rocha, J. A.; Superti, S. S.; Bonelli, R. R.; Martins, I. S.; Moreira, B. M. Colistinresistance emerges in pandrug-resistant*Klebsiellapneumoniae*epidemic clones in Rio de Janeiro, Brazil. **International Journal of Antimicrobial Agents**, v. 54, n. 5, p. 579-586, 2019. 19

6- Mathers, A. J.; Vegesana, K.; German-Mesner, I.; Ainsworth, J.; Pannone, A.; Crook, D. W.; Sifri, C. D.; Sheppard, A.; Stoesser, N.; Peto, T.; Walker, A. S.; Eyre, D. W. Risk factor for *Klebsiella pneumoniae* carbapenemases (KPC) gene acquisition and clinical outcomes across multiple bacterial species. **The Journal of Hospital Infection**, v. 104, p. 456-468, 2020.

7- Apondi, O. E.; Oduor, O. C.; Gye, B. K.; Kipkoech, M. K. High prevalence of multi-drug resistant *Klebsiella pneumoniae* in a tertiary teaching hospital in Western Kenya. **Journal of Infectious Diseases**, v., n., p. 89-85, 2016.

8- Lee, S. Y.;Kotapati, S.;Kuti, J. L.; Nightingale, C. H.;Nicolau, D. P. Impact of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella* species on clinical outcomes and hospital costs: a matched cohort study. **Infection Control Hospital Epidemiology**, v. 27, p. 1226-1232, 2006.

9- Vargas, J. M.; Moreno Mochi, M. P.; Nunez, J. M.; Cáceres, M.; Mochi, S.; Del Campo Moreno, R.; Jure, M. A. Virulence factors and clinical patterns of multiple-clone hypermucoviscous KPC-2 producing *K. pneumoniae*. **Heliyon**, v. 5, p. e01829, 2019.

10- Beceiro, A.; Tomas, M.; Bou G. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? **Clinical Microbiology Review**, v. 26, n. 2, p.185-230, 2013.

11- Ribet, D.; Cossart, P. How bacteria pathogens colonize theis hosts and invade deeper tissues. **Microbial Infections**, v. 17, n. 3, p. 173-183, 2015.

12- Rasko, D. A.; Sperandio, V. Anti-virulence strategies to combat bacteria-mediated disease. Nature Reviews Drug Discovery, v. 9, n. 2, p. 117-128, 2010.

13- Diard, M.; Baeriswyl, S.; Clermont, O.; Gouriou, S.; Picard, B.; *et al*., *Caernohabitis elegans* as a simple model to study phenotypic and genetic virulence determinants of extra-intestinal *Escherichial coli*. **Microbes and Infection**, v. 9, p. 214-223, 2007.

14- Barber, A. E.; Norton, J. P.; Wiles, T. J. Mulvey, M. A. Strengths and limitations of model systems for the study of urinary tract infections and related pathologies. **Microbiology and Molecular Biology Review**, v. 80, p. 351-367, 2016.

15- Merkx-Jacques, A.; Coors, A.; Brousseau, R.; Masson, L.; Mazza, A. Tien, Y.; Topp, E. Evaluating the pathogenic potential of environmental *Escherichia coli* by using *Carnorhabidits elegans* infection model. **Applied and Environmental Microbiology**, v. 79, p. 2435-2445, 2013.

16- Dias, C.; Ribeiro, M.; Correia-Branco, A.; Dominguez-Perles, R.; Martel, F.; Saavedra, M. J.; Simões, M. Virulence, attachment and invasion of Caco-2-cells by multidrug-resistant bacteria isolated from wild animals. **Microbial Pathogens**, v. 128, p. 230-235, 2019.

17- CLSI- Clinical Laboratories Standards Institute. Performance Standards for antimicrobial disk susceptibility tests. Approved Standard CLSI Document M2, 2018. **Clinical Laboratories Standards Institute**, Waine. PA EUA

18- CLSI- Clinical Laboratories Standards Institute. Performance Standards for antimicrobial disk susceptibility tests. Approved Standard CLSI Document M2, 2016. **Clinical Laboratories Standards Institute**, Waine. PA EUA

19- Tenover, F. C.; Arbeit, R. D.; Goering, R. V.; Mickelsen, P. A.; Murray, B. E.; Persing, D. H.; Swaminathan, B. Interpreting Chromosomal DNA restriction patterns produced by Pulsed-Field Gel Electrophoresis: Criteria for bacterial strain typing. **Journal of Clinical Microbiology**, v. 33, n. 9, p. 2233-2239, 1995.

20- Sahly, H.; Navon-Venezia, S.; Roesler, L.; Hay, A.; Carmeli, Y.; Podschun, R.; Hennequin, C.; Forestier, C.; Ofek, I. Extended-Spectrum, Beta-Lactamase production is associated with an increase in Cell invasion and expression of fimbrial adhesions in *Klebsiella pneumoniae.* **Antimicrobial Agents and Chemotherapy**, v. 52, n. 9, 2008.

21- Hennequin, C.; Robin, F. Correlation between antimicrobial resistance and virulence in *Klebsiella pneumoniae.* **European Journal of Clinical Microbiology & Infectious Diseases**, v. 35, p. 333-341, 2016.

22- Li, B.; Yi, Y.; Wang, Q.; Woo, P. C. Y.; Tan, L.; Jing, H.; Gao, G. F.; Liu, C. H. Analysis of drug resistance determinants in *Klebsiella pneumoniae* isolates from a tertiary-care hospital in Beijing, China. **PLoS One**, e42280, 2012.

23- AGÊNCIA Nacional de Vigilância Sanitária – ANVISA. Technical note nº 01/2013. Avaliableat: <http://portal.anvisa.gov.br/wps/wcm/connect/

ea4d4c004f4ec3b98925 d9d785749fbd/Microsoft+Word+-+NOTA+T%C3%89CNICA+ENTEROBACTERIAS +17+04+2013(1).pdf?MOD=AJPERES>. Access in: jun. 10, 2018.

24- Yigit, H.; Queenan, A. M.; Anderson, G. J.; Domenech-Sanchez, A.; Biddle, J. w.; Steward, C. D.; Alberti, S.; Bush, K.; Tenover, F. C. Novel Carbapenem-Hydrolyzing B-Lactamase, KPC-1 , from a Carbapenem-Resistant Strain of *Klebsiella pneumoniae*. **Antimicrobial Agents and Chemotherapy**, v. 45, n. 4, p. 1151-1161, 2001.

25- Fang, C. T.; Chuang, Y. P.; Shun, C. T.; Chang, S. C.; Wang, J. T. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. **The Journal of Experimental Medicine**, v. 199, n. 5, p. 697-705, 2004.

26- Turton, J. F.; Perry, C.; Elgohari, S.; Hampton, C. V. PCR characterization and typing of Klebsiella pneumoniae using capsular type-specific, variable number tandem repeat and virulence gene targets. **Journal of Medical Microbiology**, v. 59, n. 5, p. 541-547, 2010.

27- Bisse, S.; Fevre, C.; Passet, V.; Issenhuth-Jeanjean, S. Tournebize, R.; Diancourt, L.; Grimont, P. Virulence clones of Klebsiella pneumoniae: identification and evolutionary scenario based on genomic and phenotypic characterization. **PLoS One**, v. 4, n. 3, p. e4982, 2009.

28- Ma, L. C.; Fang, C. T.; Lee, C. Z.; Shun, C.T; Wong, J. T. Genomic heterogeneity in Klebsiella pneumoniae strains is associated with primary pyogenic liver abscess and metastatic infection. **The Journal of Infectious Disease**, v. 192, p. 117–128, 2005.

29- Merritt, J.H.; Kadouri, D.E.; O'Toole, G.A. Growing and analyzing static biofilm. **Current Protocols in Microbiology**. Chapter 1, Unit 1B; 2005.

30- Sued, B. P. R.; Pereira, P. M. A.; Faria, Y. V.; Ramos, J. N.; Binatti, V. B.; Santos, K. R. N.; Seabra, S. H.; Hirata Jr, R.; Vieira, V. V.; Mattos-Guaraldi, A. L.; Pereira, J. A. A. Sphygmomanometers and thermometers as potential fomites of *Staphylococcus haemolyticus*: biofilm formation in the presence of antibiotics. **Memórias do Instituto Oswaldo Cruz**; 2017.

31- Ribot, E. M.; Fair, M. A.; Gautom, R.; Cameron, D. N.; Swaminathan, B.; Barrett, T. J. Standardization of Pulsed-Field Gel Electrophoresis Protocols for the subtyping of Escherichia coli O157:H7, Salmonella and Shigella for PulseNet. **Foodborne Pathogens and Disease**, v. 3, n. 1, 2006.

32- Heras, J.; Dominguez, C.; Mata, E.; Pascual, V.; Lozano, C.; Torres, C.; Zarazaga, M. GelJ- a tool for analyzing DNA fingerprints gel images. **BMC Bioinformatics**, V. 16, n. 270, 2015.

33- Van Belkum, A.; Tassios, P. T.; Dijkshoorn, L.; Haeggman, S.; Cookson, B.; Fry, N. K.; Fussing, V.; Green, J.; Feil, E.; Gerner-Smidt, P.; Brisse, S.; Struelens, M. Guidelines for the validation and application of typing methods for use in bacterial

epidemiology. **Clinical Microbiology and Infectious Diseases**, v. 13, suppl. 3, p. 1-46, 2007.

34- Mattos-Guaraldi, A. L.;Formiga, L. C. Relationship of biotype and source to the hemagglutination and adhesive properties of*Corynebacterium diphtheriae*. **Brazilian Journal of Medical Biology Research**, v. 24, p. 399-406, 1991

35- Hirata Jr. R.; Souza, S. M. S.; Rocha-de-Souza, C. M.; Andrade, A. F. B.; Monteiro-Leal, L. H.; Formiga, L. C. D.; Mattos-Guaraldi, A. L.; Patterns of adherence to HEp-2 cells and actin polymerization by toxigenic *Corynebacterium diphtheria* strains. **Microbial Pathogenesis**, v. 36, n. 3, p. 125-130, 2004.

36- Souza, M. C.; Santos, L. S.; Gomes, D. L. R.; Sabbadini, P. S.; Santos, C. S.; Camello, T. C. F.; Asad, L. M. B. O.; Rosa, A. C. P.; Nagao, P. E.; Hirata Jr, R.; Mattos-Guaraldi, A. L. Agrgregative adherent strains of *Corynebacterium pseudodiphtheriticum* enter and survive within HEp-2 epithelial cells. **Memórias do Instituto Oswaldo Cruz**, v. 107, n. 4, 2012.

37- Santos, L. S.; Antunes, C. A.; Santos, C. S.; Pereira, J. A. A.; Sabbadini, P. S.; Luna, M. G.; Azevedo, V.; Hirata Jr., R.; Burkovski, A.; Asad, L. M. B. O.; Mattos-Guaraldi, A. L. *Corynebacterium diphtheriae* putative tellurite-resistance protein (CDCE8392_0813) contributes to the intracellular survival on human epithelial cells and lethality of *Caenorhabditis elegans*. **Memórias do Instituto Oswaldo Cruz**, v. 100, n. 5, 2015.

38- Antunes, C. A.; Clark, L.; Wanuske, M. T.; Hacker, E.; Ott, L.; Simpson-Louredo, L.; Luna, M. G.; Hirata Jr., R.; Mattos-Guaraldi, A. L.; Hodgkin, J.; Burkovski, A. Caenorhabditis elegans star formation and negative chemotaxis induced by infection with corynebacteria. **Microbiology Society**, v. 162, n. 1, 2016.

39- Ferreira, R. L.; Silva, B. C. M.; Rezende, G.; Nakamura-Silva, R.; Pitondo-Silva, A.; Campanini, E. B.; Brito, M. C. A.; Silva, E. M. L.; Freire, C. C. M.; Cunha, A. F.; Pranchevicius, M. C. S. High prevalence of Multidrug-resistant *Klebsiella pneumoniae* harboring several virulence and b-lactamase encoding genes in a brazilian intensive care unit. **Frontiers in Microbiology**, v. 22, n. 9, p. 3198, 2019.

40- Caneiras, C.; Lito, L.; Melo-Cristino, J.; Duarte, A. Community- and Hospital-acquired *Klebsiella pneumoniae* urinary tract infections in Portugal: Virulence and Antibiotic resistance. **Microorganisms**, 2019

41- Sahly, H.; Navon-Venezia, S.; Roesler, L.; Hay, A.; Carmeli, Y.; Podschun, R.; Hennequin, C.; Forestier, C.; Ofek, I. Extended-Spectrum, Beta-Lactamase production is associated with an increase in Cell invasion and expression of fimbrial adhesions in *Klebsiella pneumoniae.* **Antimicrobial Agents and Chemotherapy**, v. 52, n. 9, 2008.

42- Hennequin, C.; Robin, F. Correlation between antimicrobial resistance and virulence in *Klebsiella pneumoniae.* **European Journal of Clinical Microbiology & Infectious Diseases**, v. 35, p. 333-341, 2016.

43- Palmeiro. J. K.; Souza, R. F.; Schorner, M. A.; Passarelli-Araújo, H.; Grazziotin, A. L.; Vidal, N. M.; Venanacio, T. M.; Dalla-Costa, L. M. Molecular epidemiology of

multidrug-resistant Klebsiella pneumoniae isolated in a braziliantertiaty hospital. Frontiers in Microbiology, v. 10, p. 1669, 2019. 19

44- Naas T, Poirel L, Nordmann P. Minor extended-spectrum β-lactamases. **Clinical Microbiology and Infections**, v. 14, p. 42-52, 2008.

45- Bush, K.; Jacoby, G. A. Updated functional classifi cation ofβ-lactamases. **Antimicrobial Agents and Chemotherapy**, v. 54, p. 969-976, 2010.

46- Lascols, C.; Hackel, M.; Huger, A. M.; Marshall, S. H.; Bouchillon, S. K.; Hoban, D. J.; *et al*. Using Nucleic Acid Microarrays to perform Molecular epidemiology and detect novel beta-lactamases: a snapshot of ESBL throughout the world. **Journal of Clinical Microbiology**, v. 50, p. 1632-1639, 2012.

47- Oliveira, P. M. N.; Buonora, S. N.; Souza, C. L. P.; Júnior, R. S.; Silvaa, T. C.; Bom, G. J. T.; Teixeira, C. H. S.; Silva, A. R. A. Surveillanceofmultidrug-resistant bactéria in pediatricand neonatal intensivecareunits in Rio de Janeiro State, Brazil. **Revista da SociedadeBrasileira de Medicina Tropical**, e2019025, 2019.

48- Li, B.; Yi, Y.; Wang, Q.; Woo, P. C. Y.; Tan, L.; Jing, H.; Gao, G. F.; Liu, C. H. Analysis of drug resistance determinants in *Klebsiella pneumoniae* isolates from a tertiary-care hospital in Beijing, China. **PLoS One**, e42280, 2012.

49- Remya P A, Shanthi M, Sekar U. Characterisation of virulence genes associated with pathogenicity in *Klebsiella pneumoniae*.**Indian Journal of Medical Microbiology**, v. 37, p. 210-218, 2019.

50- Schroll, C.; Barken, K. B.; Krogfelt, K. A.; Struve, C. Role of type 1 and type 3 in *Klebsiella pneumoniae* biofilm formation. **BMC Microbiology**, v. 179, n. 10, 2010.

51- Aljanaby, A. A.;Alhasani, A. H. Virulence factors and antibiotic susceptibility patterns of multidrug resistance *Klebsiella pneumoniae* isolated from different clinical infections. **African Journal of Microbiology Research**, v. 10, p. 829-3, 2016.

52- Balestrino, D.; Ghigo, J. M.; Charbonnel, N.; Haagensen, J. A. J.; Forestier, C. The characterization of functions involved in the establishment and maturation of *Klebsiella pneumoniae in vitro* biofilm reveals dual roles for surface exopolysaccharides. **Environmental microbiology**, 2008.

53- Chen, D.; Li, H.; Zhao, Y.; Qiu, Y.; Xiao, L.; He, H.; Zheng, D.; Li, X.; Huang, L.; Yu, X.; Xu, N.; Hu, X.; Chen, Y. Characterization of carbapenems-resistant Klebsiella pneumoniae in a tertiary hospital in Fuzhou, China. Journal of Applied Microbiology, 2020.

54- Compain, F., Decre, D., Fulgencio, J.P., Berraho, S., Arlet, G., Verdet, C. Molecular characterization of DHA1-producing *Klebsiella pneumoniae* isolates collected during a 4-year period in an intensive care unit. **Diagnostic Microbiology and Infectious Disease**, v.80, p. 159–161, 2014.

55- Paczosa, M. K.; Mecsas, J. Klebsiella oneumoniae: going on the offense with a strong defense. Microbiology and Molecular Biology Reviews, v. 80, n. 3, p. 629-661, 2016.

56- Peixoto, R. S.; Hacker, E.; Antunes, C. A.; Weerasekera, D.; Dias, A. A. S. O.; Martins, C. A.;*et al* Pathogenic properties of a *Corynebacterium diphtheriae* strain isolated from a case of osteomyelitis. **Journalof Medical Microbiology**, v. 65, p. 1311-1321, 2016.

57- Souza, C.; Faria, Y. V.; Sant'Anna, L. O.; Viana, V. G.; Seabra, S. H.; Souza, M. C.; Vieira, V. V.; Hirata Jr., R.; Moreira, L. O.; Mattos-Guaraldi, A. L. Biofilme productionbymultiresistant*Corynebacteriumstriatum*associatedwith nosocomial outbreaks. **Memórias do Instituto Oswaldo Cruz**, v. 110, n. 2, 2015.

58- Al Akhrass F, Al Wohoush I, Chaftari AM, Reitzel R, Jiang Y, Ghannoum M, Tarrand J, Hachem R, Raad I 2012. *Rhodococcus*bacteremia in cancer patients is mostly catheter related and associated with biofilm formation. ***PLoS ONE*** *7*: e32945.

59- Baio PVP, Mota HF, Freitas AD, Gomes DLR, Ramos JN, Sant'Anna LO, Souza MC, Camello TCF, Hirata Jr R, Vieira VV, Mattos-Guaraldi AL. Clonal multidrug-resistant *Corynebacteriumstriatum* within a nosocomial environment, Rio de Janeiro, Brazil. **Memórias do Instituto Oswaldo Cruz,**v. 108, p. 23-29, 2013.

60- Gomes DL, Martins CA, Faria LM, Santos LS, Santos CS, Sabbadini PS, Souza MC, Alves GB, Rosa AC, Nagao PE, Pereira GA, Hirata Jr R, Mattos-Guaraldi AL. *Corynebacterium diphtheriae* as an emerging pathogen in nephrostomy catheter-related infection: evaluation of traits associated with bacterial virulence. **Journal of Medical Microbiology,** v.58, p. 1419-1427, 2009.

61- Cortés, G.; Álvarez, D.; Saus, C.; Albertí, S. Role of epithelial cells in defense against *Klebsiella pneumoniae* Pneumonia. **Infection and Immunity**, v. 70, n. 3, p. 1075-1080, 2002.

62- Hulse, M. L.; Smith, S.; Chi, E. Y.;Pham, A.; Rubens, C. E. Effect of type III group B streptococcal capsular polysaccharide on invasion of respiratory epithelial cells. **Infection and Immunity**, v.61, p.4835–4841, 1993.

63- Pöhlmann-Dietze, P., M. Ulrich, K. B. Kiser, G. Döring, J. C. Lee, J.-M. Fournier, K. Botzenhart, and C. Wolz. Adherence of *Staphylococcusaureus*to endothelial cells: influence of capsular polysaccharide, global regulator *agr*, and bacterial growth phase. **Infection and Immunity,** v.68, p. 4865–4871, 2000.

64- Schrager, H. M., J. G. Rheinwald, and M. R. Wessels. Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. Journal of Clinical Investigation, v. 98, p. 1954–1958, 1996.

65- Stephens, D. S., P. A. Spellman, and J. S. Swartley. Effect of the (alpha 238)-linked polysialic acid capsule on adherence of *Neisseria meningitidis* to human mucosal cells. **Journal of Infectious Diseases**, v.167, p. 475–479, 1993.

66- Mah, T. F.; O'Toole, G. A. Mechanisms of biofilm resistance to antimicrobial agents. **Trends Microbiology**, v. 9, p. 34-39, 2001.

67- Alper, S.; Mc Bride, S. J.; Lackford, B.; Freedman, J. H.; Schwartz, D. A. Specificity and complexity of the Caenorhabiditis elegans innate immune response. **Mollecular and Cellular Biology**, v. 27, p. 5544-5553, 2007.

68- Mou, T. I.; Ball, A. R.; Anklesaria, Z.; Casadei, G.; Lewis, K.; Ausubel, F. M. Identification of novel antimicrobials using live-animal identification model. **Proceedings of the National Academy of Science**, v. 103, p. 10414-10419, 2006.

69- Bialek, S.; Lavigne, J. P.; Chevalier, J.; Marcon, E.;Leflon-Guibout, V.;*et al*. Membrane efflux and influx modulate both multidrug resistance and virulence of*Klebsiella pneumoniae* in a *Caenorhabditis elegans* model. **Antimicrobial Agents and Chemotherapy**, v. 54, p. 4373–4378, 2010.

70- Coudeyras, S.;Nakusi, L.;Charbonnel, N.; Forestier, C. A tripartite efflux pump involved in gastrointestinal colonization by *Klebsiella pneumoniae* confers a tolerance response to inorganic acid. **Infection and Immunity**, v. 76 p. 4633–4641, 2008.

71- Siu, L. K.; Lin, J. C.; Gomez, E.; Eng, R.; Chiang, T. Virulence and plasmid transferability of KPC *Klebsiella pneumoniae* at the Veterans Affairs Healthcare System of New Jersey. **Microbial Drug Resistance**, v. 18, p. 380–384 2012.

72- Keynan, Y.; Rubinstein, E. The changing face of *Klebsiella pneumoniae* infections in the community. **International Journal of Antimicrobial Agents** v. 30, p. 385–389, 2007.

ANEXOS

Table 1: Nosocomial *K. pneumoniae* strains, isolated from different sites, presenting resistance profiles harboring resistance and virulence genes with ability of biofilm formation in biotic and abiotic surfaces

Strain	PFGE type	Isolation sites	Resistance profile	Resistance genes	Virulence genes	Biofilmformation	
						polystyren surface	**glass surface**
B20	A'1	Urine	AMP			MA	+
B23	A'1	Urine	ASB			SA	+
B10	B'1	Trachealsecretion	/CRX/CTX/CRO/CAZ/CPM/AMP/AMC/ASB/PPT/CIP/NOR/IMI/ERT/MER/CLO	*qnrA*	*fimH*	SA	+
B5	B'2	Pleural fluid	CFL/AMP			SA	+
B17	B'2	Trachealsecretion	CTX/CRO/CAZ/CPM/GEN/AMI/KAN/TOB/AMP/AMC/ASB/PPT/CIP/NOR/IMI/ERT/ MER/ATM/CLO/SUT/TET	*bla*$_{CTX-M}$ /*bla*$_{SHV}$/*aacc3*/*qnrA*/ *bla*$_{KpC}$	*fimH/mrkD*	SA	+
B7	C'1	Blood	/CTX/CRO/CAZ/CPM/KAN/AMP/AMC/ASB/PPT/CIP/NOR/ATM/CLO/SUT/	*bla*$_{TEM}$/*bla*$_{CTX-M}$ /*bla*$_{SHV}$/*qnrA*	*fimH/mrkD*	SA	+++
B9	C'1	Blood	CTX/CRO/CAZ/CPM/KAN/AMP/AMC/ASB/PPT/CIP/NOR/ATM/CLO/SUT/TET	*bla*$_{TEM}$ /*qnrA*	*fimH/mrkD*	SA	+++
B14	D'1	Sputum	CFL/CFZ/CRX/CTX/CRO/CAZ/AMP/ASB/SUT			SA	+++
B13	D'2	Oropharynxswab	CTX/CRO/CAZ/CPM/GEN/TOB/AMP/AMC/ASB/PPT/CIP/ATM/CLO/SUT	*bla*$_{CTX-M}$ *aacc2*/*qnrA*	*fimH/mrkD*	SA	+++
B15	D'2	Sputum	CTX/CRO/CAZ/CPM/KAN/AMP/ASB/PPT/CIP/NOR/ATM	*bla*$_{CTX-M}$ /*qnrA*	*fimH/mrkD*	SA	+++
B11	D'3	Trachealsecretion	CFLZ/CFZ/CRX/CTX/CRO/CAZ/CPM/AMP/ASB/CIP/NOR/ATM/CLO/SUT/TET	*bla*$_{CTX-M}$ /*bla*$_{SHV}$	*fimH/mrkD*	SA	+++
B12	D'3	Blood	CFLZ/CFZ/CRX/CTX/CRO/CAZ/CPM/AMP/ASB/CIP/NOR/ATM/SUT	*bla*$_{CTX-M}$ /*bla*$_{SHV}$/*aacc3*/*qnrA*	*fimH/mrkD*	SA	+++
B18	D'4	Urine	CTX/CRO/CAZ/CPM/KAN/AMP/AMC/ASB/PPT/CIP/NOR/IMI/ERT/MER/ATM/CLO/SUT	*bla*$_{TEM}$ /*qnrA*	*fimH/kfuBC*	SA	+++
B19	D'4	Urine	CTX/CRO/CAZ/CPM/GEN/KAN/TOB/AMP/AMC/ASB/PPT/CIP/NOR/ATM/CLO/SUT/TET	*bla*$_{TEM}$/ *bla*$_{CTX-M}$	*fimH/ kfuBC*	SA	+++
B4	E'1	Trachealsecretion	CTX/CRO/CAZ/CPM/GEN/KAN/AMP/AMC/ASB/PPT/CIP/NOR/IMI/ERT/MER/CLO/SUT	*bla*$_{CTX-M}$ /*bla*$_{SHV}$ / *aacc2*/ *bla*$_{KpC}$	*fimH/mrkD*	SA	++
B1	E'2	Blood	CTX/CRO/CAZ/CPM/GEN/KAN/TOB/AMP/AMC/ASB/CIP/NOR/IMI/ATM/SUT/TET	*bla*$_{TEM}$/*bla*$_{CTX-M}$ / *acc3*/*bla*$_{KpC}$	*fimH/mrkD*	SA	++
B2	E'2	Blood	CTX/CRO/CAZ/CPM/GEN/KAN/TOB/AMP/AMC/ASB/CIP/NOR/ IMI/ATM/SUT/TET	*bla*$_{TEM}$/*bla*$_{CTX-M}$ / *acc3*/*bla*$_{KpC}$	*fimH/mrkD*	SA	++

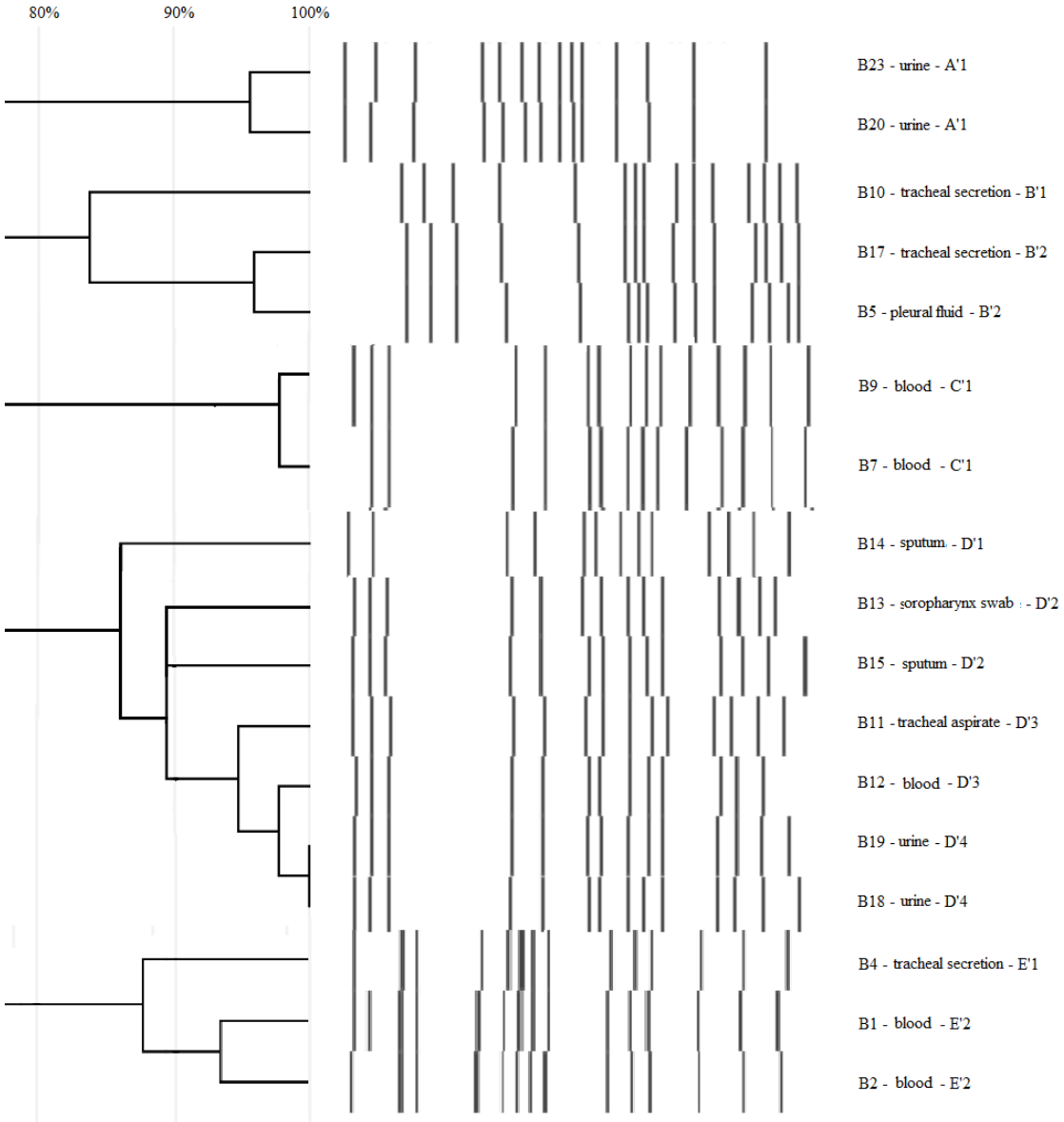
Legend: SA/ +++: strongly adherent, MA/++: moderated adherent; +: adherent bacteria appeared as a ring at the interface between the medium and the air.

Table 2: Nosocomial *K. pneumoniae* strains, isolated from different clinical sites with ability of biofilm formation in biotic and abiotic surfaces, demonstrating their ability to adherence, intracellular survival and persistence into Human lung endocarcinoma cell line A549

Strain	PFGE type	Isolation site	Human lung endocarcinoma cell line A549			Biofilm formation	
			Adherence	**Intracellular survival (1h)**	**Persistence (24h.)**	**Polystyrene Surface**	**Glass surface**
B20	A'1	Urine	34.2%	0.46%	0.2%	SA	-
B23	A'1	Urine	36.6%	0.46%	0.26%	SA	-
B10	B'1	Tracheal secretion	26.2%	0.53%	0.19%	SA	-
B5	B'2	Pleural fluid	14.3%	0.58%	0.29%	SA	-
B17	B'2	Tracheal secretion	17%	0.28%	0.07%	SA	-
B7	C'1	Blood	33.2%	0.44%	0.16%	SA	-
B9	C'1	Blood	35.7%	0.37%	0.1%	SA	+++
B10	D'1	Tracheal secretion	26.2%	0.53%	0.19%	SA	-
B14	D'1	sputum	23.4%	0.52%	0.15%	SA	+++
B13	D'2	Oropharynx swab	16%	0.48%	0.09%	SA	+++
B15	D'2	sputum	19.4%	0.7%	0.29%	SA	+++
B11	D'3	Tracheal secretion	36.2%	0.36%	0.1%	SA	+++
B18	D'4	Urine	31%	0.5%	0.23%	MA	+++
B19	D'4	Urine	32.8%	0.47%	0.25%	SA	+++
B4	E'1	Tracheal secretion	31.7%	0.4%	0.1%	SA	++
B1	E'2	Blood	36.8%	0.25%	0.08%	SA	++
B2	E'2	Blood	35.7%	0.3%	0.1%	SA	++

Legend: SA/ +++: strongly adherent, MA/++: moderated adherent; +: adherent bacteria appeared as a ring at the interface between the medium and the air.

Image 1: Pulsed Field Gel Electrophoresis demonstrating different pulsetypes of nosocomial *K. pneumoniae* isolated from different clinical sites.



Graphic 1: Infection assay measuring the survival of *C. elegans* fed on MDR *K. pneumoniae* isolated from urinary tract infection.