Molecular Epidemiology and Virulence Characteristics of *Klebsiella pneumoniae* Strains Isolated from Hospital-Associated Infections

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Abstract: *Purpose*: The present study aimed to confirm by classical and molecular laboratory methods hospital-associated outbreaks due to virulent *Klebsiella pneumoniae* strains.

Methods: Eighty three *Klebsiella pneumoniae* strains isolated from new-born patients, adults and hospital environment and devices in five hospital units, were analyzed for resistance to antibiotics, including last generation cephalosporins, sensitivity to bacteriophages and pulsed-field gel electrophoresis profiles in order to evaluate the epidemiological relatedness and their clonal spreading. Polymerase chain reaction targeting *fur* genes and several subtractive sequences (SL 002, SL 003, SL 019, SL 020, SL 021 and SL 025) was used for virulence assessment.

Results: More than 50% of strains were resistant to third generation cephalosporins and among them 69% were extended spectrum beta-lactamase producers. Phage typing associated with pulse field gel electrophoresis documented the clonal dispersion of strains. Studying the distribution of virulence sequence, our results reveal that *fur* gene is present in all strains and among the subtractive sequences the most frequent is SL 020 followed by SL 019. None of the analyzed sequences are present in all clinical isolates and none of the bacterial strains carry all these sequences pointing out the heterogeneity of *Klebsiella pneumoniae* population.

Conclusions: Phage typing method associated with pulse field gel electrophoresis typing and genetic profile for virulence indicated the occurrence of hospital associated-infections produced by *Klebsiella pneumoniae* strains. Moreover, the results reveal that virulence pattern could be used as a molecular marker in order to define strains which are involved in the process of the development of infectious diseases.

Keywords: *Klebsiella pneumoniae* infections, molecular characterization, PFGE, virulence genes, PCR.

INTRODUCTION

The genus *Klebsiella* is grouping Gram-negative opportunistic pathogens frequently isolated from bacteraemia, pneumonia, urinary tract and soft tissue infections.

Immunocompromised individuals suffering from severe diseases such as diabetes mellitus and/or chronic alcoholism, and patients with chronic cardiac diseases, pulmonary obstruction, older persons and new-born babies are the most susceptible hosts. Most of *Klebsiella* infections are hospital associated, with a high fatality rate if incorrectly treated. *Klebsiella* is the second cause of nosocomial bacteraemia and lethal sepsis in pediatric ward, especially in premature infants and intensive care units and often causes neonatal sepsis.

Murine models were used to determine host-pathogen interactions during *Klebsiella* infections, to understand the host response following infection, to detect the bacterial virulence factors which are playing an important role in the severity of infection and to identify a valuable vaccine candidate [1].

Klebsiella pneumoniae strains exhibit different virulence factors such as capsular polysaccharides, type 1 and type 3 adhesins, KPF-28 fimbriae, non fimbrial adhesins CF29K, factors involved in aggregative adhesions and siderophores [2-9]. Comparative studies were carried out to detect the correlation between different capsular serotype, mucoid or non-mucoid phenotype, and the presence of different genetic structures coding for virulence factors or harbored exclusively by virulent strains [10-12]. PCR-based subtractive hybridization technique was used to detect genetic sequences carried only by highly-virulent strains of K2 serotype of *Klebsiella pneumoniae,* and plasmid DNA analysis, amplified fragment length polymorphism (AFLP) pulsed-field gel electrophoresis (PFGE), restriction of the amplified capsular antigen gene cluster and multi locus sequence typing (http://www.mlst.net/) were used as molecular techniques to discriminate between strains [13-15].

During 2003 and the beginning of 2004 an important number of infections due to the *Klebsiella pneumoniae* strains, some of them leading to lethal sepsis, occurred in some pediatric and/or neonatal hospital units from South-Eastern region of Romania. The aim of the present study

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Table 1. Origin of *Klebsiella pneumoniae* Strains Included in Study

Hospital of Isolation	Lot Definition	Date of Isolation (M/Y)	New-Born Babies	Adults	Hospital Environment	No of Strains
H1	H1-1	06/2003	6	1	1	8
	H1-2	12/2003-02/2004	27	11	4	42
H2	H2-1	05-07/2004	10	6	0	16
	H2-2	11/2003	4	0	0	4
H3	H3	01-07/2004	5	1	0	6
H4	H4	05-10/2004	2	4	0	6
H5	H5	07/2004	1	0	0	1
Total of strains			55	23	5	83

consists in 1) evaluating the virulence potential of *Klebsiella* isolates, 2) searching a correlation between the source of isolation and the genetic profiles of strains and 3) assessing the clonal spreading of strains when a hospital-associated infection was suspected.

MATERIAL AND METHODOLOGY

Bacterial Strains

A total number of 83 *Klebsiella pneumoniae* clinical strains isolated from hospitalized new-born patients (55 strains), adults represented by accompanying mothers and/or medical care personnel (23 strains) and from hospital environment and devices in five hospital units (5 strains), were analyzed in this study (Table **1**). Strains isolated from hospitals designated H1 (n=50) and H2 (n=20) were suspected as hospital associated. According to the date of isolation, these strains were subdivided in lots H1-1, H1-2 and H2-1, H2-2, respectively. Of all strains, 31 originated from blood culture and the rest from faeces (7 strains), amniotic liquid (7 strains), respiratory tract infections (6 strains), ocular swabs (5 strains), skin swabs (6 strains), urine (5 strains), other biological samples (11 strains) and surfaces of hospital devices in contact with new-born babies (5 strains).

The strains isolated from hospitals designated as H3, H4 and H5 were included in this study as controls, for comparison purposes.

The data were presented as absolute number (n), and/or relative frequencies (%).

Strains were identified by cultural methods as *Klebsiella pneumoniae* in the clinical laboratories of the hospitals where they originated and were sent to the Reference Laboratory for further characterization.

Susceptibility Testing

The susceptibility to 12 antibiotics (Table **2**) was detected by standard diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) protocol [16] using Mueller Hinton Agar and Oxoid discs. Double disc synergy test was used for detecting the capacity of bacteria to produce extended spectrum beta-lactamases (ESBLs). The results were recorded as resistant, intermediate and susceptible using the CLSI interpretative criteria and the pattern of resistance was reported as resistotype.

Table 2. List of Antibiotics Used for Susceptibility Testing

Antibiotic	Abbreviation	Concentration/Disc
Amoxicillin+ clavulanic acid	AMC	20 + 10 µg
Imipenem	IPM	10 µg
Ceftazidime	CAZ	30 µg
Cefotaxime	CTX	30µg
Cefaclor	CEC	30µg
Amikacin	AK	30 µg
Gentamicin	CN	10 µg
Tobramicin	TOB	10 µg
Chloramphenicol	C	30µg
Nalidixic acid	NA	30 µg
Ciprofloxacin	CIP	5µg
Ofloxacin	OFX	5 µg

Bacteriophage Typing

A regional bacteriophage scheme [17] and a Romanian additional one [18] were used to identify the phage type of strains included in this study. The tests were performed with phages at routine testing dilution (RTD) and 100X RTD on nutrient agar (DIFCO) using a 1 hour broth culture. After 4 hours of incubation at 37°C, the results were recorded as confluent lysis (CL), semi-confluent lysis (SCL), and/or number of phage units and reported as phage type and/or non typable strains.

DNA Amplification

PCR was performed on crude lysate obtained from an overnight broth culture. Based on the published sequence of different subtractive genomic regions identified to be present only in virulent strains [19], primers were designed and amplicons were named SL002, SL003, SL019, SL020, Sl021 and SL025 (Table **3**). Ferric uptake regulation gene (*fur)* was also targeted using a pair of primers designed according to the published sequence [20].

The reactions were performed in a volume of 50 µl containing 200µM dNTP mix, 30 pmol of each primer, and 1.5U of Taq polymerase. Amplifications were performed in a

Table 3. Primers Used in PCR to Detect the Virulence Profiles of *Klebsiella pneumoniae* Strains

Sequence Targeted	Access Number	Reference	Primers	Nucleotide Position	Author
fur	L23871	Achenbach, LA *et al*	5'-tagcaacaggacagattccg-3' 5'-tatttttccaccgcgtcgtg-3'	400-419 852-871	This study
SL 002	AJ 276464	Lai, YC *et al*	5'-ctggacgatggataacgactc-3' 5'-acagaacctcatcacattgtcc-3'	3-23 423-444	This study
SL 003	AJ 276465	Lai, YC *et al*	5'-taaggagttttatggtgctgac-3' 5'-tcactcctgaggttatgtatc-3'	9-30 429-449	This study
SL019	AJ 276851	Lai, YC *et al*	5'-acgttacctgagcggtgttg-3' 5'-ccgcagataaaaaaacaagcga-3'	2-21 422-443	This study
SL020	AJ 276852	Lai, YC *et al*	5'-acctcgattcctgctctgaca-3' 5'-agaaggtgaaggcgatttaagc-3'	1-22 410-431	This study
SL 021	AJ 276853	Lai, YC *et al*	5'-tgcaacctgcatcagttgaag-3' 5'-tcatgcaaccaaaagtgctcg-3'	03-23 338-358	This study
SL 025	AJ 276854	Lai, YC *et al*	5'-cctgctgctatcttaagctc-3' 5'-agggatctactgatacatacc-3'	9-28 430-450	This study

My Cycler machine (Bio-Rad Laboratories, Hercules, CA, USA) in the following conditions: 4 min at 94°C for initial denaturation followed by 30 cycles of 1min at 94°C, 30 sec at 55°C for *fur*, SL 003 and SL 025, 57°C for SL 019 and SL 021, and 59°C for SL 003 and SL 020, 1 min at 72°C. A termination cycle of 5 min at 72°C was performed for each PCR.

The amplification products were electrophoresed on 2% (W/V) agarose (Certified Molecular Biology Agarose, Bio-Rad) in TBE 1X using 100 bp as molecular marker.

Molecular Typing

Pulse field gel electrophoresis (PFGE) was performed according to the standard protocol designed by Centre for Disease Control and Prevention Atlanta, and recommended within the PulseNet EUROPE Project, for Enterobacteria. The *Xba*I-restricted chromosomal DNA fragments were resolved in a 1% SeaKem Gold Agarose (Lonza Group Ltd. Switzerland) using a CHEF-Mapper system (Bio-Rad). For each gel the well-characterized strain *Salmonella* Braenderup H9812 was included as both procedure control and molecular size marker.

The electrophoretic conditions used were as follows: initial switch time, 2.16 s, final switch time 54.17 s, run time 20 h, angle 120°, gradient 6.0 V/cm, temperature 14°C, and ramping factor linear. The gel image, acquired by a CCD camera (Vilber Lourmat dark room) was analyzed using Dice similarity coefficient, UPGMA (unweighted pair group arithmetic average) algorithm and FingerPrinting II software (BioRad).

RESULTS

The Susceptibility to the Antibiotics Versus the Origin of Strains

A set of 12 antibiotics usually prescribed for *Klebsiella* infections was used to detect the presence of resistant/multiresistant strains circulating in hospitals and suspected to be at the origin of hospital associated infections. A total of 17 patterns of resistance were identified among all the 83 strains analyzed (Table **4**).

The patterns were denominated as R1-R16 and S, the last one comprising the strains susceptible to all the 12 antibiotics used. The types R1-R4 and R6-R8 were represented by strains resistant to the third generation cephalosporins. Pattern R2 was the most prevalent, 35 strains presenting this profile of resistance, and 28 of them being isolated from new-born babies. A number of 17 strains were susceptible to all antibiotics tested, 12 being isolated from adults. The other patterns were represented by fewer strains and were isolated from babies, adults and/or hospital environment. A number of 5 profiles of resistance were identified among the H1-1 strains (Fig. **1A**), R2 being represented by 3 strains, two of them isolated from new-born babies and one from a device associated to the babies care. When we analyzed the strains H1-2 (Fig. **1B**), we found that 26 of 42 strains showed the R2 type. The R2 type was also prevalent within the lot H3 of strains (Fig. **2A**).

The four strains isolated from blood specimens of babies, included in the lot H2-2, were susceptible to the all antibiotics used (Fig. **3B**), but the strains H2-1 were distributed among 7 patterns of resistance, only 2 of them being resistant to cephalosporins of third generation (Fig. **3A**).

When the double disc test was performed, a number of 29 strains were detected as ESBL producers. R2 type of resistance was displayed by 24 ESBL positive strains, and the rest were distributed between 4 resistotypes (R1=1, R3=2, R6=1 and R7=1). A number of 21 ESBL positive strains were isolated in unit H1 while no strain was ESBL positive in unit H2 (Table **5**).

Association Between Phage Type and Origin of Strains

By using the two schemes of phage typing of *Klebsiella pneumoniae* strains a correlation between phage type and the involvement of strains in nosocomial infection was detected. The 8 strains of lot H1-1 presented different profiles of sensitivity to the specific phages, being dispersed among types IIA1, IIA2 and non-typable strains. Within the same hospital unit, strains isolated later from new-born babies and designated as lot H1-2, were all identified as IB1 phage type, while those isolated from adults were identified as different

Table 4. Resistotypes Identified Among the *Klebsiella pneumoniae* Strains

No. of Resistotypes	Denomination	Pattern of Resistance	No. of Strains	%
1	R1	AMC, CAZ, CTX, CEC, CN, TOB, C, NA	1	1.21
2	R2	AMC, CAZ, CTX, CEC, CN, TOB, C	35	42.17
3	R3	AMC, CAZ, CTX, CRO, CEC, CN, TOB	1	1.21
4	R4	AMC, CAZ, CTX, CEC, CN, TOB	2	2.40
5	R5	CN, TOB, C, NA, CIP, OFX	1	1.21
6	R6	AMC, CAZ, CTX, CEC, CN	2	2.40
7	R7	AMC, CAZ, CTX, CEC, C	1	1.21
8	R8	AMC, CAZ, CTX, CEC	1	1.21
9	R9	AMC, CN, TOB, C	3	3.61
10	R10	AMC, C, NA	1	1.21
11	R11	AMC, CN, TOB	7	8.43
12	R12	CN, TOB, C	3	3.61
13	R13	AMC, CN	4	4.82
14	R14	C, NA	1	1.21
15	R15	TOB, C	1	1.21
16	R16	AMC	2	2.40
17	S	Susceptible to all 12 antibiotics	17	20.48
Total			83	100

phage types. Strains isolated from surfaces of devices associated with the ambient of new-born babies were also identified as IB1 phage type. All the strains isolated from new-born babies in hospital 2, designated as lot H2-1, were identified to be IA1 phage type while the strains isolated from accompanying mothers or hospital care personnel belonged to other phage types. Strains belonging to the lot H2-2 were all non-typable. Strains grouped in the control lots (H3, H4 and H5) showed various profiles of susceptibility to the bacteriophages.

Clonal Analysis

After *Xba*I restriction of genomic DNA and fragments separation by PFGE, the strains H1-1 showed great divergence, the genetic distance between them being up to 50%. Three strains, two from new-born babies and one from an environmental device associated to the babies care seemed to be clonal (Fig. **1A**).

All the strains H1-2 also showed up to 44% divergence, but the strains isolated from new-born babies and their environment were closely related compared with the strains isolated from accompanying mothers and hospital care personnel (Fig. **1B**).

The analysis of the strains H2-1 revealed that strains from children were closely related (97% identity) but isolates from accompanying mothers and hospital care personnel were divergent (Fig. **3A**).

As expected, strains from hospital units H3 and H4 were unrelated, each strain showing a different PFGE pattern, only two strains in H3 being identical (Fig. **2**).

Genetic Profile Associated to the Strains Virulence

The PCR screening for the presence of the selected seven genomic regions revealed a number of 13 genetic patterns of amplification, noted from GP1 to GP13 (Table **6**).

The most frequent genetic profile was GP1 (n=54) with two amplified regions, SL 020 and SL 019. These two subtractive regions were the most prevalent ones. They were identified in 81 and 74 strains respectively. The pattern GP8 was the second as frequency (n=10) strains included in this group carrying SL 020, SL 019 and SL 003 regions. The region named SL 003 was identified as the third subtractive genetic region as prevalence (19 strains). The genetic region SL 021 was detected only in one strain while gene *fur*, coding for regulation of ferric uptake, was present in all the analyzed strains.

The isolates originating in H1-1 were all GP1, except the strain isolated from the hospital environment which showed the genetic pattern 3. All the H1-2 strains isolated from new-born babies were included in GP1, while the strains isolated from accompanying personnel, care personnel and hospital environment were distributed among patterns GP1, GP2, GP4, GP5 and GP7. H2-1 strains displayed 7 genetic patterns, but the strains isolated from babies were shared by GP1 and GP8 as the strains H2-2. Isolates from H3 and H4 were included in GP1 and GP8 patterns and also in others, less frequently encountered, GP9, GP10, GP13.

DISCUSSION

Klebsiella is a well known cause of community-acquired bacterial pneumonia, but the great majority of infections are

Table 5. Relationship Between Antibiotic Susceptibility and Origin of Strains

Lot Definition	Origin of Strains	Resistotype	No. of Strains	No. of Strains ESBL Positive
H1-1	New-born babies	R2	2	2
		S	1	
		R10	1	
		R14	1	
		R6	1	
	Adults	S	1	
	Hospital environment	R2	1	1
H1-2	New-born babies	R2	23	17
		R12	3	
		R15	1	
	Adults	S	8	
		R2	2	
		R5	1	
	Hospital environment	R9	3	
		R1	1	1
H2-1	New-born babies	R11	6	
		R13	3	
		R4	1	
	Adults	S	2	
		R13	1	
		R16	2	
		R8	1	
H2-2	New-born babies	S	4	
H3	New-born babies	R2	4	3
		R7	1	1
	Hospital environment	R3	1	1
H4	New-born babies	R3	1	1
		R6	1	1
	Adults	S	1	
		R2	2	
		R11	1	
H5	New-born babies	R2	1	1
Total			83	29

between two (n=3) and five virulence genes (n= 5). Our results are in agreement with those suggesting that pathogenic *Klebsiella pneumoniae* population is highly heterogeneous and that none of the analyzed sequences are present in all clinical isolates and none of bacterial strains carries all these sequences. Most of the strains carrying SL 020 and SL 019 (pattern designated PG1) were isolated from new-born babies and this virulence pattern was correlated with other epidemiological markers (phage type and PFGE type) when the strains were suspected to be hospital-associated.

CONCLUSION

Taking in to consideration that *Klebsiella* spp. is a heterogeneous microorganism, selective and specific methods are necessary to define the strains responsible or not for infections. Phage typing method associated with PFGE typing and genetic profile for virulence indicated the occurrence of hospital associated-infections produced by *Klebsiella pneumoniae* strains. Moreover, the results reveal that virulence pattern could be used as a molecular marker for differentiating strains involved in infectious processes.

Table 6. Virulence Pattern of *Klebsiella pneumoniae* Strains

Pattern Designation	Sequences Amplified							Number of Strains
	fur	SL002	SL003	SL019	SL020	SL021	SL025	
GP1	+	-	-	+	+	-	-	54
GP2	+	-	-	+	+	-	+	1
GP3	+	-	-	+	-	-	-	1
GP4	+	-	-	-	+	-	-	2
GP5	+	-	+	-	+	-	-	3
GP6	+	-	+	+	-	-	-	1
GP7	+	-	+	+	+	-	+	2
GP8	+	-	+	+	+	-	-	10
GP9	+	+	-	-	+	-	-	3
GP10	+	+	-	+	+	-	-	2
GP11	+	+	-	+	+	+	-	1
GP12	+	+	+	-	+	-	-	1
GP13	+	+	+	+	+	-	-	2
Positive strains	83	9	19	74	81	1	3	83

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