Extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in a Neonatal Unit: Control of an Outbreak Using a New ADSRRS Technique

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Abstract

Extended-spectrum ß-lactamase (ESBL)-producing *Klebsiella pneumoniae* (EPKP) strains are frequently implicated in outbreaks in neonatal units. From April 2002 to January 2003, 149 neonates were colonized/infected with EPKP in the Neonatal Clinic of the Teaching Hospital at the Medical University of Gdańsk, Poland. A novel assay based on suppression of PCR, ADSRRS-fingerprinting, was successfully evaluated for typing EPKP isolates. The results showed that the genotypes of all outbreak-related strains were identical, which suggested that the outbreak originated from a single clone. This conclusion was confirmed by using different methods – RAPD and PFGE. The outbreak was stopped by adopting improved hygiene and instituting outbreak control measures.

K e y   w o r d s: *Enterobacteriaceae*, ESBL, PCR-fingerprinting, RAPD, ADSRRS-fingerprinting

Introduction

Klebsiella pneumoniae has been recognized as an important cause of infections in hospitalized neonates (Hart, 1993). Various environmental reservoirs have occasionally been identified in outbreaks caused by *K. pneumoniae* in such settings (Gaillot *et al*., 1998; Lalitha *et al*., 1999). Irrespective of the primary source, it seems that the most significant reservoir of the microorganism is the digestive tract of colonized patients, and that transmission occurs mostly *via* the hands of nursing staff (Coovadia *et al*., 1992; Hart, 1993). During the past decade, *K. pneumoniae* strains exhibiting resistance to newer cephalosporins due to the production of extended-spectrum β-lactamases (ESBLs) have been frequently implicated in outbreaks in pediatric hospitals and neonatal intensive care units (Bingen *et al*., 1993; Royle *et al*., 1999; Szabo *et al*., 1999; Venezia *et al*., 1995). These strains usually exhibit cross-resistance to other antibiotics, such as aminoglycosides. A report of imipenem-resistant *Klebsiella pneumoniae* is also very alarming (Ahmad *et al*., 1999). Therapeutic options are, therefore, limited. This problem is still emerging and occurring throughout the world, even at the beginning of the twenty-first century. This wide geographic spread of ESBLs was occurred due to transmission of strains between hospitals, horizontal transfer of resistance plasmids, or clonal expansion of epidemic strains. Rigorous compliance to the infection control program is one of the most important conditions in the control of outbreaks, and personnel education is a cardinal element. Moreover, education should be modified accordingly to the situation and infection control procedures should be improved. The success of the control of an outbreak depends also on the possibility of using quick, sensitive and discriminative tests as accurate epidemiological markers that permit identification of epidemic strains or spread of resistance plasmids.

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In recent years, genotyping methods have gained popularity and are frequently used to support and/or initiate epidemiological investigation. Macrorestriction analysis of genomic DNA, followed by pulsed-field gel electrophoresis (PFGE) has become the "gold standard" for molecular typing. PFGE, however, is limited in its resolving power (Gerner-Smidt *et al.*, 1998), and this contributes to difficulties with gel-to-gel and interlaboratory reproducibility (Van Belkum *et al.*, 1998). Consequently, several novel methods for DNA fingerprinting of medically important bacteria have received considerable attention for their suitability in epidemiological studies. Recently, we presented the performance and convenience of a novel assay based on the fingerprinting of bacterial genomes by amplification of DNA fragments surrounding rare restriction sites (ADSRRS-fingerprinting) for its potential usefulness in epidemiological investigation (Krawczyk *et al.*, 2003a; Krawczyk *et al.*, 2003b). This method is rapid, offers good discriminatory power and also demonstrates excellent reproducibility.

The aim of this study was to present an epidemiological investigation of the population of *K. pneumoniae* in the neonatal unit over a ten-month period. The isolates were analyzed in the context of clinical data and antimicrobial susceptibility. The performance and convenience of an ADSRRS-fingerprinting method for its potential usefulness in epidemiological investigation of *K. pneumoniae* outbreak is shown.

Experimental

Materials and Methods

Hospital setting. The outbreak occurred in the Neonatal Clinic of the Teaching Hospital of the Medical University of Gdańsk, Poland. The clinic consists of four neonatal wards: a Neonatal Intensive Care Unit, Pathology Unit, Septic Unit and Rooming-in Unit. This clinic serves an average of about 160 patients per month. Up to the year 2001, extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* (EPKP) strains were rarely isolated in the clinic. However, during 2002, a relatively high rate of isolation of EPKP strains in the clinic was observed. The infection surveillance reported here covers a period of ten months: from April 2002 to January 2003. During this period, 1582 neonatal patients were hospitalized in the clinic.

Patients and bacterial strains. After searching the computer microbiology database, we found 149 records of patients testing positive in cultures with ESBL-producing *Klebsiella pneumoniae*, and they were included in the study. The clinical data of patients were retrospectively collected from the medical records. The following sociodemographic variables and potential risk factors were assessed: gender, gestational age, mode of delivery, Apgar score, birth weight, mechanical ventilation, parenteral nutrition, length of hospital stay, type and length of antimicrobial treatment. Attempts to isolate EPKP strains from the environment were also made. Environmental screening was performed by using swabs moinstened with sterile saline and included work surfaces, sinks, incubators, solutions, and equipment used in intubation. Hand impressions were also taken in order to examine carriage of EPKP by the medical and nursing staff of the clinic.

Of all the 101 isolates of the micro-organism included in the analysis, 99 isolates were from neonates and 2 isolates were recovered from environmental examinations. Eighty-eight clinical isolates were non-repetitive (replicate) (first from each patient) and from 11 patients duplicate isolates were included in the study (from different specimens and different dates, the same specimen and different dates or different specimens and the same dates). Of all the 99 clinical isolates, 39 (39.4%) were obtained from throat swab specimens, 35 (35.4%) from rectal swab specimens, 9 (9.1%) from blood, 10 (10.1%) from urine, 5 from the respiratory tract and 1 from cerebrospinal fluid.

Infection control measures. After the reorganization of The Infection Control and Surveillance System in January 2003, the Epidemiology Department was set up. An infection control and surveillance system was modified and new priorities were established. The control of an outbreak due to ESBL-producing *Klebsiella pneumoniae* in the Neonatology Clinic was one of them. An outbreak was confirmed by epidemiological studies using molecular methods. New surveillance procedures were introduced and old procedures were improved. Extensive environmental, medical staff and patient microbiological screening were performed. Routine microbiological examinations from throat and rectal sites were performed in each newly admitted neonate at every 3 days of hospitalization from all patients without colonization/infection with the emerging strain, and at every 7 days from patients with established colonization/infections. A rigorous contact precaution was introduced, and separate nursing staff for colonized/infected neonates were designated. Five new nurses were employed in the clinic. Routine preventive use of disposable gloves for each new patient contact was implemented. Deficient invasive equipment of the Intensive Care Unit was purchased. Training sessions about surveillance methods for health care personnel (appropriate in content and vocabulary to the educational level) were organized, underlining the importance and the proper use of gloves, gown and hand hygiene. Microbiological examinations from all patients were reviewed by the Hospital Epidemiologist and referred to every day to the medical personnel of the clinic.

Identification and antimicrobial susceptibility tests. Species identification was performed with the use of a Vitek system (bioMérieux Vitek, Inc, Hazelwood, USA) and additional test (indol) recommended by the manufacturer. Susceptibility to common antibiotics was determined by disc diffusion methods with Mueller-Hinton agar according to the NCCLS. The following anti-microbials were tested: cefotaxime (30 μg), ceftazidime (30 μg), pipreracillin-tazobactam (100+10 μg), amoxicillin-clavulanate (20+10 μg), imipenem (10 μg), meropenem (10 μg), trimethoprim-sulfamethoxazol (1.25+23.75 μg), netilmicin (30 μg), amikacin (30 μg), aztreonam (30 μg) and ciprofloxacin (5 μg).

Screening and phenotypic confirmatory tests for ESBL. Routine susceptibility testing by the disc diffusion method with cefotaxime (30 μg) and ceftazidime (30 μg) was used for screening for ESBL (zone diameter: for ceftazidime = 22 mm and for cefotaxime = 27 mm).

The double disk synergy (DDS) test (Jarlier *et al.*, 1988), carried out with disks containing amoxicillin-clavulanate (20 + 10 µg), cefotaxime (30 µg) and ceftazidime (30 µg), was used as a phenotypic confirmatory test for ESBL production (increased size of inhibition zone or a new inhibition zone).

DNA isolation and typing methods. Total DNA isolation and ADSRRS-fingerprinting were performed as described previously (Krawczyk *et al.*, 2003a). The randomly amplified polymorphic DNA (RAPD) with primer RAPD-4 from the RAPD Analysis Primer Set (Pharmacia Biotech, St Albans, UK) and pulsed-field gel electrophoresis (PFGE) were performed according to the procedure described previously (Krawczyk *et al.*, 2003b).

Results

Outbreak description. One hundred and forty nine neonates hospitalized in the Neonatal Clinic of the large Teaching Hospital from April 2002 to January 2003 were colonized/infected by ESBL-producing *Klebsiella pneumoniae*. In the reported period, 86 (5.4%) neonates were colonized and 63 (4%) were found to be infected. The average percent of colonized/infected patients during each month was from 6.2% to 22.4% (Table I), and the increase in colonized infants seen in April and December 2002 was due to active surveillance. Mean gestational age of the study group was 32 weeks, most of them were born by cesarean section (69.1%) with mean birth weight about 1921 g and Apgar score 6.4 (see Table II). In about 86% of

Table I
Percent of colonized/infected patients in ten months of study period
and four months follow up

Month and year	No of hospitalized patients	No of colonized/ infected patients	Percent of colonized/ infected patients
April 2002	173	25	14.4
May 2002	166	11	6.6
June 2002	164	22	13.4
July 2002	163	19	11.7
August 2002	168	17	10.1
September 2002	135	16	11.8
October 2002	161	26	16.1
November 2002	150	21	14.0
December 2002	156	35	22.4
January 2003	146	9	6.2
February 2003	169	6	3.5
March 2003	175	12	6.9
April 2003	174	1	0.6
May 2003	175	none	0.0

Table II
Clinical data of the neonates including to the study

	Genotyping (+)	Genotyping (−)	All study group
No. of neonates	88	61	149
Gender: male	49	38	87
female	39	23	62
Gestational age, mean, wk	33.1	33.5	32
Mode of delivery: cesarean section, n (%)	62 (70.4%)	41 (67.2%)	103 (69.1%)
Apgar score at 1 min, mean	6.3	6.6	6.4
Birth weight, mean, g	1883.9	1975.5	1921.4
Mechanical ventilation, n (%)	29 (32.9%)	21 (34.4%)	50 (33.5%)
Parenteral nutrition, n (%)	76 (86.4%)	52 (85.2%)	128 (85.9%)
Length of hospital stay, mean	41,3	36.3	393
Antimicrobial treatment, n (%)	78 (88.6%)	52 (85.2%)	130 (87.2%)

them parenteral nutrition was performed in the first few days, and 87.2% of them had undergone antimicrobial treatment. More than one-third (33.5%) of the neonates needed mechanical ventilation and their mean length of hospital stay was 39.3 days (min. 4 days, max. 130 days) (Table II). ESBL-producing *Klebsiella pneumoniae* was isolated in 6.7% of patients from blood, 20.8% had urinary tract infections, and pneumonia was recognized in 12.8%. From 149 neonates included in the study, 63 (57.7%) were found to be only colonized by *Klebsiella pneumoniae* in the throat and/or alimentary canal.

Only two strains of EPKP were isolated during the environmental screening carried out several times (165 samples) in the reported time period.

Identification and antimicrobial susceptibility. All of the isolates were identified as *Klebsiella pneumoniae*. As determined by disc-diffusion antibiotic susceptibility testing, the isolates, including those two from environmental sites, exhibited almost the same pattern of resistance. The percentage susceptibility rates to cefotaxime, ceftazidime and azteronam were 0%, 99% and 0%, respectively. All isolates were susceptible to meropenem, imipenem and ciprofloxacin. However, susceptibility to pipreracillin-tazobactam varied, and 82% of isolates were susceptible. Only 8% were susceptible to amoxicillin-clavulanate, 5% to amikacin and netilmicin and 4% to trimethoprim-sulfamethoxazol.

Genotyping of isolates using ADSRRS-fingerprinting, RAPD and PFGE techniques. We investigated the clonally relatedness among outbreak ESBL-producing *Klebsiella pneumoniae* strains. One hundred and one isolates were included for typing by ADSRRS-fingerprinting, RAPD and PFGE methods. Four additional, epidemiologically unrelated EPKP strains were included for control purposes. The obtained ADSRRS-fingerprinting patterns for representative isolates are presented in Fig. 1. Each pattern consisted of approximately 12 to 15 fragments in a size range of 200 to 1000 bp. The ADSRRS-fingerprinting patterns of the 99 *K. pneumoniae* isolates recovered from patients found only one unique profile, and this confirmed that a clonal spread of the emerging pathogen was established. Two isolates recovered from environmental examinations (sink) also belonged to the same cluster of genotypes.

The RAPD and PFGE results were exactly in accordance with ADSRRS-fingerprinting patterns and revealed also one predominant pattern (results not shown).

The isolates not-related to the outbreak and reference strains showed completely different patterns by using ADSRRS-fingerprinting (Fig. 1), RAPD and PFGE (results not shown) methods.
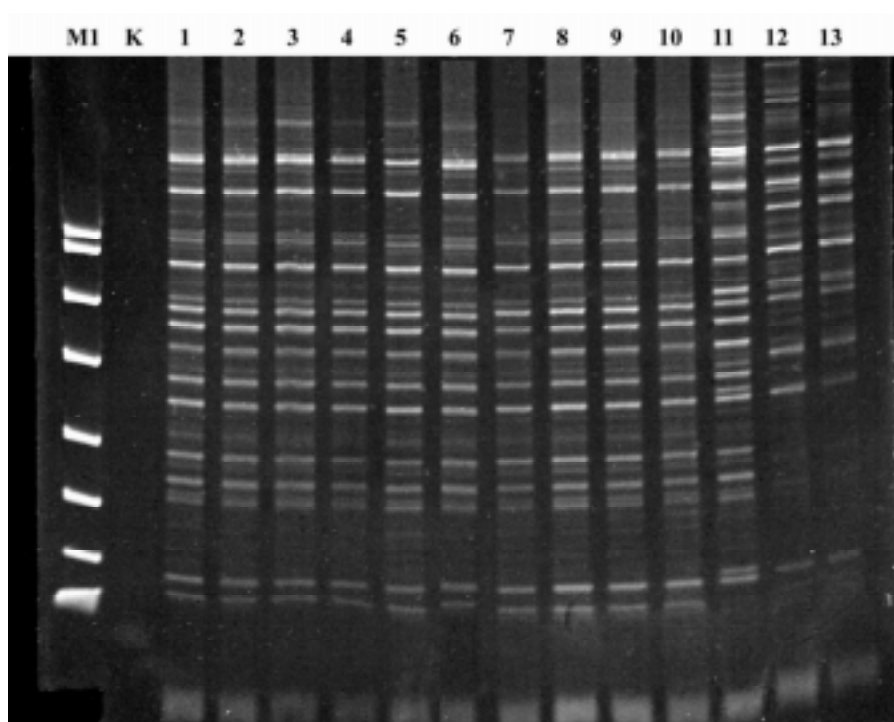

Fig. 1. ADSRRS fingerprints of the *K. pneumoniae* isolates; representative results

The lane designated M1 contained molecular mass marker (501, 489, 404, 331, 242, 190, 147 and 110 bp); lane K, negative control; lane 1–8, representative isolates from patients; lane 9–10, environmental isolates; lane 11, reference strain, lanes 12–13, related *K. pneumoniae* strains isolated from other clinic. The DNA fragments were electrophoresed in 8% polyacrylamide gel by using 1×Tris-borate EDTA running buffer at a field strength of 8 V cm^{-1}

Control measures. In the epidemiological inquest, we found that hygiene insufficiency (no adherence to the hand hygiene procedure, medical equipment sterilization, contact precautions of colonized patients) led to an outbreak, and extensive training of health care personnel was begun. In the next five months we reduced the transmission of endemic strain progressively, to succeed in May 2003 when none of the neonates were colonized/infected with ESBL-producing *Klebsiella pneumoniae.*

Discussion

Klebsiella pneumoniae often leads to nosocomial outbreaks due to its ability to spread among patients, mainly because of lack of adherence by medical personnel to infection control guidelines (Asensio *et al.*, 2000; Podschun and Ullmann, 1998; Szabo *et al.*, 1999). In general, the use of a third-generation cephalosporins was reported as a risk factor for acquisition of ESBLs (Asensio *et al.*, 2000; Jain *et al.*, 2003; Naumovski *et al.*, 1992). In our clinic, third-generation cephalosporins were used only occasionally. According to the antibiotic policy, amoxicillin with clavulanic acid, with or without aminoglicosides (mostly amikacin) was used as the first-line therapy. According to some investigators, antimicrobial combinations may also be a risk factor for colonization by multi-resistant pathogens (Pessoa-Silva *et al.*, 2003; Podschun and Ullmann, 1998). In our study 78.5% of neonates received amoxicillin with clavulanic acid and 45.6% of neonates amoxicillin/clavulanic acid with aminoglicoside (amikacin in 80.9%). However, we controlled an outbreak without changing the antibiotic policy, and, therefore, we concluded that a lack of adherence to infection control guidelines led to the spread of ESBL-producing *Klebsiella pneumoniae*, and that antibiotic treatment was only conducive in colonization of the digestive tract.

During the study period ESBL-producing *Klebsiella pneumoniae* were found in the hospital environment (sink), but we thought that it was simply another site of contamination rather than a source of pathogen. The gastrointestinal tract of neonates was the principal reservoir of the pathogen, and there was no adherence to hygiene procedures which were crucial. In spite of extensive screening of hands because we suspected transmission of the emerging strain by health care personnel, we didn't find this pathogen. However, we know it can be found for a short period of time on the skin surface (Podschun and Ullmann, 1998). During the hospital epidemiologist control study, some non-adherence to the guidelines was found: lack of contact precautions, improper use of gloves and gowns, inappropriate hand hygiene, no sterilization of nipples, wrong storage of laryngoscopes, and insufficient intensive care equipment. All of the infractions were eliminated, and the outbreak was controlled. We would like to emphasize that supplementation of deficient invasive equipment of the Intensive Care Unit and employing five new nurses was very important. Good communication between microbiologists, epidemiologists and neonatologists was crucial too, which has been emphasized previously by others (Asensio *et al.*, 2000).

Molecular epidemiology is irreplaceable in the identification of an outbreak. The clonal spread of an emerging pathogen was established, and it was very convincing to the medical personnel, which led to rigorous compliance with infection control guidelines. The origin of the resident strain and its mode of spread in the clinic were not elucidated.

Differentiation of bacterial isolates by DNA fingerprinting facilitates epidemiologic studies and disease control. In the examined *K. pneumoniae* outbreak, ADSRRS-fingerprinting, RAPD and PFGE methods showed that the genotypes of all outbreak-related strains were identical, which suggested that the outbreak originated from a single clone.

Although a particular typing method may have high discriminatory power and good reproducibility, the complexity of the method and interpretation of results, as well as the costs involved in setting up and using the method, may be beyond the capabilities of the laboratory. The choice of the molecular typing method, therefore, will depend upon the needs, skill level, and resources of the laboratory. The ADSRRS-fingerprinting is generally a simple technique with high discriminatory power and is low cost and may be most suitable for epidemiological studies (Masny and Płucienniczak, 2001). This method may be equally attractive in comparison to PFGE or AFLP for storage of genetic profiles and for the creation of reference databases of organisms to which new outbreak strains can be compared across laboratories in order to monitor changes in microbial populations.

Here we show the evaluation of a novel fingerprinting method (ADSRRS-fingerprinting) for epidemiological studies of *K. pneumoniae* outbreak. Using three genotyping methods (ADSRRS-fingerprinting, RAPD and PFGE), we showed that in an outbreak one genotype was responsible for the infections/colonisations, and although the ADSRRS-fingerprinting method may appear to be more complex than the RAPD technique,

we found it fast and reproducible, as we showed previously for epidemiological studies of *E. faecium* (Krawczyk *et al.*, 2003a) and *S. marcescens* (Krawczyk *et al.*, 2003b).

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