

# Large Oligoclonal Outbreak Due to *Klebsiella pneumoniae* ST14 and ST26 Producing the FOX-7 AmpC $\beta$ -Lactamase in a Neonatal Intensive Care Unit

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A large outbreak caused by expanded-spectrum cephalosporin-resistant *Klebsiella pneumoniae* (ESCRKP) was observed in a neonatal intensive care unit (NICU) in central Italy. The outbreak involved 127 neonates (99 colonizations and 28 infections, with seven cases of sepsis and two deaths) over a period of more than 2 years (February 2008 to April 2010). Characterization of the 92 nonredundant isolates that were available for further investigation revealed that all of them except one produced the FOX-7 AmpC-type  $\beta$ -lactamase and belonged to either sequence type 14 (ST14) or ST26. All of the FOX-7-positive isolates were resistant to cefotaxime, ceftazidime, and piperacillin-tazobactam, while 76% were susceptible to cefepime, 98% to ertapenem, 99% to meropenem, and 100% to imipenem. The two carbapenem-nonsusceptible isolates had alterations in the genes encoding outer membrane proteins K35 and K36, which resulted in truncated and likely nonfunctional proteins. The outbreak was eventually controlled by the reinforcement of infection control measures based on a multitiered interventional approach. This is the first report of a large NICU outbreak caused by ESCRKP producing an AmpC-type enzyme. This study demonstrates that AmpC-type enzyme-producing strains can cause large outbreaks with significant morbidity and mortality effects (the mortality rate at 14 days was 28.5% for episodes of sepsis), and it underscores the role of laboratory-based surveillance and infection control measures to contain similar episodes.

*Klebsiella pneumoniae* is an important opportunistic pathogen in neonatal intensive care units (NICUs), where it can cause outbreaks of infections, primarily in premature infants (1, 2). Expanded-spectrum cephalosporins (ESC) have been a cornerstone in the treatment of *K. pneumoniae* infections, especially in the NICU setting, where the repertoire of selectable anti-Gram-negative drugs is further limited by the patients' characteristics. Therefore, the emergence of ESC-resistant *K. pneumoniae* (ESCRKP) is a matter of major concern in this clinical setting.

Outbreaks caused by successful clones of ESCRKP have been reported since the early 1990s for NICUs in several countries (3–5). Production of extended-spectrum  $\beta$ -lactamases (ESBLs) is the most common mechanism of ESC resistance in *K. pneumoniae*, and most of the ESCRKP outbreaks reported for NICU settings have been caused by ESBL-producing strains (6–14). Production of other  $\beta$ -lactamases, such as AmpC-type  $\beta$ -lactamases (ACBLs), can also be responsible for ESC-resistant phenotypes in *K. pneumoniae*, but the number of reported outbreaks caused by AmpC-producing ESCRKP is considerably more limited. Thus far, only ESCRKP strains producing DHA-type or CMY-type ACBLs, co-produced with ESBLs or metallo- $\beta$ -lactamases, have been involved in a minority of NICU outbreaks of relatively small dimensions (15–17). Due to their rare occurrence, outbreaks caused by ACBL-producing *K. pneumoniae* have been less studied, and scarce information is available on incidence, clonal relationships between isolates, clinical impact, and containment measures (18).

FOX-type  $\beta$ -lactamases represent a distinct lineage of ACBLs encoded by genes originating from *Aeromonas* spp. and spreading among *Enterobacteriaceae* via conjugative plasmids (18). At least 10 variants of these enzymes have been characterized in *Entero-*

*bacteriaceae*, including *K. pneumoniae*, *Escherichia coli*, and *Klebsiella oxytoca* (<http://www.lahey.org/Studies>). Thus far, no NICU outbreaks caused by strains producing this type of ACBL have been reported. In this work, we describe a large and long-lasting NICU outbreak caused by ESCRKP producing the FOX-7 enzyme, as well as the measures adopted to control the outbreak.

(Preliminary data were presented in part as a poster at ECCMID 2009, Helsinki, Finland, 16 to 19 May 2009 [19].)

## MATERIALS AND METHODS

**Hospital setting.** The University Hospital of Siena (in central Italy) is an 880-bed teaching hospital. The NICU performs approximately 200 admissions per year and contains 14 neonatal incubators (6 intensive and 8 semi-intensive) and 8 semi-intensive beds, located in two open areas (intensive care and semi-intensive care areas, respectively). The nurse/patient ratios are 1:2 and 1:4 in the intensive care and semi-intensive care areas, respectively.

**Definitions.** Carrier state was defined as isolation of ESCRKP from a nonsterile site (e.g., from a nasopharyngeal or rectal swab) without clinical evidence of disease and no need for specific therapy. Infection was defined as isolation of ESCRKP from a normally sterile site (e.g., from a central venous catheter [CVC], blood, or cerebrospinal fluid) or from a

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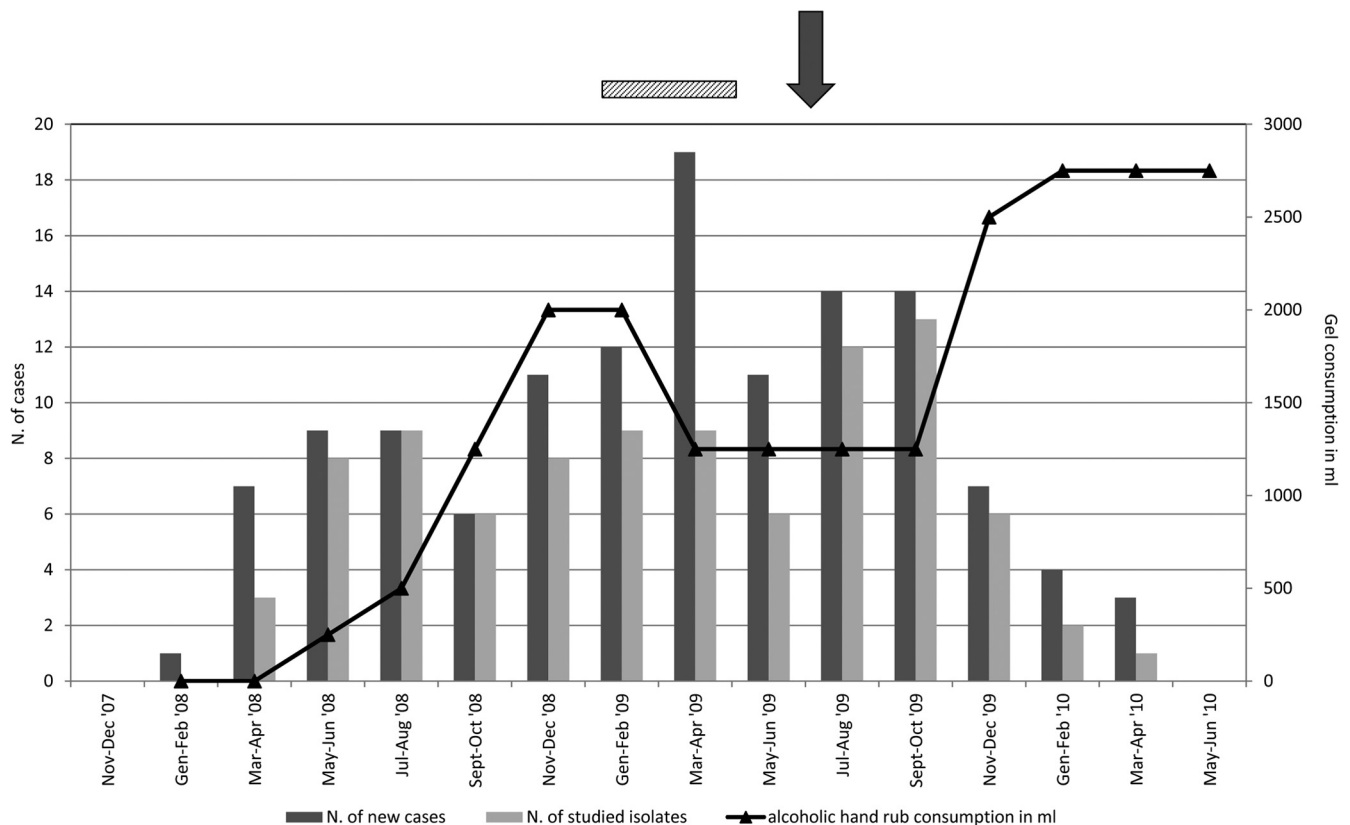
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**FIG 1** Evolution of the outbreak caused by ESC-resistant *K. pneumoniae* in the NICU. Dark gray columns, numbers of new cases (infection or colonization) reported bimonthly in the NICU; light gray columns, numbers of isolates available for analysis and investigated in this work regarding the mechanisms of resistance and clonality; black line, alcoholic hand rub gel consumption (scale at the right); hatched horizontal bar, period during which surveillance swab cultures were extended to all neonates; arrow, time of completion of the multitiered infection control intervention.

nonsterile site as a pure culture (e.g., from a conjunctival swab or lower respiratory tract specimen), concomitant with clinical diagnosis of infection by physicians caring for the patient and the need for specific therapy. Information on carriage or infection by ESCRKP was obtained from a review of clinical records.

**Infection control practices and outbreak containment measures.** Routine infection control practices in the NICU included (i) surveillance nasopharynx and rectal swab cultures to detect colonization by Gram-negative pathogens and fungi, performed on a weekly basis for all inpatients in the intensive care section, and (ii) environmental cultures from incubators, sinks, and endotracheal suctioning systems to detect the presence of microbial pathogens, performed on a weekly basis. The intervention aimed at reinforcing infection control practices to eliminate the outbreak consisted of (i) provision of alcohol-based hand rub bottles in two sizes (500 ml [at fixed positions] and 100 ml [portable]) in the ward since April 2008, (ii) extension of the surveillance nasopharynx and rectal swab cultures for detection of ESCRKP to all inpatients, on a weekly basis for 3 months, since January 2009, (iii) supplementary training for all health care workers, conducted in four internal meetings and aimed at increasing awareness of the problem and of known risk factors and educating workers about the infection control practices to be reinforced, including contact precautions and hand hygiene (alcohol-based hand rub use), in June 2009, (iv) use of reminders (posters) placed in the workplace since June 2009, and (v) performance of cross-sectional screening, using rectal swab cultures, for intestinal colonization by ESCRKP among NICU health care workers in June 2009 (Fig. 1).

During the outbreak, there were no changes in the usual cleaning practices adopted in the unit. Consumption of alcohol-based hand rub gel

was determined from the hospital pharmacy bimonthly reports and cross-checked with the records of substitution of empty bottles in their locations (for 500-ml bottles) and with the records of effective withdrawal of bottles from the ward's pharmacy by the NICU personnel (for 100-ml bottles).

**Bacterial isolates, identification, and susceptibility testing.** Microbiological data were obtained from retrospective analysis of the clinical microbiology laboratory database. Isolates that were obtained from the same patient and showed identical or very similar susceptibility patterns were considered replicates.

Identification of the isolates to the species level and antibiotic susceptibility testing were performed with the Vitek-2 automated system (bioMérieux, Marcy l'Etoile, France). MICs of selected antibiotics (imipenem, meropenem, ertapenem, cefepime, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, piperacillin-tazobactam, and tigecycline) were confirmed with Etest strips according to the instructions of the manufacturer (bioMérieux, Marcy l'Etoile, France). Susceptibility testing results were interpreted according to the criteria proposed by EUCAST ([http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST\\_files/Breakpoint\\_tables/Breakpoint\\_table\\_v\\_3.1](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_3.1)).

**Phenotypic and genotypic characterization of bacterial isolates.** ACBL production was investigated by the disk potentiation test with 3-aminophenylboronic acid (APB), as described previously (20). Production of ACBL was suspected for increases of  $\geq 5$  mm in the inhibition zone diameter around the cefotaxime- and/or ceftazidime-containing disks in the presence of APB. Cefotaxime-clavulanate and ceftazidime-clavulanate ESBL Etest strips were used for the detection of ESBL production, according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). Carbapenemase production was investigated with a spectropho-

tometric assay in crude cellular extracts, as described previously (21), using imipenem (150  $\mu$ M) as the substrate.

Genotyping by pulsed-field gel electrophoresis (PFGE) of genomic DNA was performed using the XbaI restriction enzyme and a CHEF-DRIII apparatus (Bio-Rad Laboratories), as described previously (22). Interpretation of results on the basis of comparative analysis of the PFGE patterns was performed according to current consensus criteria (23). Multilocus sequence typing (MLST) was carried out using the scheme proposed by Diancourt et al. (24); sequence types (STs) were assigned according to the online *K. pneumoniae* MLST database (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>).

**Investigation of  $\beta$ -lactamase and outer membrane protein genes.** The presence of genes encoding ACBLs was investigated by PCR using primers targeting the genes encoding the major lineages of acquired ACBLs, as described previously (25). The complete coding sequences of *bla*<sub>FOX</sub>-type genes were amplified with the primers FOX-F (5'-ATGCAA CAACGACGTGCG-3') and FOX-R (5'-GAGTCAGTTGGCCGAGTGA-3') and the following reaction conditions: 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, and a final extension step of 10 min at 72°C. Amplicons were sequenced on both strands at an external facility (Macrogen Inc., Seoul, South Korea), and the deduced protein sequences were compared with those available in the GenBank database by using the BLAST program (<http://blast.ncbi.nlm.nih.gov>). The presence of *bla*<sub>CTX-M</sub>, *bla*<sub>OXA-9</sub>, *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, and *bla*<sub>KPC</sub>  $\beta$ -lactamase genes was investigated using primers and methods described previously (26–29).

Genes encoding the OmpK35 and OmpK36 outer membrane proteins were amplified by PCR using primers and conditions described previously (30). Amplicons were sequenced on both strands at an external facility (Macrogen Inc., Seoul, South Korea), and sequences were compared with those of the *ompK35* (GenBank accession number AJ011501) and *ompK36* (GenBank accession number Z33506) genes as described previously (31, 32).

## RESULTS

**Outbreak description and containment measures.** During the period of February 2008 to April 2010, an outbreak of ESCRKP was observed in the NICU of Siena University Hospital (Fig. 1). The outbreak involved a total of 127 neonates, who were either infected ( $n = 28$ ) or colonized ( $n = 99$ ) by ESCRKP during hospital admission. Overall, 390 ESCRKP isolates were obtained from the neonates during the outbreak (average, 3.1 isolates per patient), of which 269 were from surveillance cultures (nasopharynx,  $n = 185$ ; rectum or stool,  $n = 84$ ) and 121 from clinical specimens (blood or central venous line specimens,  $n = 46$ ; lower respiratory tract specimens,  $n = 30$ ; urine samples,  $n = 11$ ; cerebrospinal fluid specimens,  $n = 4$ ; drainage specimens or swabs from other sites,  $n = 30$ ). During the same period, ESCRKP represented the vast majority (98%) of the *K. pneumoniae* isolates from the NICU.

A significant reduction in new cases was observed starting in October 2009, i.e., 3 months after completion of the infection control intervention. A complete absence of new cases was achieved in May 2010. Consumption of the alcohol-based hand rub gel, which was introduced in April 2008, increased drastically after completion of the infection control intervention (Fig. 1). All screening cultures for personnel (acceptance rate, 50%) and environmental surveillance cultures yielded negative results for ESCRKP.

**Characterization of ESCRKP isolates.** Of the total of 127 non-replicate ESCRKP isolates obtained during the outbreak period, 92 (80 from carriers and 12 from infected patients) were available for further microbiological investigations (Fig. 1). PFGE genotyping

of the 92 isolates revealed four major profiles (named A, B, C, and D) that differed from each other by more than 4 bands, with minor variants (differing by less than 4 bands) being detected for some of them (Fig. 2 and data not shown). Overall, 57 isolates belonged to PFGE type B, 24 to PFGE type A, 10 to PFGE type C, and one to PFGE type D. PFGE type A isolates were prevalent in the first part of the outbreak, while PFGE type B isolates emerged later and apparently replaced PFGE type A isolates. PFGE type C isolates were less common but were detected during the entire outbreak period. MLST analysis showed that isolates of PFGE types A and B belonged to ST14, while isolates of PFGE type C belonged to ST26 (Fig. 2).

All PFGE type A, B, and C isolates yielded positive results for disk potentiation tests with cefotaxime and ceftazidime plus APB, suggesting the production of an AmpC-type  $\beta$ -lactamase, while being negative for ESBL production. Molecular analysis revealed the presence of TEM-1 and FOX-7  $\beta$ -lactamase-encoding genes in all of the isolates. The *bla*<sub>FOX-7</sub> gene is a *bla*<sub>FOX</sub> variant that was originally characterized from sporadic isolates of *K. pneumoniae* and *Enterobacter cloacae* from the same NICU in 2004 (33) (GenBank accession number AJ703795).

The single PFGE type D isolate was positive for ESBL production and negative in the disk potentiation test with cefotaxime and ceftazidime plus APB. Molecular analysis revealed the presence of a *bla*<sub>SHV-12</sub> ESBL gene. No isolate tested positive for any of the screened carbapenemase genes (*bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, and *bla*<sub>KPC</sub>) or for *bla*<sub>CTX-M</sub>-type or *bla*<sub>OXA-9</sub> genes.

All of the FOX-7-positive isolates were resistant to cefotaxime, ceftazidime, and piperacillin-tazobactam, while 76% were susceptible to cefepime, 98% to ertapenem, 99% to meropenem, and 100% to imipenem. Of the two isolates that were resistant to ertapenem (MICs of  $>8$   $\mu$ g/ml), one also had intermediate resistance to meropenem (MIC of 4  $\mu$ g/ml) while the other exhibited a meropenem MIC of 2  $\mu$ g/ml, which is still in the susceptible range but is much higher than the modal MIC values of the other isolates ( $\leq 0.25$   $\mu$ g/ml). Concerning non- $\beta$ -lactam antibiotics, all FOX-7-positive isolates were susceptible to ciprofloxacin, while 85% were susceptible to gentamicin and 98% to tigecycline (Table 1). No differences in antimicrobial susceptibility patterns were found for isolates of different STs. The single ESBL-producing isolate was resistant to cefotaxime and ceftazidime and susceptible to all other drugs tested.

The two ertapenem-resistant isolates carried alterations in the genes encoding porins OmpK35 and OmpK36. In one isolate (that also had intermediate resistance to meropenem), the gene encoding OmpK35 was interrupted by an IS*Apu1* insertion sequence (inserted after nucleotide 770), while no significant alterations were detected in the gene encoding OmpK36. In the other isolate, the gene encoding OmpK36 carried a nonsense mutation at codon 223 (yielding a truncated protein lacking 142 amino acids at the carboxy terminus), while no significant alterations were detected in the gene encoding OmpK35. Spectrophotometric assays ruled out the production of carbapenemase activity in both isolates.

**Outcomes of infections caused by FOX-7-producing isolates.** Of the 91 studied isolates, 80 were from carriers and 11 from infected patients. Clinical diagnoses for infected infants were as follows: sepsis,  $n = 7$  (1 case with meningitis); urinary tract infections,  $n = 2$ ; conjunctivitis,  $n = 2$ . All neonates with diagnoses of sepsis were treated with carbapenems, alone or in association with gentamicin. Two infants with extremely low birth weights

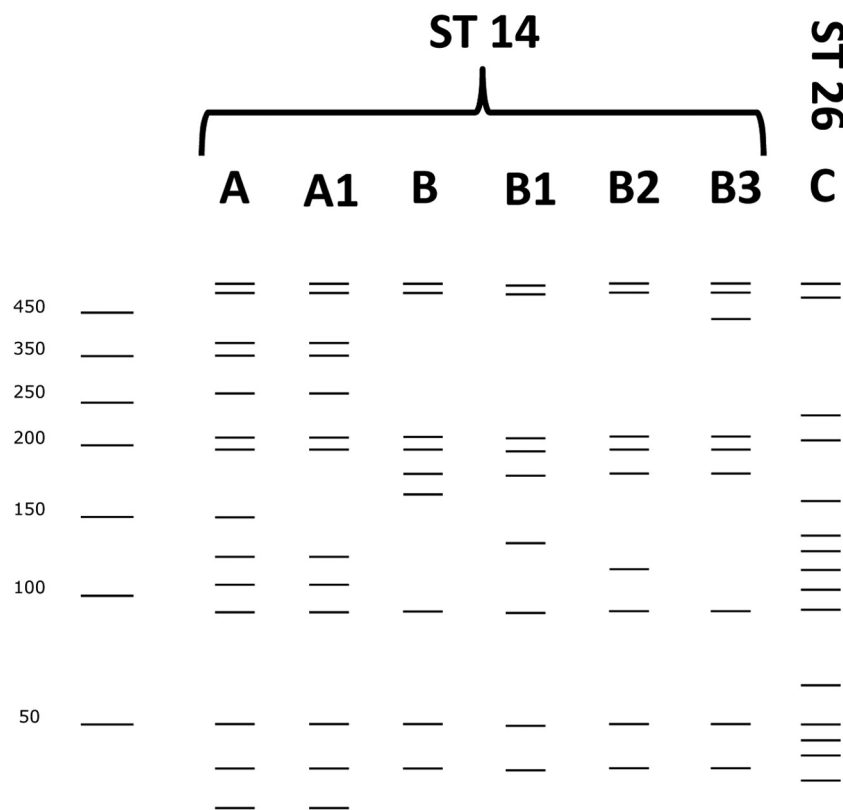


FIG 2 PFGE profiles and sequence types (STs) of the 91 FOX-7-producing isolates investigated in this work. DNA size standards are indicated on the left.

(<1,000 g) who were affected by sepsis (without meningitis) died. Considering episodes of sepsis caused by FOX-7-producing *K. pneumoniae*, the overall mortality rate at 14 days was 28.5%.

DISCUSSION

To the best of our knowledge, this is the first report of a large and long-lasting NICU outbreak caused by plasmid-mediated ACBL (pACBL)-producing *K. pneumoniae*. In fact, only sporadic cases and a few small outbreaks caused by similar strains have been described for NICU settings (15–17). This probably reflects the overall lower prevalence of pACBLs versus ESBLs among *Enterobacteriaceae*, although the difficulties in pACBL detection encountered in the clinical microbiology laboratory also could contribute

to underestimation of the circulation of these resistance determinants. This report also shows that the clinical impact of pACBL-producing *K. pneumoniae* can be comparable to that of ESBL-producing *K. pneumoniae* (10).

Among different pACBLs, the FOX-type enzymes are less prevalent than other types, such as CMY-type enzymes (18). In Italy, only one isolate of *K. oxytoca* carrying *bla*<sub>FOX-3</sub> has been characterized previously (34), while the presence of *bla*<sub>FOX-7</sub> in *K. pneumoniae* and *E. cloacae* isolates was reported previously for sporadic cases from the same NICU in 2003 to 2004 (33). To the best of our knowledge, no other cases of infection caused by FOX-7-producing *Enterobacteriaceae* have been documented. This work underscores how an infrequent resistance determinant, upon encoun-

TABLE 1 Antimicrobial susceptibility of the FOX-7-producing isolates<sup>a</sup>

Antibiotic	MIC breakpoints (μg/ml)		No. (%)			MIC (μg/ml)		
	Susceptible	Resistant	Susceptible	Intermediate	Resistant	Range	MIC <sub>50</sub>	MIC <sub>90</sub>
Cefotaxime	≤1	>2	0 (0)	0 (0)	91 (100)	32 to >32	32	32
Ceftazidime	≤1	>4	0 (0)	0 (0)	91 (100)	>32	>32	>32
Cefepime	≤1	>4	69 (76)	21 (23)	1 (1)	≤1 to 32	≤1	4
Piperacillin-tazobactam <sup>b</sup>	≤8	>16	0 (0)	1 (1)	90 (99)	16 to >64	>64	>64
Imipenem	≤2	>8	91 (100)	0 (0)	0 (0)	≤1	≤1	≤1
Meropenem	≤2	>8	90 (99)	1 (1)	0 (0)	≤1 to 4	≤1	≤1
Ertapenem	≤0.5	>1	89 (98)	0 (0)	2 (2)	≤0.5 to >32	≤0.5	≤0.5
Ciprofloxacin	≤0.5	>1	91 (100)	0 (0)	0 (0)	≤0.5	≤0.5	≤0.5
Gentamicin	≤2	>4	77 (85)	6 (6.5)	8 (8.5)	≤1 to >4	≤1	4
Tigecycline	≤1	>2	89 (98)	0 (0)	2 (2)	≤1 to >4	≤1	≤1

<sup>a</sup> The isolates belonged in ST14 (n = 81) and ST26 (n = 10).  
<sup>b</sup> The values shown are for piperacillin. The concentration of tazobactam was fixed at 4 μg/ml.

tering a successful host, can become prevalent in particular situations. In fact, the majority of studied isolates (81 of 92 isolates) belonged to ST14, which is a widespread “high-risk clone” of *K. pneumoniae* known for its ability to accumulate and to disseminate emerging resistance determinants, including those for extended-spectrum  $\beta$ -lactamases such as CTX-M-15 and for carbapenemases such as NDM-1 and KPC (35).

Control of the outbreak was apparently related to reinforcement of infection control measures based on increased awareness of the problem, hand hygiene reliance, and encouragement of alcohol-based hand rub use by health care workers. Interestingly, the simple introduction of hand rub gel soon after the beginning of the outbreak did not bring resolution. In fact, a trend to reduction in new cases was observed only upon completion of the intervention (Fig. 1), which underscores the importance of a multitered interventional approach to infection control practices. Altogether, this work confirms the key role played by infection control measures in the containment of outbreaks caused by *K. pneumoniae* in NICUs, regardless of the resistance mechanism involved (36–39).

Carbapenems represent a milestone in the treatment of infections caused by pACBL-producing *K. pneumoniae* isolates (40). In fact, all neonates affected by sepsis were treated with meropenem, alone or in association with gentamicin. For this reason, the finding of isolates with reduced susceptibility to carbapenems is a matter of concern. In those two isolates, the mechanism of resistance was due not to production of a carbapenemase but likely to reduced permeability of the outer membrane due to porin defects, in combination with ACBL production, as reported previously for *K. pneumoniae* strains producing other pACBLs (41, 42). Finally, this work underscores the key role played by the clinical microbiology laboratory in the reporting of hospital outbreaks caused by multidrug-resistant pathogens, rapid identification of emerging resistance mechanisms, and reconstruction of the epidemiological links existing between new cases, which are essential for successful infection control in health care settings.

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