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Multilocus Sequence Typing of *Klebsiella pneumoniae* Nosocomial Isolates

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A multilocus sequence typing (MLST) scheme was developed for *Klebsiella pneumoniae*. Sequences of seven housekeeping genes were obtained for 67 *K. pneumoniae* strains, including 19 ceftazidime- and ciprofloxacin-resistant isolates. Forty distinct allelic profiles were identified. MLST data were validated against ribotyping and showed high (96%) discriminatory power. The MLST approach provides unambiguous data useful for the epidemiology of *K. pneumoniae* isolates.

Klebsiella pneumoniae is an opportunistic pathogen responsible for an important proportion (4 to 8%) of nosocomial infections (14, 24). *K. pneumoniae* isolates are increasingly resistant to multiple antimicrobial agents, including quinolones (5, 23), and, owing to the production of extended-spectrum beta-lactamases (ESBL), extended-spectrum cephalosporins such as ceftazidime (22). Hospital outbreaks of *K. pneumoniae* isolates are frequent, and the interhospital dissemination of resistant strains has been described previously (1, 20).

Phenotypic methods developed for typing *Klebsiella* isolates include phage typing, bacteriocin typing, and serotyping (14). Serotyping of the capsular antigen is valuable for long-term epidemiology and reference typing (4, 16) but may not reflect accurately the genetic relationships among isolates. Multilocus enzyme electrophoresis has never been validated for the epidemiology of *Klebsiella* strains, although the diversity of some housekeeping enzymes has been demonstrated (10, 11, 21).

Molecular methods for characterization of *K. pneumoniae* isolates used for epidemiological purposes include randomly amplified polymorphic DNA (7), pulsed-field gel electrophoresis (1), and amplified fragment length polymorphism (18, 27). However, these methods are mostly used for outbreak investigation at the local level, as their interlaboratory reproducibility is difficult to achieve and as they do not generate highly informative and unambiguous data. Ribotyping is highly reproducible, especially in its automated implementation (3), and was shown to be highly discriminatory in *Klebsiella* when using EcoRI (1, 7). However, the interpretation of banding pattern variation has both practical and theoretical limitations (15).

Multilocus sequence typing (MLST) is a nucleotide sequence-based method that is adequate for characterizing the genetic relationships among bacterial isolates (12, 13, 19). It provides unambiguous and portable data that allow the implementation of multiuser international databases (17). We report the development of an MLST scheme for *K. pneumoniae* and its evaluation for characterization of nosocomial isolates.

Sixty-seven *K. pneumoniae* isolates were included. First, 39 clinical isolates that had been collected during the SENTRY Antimicrobial Surveillance Program (5) were selected randomly from different European hospitals and clinical sources. This set of isolates with diverse origins was intended to estimate the discrimination of MLST among strains with no documented epidemiological links. Second, we included 19 additional European isolates that were both ciprofloxacin-resistant (MIC $>$ 2 mg/liter) and ceftazidime-resistant (MIC $>$ 64 mg/liter) (5) and 6 isolates collected during an epidemic of ESBL-producing strains on the Caribbean island Curaçao (28). These 26 strains were included in order to estimate the diversity of genetic backgrounds among multiresistant *K. pneumoniae* strains. MICs of ceftazidime and ciprofloxacin were taken from a previous study (5). Six epidemic clusters of isolates (clusters A to F in Fig. 1) were suspected when considering the source hospital and the profile of resistance to ciprofloxacin and ceftazidime. Type strain ATCC 13883^T and the genome reference strain MGH78578 were included for comparison. The identification of all strains as *K. pneumoniae* was confirmed by *rpoB* and/or *gyrA* gene sequences (2, 6, 7).

Primer pairs were designed for PCR amplification and sequencing of internal portions of seven housekeeping genes (Table 1). Genes were selected (i) to be located far apart on the chromosome (Table 1), (ii) based on availability of PCR primers (Table 1), or (iii) for *tonB*, based on known nucleotide variation (GenBank/EMBL accession numbers AY016749 to AY016767). Other candidate genes were eliminated for technical reasons or to avoid risk of selective bias due to the use of antimicrobial agents (Table 1). Nucleotide sequences were obtained using Big Dye version 3.1 chemistry on an ABI 3700 apparatus. In order to eliminate the risk of sample mix-up, PCR and sequencing were performed using a molecular biology robot (RoboAmp 4200-PE; MWG Biotech, Courtaboeuf, France). Sequence chromatograms were edited and stored using BioNumerics version 4.01 (Applied-Maths, St. Maartens-Latem, Belgium). All nucleotides were supported by at least two sequence chromatograms. A different allele number was

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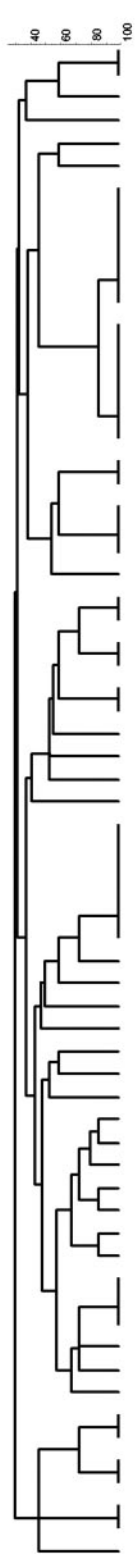

rpoB	gapA	mdh	pgi	phoE	infB	tonB	Ribotype pattern	Strain ID	Original Name	ST	RT	Country	Isolated from	CAZ	CIP	Cluster
4	4	1	1	7	4	10		SB130	18C048	1	101	Spain	RTI	0.5	16	
4	4	1	1	7	4	10		SB152	18C100	1	101	Spain	RTI	64	> 16	
4	3	1	1	9	4	17		SB210	06E037	2	111	France	UTI	> 64	2	A
6	5	1	1	9	5	11		SB132	ATCC 13883T	3	36	Unknown	Type strain	nd	nd	
5	4	1	1	1	1	6		SB1109	05D002	8	83	France	Soft tissue	0.12	0.015	
2	4	3	1	1	1	8		SB124	11E006	9	66	Italy	UTI	0.25	0.03	
1	1	1	1	1	6	1		SB2390	15505	14	77	Curacao	UTI	nd	nd	F
1	1	1	1	1	6	1		SB2397	24339	14	77	Curacao	Feces	nd	nd	F
1	1	1	1	1	6	1		SB2399	24911	14	77	Curacao	RTI	nd	nd	F
1	1	1	1	1	6	1		SB2407	35389	14	77	Curacao	UTI	nd	nd	F
1	1	1	1	1	6	1		SB2410	35850	14	77	Curacao	RTI	nd	nd	F
1	1	1	1	1	6	1		SB41	11A094	14	77	Italy	Blood	> 64	0.06	F
1	1	1	1	1	1	1		SB10	01A018	15	38	Austria	Blood	0.5	0.06	
1	1	1	1	1	1	1		SB1111	15E050	15	38	Portugal	UTI	> 64	0.5	
1	1	1	1	1	1	1		SB113	06E046	15	38	France	UTI	> 64	2	A
1	1	1	1	1	1	1		SB126	13D044	15	98	Poland	UTI	0.5	2	
1	1	1	1	1	1	1		SB20	04A025	15	38	France	Blood	0.06	0.06	
1	1	1	1	1	1	1		SB209	06A395	15	38	France	Blood	64	2	A
3	3	1	1	3	1	1		SB1103	18E092	4	63	Spain	UTI	0.5	0.03	
3	3	1	1	3	1	1		SB129	14E044	4	63	Poland	UTI	0.25	2	
3	2	1	1	3	2	3		SB108	01A114	5	6	Austria	Blood	0.06	0.03	
3	2	1	1	3	2	3		SB139	10A160	5	6	Italy	Blood	1	4	
3	2	1	1	3	2	3		SB93	14A010	5	41	Poland	Blood	64	1	
3	2	1	1	14	10	20		SB62	19A035	6	67	Switzerland	Blood	0.5	0.06	
1	2	2	1	10	1	19		SB52	16A111	35	23	Spain	Blood	> 64	0.03	D
1	2	2	1	10	1	19		SB54	16A175	35	23	Spain	Blood	> 64	0.25	D
1	2	2	1	7	1	7		SB118	09D053	36	60	Greece	UTI	> 64	4	B
1	2	2	1	7	1	7		SB138	09D080	36	60	Greece	Soft tissue	> 64	4	B
1	2	2	1	13	9	16		SB208	07A471	37	127	Germany	Blood	1	4	
1	2	2	1	13	9	16		SB4	11A023	37	127	Italy	Blood	0.12	0.03	
2	2	2	1	2	1	2		SB107	MGH 78578	38	8	Boston,MA	Ref. strain	nd	nd	
1	2	2	4	9	1	14		SB151	18A365	39	9	Spain	Blood	0.25	2	
1	2	10	1	4	1	21		SB65	20A188	40	113	UK	Blood	0.5	0.12	
1	4	7	1	9	1	12		SB28	06A093	10	123	France	Blood	<= 0.03	0.015	
4	2	2	2	6	3	4		SB112	05A096	29	30	France	Blood	0.25	0.03	
4	2	2	2	6	3	4		SB114	09A098	29	93	Greece	Blood	> 64	8	B
4	2	2	2	6	3	4		SB115	09A199	29	28	Greece	Blood	> 64	2	B
4	2	2	2	6	3	4		SB142	09A317	29	28	Greece	Blood	> 64	2	B
4	2	2	2	6	3	4		SB143	09A256	29	28	Greece	Blood	> 64	2	B
4	2	2	2	6	3	4		SB88	17A092	29	50	Spain	Blood	4	0.03	
4	2	9	1	6	3	4		SB592	09A434	31	28	Greece	Blood	> 64	1	
4	2	2	1	5	3	5		SB1102	08C003	32	84	Germany	RTI	> 64	0.03	
4	2	5	1	12	3	9		SB16	03A085	33	130	France	Blood	<= 0.03	0.03	
7	2	6	1	9	3	4		SB17	03A097	34	40	France	Blood	0.5	0.06	
8	2	1	1	12	7	4		SB19	04A022	41	104	France	Blood	0.06	0.03	
1	6	1	1	12	3	4		SB5	15A006	12	124	Portugal	Blood	0.12	0.03	
1	2	1	1	10	3	19		SB2396	24329	13	782	Curacao	Feces	nd	nd	F
4	2	2	1	4	1	4		SB1091	04C047	16	135	France	RTI	0.5	0.06	
4	2	1	1	4	1	4		SB29	07A023	17	120	Germany	Blood	0.12	0.03	
1	2	1	1	4	1	4		SB164	09A323	18	88	Greece	Blood	> 64	2	B
4	2	8	1	4	3	4		SB3	08A028	19	119	Germany	Blood	0.06	0.015	
4	2	1	1	4	3	4		SB32	08A082	20	35	Germany	Blood	0.25	0.5	
4	2	1	1	1	8	4		SB207	09A425	21	115	Greece	Blood	> 64	2	B
4	2	1	1	1	3	4		SB21	04A041	22	249	France	Blood	0.06	0.03	
4	2	1	1	9	1	12		SB14	02A029	23	114	Belgium	Blood	0.25	0.06	
4	2	1	1	9	1	12		SB42	12A041	23	855	Netherlands	Blood	0.25	0.03	
4	2	1	1	9	1	12		SB53	16A151	23	114	Spain	Blood	0.25	0.03	
4	2	1	1	3	1	2		SB12	01A067	24	39	Austria	Blood	0.06	0.03	
4	2	1	1	10	1	13		SB148	14A069	25	85	Poland	Blood	32	2	C
1	2	1	1	9	1	18		SB235	08A418	26	1	Germany	Blood	0.5	0.06	
1	2	1	3	8	6	15		SB154	22E041	42	99	Turkey	UTI	> 64	> 16	E
1	2	1	3	8	6	15		SB204	20A292	42	100	UK	Blood	> 64	> 16	
1	2	1	5	11	6	15		SB153	22A027	43	31	Turkey	Blood	> 64	> 16	E
1	2	1	5	11	6	15		SB162	24A013	43	31	Turkey	Blood	> 64	> 16	
1	2	4	3	8	1	9		SB128	14E015	44	96	Poland	UTI	> 64	8	C
1	2	4	3	8	1	9		SB149	14A076	44	96	Poland	Blood	> 64	4	C
1	2	1	6	7	1	12		SB22	05A012	45	61	France	Blood	0.12	0.12	

FIG. 1. Allelic profiles, ribotype patterns, sequence type (ST), ribotype (RT), source information, and MIC of ceftazidime (CAZ) and ciprofloxacin (CIP) of the 67 *K. pneumoniae* strains. The two first letters of the original strain name correspond to the code of the source medical center. Clinical sources were blood, respiratory tract (RTI), urinary tract (UTI), and soft tissue infections. Epidemiological clusters initially suspected on the basis of the medical center and susceptibility to CAZ (resistance breakpoint, 64 mg/liter) and to CIP (resistance breakpoint, 2 mg/liter) are indicated in the last column. All clinical strains were collected between 1997 and 2001. The UPGMA dendrogram was built from the pairwise distances between allelic profiles using BioNumerics.

TABLE 1. Gene loci included in the *Klebsiella pneumoniae* MLST scheme, PCR and sequencing primers, and variation indices for 67 strains[a]

Locus	Putative function of gene	Primer sequence[b,c]	Size (bp)	Location[d]	Temp (°C)	No. of alleles	Nucleotide diversity	Polymorphic sites (nonsynonymous substitutions)
rpoB	Beta-subunit of RNA polymerase B	VIC3: GGC GAA ATG GCW GAG AAC CA VIC2: GAG TCT TCG AAG TTG TAA CC	501	4,771,502–4,772,002	50	8	0.00166	7 (1)
gapA	Glyceraldehyde 3-phosphate dehydrogenase	gapA173: TGA AAT ATG ACT CCA CTC ACG G gapA181: CTT CAG AAG CGG CTT TGA TGG CTT	450	1,347,540–1,347,091	60	6	0.00136	5 (0)
mdh	Malate dehydrogenase	mdh130: CCC AAC TCG CTT CAG GTT CAG mdh867: CCG TTT TTC CCC AGC AGC AG	477	4,004,045–4,003,569	50	10	0.00161	10 (3)
pgi	Phosphoglucose isomerase	pgi1F: GAG AAA AAC CTG CCT GTA CTG CTG GC pgi1R: CGC GCC ACG CTT TAT AGC GGT TAA T pgi2F(seq): CTG CTG GCG CTG ATC GGC AT pgi2R(seq): TTA TAG CGG TTA ATC AGG CCG T	432	4,831,091–4,831,522	50	6	0.00092	5 (0)
phoE	Phosphoporine E	phoE604.1: ACC TAC CGC AAC ACC GAC TTC TTC GG phoE604.2: TGA TCA GAA CTG GTA GGT GAT	420	320,309–320,728	50	14	0.00727	18 (2)
infB	Translation initiation factor 2	infB1F: CTC GCT GCT GGA CTA TAT TCG infB1R: CGC TTT CAG CTC AAG AAC TTC infB2F(seq): ACT AAG GTT GCC TCC GGC GAA GC	318	3,937,568–3,937,885	50	10	0.0038	11 (0)
tonB	Periplasmic energy transducer	tonB1F: CTT TAT ACC TCG GTA CAT CAG GTT tonB2R: ATT CGC CGG CTG RGC RGA GAG	414	2,394,251–2,394,664	45	21	0.01019	14 (5)

[a] Genes *aroA, tyrB*, and *ureD* were also amplified and sequenced successfully but were not included due to lower sequence quality in general. Genes *gyrA* and *parC* used previously (7) were excluded as amino acid changes may be selected by the clinical use of quinolones.

[b] Sequencing primers were the same as the PCR primers, except when indicated.

[c] Primer sources were as follows: for *mdh*, reference 25; for *phoE*, reference 9; for pgi1F and pgi1R, reference 29; gapA173, reference 8; for *rpoB*, E. Ageron and P. Grimont, unpublished. All other primers were designed in this study.

[d] Based on the complete sequence of strain MGH78578 available at http://genome.wustl.edu/projects/bacterial/kpneumoniae/.

given to each distinct sequence within a locus, and a distinct sequence type (ST) number was attributed to each distinct combination of alleles. Allele sequences and STs are available on the public MLST web site at http://pubmlst.org/kpneumoniae. Nucleotide diversity was calculated using DNAsp version 4 (26).

All seven genes could be PCR amplified for all isolates tested. Nucleotide variation was observed at all genes, with 6 to 21 distinct alleles (Table 1), theoretically allowing more than eight million STs to be distinguished. The seven alleles obtained for genome reference strain MGH78578 were totally identical to the genome sequence at http://genome.wustl.edu. Gene *tonB* was particular in that two to four codons were deleted in a small number of strains (positions 64 to 69 in strains SB93, SB108, and SB139, positions 83 to 88 in SB1102, and positions 167 to 178 in strain ATCC13883^T). The number of variable sites per locus ranged from 5 to 18. The average nucleotide diversity (average number of nucleotide differences per site) was 0.0038. Nonsynonymous substitutions were rare (Table 1), indicating selection against amino acid changes and excluding strong selection bias on the observed allelic diversity, as is typically observed for housekeeping genes.

By combining the seven gene loci, 40 distinct sequence types (STs) were identified. Most groups of strains sharing the same ST belonged to suspected epidemiological clusters (Fig. 1). eBURST analysis (http://eburst.mlst.net/) revealed the existence of two clonal complexes, one including ST14 and ST15, the other including ST16 to -22. When considering only the isolates with no documented epidemiological link, the discriminatory index (Simpson index) was 96%. Therefore, MLST will discriminate most epidemiologically unrelated strains.

In order to further validate the ability of MLST for *K. pneumoniae* strain characterization, all 67 isolates were analyzed by ribotyping, which is known to be highly discriminatory in this species (1, 7). A total of 46 ribotypes were distinguished. Four STs (ST5, ST15, ST23, and ST42) were subdivided into two ribotype profiles (Fig. 1), whereas ST29 was subdivided into four ribotypes. Importantly, all distinct ribotypes observed within a ST were very similar (Fig. 1), probably reflecting evolution from a common ancestor. In addition, ribotype variation within an ST was consistent with geographic origin and antibiotic resistance data (Fig. 1). Simpson's index, calculated for ribotyping data on the set of isolates with no documented epidemiological link, was 98%.

When considering the 19 ceftazidime- and ciprofloxacin-resistant isolates, 11 STs (13 ribotypes) were distinguished. These STs differed among themselves by at least two genes and were distributed across the entire breadth of diversity (Fig. 1), clearly demonstrating that resistance is not restricted to a few genetic backgrounds and that it is a problem of multiple emergence rather than one of interhospital spread of a few clones. This is in agreement with the common view that ESBL plasmids are easily transferred among *K. pneumoniae* strains and that quinolone resistance can emerge during therapy.

Inspection of the suspected epidemiological clusters in the light of MLST and ribotyping data revealed that all clusters but one (cluster D) proved to be composed of at least two genotypes. In the case of cluster B, four STs were distinguished, with two groups of isolates, plus two single isolates. Thus, MLST will be useful to sort out which cases are caused by clonal spread and which are not. Importantly, the distinct STs within a cluster differed by at least two loci and also showed very distinct ribotype patterns, which excludes ST or ribotype variation being the result of microevolution from a common index case of infection.

Seven STs were observed in distinct countries (Fig. 1). Among these, ST14 corresponded to Curaçao ESBL-producing isolates and a ceftazidime-resistant Italian isolate and ST15 corresponded to ceftazidime-resistant isolates in France, Poland, and Portugal. Both STs may represent resistant *K. pneumoniae* clones that have spread across countries, possibly mediated by the transfer of hospitalized patients. Alternately, they could result from the independent acquisition of resistance by susceptible genotypes that were initially widespread, as suggested by the finding in different countries of susceptible isolates of ST4, ST5, ST23, and ST37 (Fig. 1).

Combined with precise epidemiological information and the characterization of antibiotic resistance mechanisms, MLST analysis of larger sample sets should provide a much improved understanding of the evolutionary origin and dissemination of *K. pneumoniae* multiresistant strains.

Nucleotide sequence accession numbers. Sequences were submitted to EMBL under the numbers AJ890378 to AJ890431 and AJ890476 to AJ890496.

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