Identification and characterization of ceftriaxone resistance and extended-spectrum β-lactamases in Malawian bacteraemic Enterobacteriaceae

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Objectives: To enumerate and characterize extended-spectrum β-lactamases (ESBLs) amongst ceftriaxone-resistant coliforms in Blantyre, Malawi, where third-generation cephalosporin use is currently highly restricted.

Methods: Over the period April 2004–March 2005 all ceftriaxone-resistant isolates from blood cultures were examined for the presence of ESBLs. Isoelectric focusing was performed on enzyme extracts. PCR and DNA sequencing of amplicons were used to identify the underlying genetic determinants responsible for the ESBL phenotypes. Transferability of the ESBL phenotypes was tested by conjugation to a susceptible Escherichia coli J53.

Results: Enterobacteriaceae were isolated from 1191 blood cultures, of which 19 (1.6%) were ceftriaxone resistant. Ten isolates (0.7% of all isolates) demonstrated an ESBL phenotype but only eight were characterized as three isolates were from the same patient. Genotypes SHV-11 (n = 1), SHV-12 (n = 3), SHV-27 (n = 1), TEM-63 (n = 2) and CTX-M-15 (n = 1) were detected. Plasmid transfer of the ESBL resistance phenotype was successful for all the isolates.

Conclusions: In a clinical setting of minimal cephalosporin usage there is already a diversity of ESBL genotypes. Increased use of cephalosporins in this setting is likely to result in a rapid expansion of ESBLs and their prevalence will need to be carefully monitored.

Keywords: molecular epidemiology, Africa, ESBLs

Introduction

The use of broad-spectrum penicillins and cephalosporins has been associated with the emergence of extended-spectrum β-lactamases (ESBLs) in Enterobacteriaceae. These have been described worldwide and are a major cause of nosocomial infections associated with high mortality. ESBLs have been described in Africa¹–⁷ and have considerable implications for the developing world, where there is limited access to newer antibacterial agents.

Third-generation cephalosporins are used rarely in Malawi, but their inclusion as agents of first choice in cases of community-acquired bacteraemic illness is being considered. Streptococcus pneumoniae and non-typhoidal salmonellae
(NTS) are the commonest causes of bacteremia at the Queen Elizabeth Central Hospital (QECH), a large tertiary referral hospital in Blantyre. Over recent years there has been a dramatic increase in the number of multidrug-resistant NTS and a problem of reduced susceptibility to penicillin among the pneumococci. Current policy is to treat patients with community-acquired sepsis with one or a combination of benzyl-penicillin, chloramphenicol and/or gentamicin, depending on the clinical presentation. Ceftriaxone has been intermittently available in recent years in Blantyre, first in 1997 for use in paediatric patients and in 2002 for adults participating in a clinical trial of meningitis treatment.

Following the death of a patient, attributable to a multiresistant Klebsiella pneumoniae infection, for which no therapeutic options were available, we decided to review the scale of the ESBL problem at the QECH and characterize the ESBLs produced by Enterobacteriaceae in clinically important isolates. This assessment will provide a baseline for future surveillance for ESBLs as cephalosporins become adopted as the agents of choice for community-acquired sepsis.

As part of our investigation we also chose to look for the plasmid-mediated quinolone resistance determinant qnrA, which has been associated with clavulanic acid-inhibited ESBLs in Enterobacteriaceae. Ciprofloxacin is the only alternative drug for treatment available for multiresistant NTS isolates in our setting and the presence of both resistance determinants would compromise therapy further.

**Materials and methods**

**Patients and bacterial strains**

Blood cultures were performed by the Malawi-Liverpool-Wellcome Trust Laboratories for admissions to the paediatric and adult medical services in the QECH. Samples were processed using a commercial blood culturing system (BacT Alert, BioMerieux, Lyons, France). Significant isolates were identified using standard procedures and all Enterobacteriaceae were identified to the species level using the API 20E system (BioMerieux).

**Susceptibility testing**

Disc diffusion susceptibility was performed in accordance with the British Society for Antimicrobial Chemotherapy guidelines. Antimicrobials tested in the routine laboratory include ciprofloxacin, co-trimoxazole, ampicillin, gentamicin, chloramphenicol and cephradine. Isolates reported as cephradine resistant were further examined for ESBL production and susceptibility to ceftazidime, cefoxitin and imipenem. MICs were determined using the Etest method (AB Biodisk, Solna, Sweden). ESBL detection was performed using the double disc diffusion method with cefotaxime (AB Biodisk, Solna, Sweden). ESBL detection was performed by the Malawi-Liverpool-Wellcome Trust Laboratories for admissions to the paediatric and adult medical services in the QECH. Samples were processed using a commercial blood culturing system (BacT Alert, BioMerieux, Lyons, France). Significant isolates were identified using standard procedures and all Enterobacteriaceae were identified to the species level using the API 20E system (BioMerieux).

**Conjugation experiments**

Broth matings were carried out with the clinical isolates as donors and Escherichia coli J53 (sodium azide resistant) at a bacterial cell ratio of 1:10. After 24 h, the mating mixture was centrifuged at 13000 g for 5 min and the supernatant removed (to remove any cell free β-lactamase). The mixture was resuspended in an equal volume of PBS and 30 μL was plated separately onto MacConkey agar (Oxoid Ltd, Basingstoke, UK) containing sodium azide (100 mg/L) supplemented with either cefotaxime (1 mg/L) or ceftazidime (4 mg/L). After overnight incubation, transconjugants were identified, E. coli was confirmed as the recipient and MICs were determined.

**PCR screening and DNA sequencing**

Total DNA for PCR was extracted by suspending bacteria in 5% (w/v) Chelex-100 resin slurry (Bio-Rad, Hemel Hempstead, UK) in injection grade water followed by boiling for 10 min. Samples were centrifuged (10 min at 13400 g) and used immediately or stored at –20°C. Isolates with the ESBL phenotype were examined by PCR for the presence of the blaTEM, blaSHV, blaCTX-M, and qnrA genes. Positive and negative controls were included for all the PCRs. Control DNA for the qnrA PCR was obtained from a previous study. PCR products were excised from 1% (w/v) agarose gels and purified using a commercial kit (Qiagen, GmbH). Sequence determination of amplicons for both parents and transconjugants was performed on both strands using the respective PCR primers. Sequence analysis was performed using commercial software (Lasergene, DNASTar Inc., Madison, WI, USA), and sequences were aligned to the following GenBank accession numbers: blaSHV-11, AF187732; blaSHV-12, AY940490; blaSHV-27, AF293345; blaTEM-63, AF332513; blCTX-M-15, AY044436.

**Analytical isoelectric focusing**

β-Lactamase extraction and isoelectric focusing (IEF) were performed as described previously on polyacrylamide gels containing ampholines in the pH range 3.5–9.5 (Amersham Pharmacia Biotech, Buckinghamshire, UK). Detection of cefotaxime hydrolysis by the separated proteins post-electrophoresis was achieved by overlaying the ampholine gel with agar containing cefotaxime (0.4 mg/L). The gel overlay method was used to attempt to define the differing contribution of these bands to the hydrolysis of cefotaxime as many bacteria produce both major and minor β-lactamas. After incubation for 2 h at 37°C, the agar was flooded with a McFarland standard 0.5 suspension of E. coli (NCTC 10418), re-incubated overnight and examined for bacterial growth.

**Results**

**Patients and bacterial strains**

From 1 April 2004 to 31 March 2005, 6487 blood cultures were drawn from adults, from which 649 (10%) isolates of Enterobacteriaceae were grown. Seven (1.1% of all Enterobacteriaceae) were ceftriaxone resistant, of which five had the ESBL phenotype. In each case these were community-acquired infections. One organism (H309) was isolated three times at sequential intervals from the same patient with cavitating pneumonia and thus was counted only once. In addition, there were 5964 paediatric blood cultures, from which 542 (9.1%) Enterobacteriaceae were isolated. Of the 542 isolates, 12 (2.2% of all Enterobacteriaceae) were reported to be ceftriaxone resistant, of which five had the ESBL phenotype.

Characteristics of the ESBL phenotypes are summarized in Table 1. All isolates were resistant to co-trimoxazole, amoxicillin, gentamicin, chloramphenicol and ceftazidime, as shown by disc testing. Only isolates H308 and H309 were also resistant to ciprofloxacin, but all were susceptible to imipenem. Isolates H305 and H306 (both Enterobacter cloacae) were resistant to cefotaxim. MICs of ceftazidime and cefotaxime with or without β-lactamase inhibition were determined.
clavulanate, for both original isolates and their transconjugants, are shown in Table 1.

Conjugation experiments

Transfer of the ESBL resistance phenotype to \textit{E. coli }J53 was successful for all the isolates.

\textbf{IEF, PCR and DNA sequencing}

IEF profiles of the isolates are shown in Table 1. At least two bands were detected for all isolates except H305. All isolates except H305 produced TEM-like enzymes, as evidenced by bands in the region pI 5.4–6.3. It was assumed that the band at pI 5.4, found in all isolates except H305, was most likely to represent TEM-1. This was confirmed for H309 by DNA sequencing of the amplicon from the TEM PCR. Isolates H304, H309, H310 and H311 also produced SHV-like enzymes, as evidenced by bands in the region pI 7.0–8.2. PCR screening confirmed the presence of \textit{bla}_{\text{SHV}} in isolates H304, H309, H310 and H311.

The genotypes determined by DNA sequencing are shown in Table 1. The \textit{bla}_{\text{SHV}} amplicon for H309 was not sequenced as no SHV enzymes have been reported with pIs between 7.1 and 7.5. The plasmid-mediated quinolone resistance determinant \textit{qnrA} was not detected in any of the isolates.

\textbf{Discussion}

This study suggests that ESBLs are currently found in only a small number of clinically relevant isolates in paediatric and adult patients of the QECH, Blantyre, Malawi. However, there is already a genetic diversity of ESBLs despite the highly restricted use of third-generation cephalosporins. SHV-12 was the most commonly identified genotype. This was first described in 1997\textsuperscript{18} and since then has been reported worldwide and more recently from Tunisia,\textsuperscript{4} Cameroon\textsuperscript{5} and Tanzania\textsuperscript{7} on the African continent. In our study it was isolated only from paediatric patients and was the commonest paediatric genotype, although it was carried by three different bacterial genera. The gene was shown to be transmissible in all cases and this suggests that plasmid-encoded SHV-12 may be circulating within this population. Further work is required, including studies of enteric flora, to identify risk factors for possession of this ESBL on the paediatric wards. It is interesting that there was an absence of bands detectable by IEF for the SHV-12 enzymes, and cefotaxime hydrolysis was not detected for any of these either. It may be that there were other contributors to the ESBL phenotype which were not detected or that there were low levels of these enzymes in the periplasmic space ensuring that a toxic threshold was achieved, but on protein extraction insufficient protein was released for IEF detection.

Of the other ESBL genotypes, SHV-27 has been reported previously only from Brazil\textsuperscript{19} and SHV-11 is also infrequently reported (although there is some debate as to whether SHV-11 is really an ESBL).\textsuperscript{18} CTX-M-15 has been reported worldwide, including from Cameroon\textsuperscript{6} and Tanzania\textsuperscript{7} in Africa. The patient from whom the multiresistant \textit{K. pneumoniae} carrying CTX-M-15 was isolated died due to bacterial pneumonia, as effective

\begin{table}[h]
\centering
\caption{Properties of bacterial strains and their transconjugants}
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|c|c|}
\hline
Isolate number & Case & Identification & Isoelectric points (pI) & pl value at which CTX hydrolysis was observed & Molecular identity of ESBL determinant & CAZ MIC (mg/L) & CAZ/CLA MIC (mg/L) & CTX MIC (mg/L) & CTX/CLA MIC (mg/L) \\
\hline
H304 & child & \textit{Klebsiella oxytoca} & 5.4, 7.9\textsuperscript{a} & ND & SHV-12 & 4 & <0.064 & 1 & <0.016 \\
H304 T &  &  &  &  &  &  &  &  &  \\
H305 & child & \textit{Enterobacter cloacae} & ND & ND & SHV-12 & 8 & 0.064 & 1 & 0.032 \\
H305 T &  &  &  &  &  &  &  &  &  \\
H306 & adult & \textit{E. cloacae} & 5.4, 5.6 & 5.4–5.6 & TEM-63 & >256 & 0.25 & 2 & 0.5 \\
H306 T &  &  &  &  &  &  &  &  &  \\
H307 & child & \textit{Escherichia coli} & 5.4, 5.6 & 5.4–5.6 & TEM-63 & >256 & 0.5 & 2 & 0.032 \\
H307 T &  &  &  &  &  &  &  &  &  \\
H308 & child & \textit{Klebsiella pneumoniae} & 5.4, 6.8 & ND & SHV-12 & 64 & 0.25 & 8 & 0.032 \\
H308 T &  &  &  &  &  &  &  &  &  \\
H309 & adult & \textit{K. pneumoniae} & 5.4, 6.8, 7.3 & 9.0 & CTX-M-15 & 16 & 0.125 & 128 & 0.032 \\
H309 T &  &  &  &  &  &  &  &  &  \\
H310 & adult & \textit{K. pneumoniae} & 5.4, 7.8 & ND & SHV-11 & 32 & 0.125 & 4 & 0.016 \\
H310 T &  &  &  &  &  &  &  &  &  \\
H311 & child & \textit{K. pneumoniae} & 5.4, 8.3 & 8.2–8.3 & SHV-27 & 2 & 0.125 & 64 & 0.032 \\
H311 T &  &  &  &  &  &  &  &  &  \\
\hline
\textsuperscript{a}Major bands determined by the intensity of nitrocefin staining on IEF are underlined.
\end{tabular}
\end{table}
antimicrobial chemotherapy was unavailable. Also, the CTX-M-15 gene was not detected by the nitrocefin reaction although cefotaxime hydrolysis was shown by the bioassay at a pl close to 9.0 in the expected region of CTX-M-15. We, like others, have previously observed the lack of nitrocefin hydrolysis on IEF, particularly with CTX-M-mediated β-lactamases.

It is interesting that we detected TEM-63, which was transmissible from both the E. cloacae and E. coli isolates, from both the adult and the paediatric patients. This ESBL has previously been reported only from South Africa, where it was reported initially in ESBL-producing Klebsiella spp., Proteus spp., Enterobacter spp. and E. coli strains and more recently in Salmonella strains. This suggests that there has been a spread of the TEM-63 gene within southern Africa. Of greater concern is that in all probability it is only a matter of time before it spreads into the NTS in Malawi. NTS are particularly important causes of bloodstream infections in children and in HIV-infected adults, and the rise in multidrug resistance among these bacteria shows that therapeutic options at present are limited to quinolones and cephalosporins. Although two of our isolates demonstrated ciprofloxacin resistance in association with ESBLs, it is reassuring that we did not detect the plasmid-mediated quinolone resistance determinant qnrA in any of the isolates.

We did not specifically look for AmpC-type β-lactamases in this study although isolates H305 and H306 were both resistant to cefotaxin. It is likely that the cephaparin resistance was porin mediated, as we detected no basic pl values of >9.0 on the IEF, and ESBLs were detected phenotypically in both the isolates by disc testing systems.

We have identified only small numbers of ESBLs, but it is likely that we have not fully recognized their prevalence. In our routine susceptibility testing we test only ampicillin and ceftriaxone (which is the only cephalosporin therapy available to any great extent in Malawi) and do not routinely test for ESBLs. As cefazidime was initially recommended for ESBL screening (now cefpodoxime is being recommended), it is possible that we may have missed rare isolates in this collection that are resistant to cefazidime but fully susceptible to cefotaxine. Nevertheless, our results provide a baseline from which changes in the numbers and characteristics of ESBLs can be monitored in the coming years. If the use of cefotaxime increases, as seems likely at the present time, then it is inevitable that ESBL prevalence will also increase. Unfortunately, the healthcare institutions in Malawi, as in much of the developing world, lack effective infection-control practice and infrastructure (e.g. lack of hand-washing facilities) and are permanently overcrowded. Each of these factors will serve to promote the dissemination of ESBLs. Furthermore, unlike the situation in the developed world, the financial resources to provide alternative agents such as carbapenems are lacking, and the option to tailor therapy based on antimicrobial resistance testing is unavailable in all except a handful of hospitals. Thus, it would be prudent to monitor carefully the evolving epidemiology of ESBLs with changing antibiotic practice and to act quickly on this information to control the prescription of such antibiotics if necessary.

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Transparency declarations

None to declare.

References


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