ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2011, p. 5493–5499 Vol. 55, No. 12
0066-4804/11/$12.00 doi:10.1128/AAC.05118-11
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Molecular Characteristics of KPC-Producing *Enterobacteriaceae* at the Early Stage of Their Dissemination in Poland, 2008-2009▽†

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Received 17 June 2011/Returned for modification 31 July 2011/Accepted 4 September 2011

After the first report in May 2008, the National Reference Center for Susceptibility Testing confirmed 113 cases of infection or colonization by KPC-producing members of the family *Enterobacteriaceae* in Poland by the end of 2009. The vast majority of patients were found in 18 hospitals; three patients were diagnosed at outpatient clinics. Most of the institutions were in the Warsaw area, including three hospitals with the highest numbers of cases. When available, the data on previous hospitalizations often indicated that these hospitals were the probable acquisition sites; one patient arrived from New York. The group of 119 unique isolates consisted of *Klebsiella pneumoniae* ($n = 114$), followed by *Klebsiella oxytoca* ($n = 3$), and *Escherichia coli* ($n = 2$). The *K. pneumoniae* isolates were dominated by the clone sequence type 258 (ST258) ($n = 111$); others were ST11 and ST23. The ST258 group was heterogeneous, with 28 pulsed-field gel electrophoresis (PFGE) subtypes, ~25 plasmid profiles, and nine β-lactamase patterns differing by KPC variants (KPC-2 mainly), and SHV-12, CTX-M-3, and TEM-1-like enzymes. Plasmids carrying *bla*$_{KPC}$ genes varied in size (~48 to 250 kb), structure, and conjugation potential. Transferable IncFII$_K$ plasmids of ~110 to 160 kb, probably pKpQIL or its derivatives, were observed in all *K. pneumoniae* clones and in *K. oxytoca*. Also prevalent were nontypeable pETKp50-like plasmids of ~50 kb, found in *K. pneumoniae* ST258 and *E. coli* isolates (ST93 and ST224). Two *K. pneumoniae-E. coli* pairs from single patients might represent the *in vivo* transfer of such plasmids. The striking diversity of KPC producers at the early stage of dissemination could result from several introductions of these bacteria into the country, their multidirectional evolution during clonal spread, and transfer of the plasmids.

Resistance to carbapenems in members of the family *Enterobacteriaceae* has become a matter of the highest concern in recent years (6, 37). It has been associated largely with various carbapenem-hydrolyzing β-lactamases, including KPC-like enzymes, which hydrolyze all β-lactams that are in use (28, 32). Of several variants identified since the first report (43), KPC-2 and KPC-3 have been the most frequent types, and *Klebsiella pneumoniae* has been the main producer species (28, 32). *bla*$_{KPC}$ genes are located on plasmids with different replicon types (e.g., IncFII, IncL/M, IncN, IncR, and ColE1), size (~12 to ~180 kb), conjugation ability, and resistance repertoire (1, 10, 16, 18, 20, 24, 25, 27). The direct context of *bla*$_{KPC}$ is formed by Tn*4401*-like transposons of some structural diversity (10, 22, 23, 27, 29). KPC producers vary in their level of resistance to β-lactams which may increase owing to modifications in *bla*$_{KPC}$ promoters, *bla*$_{KPC}$ duplications, acquisition of other β-lactamase genes, or porin alterations (10, 16, 22, 27, 43). The presence of non-β-lactam resistance mechanisms and the fact that bacteria commonly possess these mechanisms means that KPC producers are often susceptible to few drugs, e.g., gentamicin, amikacin, and/or colistin (32).

Since the late 1990s, KPC-producing organisms have spread in the United States (35), while since 2005, their expansion has been observed in Israel (37, 38) and worldwide (32). Although KPCs have been found in numerous *K. pneumoniae* clones and in other species (1, 9, 10, 18, 23, 25, 38), this spread has been strongly associated with *K. pneumoniae* sequence type 258 (ST258) and related clones, like ST11 (1, 3, 23, 25, 34, 36). Identified in many countries and continents, this hyperepidemic clonal complex carries either *bla*$_{KPC-2}$ or *bla*$_{KPC-3}$ on different plasmids, such as the ~110-kb IncFII$_K$ pKpQIL, identified and fully sequenced in Israel (25).

The aim of this study was to analyze the dissemination of KPC producers in Poland, following the first report in May

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▽ Published ahead of print on 19 September 2011.

2008 (3). Isolates identified in 2008-2009 were subjected to typing and plasmid and β-lactamase analyses.

MATERIALS AND METHODS

Clinical isolates. A total of 119 KPC-producing *Klebsiella pneumoniae* (n = 114), *Klebsiella oxytoca* (n = 3), and *Escherichia coli* (n = 2) isolates were collected in 2008 and 2009 (n = 33 and 86, respectively) by the National Reference Center for Susceptibility Testing (NRCST) as a part of its surveillance activity (Table 1). Most of these isolates (n = 115) were from patients in 18 hospitals in Poland, including 12 hospitals in the Warsaw area (Fig. 1) that were tertiary care teaching, tertiary care specialist, secondary care regional, or primary care local institutions. One isolate was cultured from a nursing home resident upon her admission to hospital 11 (h11) (in Warsaw suburbs), and three others were from outpatients (two in Warsaw). The isolates originated from 113 patients; in some cases, the isolates from one patient gave different species (two pairs of *K. pneumoniae* and *E. coli*) or typing results, which were included in the study. Ten hospitals sent 1 or 2 organisms each, while others had multiple KPC isolations, including h3, h4, and h5 with 19, 24, and 23 isolates, respectively. Many isolates were from infections, namely, urinary tract (urine samples; n = 61), invasive (blood or peritoneal fluid samples; n = 22), respiratory tract (bronchoalveolar lavage samples; n = 8), or skin and soft tissue (n = 6) infections. The other isolates represented probable cases of colonization of gastrointestinal (stool samples or rectal swabs; n = 17) or respiratory (sputum samples or nasopharyngeal swabs; n = 5) tracts. Species were identified with the Vitek 2 system (bioMérieux, Marcy l'Etoile, France).

KPC detection and genetic context of bla_{KPC} genes. The detection of KPC was assessed by the disk test with phenylboronic acid (12) and confirmed by PCR (30). bla_{KPC} amplicons were cut with RsaI (Fermentas, Vilnius, Lithuania) to distinguish between bla_{KPC-2}- and bla_{KPC-3}-like genes (26) and directly sequenced for selected isolates. The location of bla_{KPC} within Tn*4401*-like elements was analyzed by PCR mapping as previously proposed (29).

Identification of other β-lactamases. β-Lactamase profiling was performed by isoelectric focusing (5). Genes coding for CTX-M-1- and SHV-like extended-spectrum β-lactamases (ESBLs) (bla_{CTX-M} and bla_{SHV}, respectively), and TEM-type enzymes (bla_{TEM}) were amplified and sequenced as described previously (2, 13).

Typing. Pulsed-field gel electrophoresis (PFGE) was performed as reported previously (39), using the XbaI enzyme (Fermentas); DNA patterns were analyzed visually by using the criteria of Tenover et al. (40). *K. pneumoniae* and *E. coli* isolates were subjected to multilocus sequence typing (MLST) as described previously (11, 42); the *Klebsiella pneumoniae* MLST database (www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) and the *Escherichia coli* MLST Database (http://mlst.ucc.ie/mlst/dbs/Ecoli) were used for assigning sequence types (STs).

Plasmid analysis. Plasmid profiling was carried out by PFGE of total DNA from isolates that had been cut with nuclease S1 (New England BioLabs, Beverly, MA) (36). After electrophoresis, DNA was blotted onto Hybond-N+ (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and hybridized with a bla_{KPC} PCR probe, using the ECL Random-Prime Labeling and Detection system (Amersham Pharmacia Biotech). Mating was done with *E. coli* A15 Rif^r (5); transconjugants were selected with 100 μg/ml rifampin (Polfa Tarchomin, Warsaw, Poland) and 0.5 μg/ml imipenem (Merck, Sharp & Dohme Research, Rahway, NJ) or 2 μg/ml cefotaxime (Polfa). Plasmid DNA from the isolates, purified with the Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany), was electroporated into *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA), and selected with 0.5 μg/ml imipenem. Plasmids from the *E. coli* recombinants were characterized by PstI fingerprinting (2) and by PCR-based replicon typing (PBRT) (7); the IncR-type replicon was detected as described previously (27) and sequenced. Plasmids of the pKpQIL type (25) were studied by PCR mapping covering seven regions, with primers designed on the basis of the pKpQIL sequence (GenBank accession no. GU595196) (Table 2).

Susceptibility testing. The MICs of antimicrobial agents were evaluated with Etest strips (AB bioMérieux, Solna, Sweden) or M.I.C. Evaluator strips (Oxoid, Basingstoke, United Kingdom). The results were interpreted according to the CLSI guidelines (8); for tigecycline and colistin, the criteria of EUCAST were applied (www.eucast.org).

RESULTS

Epidemiology in Warsaw area. The first KPC-positive *K. pneumoniae* isolates in Poland were identified by the NRCST in May-June 2008; the patient was hospitalized on two different occasions in two Warsaw centers (h1 and h2) (3). Soon after, the next non-carbapenem-susceptible enteric isolates were sent by Polish hospitals for reference diagnostic analysis; by the end of 2008, 33 KPC-producing isolates from 31 patients in five Warsaw centers were confirmed. Hospitals h1 and h2 had two cases and one case, respectively, but the other hospitals recorded clusters of cases (h3, h4, and h5 with 19, 6, and 5 patients, respectively), comprising patients in different wards infected or colonized mainly with *K. pneumoniae* (three *K. oxytoca* cases in h5). In 2009, the five centers continued to identify KPC-positive *K. pneumoniae* cases, with h3, h4, and h5 being the most affected (h4, 18 patients; h5, 15 patients; h3 stopped confirming cases at the NRCST). From two patients in h5, both *K. pneumoniae* and *E. coli* isolates were recovered. Five more Warsaw centers (h6 to h10) and two in the suburbs (h11 and h12) recorded KPC cases. Finally, two patients were diagnosed in Warsaw outpatient clinics (a1 and a2). The data on the patients' movement in the area often could not be obtained; in other cases, the information was fragmentary or indicated several hospitals, both the affected ones and those that did not report KPC-producing isolates. In h11, which started screening all patients at admission in early 2009, KPC-producing isolates were identified in a patient previously treated in h4, a nursing home patient, and three dialysis outpatients. The latter patients had complicated histories of earlier hospitalizations, and where they acquired KPC-producing *K. pneumoniae* is unclear.

Epidemiology outside Warsaw. In May 2009, the first KPC-producing *K. pneumoniae* isolate was identified in a hospital in Gdańsk, Poland (h13), and by the end of 2009, five other centers (h14 to h18) and one outpatient clinic (a3) recorded such organisms. These were mainly single or a few cases, except for a hospital in Płock (h15) where an outbreak with six patients occurred. The first patients in h13 and h16 arrived from Warsaw h3, the outbreak in h15 commenced with a patient from h5, while the patient from a3 had stayed in h12. The patient in Olsztyn, Poland (h18) had been hospitalized in New York. Only two cases (h14 and h17) were not linked to previous care elsewhere.

Typing. All but three *K. pneumoniae* isolates belonged to a single PFGE type, type A, split into 28 subtypes, A1 to A28 (Table 1). Similarity between two patterns varied from ~72% to 94% (Dice coefficient calculated manually). Subtypes A4 and A3 were highly prevalent, grouping 39 and 21 isolates from nine and eight sites, respectively. Of the hospitals in Warsaw that were the most affected, h5 had a relatively homogeneous population of *K. pneumoniae* KPC (subtypes A4 and A24 mostly), while others were notably diverse. The isolate from Olsztyn, Poland, and New York was of unique subtype (A17). MLST classified 16 selected type A isolates with the ST258 clone. The three remaining *K. pneumoniae* isolates were split into two other PFGE types and STs. The single ST23 strain was the only organism from a hospital in Kielce, Poland (h17). Two isolates from a Warsaw hospital (h6) represented ST11. The three *K. oxytoca* isolates from h5 produced identical PFGE patterns. In contrast, the two *E. coli* isolates from the same site differed from each other and were classified as clones ST93 and ST224.

TABLE 1. KPC-producing isolates and their most important characteristics[a]

Species	Medical center (n)[b]		Total no. of isolates	PFGE subtype	MLST[c]	KPC type[d]	ESBL (n)[b,d]	Other acquired β-lactamases (n)[b,d]	Size (kb) of bla_{KPC} plasmid(s)	bla_{KPC} plasmid(s) in $R^{+c,e}$			Tn*4401*	Size and type of ESBL plasmid(s) in $R^{+c,e,g}$
	In the Warsaw area	In other areas								Size (kb)[f]	PstI fingerprinting restriction pattern	Replicon type[g]		
K. pneumoniae	h1 (4), h4 (1)		5	A1	ST258	2	SHV-12	TEM-1	∼110	∼110	c	FII_K and FIB	a	∼40 kb, NT
	h4 (2)		2	A2	ND	2	SHV-12	TEM-1*	∼160 and ∼250	–	ND	ND	a	∼40 kb, NT
	h2 (2), h3 (7), h4 (1), h7 (1), h11 (6), h12 (1)	h13 (2) and a3 (1)	21	A3	ST258	2	SHV-12 (13) and SHV-12 and CTX-M-3 (7)	TEM-1*	∼110	ND	ND	ND	a	ND
	h4 (14), h5 (11), h9 (2), h10 (2), h11 (1), a1 (1), a2 (1)	h14 (1) and h15 (6)	39	A4	ST258	2	CTX-M-3 (6)	TEM-1* (7)	∼50	∼50	b	NT	a	∼90 kb, L/M
	h4 (1)		1	A5	ND	2*	SHV-12*	TEM-1*	∼110 and ∼230	ND	ND	ND	a	ND
	h3 (4), h4 (1), h6 (1)		6	A6	ND	2*	SHV-12	TEM-1*	∼110	ND	ND	ND	a	ND
	h3 (2)		2	A7	ND	2	SHV-12*	TEM-1*	∼48 and ∼110	∼110	c	FII_K and FIB	a	ND
	h11 (1)		1	A8	ND	2*	SHV-12* and CTX-M-3	TEM-1*	∼110	ND	ND	ND	a	ND
	h3 (1)		1	A9	ND	2*	SHV-12*	TEM-1*	∼110	ND	ND	ND	a	ND
	h3 (1)		1	A10	ND	2	SHV-12	TEM-1*	∼130	∼130	c	FII_K and FIB	a	∼40 kb, NT
	h3 (1)		1	A11	ST258	2	CTX-M-3	TEM-1*	∼120	∼120	c	FII_K and FIB	a	∼90 kb, L/M
	h3 (1)		1	A12	ND	2	SHV-12	TEM-1*	∼125	∼125	c	FII_K and FIB	a	∼40 kb, NT
	h2 (1)		1	A13	ST258	3	CTX-M-3	TEM-1*	∼48 and ∼70	∼48	a	R	b	∼90 kb, L/M
	h3 (1)		1	A14	ND	2*	SHV-12*	TEM-1*	∼110	ND	ND	ND		ND
	h3 (1)		1	A15	ST258	2	SHV-12	–	∼200	–	ND	ND	a	∼40 kb, NT
	h1 (2)		2	A16	ST258	2	SHV-12	TEM-1*	∼110 and ∼220	∼110	c	FII_K and FIB	a	∼40 kb, NT
	–	h18 (1)	1	A17	ST258	3	–	TEM-1*	∼110	∼110	c	FII_K and FIB	a	–
	h2 (1), h6 (1)	h16 (1)	3	A18	ST258	2*	SHV-12* and CTX-M-3	TEM-1*	∼110	ND	ND	ND	a	∼90 kb, L/M
	h1 (1), h7 (1), h12 (1)		3	A19	ST258	2	SHV-12	TEM-1*	∼110 and ∼230	∼110	c	FII_K and FIB	a	∼40 kb, NT
	h1 (1), h2 (2), h11 (1)		4	A20	ND	2*	–	TEM-1*	∼110	ND	ND	ND	a	–
	h4 (1) and h8 (1)		2	A21	ST258	2*	SHV-12*	–	∼110	ND	ND	ND	a	ND
	h6 (1)		1	A22	ST258	2*	SHV-12*	TEM-1*	∼160	ND	ND	ND	a	ND
	h7 (1)		1	A23	ST258	2	SHV-12 and CTX-M-3	TEM-1*	∼160	∼160	d	FII_K and FIB	a	∼40 kb, NT; ∼90 kb, L/M
	h5 (5)		5	A24	ND	2*	–	–	∼50	∼50	b	NT	a	–
	h4 (1)		1	A25	ND	2	SHV-12	TEM-1*	∼250	–	ND	ND	a	∼40 kb, NT
	h4 (1)		1	A26	ND	2	SHV-12	–	∼160	∼160	d	FII_K and FIB	a	∼40 kb, NT
	h5 (2)		2	A27	ST258	2*	–	–	∼50	ND	ND	ND	a	–
	h4 (1)		1	A28	ND	2	SHV-12	TEM-1*	∼160	∼160	d	FII_K and FIB	a	∼40 kb, NT
	–	h17 (1)	1	B	ST23	2*	–	TEM-1*	∼110	ND	ND	ND	a	–
	h6 (2)		2	C	ST11	2	SHV-12* and CTX-M-3	TEM-1*	∼110	∼110	c	FII_K and FIB	a	∼90 kb, L/M
K. oxytoca	h5 (3)		3	a	ND	2	–	TEM-1*	∼110	∼110	c	FII_K and FIB	a	–
E. coli	h5 (1)		1	α	ST224	2	–	TEM-1*	∼50	∼50	b	NT	a	–
	h5 (1)		1	β	ST93	2	–	TEM-1*	∼50	∼50	b	NT	a	–

[a] The isolates are ordered in the table according to the species and PFGE data.
[b] n is the number of isolates from a particular medical center or number of isolates producing a particular type of β-lactamase.
[c] ND, not determined.
[d] The isolates for which sequencing was not performed are indicated by asterisks; the identification was based on the PCR and RsaI digestion data (bla_{KPC} genes) or the isoelectric focusing, PCR, and sequencing data obtained for other isolates (bla_{SHV} and bla_{TEM} genes).
[e] R^+, *E. coli* A15 transconjugant or *E. coli* TOP10 transformant.
[f] –, neither transconjugant nor transformant was obtained for these isolates.
[g] NT, nontypeable by PBRT (7).

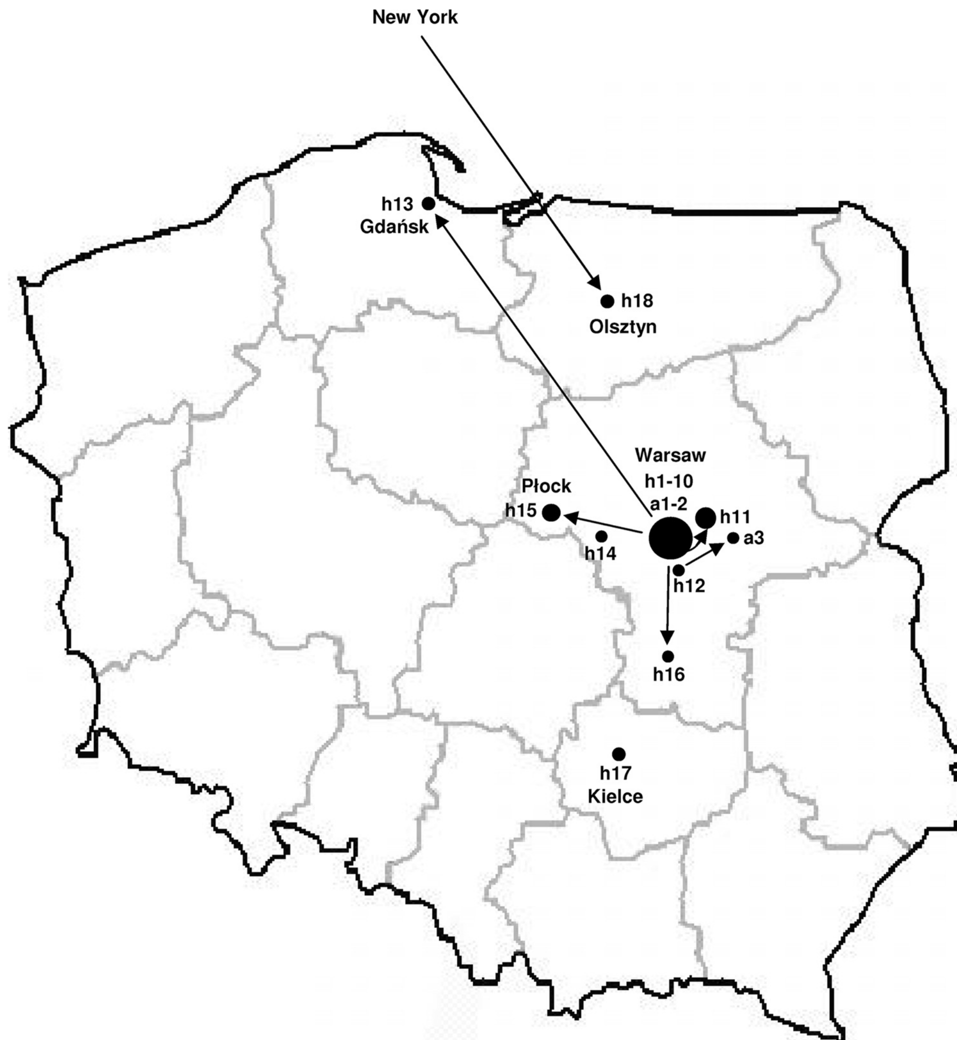

FIG. 1. Map of Poland with the geographic locations of the medical centers in which KPC-positive isolates were identified. h1 to 18, hospitals; a1 to 3, outpatient clinics. The arrows show the movement of patients with KPC-producing organisms between locales.

β-Lactamase content. Two unique *K. pneumoniae* ST258 isolates carried bla_{KPC-3} (subtype A13 from Warsaw h2 and subtype A17 from Olsztyn/New York); all the others likely had bla_{KPC-2}, confirmed by sequencing for 19 isolates (Table 1). Twenty-two *K. pneumoniae* isolates also expressed β-lactamases encoded by $bla_{CTX-M-1}$-like genes; the genes were found to be $bla_{CTX-M-3}$ in 11 isolates. Fifty-nine *K. pneumoniae* isolate produced enzymes correlating with amplicons of $bla_{SHV-5/12}$-like genes, identified as bla_{SHV-12} in 13 isolates. CTX-M-3 and SHV-12 were coexpressed in 14 isolates. Seventy-six isolates had β-lactamases corresponding to bla_{TEM-1}-like genes. The β-lactamase profiles correlated with the PFGE types/subtypes; only the *K. pneumoniae* ST258 subtypes A4 and A3 varied regarding the presence of CTX-M-3, SHV-12, and/or TEM-1.

Plasmids with bla_{KPC} genes. Plasmid DNA of the isolates revealed high variation with around 30 S1 profiles (data not shown). The profiles consisted of 1 to 4 molecules varying in size from ~40 kb to ~350 kb and in general correlated with PFGE types/subtypes and β-lactamase patterns. All S1 profiles were hybridized with the bla_{KPC} probe, revealing a number of molecules split into five size groups, of ~48 to 50 kb, ~70 kb, ~110 to 130 kb, ~160 kb and ~200 to 250 kb (Table 1). In six profiles, two plasmids carried bla_{KPC}. The ~110- to 130-kb molecules were observed alone or with other sized molecules in 62 isolates of ~15 S1 profiles from *K. pneumoniae* ST11, ST23, and ST258 of 17 subtypes (including the A17 KPC-3 isolate from Olsztyn/New York), and *K. oxytoca* isolates. The second common plasmid was of ~50 kb, identified in 46 *K. pneumoniae* ST258 isolates of three subtypes and two S1 profiles, and *E. coli* isolates.

Twenty-four isolates with all bla_{KPC} plasmids, including *K. oxytoca* and both *E. coli* and *K. pneumoniae* pairs from the same patient, were used in mating, which worked for 13 isolates. Then, DNA from the nonmating isolates was subjected to electroporation, which yielded seven transformants (Table 1). Plasmids from all 20 *E. coli* recombinants had four types of PstI restriction patterns, namely, type a for the plasmid of ~48 kb, type b for plasmids of ~50 kb, type c for plasmids of ~110 to 130 kb, and type d for plasmids of ~160 kb (patterns shared at least 80% or 90% of bands of >1.5 kb within a type). The

TABLE 2. Pairs of primers used in the PCR mapping assay analyzing the pKpQIL-like plasmids

Primer	Sequence (5′ → 3′)	Annealing positions[a]	Product size (bp)	pKpQIL fragment analyzed[a]	Result[b]
RepAF RepAR	GCTGAGCGTAAATCTCAC CTCAACCTCACGCTTCAG	4439–4456 4580–4597	159	IncFII$_K$ replicon region (FII$_K$ *repA* gene)	+
RepAF ORF8R	GCTGAGCGTAAATCTCAC CAGACGTGATTTTCTGACCAC	4439–4456 5915–5935	1,497	Junction between the FII$_K$ replicon region and the pNYC-derived region	+
ORF11R tnpRF	GCATGTTTGAACTGGTCAG CGACGTCGATGTATTTGCATG	8347–8365 9008–9028	682	Junction between Tn*4401* and its 5′ flank within the pNYC region	+
IS26LF TEM-F	GTCGGTGGTGATAAAC TTACTGTCATGCCATCC	25556–25571 28386–28402	2,847	IS*26* insertion within *1331* the pKPN4-derived Tn	+/−
OXA-9F TEM-F	CTGCTGCATATGTTGGTG TTACTGTCATGCCATCC	26542–26559 28386–28402	1,861	Junction between *bla*$_{OXA-9}$ and *bla*$_{TEM-1}$ within the pKPN4 Tn*1331*	+/−
rep2R rep2F	TCTGCTGGTTATTGGGTGAG GTCTTCGCAGCACAACTATC	53337–53356 53629–53648	312	FIB-like *repA* gene within the pKPN4 region	+
TraXF ORF2R	CGCAATGTTCTATGCTGTG CAGAATAACTGCTGCTCAG	112918–112936 838–856	1,576	Junction between the pKPN4 region (*traX* gene) and the FII$_K$ replicon region	+/−

[a] Position numbering and the description of plasmid regions is according to reference 25 and the pKpQIL sequence (GenBank accession no. GU595196).
[b] Symbols: +, PCR worked with all plasmids analyzed; +/−, PCR worked, but not with all plasmids analyzed.

fingerprints of type c matched well with the fingerprint of the pKpQIL plasmid with *bla*$_{KPC-3}$ from Israel (25), which was generated *in silico*. The type b plasmids of ~50 kb in the *E. coli* and *K. pneumoniae* isolates from the same patient had very similar fingerprints. Plasmids that did not transfer were those of ~70 kb and ~200 to 250 kb.

The PCR assay checking for several pKpQIL regions (Table 2) confirmed that plasmids of ~110 to 130 kb represented several variants of that structure (25); moreover, the ~160-kb plasmids also behaved in a similar way. The IncFII$_K$-type *repA* gene (25, 41), amplifiable also with PBRT primers for FII$_{AS}$ (7), was always observed, so was the additional replicon FIB. The position of FII$_K$ *repA* was stable regarding the downstream region with Tn*4401* but not with respect to the upstream *tra* operon (25). The plasmids of ~50 kb and ~48 kb were nontypeable by PBRT (7); however, the latter plasmid carried the IncR-type *repB* gene (27).

Plasmids with *bla*$_{CTX-M-3}$ and *bla*$_{SHV-12}$ genes. Nineteen isolates with CTX-M-3 ($n = 6$) or SHV-12 ($n = 13$) were used in conjugation assays (Table 1); only two of the SHV-12 producers did not mate. The ~90-kb plasmids from the CTX-M-3 transconjugants had PstI fingerprints and L/M-type replicons like the pCTX-M3-type molecules (15). The ~40-kb plasmids from SHV-12 strains showed identical fingerprints and were nontypeable by PBRT (3).

Genetic context of *bla*$_{KPC}$ genes. All but one of a wide group of isolates tested carried the Tn*4401*a element (Table 1), with a 100-bp deletion between IS*Kpn7* and *bla*$_{KPC}$ (29). Only the *K. pneumoniae* ST258 A13 KPC-3 isolate from Warsaw h2 had the Tn*4401*b variant.

Susceptibility testing. Thirty-seven representative isolates were subjected to susceptibility testing (see Table S1 in the supplemental material). All were resistant to penicillins, penicillin-inhibitor combinations, ceftazidime, and aztreonam. Considering the confluent growth around antibiotic strips, the MICs of carbapenems varied (MICs, 0.5 to >32 μg/ml); however, multiple in-ellipse colonies were usually observed (32). Except for *K. pneumoniae* ST23, *K. oxytoca*, and *E. coli* ST93, all isolates were resistant to ciprofloxacin. Amikacin and gentamicin were often active; however, *K. pneumoniae* ST11 and some ST258 isolates were resistant to these two drugs. All the isolates tested were susceptible to colistin; 18 isolates were intermediate to tigecycline.

DISCUSSION

This study shows the early stage of KPC-producing *K. pneumoniae* dissemination in Poland in 2008-2009. Although microbiology laboratories are not obliged to send pathogens on the alert or watch list to the NRCST, years of experience and good reciprocal contacts allow us to assume that the isolates reflected the situation well. It is impossible to know whether the KPC-producing *K. pneumoniae* history in Poland commenced with the first report of *K. pneumoniae* ST258 in hospitals h1 and h2 in May-June 2008 (3). This organism belonged to PFGE subtype A1 and had plasmids of ~40 kb (*bla*$_{SHV-12}$), ~110 kb (*bla*$_{KPC-2}$ and *bla*$_{TEM-1}$), and ~200 kb. The following 30 *K. pneumoniae* ST258 isolates, collected by the end of 2008, exhibited 13 PFGE subtypes, six β-lactamase patterns, and all *bla*$_{KPC}$ plasmid types (~10 S1 profiles), and only three isolates (two from h1) were like the original ones. It is likely that there were several occurrences of *K. pneumoniae* ST258 in Warsaw hospitals, possibly also before May 2008. The notable rate of asymptomatic carriage and the diagnostic problems and lack of awareness at the time could have caused KPC-producing organisms to be overlooked.

In 2008, the NRCST started promulgating practical knowledge of KPC-producing bacteria and subsequently published national guidelines for the laboratory detection and infection control (www.korld.edu.pl). The increased awareness but also the progressive spread resulted in the higher number of isolations in 2009 (86 isolates; 82 patients). A remarkable role has

been played by Warsaw hospitals h3, h4, and h5, which have become sites where KPC-producing bacteria are endemic and export KPC strains to other places. It is hard to understand the situation in hospitals with several separate KPC cases (e.g., h1, h2, or h11) that could be due to new entries and/or "leaks" in infection control systems. Several institutions seem to have counteracted the KPC spread well as a result of strict precaution procedures implemented just after identification of the index case.

The analysis showed serious difficulties in tracking the dissemination of KPC-producing *K. pneumoniae*. Although most of the isolates (97.4%) belonged to the pandemic clone ST258, they revealed high diversity in PFGE subtypes, S1 profiles, bla_{KPC}-carrying plasmids, and β-lactamase patterns. All these differences confused the matter; only in some cases were clear situations of ongoing spread (e.g., subtype A3 in h3 or A4 in h4 and h5) or interhospital transfer (A4 in h5 and h15) observed. In general, it was difficult to sort out whether an ST258 organism was introduced independently into a hospital or the country (for example, the A17 organism was introduced into Olsztyn, Poland, from New York) or evolved from another one at the site by changes in chromosomal and/or plasmid DNA. The ST258 intrinsic diversity has been observed in other studies; however, isolates with around ~80% similarity in PFGE and significant differences in plasmids were collected over rather large areas with longer KPC history so far (14, 23). To our knowledge, the heterogeneity level described here has been extreme for the time scale and geographic scale of this study.

Plasmids with the bla_{KPC} genes largely contributed to this diversity by size, structure, and transfer ability. The major group were the conjugative pKpQIL-type molecules (IncFII$_K$) originally identified in ST258 in Israel, dominating KPC-3-producing *K. pneumoniae* in that country (24, 25). By size one may suspect that such plasmids with bla_{KPC-2} or bla_{KPC-3} have been present in ST258 in other countries (36); moreover, plasmids identified by PBRT as with replicon FII$_{AS}$ (10) might also belong to this group (primers for FII$_{AS}$ amplify the pKpQIL FII$_K$ *repA*). In Poland, the pKpQIL-like plasmids were observed in all the *K. pneumoniae* clones and *K. oxytoca* and showed variety in size and structure. Except for the isolate with bla_{KPC-3} from Olsztyn/New York, all the other isolates had bla_{KPC-2}. This study revealed high plasticity of the pKpQIL-like plasmids and further proved their crucial role in the KPC spread, with no strict correlation with *K. pneumoniae* ST258 as reported (31).

Other prevalent plasmids were those of ~50 kb, observed first in another study on five *K. pneumoniae* isolates from h4 and designated pETKp50 (44). Compared to pKpQIL, the pETKp50-like molecules were present in a more uniform group of isolates. Of six isolates tested, only two yielded transconjugants, and they were actually a pair of *E. coli* and *K. pneumoniae* from one patient. This result showed some conjugative potential for pETKp50 and might illustrate yet another case of *in vivo* transfer of a bla_{KPC} plasmid (4, 17). Although ~50-kb plasmids were found in other countries, it is difficult to compare these because of their nontypeability or lack of replicon data (10, 24). IncR plasmids with bla_{KPC-2} have been identified in the United States (27), but again the data reported do not allow us to compare these plasmids with the ~48-kb bla_{KPC-3} plasmid from the unique A13 isolate. The presence of several bla_{KPC} plasmid types was observed on a country or global scale (1, 10, 18, 24); however, to our knowledge, the molecules of ~200 to ~250 kb have been the largest ones identified so far. Again, it is hard to reveal how far this variety resulted from plasmid rearrangements during spread in Poland or from independent introductions of KPC-producing organisms. Most probably, the pKpQIL-like molecules illustrated both possibilities, and the presence of two bla_{KPC} plasmids in some isolates was indicative of Tn*4401* transposition between these plasmids.

KPC producers also varied by other acquired β-lactamases and plasmids. Similar to other countries (14, 21, 33, 36), many *K. pneumoniae* isolates (ST11 and ST258) produced SHV-12, encoded by genes carried on nontypeable plasmids of ~40 kb. Specific to Poland was the presence of CTX-M-3, which was also observed in the earlier report (44). CTX-M-3, encoded by highly conjugative IncL/M plasmids of the pCTX-M3 family (15), has been the most widespread ESBL in the country (2, 13). Indeed, our results showed that pCTX-M3-like plasmids have already penetrated into KPC-producing *K. pneumoniae* ST11 and ST258 populations in several hospitals.

This has been one of the few reports showing a comprehensive view of the spread of KPC-producing *K. pneumoniae* on a country-wide scale (23, 24), with striking complexity considering its early stage. This study confirmed and expanded previous data on the two crucial factors of the "success" of KPC-producing *K. pneumoniae*, namely, the *K. pneumoniae* ST258 clone and the pKpQIL-like plasmids. The multidimensional heterogeneity of ST258, in the context of its global spread, causes difficulties in outbreak investigations and tracking KPC-producing organisms, indicating the need of more precise typing, as well as the superior role of detailed clinical-epidemiological data. In Europe, Poland is one of the countries most affected by KPC producers (19); the increased awareness shown by many hospital infection control teams offers hope that Poland will not become the next region where these alarming organisms are endemic.

ACKNOWLEDGMENTS

We are very thankful to Alessandra Carattoli and Izabela Kern-Zdanowicz for helpful consultations.

This work was partially financed by the grant Narodowy Program Ochrony Antybiotyków (NPOA) from the Polish Ministry of Health and grants SPUB MIKROBANK and 934/6. PR UE/2009/7 from the Polish Ministry of Science and Higher Education.

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