Type of the Paper (Article)

Molecular Insights into β-Lactams Resistance in *Klebsiella pneumoniae* Clinical Isolates with a Focus on Multidrug Resistance and Virulence

Lavouisier F.B. Nogueira#, Marília S. Maia#, Marco A.F. Clementino#, Ila F.N. Lima, Jorge L.N. Rodrigues, Luciana V.C. Fragoso, Glairta S. Costa, Jose Q.S. Filho, Alexandre Havt, Deiziane V.S. Costa, Lyvia M.V.C. Magalhães, Dilza Silva, Nicholas E. Sherman, José K. Sousa* and Aldo A.M. Lima*

# - These authors contributed equally to this work.

* - Corresponding authors.

Abstract: *Klebsiella pneumoniae* is associated with high resistance to antimicrobials and is common in isolates from colonization and healthcare-associated infections (HAIs). This study aims to develop assays to detect resistance genes belonging to the *bla* family and investigate metabolic pathways in resistant isolates of *K. pneumoniae*. The genes of the subfamilies included were: *bla*SHV, *bla*TEM, *bla*NDM, *bla*KPC, *bla*GES, *bla*CTX-M and relevant variants of the *bla*OXA subfamily. Mass spectrometry data were acquired on the Orbitrap IDX spectrometer (Thermo) connected to the Vanquish UPLC system. Isolates of *K. pneumoniae* (N = 122) were obtained from clinical samples from 04/23/2019 to 05/29/2021. A high prevalence of resistance to penicillins, cephalosporins and carbapenems was found among the isolates. The identified genotypic profile showed a high prevalence of genes belonging to Ambler's classes of beta-lactamases A, B and D. In the metabolomic study, the N-fructosyl isoleucine metabolite was identified increased in multidrug-resistant (MDR) strains of *K. pneumoniae* compared to strains

susceptible to antimicrobials. In conclusion, the assays developed were efficient in detecting the main genes of the *bla* family of resistance in *K. pneumoniae*. The use of the pentose phosphate metabolic pathway suggests a beneficial regulation of bacterial growth, and colonization in MDR *K. pneumoniae* strains, with may indicate the use of this pathway as a virulence mechanism in resistant strains.

KEYWORDS: Antimicrobial resistance; multidrug-resistant; β-lactamases; qPCR; molecular diagnosis; *Klebsiella pneumoniae*.

INTRODUCTION

Bacterial resistance to antimicrobial agents is a global public health problem that leads to an increase in the cost of treatment, length of stay, morbidity and mortality of hospitalized patients, especially in intensive care units (ICU).[1,2] In this sense, understanding the emergency mechanisms of regulation and dissemination of resistance to antimicrobial agents in the pathogenesis of bacterial colonization and/or infection can be of critical importance in preventing and controlling this problem.

Klebsiella pneumoniae is a Gram-negative γ-proteobacteria bacteria, belonging to the *Enterobacteriacae* family, and is generally viewed as an opportunistic microorganism, carrying several virulence factors and capable of accumulating resistance genes to various classes of antimicrobials. It is commonly related to cases of colonization and/or healthcare-associated infections (HAIs) and has been identified as an etiological agent in pneumonia, urinary tract infections (UTI), soft tissue and surgical wound infections, bacteremia, and sepsis.[3]

It is estimated that *K. pneumoniae* is responsible for approximately 10% of all cases of HAIs, and of these, 32.8% are caused by strains resistant to multiple

antimicrobial drugs. However, studies indicate that the rate of isolated strains presenting resistance to antimicrobials has increased over the years.[4,5]

K. pneumoniae has been associated with the ability to overcome colonization resistance imposed by the gastrointestinal microbiota[6] and has been reported as an emerging Multidrug-Resistant microorganism of emergency priority by the World Health Organization (WHO) for the development of new therapies.[7] Epidemiological data have demonstrated that *K. pneumoniae* can translocate from the gastrointestinal tract to other sterile sites of the same host or other patients through the fecal-oral route. What makes clear the clinical relevance of this microorganism. [8]

One of the most likely causes of the increasingly frequent emergence of bacterial strains resistant to one or more antibiotics is the excessive and sometimes incorrect use of antimicrobials. The relatively long time required to identify the pathogen by traditional methods, as well as for the results of the antimicrobial susceptibility test (AST), forces the clinician to use broad-spectrum drugs empirically, increasing selective pressure, which ends up benefiting pathogens genetically capable of adapting to the adverse environment.[1,9]

Possible answers to reduce the time needed to obtain a resistance profile, and to investigate new approaches in treatment, is the development of molecular methodologies, that can make the identification of resistance faster, and also be more sensitive than the traditional methodologies.[10] And the investigation of metabolic differences between sensitive and resistant strains, that can lead to metabolites or pathways that can be used as targets for the development of new treatments and strategies for dealing with multidrug-resistant (MDR) microorganisms. [11-12]

Beta-lactams are the class of antimicrobials most affected by resistance in general, which is conferred in gram-negatives, mainly by the *bla* gene family, this group of genes encodes enzymes called beta-lactamases, which can neutralize the action of beta-lactams through hydrolysis of the beta-lactam ring.[13–15]

In this study it was investigated the phenotype of resistance, the genotypic profile of the *bla* family of β-lactamases, and the metabolic differences between sensitive and MDR *K. pneumoniae* strains, isolated from patients admitted to the ICU in a university hospital in the city of Fortaleza-CE, Brazil.

We aim to develop a molecular assay, capable of identifying all variants of the most relevant genes of the *bla* family, these being: *bla*SHV, *bla*TEM, *bla*NDM, *bla*KPC, *bla*GES and *bla*CTX-M. In addition, also was included the most epidemiologically relevant variants of the *bla*-OXA subfamily. Thus, creating a set of primers capable of detecting the presence of hundreds of resistance genes with a reduced number of reactions. Were also investigated the main differences between the metabolites up and down regulated in sensitive and MDR strains of *K. pneumoniae*, both from the intracellular medium and the supernatant among themselves and in comparison, with the control medium.

RESULTS

Selection of bacterial samples. A total of 249 samples of Gram-negative bacteria resistant to beta lactam antimicrobials were collected from 04/23/2019 to 05/29/2021, those were identified, and the most prevalent microorganism found was *Klebsiella pneumoniae* 48.99% (122/249), been 21,8% from infections cases and 78,2% from colonization cases.

Identification of the phenotypic profile of beta-lactams resistance of *K. pneumoniae* strains. The bacteria isolates included in the study were tested against a wide range of beta-lactams drugs, and the presence of a high percentage of isolates resistant to these drugs was verified, which included **Penicillins**: ampicillin/sulbactam (92.00%) and piperacillin/tazobactam (88.46%), **Cephalosporins**: cefepime (83.02%), cefoxitin (73.68%), ceftazidime (86.54%), ceftazidime/avibactam (11 .76%), ceftriaxone (83.02%), cefuroxime (90.38%) and cefuroxime axetil (81.82%) and **Carbapenems**: ertapenem (44.00%), imipenem (71.70%) and meropenem (69.81%), as shown in **Figure 1**.

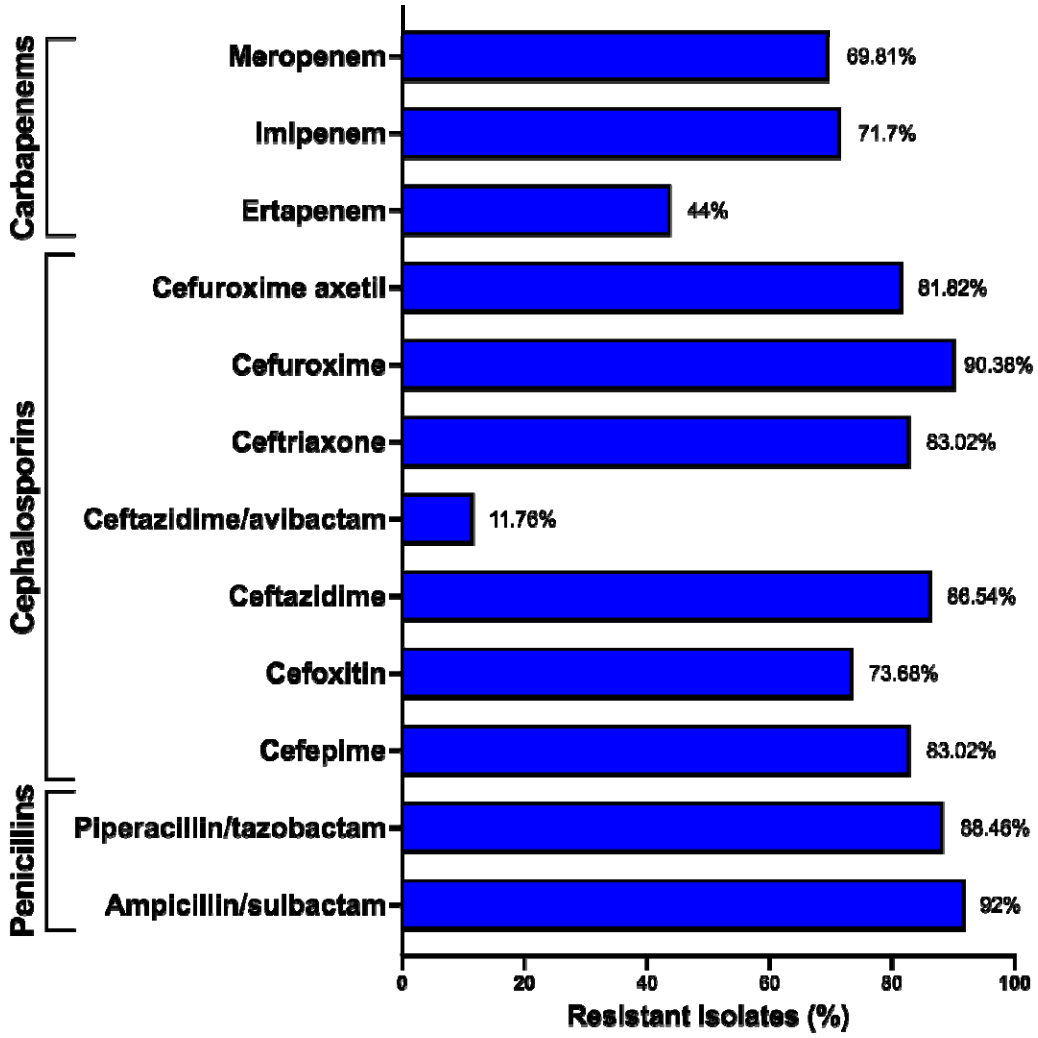


Figure 1. Prevalence of *K. pneumoniae* isolates resistant to the beta-lactams evaluated (Penicillins, Cephalosporins and Carbapenems), demonstrating a phenotypic profile of resistance to multiple beta-lactams drugs, in approximately 70% of the isolates, with the exception of ceftazidime/avibactam and ertapenem.

Primers design. After selection on the platforms: Comprehensive Antibiotic Resistance Data-base (CARD) and National Center for Biotechnology and Information (NCBI, USA), and subsequent compilation and analysis of FASTA sequences, it was possible to identify consensus sequences common to all sequences available in the databases for each of the genes included in the study: *bla*SHV (N = 156), *bla*TEM (N = 167), *bla*NDM (N = 27), *bla*KPC (N = 208), *bla*GES (N = 25), and *bla*CTX-M (N = 144). Except for the *blaOXA* gene, for which the most clinically relevant sequences were used *(N = 203).*

The *bla*CTX-M and *bla*OXA genes, due to their diversity and high degree of genetic variation between homologous sequences, were grouped into clades, and divided into groups. the *bla*CTX-M gene group was named according to the international standardization for this gene with the CTX-M 1*like* been divided into two groups due to its high number or sequences and the genetic variation between them. CTX-M 1.1*like* (N = 42), CTX-M 1.2*like* (N = 16), CTX-M 2*like* (N = 23), CTX-M 8*like* (N = 14) and CTX-M 9*like* (N = 49), while the *blaOXA* gene was divided into the OXA-*23like* (N = 25), OXA-*24/40like* (N = 8), OXA-*48like* (N = 17) and OXA-*51like* (N = 153), as can be seen in **Figures 2** and **3**.

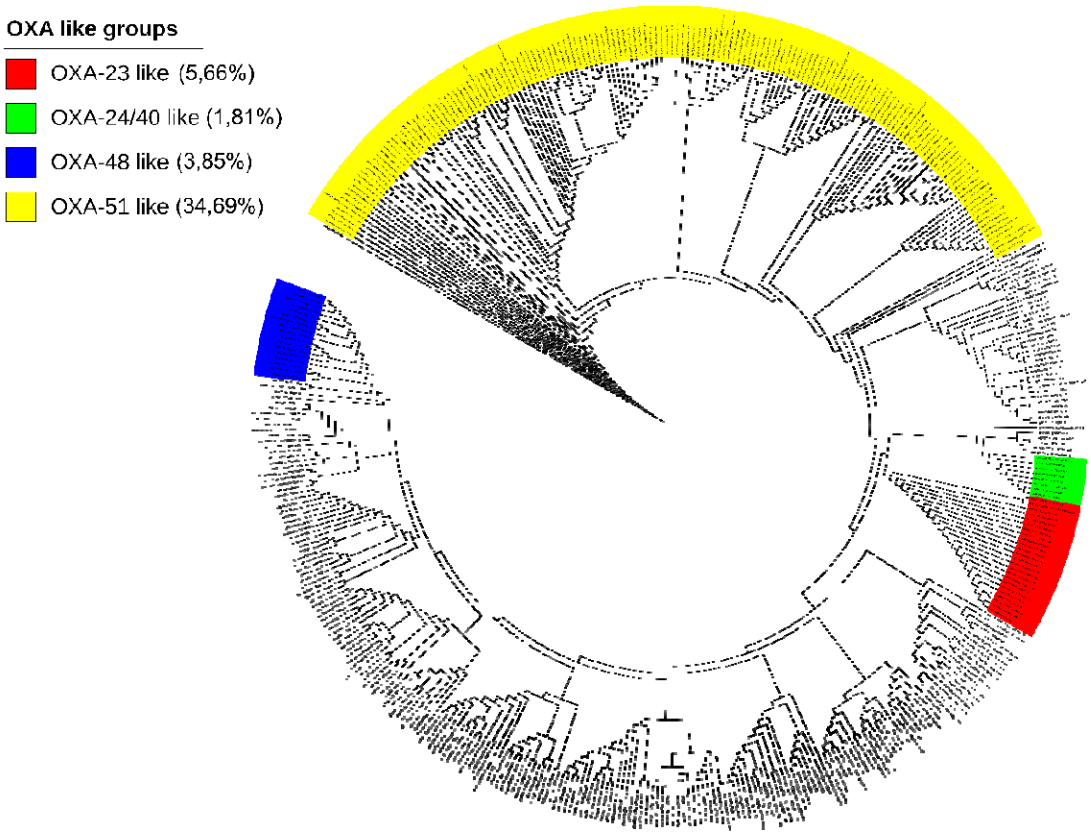


Figure 2. Cladogram of the *bla*OXA subfamily showing the most epidemiologically relevant groups, covered by the primers developed, and the percentage of sequences identifiable by each primer in comparison with the total number of the *bla*OXA family (N = 441) at the time of publication of this work.

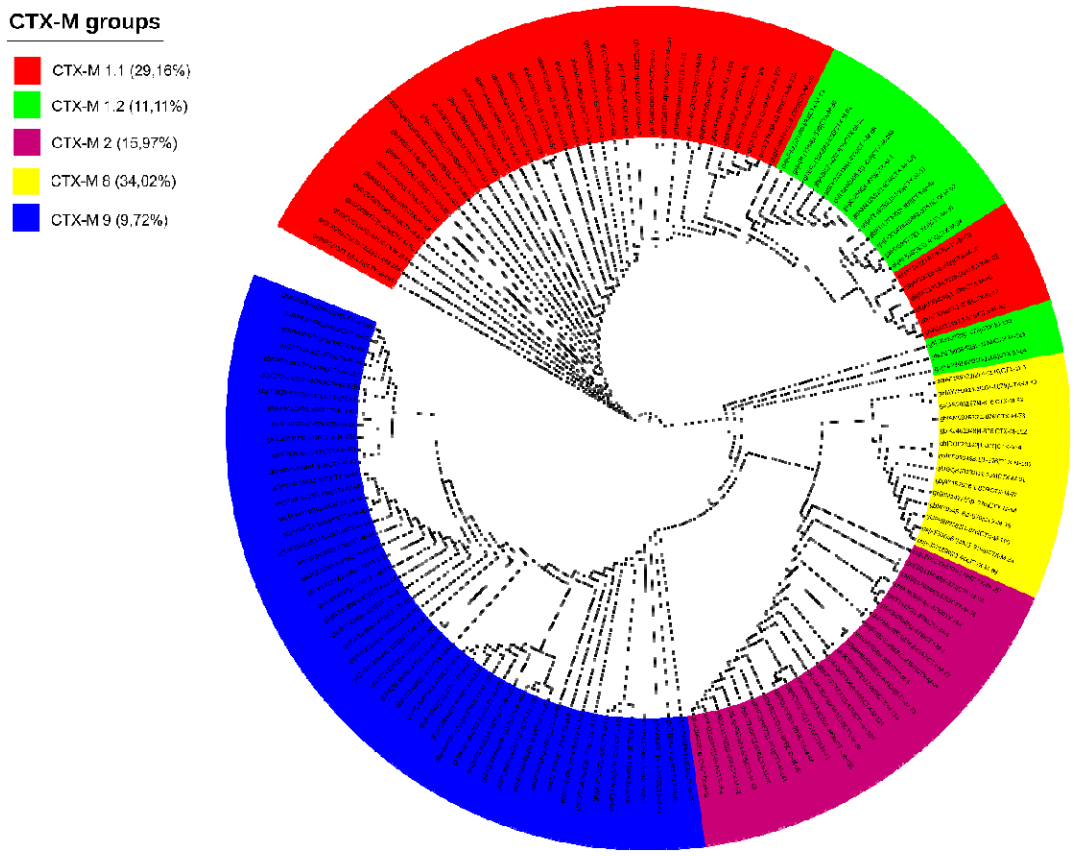


Figure 3. Cladogram with all sequences from the *bla*CTX-M subfamily divided into groups, illustrating the identifiable sequences, and the percentage of sequences detectable by each primer in comparison with the total number of the *bla*CTX-M family (N = 144) until the time of publication of this work.

A total of 14 pairs of primers were developed, which together have the capacity to detect 929 variants of resistance genes belonging to the *bla* gene family. The primer sequences for genes with conserved and non-conserved sequences can be found in **Tables 1** and **2**, respectively.

143 **Table 1.** Primer sequences for conserved genes

Name of gene	Number of variations detectable	Primer name	Sequence	Primer size
*bla*SHV	156	SHV-F	ATTATCTCCCTGTTAGCCACCC	22
		SHV-R	GTTTAATTTGCTCAAGCGGCTG	22
*bla*TEM	167	TEM-F	ACCCAGAAACGCTGGTGAAA	20
		TEM-R	GGGGCGAAAACTCTCAAGGA	20
*bla*NDM	27	NDM-F	GAAGCTGAGCACCGCATTAG	20
		NDM-R	CCATTTGCTGGCCAATCGTC	20
*bla*KPC	208	KPC-F	TCGCGGAACCATTCGCTAAA	20
		KPC-R	GAATGAGCTGCACAGTGGGA	20
*bla*GES	25	GES-F	GCCCAGGAGAGAGATTACGC	20
		GES-R	CTTGACCGACAGAGGCAACT	20

144

145

146 **Table 2.** Primer sequences for non-conserved genes, divided into clades.

	Name of gene	Number of variations detectable	Primer name	Sequence	Primer size
*bla*CTX-M	*bla*CTX-M 1.1*like*	42	CTXM1.1L-F	GATTGCGGAAAAGCACGTCA	20
			CTXM1.1L-R	TTCATCGCCACGTTATCGCT	20
	*bla*CTX-M 1.2*like*	16	CTXM1.2L-F	CGCCGCTGATTCTGGTCA	18
			CTXM1.2L-R	TGACGATTTTAGCCGCCGAC	20

	bla*CTX-M 2*like	23	CTXM2L-F	ATGGCGCAGACCCTGAAAAA	20
			CTXM2L-R	CTGCCGGTTTTATCGCCCA	19
	bla*CTX-M 8*like	14	CTXM8L-F	CGCTCAACACCGCGATCC	18
			CTXM8L-R	ATCCCCGACAACCCACGAT	19
	bla*CTX-M 9*like	49	CTXM9L-F	CGTGGCTCAAAGGCAATACG	20
			CTXM9L-R	TCTGTTGCGGCTGGGTAAAA	20
***bla*OXA**	***bla*OXA-23*like***	24	OXA23L-F	GCTCTAAGCCGCGCAAATAC	20
			OXA23L-R	TGACCTTTTCTCGCCCTTCC	20
	bla*OXA-24/40*like	8	OXA24/40L-F	TGCCGATGACCTTGCACATA	20
			OXA24/40L-R	CCATTAGCTTGCTCCACCCA	20
	bla*OXA-48*like	17	OXA48L-F	:GGTAGCAAAGGAATGGCAAG	21
			OXA48L-R	GGGCGATCAAGCTATTGGGA	20
	bla*OXA-51*like	153	OXA51L-F	GATCGGCCTTGAGCACCATA	20
			OXA51L-R	GCCATAACCAACACGCTTCA	20

***In silico* primers validation.** The parameters obtained for each primer after analysis using the Primer-BLAST® (NCBI, USA) and Sequence Manipulation Suite (SMS): PCR Primer Stats software are available in **Table 3**.

Table 3. Parameters obtained by *in silico* validation of the developed primers.

Gene	Primer name	Tm (C°)	GC%	Self complementarity	Self 3' complementarity	Produt length
***bla*SHV**	SHV-F	59,2	50,0	3	0	70
	SHV-R	59,3	45,4	5	3	
***bla*TEM**	TEM-F	60,1	50,0	4	0	110

	TEM-R	59,9	55,0	2	1	
***bla*NDM**	NDM-F	59,3	55,0	5	1	86
	NDM-R	60,1	55,0	6	2	
***bla*KPC**	KPC-F	60,3	50,0	4	2	128
	KPC-R	60,3	55,0	5	1	
***bla*GES**	GES-F	59,9	60,0	3	2	94
	GES-R	59,9	55,0	3	2	
bla*CTX-M 1.1*like	CTXM1.1L-F	59,7	50,0	4	1	87
	CTXM1.1L-R	60,1	50,0	4	2	
bla*CTX-M 1.2*like	CTXM1.2L-F	60,1	61,1	3	2	90
	CTXM1.2L-R	61,0	55,0	3	3	
bla*CTX-M 2*like	CTXM2L-F	60,5	50,0	4	0	155
	CTXM2L-R	60,7	57,8	4	1	
bla*CTX-M 8*like	CTXM8L-F	61,5	66,6	4	2	197
	CTXM8L-R	60,9	57,8	3	2	
bla*CTX-M 9*like	CTXM9L-F	59,9	55,0	3	2	180
	CTXM9L-R	60,1	50,0	3	0	
bla*OXA-23*like	OXA23L-F	60,0	55,0	4	0	129
	OXA23L-R	59,9	55,0	2	0	
bla*OXA-24/40*like	OXA24/40L-F	59,7	50,0	4	2	177
	OXA24/40L-R	60,0	55,0	4	0	
bla*OXA-48*like	OXA48L-F	59,8	52,3	3	0	183
	OXA48L-R	59,8	55,0	4	0	
bla*OXA-51*like	OXA51L-F	59,8	55,0	4	2	199
	OXA51L-R	59,1	50,0	2	1	

152

153 **Testing, optimization, and standardization of *in vitro* reactions.** All primers
154 were tested using positive controls developed *in house*, and it was possible to verify
155 that the ideal annealing temperature (Ta) for the developed primer pairs was 61°C,
156 except for the primer referring to the *bla*NDM gene, for which the ideal Ta was 64°C.
157 The reactions were then evaluated for their specificity and stability by checking the
158 Melting temperature (Tm). The melting curves are available in the supplementary
159 material **Figure S1**.

160 **Efficiency curve** It was found that all tested primers presented an efficiency
161 rate ≥93.21 and ≤101.28%, R^2 ≥0.99, and detection limit between ≈2 and ≈13 copies/μL as
162 shown in **Table 4**.

163 **Table 4.** Results of the efficiency curves of the developed primers.

Gene name	Efficiency (%)	Correlation coefficient (R^2)	Threshold	Detection limit (Copies/μL)	Melting Peak (Tm°C)
*bla*SHV	96,15	0,992	0,60	≈2	86,7
*bla*TEM	95,32	1,000	0,70	≈2	81,5
*bla*NDM	99,90	0,999	0,48	≈2	85,7
*bla*KPC	100,44	0,992	0,24	≈2	85,7
*bla*GES	100,21	0,997	0,27	≈2	81,7
*bla*CTX-M 1.1*like*	95,75	0,999	0,40	≈13	84,3
*bla*CTX-M 1.2*like*	100,82	0,994	0,32	≈13	83,2
*bla*CTX-M 2*like*	98,09	0,998	0,30	≈2	86,4
*bla*CTX-M 8*like*	95,32	1,000	0,10	≈13	88,4
*bla*CTX-M 9*like*	97,82	0,996	0,10	≈2	89,0

*bla*OXA-23*like*	99,51	1,000	0,15	≈2	78,4
*bla*OXA-24/40*like*	101,82	0,988	0,15	≈13	78,7
*bla*OXA-48*like*	93,21	1,000	0,20	≈13	80,29
*bla*OXA-51*like*	95,51	0,993	0,55	≈2	82,0

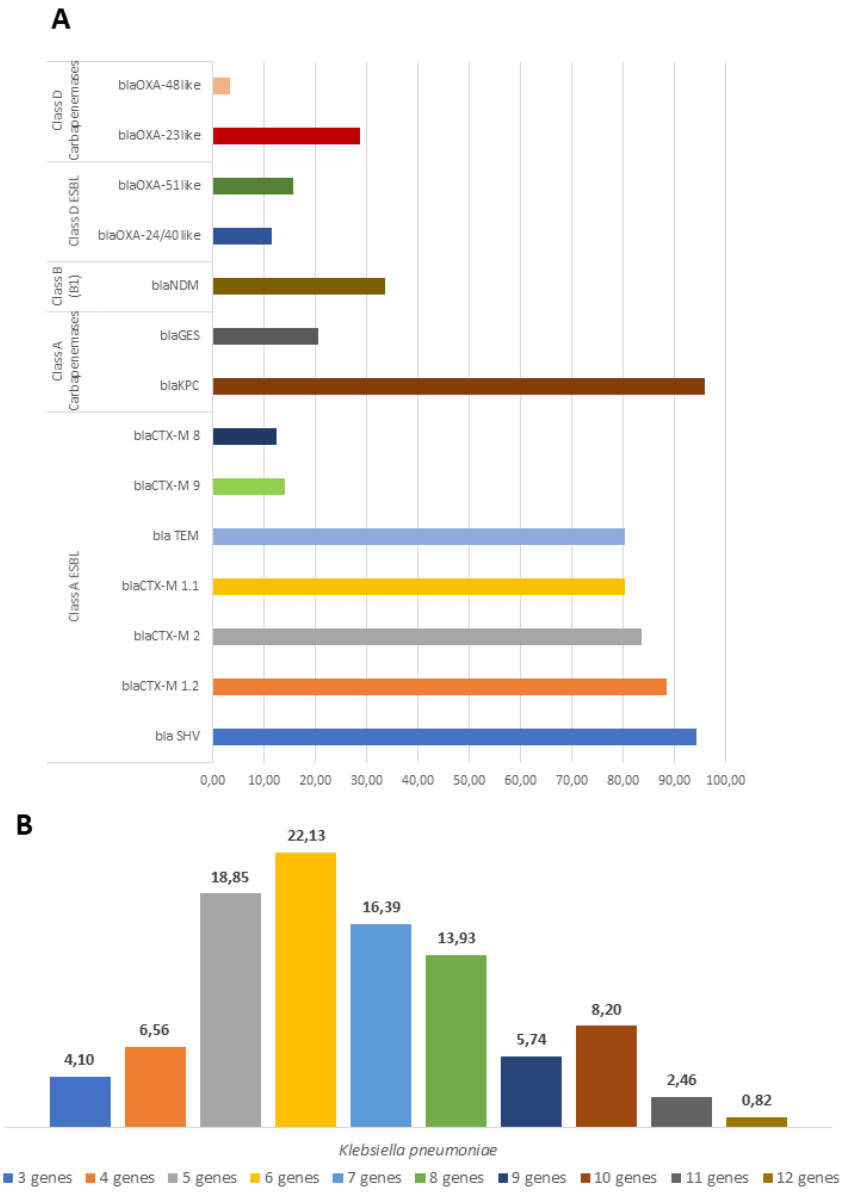
Identification of the genetic resistance profile of *K. pneumoniae* isolates. The 122 *K. pneumoniae* isolates were tested against the 14 pairs of primers developed, and it was possible to verify a high prevalence of beta-lactam resistance genes among the isolates analyzed. The most prevalent genes detected were, respectively: *bla*KPC (95.90%); *bla*SHV (94.26%); *bla*CTX-M 1.2*like* (88.52%); *bla*CTX-M 2*like* (83.61%); *bla*CTX-M 1.1*like* (80.33%); *bla*TEM (80.33%); *bla*NDM (33.61%); *bla*OXA-23*like* (28.69%); *bla*GES (20.49%); *bla*OXA-51*like* (15.57%); *bla*CTX-M 9*like* (13.93%); *bla*CTX-M 8*like* (12.30%); *bla*OXA-24/40*like* (11.48%) *and* *bla*OXA-48*like* (3.28%). The results can be seen in **Figure 4A**, grouped according to Ambler's classification.

It is important to note that not all variants of each group of genes analyzed belong to a single class in the Ambler's classification, such as *bla*TEM and *bla*SHV genes, where not all variants have activity as extended spectrum beta-lactamases (ESBL) for example, however, due to the grouping of all sequences in a single primer, the separation of these sequences into other groups would be impossible, so the grouping of genes was carried out globally in a way that covered the mains classification of the subfamily in question.

Through the data obtained by the qPCR reactions developed, it was also possible to verify that the isolates analyzed during this study had the presence of

183 several genes belonging to the *bla* family accumulated, with values varying from 3 to

184 12 of the tested genes, present per isolate, as shown in **Figure 4B**.



185

186 **Figure 4**. **(A)** Prevalence of beta-lactams resistance genes among the analyzed isolates,

187 grouped according to the Ambler classification. **(B)** The percentage of *K. pneumoniae*

188 isolates showing accumulation of genes encoding beta-lactamases belonging to the *bla*

189 family.

Comparisons of metabolomic profiles of *K. pneumoniae* isolates. In total, 241 metabolites were identified in positive mode and 116 metabolites identified in negative mode. Among these, 309 metabolites were identified by combining data from both acquisition modes (positive and negative) without redundancy. The principal component analysis (PCAs) and the heatmap analysis between the metabolites in supernatant of susceptible *K. pneumoniae* versus the control culture medium showed complete separation of the main vectors of the PCA plots and differential upregulation or downregulation between the two experimental groups (**Figure 5**). Among these metabolites, 25 were upregulated or downregulated. Interestingly, metabolites related to the synthesis of nitrogenous bases, protein synthesis and energy supply to the bacterial cell are significantly increased in supernatant derived from susceptible *K. pneumoniae* (**Figures S2, S3 and S4**). Similar findings were detected between MDR *K. pneumoniae* strains and control culture medium (**Figures S5, S6 and S7**).

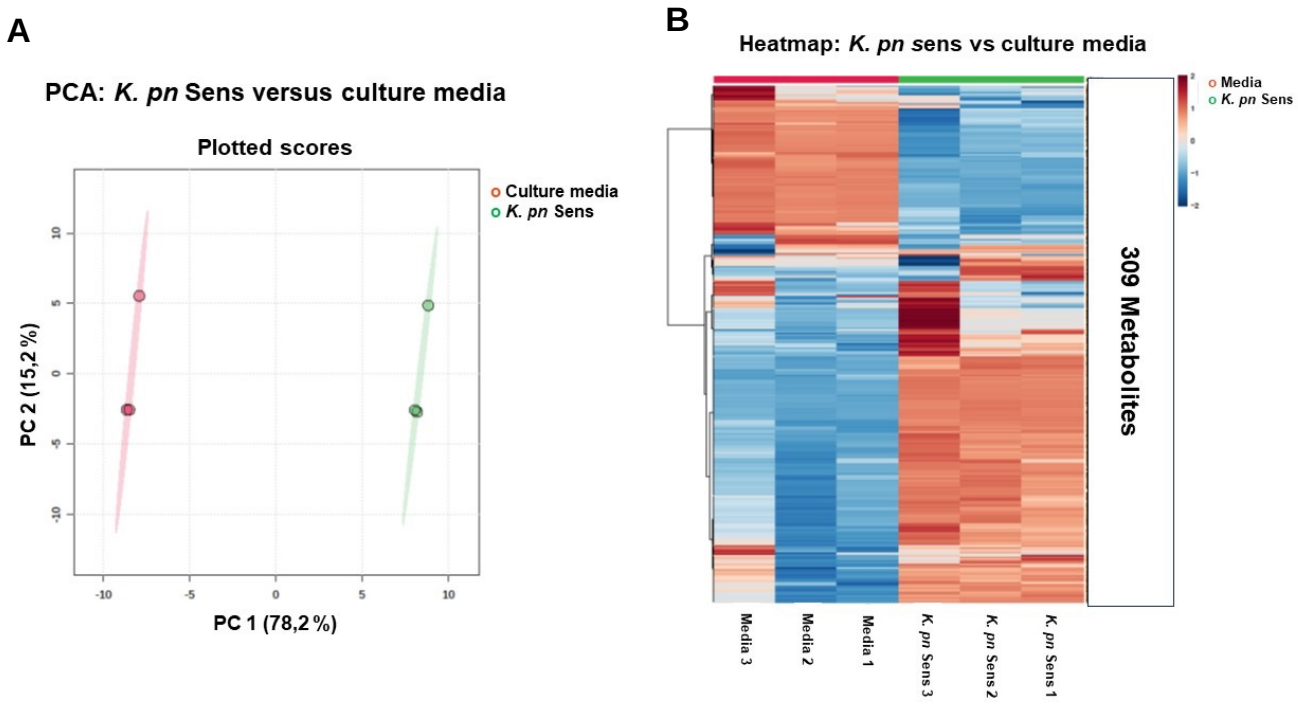


Figure 5. Comparison between susceptible *K. pneumoniae* supernatants (Sens) versus culture media. **(A)** Plot of the principal component analysis (PCAs) and **(B)** the heatmap of the comparison of metabolites between the supernatant of susceptible *K. pneumoniae* versus the control culture media.

The principal component analysis (PCAs) and the heatmap analysis of metabolites in supernatants from *K. pneumoniae* MDR versus susceptible isolates exhibited complete separation of the main vectors of the PCA plots (**Figure 6A**) and differences on levels of metabolites (**Figure 6B**). The analysis of deregulated metabolites between *K. pneumoniae* MDR versus susceptible isolates showed increased N-fructosyl isoleucine in the MDR *K. pneumoniae* isolates ($p < 0.05$, **Figure 6C**).

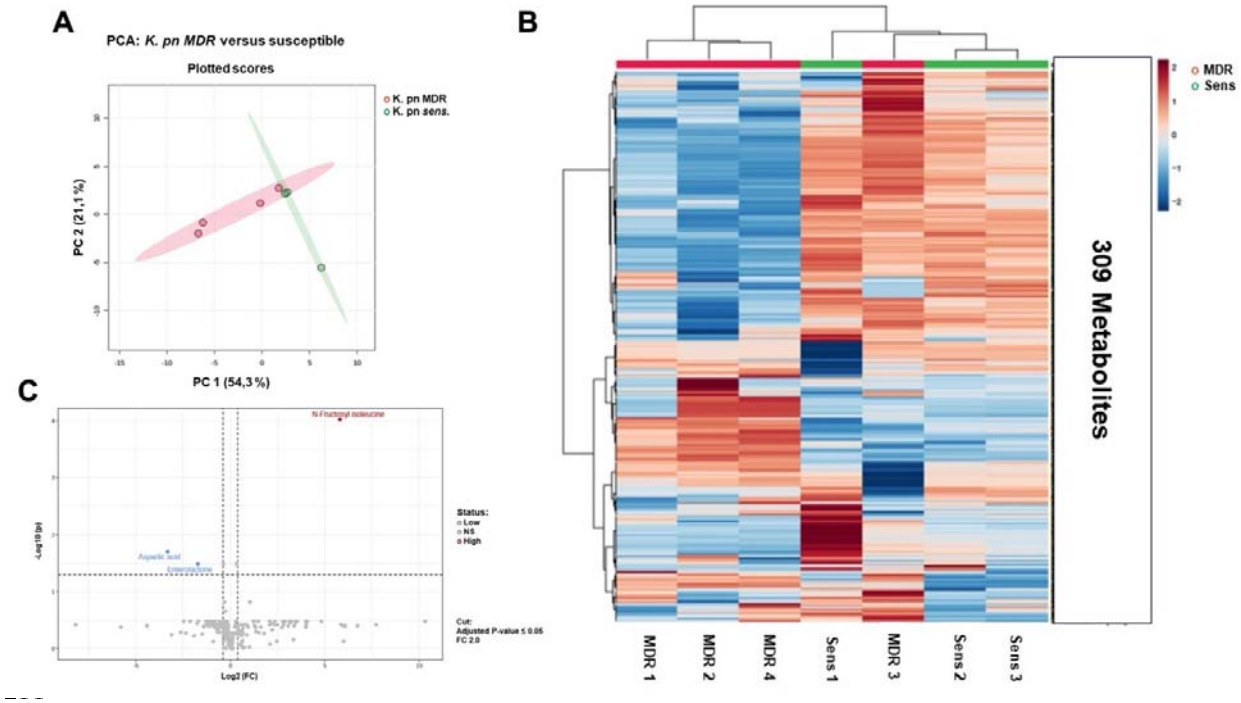


Figure 6. Comparison of supernatants from *K. pneumoniae* MDR versus susceptible (Sens). **(A)** Plots of the principal component analysis (PCAs) and **(B)** the heatmap of the comparison of metabolites between the supernatant of *K. pneumoniae* MDR versus

susceptible (Sens). **(C)** Plots of dysregulated metabolites between supernatants of *K. pneumoniae* MDR and susceptible.

Analysis of the intracellular metabolites of susceptible versus MDR K. pneumoniae showed differences in the PCAs vectors (**Figure 7A**), heatmap (**Figure 7B**). and increased N-fructosyl isoleucine levels in the MDR K. pneumoniae isolates ($p<0.05$, **Figure 7C**). **Figure 8** shows the PCAs and heatmap plots of supernatant from sensitive and MDR K. pneumoniae.

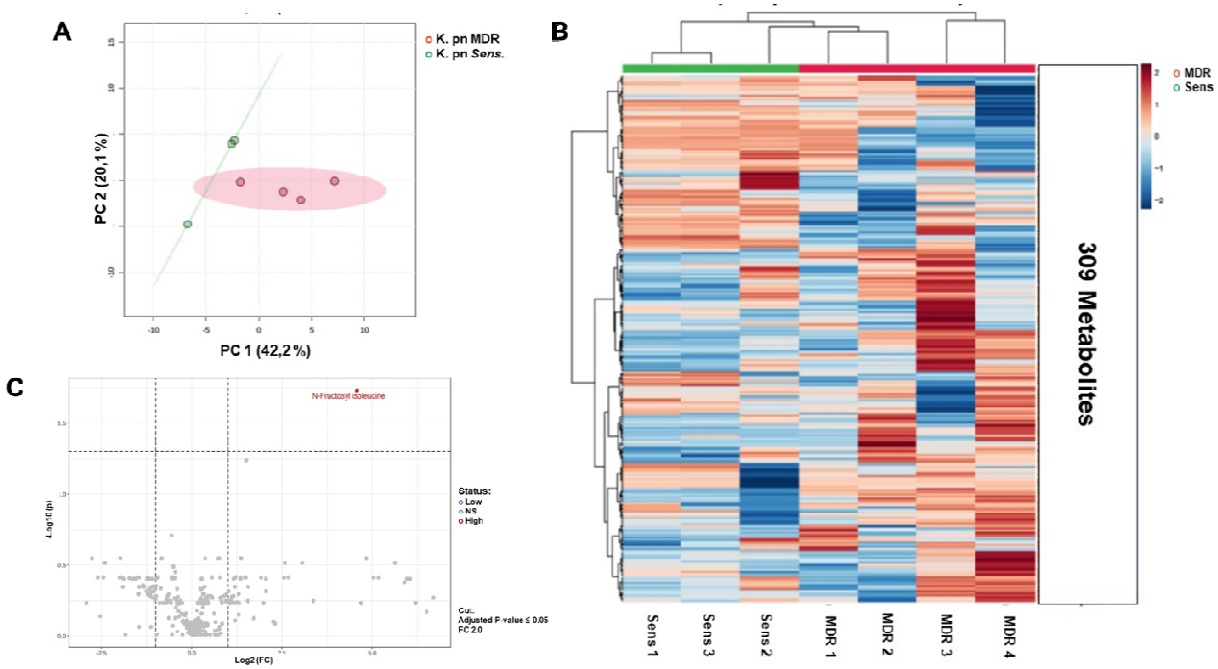


Figure 7. Comparison of Intracellular media of *K. pneumoniae* MDR versus susceptible (Sens). **(A)** Principal Component Analysis (PCAs) plots and **(B)** Heatmap of the Intracellular Environment of *K. pneumoniae* MDR and susceptible. **(C)** Plots of dysregulated metabolites between the intracellular media of *K. pneumoniae* MDR and susceptible.

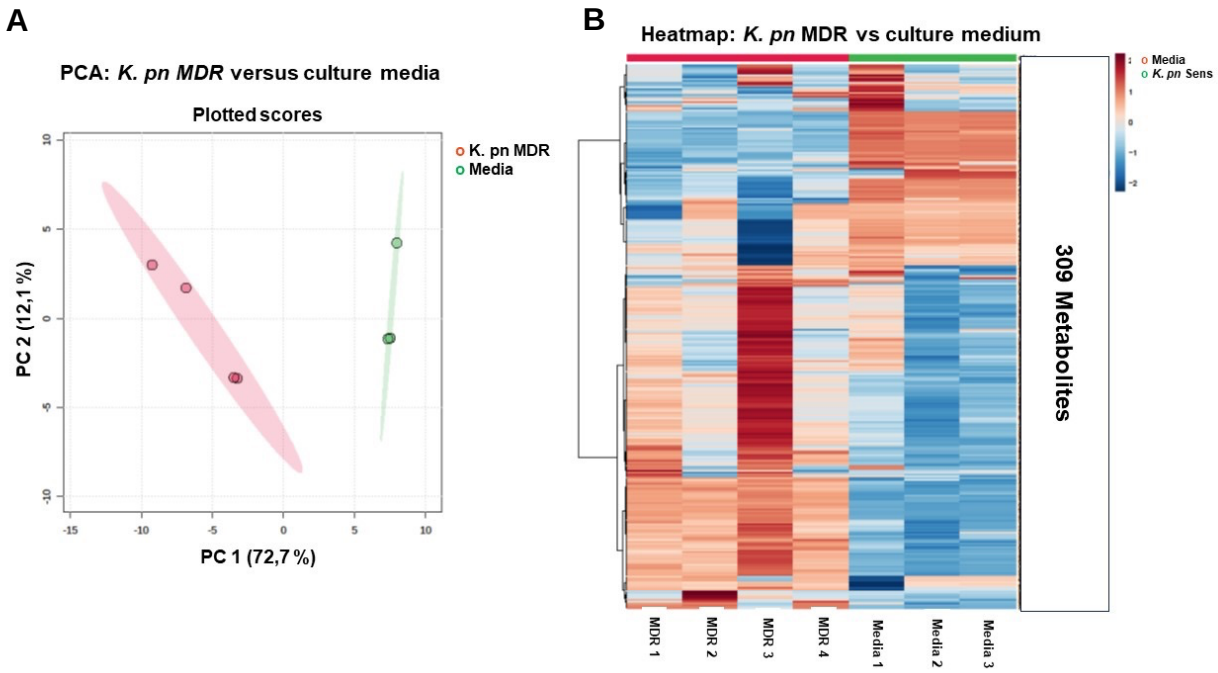


Figure 8. Comparison of *K. pneumoniae* Multidrug-Resistant (MDR) supernatants versus culture media. (A) Principal component analysis (PCAs) plots and (B) Heatmap of the supernatant of *K. pneumoniae* susceptible and MDR. Observe the separation of the main vectors of the (PCAs) plots and details of the differences in the heatmap between the two experimental groups.

DISCUSSION

During the period analyzed in the study, 249 bacterial isolates resistant to two or more classes of antimicrobials used in TSA were identified. Of these resistant isolates, 48.99% (122/249) belonged to the species *Klebsiella pneumoniae*, being this microorganism the most prevalent among those identified.

This data is consistent with what is seen in Egypt, where when evaluating 186 samples from Cairo hospitals, *K. pneumoniae* was found as the main gram-negative bacterial agent representing 40.9% (76/186) of the identified microorganisms, and

among these isolates obtained, a high prevalence of resistance to beta-lactams, quinolones, and sulfonamides was identified (89.4%, 89.4% and 87.1%) respectively.[16]

The phenotypic profile of the *K. pneumoniae* isolates analyzed in the present study demonstrated high levels of resistance to the beta-lactams drugs tested in all classes evaluated (penicillins, cephalosporins and carbapenems). Although the resistance profile of strains may vary depending on the test location and the conditions to prevent spread, the resistance to beta-lactams among *Enterobacteriaceae* is known to be high, as demonstrated in a study carried out in Brazil, with samples of human and veterinary origin where 62.85% and 54.28% of *Enterobacteriaceae* isolated were resistant to amoxicillin/clavulanate and cefazolin respectively.[17]

In studies carried out in Egypt, evaluating isolates of *K. pneumoniae* from food sources, and in Tunisia and Iran evaluating isolates from hospital samples, the presence of high resistance to beta-lactams can be verified for ampicillin/sulbactam (93%), ceftazidime (95.5%), cefoxitin (95.5%), cefotaxime (93.2%), amoxicillin/clavulanate (86.4%), ertapenem (90.9%) and temocillin (84.0%).[18, 19, 20]

Likewise, in a study carried out with 102 isolates obtained from two Portuguese hospitals, the presence of resistance rates > 90% was verified for all beta-lactam drugs tested, except for cephalosporins: cefoxitin (40.2%), cefotetan (68.6%) and carbapenems: ertapenem (23.5%), imipenem (32.4%), meropenem (34.3%) and doripenem (33.3%).[21]

This result is consistent with that found in this study, where the beta-lactam drugs that showed a lower resistance rate belonged to the same classes mentioned, namely: cefoxitin (73.68%), ceftazidime/avibactam (11.76%), ertapenem (44.00%), imipenem (71.70%) and meropenem (69.81%).

Resistance to beta-lactams is conferred in Gram-negatives mainly by genes belonging to the *bla* family. This group of genes is composed of dozens of subfamilies and hundreds of genetic subvariants in each family, the molecular panel developed managed to encompass some of the most predominant and clinically relevant subfamilies among clinical isolates, identified in several studies, namely: *bla*SHV, *bla*TEM, *bla*NDM, *bla*KPC, *bla*GES, *bla*CTX-M and *bla*OXA.[22–24]

After analysis using the Primer-BLAST® (NCBI, USA) and Primer Stats Sequence Manipulation Suite (SMS) software, it was verified that the developed primers have specificity for the sequences used in the alignment, melting temperature (Tm) between 59 29 °C and 61.55 °C, GC% concentration varying between 45.45% and 66.67% in addition to having complementary base numbers ≤ 6 in the analysis of dimer and hairpin formation. The values obtained for these parameters therefore demonstrate that they are compatible with those obtained in reference studies.[25–27]

Due to the high number of sequences included in the development of primers, many of the alternatives obtained through Primer-BLAST® (NCBI, USA) did not meet the stability requirements regarding the formation of secondary structures (self-annealing and hairpins), In order to choose the options with the best performance in relation to these parameters, the GC clamp was relaxed so that the primers SHV-F, SHV-R, CTXM1.2L-R and CTXM2L-R obtained 4 G or C residues within the last 5 nucleotides of the 3' end, the primers CTXM9L-R and CTXM2L-F did not present C or G residues within the last 5 nucleotides of the sequence.

This fact could lead to the formation of strong bonds and increase the Tm value in primers with more than 3 terminal GC residues, and cause inhibition of amplification due to the presence of weak bonds when there is an absence of terminal

293 CG [28,29]. Such changes, however, were not evident when the primers were tested *in*
294 *vitro*.

295 The variety of sequences detectable by the developed primers makes in vitro
296 validation using positive controls for all sequences virtually impossible. In silico
297 analysis allowed the evaluation of all tested sequences quickly, cheaply and constantly,
298 since the developed primers can be tested against new variants of the target genes.[26–28]

299 In vitro analyzes were carried out using SYBR Green master mix, the melting
300 curve was therefore used as a parameter to evaluate the specificity of the reactions.
301 And it was verified that for all tested primers a single melting peak was found,
302 indicating the formation of a single amplicon. And excluding the presence of
303 secondary structures (primer dimers and hairpins).

304 The analysis of the melting curve and Tm is considered fundamental to test the
305 specificity of reactions that use SYBR Green as an amplification indicator, because
306 unlike reactions that use specific probes, which emit fluorescence only when the
307 reaction occurs at the expected target. The SYBR Green works by binding to any
308 double-stranded fragment formed, thus emitting fluorescence. Therefore, only by
309 analyzing the melting curve and the specific Tm of the amplicon is it possible to
310 determine whether the reaction was successful.[29–31]

311 The efficiency curve and its parameters were used to evaluate the performance
312 of qPCR reactions, by checking how efficiently the targets were amplified in each PCR
313 cycle.[32, 33] When evaluating the activity of primers in qPCR reactions using the
314 efficiency curve, it was found that all primers analyzed in this study presented
315 efficiency values >90% and <110% and correlation coefficients (R^2) >0.9, thus being in
316 accordance with the parameters presented in reference documents present in the

317 literature.[33] It was also possible to verify a high detection capacity even at low
318 concentrations of genetic material since the detection limit obtained varied between ≈2
319 and ≈13 copies/μL of the target gene used as control.

320 Several studies report the growth in the prevalence of the *bla*KPC gene, with
321 this being the most detected carbapenemase worldwide. The values found vary
322 according to the location. The main variant of the *bla*KPC gene detected is *bla*KPC-2,
323 with prevalence values varying from 1.2% to 51.6% among enterobacteria in studies
324 carried out in the United States of America and China respectively.[34, 35] This gene is
325 found more frequently in isolates of *K. pneumoniae*, many studies report high
326 prevalence rates in this species ranging from 17.2% to 64.6%.[4, 35, 36, 37]

327 In a study conducted in an intensive care unit in the northeast region of Brazil,
328 it was shown that of 25 isolates of *K.* pneumoniae obtained during the research, 100%
329 exhibited the presence of the *bla*KPC gene. [38]

330 The values found in this paper for the *bla*KPC gene may reflect the fact that the
331 primer developed detected all variants of the gene, as well as the higher rates of
332 resistance usually found in intensive care unities (ICU).[39]

333 The results found in this work are in agreement with other studies performed at
334 the northeast of Brazil[38], what can represent an endemic presence of the *bla*KPC gene
335 among *K. pneumoniae* isolates in this Brazilian region.

336 The *bla*TEM and *bla*SHV genes were the first ESBL identified, and together with
337 the *bla*CTX-M subfamily of genes are considered the most prevalent ESBL's. These
338 genes are widely distributed among enterobacteria; in a study carried out in Sudan, the
339 *bla*TEM, *bla*CTX-M and *bla*SHV genes were identified in 86.0%, 78.0% and 28.0%
340 respectively.[40]

In a work carried out in ICUs in Chile, it was possible to verify the presence of these genes with prevalence rates of 81.0%, 84.7% and 73.0% for *bla*SHV, *bla*CTX-M-1 and *bla*TEM, where a higher prevalence of the *bla*SHV gene was found when compared to the study carried out in Sudan.[41]

In Brazil, it is also possible to verify the presence of high prevalence rates of these genes among *K.* pneumoniae isolates from ICU, with studies indicating rates of 100%, 96% and 72% for *bla*TEM, *bla*SHV and *bla*CTX-M1 respectively. [38] These results agree with those evidenced in this study for the K. pneumoniae isolates tested.

The *bla*NDM gene is considered the second most prevalent carbapenemase producing gene in the world, behind only the *bla*KPC gene. In this study, the presence of the metallo beta-lactamase NDM gene was verified in (33.61%) of the isolates. Similar results were evidenced in China, where the presence of this gene was verified in 35.7% of 935 carbapenem-resistant enterobacteria tested.[34]

The prevalence found in this study for the *bla*NDM gene is still below what was found in other studies carried out in Brazil in the states of São Paulo and Sergipe, where the authors found a prevalence of 70 and 50% respectively in *K. pneumoniae* isolates. [42, 43]

Regarding oxacillinases, a low prevalence of the *bla*OXA-48*like* gene was observed (3.28%). This gene may be present in all *Enterobacteriaceae*; however, it is more prevalent in strains of *K. pneumoniae*.[21]

The values found in the literature for the presence of this gene in *Enterobacteriaceae* are very divergent, with marked variations related to the location of the analysis, these values vary from 7.3% in China to 83.3% in Morocco.[34, 44] In a study conducted in Brazil where 4,451 isolates of *Enterobacteriaceae* were analyzed, the

365 *bla*OXA-48*like* gene was detected in only 2.5% of the isolates tested.[45] In this way, the
366 result obtained is compatible with what was previously identified in Brazil, and close
367 to what is seen in China.

368 The presence of variants of the *bla*OXA gene (*bla*-OXA-51*like* (15.57%), *bla*OXA-
369 24/40*like* (11.48%) and *bla*OXA-23*like* (28.69%)) was also observed in the isolates tested,
370 these genes were long considered exclusive to strains of *Acinetobacter baumannii*,
371 however studies have demonstrated the presence of these genes in other
372 *Enterobacteriaceae*, including *K. pneumoniae*.[46] Results regarding the prevalence of these
373 genes in strains of *K. pneumoniae* are still scarce, however, in a study conducted in
374 Bahrain in the Persian Gulf, the *bla*OXA-*51* and *bla*OXA-*23* genes were identified
375 respectively in 45.8% and 41.6%. % of *K. pneumoniae* isolates analyzed.[47]

376 It is believed that many of the beta-lactamases that today can be found
377 contained in mobile genetic elements had their origin in chromosomes of other
378 bacteria, as occurred with the SHV type variants that derived from the *K. pneumoniae*
379 chromosomal SHV-1, and the CTX-M type variants that appear to have originated from
380 the chromosomal CTX-M of *Kluyvera* spp[21]. This change in the presentation of these
381 genes, from chromosomal to mobile elements, may explain the appearance of genes
382 such as chromosomal *bla*OXA present in *A. baumannii* strains on plasmids widespread
383 in other species.

384 It was possible to verify the presence of isolates presenting multiple genes
385 coding for beta-lactamases, from 3 to 12 of the genes analyzed. Studies point to a high
386 prevalence among *Enterobacteriaceae* of the accumulation of genes from the *bla* family,
387 mainly genes belonging to ESBL, OXA beta-lactamases and carbapenemases such as
388 *bla*KPC and *bla*NDM. This phenomenon is more evident in hospital environments

where selective pressure favors microorganisms carrying resistance genes and where there is a high rate of sharing of these genes.[48,49]

Investigations into metabolomic modulation in *K. pneumoniae* have demonstrated a promising area for understanding resistance to antimicrobial agents, developing new therapies, and for studying bacterial virulence. For example, significant inhibition of the pentose phosphate pathway, citrate cycle, amino acid and nucleotide metabolism was observed to be beneficial during treatment of MDR *K. pneumoniae* using the bacteriophage-polymyxin combination, showing the potential for new therapeutic targets and pathways metabolic processes to improve the effectiveness of treatment with available antimicrobial agents.[50,51]

This study is in accordance with others that highlighted the importance of the pentose phosphate pathway in MDR *K. pneumoniae* strains.[50] This finding was present in both media, supernatant and intracellular in MDR isolates, compared with *K. pneumoniae* isolates susceptible to antimicrobial agents. The significant presence of the metabolic N-fructosyl isoleucine ($C_{12}H_{23}NO_7$; MW: 293.316) in the supernatant and intracellular media of *K. pneumoniae* MDR indicates the expression of the fructose degradation enzyme gene (*frwC*; fructose-specific phosphotransferase system), which is involved in regulating bacterial growth, virulence and overcoming colonization resistance due to the use of alternative carbon source from fructose.[52, 53]

Metabolomics has proven to be a promising approach in the study of antimicrobial resistance. Its use allows for the comprehensive investigation of metabolic changes associates with different resistance mechanisms, such as alternative energy-obtaining pathways, modifications that alter the cell wall, communication

412 between bacterial cells, and changes in the colonization capacity of these
413 microorganisms.[54, 55]

414 The identification of these metabolic differences plays an important role in the
415 Discovery of new therapeutic targets and strategies for investigating the reversal of
416 resistance in these pathogens.[55-57] The combination of genetic and metabolomic
417 techniques therefore contributes to a comprehensive view of resistance mechanisms in
418 bacteria. [56,57]

419 The presence of N-fructosyl isoleucine in resistant strains, and the metabolic
420 benefit that its presence appears to generate in increasing colonization capacity,
421 together with the selective pressure found in ICU environments due to the constant use
422 of antimicrobials may be related to the high rates of resistant isolates disseminated
423 among patients in this environment.

424 The metabolic evaluation in turn points to a metabolic advantage in resistant
425 strains compared to sensitive ones, which goes beyond the resistance capacity itself.[58]

426 This study has some limitations, such as the exploration of only *bla* family
427 genes, limiting a broader understanding of resistance mechanisms to antimicrobial
428 agents. On the other hand, it presents a broad methodology for exploring the
429 important *bla* family genes most associated with resistance to beta-lactams in *K.*
430 *pneumonia*. The metabolic findings showed a specific indication that suggests an
431 association with virulence and better proteomic studies will be necessary to associate
432 with potential mechanisms of resistance to antimicrobial agents. Furthermore,
433 additional preclinical studies will be necessary to determine and more specifically
434 validate the virulence associated with the pentose phosphate pathway in *K.*
435 *pneumoniae*.

436

437

438 **CONCLUSIONS**

439 The results of this work show the high prevalence of resistance to antimicrobial
440 agents in isolates of *K. pneumoniae* within the hospital environment, and that this
441 phenotypic pattern exhibited a great concern as it limits the therapeutic options
442 available, directly implicating the patient's prognosis.

443 The assays developed were within the quality criteria for qPCR reactions using
444 SYBR Green master mix and were efficient in detecting the beta-lactams resistance
445 genes from the *bla* family evaluated in this study. The genotypes of the *bla* genes
446 obtained are relevant and worrying for the local hospital scenario, and compatible with
447 the identified phenotypic profile.

448 The identification of the metabolic N-fructosyl isoleucine in the supernatant
449 and intracellular media of *K. pneumoniae* MDR indicates the expression of the fructose
450 degradation enzyme gene, which is involved in regulating bacterial growth, virulence
451 and overcoming resistance to colonization due to the use of an alternative carbon
452 source from fructose.

453 **MATERIALS AND METHODS**

454 **Obtaining bacterial isolates and identification.** For the following study,
455 samples were collected as part of the clinical investigation of patients admitted to the
456 ICU of a tertiary care health unit in the city of Fortaleza-CE. Were included in the work
457 those microorganisms that were identified as gram-negative bacteria, resistant to two

458 or more groups of antimicrobial agents, including subclasses of β-lactams,
459 fluoroquinolones and aminoglycosides.

460 The bacterial isolates were identified and tested for their susceptibility to
461 antimicrobials using the automated VITEK® 2 Compact method (BioMérieux, Marcy
462 l'Etoile, France), according to the manufacturer's recommendations. Minimum
463 inhibitory concentrations were interpreted according to the Clinical and Laboratory
464 Standards Institute (CLSI). For quality control of sensitivity tests, strains from the
465 American Type Culture Collection (ATCC) were used. Specimens that had a resistance
466 profile that fit the research objectives were included in the study.

467 **Extraction of bacterial DNA.** To extract the genetic material, the Wizard
468 Genomic DNA Purification extraction and purification kit (Promega, Madison, USA)
469 was used, according to the manufacturer's recommendations.

470 After extraction, all samples were then quantified by spectrophotometry using
471 the NanoDropTM 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and
472 stored in a -80°C freezer until used in the experiments.

473 **Selection of genes used in the study and obtaining FASTA sequences.** For
474 greater coverage of the genetic profile of resistance to β-lactams in gram-negatives,
475 genes with relevant prevalence epidemiology, belonging to the *bla* gene family, were
476 part of the study, and these included the genes*: blaSHV, blaTEM, blaNDM, blaKPC,*
477 *blaGES, blaCTX-M and blaOXA*. The sequences used were obtained through the
478 Comprehensive Antibiotic Resistance Database (CARD) and National Center for
479 Biotechnology information (NCBI) platforms, which compiles and organizes the

480 resistance gene sequences available in GenBanck, the list of identifiable sequences is
481 available in the supplementary material Text S1.

482 **Primer design.** To design the primers, all variant sequences of each gene
483 included in the study available on the CARD and NCBI platforms were gathered, the
484 sequences were aligned using the Clustal Omega software, and the alignments were
485 analyzed using the SnapGene software.

486 Consensus regions with homology ≥95% were selected and these were used to
487 design the primers using the Primer Blast platform from the National Center for
488 Biotechnology and Information (NCBI, USA). The consensus sequences obtained is
489 available in the supplementary material Text S2.

490 Genes with few conserved regions, for which it was not possible to obtain
491 consensus sequences with homology ≥95%, were separated into clades, and primers
492 were then designed for the sequences by phylogenetic grouping, complying with a
493 minimum of 95% similarity.

494 ***In silico* validation of the developed primers.** All primers developed were
495 validated *in silico* regarding their specificity, structure, formation of primer-dimers and
496 hairpins using the Primer-BLAST® (NCBI, USA) and Sequence Manipulation Suite
497 (SMS): PCR Primer Stats platforms.

498 **Testing, optimization, and standardization of primers.** The reactions were
499 standardized with the use of positive controls developed in-house, through
500 amplification of genetic material, isolation, and purification of amplicons, originating
501 from isolates phenotypically resistant to beta-lactams, and negative control (water
502 DNase/RNase free). They were carried out using a CYBR Green master mix (Promega,

503 Madison, USA), the initial results were evaluated regarding the melting temperature
504 (Tm), to confirm the specificity of the amplicons.

505 To determine the most efficient qPCR conditions, to reduce the existence of
506 non-specificity and facilitate the interpretation of the results, the concentration gradient
507 and annealing temperature (Ta) of the primers were performed. The qPCR reaction
508 conditions included a hot start step at 95°C for 2 minutes, followed by 35 cycles
509 consisting of a denaturation step for 15 seconds at 95°C, and an annealing/extension
510 step for 1 minute at Ta. specific to each primer, all reactions went through a final
511 melting curve step, with a temperature variation of 60 to 95°C with an increase of
512 0.05°C/sec.

513 A 9-point efficiency curve was performed with a dilution factor of 1:8, for each
514 primer developed, with concentrations ranging from ≈27,438,596 to ≈2 (copies/μL),
515 through this procedure it was possible to determine the values of threshold, evaluate
516 the efficiency (%), the correlation coefficient (R^2) and the limit of detection of each
517 primer.

518 **Detection of resistance-related genes by molecular biology.** The *K. pneumoniae*
519 isolates obtained in the study were tested against the developed primers. Melting
520 curve analysis of all reactions was used to evaluate the specificity of the results of the
521 isolates in comparison to the specific melting temperature (Tm) of the positive control.

522 **Preparation of *K.* pneumoniae samples for metabolomic evaluation.** Isolates
523 of *K. pneumoniae* susceptible and multidrug resistant to antimicrobial agents obtained
524 in this work were submitted for metabolomic analysis. For this experiment, seven
525 samples of the culture supernatant were designed: one sample of the culture medium,
526 two samples of the culture supernatant of susceptible *K. pneumoniae* (biological

527 replicates), four samples of the culture supernatant of multidrug-resistant *K.*
528 *pneumoniae* (MDR) for untargeted metabolomics determination and analysis. Samples
529 of the intracellular medium of the *K. pneumoniae* culture, two samples of susceptible *K.*
530 *pneumoniae* (biological replicates) and four samples of MDR *K. pneumoniae* were also
531 used for untargeted metabolomic analysis. Technical replicates were performed for
532 susceptible media and samples to allow for a pilot statistical comparison.

533 **Methodology for extracting *K. pneumoniae* culture supernatant.** To 200 µL of
534 *K. pneumoniae* culture medium or control medium, 800 µL of 80% methanol was added
535 at -20 °C. The samples were vortexed and incubated at -20°C for 2 hours for protein
536 precipitation. The samples were centrifuged for 10 min at 4°C at 10,000 g (Benchtop
537 refrigerated centrifuge 10k RPM Eppendorf) and the supernatants were transferred to
538 new tubes. The samples were dried under vacuum (Speed Vac Thermo fisher) for
539 approximately 4-5 hours. Dried samples were redissolved in 100 µL of 0.1% formic
540 acid in water containing Metabolomics QReSS labeled heavy standards diluted 100X.
541 (https://www.isotope.com/userfiles/files/assetLibrary/MET_RSCH_QReSS.pdf). A
542 quality control (QC) was done, combining 10 µL of each sample. After the QC test in
543 the mass spectrometer, the samples were diluted 10x to be analyzed. The injection
544 volume of each sample was 10 µL per ionization mode.

545 **Methodology for extractions from the intracellular medium of *K. pneumoniae***
546 **culture.** To each tube, 750 µL of cold chloroform:methanol (2:1) mixture at -20 °C was
547 added, vortexed and transferred to tubes reinforced with a ball beater. Cells were
548 disrupted in a bead beater with steel balls for 3 min at an intensity of 5 (Bead Ruptor
549 Elite Omni International Material). The tubes were shaken vigorously for 30 min at 4°C
550 in a temperature-controlled thermal shaker (Thermomixer Eppendorf). 400 µL of water

was added, shaken vigorously, and centrifuged for 10 min at 10,000 rpm (Benchtop refrigerated centrifuge 10k RPM Eppendorf) for phase separation. The upper aqueous/methanolic phase was saved as a mixture of soluble metabolites and transferred to Eppendorf tubes. Soluble metabolites were dried under vacuum for 3-4 h (Speed Vac Thermo Fisher). Before running, samples were reconstituted in 100 μL of 0.1% formic acid in water containing 100X diluted Metabolomics QReSS labeled heavy standards (https://www.isotope.com/userfiles/files/assetLibrary/MET_RSCH_QReSS.pdf). The QC sample was prepared with 10 μL of each sample. The injection volume of each sample was 10 μL.

Analysis by Ultra Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry (UPLC-MS/MS) of metabolites. MS data were acquired on the Orbitrap IDX spectrometer (Thermo) connected to the Vanquish UPLC system. Soluble metabolites were separated using a Waters BEH C18 column (100 x 2.1 mm, 1.9 μm) operated at 30 °C and a flow rate of 250 μL/min. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in 90% methanol. Bulk scan range: 67-1000 at 120,000 resolutions with 0.6 sec scan range. The 10 most intense ions in each full scan were selected for fragmentation and MS2 spectra were acquired at a resolution of 30,000 and scaled collision dissociation fragmentation energy of 25, 30, 35 was used.

UPLC gradient. The total time used in the UPLC gradient runs was 15 minutes. The flow used was 0.250 mL/min and the following gradient in the percentage of mobile phase B was 50% at 8 min, 98% at 9 min. maintaining until a time of 13 min., ending with 0% between a time of 13.1 – 15 min.

Verification settings in the mass spectrum. The configurations used in the verification through the mass spectrum were orbit trap of the mass spectrum in the master scan, with an orbitrap detector with a resolution of 120,000. We used quadrupole isolation with a scanning range between 67-1000 m/z, using the frequency lens at 60%. The automatic gain control target was customized and normalized to 25%. The maximum injection time mode has been customized with a maximum injection time of 50 ms. MIcroscans of 1 and profile with positive polarity. Font fragmentation has been disabled.

Data acquisition, metabolic identification, and data analysis. Once the data was acquired, the samples were analyzed using the open-source software MS-DIAL. More details about the software can be obtained here: http://prime.psc.riken.jp/compms/msdial/main.html.[59,60] Three blank samples were included in the analysis to identify background ions and remove them later. Samples acquired using MS1 full scan used for quantification and data-dependent acquisition (DDA) mode were used for spectral identification of metabolites. Tolerance MS1 was set to 0.01 Da and MS2 set to 0.025 Da. For peak harvesting, the mass slice width was set to 0.1. For peak alignment, the maximum retention time tolerance was set at 0.5 min, the MS1 tolerance was set at 0.01. Peaks were identified by searching MS2 spectra in the public MS-DIAL database downloaded in January 2023 (324,191 records for positive mode and 64,669 entries for negative mode) using a mass tolerance of 0.01 Da for MS1 and 0.05 Gives to MSMS with an identification cutoff point of 80% http://prime.psc.riken.jp/compms/msdial/main.html#MSP. Additionally, peaks were also searched against the core Biomolecular Analysis Facility's internal IROA library (both positive and negative mode) with a mass tolerance of 0.02 and identification

599 cutoff of 85%. The data was manually inspected and identifications without MS2 were
600 filtered, except for identifications of the IROA molecule.
601
602
603
604 **Supplementary Materials**: The following supporting information: Figures S1-S7; Text
605 S1; Text S2.
606 **Figure supplement legend:**
607 **Figure S1** – Primer's specificity evaluation through Melting curve: (A) SHV, (B)
608 KPC, (C) NDM, (D) TEM, (E) GES, (F) OXA-23*like*, (G) OXA-24/40like, (H) OXA-48like,
609 (I) OXA-51like, (J) CTX-M 1.1*like*, (K) CTX-M 1.2*like*, (L) CTX-M 2*like*, (M) CTX-M 8*like* e
610 (N) CTX-M 9*like*.
611 **Figure S2** - Plot analysis of the 25 main metabolites from the supernatant of
612 susceptible *K. pneumoniae* in general without separation by up- or down-regulation of
613 the analytes. Note that in the supernatant of *K. pneumoniae* compared to the culture
614 media, metabolites related to the synthesis of nitrogenous bases, protein synthesis and
615 energy supply to the bacterial cell are significantly increased.
616 **Figure S3** - Plot of the analysis of the 25 main metabolites from the supernatant
617 of susceptible *K. pneumoniae*, separated because they were up-regulated in relation to
618 the control culture media.
619 **Figure S4** - Plot of the analysis of the 25 main metabolites from the supernatant
620 of susceptible *K. pneumoniae*, separated because they were down-regulated in relation
621 to the control culture media.

Figure S5 - Plot of the analyzes of the 25 main metabolites from the supernatant of Multidrug-Resistant (MDR) *K. pneumoniae* in general without separation by up- or down-regulation in the analytes. Note that in the intracellular media of *K. pneumoniae* compared to the culture medium, metabolites related to the synthesis of nitrogenous bases, protein synthesis and energy supply to the bacterial cell are significantly increased.

Figure S6 - Plot of the analysis of the 25 main metabolites from the supernatant of *K. pneumoniae* MDR, separated by being up-regulated in relation to the control culture media.

Figure S7 - Plot d of the analysis of the 25 main metabolites from the supernatant of *K. pneumoniae* MDR, separated because they were down-regulated in relation to the control culture media.

AUTHOR INFORMATION

Corresponding Authors:

Aldo A.M. Lima - Institute of Biomedicine, Faculty of Medicine, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil. Postgraduate Medical Microbiology Program, Department of Medicine, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil. https://orcid.org/0000-0002-0299-1747

Email: alima@ufc.br

José Kleybson de Sousa – Department of Pediatrics Gastroenterology, UVA Hospital, University of Virginia, Charlottesville, Virginia 22903, United States; https://orcid.org/0000-0003-3512-484X

Email: ztx7ex@virginia.edu;

Authors

Lavouisier F.B. Nogueira - Institute of Biomedicine, Faculty of Medicine, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil. Postgraduate Medical Microbiology Program, Department of Medicine, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil. https://orcid.org/0000-0002-5008-8181

Marília S. Maia - Institute of Biomedicine, Faculty of Medicine, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil. Postgraduate Medical Microbiology Program, Department of Medicine, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil. https://orcid.org/0000-0002-7005-7841

Marco A.F Clementino - Institute of Biomedicine, Faculty of Medicine, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil. Postgraduate Medical Microbiology Program, Department of Medicine, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil. https://orcid.org/0000-0002-0628-8047

Ila F.N. Lima – Hospital Universitário Walter Cantídio, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil.

Jorge L.N. Rodrigues - Hospital Universitário Walter Cantídio, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil.

Luciana V.C. Fragoso - Hospital Universitário Walter Cantídio, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil.

666 Glairta S. Costa - Hospital Universitário Walter Cantídio, Federal University of Ceara,
667 Fortaleza-Ceara 60430-270, Brazil.

668 Jose Q.S. Filho - Institute of Biomedicine, Faculty of Medicine, Federal University of Ceara,
669 Fortaleza-Ceara 60430-270, Brazil. https://orcid.org/0000-0002-9619-4553

670 Alexandre Havt - Institute of Biomedicine, Faculty of Medicine, Federal University of Ceara,
671 Fortaleza-Ceara 60430-270, Brazil. Postgraduate Medical Microbiology Program, Department
672 of Medicine, Federal University of Ceara, Fortaleza-Ceara 60430-270, Brazil.
673 https://orcid.org/0000-0002-4546-2976

674 Deiziane V.S. Costa - Infectious Diseases and International Health, University of Virginia,
675 Charlottesvillle, VA 22908. https://orcid.org/0000-0001-6402-8908

676 Lyvia M.V.C. Magalhães - Institute of Biomedicine, Faculty of Medicine, Federal University
677 of Ceara, Fortaleza-Ceara 60430-270, Brazil. https://orcid.org/0000-0001-6990-4903

678 Dilza Silva - Biomolecular Analysis Facility, School of Medicine, University of Virginia,
679 Charlottesvillle, VA 22908. https://orcid.org/0000-0002-6519-346X

680 Nicholas E. Sherman - Biomolecular Analysis Facility, School of Medicine, University of
681 Virginia, Charlottesvillle, VA 22908. https://orcid.org/0000-0003-3206-3113

682

683 **Author Contributions**:

- 684 Bibliographic review and data collection, Lavouisier F.B. Nogueira, Marília S.
685 Maia, Marco A.F Clementino and Aldo A.M. Lima.

- Methodology, Lavouisier F.B. Nogueira, Marília S. Maia, Marco A.F Clementino, Alexandre Havt, Ila F.N. Lima, Jorge L.N. Rodrigues, Luciana V. C. Fragoso and Aldo A. M. Lima.
- Bioinformatics, Lavouisier F.B. Nogueira and Marco A.F Clementino.
- In silico analyses, Lavouisier F.B. Nogueira, Marco A.F Clementino.
- *In vitro* validation of primers and sample testing, Lavouisier F.B. Nogueira, Marília S. Maia.
- Metabolomics, Datascience and data Bank, Jose Q.S. Filho; Deiziane V.S. Costa, José K. Sousa, Lyvia M.V.C. Magalhães, Dilza Silva, Nicholas E. Sherman and Aldo A.M. Lima
- Written review and editing, Lavouisier F.B. Nogueira.
- Supervision, Alexandre Havt and Aldo A.M. Lima.
- Project administration, Aldo A.M. Lima.
- Resource acquisition, Aldo A.M. Lima.

Funding: This research was funded by CNPq (http://www.cnpq.br), grant numbers 402607/2018-0 and 408549/2022-0 and FUNCAP (https://www.funcap.ce.gov.br/), grant number: OFÍCIO Nº 102/2021 – DINOV.

Acknowledge: This work used the following equipment's (Benchtop refrigerated centrifuge 10k RPM Eppendorf; Speed Vac Thermo fisher; Bead Ruptor Elite Omni International Material; Thermomixer Eppendorf; Orbitrap IDX spectrometer (Thermo) connected to the Vanquish UPLC system) available in the Biomolecular Analysis Core Facility which is supported by the University of Virginia School of Medicine, Research Resource Identifiers (RRID):SCR_025476.

Data Availability Statement: The data and reports relating to this study will be available via direct request via email to the corresponding author.

Conflicts of Interest: The authors declare that there are no financial or personal conflicts of interest that may have influenced the work.

REFERENCES

(1) Munita, J. M.; Arias, C. A. Mechanisms of Antibiotic Resistance. *Microbiol Spectr* **2016**, *4* (2). https://doi.org/10.1128/MICROBIOLSPEC.VMBF-0016-2015.

(2) Frieri, M.; Kumar, K.; Boutin, A. Antibiotic Resistance. *J Infect Public Health* **2017**, *10* (4), 369–378. https://doi.org/10.1016/J.JIPH.2016.08.007.

(3) Holt, K. E.; Wertheim, H.; Zadoks, R. N.; Baker, S.; Whitehouse, C. A.; Dance, D.; Jenney, A.; Connor, T. R.; Hsu, L. Y.; Severin, J.; Brisse, S.; Cao, H.; Wilksch, J.; Gorrie, C.; Schultz, M. B.; Edwards, D. J.; Van Nguyen, K.; Nguyen, T. V.; Dao, T. T.; Mensink, M.; Le Minh, V.; Nhu, N. T. K.; Schultsz, C.; Kuntaman, K.; Newton, P. N.; Moore, C. E.; Strugnell, R. A.; Thomson, N. R. Genomic Analysis of Diversity, Population Structure, Virulence, and Antimicrobial Resistance in Klebsiella Pneumoniae, an Urgent Threat to Public Health. *Proc Natl Acad Sci U S A* **2015**, *112* (27), E3574–E3581. https://doi.org/10.1073/PNAS.1501049112/SUPPL_FILE/PNAS.1501049112.SD01.XLSX.

(4) Asri, N. A. M.; Ahmad, S.; Mohamud, R.; Hanafi, N. M.; Zaidi, N. F. M.; Irekeola, A. A.; Shueb, R. H.; Yee, L. C.; Noor, N. M.; Mustafa, F. H.; Yean, C. Y.; Yusof, N. Y. Global Prevalence of Nosocomial Multidrug-Resistant Klebsiella Pneumoniae: A Systematic Review and Meta-Analysis. *Antibiotics* **2021**, *10* (12). https://doi.org/10.3390/ANTIBIOTICS10121508.

(5) Sharma, A.; Thakur, A.; Thakur, N.; Kumar, V.; Chauhan, A.; Bhardwaj, N. Changing Trend in the Antibiotic Resistance Pattern of Klebsiella Pneumonia Isolated From Endotracheal Aspirate Samples of ICU Patients of a Tertiary Care Hospital in North India. *Cureus* **2023**, *15* (3). https://doi.org/10.7759/CUREUS.36317.

(6) Cortés, G.; Borrell, N.; De Astorza, B.; Gómez, C.; Sauleda, J.; Albertí, S. Molecular Analysis of the Contribution of the Capsular Polysaccharide and the Lipopolysaccharide O Side Chain to the Virulence of Klebsiella Pneumoniae in a Murine Model of Pneumonia. *Infect Immun* **2002**, *70* (5), 2583–2590. https://doi.org/10.1128/IAI.70.5.2583-2590.2002/FORMAT/EPUB.

(7) Struve, C.; Bojer, M.; Krogfelt, K. A. Identification of a Conserved Chromosomal Region Encoding Klebsiella Pneumoniae Type 1 and Type 3 Fimbriae and Assessment of the Role of Fimbriae in Pathogenicity. *Infect Immun* **2009**, *77* (11), 5016–5024. https://doi.org/10.1128/IAI.00585-09/FORMAT/EPUB.

(8) Lin, C. T.; Chen, Y. C.; Jinn, T. R.; Wu, C. C.; Hong, Y. M.; Wu, W. H. Role of the CAMP-Dependent Carbon Catabolite Repression in Capsular Polysaccharide Biosynthesis in Klebsiella Pneumoniae. *PLoS One* **2013**, *8* (2), e54430. https://doi.org/10.1371/JOURNAL.PONE.0054430.

(9) Davies, J.; Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiol Mol Biol Rev* **2010**, *74* (3), 417. https://doi.org/10.1128/MMBR.00016-10.

(10) Salipante, S. J.; Jerome, K. R. Digital PCR-An Emerging Technology with Broad Applications in Microbiology. *Clin Chem* **2020**, *66* (1), 117–123. https://doi.org/10.1373/CLINCHEM.2019.304048.

(11) Fortuin, S.; Soares, N. C. The Integration of Proteomics and Metabolomics Data Paving the Way for a Better Understanding of the Mechanisms Underlying Microbial Acquired Drug Resistance. Frontiers in Medicine 2022, 9. https://doi.org/10.3389/fmed.2022.849838.

(12) Zeng, C.; Tandhanskul, A.; Krungkaew, S.; Likhanapaiboon, T.; Kasayapanan, W.; Yasurin, P.; Tang, J.; Phusantisampan, T.; Tawai, A. Research Progress on Using Omics Technology to Examine the Antimicrobial Mechanisms of Natural Active Substances. Applied Science and Engineering Progress 2024. https://doi.org/10.14416/j.asep.2024.07.003.

(13) Bush, K.; Bradford, P. A. β-Lactams and β-Lactamase Inhibitors: An Overview. *Cold Spring Harb Perspect Med* **2016**, *6* (8). https://doi.org/10.1101/CSHPERSPECT.A025247.

(14) Ferreira, A. M.; Martins, K. B.; Silva, V. R. da; Mondelli, A. L.; Cunha, M. de L. R. de S. da. Correlation of Phenotypic Tests with the Presence of the BlaZ Gene for Detection of Beta-Lactamase. *Braz J Microbiol* **2017**, *48* (1), 159–166. https://doi.org/10.1016/J.BJM.2016.10.011.

(15) Zhu, L. J.; Pan, Y.; Gao, C. Y.; Hou, P. F. Distribution of Carbapenemases and Efflux Pump in Carbapenem-Resistance Acinetobacter Baumannii. *Ann Clin Lab Sci* **2020**, *50* (2).

(16) Makharita, R. R.; El-Kholy, I.; Hetta, H. F.; Abdelaziz, M. H.; Hagagy, F. I.; Ahmed, A. A.; Algammal, A. M. Antibiogram and Genetic Characterization of Carbapenem-Resistant Gram-Negative Pathogens Incriminated in Healthcare-Associated Infections. *Infect Drug Resist* **2020**, *13*, 3991–4002. https://doi.org/10.2147/IDR.S276975.

(17) Santos, A. L.; Dos Santos, A. P.; Ito, C. R. M.; de Queiroz, P. H. P.; de Almeida, J. A.; Júnior, M. A. B. de C.; de Oliveira, C. Z.; Avelino, M. A. G.; Wastowski, I. J.; Gomes, G. P. L. A.; E Souza, A. C. S.; Vasconcelos, L. S. N. de O. L.; Santos, M. de O.; da Silva, C. A.; Carneiro, L. C. Profile of Enterobacteria Resistant to Beta-Lactams. *Antibiotics* **2020**, *9* (7), 1–18. https://doi.org/10.3390/ANTIBIOTICS9070410.

(18) Abdel-Rhman, S. H. Characterization of β-Lactam Resistance in K. Pneumoniae Associated with Ready-to-Eat Processed Meat in Egypt. *PLoS One* **2020**, *15* (9), e0238747. https://doi.org/10.1371/JOURNAL.PONE.0238747.

785 (19) Messaoudi, A.; Mansour, W.; Jaidane, N.; Chaouch, C.; Boujaâfar, N.; Bouallègue, O.
786 Epidemiology of Resistance and Phenotypic Characterization of Carbapenem Resistance
787 Mechanisms in Klebsiella Pneumoniae Isolates at Sahloul University Hospital-Sousse,
788 Tunisia. *Afr Health Sci* **2019**, *19* (2), 2008–2020. https://doi.org/10.4314/AHS.V19I2.24.
789 (20) Farhadi, M.; Ahanjan, M.; Goli, H. R.; Haghshenas, M. R.; Gholami, M. High Frequency of
790 Multidrug-Resistant (MDR) Klebsiella Pneumoniae Harboring Several β-Lactamase and
791 Integron Genes Collected from Several Hospitals in the North of Iran. *Annals of Clinical*
792 *Microbiology and Antimicrobials* **2021**, *20* (1). https://doi.org/10.1186/s12941-021-00476-1.
793 (21) Oliveira, R.; Castro, J.; Silva, S.; Oliveira, H.; Saavedra, M. J.; Azevedo, N. F.; Almeida, C.
794 Exploring the Antibiotic Resistance Profile of Clinical Klebsiella Pneumoniae Isolates in
795 Portugal. *Antibiotics (Basel)* **2022**, *11* (11). https://doi.org/10.3390/ANTIBIOTICS11111613.
796 (22) Bush, K.; Bradford, P. A. Epidemiology of β-Lactamase-Producing Pathogens. *Clin*
797 *Microbiol Rev* **2020**, *33* (2). https://doi.org/10.1128/CMR.00047-19.
798 (23) Castanheira, M.; Simner, P. J.; Bradford, P. A. Extended-Spectrum β-Lactamases: An
799 Update on Their Characteristics, Epidemiology and Detection. *JAC Antimicrob Resist* **2021**,
800 *3* (3). https://doi.org/10.1093/JACAMR/DLAB092.
801 (24) Gundran, R. S.; Cardenio, P. A.; Villanueva, M. A.; Sison, F. B.; Benigno, C. C.;
802 Kreausukon, K.; Pichpol, D.; Punyapornwithaya, V. Prevalence and Distribution of
803 BlaCTX-M, BlaSHV, BlaTEM Genes in Extended- Spectrum β- Lactamase- Producing E.
804 Coli Isolates from Broiler Farms in the Philippines. *BMC Vet Res* **2019**, *15* (1).
805 https://doi.org/10.1186/S12917-019-1975-9.
806 (25) Queiroz, J. A. da S.; Alves, L. S.; Dall'acqua, D. S. V.; Souza, L. F. B.; Faculdades
807 Integradas Aparício Carvalho. Porto Velho, R. Brasil.; Faculdades Integradas Aparício
808 Carvalho. Porto Velho, R. Brasil.; Fundação Oswaldo Cruz. Porto Velho, R. Brasil. / F. I.
809 A. Carvalho. P. V. R. Brasil.; Faculdades Integradas Aparício Carvalho. Porto Velho, R.

Brasil. Desenho e Validação de Primers In Silico Para Detecção Do Vírus Sincicial Respiratório Humano. *Revista FIMCA* **2017**, *4* (1), 17–30.

(26) Kumar, A.; Chordia, N. In Silico PCR Primer Designing and Validation. *Methods Mol Biol* **2015**, *1275*, 143–151. https://doi.org/10.1007/978-1-4939-2365-6_10.

(27) Rodríguez, A.; Rodríguez, M.; Córdoba, J. J.; Andrade, M. J. Design of Primers and Probes for Quantitative Real-Time PCR Methods. *Methods in Molecular Biology* **2015**, *1275*, 31–56. https://doi.org/10.1007/978-1-4939-2365-6_3.

(28) van Weezep, E.; Kooi, E. A.; van Rijn, P. A. PCR Diagnostics: In Silico Validation by an Automated Tool Using Freely Available Software Programs. *J Virol Methods* **2019**, *270*, 106–112. https://doi.org/10.1016/J.JVIROMET.2019.05.002.

(29) Yang, J.; Kemps-Mols, B.; Spruyt-Gerritse, M.; Anholts, J.; Claas, F.; Eikmans, M. The Source of SYBR Green Master Mix Determines Outcome of Nucleic Acid Amplification Reactions. *BMC Res Notes* **2016**, *9* (1), 1–10. https://doi.org/10.1186/S13104-016-2093-4/FIGURES/4.

(30) Wang, Y.; Li, W.; Guo, X.; Zhang, D.; Sun, J.; Fu, Z.; Liu, G.; Li, Y.; Jiang, S. Development of SYBR Green I-Based Polymerase Chain Reaction for Feline Bocavirus 1 Detection. *3 Biotech* **2021**, *11* (2). https://doi.org/10.1007/S13205-020-02577-8.

(31) Desriani; Azamris; Ghaissani, S. S.; Kinanti, S. R.; Warisman, M. A.; Fitria, N. Design and Characterization of a SYBR Green I-Based Melting Curve Method for Investigation of HER2I655V Polymorphism in Breast Cancer. *J Genet Eng Biotechnol* **2021**, *19* (1). https://doi.org/10.1186/S43141-020-00108-9.

(32) Svec, D.; Tichopad, A.; Novosadova, V.; Pfaffl, M. W.; Kubista, M. How Good Is a PCR Efficiency Estimate: Recommendations for Precise and Robust QPCR Efficiency Assessments. *Biomol Detect Quantif* **2015**, *3*, 9–16. https://doi.org/10.1016/J.BDQ.2015.01.005.

(33) Thermo Fisher Scientific. *Real-Time PCR: Understanding Ct - US*. https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/real-time-pcr-understanding-ct.html (accessed 2024-05-05).

(34) Han, R.; Shi, Q.; Wu, S.; Yin, D.; Peng, M.; Dong, D.; Zheng, Y.; Guo, Y.; Zhang, R.; Hu, F. Dissemination of Carbapenemases (KPC, NDM, OXA-48, IMP, and VIM) Among Carbapenem-Resistant Enterobacteriaceae Isolated From Adult and Children Patients in China. *Front Cell Infect Microbiol* **2020**, *10*, 314. https://doi.org/10.3389/FCIMB.2020.00314.

(35) Marschall, J.; Tibbetts, R. J.; Dunne, W. M.; Frye, J. G.; Fraser, V. J.; Warren, D. K. Presence of the KPC Carbapenemase Gene in Enterobacteriaceae Causing Bacteremia and Its Correlation with In Vitro Carbapenem Susceptibility. *J Clin Microbiol* **2009**, *47* (1), 239. https://doi.org/10.1128/JCM.02123-08.

(36) Kharazmkia, A.; Amirizadeh, M.; Goudarzi, Z.; Birjandi, M.; Barfipoursalar, A.; Mir, S. Prevalence of KPC-Producing Bacteria in Negative Gram of Clinical Samples Obtained from Patients. *Annals of Medicine and Surgery* **2022**, *77*, 103690. https://doi.org/10.1016/J.AMSU.2022.103690.

(37) Pawłowska, I.; Ziółkowski, G.; Jachowicz-Matczak, E.; Stasiowski, M.; Gajda, M.; Wójkowska-Mach, J. Colonization and Healthcare-Associated Infection of Carbapenem-Resistant Enterobacteriaceae, Data from Polish Hospital with High Incidence of Carbapenem-Resistant Enterobacteriaceae, Does Active Target Screening Matter? *Microorganisms* **2023**, *11* (2). https://doi.org/10.3390/MICROORGANISMS11020437.

(38) Ferreira, R. L.; da Silva, B. C. M.; Rezende, G. S.; Nakamura-Silva, R.; Pitondo-Silva, A.; Campanini, E. B.; Brito, M. C. A.; da Silva, E. M. L.; Freire, C. C. de M.; Cunha, A. F. da; Pranchevicius, M.-C. da S. High Prevalence of Multidrug-Resistant Klebsiella Pneumoniae Harboring Several Virulence and β-Lactamase Encoding Genes in a

Brazilian Intensive Care Unit. *Frontiers in Microbiology* **2019**, *9*. https://doi.org/10.3389/fmicb.2018.03198.

(39) Kernéis, S.; Lucet, J. C.; Santoro, A.; Meschiari, M. Individual and Collective Impact of Klebsiella Pneumoniae Carbapenemase (KPC)-Producing K. Pneumoniae in Patients Admitted to the ICU. *J Antimicrob Chemother* **2021**, *76* (Suppl 1), I19–I26. https://doi.org/10.1093/JAC/DKAA494.

(40) Dirar, M. H.; Bilal, N. E.; Ibrahim, M. E.; Hamid, M. E. Prevalence of Extended-Spectrum β-Lactamase (ESBL) and Molecular Detection of Bla TEM, Bla SHV and Bla CTX-M Genotypes among Enterobacteriaceae Isolates from Patients in Khartoum, Sudan. *Pan Afr Med J* **2020**, *37*, 1–11. https://doi.org/10.11604/PAMJ.2020.37.213.24988.

(41) Pavez, M.; Troncoso, C.; Osses, I.; Salazar, R.; Illesca, V.; Reydet, P.; Rodríguez, C.; Chahin, C.; Concha, C.; Barrientos, L. High Prevalence of CTX-M-1 Group in ESBL-Producing Enterobacteriaceae Infection in Intensive Care Units in Southern Chile. *Braz J Infect Dis* **2019**, *23* (2), 102–110. https://doi.org/10.1016/J.BJID.2019.03.002.

(42) Camargo, C. H.; Yamada, A. Y.; Souza, A. R. de; Reis, A. D.; Santos, M. B. N.; Assis, D. B. de; Carvalho, E. de; Takagi, E. H.; Cunha, M. P. V.; Tiba-Casas, M. R. Genomic Diversity of NDM-Producing Klebsiella Species from Brazil, 2013–2022. Antibiotics 2022, 11 (10), 1395.https://doi.org/10.3390/antibiotics11101395.

(43) Vivas, R.; Dolabella, S. S.; Barbosa, A. A. T.; Jain, S. Prevalence of Klebsiella Pneumoniae Carbapenemase - and New Delhi Metallo-Beta-Lactamase-Positive K. Pneumoniae in Sergipe, Brazil, and Combination Therapy as a Potential Treatment Option. *Revista da Sociedade Brasileira de Medicina Tropical* **2020**, *53*. https://doi.org/10.1590/0037-8682-0064-2020.

(44) Belouad, E. M.; Benaissa, E.; El Mrimar, N.; Bssaibis, F.; Maleb, A.; Elouennass, M. Predominance of OXA-48 Carbapenemase-Producing Enterobacterales in a Moroccan Hospital. *Int J Microbiol* **2023**, *2023*. https://doi.org/10.1155/2023/8581883.

886 (45) Magagnin, C. M.; Rozales, F. P.; Antochevis, L.; Nunes, L. S.; Martins, A. S.; Barth, A. L.;
887 Sampaio, J. M.; Zavascki, A. P. Dissemination of Bla OXA-370 Gene among Several
888 Enterobacteriaceae Species in Brazil. *Eur J Clin Microbiol Infect Dis* **2017**, *36* (10), 1907–
889 1910. https://doi.org/10.1007/S10096-017-3012-X.
890 (46) Evans, B. A.; Amyes, S. G. B. OXA β-Lactamases. *Clin Microbiol Rev* **2014**, *27* (2), 241–263.
891 https://doi.org/10.1128/CMR.00117-13/ASSET/DF7BB1F6-F6E8-4208-8829-
892 B01CD28A0242/ASSETS/GRAPHIC/ZCM9990924610006.JPEG.
893 (47) Shahid, M.; Ahmad, N.; Saeed, N. K.; Shadab, M.; Joji, R. M.; Al-Mahmeed, A.; Bindayna,
894 K. M.; Tabbara, K. S.; Dar, F. K. Clinical Carbapenem-Resistant Klebsiella Pneumoniae
895 Isolates Simultaneously Harboring Bla NDM-1, Bla OXA Types and QnrS Genes from the
896 Kingdom of Bahrain: Resistance Profile and Genetic Environment. *Front Cell Infect*
897 *Microbiol* **2022**, *12*. https://doi.org/10.3389/FCIMB.2022.1033305.
898 (48) Manandhar, S.; Zellweger, R. M.; Maharjan, N.; Dongol, S.; Prajapati, K. G.; Thwaites, G.;
899 Basnyat, B.; Dixit, S. M.; Baker, S.; Karkey, A. A High Prevalence of Multi-Drug Resistant
900 Gram-Negative Bacilli in a Nepali Tertiary Care Hospital and Associated Widespread
901 Distribution of Extended-Spectrum Beta-Lactamase (ESBL) and Carbapenemase-
902 Encoding Genes. *Ann Clin Microbiol Antimicrob* **2020**, *19* (1), 1–13.
903 https://doi.org/10.1186/S12941-020-00390-Y/FIGURES/5.
904 (49) Awosile, B. B.; Agbaje, M.; Adebowale, O.; Kehinde, O.; Omoshaba, E. Beta-Lactamase
905 Resistance Genes in Enterobacteriaceae from Nigeria. *Afr J Lab Med* **2022**, *11* (1).
906 https://doi.org/10.4102/AJLM.V11I1.1371.
907 (50) Han, M. L.; Nang, S. C.; Lin, Y. W.; Zhu, Y.; Yu, H. H.; Wickremasinghe, H.; Barlow, C. K.;
908 Creek, D. J.; Crawford, S.; Rao, G.; Dai, C.; Barr, J. J.; Chan, K.; Turner Schooley, R.;
909 Velkov, T.; Li, J. Comparative Metabolomics Revealed Key Pathways Associated with the
910 Synergistic Killing of Multidrug-Resistant Klebsiella Pneumoniae by a Bacteriophage-

911 Polymyxin Combination. *Comput Struct Biotechnol J* **2022**, *20*, 485–495.
912 https://doi.org/10.1016/J.CSBJ.2021.12.039.

913 (51) Kumar, A.; Singh, S.; Gupta, S. K.; Kumar, S.; Kumar, S.; Singh, R.; Thakur, L.; Kumar, M.;
914 Kapil, A.; Kumar, Y.; Kumar, N. Identification of Metabolite Extraction Method for
915 Targeted Exploration of Antimicrobial Resistance Associated Metabolites of Klebsiella
916 Pneumoniae. *Sci Rep* **2022**, *12* (1). https://doi.org/10.1038/S41598-022-12153-0.

917 (52) Hudson, A. W.; Barnes, A. J.; Bray, A. S.; Ornelles, D. A.; Zafar, M. A. Klebsiella
918 Pneumoniae L-Fucose Metabolism Promotes Gastrointestinal Colonization and
919 Modulates Its Virulence Determinants. *Infect Immun* **2022**, *90* (10).
920 https://doi.org/10.1128/IAI.00206-22.

921 (53) Lin, D.; Fan, J. M.; Wang, J.; Liu, L.; Xu, L.; Li, F.; Yang, J.; Li, B. The Fructose-Specific
922 Phosphotransferase System of Klebsiella Pneumoniae Is Regulated by Global Regulator
923 CRP and Linked to Virulence and Growth. *Infect Immun* **2018**, *86* (8).
924 https://doi.org/10.1128/IAI.00340-18.

925 (54) Aminov, R. Metabolomics in Antimicrobial Drug Discovery. *Expert Opinion on Drug*
926 *Discovery* **2022**, *17* (9), 1047–1059. https://doi.org/10.1080/17460441.2022.2113774.

927 (55) Kok, M.; Maton, L.; van der Peet, M.; Hankemeier, T.; van Hasselt, J. G. C. Unraveling
928 Antimicrobial Resistance Using Metabolomics. *Drug Discovery Today* **2022**, *27* (6), 1774–
929 1783. https://doi.org/10.1016/j.drudis.2022.03.015.

930 (56) Soni, V.; Wang, Z.; Singh, V. Editorial: Bacterial Metabolomics Approach towards
931 Antimicrobials and Resistance. *Frontiers in Microbiology* **2023**, *14*.
932 https://doi.org/10.3389/fmicb.2023.1222594.

933 (57) Abril, A. G.; Quintela-Baluja, M.; Villa, T. G.; Calo-Mata, P.; Barros-Velázquez, J.; Carrera,
934 M. Proteomic Characterization of Virulence Factors and Related Proteins in Enterococcus
935 Strains from Dairy and Fermented Food Products. International Journal of Molecular
936 Sciences 2022, 23 (18), 10971. https://doi.org/10.3390/ijms231810971.

(58) Azhar, F.; Busharat, M.; Chaudhary, S. R.; Waheed, Z.; Jamil, M. Metabolomics in Drug Discovery: Restoring Antibiotic Pipeline. *Asian Pacific Journal of Tropical Biomedicine* **2023**, *13* (9), 378. https://doi.org/10.4103/2221-1691.385568.

(59) Tsugawa, H.; Cajka, T.; Kind, T.; Ma, Y.; Higgins, B.; Ikeda, K.; Kanazawa, M.; Vandergheynst, J.; Fiehn, O.; Arita, M. MS-DIAL: Data-Independent MS/MS Deconvolution for Comprehensive Metabolome Analysis. *Nature Methods 2015 12:6* **2015**, *12* (6), 523–526. https://doi.org/10.1038/nmeth.3393.

(60) Tsugawa, H.; Nakabayashi, R.; Mori, T.; Yamada, Y.; Takahashi, M.; Rai, A.; Sugiyama, R.; Yamamoto, H.; Nakaya, T.; Yamazaki, M.; Kooke, R.; Bac-Molenaar, J. A.; Oztolan-Erol, N.; Keurentjes, J. J. B.; Arita, M.; Saito, K. A Cheminformatics Approach to Characterize Metabolomes in Stable-Isotope-Labeled Organisms. *Nat Methods* **2019**, *16* (4), 295–298. https://doi.org/10.1038/S41592-019-0358-2.