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Major Article

Investigation of class 1 integrons in *Klebsiella pneumoniae* clinical and microbiota isolates belonging to different phylogenetic groups in Recife, State of Pernambuco

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ABSTRACT

Introduction: The high prevalence of *Klebsiella pneumoniae* infections is related to the ability of *K. pneumoniae* to acquire and disseminate exogenous genes associated with mobile elements, such as R plasmids, transposons and integrons. This study investigated the presence of class 1 integrons in clinical and microbiota isolates of *K. pneumoniae* belonging to different phylogenetic groups and correlated these results with the antimicrobial resistance profiles of the studied isolates. **Methods:** Of the 51 isolates of *K. pneumoniae* selected for this study, 29 were from multidrug-resistant clinical isolates, and 22 were from children's microbiota. The susceptibility profile was determined using the disk diffusion method, and class 1 integrons were detected through polymerase chain reaction (PCR). **Results:** The results showed that none of the 22 microbiota isolates carried class 1 integrons. Among the 29 clinical isolates, 19 (65.5%) contained class 1 integrons, and resistance to sulfamethoxazole/trimethoprim was identified in 18 of these isolates (94.7%). Among the *K. pneumoniae* isolates with class 1 integrons, 47% belonged to the KpI phylogenetic group, and one isolate (14.3%) carrying these genetic elements belonged to the KpIII group. **Conclusions:** The wide variety of detected class 1 integrons supports the presence of high rates of antimicrobial resistance, genetic variability, and rapid dissemination of beta-lactamase genes among *K. pneumoniae* clinical isolates in recent years in hospitals in Recife-PE, Brazil. The findings of this study indicate that the surveillance of *K. pneumoniae* integrons in clinical isolates could be useful for monitoring the spread of antibiotic resistance genes in the hospital environment.

Keywords: Class 1 integron. *Klebsiella pneumonia.* Phylogenetic groups.

INTRODUCTION

Klebsiella pneumoniae is a bacillus that is commonly associated with serious nosocomial infections, such as septicemia, pneumonia, urinary tract infections, and meningitis[1-3]. This species is classified into three phylogenetic groups, KpI, KpII, and KpIII, based on nucleotide variations in the constitutively expressed genes *gyrA*, *parC*, and *rpoB*[4,5]. Mobile genetic elements, such as integrons, contribute to the evolution and dissemination of multidrug resistance genes *(bla_{CTX-M}, bla_{IMP},* and bla_{GES}) in *K. pneumoniae* through horizontal or vertical transfer[6-9]. The mobile class 1 integrons are predominantly found in clinical isolates and are associated with transposon Tn21. Class 1 integrons are composed of two conserved regions, a 3′ conserved segment (3′ CS) and a 5′ conserved segment (5′ CS), as well as an internal variable region containing gene cassettes that encode antimicrobial resistance determinants. The 3′ CS segment contains the *qacE* Δ *1* and *sul* 1 genes, which confer resistance to ethidium bromide and quaternary ammonium compounds and to sulfonamide, respectively[10-12]. These genetic elements can be found in different species of Gram-negative bacteria[13-17] from hospital environments. Based on the amino acid sequence of the IntI protein, five classes of mobile integrons have been described[18]. Classes 1, 2, and 3 are the most commonly detected[19]. Class 1 integrons are the most widespread and have been frequently found in extended-spectrum β-lactamase (ESBL)-producing clinical isolates of *Enterobacteriaceae*, including *K. pneumoniae*[6,13,16,20,21]. Class 2 integrons occur less frequently in ESBL-producing *Escherichia coli* and *K. pneumoniae*, and class 3 integrons are rarely found in ESBL-producing *K. pneumoniae*[21,22].

Class 1 and class 2 integrons have also been found in *Escherichia coli* from fecal samples from healthy individuals in Spain, indicating that individuals from the community may serve as reservoirs for these genetic elements [23]. Nevertheless, no published study has correlated the presence of these genetic elements with *K. pneumoniae* phylogenetic groups or with microbiota isolates. Thus, the present study evaluated the presence of class 1 integrons in clinical and microbiota isolates of *K. pneumoniae* from different phylogenetic groups in Recife, Brazil, and it correlated their presence with the antimicrobial resistance profiles displayed by the isolates.

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METHODS

Bacterial isolates

A total of 51 *K. pneumoniae* isolates already typed for phylogenetic groups[24] were analyzed for the presence of class 1 integrons. Twenty-nine of these isolates were from hospitalized patients from the City of Recife, Pernambuco, Brazil, and 22 were from normal oropharyngeal and fecal microbiota from healthy 3- to 4-year-old children attending a day care center called *Lar Fabiano de Cristo* in the Várzea district of Recife, Brazil **(Table 1)**. All the cultures were stored in 20% glycerol at -70°C and were grown at 37ºC for 18h in brain-heart infusion broth (BHI) or Luria-Bertani broth (LB).

Antibiotic susceptibility analysis

Antibiotic susceptibility was tested on Mueller-Hinton agar using the disk diffusion method Clinical and Laboratory Standards Institute (CLSI)[25]. The following antibiotics were tested: amoxicillin-clavulanate (AMC), amoxicillin (AMO), amikacin (AMI), ampicillin (AMP), aztreonam (ATM), ceftazidime (CAZ), cefotaxime (CTX), cefepime (CPM), ciprofloxacin (CIP), chloramphenicol (CLO), streptomycin (EST), gentamicin (GEN), imipenem (IPM), kanamycin (KAN), nalidixic acid (NAL), sulfamethoprim (TSP), and tetracycline (TET).

Genomic DNA extraction

Genomic deoxyribonucleic acid (DNA) was extracted from direct colony suspensions in 200µl of distilled water. The suspensions were heated at 100°C for 10min and centrifuged (5min/10,000xg), and 100µl of the recovered supernatant was stored at -20ºC until use.

Detection of class 1 integrons through PCR

Amplification reactions were prepared in a total volume of 25µl containing 1ng of genomic DNA, 2 units of *Taq* DNA polymerase (Promega), 200µM of each deoxyribonucleotide triphosphate (dNTP) (Promega), 1.5mM $MgCl_2$, 1µM of each primer (3'CS (AAGCAGACTTGACCTGAT) and 5'CS (GGCATCCAAGCAGCAAG), and 1X reaction buffer[26]. The cycling conditions were one cycle of 5 min at 95 °C; 30 cycles of 1 min at 95°C, 1min at 65°C, and 1min at 72°C; and one cycle of 10min at 72°C. The amplification products were visualized in 1% agarose gels in Tris-borate buffer (TBE) buffer.

RESULTS

Class 1 integrons

Among the 51 *K. pneumoniae* clinical and microbiota isolates analyzed in this work, 19 (37.2%) carried class 1 integrons. Nineteen (65.5%) of the 29 clinical isolates carried class 1 integrons, as indicated by amplicons ranging from 750 to >2,080bp. None of the 22 microbiota isolates carried these genetic elements **(Table 1)**. Three clinical isolates (K5A, K10P, and K14P) contained two integrons each, with amplicon sizes ranging from 750 to >2,080bp. The examination of the relationship between the phylogenetic groups and the presence of class 1 integrons revealed that 16 (80%) of the clinical isolates carrying class 1 integrons belonged to the KpI phylogenetic group. Clinical isolates from 1998 and 1999 exhibited lower integron frequencies compared with those from 2007 and 2008 **(Table 1)**.

Antimicrobial resistance

The identified *K. pneumoniae* isolates with class 1 integrons exhibited the following high levels of resistance to antibiotics: AMP 100% (n=19); AMO 57.9% (n=11); AMC 42.1% (n=8); CTX 94.7% (n=18); CAZ 73.7% (n=14); CPM 47.4% (n=9); and IPM and meropenem 36.8% (n=7). In addition, resistance to sulfamethoxazole associated with trimethoprim was observed in 94.7% (n=18) of these isolates, and resistance to monobactam aztreonam (ATM) was observed in 73.7% (n=14) of these isolates. The *K. pneumoniae* isolates were more susceptible to aminoglycosides, fluoroquinolones, and CLO than to penicillin, cephalosporin, sulfa, or monobactams. Eighteen (94.7%) of the *K. pneumoniae* isolates carrying class 1 integrons exhibited simultaneous sulfamethoxazole/trimethoprim resistance. K10-R was the only isolate with one class 1 integron and no resistance to sulfamethoxazole/trimethoprim. Conversely, the K12-A and K20-P isolates did not contain class 1 integrons but displayed resistance to sulfamethoxazole/trimethoprim **(Table 1)**.

DISCUSSION

This study investigated the presence of class 1 integrons in clinical and microbiota isolates of *K. pneumoniae* from different phylogenetic groups and correlated these results with the antimicrobial resistance profiles of the studied isolates. Few studies have evaluated the presence of class 1 integrons in *K. pneumoniae* isolates from the Brazilian northeast[27]. Chagas et al.[20] showed that all *K. pneumoniae* ESBL-producing isolates from Rio de Janeiro and São Paulo, Brazil carried class 1 integrons. Ahangarzadeh Rezaee et al.[13] found that integrons were widely prevalent in clinical isolates of *K. pneumoniae* from northwestern Iran and were associated with multidrug resistance. In the present study, class 1 integrons were identified in clinical isolates only; these results are similar to those reported by Dalsgaard et al.[11] and confirm that integrons are predominantly found in clinical isolates.

Because no integrons were identified in the studied *K. pneumoniae* microbiota isolates from healthy individuals, we suggest that they are not reservoirs for class 1 integrons. Thus, *K. pneumoniae* clinical isolates are the main reservoirs for these genetic elements and disseminate them to other bacterial species through horizontal or vertical transfer in the hospital environment. Most of the clinical isolates carried one integron; however, the K5A, K10P, and K14P isolates each contained two integrons. This result is consistent with reports by Penteado et al.[28] and Lopes et al.[27], who observed additional integrons in isolates and suggested the presence of high diversity in these genetic elements in *K. pneumoniae* isolates from Recife. The 3´ segment of class 1 integrons contains the *sul* 1 gene,

TABLE 1 - Specimens, origins, phylogenetic groups, presence of class 1 integrons, and antimicrobial resistance profiles of *Klebsiella pneumoniae* isolates from Recife, Brazil.

Isolates[a]	Specimen[b]	Phylogenetic group	Origin[c]	Year of isolation	Integron[bp]	Antimicrobial resistance[d]
K1A	Hosp.	Kp I	Catheter	2007	1,800	AMO, AMP, NAL, AMC, ATM, CAZ, CTX, CLO (I), SZT
K2A	Hosp.	Kp I	Urine	2007	1,600 to >2,080	AMO, AMP, NAL, AMC, ATM, CAZ, CTX, TET, SZT
K3A	Hosp.	Kp III	Wound	2007	> 2,080	AMO, AMP, NAL (I), AMC, CTX (I), TET, SZT
K4A	Hosp.	Kp III	Trach. aspirate	2007	Negative	AMO, AMP, NAL, CTX (I), TET
K5A	Hosp.	Kp I	Urine	2007	1,300 to 2,080	AMO, AMP, AMC, ATM, CAZ, CTX, TET, CLO, SZT
K7A	Hosp.	Kp I	Trach. aspirate	2007	1,800	AMO, AMP, NAL, AMC, ATM, CAZ, CTX, TET, SZT
K8A	Hosp.	Kp I	Blood	2007	1,500	AMO, AMP, NAL (I), TET, SZT
K9A	Hosp.	Kp I	Trach. Aspirate	2007	2,000	AMO, AMP, AMC, ATM, CAZ, CTX, CLO, SZT
K10A	Hosp.	Kp I	Urine	2007	2,080	AMO, AMP, NAL, AMC, ATM, CAZ, CTX, TET, CLO, SZT
K12A	Hosp.	Kp I	Urine	2007	Negative	AMO, AMP, SZT
K1P	Hosp.	Kp I	Urine	2008	1,800	AMP, ATM, CPM, CTX, CAZ, IPM, SZT
K4P	Hosp.	Kp II	Sputum	2008	Negative	AMP
K5P	Hosp.	Kp I	Blood	2008	1,800	AMP, ATM, CPM, CTX, CAZ, IPM, SZT
K8P	Hosp.	Kp I	Urine	2008	1,800	AMI, AMP, ATM, CPM, CTX, CAZ, GEN, IPM, SZT
K10P	Hosp.	Kp I	Trach. aspirate	2008	1,600 to 750	AMP, ATM, CPM, CTX, CAZ, CIP, IPM, SZT
K12P	Hosp.	Kp I	Urine	2008	1,800	AMP, ATM, CPM, CTX, CAZ, CIP, GEN, IPM, SZT
K13P	Hosp.	Kp I	Trach. aspirate	2008	1,600	AMP, ATM, CPM, CTX, CAZ, CIP, GEN, IPM, SZT
K14P	Hosp.	Kp I	Blood	2008	1,800 to >2,080	AMP, ATM, CPM, CTX, CAZ, CIP, GEN, SZT
K15P	Hosp.	Kp I	Urine	2008	1,500	AMP, ATM, CPM, CTX, CAZ, CIP, IPM, SZT
K20P	Hosp.	Kp I	Urine	2008	Negative	AMP, ATM, CPM, CTX, CAZ, CIP, GEN, IPM, SZT
K10-R	Hosp.	Kp II	Trach. aspirate	1998	1,300	AMP, AMO, CLO (I), TET (I), EST (I), CTX (I)
K15-R	Hosp.	Kp I	Blood	1998	Negative	AMI (I), AMP, AMO, AMC, CLO, CTX, GEN, KAN(I), TET(I)
K16-R	Hosp.	Kp I	Urine	1998	> 2,000	AMP, AMO, AMC(I), CLO, CPM, GEN, KAN, EST, ATM, CTX, SZT
K18-R	Hosp.	Kp II	Urine	1998	Negative	AMP, AMO, TET (I)
K20-R	Hosp.	Kp II	Urine	1998	Negative	AMP, AMO, TET (I), CTX (I)
K7-C	Hosp.	Kp II	Urine	1999	Negative	AMP, AMO, CTX(I)
K10-C	Hosp.	Kp I	Urine	1999	Negative	AMP, AMO, AMC (I), CLO, TET (I), NAL, ATM, CAZ (I), CTX (I)
K12-C	Hosp.	Kp II	Urine	1999	750	AMP, AMO, CLO, TET, CTX (I), SZT
K17-C	Hosp.	Kp III	Urine	1999	Negative	AMP, AMO, CLO (I)
K3.1-F	Microb.	Kp III	Fecal	2004	Negative	KAN (I)
K3.2-F	Microb.	Kp II	Fecal	2004	Negative	AMP, AMO, KAN (I)
K6.3-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO, EST (I)
K7.1-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO
K7.2-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO, KAN (I), EST (I)
K10.1-F	Microb.	Kp II	Fecal	2004	Negative	AMP, AMO
K10.2-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO, TET
K13.3-F	Microb.	Kp II	Fecal	2004	Negative	AMP, AMO, KAN (I), EST (I)
K21.1-F	Microb.	Kp III	Fecal	2004	Negative	AMO (I), EST (I), KAN (I)

Continues

TABLE 1 - Continuation.

Isolates[a]	Specimen[b]	Phylogenetic group	Origin[c]	Year of isolation	Integron[bp]	Antimicrobial resistance[d]
K21.2-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO
K24.1-F	Microb.	Kp II	Fecal	2004	Negative	KAN (I), TET
K24.2-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO
K51.1-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO
K58.1-F	Microb.	Kp I	Fecal	2004	Negative	AMO (I), EST (I), KAN (I)
K58.2-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO, KAN (I)
K63.1-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO, KAN (I)
K63.2-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO, KAN (I), EST (I)
K68-F	Microb.	Kp I	Fecal	2004	Negative	AMP, AMO, KAN (I)
K22-ORO	Microb.	Kp I	Oropharyngeal	2004	Negative	AMP, AMO, KAN (I)
K106.1-ORO	Microb.	Kp III	Oropharyngeal	2003	Negative	AMO, AMP
K2.3-ORO	Microb.	Kp III	Oropharyngeal	2003	Negative	AMO
K112.1-ORO	Microb.	Kp I	Oropharyngeal	2003	Negative	AMO, AMP

Kp: *Klebsiella pneumonia;* bp: base pairs; IPM: imipenem; KAN: kanamycin; AMO: amoxicillin; AMP: ampicillin; NAL: nalidixic acid; AMC: amoxicillin-clavulanate; ATM: aztreonam; CAZ: ceftazidime; CTX: cefotaxime; CLO: chloramphenicol; SZT: trimethoprim/sulfamethoxazole; TET: tetracycline; EST: streptomycin; I: intermediate susceptibility. KAN: kanamycin. [a]Isolates: K: *K. pneumoniae*; A: public hospital; R: public hospital; C: public hospital; P: private hospital; F: fecal; ORO: oropharyngeal. [b]Specimen: Hosp: hospital; Microb: microbiota. [c]Origin: Trach: tracheal. [d]Resistance profile.

which encodes sulfonamide resistance. The K10-R isolate carried a class 1 integron but did not exhibit sulfamethoxazole/trimethoprim resistance. The most likely reason for this result is that this isolate does not express the *sul1* gene. Our study confirmed a correlation between sulfamethoxazole/trimethoprim resistance and the presence of class 1 integrons: 94.7% of the analyzed isolates carried class 1 integrons and exhibited sulfamethoxazole/trimethoprim resistance[29,30]. The K12-A and K20-P isolates did not carry class 1 integrons but displayed sulfamethoxazole/trimethoprim resistance. The *sul1* gene encodes resistance to sulfonamides only, and we evaluated the associated sulfamethoxazole/trimethoprim. The most common mechanism of trimethoprim resistance involves variants of the dihydrofolate reductase (DFR), and the absence of class 1 integrons in these two isolates suggests that they may express the *dfrA* gene and that this gene could be located in other genetic elements, such as plasmids, transposons and chromosomes, which could explain our findings[31-34]. We observed that isolates belonging to the KpI phylogenetic group had the highest frequency of class 1 integrons; some class 1 integrons were observed in isolates from the KpII group, but very few were observed in isolates from the KpIII group. This high frequency of class 1 integrons is one of the factors explaining why isolates from the KpI group presented the highest level of resistance, followed by isolates from the KpII group and isolates from the KpIII group[4]. We also observed that the frequency and diversity of class 1 integrons in the *K. pneumoniae* clinical isolates in Recife increased between 1998 and 1999 and between 2007 and 2008. This result is consistent with the observed rapid dissemination of beta-lactamase genes along with resistance to extended-spectrum cephalosporins and carbapenems among *K. pneumoniae* clinical isolates in Recife in recent years[35]. In this study, the diversity of class 1 integrons in *K. pneumoniae* isolates from Recife, Brazil favors the dissemination of resistance among *K. pneumoniae* and other species in the hospital environment. Moreover, this study also indicates that the surveillance of *K. pneumoniae* integrons in clinical isolates could be useful for monitoring the spread of antibiotic resistance genes in the hospital environment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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