Original Article

Investigation of clonal relationships of *K. pneumoniae* isolates from neonatal intensive care units by PFGE and rep-PCR

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Abstract

Introduction: Clonal relationships of *Klebsiella pneumoniae* strains obtained during an epidemic and after a one-year post-epidemic (non-epidemic) period in the same neonatal intensive care unit (NICU) using pulsed-field gel electrophoresis (PFGE) and repetitive polymerase chain reaction (rep-PCR) by the DiversiLab (DL) system were investigated, and the results of both molecular techniques were evaluated.
Methodology: Fifteen *K. pneumoniae* strains were included in this study. All identified bacterial strains were confirmed by 16S rDNA sequencing and analyzed by PFGE and the DL system.
Results: According to the PFGE results, 15 isolates showed 10 different band profiles. Nine of these 15 isolates were included in one of the formed clusters, and the remaining six isolates were not included in any of them. According to the DL system results, 15 isolates showed two different clusters, with three strains in one cluster and four strains in the other. The remaining strains could not be placed any one of the clusters. PFGE was used as the gold standard based on its strong genetic discriminatory power. The DL system results showed that PFGE missed the relationship of the two epidemic-related strains and demonstrated one epidemic-unrelated strain to be epidemic related.
Conclusions: Both systems may easily be used for clonal relationships of *K. pneumoniae* strains. The DL system was clearly more rapid and convenient than PFGE, but its discriminatory power seemed to be inferior to that of PFGE based on 15 *K. pneumoniae* strains.

Introduction

Nosocomial infection in the neonatal intensive care unit (NICU) is a significant cause of morbidity and mortality [1,2]. Gram-negative bacteria (GNB) cause 20% to 40% of all cases of late-onset sepsis in the NICU and are frequently associated with adverse clinical outcomes [3,4]. The major Gram-negative pathogens associated with nosocomial infections are saprophytic or commensal microbes, such as *Klebsiella* spp. [5]. The ability of these organisms to spread rapidly often leads to nosocomial epidemics, especially in the NICU [6-10].

Investigation of a presumed *Klebsiella* spp. epidemic often requires strain-typing data to identify epidemic-related strains. Traditional techniques for typing *K. pneumoniae* are based on phenotypic characteristics and include biotyping, antibiogram typing, O-serotyping, and bacteriocin and phage typing; however, all of these techniques have poor discriminatory power [11]. Alternatively, molecular methods such as plasmid profile analysis, ribotyping, fragment restriction endonuclease analysis, multilocus sequence typing, gene sequencing, pulsed-field gel electrophoresis (PFGE), and repetitive sequence-based polymerase chain reaction (rep-PCR) are available for strain typing [12-20].For analysis of an epidemic, strain typing of the isolates should be performed using at least one of these molecular methods.

The aims of this study were to analyze the clonal relationships of *K. pneumoniae* strains obtained during an epidemic and during the one-year post-epidemic period (non-epidemic period) in the same NICU using PFGE and rep-PCR and to evaluate the results of both molecular techniques.

Methodology

NICU epidemic and sample collection

An epidemic occurred in the 17-bed NICU of the Training and Research Hospital of Sakarya University. The emergence of an epidemic was suspected after isolation of *K. pneumoniae* from six different cultures of four patients' samples in the NICU from 13 to 15 May, 2013. Two catheter cultures from two patients, one blood and one cerebrospinal fluid culture from one patient, and one blood and one stool culture from one patient, yielded *K. pneumoniae*. The culture results were shared with the infection control committee of the hospital. Additional culture samples were subsequently needed to accurately determine the presence of an epidemic. New culture samples were thus gathered from six other patients hospitalized in the NICU during the same period, from all NICU workers, and from 70 different locations within the NICU environment. Six additional *K. pneumoniae* strains were obtained from the stool samples of six patients hospitalized during the same period, and these strains were accepted to be colonization. Finally, 12 *K. pneumoniae* strains were isolated. The epidemic was restricted to four patients, and precautions recommended by the infection control committee of the hospital were implemented.

The NICU was closely monitored during the one-year post-epidemic period (non-epidemic period), and seven more *K. pneumoniae* strains were isolated from clinical samples of five patients. The samples were as follows: one urine sample from one patient, three blood samples from three patients, and two blood and one wound sample from one patient. Eventually, 19 *K. pneumoniae* strains from 15 patients were isolated, and the clonal relationships of 15 of these strains were explored in the present study. The remaining four *K. pneumoniae* strains were excluded because when more than one strain was obtained from the same patient on the same day, only one strain was included. All isolates were numbered 1 to 15. Strains 1 to 10 were isolated during the epidemic, and strains 11 to 15 were isolated during the one-year post-epidemic period. No strains were isolated during the last six months of the post-epidemic period.

Bacterial identification and antimicrobial susceptibility

Isolated strains were inoculated into triple sugar iron agar, lysine iron agar, motility-indole-ornithine medium, citrate agar, phenylalanine agar, and Christensen urea agar. The results were recorded after the incubation (18 to 24 hours at 37°C) [21].

The antimicrobial susceptibility was performed using the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar, as described in Clinical and Laboratory Standard Institute (CLSI) guidelines [22].

In addition to conventional bacterial identification and antimicrobial susceptibility methods, the VİTEK 2 automated system (Biomerieux, Marcy l'Etoile, France) was used.

16S rDNA sequencing

All identified bacterial strains were confirmed by 16S rDNA sequencing. After harvesting the *K. pneumoniae* stains from cultured media, the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) was used to extract the bacterial DNA. The ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, California, USA) was used to analyze the partial nucleotide sequences of 16S rDNA. The Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nim.nhi.gov) analysis results of the partial nucleotide sequences data obtained from the genetic analyzer were assessed using the National Center for Biotechnology Information (NCBI) web page. *K. pneumoniae* strain AUH-BG208 was used as the reference strain in the neighbor-joining analyses.

PFGE

PFGE typing was performed for 10 *K. pneumoniae* isolates during the epidemic. A second analysis of all 15 isolates was then performed, which included the strains isolated after the epidemic. The protocol suggested by Durmaz *et al*. [23] was used for PFGE typing of the *K. pneumoniae* isolates.

DNA banding patterns were analyzed using BioNumerics software, version 5.10. The Dice coefficient was chosen to calculate pair-wise similarities, and the unweighted pair-wise grouping with mathematical averages (UPGMA) algorithm was chosen to construct dendograms. Both position tolerance and optimization were set at 1%. According to the Dice similarity coefficient, $\geq 90\%$ similarities were accepted as same PFGE types, and PFGE types were also defined by lower cut-off values, $\geq 85\%$ similarity, and $\geq 80\%$ similarity.

Rep-PCR

Repetitive PCR analyses were managed using the DiversiLab (DL) bacterial typing system, a semiautomated typing system based on repetitive extragenic palindromic sequence-based PCR (rep-PCR) (bioMérieux, Marcy l'Etoile, France). The obtained DNA was quantified by spectrophotometry

with a Gene Quant 1300 (GE Healthcare Europe GmbH, Milan, Italy) and diluted to 25 to 50 ng/mL. Diluted DNA was amplified using the *Klebsiella* fingerprinting kit (bioMérieux, France) according to the manufacturer's instructions. Amplicons were analyzed using a 2100 Bioanalyzer (Agilent Technologies,Santa Clara, USA). For this procedure, a microfluidics chip (LabChip device; Caliper Technologies, Hopkinton, MA, USA) that separates DNA fragments of different sizes was used, resulting in chromatograms with peaks for each amplicon. Analysis was performed by internet-based DL software, version 3.4, which creates virtual gel images and uses the band-based modified Kullback-Leibler distance to calculate percentage similarities. The automatically generated dendograms, similarity matrices, electropherograms, virtual gel images, scatter plots, and selectable demographic fields were used for interpretation.

DNA band patterns from the DL system were analyzed using the DL analysis software (bioMérieux, France) with the Pearson correlation coefficient to determine the distance matrices and the UPGMA for similarities and cluster analysis, respectively. Homologies of > 97% and 95% to 97% were regarded as the parent clone (indistinguishable) and similar isolates, respectively, according to the manufacturer's recommendations and results of longitudinal studies

and comparisons. A similarity value of < 95% was considered to indicate a difference [24].

Results

Fifteen newborns were included in this study. Four of them were born prematurely, and six were female. The mean gestational age of the patients was 33.10 weeks, and the mean birth weight was 1,864 g (Table 1). In case of suspected infection, appropriate doses of ampicillin/gentamicin were given to patients as empirical antimicrobial treatment protocol. The antimicrobial treatment regimen was reordered according to antimicrobial susceptibility test results.

Sequencing and neighbor-joining analyses of the 15 *K. pneumoniae* strains demonstrated that all strains and the reference strain had 91% to 97% similarity.

When the strains isolated during the epidemic were evaluated according to antibiotic susceptibility patterns, the susceptibility patterns of strains 1, 5, 7, 8, 9, and 10; 2 and 3; 11 and 14; and 12 and 15 were identical. Strains 4, 6, and 13 showed no similarity with the other strains in terms of susceptibility. Strains 1 (isolated from blood and stool samples), 4 (catheter), 11 (urine), 13 (blood), and 14 (blood) were determined to be extended spectrum beta-lactamase (ESBL) positive. Among the ESBL-positive strains, only strains 11 and 14 showed the same antibiotic susceptibility patterns. All strains were susceptible to

Table 1. Patients' demographic data

Patient no	Diagnosis	Gender	Gestational age (weeks)	Weight (grams)	Sample	Outcome	Antibiotic treatment	Day of infection	Stay in hospital (days)
1	Transient tachypnea of the newborn	Male	36	2,300	Blood + stool	Exitus*	Yes	2	37
2	Premature + respiratory fetal distress	Female	26	660	Catheter	Recovery	Yes	17	33
3	Premature + respiratory fetal distress + low gestational weight	Male	27	560	Blood + CSF	Exitus*	Yes	33	35
4	Premature	Female	26	650	Catheter	Exitus**	Yes	17	19
5	Malnutrition	Male	40	3,300	Stool	Recovery	None	-	61
6	Low gestational weight	Female	38	2,540	Stool	Recovery	None	-	7
7	Respiratory fetal distress	Male	33	1,680	Stool	Recovery	None	-	13
8	Jaundice	Female	37	3,570	Stool	Recovery	None	-	7
9	Premature	Male	31	1,370	Stool	Recovery	None	-	23
10	Preeclamptic mother	Male	32	1,660	Stool	Recovery	None	-	24
11	Septicemia	Female	36	2,170	Urine	Recovery	Yes	5	27
12	Premature	Female	27	590	Blood	Exitus**	None	***	12
13	Premature + preeclamptic mother	Male	28	1,030	Blood	Exitus**	None	***	7
14	Respiratory fetal distress	Male	38	3,490	Blood	Recovery	Yes	10	24
15	Diaphragmatic hernia	Male	41	3,380	2 Blood + 1 wound	Recovery	Yes	27	83

*Deaths due to sepsis caused by epidemic strains. **Deaths due to sepsis caused by non-epidemic strains. ***These patients died before bacterial identification.

carbapenems (imipenem, meropenem, ertapenem, and doripenem), colistin, amikacin, and ciprofloxacin.

According to the PFGE results, 15 isolates showed 10 different band profiles (pulsotypes). Nine of these 15 isolates were included in one of the formed clusters, and the remaining six isolates were not included in any cluster. The clustering rate of the isolates was 60% (9 of 15 isolates). The largest cluster was cluster B, with seven members; cluster A had two members, and the other clusters had one member. The strains isolated from clinical and stool samples (considered to be colonization) during the epidemic were found to be clonally related and placed in cluster B. Cluster B was divided in two subclusters: subcluster B contained strains 1, 2, 3, 5, and 8, which were clonally indistinguishable, and subcluster B1 contained strains 6 and 10, which were closely related to members of B. Strains 11 and 14, located in cluster A, were found to be closely related to each other. These isolates were not associated with the epidemic strains; additionally, they were isolated from two different patients on the same day during a non-epidemic period. Another important finding was that, except for strains 4 and 7, which were excluded, all strains isolated during the epidemic and all strains isolated from stool samples were obviously clonally related (Figure 1).

The rep-PCR results were as follows. The epidemic-related strains 1 and 2 were indistinguishable, with 97.7% similarity, and strain 3 was related to them with $\geq 95\%$ similarity (cluster I). The strains obtained during the course of the epidemic (strains 5, 8, and 9, considered to be colonization), were found to be related to strain 11, which was cultivated during the non-epidemic period (Cluster Ia). Of strains 11 and 14, both from the non-epidemic period, strain 11 was an epidemic-related strain and strain 14 was not (similarity rate of 90%–95%). The other strains (strains 4, 7, 10, 12, 13, and 15) were identified as clonally unrelated to the epidemic strains (Figure 2).

Discussion

K. pneumoniae, which causes various infections, is an opportunistic pathogen that persists on environmental surfaces within hospitals and can colonize the intestines, skin, and respiratory tract. It can also spread easily via the hands of healthcare workers, which likely facilitates nosocomial spread because *K. pneumoniae* can survive for several hours on the hands. Colonized individuals, who can be long-term asymptomatic carriers, act as reservoirs for

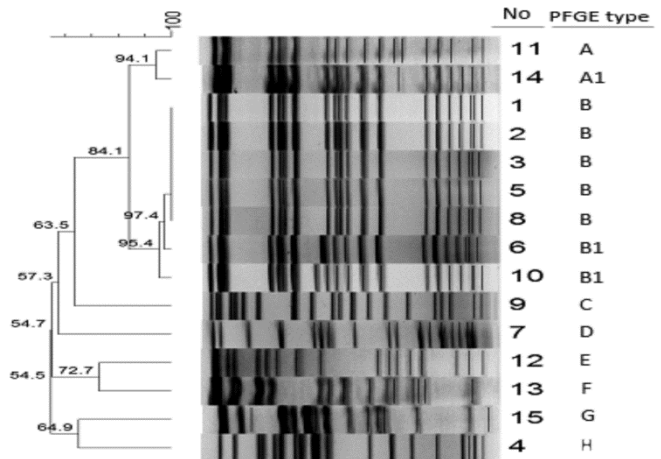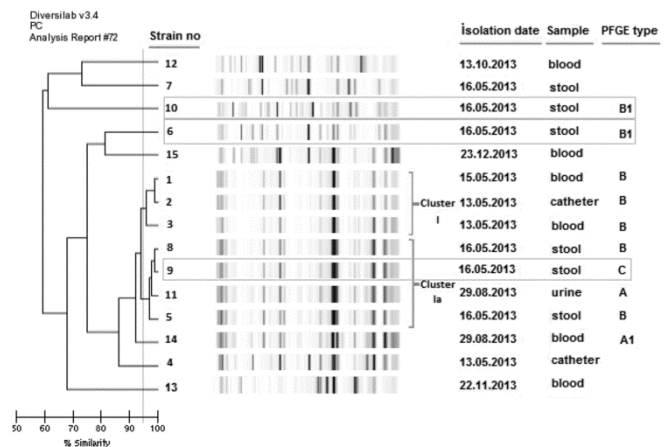**Figure 1.** PFGE image of the *K. pneumoniae* strains

Figure 2. Dendogram of the rep-PCR (DL system) results of the *K. pneumoniae* strains

continuous transmission, making the spread of bacteria and control of epidemics difficult. These characteristics have led to the placement of *K. pneumoniae* in the group of important bacteria for NICUs [10,11].

Traditional epidemiological methods are insufficient for typing of microorganisms. Particularly in the case of an epidemic, molecular epidemiological methods, which are superior to traditional methods, should be used in conjunction with traditional methods. Rapid detection of microbial clonal relationships prevents the spread of multidrug-resistant pathogens and allows for the monitoring of infection control measures, although these methods have both advantages and disadvantages. In the present study, clinical isolates of *K. pneumoniae* strains collected during a *K. pneumoniae* epidemic in the NICU of our

hospital and the one-year post-epidemic period were investigated by PFGE and rep-PCR (DL system). The two methods were evaluated to determine their ability to detect clonal relationships among the isolated strains.

The antimicrobial susceptibility profiles (as phenotypic patterns) divided the strains into four groups. Two *K. pneumoniae* strains (strains 2 and 3) had identical antimicrobial susceptibility profiles. Strain 1 was in the same group as strains 5, 7, 8, 9, and 10, which were considered to be colonization. The strains isolated during the non-epidemic period (strains 11 and 14) exhibited identical antimicrobial susceptibility profiles, and both were evaluated as unrelated by molecular techniques. Strain 4 was obtained during the epidemic, strain 6 was considered to be colonization, and strain 13 was obtained during the non-epidemic period; all three had singleton antimicrobial susceptibility profiles. Strains 4 and 13 were considered to be unrelated to the epidemic, but strain 6 was related and located in cluster B by PFGE. This relationship was not observed by rep-PCR. Although strains 7, 12, and 15 were placed in one of the four phenotypic groups, they were not placed in any molecular groups by either technique. This investigation confirmed that depending on phenotypic patterns such as the type of sample, date of sampling, and antimicrobial susceptibility profile, traditional epidemiological data cannot be used to identify and monitor an epidemic [25].

The PFGE results of the first 10 *K. pneumoniae* strains cultivated during the epidemic were as follows. With the exception of strains 4, 7, and 9 (the last two strains were considered to be colonization), all strains were determined to be related and placed in clusters B and B1. Strains 1, 2, 3, 5, and 8 were indistinguishable; strains 6 (97.4% similarity) and 10 (95.4% similarity) were considered to be very closely related to the indistinguishable strains. Strains 11 and 14 were isolated from two patients during the non-epidemic period. These strains were related to each other with 94.1% similarity (clusters A, A1). Groups A and B were related, with 84.1% similarity. Previous studies have used PFGE results as low as 80% to 85% to evaluate genetic relationships [24,26]. In this respect, the strains from the non-epidemic period may have been related to the epidemic strains. Consequently, a second mini-epidemic limited to these two patients in the NICU likely occurred during the non-epidemic period.

In the hospital setting, clinical microbiological laboratory tests to identify epidemic strains are generally based on fingerprinting methods such as PFGE, which is considered the gold standard. PFGE has strong genetic discriminatory power. Nevertheless, there are some limitations to the use of PFGE, such as its time-consuming nature and requirement for rigorous standardization and experienced personnel to achieve reproducible results that are comparable over time and among locations. Furthermore, there is no consensus on the nomenclature for PFGE patterns and no international database available for comparison [14,24,27-29]. In the present investigation, we used PFGE and rep-PCR for fingerprinting of 15 *K. pneumoniae* strains and evaluated the results of the two systems. PFGE was accepted as the gold standard method because of its high discriminatory power. We obtained identical results with two PFGE studies: the first immediately after the epidemic with 10 *K. pneumoniae* strains, and the second after one year post-epidemic with 15 *K. pneumoniae* strains.

The DL system revealed that strains 1, 2, and 3, which were from the epidemic period; strains 5, 8, and 9, which were considered to be colonization; and strain 11, which was from the non-epidemic period, had the same genetic patterns, with 95% to 100% similarity (clusters I and Ia). Although strain 9 was correlated with the epidemic strains by the DL system, it was not correlated according to PFGE. The DL system evaluates strains with < 95% similarity as being different. Some researchers have suggested that in any epidemic, this value can be reduced to 90% on analysis of the genotype results from the DL system [26]. Accordingly, the non-epidemic strain 14 was similar to the strains in clusters I and II with a ratio of 90% to 95%. Strains 6 and 10 were determined to be epidemic-related strains by PFGE (cluster B1), but rep-PCR revealed that these strains had a very weak relationship at < 90% similarity. Strains determined to be unrelated to the epidemic by PFGE were strains 4, 7, 9, 12, 13, and 15, while by rep-PCR were 4, 6, 7, 10, 12, 13, and 15. Thus, it is clear that the 15 epidemic and non-epidemic isolates were not identical between the PFGE and rep-PCR systems. When the relationship level was reduced to 90% from 95%, the rep-PCR results indicated that strains 11 and 14 were epidemic-related strains. Thus, the main difference between the two methods arose from grouping strains 6, 9, and 10, which were considered to be colonization. Although PFGE evaluated strains 6 and 10 as related to the epidemic, the DL system did not. Additionally, while PFGE showed that strain 9 was not associated with the epidemic, the DL results showed the contrary. Brolund *et al.* [24] indicated that

DL can be used to eliminate an unrelated strain in any monitoring study, but the power of this system is not sufficient to discriminate clonally related strains. Confirmation of the results of the DL system by a more powerful system is required. Although we worked with a very limited number of samples, the results were in agreement with the conclusion of Brolund *et al.* [24].

The DL system is a rep-PCR technology that offers semiautomated, easy-to-use, high-throughput, rapid bacterial strain typing with poor reproducibility. It may be a suitable alternative to PFGE analysis for epidemic investigation of healthcare-associated pathogens such as methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Klebsiella* spp., and has been proven to be a rapid and reliable method for molecular analysis of nosocomial epidemics [26]. Nevertheless, although this method was recently validated for typing of *Acinetobacter* spp., *Escherichia coli*, and *Klebsiella* spp., it was found to be insufficiently discriminative for typing of *S. aureus* [30,31]. This method can be easily introduced into routine settings and requires less hands-on time than does PFGE. Its use is also facilitated by an associated website that allows for easy analysis and visualization of the data.However, comparisons among centers have not yet been performed[14].The DL system has also been used in previous studies. In some studies, the DL cut-off value was lowered to 90% to establish results fully compatible with those of PFGE for the detection of clonal relationships among *Klebsiella* spp. isolates. A very small number of *K. pneumoniae* isolates was examined in this study, and even in cases of a lowered cut-off value, the two methods were not almost fully compatible [24,26].

The ideal typing method should be rapid, easy to perform, high-throughput, and applicable to a wide range of microorganisms. It should also meet performance criteria, such as full typeability, reproducibility, high discriminatory power, and concordance with validated typing methods as well as consistency with underlying subspecies genetic population structures [30,32,33]. However, such an ideal molecular typing method does not yet exist. Therefore, faster, simpler, and more easily standardized epidemiological typing methods are necessary for use in clinical microbiology laboratories [24].

Conclusions

The *K. pneumoniae* strains in the epidemic described herein originated from patients in our NICU. The patients harbored the bacteria as colonization and/or infection agents. The results from the non-epidemic period suggested that a mini-epidemic, caused by *K. pneumoniae* strains related to the epidemic strains, occurred; this was determined by PFGE and the DL system, respectively, when the results were adjusted to 90% from 95% similarity. Additionally, the epidemic strains may not have been completely eradicated. While the DL system missed the relationship of the two epidemic-related strains, the system software showed one strain to be being epidemic related. These two strains were related to the epidemic, and the other was not. The DL system was clearly more rapid and convenient than PFGE, but its discriminatory power seemed to be inferior to that of PFGE on the basis of 15 *K. pneumoniae* strains. This is in agreement with the findings of other researchers [24,26,34].

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