Prevalence of extended-spectrum β -lactamase-producing Gram-negative bacteria in septicaemic neonates in a tertiary care hospital

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The present study was undertaken to investigate the high incidence of multiresistant Gram-negative bacilli causing neonatal septicaemia. Samples of neonatal blood from 728 suspected cases were obtained in brain heart infusion broth with sodium polyanethol sulfonate. All Gram-negative rods isolated were subsequently subjected to routine antimicrobial susceptibility testing and tests for extended-spectrum β -lactamase (ESBL) production, as per NCCLS recommendations. ESBL was detected in 86.6 % of *Klebsiella* spp., 73.4 % of *Enterobacter* spp. and 63.6 % of *Escherichia coli* strains. It was also observed that 74.4–80.9 % of these ESBL producers were resistant to cefotaxime and 47.6–59.5 % were resistant to ceftazidime in routine susceptibility testing. Some ESBL producers (36.3–61.5 %) were found to be susceptible to either or both cephalosporins used in this study. It is concluded that indiscriminate use of third-generation cephalosporins may be responsible for the selection of ESBL-producing multiresistant strains in the neonatal intensive-care unit (NICU).

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INTRODUCTION

Resistance to antimicrobials is more common where they are used with greatest frequency (Ellner *et al.*, 1987); intensive-care units are one of those places. In this study, we focused on the neonatal intensive-care unit (NICU). We chose septicaemic neonates as the subjects of our study, as we observed that the positivity rate of blood cultures was appreciably high, with a predominance of multiresistant Gram-negative rods among the isolates. The extensive use of third-generation cephalosporins as first-line drugs in these cases added to our concern. There have been many instances of Gram-negative outbreaks in neonatal units around the world (Voss *et al.*, 1994; McDonald *et al.*, 1998; Moolenaar *et al.*, 2000). The situation may be further complicated by multiresistant isolates. Such multiresistance in Gram-negative bacteria may be associated with the production of extended-spectrum β -lactamase (ESBL). Until the mid-1980s, resistance to β -lactam antibiotics was known to be limited to organisms with inducible chromosomal β -lactamase genes; this form of resistance is not transmissible. Consequently, it came as an unwelcome surprise when a species of *Klebsiella* with plasmid-mediated resistance to extended-spectrum cephalosporins was isolated in Germany in 1983, and in the following year, similar resistance in *Klebsiella* was reported

from France. It was found that in the former case, the resistance was due to a new β -lactamase that differed from the enzyme SHV-1 by a single mutation, and was therefore named SHV-2 (Goussard *et al.*, 1991). The enzyme detected in France was named CTX-1, and was found to be a mutant TEM enzyme (Sirot *et al.*, 1987). These enzymes, also called ESBLs, are produced exclusively by Gram-negative bacteria and are active against extended-spectrum cephalosporins, aztreonam, narrow-spectrum cephalosporins and anti-Gram-negative-bacterium penicillins (Philippon *et al.*, 1989; Jacoby & Medeiros, 1991). At present, at least 67 TEM-derived and 12 SHV-derived ESBLs have been described (e.g. Jacoby & Medeiros, 1991; Naumovski *et al.*, 1992). More ESBLs are being added to the list, e.g. SHV-13 (Yuan *et al.*, 2000) and SHV-24 (Kurokawa *et al.*, 2000), and an increasing number of ESBL-producing bacteria is being reported. Neonates are particularly vulnerable to infection, so any delay in the initiation of empirical therapy or wrong choice of antibiotics is to be avoided. Keeping these facts in mind, we designed our study to investigate the prevalence of ESBL-producing Gram-negative rods in septicaemic neonates.

METHODS

All septicaemic neonates admitted to the NICU of King George's Medical College were prospectively enrolled over a period of 1 year. Blood (1–2 ml) was collected from each patient, and inoculated into

Abbreviations: ESBL, extended-spectrum β -lactamase; NICU, neonatal intensive-care unit.

5 ml brain heart infusion broth with 0.025 % sodium polyanethol sulfonate (Himedia Laboratories). A second sample was collected from a different site after 1 h to rule out contaminating flora. The broth was incubated aerobically at 37 °C. A blind subculture was done after 18 h; if no growth was obtained, the bottles were examined daily for 7 days. Any sign of growth was followed up by subculture. Media used for subculturing included chocolate agar, 5 % sheep blood agar and MacConkey agar (Himedia Laboratories). Isolates were identified using standard biochemical tests (Collee *et al.*, 1996).

Antimicrobial susceptibility tests. Susceptibility tests were performed using the Kirby–Bauer disc diffusion method and following NCCLS guidelines (National Committee for Clinical Laboratory Standards, 2000). Antimicrobials used were ampicillin (10 µg per disc), amoxicillin (10 µg per disc), gentamicin (10 µg per disc), amikacin (30 µg per disc), cefotaxime (30 µg per disc), ceftazidime (30 µg per disc), ciprofloxacin (5 µg per disc), cotrimoxazole (1.25 µg trimethoprim per disc/23.75 µg sulfamethoxazole per disc) and tetracycline (30 µg per disc). The discs were obtained from Himedia Laboratories.

Screening for ESBL. This was done as part of the routine susceptibility testing, according to criteria recommended by the NCCLS. Two discs, ceftazidime (30 µg) and cefotaxime (30 µg), were used. An inhibition zone of ≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime indicated that the strain probably produced ESBL.

Phenotypic confirmatory test for ESBL production. This was done as per NCCLS recommendations, on Mueller–Hinton agar. Four discs, containing cefotaxime (30 µg), cefotaxime/clavulanic acid (30 µg/10 µg), ceftazidime (30 µg) and ceftazidime/clavulanic acid (30 µg/10 µg), were used. A ≥ 5 mm increase in zone diameter for either antimicrobial tested in combination with clavulanic acid versus its zone when tested alone confirmed ESBL production. Discs containing clavulanic acid were prepared and stored according to NCCLS guidelines.

RESULTS

Blood samples for culture were obtained from 728 suspected cases of neonatal septicaemia, of which 346 (47.5 %) were positive. The total number of pathogenic isolates was 400, of which 50 were *Candida* spp. and 350 were bacterial isolates. Four samples showed polymicrobial growth. The Gram-negative bacterium most commonly isolated was *Klebsiella* spp. (24.6 %), followed by *Enterobacter* spp. (22.9 %), *Escherichia coli* (14.0 %), *Pseudomonas* spp. (2.9 %), *Citrobacter* spp. (1.7 %) and *Acinetobacter* spp. (1.7 %). Thus, 67.7 % of the isolates were Gram-negative rods and the rest comprised Gram-positive bacteria, such as *Staphylococcus aureus* (14.0 %), coagulase-negative *Staphylococcus* (16.6 %) and *Enterococcus* spp. (1.7 %).

All Gram-negative rods were screened and tested for ESBL production. ESBL was detected in 75 (87.2 %) isolates of *Klebsiella* spp., 58 (72.5 %) isolates of *Enterobacter* spp., 32 (65.3 %) isolates of *E. coli*, two (33.3 %) isolates of *Acinetobacter* spp. and in none of the isolates of *Citrobacter* or *Pseudomonas* spp.

When the results of the initial screening test for the three major Gram-negative isolates were compared with the results of the confirmatory test for ESBL (Table 1), it was found that more than 78 % of isolates that were positive in the screening test were also positive by the confirmatory testing. Some of the isolates that were positive in the screening test were negative for ESBL production when tested by the confirmatory method. All isolates that were negative by the screening test were found to be non-ESBL producers by the confirmatory test.

Routine susceptibility testing failed to predict ESBL production in 37.5 % of *E. coli*, 63.1 % of *Klebsiella* spp. and 48.0 % of *Enterobacter* isolates. These strains showed some degree of susceptibility to both cefotaxime and ceftazidime in routine susceptibility testing (Table 2). Most isolates (78.7–94.0 %) that were resistant to both cephalosporins were ESBL producers, while 5.9–21.2 % were not.

Analysis of antimicrobial-resistance patterns (Fig. 1) showed that resistance to β -lactam and non- β -lactam agents is more frequent in ESBL-producing strains than in those which did not produce the enzyme.

A follow-up of the culture-proven cases of neonatal septicaemia during their hospital stay revealed that 38 % of the patients died and the rest were discharged after recovery. The death rate was even higher (46.3 %) with Gram-negative septicaemia (Table 3). More than 60 % of the newborns harbouring ESBL-producing bacteria in their blood expired, as opposed to 35.7 % of those that were infected with non-ESBL-producing strains.

DISCUSSION

The microbiological spectrum of neonatal septicaemia shows marked geographical variations. In tropical areas, early-onset infections may be caused by multiresistant hospital-acquired bacteria, which are transmitted during the perinatal period. These organisms are usually resistant genera of the family

Table 1. ESBL-producing Gram-negative bacilli: screening vs confirmatory test

Percentage of total isolates is given in parentheses.

Confirmatory test result	Isolates positive on screening test			Isolates negative on screening test		
	<i>E. coli</i>	<i>Klebsiella</i> spp.	<i>Enterobacter</i> spp.	<i>E. coli</i>	<i>Klebsiella</i> spp.	<i>Enterobacter</i> spp.
Positive	32 (78.0)	75 (87.2)	58 (78.3)	0	0	0
Negative	9 (21.9)	11 (12.7)	16 (21.6)	8 (100)	0	6 (100)

Table 2. Efficacy of routine susceptibility testing in detection of ESBL production

Isolates are defined as resistant if diameter of inhibition zone $\leq$ 14 mm, and as sensitive if diameter of zone $>$ 14 mm. Percentage of total isolates is given in parentheses.

Confirmatory test result	Isolates resistant to either or both cephalosporins			Isolates sensitive to both cephalosporins		
	<i>E. coli</i>	<i>Klebsiella</i> spp.	<i>Enterobacter</i> spp.	<i>E. coli</i>	<i>Klebsiella</i> spp.	<i>Enterobacter</i> spp.
Positive	26 (78.7)	63 (94.0)	46 (83.6)	6 (37.5)	12 (63.1)	12 (48.0)
Negative	7 (21.2)	4 (5.9)	9 (16.3)	10 (62.5)	7 (36.8)	13 (52.0)

Enterobacteriaceae, *Pseudomonas* spp. and staphylococci (Begue, 1991). The spectrum of bacteria in our hospital is comparable to that of the National Neonatal Perinatal Network Database report. Our most frequent isolates were *Klebsiella* spp. (24.6%), in accordance with other Indian studies (Das *et al.*, 1999; Kapoor & Sumathi, 2000). The prevalence of *Enterobacter* sepsis was alarming; a report from Pakistan in 1996 expressed concern about increasing *Enterobacter* sepsis (Bhutta, 1996). The overall incidence of septicaemia confirmed by culture in our laboratory was 47.5%; reports from India and other countries show that the incidence varies between 36 and 55% (Gaynes *et al.*, 1996; Ako-Nai *et al.*, 1999; Das *et al.*, 1999).

The high percentage of ESBL-producing isolates may be due to the selective pressure imposed by extensive use of antimicrobials in the intensive-care unit. Some earlier reports have quoted a lower prevalence of ESBL-producing isolates, compared to that of the present study (Emery & Weymouth, 1997; Vercauteren *et al.*, 1997). Distinct regional variations have been detected in the incidence of ESBL-producing isolates, and it is often a local problem (Fluit *et al.*, 1998). A study from central India reported that 76.5% of *Klebsiella* isolates resistant to third-generation cephalosporins were ESBL producers, as confirmed by the double-disc synergy test (Hansotia *et al.*, 1997). Another study from southern India reported an incidence of 58.06% for ESBL-producing *E. coli*, and 57.14% for ESBL-producing *Enterobacter* spp. (Ananthakrishnan *et al.*, 2000). In our study, two isolates of *Acinetobacter* spp. showed the presence of ESBL; as we tested only six isolates, it is difficult to reach any conclusion regarding the ESBL-producing potential of this bacterium. None of the isolates of *Pseudomonas* spp. were positive for ESBL production by the method we used; whether they were actually non-producers, or whether some of them did produce β -lactamases that were not inhibited by clavulanate, needs to be investigated. The majority of the isolates positive on screening were confirmed to be ESBL producers, although some were negative for ESBL production by confirmatory testing. It may well be the case that these latter groups could be treated with β -lactam agents. As opposed to the group that were positive on screening, none of the isolates that were negative on screening behaved otherwise in the confirmatory tests, i.e. all of them were negative

for ESBL production by the confirmatory method, as expected. As far as the routine susceptibility test is concerned, the majority of the resistant isolates were ESBL producers, but a large number of those that showed susceptibility to either or both cephalosporins (37.5–63.1%) in the routine susceptibility testing were actually positive in the confirmatory test for ESBL. Many other workers have reported similar results (Thomson & Sanders, 1992; Sanders *et al.*, 1996). Whilst these strains remain susceptible to cefotaxime or ceftazidime *in vitro*, there is little doubt that these drugs are rarely successful in treating infections caused by ESBL-producing members of the family *Enterobacteriaceae*, unless the infection is limited to the urinary tract (Sanders *et al.*, 1996). To sum up the results of the screening and routine susceptibility tests in predicting ESBL production, it is important to mention that for the screening test, negative results are a better guide than positive results. Following all positive results might lead to unnecessary avoidance of conventional β -lactams in a good number of cases. In the case of routine susceptibility testing, negative predictions are to be taken with caution as a large number of ESBL producers can be missed. This might give disastrous results of uncontrolled septicaemia if treated with β -lactam agents.

When the antimicrobial-resistance patterns of the isolates were reviewed, we found that among the ESBL producers, more than 74% were resistant to cefotaxime and up to 59.5% were resistant to ceftazidime. Cefotaxime was found to be the most common cephalosporin administered to the neonates in our NICU.

All ESBL producers were resistant to ampicillin, more than 44% to cotrimoxazole, more than 88% to tetracycline and more than 76% to gentamicin. Such wide resistance spectra of ESBL producers, including resistance to drugs such as sulfonamides, trimethoprim and aminoglycosides, have been observed by many others (Jett *et al.*, 1995; Villa *et al.*, 2000; Subha *et al.*, 2001). One study reported that ciprofloxacin resistance and ESBL production in *Klebsiella pneumoniae* are closely associated (Paterson *et al.*, 2000). They found that, globally, 18% of ESBL producers were resistant to ciprofloxacin. Our results also showed that a greater percentage of ESBL producers than non-producers were resistant to ciprofloxacin. Such a broad resistance spectrum is a

Fig. 1. Antimicrobial-resistance patterns of clinical isolates of: (a) *Escherichia coli*; (b) *Klebsiella* spp.; (c) *Enterobacter* spp. Filled bars represent total resistant organisms, hatched bars represent resistant ESBL producers and open bars represent resistant ESBL non-producers. Ap, Ampicillin; Ax, amoxicillin; Co, cotrimoxazole; T, tetracycline; G, gentamicin; Ak, amikacin; Cf, cefotaxime; Cz, ceftazidime; Cp, ciprofloxacin.

cause for concern and necessitates the restricted use of extended-spectrum cephalosporins, and a trial of other suitable alternatives. The cost of the antibiotics has always been a limiting factor in therapy planning; keeping this in

Table 3. ESBL production and outcome of Gram-negative neonatal septicaemia

Outcome	Cases with isolates producing ESBL (%)	Cases with isolates not producing ESBL (%)
Survival	38.7	64.2
Death	61.2	35.7

mind, it is worth noting that in comparison to other antibiotics, resistance to amikacin and ciprofloxacin was less frequent. A recent study has found ciprofloxacin to be highly effective in treating multiresistant Gram-negative infections, including use in premature and extremely low-birth-weight infants (Khaneja *et al.*, 1999). Ciprofloxacin also acts against staphylococci.

In our region, testing for ESBL production is not routinely done by most centres. This may allow the dissemination of ESBL-producing strains within and between hospitals to remain undetected for long periods. The consequence can be serious outbreaks, particularly in the intensive-care units.

The overall mortality rate in neonates with septicaemia is high (38.0 %) in our NICU (Table 3). It was even higher in cases of Gram-negative septicaemia, particularly the cases from which ESBL-producing isolates were recovered. These patients showed discouraging results with the antimicrobial therapy. Keeping in mind the high prevalence of ESBL-producing bacteria in our NICU, we feel it is extremely important to implement a revised strategy of empirical therapy and to monitor ESBL production routinely in NICU isolates, as well as isolates from other wards. Once the availability of clavulanic acid is ensured, only a simple disc diffusion test will be necessary to screen ESBL production.

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