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High prevalence of extended spectrum beta-lactamase (ESBL) producers in fatal cases of pediatric septicemia among the Enterobacteriaceae in the pediatric hospital of Annaba, Algeria

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The aim of this study was to determine the prevalence of extended spectrum beta-lactamase (ESBL) producers and to perform molecular typing of ESBL-encoding genes in Enterobacteriaceae from clinical isolates recovered from blood samples of children in Annaba, Algeria. A total of 42 clinical isolates were collected from March 2010 to July 2011 from the pediatric hospital of Annaba. The strains were identified by phenotypic tests and the ESBL-encoding genes was accomplished by PCR amplification and sequencing. Among these isolates, 10 ESBL isolates had CTX-M-15, 13 had TEM-1, one isolate had TEM-136, 2 were positive for SHV-11, 8 had SHV-12, 3 had SHV-28, 1 isolate contained SHV-32, and 1 isolate had SHV-133. A total of 45.2% of the patients died. The high prevalence of ESBL producers among Enterobacteriaceae, along with the observation of 19 fatal cases, is worrisome; therefore, we believe that national surveillance of antibiotic resistance should be urgently implemented in Algeria.

Key words: Antibiotic resistance, Enterobacteriaceae, Algeria, septicemia.

INTRODUCTION

Antibiotic resistance has become a global health problem during the past two decades (Rolain et al., 2012). Combination of issues has created conditions that lead to the dissemination and selection for resistant bacteria, such as population density, uncontrolled use of antibiotics, lack of clean water supply, and lack of proper treatment for sewage and industrial effluents (Bush et al., 2011).

Most of the extended spectrum beta-lactamases (ESBLs) detected in Enterobacteriaceae were of the TEM and SHV types until 1989, when a new type of ESBL of the CTX-M family with a high level of resistance to cefotaxime was discovered in *Escherichia coli* in Germany (Anastay et al., 2012). This ESBL type had previously been described in other species of Enterobacteriaceae in 1991 and, specifically, in *Salmonella enterica* in France, Argentina, Senegal and Algeria; therefore, antibiotic resistance associated with the acquisition of ESBLs, CTX-M-15 in particular, is emerging at a spectacular rate (Edelstein et al., 2003; Weill et al., 2004; Touati et al., 2008); and is considered one of the most frequently

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occurring ESBLs in the world (Anastay et al., 2012).

The economic and human cost caused by ESBLs is increasing at an alarming rate. For instance, in 2007, antibiotic-resistant infections were responsible for 2,500 deaths in Europe with a loss of 2.5 million Euros and supplementary hospital costs that exceeded 1.5 billion Euros (Bush et al., 2011). The empirical treatment has a high incidence, particularly due to the widespread prescription of third generation cephalosporins, (C3Gs), which result in high levels of ESBL dissemination. The occurrence of ESBLs is often overlooked due to inadequate detection techniques, which lead to an underestimation of their prevalence at both the local and national levels (De Kraker et al., 2011).

Since 2006, few reports have been published on the current situation in Algeria. Little information is available on pediatric infections caused by extended spectrum β-lactamases, particularly from children whose infections lead to sepsis- a major public health crisis (Nedjai et al., 2012; Oteo et al., 2012). Several studies have shown that sepsis is a major cause of mortality in hospitalized patients, and about $17 billion is spent annually on its treatment (Allareddy et al., 2012).

According to the World Health Organization (WHO year), sepsis is the fourth leading cause of death in children younger than four years of age (Bryce et al., 2005). In developing countries, the rate of sepsis in children in pediatric intensive care units (PICUs) is higher than 50% (Khan et al., 2012).

Information regarding the molecular epidemiology and the current knowledge about the prevalence of ESBLs in Enterobacteriaceae isolated from blood culture samples from children in Algeria is cited in this paper. This study is the first in Algeria that addresses pediatric sepsis mortality caused by Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial isolates

A total of 42 non-replicated Enterobacteriaceae isolates were collected from pediatric blood culture samples with citrate broth at the Laboratory of Microbiology, the Pediatric Hospital of Annaba (Algeria) from March 2010 to July 2011. The identification of Enterobacteriaceae species was performed using the API 20 E system (Biomerieux, Marcy l'etoile, France) and confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Seng et al., 2009). The number of patients with sepsis caused by bacterial isolates of different ESBL types and the clinical outcomes for these patients are shown in Table 1.

Antimicrobial susceptibilities

Antimicrobial susceptibility was determined for the following 25 antimicrobial agents by the disk diffusion method on Mueller-Hinton agar according to the French Society for Microbiology guidelines (Soussy et al., 2012): piperacillin (75 µg), ticarcillin (75 µg), amoxicillin/clavulanic acid (20/10 µg), piperacillin/tazobactam (75/10 µg), cefazolin (30 µg), cefoxitin (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), Aztreonam (30 µg), imipenem (10 µg), amikacin (30 µg), gentamicin (15 µg) nalidixic acid, (30 µg), ofloxacin (5 µg), pefloxacin (5 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), tetracycline (30 µg), colistin (50 µg), nitrofurantoin (300 µg), trimethoprim/ sulfamethoxazole (1.25/23.75 µg) and fosfomycin (50 µg).

Phenotypic detection of ESBL

Isolates showing an inhibition zone size of ≤ 18 mm with cefoxitin or 22 mm with ceftazidime were identified as potential ESBL producers, as recommended by the Antibiogram Committee of the French Microbiology Society (CA-SFM) recommendations and were selected for the sequencing of the genes encoding CTX-M, TEM and SHV. Screening of the 42 strains to test for ESBL secretion in Enterobacteriaceae strains was performed by double disk synergy test (DDST) as described by Jarlier et al. (1988) using a central amoxicillin + clavulanic acid disk 20 mm away from cefotaxime, ceftazidime or Aztreonam disks. The presence of ESBLs was indicated by a champagne cork aspect.

Detection of resistance genes

Detection of the ESBL genes encoding CTX-M, TEM, and SHV was performed with total DNA; briefly, a fresh bacterial colony was suspended in 200 µl of sterile deionized water and stored at -20°C for PCR assays. A commercial Master Mix was used (Quantitect Probe PCR Master mix, Qiagen), and the primers used are given in Table 2. Amplification was carried out on a DNA thermal cycler (Multigene Labnet International, Inc.) as follows: initial denaturation at 95°C for 5 min; 35 cycles of 94°C for one min, 55°C for 50 s, 72°C for 1 min; and a final elongation step at 72°C for 7 min (Edelstein et al., 2003; Kruger et al., 2004; Yagi et al., 2000).

The amplicons were visualized after electrophoresis at 150 V for 30 min on a 1% agarose gel containing ethidium bromide and visualized with an E-BOX VILBER, printed by a Sony digital graphic printer UP897, and sequenced using the Big Dye R Terminator V3.1 Cycle sequencing kit. The sequences were synthesized with an ABI 3100 automated Sequencer (Applied, Biosystems, Foster City, CA) and then analyzed with Codon code aligner software; comparisons with known sequences were made using the BLAST software provided by the National Center of Biotechnology Information (NCBI).

RESULTS

A septicemia was documented in 42 inpatients during the study period. The patients had a median age of 2 years and 10 months (range 2 months to 14 years); 32 (76.19%) were male. Various conditions were diagnosed among patients, 26.19% had leukemia; 14.28% had lymphoma; 11.90% had respiratory distress and cystitis; 9.52% had leishmaniasis Kala Azar; 7.14% had acute pyelonephritis; 4.76% had anemia, chronic obstructive-broncholitis and ND; and 2.38% had meningitis and rheumatic fever. A total of 45.23% of cases were fatal.

Among the 42 Enterobacteriaceae blood culture strains collected, 34 had ESBL phenotype; therefore, 80.95% of the isolates were defined as extended-spectrum β-lactamase (ESBL) producers according to DDST method. The isolates tested and the rates of ESBLs producers were

Table 1. Characterization of clinical isolates of *Klebsiella pneumoniae, Enterobacter cloacae, Escherichia coli, and Serratia marcescens* from blood samples

Patient profile						CTX-M	TEM	SHV	Outcome
code	Sex	Age (months)	Reasons for hospitalization	Strain	Antibiotic phenotype	Type	Type	Type	
[1]	M	2	Cystitis	*K. pneumoniae*	ESBL		-	SHV-12	Recovery
[2]	F	3	Severe chronic obstructive bronchiolitis	*K. pneumoniae*	ESBL	CTX-M-15	-	-	Recovery
[3]	M	5	RF	*K. pneumoniae*	ESBL	CTX-M-15	-	SHV-11	Recovery
[4]	M	6	ALL	*K. pneumoniae*	ESBL	CTX-M-15	-	SHV-12	Recovery
[5]	M	7	Acute pyelonephritis	*K. pneumoniae*	ESBL	CTX-M-15	TEM-1	SHV-12	Recovery
[6]	M	9	Respiratory distress	*K. pneumoniae*	ESBL		-	SHV-32*	Death
[7]	M	10	Leukemia	*K. pneumoniae*	ESBL	CTX-M-15	-	SHV-28*	Death
[8]	M	10	ALL	*K. pneumoniae*	ESBL		-	SHV-11*	Recovery
[9]	M	12	ALL	*K. pneumoniae*	ESBL		-	-	Recovery
[10]	F	18	Lymphoma	*K. pneumoniae*	ESBL		-	SHV-133*	Death
[11]	F	24	ND	*K. pneumoniae*	CASE	CTX-M-15	-	-	Recovery
[12]	M	24	Respiratory distress	*K. pneumoniae*	WILD TYPE		-	SHV-28	Death
[13]	F	30	Leukemia	*K. pneumoniae*	ESBL		-	-	Death
[14]	M	36	Leukemia	*K. pneumoniae*	CASE+PASE		TEM-1	-	Death
[15]	M	48	Lymphoma	*K. pneumoniae*	ESBL		-	-	Death
[16]	M	48	Lymphoma	*K. pneumoniae*	ESBL		-	-	Death
[17]	M	48	Chronic obstructive broncholitis	*K. pneumoniae*	WILD TYPE		-	-	Recovery
[18]	M	60	Meningitis	*K. pneumoniae*	ESBL		-	-	Recovery
[19]	M	60	Cystitis	*K. pneumoniae*	ESBL		TEM-1	SHV-28	Recovery
[20]	F	72	Acute pyelonephritis	*K. pneumoniae*	ESBL		-	-	Recovery
[21]	M	72	Lymphoma	*K. pneumoniae*	ESBL+CASE	CTX-M-15	-	-	Recovery
[22]	M	156	Respiratory distress	*K. pneumoniae*	ESBL	CTX-M-15	-	-	Death
[23]	M	168	ND	*K. pneumoniae*	WILD TYPE		-	-	Recovery
[24]	M	3.3	Leukemia	*K. pneumoniae*	ESBL		TEM-1	SHV-12	Death
[25]	F	3	Cystitis	*E. cloacae*	ESBL+CASE	CTX-M-15	TEM-1	SHV-12	Recovery
[26]	F	5	Leishmaniasis kala-azar	*E. cloacae*	PASE		-	-	Recovery
[27]	F	5	ALL	*E. cloacae*	ESBL		TEM-1	-	Recovery
[28]	M	5	Anemia	*E. cloacae*	ESBL+CASE		TEM-1	-	Recovery
[29]	M	8	Pulmonary + Anemia	*E. cloacae*	CASE HYPER PRODUCT		TEM-1	SHV-12	Recovery
[30]	F	24	Leishmaniasis kala-azar	*E. cloacae*	ESBL+CASE	CTX-M-15	TEM -136*	SHV-12	Death
[31]	M	24	Acute pyelonephritis	*E. cloacae*	ESBL+CASE		TEM-1	-	Death
[32]	M	36	Respiratory distress	*E. cloacae*	ESBL+CASE		TEM-1	-	Death
[33]	M	36	Cystitis	*E. cloacae*	ESBL+CASE		TEM-1	SHV-12	Death
[34]	M	48	Lymphoma	*E. cloacae*	ESBL+CASE		TEM-1	SHV-12	Death
[35]	M	108	Leukemia	*E. cloacae*	ESBL+CASE		-	-	Death

Patient profile						CTX-M	TEM	SHV	Outcome
code	Sex	Age (months)	Reasons for hospitalization	Strain	Antibiotic phenotype	Type	Type	Type	
[36]	M	2	Leishmaniasis kala-azar	*E.coli*	ESBL		-	-	Death
[37]	M	24	Leishmaniasis kala-azar	*E.coli*	PASE		TEM-1	-	Recovery
[38]	F	168	ALL	*E.coli*	ESBL		-	-	Recovery
[39]	M	8	Cystitis	*S. marcescens*	ESBL+CASE		-	-	Recovery
[40]	M	8	Respiratory distress	*S. marcescens*	ESBL		-	-	Death
[41]	M	12	Lymphoma	*S. marcescens*	ESBL+CASE		-	-	Death
[42]	M	12	Anemia	*S. marcescens*	ESBL		-	-	Recovery

Table 2. Primers and probes used in this study.

Target	Primer name	Primer sequence	Amplicon size (bp)	Reference/source
CTX-M	CTX Szabo R	TGTGCAGYACCAGTAARGTKATGGC	334	Edelstein et al., 2003
	CTX Szabo F	TCACKCGGRTCGCCNGGRAT		
TEM	TEM Szabo R	ATGAGTATTCAACATTTCCGTG	862	Kruger et al., 2004
	TEM Szabo F	TTACCAATGCTTAATCAGTGAG		
SHV	SHV Szabo R	ATTTGTCGCTTCTTTACTCGC	998	Yagi et al., 2000
	SHV Szabo F	TTTATGGCGTTACCTTTGACC		

as follow: *Serratia marcescens* (n=4), 100%; *Enterobacter cloacae* (n=11), 81.81%; *Klebsiella pneumonia* (n=19), 79.16%; and *Esherichia coli* (n=3), 66.66%. The antimicrobial resistance pattern of the isolates is shown in Figure 1. PCR amplification using specific primers, and sequencing showed that one ESBL-producing isolate of *E. cloacae* contained TEM-136, and five *K. pneumoniae* had the following genes: SHV-28 (n=3), SHV-32 (n=1) and SHV-133 (n=1).

Seventeen of 34 (50%) patients with ESBL-producing isolates died while in the hospital within 20 days after admission, and 2 of 8 (25%) patients with non-ESBL-producing isolates died within 10 days after admission. Although the mortality associated with non-ESBL-producing isolates is greatest during the first days after admission, the mortality associated with sepsis due to ESBL-producing isolates peaked more than 20 days after admission. Patients with septicemia due to ESBL-producing strains had a significantly higher fatality rate than those with non-ESBL-producing isolates (50% versus 25%, P=0.038).

DISCUSSION

Our understanding of the origin and spread of antibiotic resistance in the microbial community remains low. Antibiotic resistance genes can be found in areas with only minimal or hypothetical antibiotic exposure, such as municipal waste water treatment plant effluents in the Czech Republic, aquaculture facilities in northwestern Wisconsin, USA, and in human communities living in very remote areas (Peruvian Amazonas) (Rolain et al., 2012; Dolejska et al., 2011; Seyfried et al., 2010 ; Bartoloni et al., 2009).

There is a lack of epidemiological data in the Maghrebian countries, and very few studies are published concerning ESBLs among the Enterobacteriaceae (Bourjilat et al., 2011; Lahlaoui et al., 2012) (Table 3). Comparing data from Mediterranean regions according to the resistance surveillance system of 2009 (EARSS), the incidence of ESBL-producing *K. pneumoniae* (ESBL-KP) lies between 73 and 100% in the following countries: Greece, Hungary, Latvia, Lithuania, Romania, Bulgaria, and Macedonia. Regarding ESBL-producing *E. coli* (ESBL-EC), the incidence ranges between 85 and 100% in more than half of the reporting countries, including Germany, France, Belgium, Denmark, Norway, Finland, Ireland, Spain, UK, Italy, Portugal, Greece and Austria. (EARS-Net., 2009).

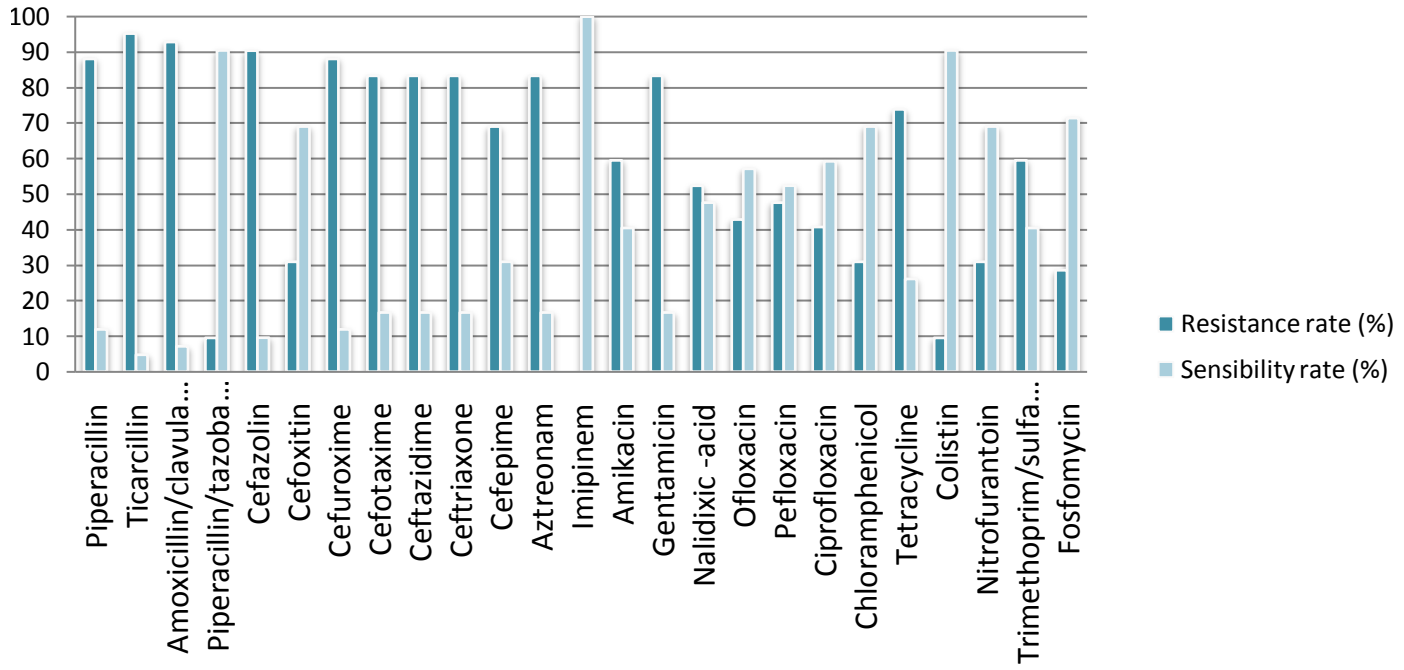

Figure 1. Results of antibiotic susceptibility for ESBL-producing *Klebsiella pneumoniae, Enterobacter cloacae*, *Escherichia coli* and *Serratia marcescens* from blood samples.

An analysis of our results showed that the rate of ESBL-producing *K. pneumoniae* (79,16%) was significantly higher compared to results reported in 2008 in Algiers with a prevalence of 19.9% for ESBL-producing *K. pneumoniae* (Messai et al., 2008), and compared to results reported in October 2011 in Algeria with a prevalence of 26.8% for these strains (Nedjai et al., 2012). TEM-136 has previously been reported in *K. pneumoniae* from Italy; (Bagattini et al., 2006) however, this is the first report of the TEM-136 genotype in the African continent, particularly in Annaba, Algeria. SHV-28 was first discovered in Tanzania (Ndugulile et al., 2005) and has since been reported in many parts of the world, such as Italy, United-Arab-Emirates, Brazil and China (Perilli et al., 2011; Alfaresi et al., 2011; Veras et al., 2011; Shi et al., 2009). However, this is the first detection of SHV-28 in Algeria. Also, we report the first isolation of SHV-32 in our country. This last one has been found in Palestine, Canada and Spain (Hussein et al., 2009; Melano et al., 2006; Chaves et al., 2001). Furthermore, our study is the first report of the high prevalence of ESBL-producers, including TEM-136, SHV-28 and SHV-32, among Enterobacteriaceae from blood cultures ofchildren. We show that TEM and SHV are widespread among Enterobacteriaceae isolates that are multidrug resistant**.** We also demonstrate that sepsis caused by these Enterobacteriaceae is associated with very high fatality rates (Blomberg et al., 2005). Patients with sepsis due to ESBL-producing organisms had a significantly higher fatality rate than those with non-ESBL-producing strains. The prescription of several types of β-lactamases, particularly C3G; self-medication; a long stay in the hospital; a prescription by unqualified health professionals; the late initiation of the appropriate treatment; inappropriate chemotherapy; the presence of a central venous, mechanical ventilation; and a longer time for admission to blood culture were the major significant risk factors for infection with ESBLs and significantly associated with a fatal outcome. There was no independent risk factor associated with a higher mortality rate (Tuon et al., 2011; Hsieh et al, 2010). The hospitalization rate for sepsis has almost doubled in the last decade (Khan et al., 2012); the frequency of sepsis was slightly higher in the present study as compared to previous studies that showed a lower mortality rate in children with septicemia caused by ESBL-producing strains (Kim et al., 2002; Zaoutis et al., 2005). The rise in the mortality rate is generally considered not to be due to increased disease severity in patients presenting with ESBL-producing organisms but rather due to higher rates of treatment failure because health care settings, especially in developing countries, do not follow any specific policy for antibiotic use (Khan et al., 2012).

Sepsis represents a current problem in Annaba Sainte-Therese's Hospital. Problems associated with sepsis include infection with multidrug-resistant Enterobacteriaceae (especially ESBLs), which are difficult to treat and are associated with increased mortality. Additionally, the emergence of a combination of resistance genes in Enterobacteriaceae may pose a public health risk, thus substantially restricting the therapeutic alternatives. We believe that a change in the empirical approach to treatment is necessary, mainly with regards to patients

Table 3. Table summarizing the genes encoding ESBLs in samples from Algeria

Clinical strain	Number of strains	ESBL		City	Reference
		SHV	CTX-M		
Klebsiella pneumoniae	2	ND	CTX-M -15	Bejaia	Touati et al., 2006
Escherichia coli	1	ND	CTXM-15		
Enterobacter cloacae	2	ND	CTX-M-15 (2)	Bejaia	Touati et benalloua 2006
Escherichia coli	3	ND	CTX-M-15 (3)	Algiers	Messai et al., 2006
Escherichia coli	16	ND	CTX-M-15 (13) CTX-M-3 (3)	Algiers	Ramdani-Bouguessa et al., 2006
Klebsiella pneumoniae	39	ND	CTX-M-1 (25)		
Klebsiella pneumoniae	39	ND ND	CTX-M -3 CTX-M- 15	Algiers	Messai et al., 2008
Enterobacter cloacae	25	SHV-12(4)	CTX-M-3 (9) CTXM-15 (1)	Algiers	Iabadene et al., 2008
Escherichia coli	1	ND	CTX-M -15		
Klebsiella pneumoniae	1	ND			
Serratia marcescens	1	ND	CTX-M-15	Algiers	Iabadene et al., 2009
Proteus mirabilis	1	ND	CTX-M-15		
Enterobacter cloacae	2	SHV-12			
Klebsiella pneumoniae	3	SHV-11	CTX-M-28 (2)		
Enterobacter cloacae	3				
Proteus mirabilis	7	ND		Annaba	Meradi et al., 2011
Escherichia coli	7	ND			
Morganella morganii	2	ND	ND		
Proteus vulgaris	3	ND	ND		
Klebsiella pneumoniae	24	SHV-11(2) SHV-12(3) SHV-28(3) SHV-32(1) SHV-133(1)	CTX-M-15(8)	Annaba	This study
Enterobacter cloacae	11	SHV-12(5)	CTX-M-15(2)		
Escherichia coli	3	ND	ND		
Serratia marcescens	4	ND	ND		

with sepsis during hospitalization, which can decrease the global mortality of patients infected by ESBL-producing Enterobacteriaceae and that the implementation of a strict hospital infection control policy, including efforts to promote a judicious use of antibiotics, are needed. Continuous monitoring of ESBL-producing Enterobacteriaceae in the community and in the hospital setting is also required, as is the early detection of sepsis, which could be one of the major keys to reducing the high mortality rate of sepsis in developing countries.

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