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Extended Spectrum β-Lactamases Producing *Klebsiella pneumoniae* from the Neonatal Intensive Care Unit at the University Teaching Hospital in Lusaka, Zambia

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Abstract

Klebsiella pneumoniae is one of the major causes of blood-stream infections in neonatal intensive care units. Treatment has been a challenge due to the development of multidrug resistant strains, which are mainly attributed to the ability of the organism to produce extended spectrum β-lactamases that confer resistance to second and third generation cephalosporins. This laboratory-based cross-sectional study was aimed at determining the extent of extended spectrum β-lactamase production among invasive *K. pneumoniae* isolates from blood culture specimens at the University Teaching Hospital in Lusaka. The production of the extended spectrum β-lactamases was detected using the combination disc method, and by detecting genes encoding extended spectrum β-lactamases using Polymerase Chain Reaction. The drug resistance profile was determined using the Kirby-Bauer disc diffusion method against tetracycline, chloramphenicol, amikacin, gentamicin, co-trimoxazole, ciprofloxacin, cefotaxime, ceftazidime, cefpodoxime and imipenem. All the 45 isolates were found to be ESBL producers, and out of these 33/45 (73%) were found to have detectable ESBL-encoding genes: Bla_{SHV} (27/45, 60%) and Bla_{TEM} (6/45, 13%). No Bla_{CTX-M}gene was detected. Antimicrobial susceptibility testing revealed a high frequency of antimicrobial resistance: cefotaxime (100%), ceftazidime (100%), cefpodoxime (100%), co-trimoxazole (100%), tetracycline (100%),gentamycin (97.8%) and chloramphenicol (97.8%), and ciprofloxacin (95.6%). The antimicrobial resistance profile indicated that all the isolates were multidrug resistant, with each being resistant to at least 5 antibiotics. However, all the isolates were susceptible to amikacin andimipenem. In conclusion, there is high prevalence extended spectrum β-lactamases producing invasive *K. pneumoniae* isolates, which are also multidrug resistant, in the intensive care unit at the University Teaching Hospital. It is, therefore, recommended that all *K. pneumoniae* isolates should be screened for production of extended spectrum β-lactamases, and that infection control measures should be instituted at the University Teaching Hospital to curtail this problem.

Key Words: *Klebsiella pneumoniae*, ESBLs, Multi-drug resistance, Neonatal intensive care unit

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Introduction

Klebsiella pneumoniae is the second most common cause of community and hospital acquired Gram-negative bloodstream infections after *Escherichia coli* [1, 2]. The importance of this organism in health-care settings has been increasing due to the emergence and progressive spread of multidrug resistance, specifically the extended-spectrum β-lactamase (ESBL)-producing strains [3-7]. Multidrug-resistant (MDR) *K. pneumoniae* strains cause frequent grievous bacteraemia and septicaemia by producing ESBL and cephalosporinase enzymes, and also using other mechanisms [8, 9].

ESBLs are plasmid-encoded enzymes which can result from mutations in the TEM-1, SHIV-1 and OXA β-lactamase genes [5, 10]. They hydrolyse β-lactams, including third generation cephalosporins with an oxyimino side chain, as well as oxyimino-monobactamaztreonam, thus conferring resistance to these antibiotics [11, 12]. Mobile genetic elements such as insertion sequences, transposons and conjugative, which harbour the β-lactamase genes, mediate the transfer of these genes in and between bacterial species [5, 13, 14].

Outbreaks of ESBL-producing organisms have been described, and most of these organisms result in an increased number in mortality cases [15-18]. One of the major contributing factors in these outbreaks is poor adherence to infection control policies in health-care settings [19-21]. Infants, particularly those with a low gestational age or a low birth weight, are at greater risk for developing health care associated infections in neonatal intensive care units (NICUs) due to their prolonged hospitalisation and exposure to central venous catheters, mechanical ventilation, total parenteral nutrition, and long-term antimicrobial therapy with third-generation cephalosporins [19, 22-25].

Klebsiella species are the most commonly implicated pathogen in neonatal sepsis outbreaks [22, 26, 27]. However, in developing countries there is a paucity of data on *Klebsiella* infection outbreaks in NICUs, and this may be attributed to the limited diagnostic capacity in these countries. In Zambia, no such studies have been conducted. In 2013 records in the NICU at the University Teaching Hospital in Lusaka indicated that the rate of neonatal sepsis was as high as 40%, and most of the blood culture samples yielded *K. pneumoniae* as the predominant organism, accounting for about 30% of the isolates. Resistance to the third generation cephalosporins was observed amongst these isolates. This study, therefore, was aimed at detecting ESBL production in *K. pneumoniae* isolated from blood cultures of neonates from the NICU, and determining their antimicrobial susceptibility patterns.

Materials and Methods

Bacterial Isolates: A total of 45 *K. pneumoniae* isolates, obtained from blood culture (1-4ml) specimens from of neonates admitted to the NICU at the University Teaching Hospital (UTH) in Lusaka, Zambia as part of the routine hospital care, were analysed. The UTH, a 2000-bedhospital, is the largest referral hospital in the country. We used the Bacteriology Laboratory's blood culture database to identify cases. The hospital's protocol for obtaining blood cultures advises clinicians to obtain one paediatric blood culture bottle from any neonate suspected of having systemic infection. A probable case was defined as *Klebsiella* species isolated from blood by the laboratory from an infant aged <28 days who was admitted to the NICU between January 2013 to December 2013.

Bacterial Isolation and Identification:
K. pneumoniae was isolated from blood culture samples that were submitted to the bacteriology laboratory for routine investigations. Blood stream infection was detected using the BACTEC Blood Culture System (Becton Dickinson, USA) and subcultured on blood, chocolate, and MacConkey agar plates (Oxoid Ltd, Basingstoke, Hampshire, England) and incubated at 37^{o}C for 18-24 hours. *K. pneumoniae* isolates were identified by standard microbiological methods including lactose fermentation, use of triple sugar iron agar, lysine iron agar, sulphur indole motility, citrate utilisation and urease tests (Oxoid Ltd. Basingstoke, Hampshire, England). Results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines [28].

Antimicrobial Susceptibility Testing: Antimicrobial susceptibility testing was performed by the Kirby-Bauer Disc Diffusion method using the following antibiotics according to CLSI guidelines (CLSI, 2011): 30µg amikacin, 5µg ciprofloxacin, 30µg chloramphenicol, 30µg tetracycline, 30µg cotrimoxazole, 30µg gentamicin, 30µg cefotaxime, 10µg cefpodoxime, and 30µg ceftazidime (Mast Diagnostics Ltd, Merseyside, UK).

Detection of Extended Spectrum β-Lactamases

Phenotypic ESBL Screening: The isolates were screened for ESBL production using the Kirby-Bauer method by testing them against cefpodoxime, ceftazidime and cefotaxime (Mast Diagnostics Ltd, Merseyside, UK) as indicator cephalosporins on Muller-Hinton agar plate (Oxoid Ltd. Basingstoke, Hampshire, England), and incubated at 37^{o}C.*Klebsiella pneumoniae* ATCC 700603 was used as the standard control strain. Production of ESBLs was confirmed phenotypically by using the combination disc method using the above cephalosporins in addition to clavulanic acid at 37^{o}C for 18-24 hours.

PCR Detection of β-lactamase Genes

DNA Preparation: Bacterial DNA was extracted on the easy Mag instrument (bioMerieux Inc, Durham, NC, USA) using the "off-board lysis" protocol as recommended by the manufacturer. A loopful of

bacteria was emulsified in nuclease free water and lysed using the "off-board lysis" protocol, after which 400µl of the lysed sample was transferred to the sample strip well. The sample strips were then loaded onto the easyMAG machine and the extraction process was performed according to the manufacturer's protocol. DNA was eluted in a final volume of 50µl.

PCR Amplification of ESBL genes: The PCR was carried out in a final volume of 25µl consisting of 2µl of DNA and 12.5µl of 1x Mastermix (Fermentas Life Sciences, Glen Burnie, MD, USA), 2.5µl of 5µM of each primer (Table 1), and 5.5µl of molecular grade water (PCR water). DNA from *K. pneumoniae* type strain ATCC 700603 was used as a positive control, while de-ionised water was used as a negative control. The cycling conditions were: initial denaturation at 95°C for 5 minutes, and then subjected to 35 cycles of amplification for 30s at 95°C, 30s at 60°C, 30 at 72°C, followed by a 10min extension step at 72°C on an Applied Biosystems PCR system 2700 Thermocycler (GeneAmp, USA). The annealing temperatures for Bla_{SHV}, Bla_{CTX-M} and Bla_{TEM} primers (Table 1) were 60°C, 58°C and 56°C, respectively. The presence of the 931bp Bla_{TEM}, 909bp Bla_{CTX-M} and 868bla$_{SHV}$ amplified product was detected by electrophoresis of 5µl of the amplicon on a 1.5% agarose gel **(**Sigma Chemical Co., St Louis, USA).

Ethics Consideration: Ethics approval for this study was obtained from the University of Zambia Biomedical and Research Ethics Committee. Permission to use archived bacterial isolates was obtained from the management at the University Teaching Hospital. Study numbers were used to identify the bacterial isolates. Results for antimicrobial susceptibility testing were promptly reported to the attending physician for patient care.

Results

Detection of ESBL Production

All the 45 (100%) *K. pneumoniae* isolates which were screened for ESBL production were resistant to cefotaxime, ceftazidime and cefpodoxime (Figure 1), suggesting that they were all ESBL producers.

PCR detection of ESBL-encoding genes

All the isolates ESBL-positive isolates were subjected to PCR screening of genes encoding ESBLs, $Bla_{CTX,}$ Bla_{TEM} and Bla_{SHV}. Of all the 45 ESBL producing isolates, 33/45 (73%) were found to have detectable ESBL-encoding genes: 27/45 (60%) were Bla_{SHV}, while 6/45 (13%) were Bla_{TEM}. No Bla_{CTX-M} was detected (Figure 2). Figure 3 shows evidence of the PCR detection of the above-named genes.

Determination of Antimicrobial Susceptibility Patterns

Antimicrobial susceptibility testing revealed a high frequency of antimicrobial resistance: cefotaxime (100%), ceftazidime (100%), cefpodoxime (100%), co-trimoxazole (100%), tetracycline (100%), gentamycin (97.8%) and chloramphenicol (97.8%), and resistance to ciprofloxacin (95.6%). However, all the isolates were susceptible to amikacin and imipenem (Figure 4). The antimicrobial resistance profile indicated that all (45, 100%) the isolates were multidrug resistant, with each being resistant to at least five antibiotics (Table 2).

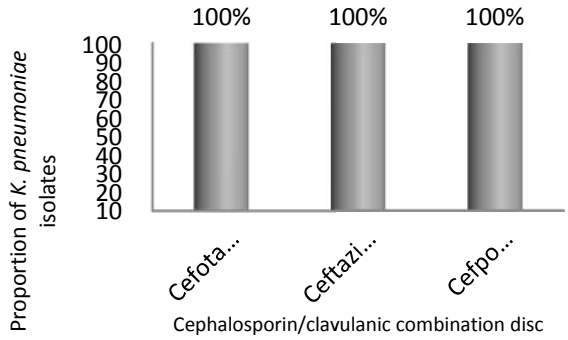

Figure 1: Proportion of *K. pneumoniae* isolates positive for ESBL production as yielded by the phenotypic screening and confirmatory tests using cefotaxime, ceftazidime and cefpodoxime

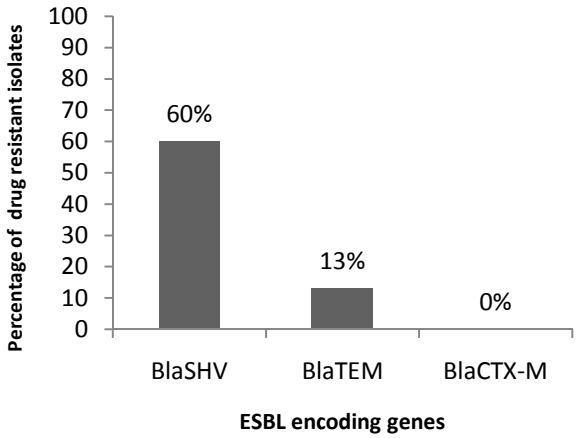

Figure 2: ESBL-encoding genes detected among the ESBL-producing *K. pneumoniae* isolates

A B

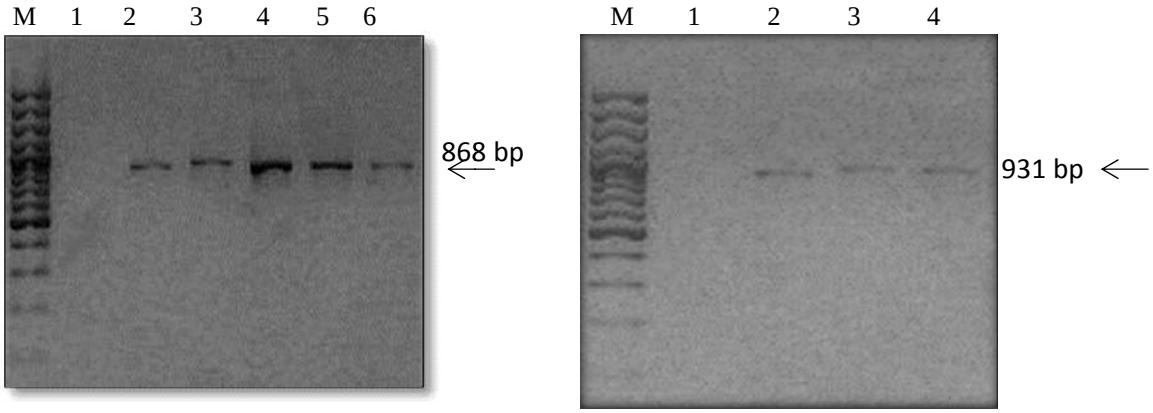

Figure 3: PCR detection of the ESBL genes. A): Detection of Bla_{SHV}ESBL genes. Lane M: 100bp DNA Marker; Lane 1: Negative control; Lane 2: ATCC Positive control, Lanes 3-6: Isolates Positive for Bla_{SHV}gene. B): PCR Detection of Bla_{TEM} ESBL genes. Lane M: 100bp DNA Marker, Lane1: Negative control, Lane 2: Positive control, Lane 3 – 4: Positive isolates for Bla_{TEM}

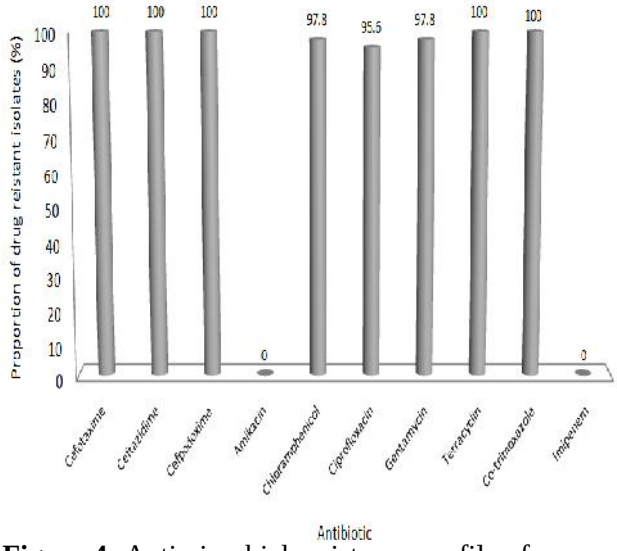

Figure 4: Antimicrobial resistance profile of *K. pneumoniae* to different antibiotics

Table 2: Antimicrobial resistance pattern for *K. pneumoniae* isolates causing bloodstream infections

Antibiotics Tested	Frequency	(%)
T, TS, CTX, CAZ, CPD	1	2.2
T, TS, CTX, CAZ, CPD, GM	3	6.7
T, TS, CTX, CAZ, CPD, GM, C	3	6.7
T, TS, CTX, CAZ, CPD, GM, CIP	12	26.7
T, TS, CTX, CAZ, CPD, GM, CIP, C	26	57.7
TOTAL	45	100

Key: CTX = Cefotaxime; CAZ = Ceftazidime; CPD = Cefpodoxime; GM = Gentamycin; CIP = Ciprofloxacin; T = Tetracycline; TS= Cotrimoxazole

Target	Primer	Sequence	Product Size (bp)
Bla_{TEM}	TEM-F TEM-R	5'-TCCgCTCATgAgACAATAACC-3' 5'-TTggTCTgACAgTTACCAATgC-3'	931
Bla_{SHV}	SHV-F SHV-R	5'-TggTTATgCgTTATATTCgCC-3' 5'-ggTTAgCgTTgCCAgTgCT-3'	868
Bla_{CTX-M}	CTX-F CTX-R	5-'TCTTCCAgAATAAggAATCCC-3' 5'-CCgTTTCCgCTATTACAAAC-3'	909

Table 1: Primers used in the amplification of the ESBL encoding genes

Discussion

This study was aimed at detecting ESBLs in invasive *K. pneumoniae* isolates at the University Teaching Hospital. ESBLs are a leading cause of resistance to β-lactam antibiotics among *Enterobacteriaceae* and their prevalence and incidence is increasing worldwide, especially in many hospital settings [29, 30]. ESBL-producing *K. pneumoniae* are an important cause of nosocomial infections in neonatal intensive care units worldwide

[5, 17, 18]. In our study, all the isolates of *K. pneumonia* analysed were found to express ESBL enzymes, and this is troubling. A similar study carried out in Thailand also indicated a high (87.3%) prevalence of ESBL-producing *K. pneumonia* [15]. The high prevalence of ESBL in our study may suggest over prescription of third generation cephalosporins at the UTH and the spread of these organisms due to inappropriate unhygienic habits amongst health-care workers. However, since UTH is a referral hospital, this could reflect inappropriate use of antibiotics at the clinics and hospitals from which the patients are referred. In addition, this may suggest wide-spread dissemination of these ESBL strains in the NICU, probably in many other parts of the hospital. A number of studies have shown that the length of hospitalisation, use of central venous catheters, mechanical ventilators, and over-prescription of antibiotics are risk factors for the acquisition and spread of ESBL-producing organism [31-34]. Therefore, such an alarming prevalence of these organisms indicates the urgent need for mitigation strategies in order to prevent further spread of these bacterial strains.

In this study, ESBL encoding genes were detected in only 73% of the ESBL-producing isolates, and this could be attributed to two reasons. One possible explanation is that phenotypic methods, such as that used in this study, are less specific in comparison to molecular methods[35]. A study in the Netherlands, on the molecular characterisation and phenotypic confirmation of ESBLs in *K. pneumoniae* and *E. coli* showed that some of the phenotypically positive ESBL-producers were PCR-negative for the ESBL genes that were tested for [5]. It could have also been attributed to the production of ESBLs other than CTX-M, SHV and TEM, which in this study, were not screened for. There are several other ESBLs that are found most significantly in other members of the *Enterobacteriaceae* family, e.g. OXY found in *Pseudomonas*species and others which belong to classes B, C and D ESBLs [12]. These enzymes fit the definition of ESBLs implying that they have the capacity to hydrolyse the oxyimino groups of second and third generation, and induce resistance to their respective cephalosporins. In another study aimed at determining the prevalence of Bla_{SHV}, Bla_{CTX-M} and Bla_{TEM}, it was found that there were isolates which were confirmed phenotypically to produce ESBLs but were PCR negative [36]. This was also attributed to the production of ESBLs other than Bla_{SHV}, Bla_{CTXM} and Bla_{TEM}. In this study, only Bla_{SHV} and Bla_{TEM} genes were detected. Of these two genes, Bla_{SHV} was the most predominant gene. In some parts of the world, Bla_{TEM} and Bla_{CTX-M} have been found to be the most prevalent genes [30, 35, 36]. There are variations in the distribution of ESBLs world-wide and some ESBLs are not present in certain geographical areas, while they are more prevalent in other areas [12]. In Indonesia, Severin and colleagues [37]in a similar study detected Bla_{SHV} and Bla_{CTX-M}, but did not any Bla_{TEM}, and this was attributed to variations in epidemiological distribution of ESBLs.

When the *K. pneumoniae* isolates were subjected to antimicrobial susceptibility testing, all of them showed a multidrug resistance pattern, with each them being resistant to at least four antibiotics. Such a high level of resistance presents a great challenge in treatment options for infections caused by this organism, and may result in a rampant increase in mortality cases at the UTH. Studies from other parts of the world show that ESBL-producers are usually multi-drug resistant, and this is can be attributed to other drug resistance genes carried on plasmids that harbour the ESBL-encoding genes [12, 30, 38, 39].

Nevertheless, all the *K. pneumoniae* isolates tested in this study were sensitive to amikacin and imipenem. This corresponds to the findings of many similar studies which showed that ESBL-producing *K. pneumoniae* are often susceptible to amikacin and imipenem [15, 39, 40]. Most significantly, the sensitivity of all the isolates to imipenem suggested that there were no carbapenem-resistant *K. pneumoniae* in the NICU at the UTH. Therefore, amikacin and imipenem still remain good treatment optionsfor *K. pneumoniae*-associated infections at the UTH. However, the increasing world-wide reports of carbapenem-resistant strains of *K. pneumoniae* point to the need for surveillance systems against these strains.

This study had a number of limitations. The study only involved isolates from the UTH, and therefore, the findings do not represent the national picture. Furthermore, it was not possible to determine the clinical outcomes of the affected infants due to lack of access to patient records. It was also not possible to establish the source of infection due to lack of logistical support at the hospital. Thirdly, the sample size was not large enough to reflect the true picture of the problem.

We recommend that infection control measures such as proper hand-washing practices, improved aseptic technique for clinical procedures

and a general heightening of infection control awareness be instituted at the UTH. Further work is warranted to study *K. pneumonia* isolates from other parts of Zambia. This will give an accurate picture of the burden of ESBL and MDR problem in the country. Additional studies are also needed to define the molecular epidemiology of this important pathogen. This will help in determining the source of infection.

Conclusion

There is a high prevalence of ESBL-producing *K. pneumoniae* in the NICU at the UTH. Bla_{SHV} was the predominant gene detected that encodes for ESBLs. All the ESBL-producing *K. pneumoniae* isolates were multi-drug resistant to fluoroquinolones, aminoglycosides, sulphonamides, tetracycline and chloramphenicol. This presents very limited treatment options for the patients at the hospital. However, all the ESBL-producing isolates were sensitive to amikacin and imipenem, making them good treatment options. There is an urgent need for the hospital to implement strict infection control measures in order to eliminate infections caused by *K. pneumoniae*. This was the first study of its kind to be carried out in Zambia.

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Conflict of interest: The declare no conflicts of interest.

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