Ertapenem Resistance among Extended-Spectrum- β -Lactamase-Producing *Klebsiella pneumoniae* Isolates[▽]

Azita Leavitt,¹ Inna Chmelnitsky,¹ Raul Colodner,² Itzhak Ofek,³
Yehuda Carmeli,¹ and Shiri Navon-Venezia^{1*}

The Laboratory for Molecular Epidemiology and Antibiotic Research, Division of Epidemiology, Tel Aviv Sourasky Medical Center—Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel¹; Clinical Microbiology Laboratory, Ha'Emek Medical Center, Afula, Israel²; and Department of Clinical Microbiology and Immunology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel³

Received 7 April 2008/Returned for modification 19 September 2008/Accepted 3 February 2009

Ertapenem resistance in *Klebsiella pneumoniae* is rare. We report on an ertapenem-nonsusceptible phenotype among 25 out of 663 (3.77%) extended-spectrum- β -lactamase (ESBL)-producing *K. pneumoniae* isolates in a multicenter Israeli study. These isolates originated from six different hospitals and were multiclonal, belonging to 12 different genetic clones. Repeat testing using Etest and agar dilution confirmed ertapenem nonsusceptibility in only 15/663 (2.3%) of the isolates. The molecular mechanisms of ertapenem resistance in seven single-clone resistant isolates was due to the presence of ESBL genes (CTX-M-2 in four isolates, CTX-M-10 and OXA-4 in one isolate, SHV-12 in one isolate, and SHV-28 in one isolate) combined with the absence of OMPK36. Seven of 10 isolates initially reported as ertapenem nonsusceptible and subsequently classified as susceptible showed an inoculum effect with ertapenem but not with imipenem or meropenem. Population analysis detected the presence of an ertapenem-resistant subpopulation at a frequency of 10^{-6} . These rare resistant subpopulations carried multiple ESBL genes, including TEM-30, SHV-44, CTX-M-2, and CTX-M-10, and they lacked OMPK36. The clinical and diagnostic significance of the results should be further studied.

Klebsiella pneumoniae has been found to be the most common species to produce extended-spectrum β -lactamases (ESBLs) (6), and in some countries the prevalence of ESBL production approaches 50% (25). Antimicrobial coresistance among these ESBL-producing isolates limits the number of drugs that are useful against these strains (29), leaving carbapenems to be the most reliable agents (19, 35). In Tel Aviv Medical Center, 45% of *K. pneumoniae* isolates have an ESBL-producing phenotype (24), and ertapenem is an optional therapy for nosocomial infections caused by these pathogens.

Ertapenem is a 1- β -methyl carbapenem that reached clinical use in 2001 (30). It is highly active against ESBL-producing and high-level AmpC-producing gram-negative bacteria (22) and is an important agent to treat these infections, as it is not likely to lead to carbapenem resistance in *Pseudomonas* (8, 23).

The emergence of ertapenem resistance in *K. pneumoniae*, which is not related to the production of carbapenemases such as metalloenzymes or KPC enzymes, is rare and has been studied only in single cases (36). In these cases, resistance was associated with the production of an ESBL and deficiency in the expression of the outer membrane proteins (OMPs) OMPK35 and OMPK36 (17, 37). Reports on the incidence of ertapenem resistance are limited; however, one report on ESBL-producing *K. pneumoniae* isolates collected from intra-abdominal infections found that 10.9% were ertapenem resistant (26).

The accurate susceptibility testing of *K. pneumoniae* isolates to ertapenem is critical for choosing the appropriate antibiotic therapy for treating infections caused by ESBL-producing strains. It has been recommended previously that imipenem and meropenem can be surrogate markers for susceptibility to ertapenem (15), although difficulties in attaining precise susceptibility testing results for imipenem and meropenem in clinical microbiology laboratories have been described previously (34) due to the degradation of the drug, which leads to possible false susceptibility results (13, 31, 33, 36), even when automated systems such as MicroScan and Vitek systems are used (6, 7). No studies were performed regarding ertapenem susceptibility testing.

In a previous nationwide surveillance study in Israel, 663 ESBL-producing *Klebsiella pneumoniae* isolates were screened for their susceptibility properties. Ninety-five percent of these isolates were susceptible to ertapenem, and 98.8 and 95% were susceptible to imipenem and meropenem, respectively (12). With the high prevalence of ESBLs in our country and the need for optional antibiotic therapies, we aimed to explore the molecular mechanisms that render these isolates resistant to ertapenem. Moreover, we aimed to explain discrepancies in ertapenem MIC testing using agar-based susceptibility testing methods and to examine the effect of exposure to ertapenem on these isolates.

MATERIALS AND METHODS

Study design. A total of 663 *Klebsiella pneumoniae* isolates with confirmed ESBL-producing phenotypes were collected over a 6-month period between January and December 2004 from 10 major Israeli hospitals. ESBL detection tests were performed based on the Kirby-Bauer disk diffusion test, which was described previously (24). The isolates were subjected to MIC testing by Etest for

* Corresponding author. Mailing address: Division of Epidemiology, Aviv Sourasky Medical Center, 6 Weizmann St., Tel Aviv 64239, Israel. Phone: 972 3 692 5644. Fax: 972 3 697 4966. E-mail: shiri_nv@tasmc.health.gov.il.

[▽] Published ahead of print on 11 February 2009.

various antibiotics, including the carbapenems imipenem, meropenem, and ertapenem (12). Twenty-five of these *Klebsiella* isolates were found to be nonsusceptible to ertapenem (MIC, ≥ 8) and were the subject of this study.

PAGE. The genetic relatedness of all ertapenem-nonsusceptible *K. pneumoniae* isolates was analyzed by pulsed-field gel electrophoresis (PFGE). Bacterial DNA was prepared and cleaved with 20 U SpeI endonuclease (New England Biolabs, Boston, MA) as previously described (28), and DNA macrorestriction patterns were compared visually and interpreted according to the criteria established by Tenover et al. (34).

Susceptibility testing. All 25 ESBL-producing isolates that were identified as ertapenem nonsusceptible in a previous study (12) were subjected to a repeat MIC testing using Etest and agar dilution in the current study. Ertapenem MIC testing was performed according to the Clinical and Laboratory Standards Institute guidelines (11) using cation-adjusted Mueller-Hinton (MH) agar (Hy-Labs, Rehovot, Israel) supplemented with increasing amounts of ertapenem (Merck Research Laboratories, Rahway, NJ). The tested inoculum used in all susceptibility testing was 0.5 McFarland unless stated otherwise. Ertapenem MICs were determined in the presence and absence of 2 $\mu\text{g/ml}$ clavulanic acid or 0.4 mM EDTA to determine the contribution to the resistance of class A and class B β -lactamases, respectively.

Study of isolates with discrepant ertapenem susceptibility results. Ertapenem susceptibility discrepancies (i.e., a greater than onefold dilution difference) between repeated agar-based testing methods were further explored by examining the effect of the inoculum size on the susceptibility testing of carbapenems and by performing population analysis studies.

Inoculum effect experiments. Organisms were grown on MH agar plates overnight. An inoculum (10^8 CFU/ml) was prepared by suspending a sufficient number of colonies in MH broth to achieve a 0.5 McFarland suspension (corresponding to an optical density at 600 nm of 0.1). Susceptibility testing using Etest and agar dilution methods was performed with inocula containing 10^5 and 10^7 CFU. A positive inoculum effect was defined as an eightfold or greater increase in the ertapenem MIC on testing with the higher inoculum (20).

Population analysis. Cultures were grown overnight in MH broth and were serially diluted with saline. A 100- μl volume of each dilution was plated on freshly prepared ertapenem-containing MH plates (0.25 to 64 $\mu\text{g/ml}$). Colonies were counted after overnight incubation at 37°C, and the viable count was plotted against the ertapenem concentration (38).

β -Lactamase analysis. The production of ertapenem-hydrolyzing enzymes by all ertapenem-resistant isolates was analyzed with an ertapenem inactivation bioassay (40) performed on MH agar plates. A suspension of *Escherichia coli* ATCC 25922 equivalents to a 0.5 McFarland standard was inoculated on a large MH agar plate, as described for disk diffusion. Five evenly spaced ertapenem disks then were applied to the plate, four on the periphery and one in the center of the plate. Crude extract of the organism to be tested for the presence of carbapenemase was prepared by sonication, and a loop was used to make a 15-mm streak of crude extract on each side of the ertapenem disk on the periphery of the plate (the center disk served as the control). Four different organism suspensions were used on each plate. The KPC-3-producing clinical strain of *Klebsiella pneumoniae* (strain 490) and *E. coli* ATCC 25922 were used as positive and negative controls, respectively, in bioassays and hydrolysis assays for the detection of carbapenem-hydrolyzing activity. The plates were incubated at 37°C for 18 to 20 h. Alterations in the shape of the zones of inhibition around the test organism were examined. Screening for the production of metallo- β -lactamase was performed by disk approximation tests using EDTA and 2-mercaptopyruvic acid (2). The presence of β -lactamases with ertapenem-hydrolyzing activity of selected ertapenem-resistant clones was further analyzed by performing ertapenem hydrolysis assays. The hydrolysis of 0.1 mM imipenem and ertapenem (Merck, Hoesdendon, United Kingdom) was monitored in crude extracts of these isolates by UV spectrophotometry at 299 and 294 nm, respectively, in 10 mM phosphate buffer (pH 7.0) (9). Activity was standardized relative to the protein concentration, which was determined by the Bio-Rad protein microassay using the Bradford method. Bovine serum albumin was used as the standard. Ertapenem hydrolysis assays were performed in the presence of EDTA (0.1 mM; pH 8.0).

Molecular analysis. The identification of *bla* ESBL genes, *bla*_{KPC}, and plasmid-mediated AmpC genes in all isolates was determined by PCR on 1 μl cell lysate using specific primers designed for identifying β -lactamase genes, including *bla*_{TEM}, *bla*_{SHV} (28), *bla*_{OXA} (1, 10, 16, 27), *bla*_{CTX-M} (32), *bla*_{SPM} (9), *bla*_{ACT}, *bla*_{MIR} (3), *bla*_{CMY}, *bla*_{FOX}, *bla*_{MOX} (21), and *bla*_{KPC} (7). The PCR conditions were as follows: 15 min at 95°C; then 35 cycles of 1 min at 94°C, 2 min at 68°C, and 3 min at 72°C; and finally an extension step of 10 min at 72°C. PCRs were performed with HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), and the resulting PCR products were analyzed in a 1% agarose gel with ethidium

bromide staining and UV light. PCR products were sequenced and analyzed with an ABI PRISM 3100 genetic analyzer (PE Biosystems) using the DNA Sequencing Analysis Software and 3100 Data Collection Software, version 1.1. The nucleotide and the deduced protein sequences were analyzed and compared using software available via the Internet at the NCBI web site (<http://www.ncbi.nlm.nih.gov/>).

OMP analysis. OMPs were prepared from selected ertapenem-resistant and -susceptible isolates grown in MH broth in the absence or presence of 4 $\mu\text{g/ml}$ ertapenem according to the method of Wu et al. (39). Cell membrane proteins were obtained by disrupting a logarithmic-phase culture with a VCX 600 sonicator (Misonix). Cell debris was removed by centrifugation at 8,000 rpm for 20 min at 4°C, and the supernatant was subjected to ultracentrifugation at 40,000 rpm for 1 h at 4°C to collect the membranes. Membranes were solubilized in 1.5% sodium lauryl sarcosinate for 30 min at room temperature. The suspension was centrifuged at 8,000 rpm for 45 min at 4°C, and the pellet containing the OMPs was resuspended in 100 μl 0.05 M phosphate buffer (pH 7.0). Protein concentrations were determined using Bradford assay, and equal amounts of protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed according to the method of Bradford et al. (4), using a Mini protein cell electrophoresis system apparatus with prepared 10% polyacrylamide gels (Bio-Rad). Samples were boiled for 5 min prior to being loaded and then were separated at a constant voltage of 150 V in a running buffer of 1 $\times$ Tris-glycine-SDS (Bio-Rad) and visualized by Coomassie blue staining (Gibco-BRL). After electrophoresis, protein bands of interest were excised from the gel, washed in a 200 mM NH_4HCO_3 -50% acetonitrile solution, and dried in a SpeedVac. Protein were rehydrated in a 20- $\mu\text{g/ml}$ trypsin solution (Promega, Madison, WI) and incubated for 16 h at 37°C. Peptides were extracted from gel slices by diffusion in water and were identified by liquid chromatography-mass spectrometry/mass spectrometry (MS/MS) using an Ultimate Nano high-performance liquid chromatography system (LC Packings, Amsterdam, The Netherlands) and a Qstar Pulsar mass spectrometer (Applied Biosystems, Foster City, CA). The MS data were analyzed using the Mascot protein identification software (Matrix Science, London, United Kingdom). For the OMP analysis of ertapenem-susceptible strains possessing an inoculum effect, prior to protein analysis bacteria were grown in MH broth in the presence of 4 $\mu\text{g/ml}$ ertapenem to obtain only the resistant population.

RESULTS

Occurrence of ertapenem resistance among *Klebsiella pneumoniae* isolates and their clonal relatedness. Twenty-five of 663 ESBL-producing *Klebsiella pneumoniae* strains (3.77%), collected from six hospitals in Israel, were found to be resistant (18 isolates; 50% minimum inhibitory concentration [MIC_{50}], ≥ 16 $\mu\text{g/ml}$) or intermediate (7 isolates; MIC, 4 to 8 $\mu\text{g/ml}$) to ertapenem by Etest (Tables 1 and 2). All isolates but one (isolate 20; MIC, >32 $\mu\text{g/ml}$) were susceptible to imipenem (MIC₅₀, 1 $\mu\text{g/ml}$). As for meropenem, four isolates were intermediate (MIC, 6 to 8 $\mu\text{g/ml}$), and one isolate (isolate 20) was resistant (MIC, >32 $\mu\text{g/ml}$; MIC₅₀, 3 $\mu\text{g/ml}$) (Table 1). Ertapenem-resistant isolates originated from blood (12), wounds (6), bone marrow (1), catheters (2), and peritoneal fluid (1).

All 25 ertapenem-nonsusceptible isolates were subjected to genotyping, which revealed 12 different genetic clones; clone A was the most prevalent PFGE clone, being present in 8 out of the 25 isolates (32%) and detected in four of the six studied hospitals. In hospital 1 the dominant clone was A, but in hospitals 2 and 5 the dominant clones were K and D, respectively, clones that were absent in other hospitals (Table 1).

Ertapenem susceptibility testing. The 25 ertapenem-nonsusceptible isolates included in this study were collected from different hospital clinical laboratories throughout Israel. When these ESBL-producing isolates arrived in our laboratory, the

TABLE 1. Twenty-five ESBL-producing *K. pneumoniae* isolates included in this study^a

<i>K. pneumoniae</i> isolate	Hospital	PFGE clone	Carbapenem MIC (μg/ml)				
			Initial Etest ^b			ERT MIC ^c	
			ERT	IMP	MP	Second Etest	Agar dilution
2	1	A	8	0.75	0.125	0.125	2
3	1	A'	>32	4	>32	24	32
4	1	G	32	1.0	1.5	0.38	2
5	1	H	16	4	4	3	2
6	1	A'	8	0.25	0.125	0.19	<1
7	1	B	16	0.5	4	0.38	<1
9	1	J	32	4	2	1	1
10	2	G	>32	0.25	0.047	0.38	1
11	2	K	16	0.75	2	>32	32
12	2	K''	>32	1.5	3	24	32
13	2	K'	32	1.5	6	>32	64
14	3	M	6	0.25	0.064	1	<1
15	3	N	12	0.75	1.5	6	8
16	3	A	16	1	4	4	16
17	4	F	>32	1	4	>32	16
18	4	E	6	0.38	1.5	12	8
20	5	D	>32	>32	>32	>32	64
21	5	A	>32	2	6	12	16
22	5	A	>32	2	6	>32	32
23	5	D	8	1	8	>32	4
24	5	D	32	1	3	0.25	1
26	5	A	6	0.19	2	8	16
27	5	D	8	0.38	1	1	<1
94	6	A	12	0.19	0.75	8	8
160	6	I	32	4	8	>32	32

^a MICs in boldface designate isolates that changed their ertapenem status from resistance to susceptible. ERT, ertapenem; IMP, imipenem; MP, meropenem.

^b Performed in the initial isolating hospital's clinical laboratory.

^c Repeated ertapenem susceptibility testing was performed in our laboratory for all of the ertapenem-nonsusceptible isolates, as described in Materials and Methods. MICs presented are the averages from three independent measurements.

MIC testing of carbapenems was repeated and revealed discrepancies (Table 1).

Of the 25 isolates with initial ertapenem MICs of >4 μg/ml, 10 isolates (40%) were defined as susceptible to ertapenem (MIC, 0.125 to 4 μg/ml) upon repetitive testing by both Etest and agar dilution, 2 were intermediate, and 13 were resistant (Table 2). Results from repetitive MIC testing for imipenem and meropenem did not vary or varied by one dilution.

TABLE 2. Summary of ertapenem susceptibility testing

Test and resistance status ^b	No. of isolates	MIC range (μg/ml)	MIC ₅₀ (μg/ml)	% of total ^a
Initial Etest				
S	0			0
I	3	6	0	0.4
R	22	8–>32	32	3.3
Second Etest				
S	10	0.125–4	0.38	1.5
I	2	4–6		0.3
R	13	8–>32	>32	2
Agar dilution				
S	10	<1–2	1	1.5
I	1	4		0.1
R	14	8–64	32	2.1

^a Calculated out of 663 ESBL-producing *K. pneumoniae* isolates tested.

^b S, susceptible; I, intermediate; R, resistant.

TABLE 3. Effect of inoculum size on ertapenem susceptibility testing of 10 ertapenem-susceptible strains

<i>K. pneumoniae</i> isolate no.	Ertapenem MIC (μg/ml) at the tested inoculum (CFU)			
	Etest		Agar dilution	
	10 ⁵	10 ⁷	10 ⁵	10 ⁷
2	0.19	0.5	1	4
4	0.5	6	1	4
5	0.25	16	1	64
6	0.5	16	0.250	16
7	0.25	16	0.250	32
9	1	3	1	16
10	0.5	16	4	64
14	0.25	1.5	0.5	32
24	0.25	8	2	32
27	0.5	>32	0.5	32

Moreover, significant discrepancies in ertapenem susceptibility results were observed in different Etest measurements as a result of the appearance of resistant colonies within the zone of inhibition. The discrepancies observed in MIC testing did not correlate with a specific genetic clone or clones. Overall, we found reasonable agreement between Etest and agar dilution testing results (Table 2); however, in three isolates we noted discrepancy in MICs between Etest and agar dilution susceptibility results (a difference greater than two dilutions).

The 10 isolates initially classified as nonsusceptible and later found to be susceptible were subjected to a further detailed analysis. The other 13 isolates that showed constant ertapenem-resistant profiles (2% of the ESBL-producing *Klebsiella* isolates) were categorized as ertapenem-resistant isolates. Seven of these that represented single-clone isolates were further studied for mechanisms of ertapenem resistance.

Inoculum effect studies. Ten isolates reported as ertapenem resistant initially and susceptible on repetitive detection (Table 1) were subjected to inoculum effect experiments. Seven of 10 isolates tested (70%) showed an inoculum effect (a greater than eightfold increase in the ertapenem MIC); the MIC₅₀ increased from 0.5 to 8 μg/ml by Etest and from 2 to 32 μg/ml by agar dilution (Table 3). All 10 isolates showing a positive inoculum effect for ertapenem were tested for their susceptibility to imipenem and meropenem at high inoculum sizes; the imipenem MIC increased with inoculum size (from a MIC₅₀ of 0.19 μg/ml to a MIC₅₀ of 0.5 μg/ml) but an inoculum effect was not observed. As for meropenem, the MIC₅₀ increased from 0.064 μg/ml to 1.5 μg/ml at a high inoculum, and four of seven isolates showed an inoculum effect; however, none of them changed their classification from susceptible to intermediate or resistant.

Population analysis studies. To examine the possibility of the existence of an ertapenem-resistant subpopulation, two isolates that showed an inoculum effect on ertapenem (isolates 5 and 7) were subjected to population analysis. The distributions of the populations of the two isolates in terms of their resistance to ertapenem were compared to those of two isolates that did not possess an inoculum effect, one of which was an ertapenem-resistant isolate (MIC, 64 μg/ml) and one of which was an ertapenem-susceptible isolate (MIC, <1 μg/ml) (Fig. 1). Population analysis revealed the existence of an ertapenem-resistant subpopulation at a frequency of 1.5 ×

FIG. 1. Population analysis of four ESBL-producing *Klebsiella pneumoniae* strains with and without an inoculum effect. The distribution of the resistant subpopulation among the population of two ertapenem-sensitive strains that possess an inoculum effect (strains 5 and 7) differed from the population distribution of a stable ertapenem-resistant strain (strain 490) and that of an ertapenem-sensitive strain (strain 8). Strains 5 and 7 showed the preexistence of a rare ertapenem-resistant subpopulation absent in strain 8 that may lead to discrepancies in ertapenem susceptibility test results for the inoculum effect strains.

10^{-6} , demonstrating the preexistence of rare ertapenem-resistant subpopulations among ertapenem-susceptible isolates that initially demonstrated resistant MICs.

Molecular mechanisms of ertapenem resistance. (i) Analysis of β -lactamases. Seven single-clone ertapenem-resistant isolates and the two isolates that possessed an inoculum effect with ertapenem were chosen for the molecular characterization of the resistance mechanisms involved in ertapenem resistance. All isolates possessed ESBL genes. Seven isolates possessed CTX-M-type genes (CTX-M-2, isolates 3, 5, 7, 15, 18, and 160; CTX-M-10, isolates 5, 7, and 11), four possessed SHV-type genes (SHV-44, isolates 5 and 7; SHV-12, isolate 17; and SHV-28, isolate 20); isolates 5 and 7 possessed TEM-30, and isolate 11 also possessed OXA-4. All isolates produced additional β -lactamases, such as SHV-1 (five isolates), TEM-1 (one isolate), and OXA-2 (four isolates). Other β -lactamases belonging to Ambler class A, including *bla*_{KPC}, class B, or plasmid-mediated AmpC- or OXA-type carbapenemases, were not detected. Crude enzyme preparations from all isolates did not exhibit ertapenem-hydrolyzing activity in bioassays and did not hydrolyze ertapenem and imipenem according to spectrophotometric assays; the result for the control isolate car-

rying KPC was 46.5 mU/mg (where *U* = micromoles of imipenem/minute).

(ii) Outer membrane analysis. OMP analysis was performed on seven stable ertapenem-resistant strains, on two susceptible isolates possessing an inoculum effect, and on *K. pneumoniae* ATCC strain 13883 (Fig. 2A). SDS-PAGE revealed the presence of two main OMPs, with apparent molecular masses of 35 and 36 kDa, in ertapenem-susceptible strains and demonstrated the absence of OMP36 in six of the seven stable ertapenem-resistant isolates. An analysis of OMP36 by MS identified this OMP as OMPK36. OMP35 (with an apparent molecular mass of 35 kDa) was identified as OMPA. The OMP profiles obtained from two ertapenem-susceptible isolates that showed an inoculum effect were similar to those of ertapenem-susceptible isolates (Fig. 2A). OMP analysis, which was performed on these two strains after growth in the presence of ertapenem, revealed a lack of OMPK36, similarly to the OMP pattern of resistant strains, confirming the existence of an ertapenem-resistant subpopulation in the presence of the antibiotic (Fig. 2B).

DISCUSSION

This study describes the occurrence of a 3.7% rate of ertapenem resistance among a large collection of ESBL-producing *K. pneumoniae* hospital isolates in Israel. Most of these isolates were susceptible to imipenem and/or meropenem. Ertapenem resistance existed in multiple genetic clones of *K. pneumoniae* from various origins and was not due to clonal spread.

The molecular characterization of these ertapenem-nonsusceptible isolates revealed a lack of carbapenemases and indicated the presence of ESBL genes together with changes in permeability as a result of OMP changes. This mechanism was reported earlier for clinical strains of *K. pneumoniae* carrying SHV-2 and CTX-M enzymes (14, 17, 37). Moreover, it has been shown that other ESBL genes (SHV and CTX-M) as well as β -lactamases such as OXA-2 confer ertapenem resistance when introduced on a plasmid into a cured *K. pneumoniae* strain (18). Our ertapenem-resistant strains carried various ESBL genes, such as CTX-M-2 (not reported previously), CTX-M-10, SHV-12, and SHV-28 (not reported previously), together with the loss of OMPK36. One isolate, isolate 18

FIG. 2. (A) OMP profiles of ertapenem-sensitive *K. pneumoniae* isolates and ertapenem-resistant isolates (ertapenem-S and ertapenem-R, respectively). OMPK36 was present in ertapenem-sensitive isolates (*K. pneumoniae* ATCC strain 13883 [ATCC] and isolates 5 and 7, the two sensitive isolates with a positive inoculum effect). M, molecular size marker. (B) The OMP profile of isolate 7 (possessing an inoculum effect) with ertapenem (7+ERT) and without ertapenem (7) in growth medium demonstrated a lack of OMPK36 in the presence of the antibiotic similarly to the OMP pattern of resistant strains, supporting the preexistence of a resistant subpopulation that remains viable in the presence of ertapenem.

(MIC, 12 µg/ml), possessed CTX-M-2 and also had OMPK36 (Fig. 2A), suggesting that another mechanism is involved in ertapenem resistance and that this strain should be further investigated.

Significant discrepancies in ertapenem susceptibility testing using agar-based methods were observed in this study. These discrepancies varied with inoculum size and were found to be related to the existence of an ertapenem-resistant subpopulation that carried various ESBL genes, such as CTX-M-2, CTX-M-10, TEM-30, or SHV-44, and their permeability was affected by ertapenem-selective pressure. This is the first study to investigate discrepancies regarding ertapenem susceptibility testing and is the first to demonstrate the effect of ertapenem on the in vitro selection of resistant subpopulations.

This study defines two appearances of ertapenem resistance in ESBL-producing *K. pneumoniae* isolates. (i) Strains that have a resistant phenotype under various testing conditions and independently of the inoculum size (2% of the ESBL-producing isolates tested); these strains were ESBL producers and lacked OMPK36. (ii) Strains that were susceptible to a standard inoculum but show an inoculum effect (1.5% of the ESBL-producing isolates tested); these strains were found to have a rare preexisting resistant subpopulation due to the combination of common and rare ESBLs and OMPK36.

Since ertapenem increasingly is used to treat ESBL infections, these findings should be explored further. While it is clear that the strains that are ertapenem resistant under various test conditions should be reported as resistant and not be treated with ertapenem, it is not obvious how to report and treat the strains that are susceptible under standard testing conditions but have rare ertapenem-resistant subpopulations (1:1,000,000). Although rare, the correct detection of these strains is important. We tried to define phenotypic markers that may assist in differentiating between the inoculum effect isolates and the truly ertapenem-resistant isolates; the latter had meropenem MICs of 2 µg/ml or greater, while none of the isolates that possessed an inoculum effect had meropenem MICs of 1 µg/ml or above. The imipenem MIC was not a good indicator for distinguishing between them, as the imipenem MICs for all of these isolates actually were low in most cases.

Understanding the clinical significance of these strains is important. ESBL producers often are multidrug resistant, and if ertapenem can be used to treat these infections, the erroneous reporting of these isolates as resistant may limit significantly the treatment options. On the other hand, if these strains are more likely to fail ertapenem treatment, the misdiagnosis of this phenotype may have grave consequences. Further studies, including in vivo experimental modeling, using these strains at various inoculum sizes with ertapenem therapy may shed light on the clinical implications of these strains.

Our study confirms that among ESBL-producing *K. pneumoniae* isolates, a small (2%) but likely important population of ertapenem-resistant strains exists due to ESBL production combined with OMPK36 loss. Another small population (1.5%) has a rare preexisting subpopulation that may lead to false resistance test results when a high inoculum is used. The diagnostic implications of these findings need to be resolved, and clinical correlates are warranted.

ACKNOWLEDGMENTS

This work was supported by a grant from the Public Committee for the Designation of Estate Funds Ministry of Justice State of Israel.

This work was performed for the partial fulfillment of the requirements for the Ph.D. degree of A.L.

REFERENCES

1. Afzal-Shah, M., N. Woodford, and D. M. Livermore. 2001. Characterization of OXA-25, OXA-26, and OXA-27, molecular class D β-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* **45**:583–588.
2. Arakawa, Y., N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, and M. Goto. 2000. Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria using thiol compounds. *J. Clin. Microbiol.* **38**:40–43.
3. Bauvois, C., A. S. Ibuka, A. Celso, J. Alba, Y. Ishii, J. M. Frere, and M. Galleni. 2005. Kinetic properties of four plasmid-mediated AmpC β-lactamases. *Antimicrob. Agents Chemother.* **49**:4240–4246.
4. Bradford, P. A., C. Urban, N. Mariano, S. J. Projan, J. J. Rahal, and K. Bush. 1997. Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase, and the loss of an outer membrane protein. *Antimicrob. Agents Chemother.* **41**:563–569.
5. Bradford, P. A. 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* **14**:933–951.
6. Bratu, S., M. Mooty, S. Nichani, D. Landman, C. Gullans, B. Pettinato, U. Karumudi, P. Tolane, and J. Quale. 2005. Emergence of KPC-possessing *Klebsiella pneumoniae* in Brooklyn, New York: epidemiology and recommendations for detection. *Antimicrob. Agents Chemother.* **49**:3018–3020.
7. Bratu, S., D. Landman, R. Haag, R. Recco, A. Eramo, M. Alam, and J. Quale. 2005. Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* in New York City. *Arch. Intern. Med.* **165**:1430–1435.
8. Carmeli, Y., S. Lidji, E. Shabtai, S. Navon-Venezia, and M. J. Schwaber. 2007. Abstr. 47th Intersci. Conf. Antimicrob. Agents Chemother., abstr. K-396.
9. Castanheira, M., M. A. Toleman, R. N. Jones, F. J. Schmidt, and T. R. Walsh. 2004. Molecular characterization of a β-lactamase gene, *bla*_{GIM-1}, encoding a new subclass of metallo-β-lactamase. *Antimicrob. Agents Chemother.* **48**:4654–4661.
10. Chmelnitsky, I., Y. Carmeli, A. Leavitt, M. J. Schwaber, and S. Navon-Venezia. 2005. CTX-M-2 and a new CTX-M-39 enzyme are the major extended-spectrum β-lactamases in multiple *Escherichia coli* clones isolated in Tel Aviv, Israel. *Antimicrob. Agents Chemother.* **49**:4745–4750.
11. Clinical and Laboratory Standards Institute. 2005. Performance standards for antimicrobial susceptibility testing. Fifteenth informational supplement, M100–S15. Clinical and Laboratory Standards Institute, Wayne, PA.
12. Colodner, R., Z. Samra, N. Keller, H. Sprecher, C. Block, N. Peled, T. Lazarovitch, R. Bardenstein, O. Schwartz-Harari, and Y. Carmeli. 2007. First national surveillance of susceptibility of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella* spp. to antimicrobials in Israel. *Diagn. Microbiol. Infect. Dis.* **57**:201–205.
13. Doern, G. V., A. B. Brueggemann, R. Perla, J. Daly, D. Halkias, R. N. Jones, and M. A. Saubolle. 1997. Multicenter laboratory evaluation of the bioMérieux VITEK antimicrobial susceptibility testing system with 11 antimicrobial agents versus members of the family *Enterobacteriaceae* and *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* **35**:2115–2119.
14. Elliott, E., A. J. Brink, J. van Greune, Z. Els, N. Woodford, J. Turton, M. Warner, and D. M. Livermore. 2006. In vivo development of ertapenem resistance in a patient with pneumonia caused by *Klebsiella pneumoniae* with an extended-spectrum beta-lactamase. *Clin. Infect. Dis.* **42**:e95–e98.
15. Friedland, I. R., R. Isaacs, L. A. Mixson, M. Motyl, and G. L. Woods. 2002. Use of surrogate antimicrobial agents to predict susceptibility to ertapenem. *Diagn. Microbiol. Infect. Dis.* **43**:61–64.
16. Héritier, C., L. Poirel, D. Aubert, and P. Nordmann. 2003. Genetic and functional analysis of the chromosome-encoded carbapenem-hydrolyzing oxacillinase OXA-40 of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* **47**:268–273.
17. Jacoby, G., P. Han, and J. Tran. 1997. Comparative in vitro activities of carbapenem L-749,345 and other antimicrobials against multidrug-resistant gram-negative clinical pathogens. *Antimicrob. Agents Chemother.* **41**:1830–1831.
18. Jacoby, G. A., D. M. Mills, and N. Chow. 2004. Role of β-lactamases and porins in resistance to ertapenem and other β-lactams in *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **48**:3203–3206.
19. Jacoby, G. A., and L. S. Munoz-Price. 2005. The new β-lactamases. *N. Engl. J. Med.* **352**:380–391.
20. Kang, C. I., H. Pai, S. H. Kim, H. B. Kim, E. C. Kim, M. D. Oh, and K. W. Choe. 2004. Cefepime and the inoculum effect in tests with *Klebsiella pneumoniae* producing plasmid-mediated AmpC-type β-lactamase. *J. Antimicrob. Chemother.* **54**:1130–1133.
21. Lee, S. H., J. Y. Kim, S. K. Lee, W. Jin, S. G. Kang, and K. J. Lee. 2000.

- Discriminatory detection of extended-spectrum β -lactamases by restriction fragment length dimorphism-polymerase chain reaction. *Lett. Appl. Microbiol.* **31**:307–312.
22. Livermore, D. M., K. J. Oakton, M. W. Carter, and M. Warner. 2001. Activity of ertapenem (MK-0826) versus *Enterobacteriaceae* with potent β -lactamases. *Antimicrob. Agents Chemother.* **45**:2831–2837.
  23. Livermore, D. M., A. M. Sefton, and G. M. Scott. 2003. Properties and potential of ertapenem. *J. Antimicrob. Chemother.* **52**:331–344.
  24. Navon-Venezia, S., O. Hammer-Munz, D. Schwartz, D. Turner, B. Kuzmenko, and Y. Carmeli. 2003. Occurrence and phenotypic characteristics of extended-spectrum β -lactamases among members of the family *Enterobacteriaceae* at the Tel-Aviv Medical Center (Israel) and evaluation of diagnostic tests. *J. Clin. Microbiol.* **41**:155–158.
  25. Paterson, D. L., W. C. Ko, A. Von Gottberg, S. Mohapatra, J. M. Casellas, H. Goossens, L. Mulazimoglu, G. Trenholme, K. P. Klugman, R. A. Bono, L. B. Rice, M. M. Wagener, J. G. McCormack, and V. L. Yu. 2004. Antibiotic therapy for *Klebsiella pneumoniae* bacteremia: implications of production of extended-spectrum beta-lactamases. *Clin. Infect. Dis.* **39**:31–37.
  26. Paterson, D. L., F. Rossi, F. Baquero, P. Hsueh, G. L. Woods, V. Satishchandran, T. A. Snyder, C. M. Harvey, H. Teppler, M. J. DiNubile, and J. W. Chow. 2005. In vitro susceptibilities of aerobic and facultative gram-negative bacilli isolated from patients with intra-abdominal infections worldwide: the 2003 study for monitoring antimicrobial resistance trends (SMART). *J. Antimicrob. Chemother.* **55**:965–973.
  27. Poirel, L., S. Marque, C. Heritier, C. Segonds, G. Chabanon, and P. Nordmann. 2005. OXA-58, a novel class D β -lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* **49**:202–208.
  28. Schlesinger, Y., S. Navon-Venezia, I. Chmelnitsky, O. Hammer-Münz, A. Leavitt, H. S. Gold, M. J. Schwaber, and Y. Carmeli. 2005. Characterization of extended-spectrum β -lactamases (ESBLs) among *Enterobacter* isolates in Tel Aviv. *Antimicrob. Agents Chemother.* **49**:1150–1156.
  29. Schwaber, M. J., S. Navon-Venezia, D. Schwartz, and Y. Carmeli. 2005. High levels of antimicrobial core-resistance among extended-spectrum- β -lactamase-producing *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* **49**:2137–2139.
  30. Shah, P. M., and R. D. Isaacs. 2003. Ertapenem, the first of a new group of carbapenems. *J. Antimicrob. Chemother.* **52**:538–542.
  31. Steward, C. D., D. Wallace, S. K. Hubert, R. Lawton, S. K. Fridkin, R. P. Gaynes, J. E. McGowan, Jr., and F. C. Tenover. 2000. Ability of laboratories to detect emerging antimicrobial resistance in nosocomial pathogens: a survey of Project ICARE laboratories. *Diagn. Microbiol. Infect. Dis.* **38**:59–67.
  32. Steward, C. D., J. K. Rasheed, S. K. Hubert, J. W. Biddle, P. M. Raney, G. J. Anderson, P. P. Williams, K. L. Brittain, A. Oliver, J. E. McGowan, Jr., and F. C. Tenover. 2001. Characterization of clinical isolates of *Klebsiella pneumoniae* from 19 laboratories using the National Committee for Clinical Laboratory Standards extended-spectrum β -lactamase detection methods. *J. Clin. Microbiol.* **39**:2864–2872.
  33. Steward, C. D., J. M. Mohammed, J. M. Swenson, S. A. Stocker, P. P. Williams, R. P. Gaynes, J. E. McGowan, Jr., and F. C. Tenover. 2003. Antimicrobial susceptibility testing of carbapenems: multicenter validity testing and accuracy levels of five antimicrobial test methods for detecting resistance in *Enterobacteriaceae* and *Pseudomonas aeruginosa* isolates. *J. Clin. Microbiol.* **41**:351–358.
  34. Tenover, F. C., R. K. Kalsi, P. P. Williams, R. B. Carey, S. Stocker, D. Lonsway, J. K. Rasheed, J. W. Biddle, J. E. McGowan, Jr., and B. Hanna. 2006. Carbapenem resistance in *Klebsiella pneumoniae* not detected by automated susceptibility testing. *Emerg. Infect. Dis.* **8**:1209–1213.
  35. Tumbarello, M., M. Sanguinetti, E. Montuori, E. M. Trecarichi, B. Posteraro, B. Fiori, R. Citton, T. D'Inzeo, G. Fadda, R. Cauda, and T. Spanu. 2007. Predictors of mortality in bloodstream infections caused by extended-spectrum- β -lactamase-producing *Enterobacteriaceae*: importance of inadequate initial antimicrobial treatment. *Antimicrob. Agents Chemother.* **51**:1987–1994.
  36. White, R. L., M. B. Kays, L. V. Friedrich, E. W. Brown, and J. R. Koonce. 1991. Pseudoresistance of *Pseudomonas aeruginosa* resulting from degradation of imipenem in an automated susceptibility testing system with predried panels. *J. Clin. Microbiol.* **29**:398–400.
  37. Woodford, N., J. W. Dallow, R. L. Hill, M. F. Palepou, R. Pike, M. E. Ward, M. Warner, and D. M. Livermore. 2007. Ertapenem resistance among *Klebsiella* and *Enterobacter* submitted in the UK to a reference laboratory. *Int. J. Antimicrob. Agents* **29**:456–459.
  38. Wootton, M., R. A. Howe, R. Hillman, T. R. Walsh, P. M. Bennett, and A. P. MacGowan. 2001. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital. *J. Antimicrob. Chemother.* **47**:399–403.
  39. Wu, T. L., L. K. Siu, L. H. Su, T. L. Lauderdale, F. M. Lin, H. S. Leu, T. Y. Lin, and M. Ho. 2001. Outer membrane protein change combined with co-existing TEM-1 and SHV-1 β -lactamases lead to false identification of ESBL-producing *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* **47**:755–761.
  40. Yigit, H., A. M. Queenan, G. J. Anderson, A. Domenech-Sanchez, J. W. Biddle, C. D. Steward, S. Ablert, K. Bush, and F. C. Tenover. 2001. Novel carbapenem-hydrolyzing β -lactamase KPC-1 from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **45**:1151–1161.