

Biotechnology

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Comparative assessment of five clinical *Klebsiella* isolates in terms of antibiotic resistance and plasmid profiles

Beş farklı klinik *Klebsiella* türünün antibiyotik dirençliliği ile plazmid profillerinin karşılaştırmalı olarak değerlendirilmesi

Abstract: Objective: This study is firstly aimed to biotype and to investigate the antibiotic resistance profiles of *Klebsiella* strains which were isolated from clinical materials. Also plasmid profile types of *Klebsiella* species were investigated and the results were compared with each other.

Methods: *Klebsiella* strains were biotyped by BBL Enterotube II and the antibiotic resistance was tested by Kirby Bauer disc diffusion method. In order to analyse *Klebsiella* strains genotypically, plasmid DNA's of them were isolated and generated plasmid profile types determined by Dice coefficients of similarity.

Results: According to biotyping, *Klebsiella* strains were distributed to five different species (*K. pneumoniae*, *K. ornithinolytica*, *K. oxytoca*, *K. terrigena*, *K. rhinoscleromatis*). As well as *K. pneumoniae* (37.33%) was the most isolated strain, *K. terrigena* (8.95%) which is not found to be investigated as much as other strains in the literature, was also observed. Additionally, it is determined that *Klebsiella* strains were resistant to at least one and at most seven antibiotics. With a similarity coefficient of 84%, it was observed that five different *Klebsiella* species displayed 17 different plasmid profile types. Among these profiles, P1 (52.23%) was the most observed type which exhibits >10 kbp plasmid DNA band and this profile was isolated from all strains.

Conclusion: Finally, it is observed that antibiotic resistance can be due to the plasmid or chromosomal sources and different strains of the same genus may exhibit the same plasmid profile because of the plasmid transformation from one strain to another.

Keywords: Antibiotic resistance, biotyping, *Klebsiella* species, plasmid profile type

Özet: Amaç: Bu çalışma öncelikle klinik materyallerden izole edilen *Klebsiella* türlerinin biyotiplendirilmesi ve antibiyotik direnç profillerinin belirlenmesini amaçlamaktadır. Bunun yanı sıra çalışmamızda *Klebsiella* türlerinin plazmid profilleri incelenmiş ve antibiyotik direnç profilleri ile karşılaştırılarak yorumlanmıştır.

Metod: *Klebsiella* türleri BBL Enterotube II kullanılarak biyotipleme yapıldı ve antibiyotik dirençlilikleri Kirby Bauer disk difüzyon metodu kullanılarak test edildi. *Klebsiella* suşlarının genotipik olarak analiz edilmesi için söz konusu türlerin plazmid DNA'ları izole edildi ve oluşturulan plazmid tipleri benzerlik katsayısı kullanılarak belirlendi.

Bulgular: Biyotipleme sonuçlarına göre *Klebsiella* türlerinin 5 farklı türe (*K. pneumoniae*, *K. ornithinolytica*, *K. oxytoca*, *K. terrigena*, *K. rhinoscleromatis*) ayrıldığı belirlendi. *K. pneumoniae* (%37.33) türünün en fazla izole edilen tür olduğu, aynı zamanda literatürde diğer *Klebsiella* türlerine kıyasla daha az çalışılmış olan *K. terrigena*'nın (%8.95) da çalışmamızda izole edildiği gözlemlendi. Ayrıca, *Klebsiella* türlerinin en az 1 en fazla 7 antibiyotiğe dirençli oldukları belirlendi. 5 farklı *Klebsiella* türünün %84 benzerlik katsayısı ile 17 farklı plazmid profil tipine ayrıldığı saptandı. Bu profiller içerisinde >10 kbp plazmid

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DNA bantı içeren P1 (%52.23) plazmid profil tipinin *Klebsiella* türlerinin hepsinde en fazla izole edilen tip olduğu belirlendi.

Sonuç: Sonuç olarak, antibiyotik direncinin plazmid veya kromozomal kaynaklı olabileceği ve aynı cins içerisinde yer alan farklı türlerin, bir türden diğer türe aktarılan plazmidler nedeniyle aynı plazmid profil tipini gösterebileceği saptandı.

Anahtar Kelimeler: Antibiyotik direnci, biyotipleme, *Klebsiella* türleri, plazmid profil tipi

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Introduction

The *Klebsiella* genus belongs to the family of *Enterobacteriaceae* and includes different species such as *K. pneumoniae*, *K. ozaneae*, *K. rhinoscleromatis*, *K. oxytoca*, *K. planticola*, *K. terrigena* and *K. ornithinolytica* [1,2]. Although there is a limited number of *Klebsiella* *sp.* strains in the normal flora, the members of this genus are usually known to cause septicemia, pneumoniae and urinary tract infections. Furthermore, *Klebsiella* *sp.* strains are often the causative agents of nosocomial infections which may cause mortality and morbidity [1,3–6].

Bacterial resistance to antimicrobial agents increases and spreads rapidly among nosocomial and community-acquired pathogens [7]. Recently, it is estimated that the increase in antibiotic resistance may develop due to the presence of multidrug resistance encoded transferable plasmids in *Enterobacteriaceae* family and spread of these plasmids between enterobacterial species. Therefore, the antibiotic resistance has become an important health problem [8]. Likewise, increase in antibiotic resistance in *Klebsiella* species has been marked [6].

In order to determine a certain strain, identification of plasmid profiles can be used as serotype specific reference pattern. For investigating epidemic diseases caused by bacteria which show similar biochemical and antibiotic susceptibility profiles, plasmid profile analysis is accepted as a useful method [9].

We aimed to find antibiotic resistance patterns and plasmid profiles of *Klebsiella* *sp.* strains isolated from clinical materials. We compared our findings with the previous studies in the relevant literature. Our findings demonstrated that the plasmid content and resistance

patterns have potential to be used in *Klebsiella* *sp.* acquired infections and also these findings are also important to take precautions against the transposition of antibiotic resistance plasmids between different *Klebsiella* species in order to prevent the formation of multi drug resistance encoded transferable plasmids.

Materials and Methods

Bacterial strains and biotyping

In this study, *Klebsiella* *sp.* strains were isolated from clinical materials (blood, urine and tracheal aspirate). The isolates were identified by standard phenotypical methods [10]. All isolated strains were inoculated in to the Brain Heart Infusion Broth media including 10% glycerol and stored at –20°C for later analysis.

In addition to phenotypical analysis, BBL Enterotube II Tube Test (Fisher Scientific, USA) was also used to identify *Klebsiella* strains. By interpretation combining phenotypically identification test results and BBL Enterotube II test results together, biotyping was done [10].

Antibiotic resistance testing and antibiotyping

In order to determine antibiotic resistance of *Klebsiella* *sp.* strains against eight different antibiotics (Amikacin: 30µg, Tobramycin: 10µg, Cefaclor: 30µg, Ceftazidime: 30µg, Cefotaxime: 30µg, Ceftriaxone: 30µg, Ampicillin: 10µg, Amoxicillin/clavulanic acid: 20/10µg) Kirby-Bauer disc diffusion method was used. The strains were classified as Resistant (R), Intermediate (I) or Sensitive (S) according to the zone table established by CLSI (Clinical and Laboratory Standards Institute) [11]. Antibiotyping was done by grouping *Klebsiella* *sp.* strains which showed the same antibiotic resistance pattern.

Plasmid DNA isolation

In order to isolate plasmid DNA, *Klebsiella* *sp.* strains were inoculated in to the Luria Bertani broth media and were incubated at 37°C and 150 rpm in a shaker incubator (Certomat BS-I, Sartorius). After the incubation period, plasmid DNA's of *Klebsiella* strains were isolated by using High Pure Plasmid Isolation Kit (Roche Applied Science, Germany).

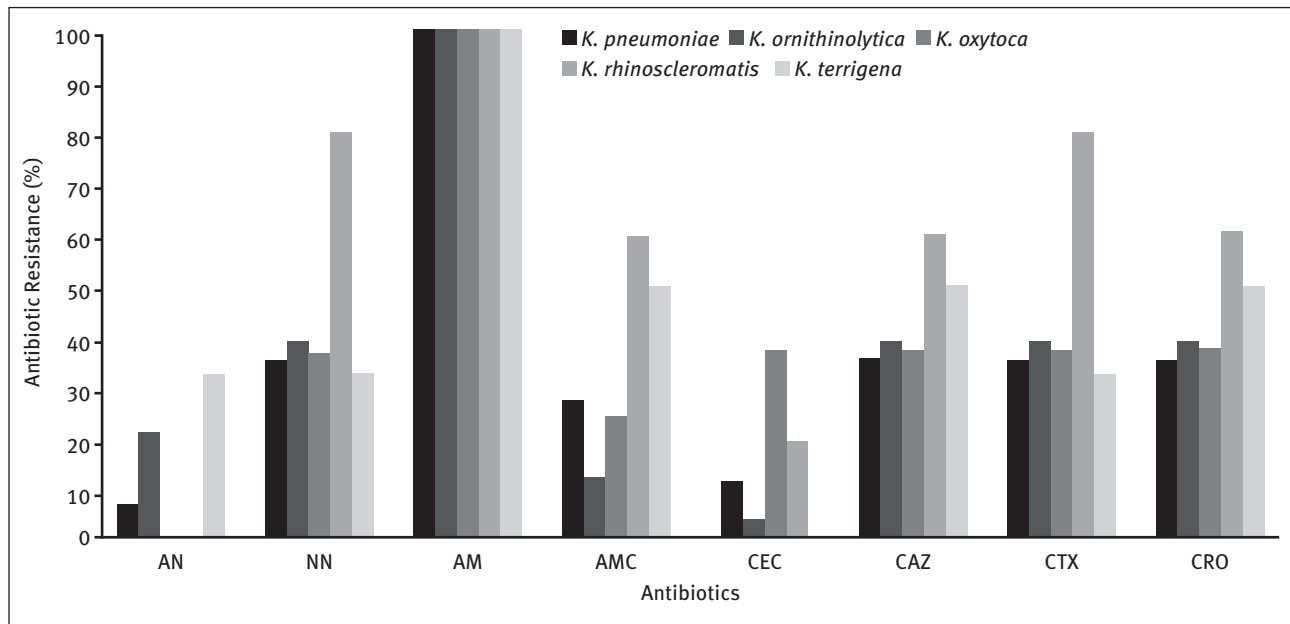


Figure 1: The percentage of antibiotic resistance of *Klebsiella* species. (AN: Amikacin; NN: Tobramycin; AM: Ampicillin; AMC: Amoxicillin/clavulanic acid; CEC: Cefaclor; CAZ: Ceftazidime; CTX: Cefotaxime; CRO: Ceftriaxone).

Gel electrophoresis and data analysis

Plasmid DNA fragments were separated by gel electrophoresis in a 1% (w/v) agarose in 1X TBE buffer (0.9 M Tris Base, 0.9 M Boric Acid and 0.02M EDTA). One kbp DNA ladder (ThermoFisher Scientific, USA) was loaded in the agarose gel and plasmid DNA fragments were separated by electrophoresis for 5h, at 50V. Gels were stained with ethidium bromide and photographed by using Gel Logic 200 Molecular Imaging System (Kodak; Rochester). The band profiles of *Klebsiella sp.* strains were analysed with NTSYS-pc (version 2.1; USA) program. The dendrogram was constructed by using the unweighted pair group method with arithmetic mean (UPGMA) and using Dice coefficients of similarity. By interpreting the dendrogram, *Klebsiella sp.* strains with a similarity coefficient of 84% were grouped in a cluster. The discriminatory power of plasmid profile typing was calculated by using the Hunter-Gaston discriminatory index.

Results

The results of biotyping showed that *Klebsiella sp.* strains were distributed to five different species. While *K. pneumoniae* (37.33%) strains were the most common biotype, the other biotypes were *K. ornithinolytica* (34.32%), *K. oxytoca* (11.94%), *K. terrigena* (8.95%) and *K. rhinoscleromatis* (7.46%).

Antibiotic resistance patterns of five different *Klebsiella* species against eight different antibiotics, it shows that all the strains were resistant to ampicillin. Also these strains showed resistance to at least one (ampicillin) and at most seven antibiotics (ampicillin, amoxicillin/clavulanic acid, ceftazidime, cefotaxime, ceftriaxone, tobramycin, amikacin or cefaclor). Resistance values for each antibiotic were calculated (Figure 1).

Based on the antibiotic resistance patterns, seven different antibiotic groups were generated (designated as A-G). It was observed that antibiotic A which showed only ampicillin (in penicillin group) resistance, was the most predominant antibiotic type (58.2%) within all *Klebsiella sp.* strains. Antibiotic resistance patterns and the percentage of antibiotic type profiles of *Klebsiella* species are shown in Table 1.

In order to compare antibiotic resistance patterns and plasmid DNA profiles of five different *Klebsiella* species with each other, plasmid profile analysis was done. With a similarity coefficient of 84%, it was observed that five different *Klebsiella* species displayed 17 different profile type (Figure 2) and also these strains exhibit at least one and at most seven plasmid DNA bands (Figure 3 a–c).

According to these results, it was found that P1 which exhibits >10kbp DNA band, was predominant (52.23%). P15 (only isolated from *K. ornithinolytica*) and P17 (isolated from *K. pneumoniae* and *K. rhinoscleromatis* species) had the highest number of plasmids (Table 2). All the strains which displayed P17 profile were grouped in antibiotic G

Table 1: Antibiotic resistance patterns and the percentage of antibiotype profiles of *Klebsiella* species.

Antibiotype	Antibiotic resistance patterns	<i>K. pneumoniae</i>	<i>K. ornithinolytica</i>	<i>K. oxytoca</i>	<i>K. rhinoscleromatis</i>	<i>K. terrigena</i>	All <i>Klebsiella</i> strains (%)
A	AM	64%	60.86%	62.5%	40%	33.33%	58.2%
B	AN,AM,CAZ,CTX,CRO,NN	4%	13.04%	0%	0%	0%	5.97%
C	AM,CAZ,CTX,CRO,NN	4%	8.69%	0%	0%	0%	4.47%
D	AM,CEC,CAZ,CTX,CRO,NN	0%	4.34%	12.5%	0%	0%	2.98%
E	AN,AM,AMC,CAZ,CTX,CRO,NN	4%	4.34%	0%	0%	33.33%	5.97%
F	AM,AMC,CEC,CAZ,CTX,CRO,NN	12%	0%	25%	20%	0%	8.95%
G	AM,AMC,CAZ,CTX,CRO,NN	12%	8.69%	0%	40%	33.33%	13.43%

AN: Amikacin; NN: Tobramycin; AM: Ampicillin; AMC: Amoxicillin/clavulanic acid; CEC: Cefaclor; CAZ: Ceftriaxime; CTX: Cefotaxime; CRO: Ceftriaxone.

Table 2: Characteristic properties of plasmid profile analysis tested *Klebsiella* species.

Plasmid profile type	Size of plasmid DNA bands (kbp)	Number of plasmid DNA bands	<i>K. pneumoniae</i>	<i>K. ornithinolytica</i>	<i>K. oxytoca</i>	<i>K. rhinoscleromatis</i>	<i>K. terrigena</i>	All <i>Klebsiella</i> strains (%)
P1	>10	1	56%	47.82%	50%	40%	66.67%	52.23%
P2	>10, 1.5	2	0%	8.69%	0%	0%	0%	2.98%
P3	>10, 2.5	2	4%	0%	0%	0%	0%	1.49%
P4	>10, 4, 2	3	0%	8.69%	0%	0%	0%	2.98%
P5	>10, 2	2	0%	0%	0%	20%	0%	1.49%
P6	>10, 4	2	20%	4.34%	25%	20%	0%	13.43%
P7	>10, 4, 3	3	0%	0%	12.5%	0%	0%	1.49%
P8	>10, 5	2	0%	4.34%	0%	0%	0%	1.49%
P9	>10, 5, 3.5	3	0%	4.34%	0%	0%	16.67%	2.98%
P10	>10, 5, 3	3	0%	4.34%	0%	0%	0%	1.49%
P11	>10, 8, 6, 5	4	0%	4.34%	0%	0%	0%	1.49%
P12	>10, 3.5, 3	3	0%	4.34%	0%	0%	0%	1.49%
P13	>10, 6, 3, 2	4	0%	0%	0%	0%	0%	1.49%
P14	>10, 6, 3.5, 3, 2, >1.5	6	0%	0%	0%	0%	16.67%	1.49%
P15	>10, 5, 4, 3.5, 3, 2.5, 2	7	0%	8.69%	0%	0%	0%	2.98%
P16	>10, 2.5, 2, >1.5	4	16%	0%	0%	0%	0%	5.97%
P17	>10, 6, 5, 3.5, 2.5, 2, >1.5	7	4%	0%	0%	20%	0%	2.98%

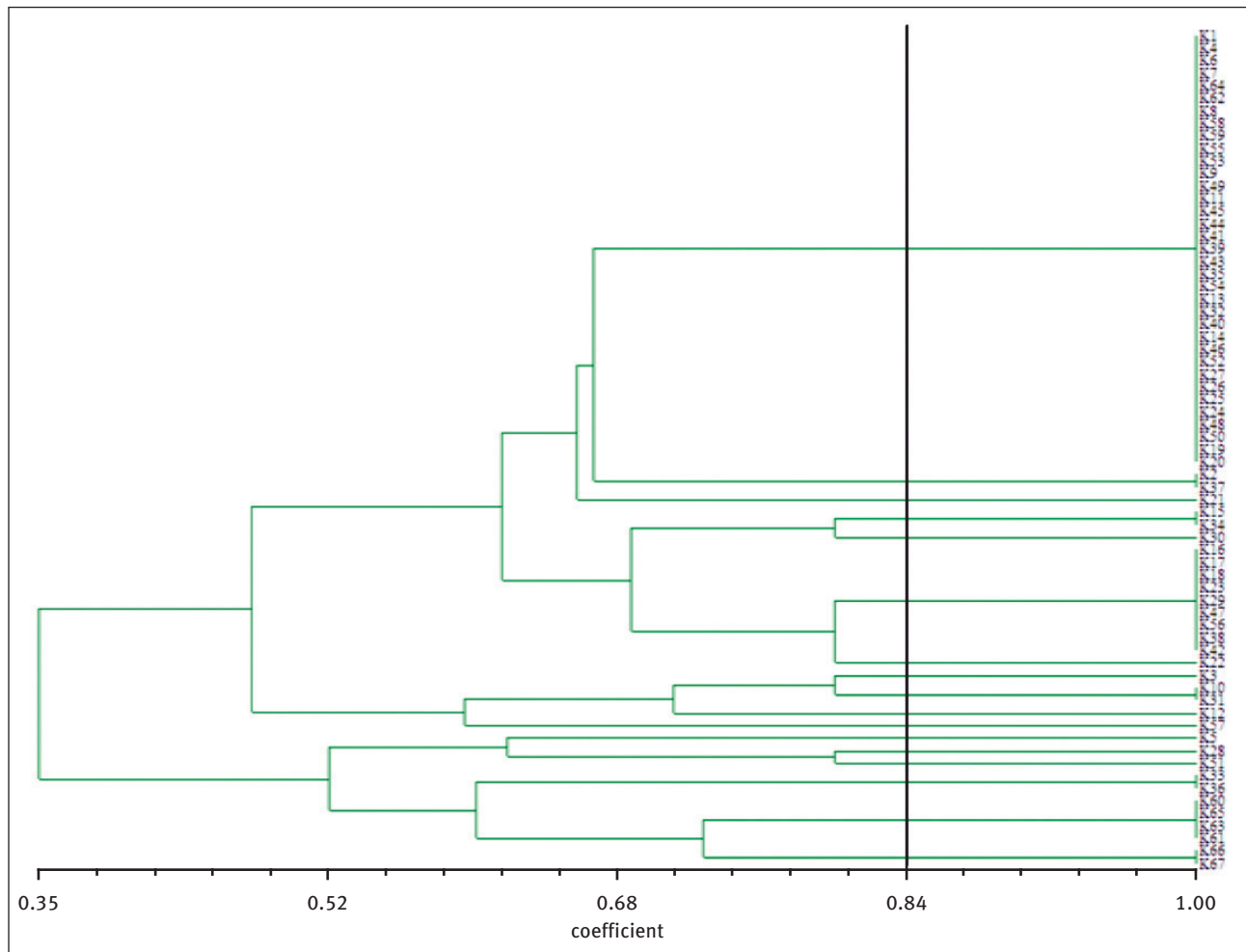


Figure 2: The dendrogram of plasmid profile analysis of *Klebsiella* species with a similarity co-efficient of 84%. (K1-K15, K33-K38, K57, K58: *K. ornithinolytica*; K16-K21, K39-K49, K59-K66: *K. pneumoniae*; K22-K29: *K. oxytoca*; K30, K54-K56, K67: *K. rhinoscleromatis*; K31-K32, K50-K53: *K. terrigena*).

(resistant to all antibiotics which belong to penicillin and cephalosporin groups and also tobramycin which belongs to amino glycoside's). Also, the discriminatory power of plasmid profile analysis of five different *Klebsiella* species was calculated and found as 0.71 [12].

The plasmid profile analysis indicated that all of the strains included a 3.5 kbp band. These strains, were resistant to ceftazidime, cefotaxime (in the third generation of cephalosporin's), tobramycin (in amino glycoside's) and ampicillin (which belongs to penicillin's). Due to the other strains which were also resistant to tobramycin, but not include the 3.5 kbp band, we infer that this band harbors the cephalosporin resistance functions. K51 which includes 3 kbp plasmid band among *K. terrigena* strains, showed antibiotype E (resistant to ampicillin in penicillin group and all cephalosporin's and amino glycosides) and has a common 3.5 kbp plasmid DNA band with the other *K. terrigena* strains with the same antibiotype. All of the

strains (K31, K36, K66 and K67) having 5 kbp plasmid DNA band, were also resistant to tobramycin, ceftazidime, cefotaxime, ceftriaxone (in amino glycosides, penicillin's and in the third generation of cephalosporin's). Therefore, it is supposed that this DNA band coded amoxicillin/clavulanic acid resistance functions. The plasmid profile type, plasmid band size and plasmid band number of five different *Klebsiella* species are summarized in Table 2.

Discussion

Biotyping of *Klebsiella* sp. strains are important because they are among the widely encountered pathogens in nosocomial infections. Here we investigated and compared antibiotic resistance, antibiotype and plasmid profile types of different *Klebsiella* species which were not analyzed together previously.

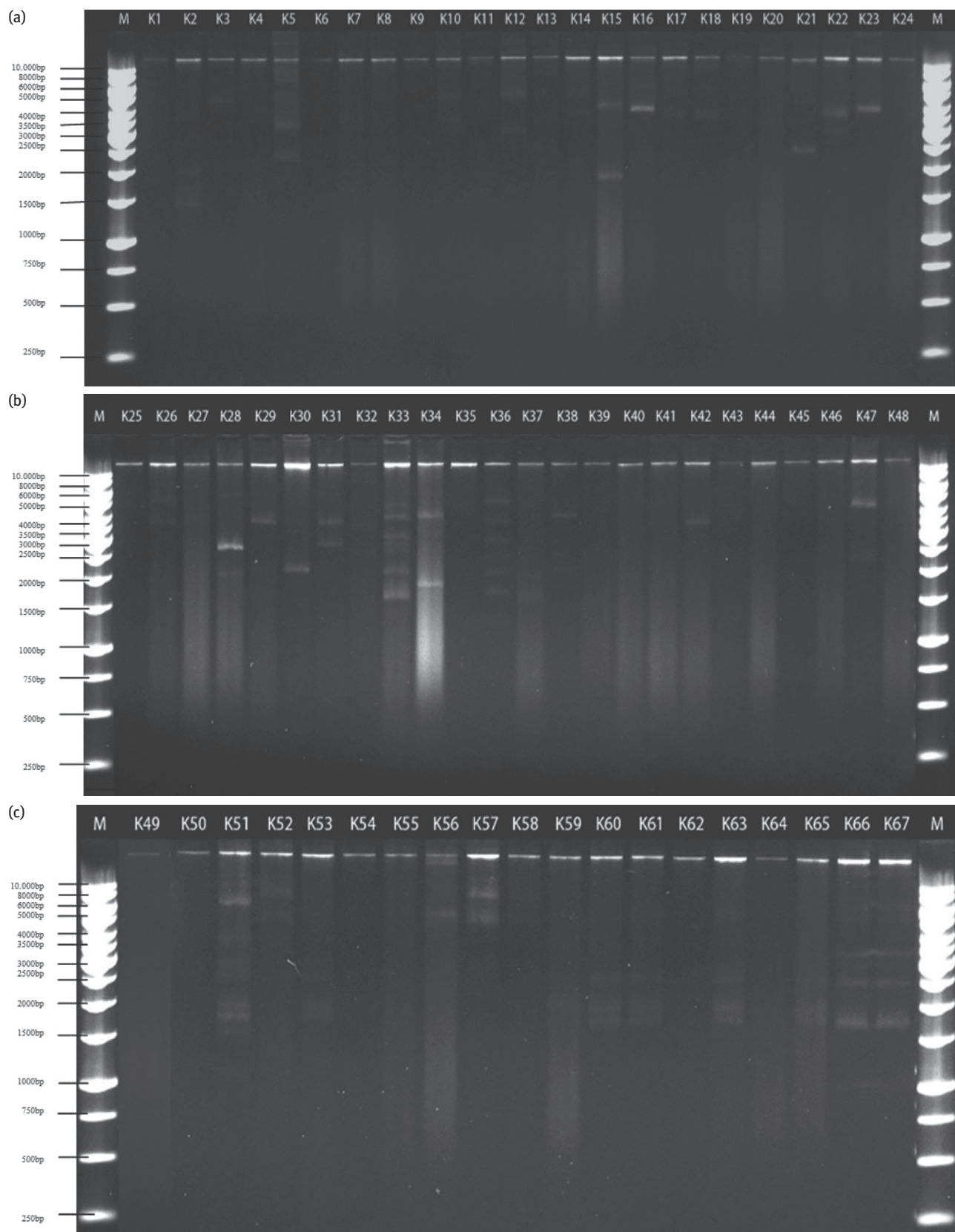


Figure 3: Plasmid patterns of *Klebsiella* strains in 1% (w/v) agarose gel. (a) K1-K15: *K. ornithinolytica*; 16-K21: *K. pneumoniae*; K22-K24: *K. oxytoca*. (b) K25-K29: *K. oxytoca*; K30: *K. rhinoscleromatis*; K31-K32: *K. terrigena*; K33-K38: *K. ornithinolytica*; K39-K48: *K. pneumoniae*. (c) K49, K59-K66: *K. pneumoniae*; K50-K53: *K. terrigena*; K54-K56, K67: *K. rhinoscleromatis*; K57-K58: *K. ornithinolytica*.

In this study, we found that *Klebsiella* strains isolated from clinical materials, fell into different biotypes (*K. pneumoniae*, *K. ornithinolytica*, *K. oxytoca*, *K. terrigena*, *K. rhinoscleromatis*) and *K. pneumoniae* (37.33%) was the predominant biotype. Likewise in several other reports, *K. pneumoniae* strains have been found to be the most common biotype [4,13,14]. According to the results of different researches, “The National Nosocomial Infection Surveillance System” stated that, *K. pneumoniae* is the fifth common agent of nosocomial urinary tract infections [15] and the second common agent of gram negative bacterial infections [3,13,16]. Furthermore, this pathogen causes mortality on new born and adults and the treatment of *Klebsiella* sp. acquired infections cannot be carried out effectively because of the multidrug resistant strains [4].

Along with *K. pneumoniae*; *K. ornithinolytica*, *K. oxytoca* and *K. rhinoscleromatis* were also identified by biotyping in this study. Owing to their infections in respiratory and urinary tract as community-acquired and nosocomial agents, it is probable to see these species in clinical materials. In addition; *K. pneumoniae*, *K. oxytoca* and the other species of *Klebsiella* are also isolated from the patients of urology, neonatal intensive and geriatric units as a nosocomial infection agent in previous reports [17]. In this study, we encountered *K. terrigena* which was firstly isolated from soil and water [18] and was seen first in a 45-year-old, liver transplantation patient in 2007 [19]. After that, this strain was isolated as an infection agent from a 69-year-old patient, following *Clostridium difficile* and *K. pneumoniae* infections. Compared to other species of *Klebsiella* genus, there is limited information about *K. terrigena* in the literature [20]. Since, comparison of antibiotic resistance and plasmid profiles of *K. terrigena* have not been done before, we investigated and established the above mentioned properties of the species in our study.

Antibiotic resistance patterns are regarded as useful guides for empirical treatment, typing methods and the spread of antibiotic resistance determinants [21]. We determined that the antibiotic resistance profiles of five different *Klebsiella* species and evaluated comparatively. When the antibiotic resistance profiles of *Klebsiella* sp. strains were examined, it was seen that *K. oxytoca* and *K. rhinoscleromatis* strains were susceptible to amikacin while the resistance to this antibiotic was found as 13.43% among all *Klebsiella* sp. strains. Similarly, the other researchers investigating the resistance against amikacin, also found that this antibiotic was an alternative for the treatment of *Klebsiella* sp. acquired infections [3,22,23]. The antibiotics which belong to cephalosporins were also examined in this study and ceftazidime resistance was found as 40.29%, comparable with the previous studies,

that have reported 36% and 36.7% resistance [5,15]. Cefaclor, another antibiotic in the group, and it is seen that, all *K. terrigena* strains were susceptible to this antibiotic. *K. pneumoniae* strains showed 36% against ceftriaxone and our findings agreed with Lina et al.’s [5] study which was also done with *K. pneumoniae* regarding ampicillin and amoxicillin/clavulanic acid (in penicillin group) resistance. We found that all the strains were resistant to ampicillin (100%) and this result agreed with previous reports on the matter [3,24].

According to plasmid profile analysis, P1 which exhibits only one plasmid DNA band with a molecular weight of >10 kbp, was found to be the most common plasmid in our *Klebsiella* isolates. However 91.42% of the strains showing P1 were grouped in antibiotype A, the other strains showing P1 were grouped in different antibiotypes. This agreed with Jazani et al.’s [25] findings, indicating in this case the antibiotic resistance functions are likely to be on the chromosomes. Furthermore, we observed that some of the *Klebsiella* sp. strains, including a single plasmid band, were resistant to only (ampicillin) but the others were resistant to seven antibiotics (ampicillin, amoxicillin/clavulanik acid, ceftazidime, cefotaxime, ceftriaxone, tobramycin, amikacin or cefaclor). Similarly this implied that, in some strains the antibiotic resistance encoding genes on the chromosomes. Agreeing with our results, Nsofor and Iroegbu [26] were found that some strains were resistant to 4 antibiotics and included more than one plasmid bands, the others were resistant to much more antibiotics but included one or two plasmid bands only.

The size of plasmid DNA bands of the five *Klebsiella* species was also investigated in our study. We found that while 1.5 kbp and 8 kbp plasmid DNA bands were isolated from only *K. ornithinolytica* species, >10 kbp, 6kbp and 2 kbp plasmid DNA bands were isolated from all *Klebsiella* species. We have observed that different *Klebsiella* species exhibited the same plasmid DNA bands as well (Table 2). This implied that plasmids, carrying antimicrobial resistance genes enabled the transfer of genetic materials the bacterial species [27] and as a result, the same plasmid band may be observed in the different strains of the same genus.

Conclusions

Our study indicates that different *Klebsiella* species exhibit various number of plasmid DNA bands and also different species of the same genus may exhibit the same plasmid DNA likely to be the result of plasmid transfer among strains. Additionally, the number of plasmid DNA bands

does not give a significant clue about antibiotic resistance patterns. Our results also indicated that some antibiotic resistance functions chromosomally encoded. Finally, because of being a serious nosocomial infection agent, it is very important to take precautions against the transposition of antibiotic resistance plasmids between different *Klebsiella* species in order to prevent the formation of multidrug resistance encoded transferable plasmids.

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Conflict of Interest: The authors have no conflict of interest.

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