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Research Article

The prevalence of TEM and SHV genes among Extended-Spectrum Beta-Lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*

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Background: Antimicrobial resistance to cephalosporin, penicillin and aztreonam is mediated by Extended-Spectrum Beta-Lactamases (ESBL) via hydrolysis of antibiotics. The most common bacteria associated with ESBL among the Enterobacteriaceae are *Escherichia coli* and *Klebsiella pneumonia*. Pathogenic *Escherichia coli* is associated with diarrhoea affecting mostly elderly, children under five years and the immunocompromised. There are a number of antibiotic regimens for treatment among them cephalosporins. There is reported increase in microbial resistance to cephalosporin use and the resistance is mediated by either TEM or SHV genes.

Objective: To investigate the prevalence of ESBL-producing *E. coli* and *Klebsiella pneumonia* from patients presenting with diarrhea in Machakos District Hospital, Kenya.

Methods: Bacterial isolates were identified to species level by biochemical methods and tested for sensitivity to twelve different antibiotics including cephalosporins, aminoglycosides and quinolones. Those resistant to cephalosporins with a zone diameter of ≤20 mm were tested phenotypically for Extended Spectrum Beta Lactamase (ESBL) phantom development and confirmed by MicroScan. Resistant strains to cephalosporin were further tested for presence and frequency of TEM and SHV genes.

Results: Out of the 200 *K. pneumonia* and 100 *E.coli* tested, 18 (6%) were positive for ESBL production phenotypically. These 18 (100 %) isolates demonstrated phantom phenomena phenotypically. Eight (4%) and 2 (1%) of the 200 *K. pneumonia* isolates had TEM and SHV resistant genes, respectively. There were 5 (5%) TEM and 3 (3%) SHV detected from 100 *E. coli* isolates. The 18 phenotypically detected and E-test-positive strains (10 Klebsiella spp. and 8 *E. coli*) were retested with VITEK (GNS-532 card), and 17 of these strains (94.4%) were subsequently found to be ESBL positive. One strain (5.6%) tested ESBL negative by VITEK. The cefotaxime ESBL strip detected the presence of ESBL activity in these 18 phenotypically-positive strains.

Discussion: The detection of ESBL-producing *E. coli* and Klebsiella isolates from Machakos District Hospital was 6%. The findings point out the need for continuous surveillance to determine prevalence of ESBL-producing Enterobacteria strains for better management of diarrheal illness.

Key words: Extended spectrum Beta Lactamases; Cephalosporin resistance genes; Enterobacteriaceae

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1. Introduction

Extended-spectrum beta-lactamases (ESBL) are enzymes that confer resistance to penicillins, third generation cephalosporins and aztreonam via hydrolysis of the antibiotics. These are inactivated by beta-lactamase inhibitors such as clavulanic acid (Paterson and Bonomo, 2005). *Escherichia coli* and *K. pneumoniae* are the most common ESBL producing bacterial species. However, ESBL has also been detected in other species of *Enterobacteriaceae* and *Pseudomonadaceae* (Freitas et al, 2003). Infections due to ESBL-producing *Enterobacteriaceae* should not be treated with beta-lactam antibiotics due to the risks of therapeutic failure and increased infections that could result in death (Paterson and Bonomo, 2005). Production of ESBL enzymes is an important mechanism of beta-lactam resistance in *Enterobacteriaceae* (Sanguinetti et al, 2003). Early detection of multiresistant bacteria is therefore vital indirecting treatment options and isolation of patients, which is geared towards minimizing spread of these pathogens and also to prevent nosocomial infections and outbreaks in the community (Paterson and Bonomo, 2005, Junior et al, 2004). The study aimed at evaluating the prevalence of ESBL producing *E. coli* and *K. pneumoniae* and to detect the presence of TEM and SHV genes among ESBL producing bacteria. The study enrolled subjects from all ages presenting to Machakos District Hospital for treatment of diarrhea.

2. Methodology

2.1 Patient recruitment

Eligible for inclusion into the study were patients with three or more episodes of loose, watery, mucoid or bloody diarrhea in a day; those who had given consent to participate in the study and those who had not taken any antibiotic before visiting the hospital. The details of study was explained to the patient by the study nurse. Every third patient was recruited into the study after procurement of informed consent.

2.2 Sample collection

After the patient or guardian had been thoroughly briefed about the study and given signed consent to participate, the patient was given a sterile stool cup to collect a single stool sample. In case of a minor, the guardian or parent gave the consent. Care was taken during stool collection to avoid contamination with urine, soil or water. Part of the collected stool was transferred into Carry Blair transport medium and shipped to Kenya Medical Research Institute Microbiology laboratory for processing and identification.

2.3 Bacteria identification:

The stool samples upon arrival at the laboratory were macro examined, recorded and plated immediately on MacConkey for 18 to 24 h at 37°C. Five lactose fermenters and one mucoid colony from each MacConkey plate were sub cultured for purity on nutrient agar and then used for biochemical testing (Heuvelink et al 1995). The biochemical used were [triple sugar iron (TSI), motility, indole and ornithine (MIO), and Simmon's citrate (SC)]… The positive results for positive *E. coli* were read as TSI: A/A, ± Gas and -H2S. MIO: + motility, +indole, ±ornithine; Simmon's citrate: negative. *K. pneuomonia* was confirmed as TSI: A/A, + Gas and -H2S. MIO: + motility, +indole, +ornithine; Simmon's citrate:positive.

2.4 Antibiotic susceptibility testing

Following identification the isolates were purified again on nutrient agar and tested for antimicrobial susceptibility including beta lactams, aminoglycosides, penicillins and quinolones. Antimicrobial susceptibility testing was carried out on 12 different antibiotics based on treatment of Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). Those that were resistant to third genaration cephalosporins were tested for the presence of the extended spectrum beta lactamases using double disc diffusion (DDD), E-test, VITEK and finally PCR for detection of TEM and SHV resistance genes.

The antibiotic susceptibility profiles were performed according to the Kirby-Bauer disk diffusion method and interpreted based on the CLSI guidelines (CLSI, 2012). Briefly: freshly prepared bacteria were emulsified into a normal saline standard suspension that was adjusted to equivalent turbidity to 0.5 McFarland standard. This was spread on freshly prepared and quality controlled Mueller Hinton agar and spread evenly using a curved rod. The 12 antibiotics were applied on the plate with bacteria using a disc dispenser and allowed to settle for 10 minutes before incubating at 37 °C for 18-24 hours. Following incubation, the susceptibility results were read and interpreted based on CLSI standards (CLSI, 2012) as resistant, intermediate or susceptible.

E-test

A bacteria suspension of equivalent turbidity to 0.5 McFarland standard was spread on freshly prepared Mueller Hinton and spread evenly using a glass rod. Sterile E-strips embedded with different antibiotics were applied on the inoculated plate and pressed gently with sterile forceps onto the plate before incubating for 18-24 hours. Following incubation, the plates were read and interpreted base on the CLSI standard (CLSI, 2012) as resistant, intermediate or susceptible.

MicroScan

For MicroScan, a 0.5 McFarland equivalent of purified bacteria suspension was placed in to the 96 well plate and incubated in the MicroScan automated microbiological system for 18-24 hours. The MicroScan automatically interpreted the susceptibility results and flagged all bacteria that had cephalosporin resistance.

VITEC testing

Each isolate tested using double disc diffusion and E-test was confirmed by VITEK. The VITEK system with the ESBL test panel was contained in the NO45 card (all from BioMérieux). The panel had six wells containing cefepime at 1.0 µg/ml, cefotaxime at 0.5 µg/ml, and ceftazidime at 0.5 µg/ml, alone and in combination with CA (10, 4, and 4 µg/ml, respectively), and growth in each well was quantitatively assessed by means of an

optical scanner. The proportional reduction in growth in wells containing cephalosporin plus CA compared with those containing the cephalosporin alone was considered indicative of ESBL production. Quality control strains (*E. coli* ATCC 25922, *E. coli* ATCC 35218, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853) were included in each run.

2.5 Phenotypic detection of ESBL

The DDD method employed four discs; cefotaxim (30 µg), cefotaxim/clavulanate (10 µg), ceftazidime (30 µg) and ceftazidime/clavulanate (10 µg) disks (Oxoid) and interpreted according to the standards established by the Clinical and Laboratory Standards (CLSI). A broth culture of the test organism was adjusted to a 0·5 McFarland standard and inoculated onto freshly made and quality controlled Mueller–Hinton agar (Oxoid: Basingstoke, UK). The combination discs and the corresponding standard cephalosporin discs were placed at a distance of (20mm) from each other on the plate. The plates were incubated at 37 °C for 18 hrs aerobically before the zone sizes were recorded. A positive result was indicated by a zone-size difference of ≤5 mm diameter between the combination disc and the corresponding standard antibiotic disc with phantom development. Minimum Inhibitory concentrations were carried out using both E-test and the MicroScan where the detection in resistance of Cephalosporins were recorded and flagged for the presence of ESBL. For all ESBL detection methods, the known ESBL-producing *E. coli* strains SA1636 (TEM-3) and SA1652 (SHV-2) were used as positive controls. *E. coli* NCTC 10418 was used as a negative control.

2.6 Genotypic detection of ESBL

Purified colonies of ESBLs producing *K. pneumonia* and *E. coli* were suspended in TE buffer and their DNA extracted by boiling (van Soolingen et al, 1991). The SHV and TEM genes were detected as described previously (Kariuki et al, 2002, del Carmen Rodríguez et al, 2004) briefly, specific primers for the genes (forward primer 5´-TCAGCGAAAAACACCTTG -3´; Reverse primer 5´-CCCGCAGATAAATCACCA -3´ for SHV gene; forward primer 5´-GAGTATTCAACATTTCCGTGTC -3´; reverse primer 5´-TAATCAGTGAGGCACCTATCTC -3´ for TEM gene) were used for PCR amplification that produced 498 bp and 861 bp PCR products for SHV and TEM genes, respectively (Integrated DNA Technologies, Inc. Coralville, USA), as described by Paterson (Paterson, 2000). The PCR mixture consisted of 10 pmol of each primers, 1 µl DNA sample (3 µg/µl), 1.5 mM MgCl2, 0.2 mM each dNTP, and 5 u Taq DNA polymerase (Cinagen, Iran) in a total number of 50 µl of PCR reaction. Amplification of SHV and TEM genes was performed as follows: initial denaturation at 95 °C for 2 min and 35 cycles of 1 min at 95 °C, 30 sec at 60 °C (annealing), 1 min at 72 °C (extension) and five min at 72 °C for the final extension. The amplified product was submitted for electrophoresis in 2% agarose gel (BioRad) with ethidium bromide (Invitrogen) (0.5 µg/mL) for 1 hour (6 Volts/cm), and visualized under UV light and results documented.

2.7 Ethical considerations

Ethical approval for the study was granted by the Kenya Medical Research Institute - Ethical Review Committee (Ref. No.: SSC 989).

3. Results

Patients Age distribution

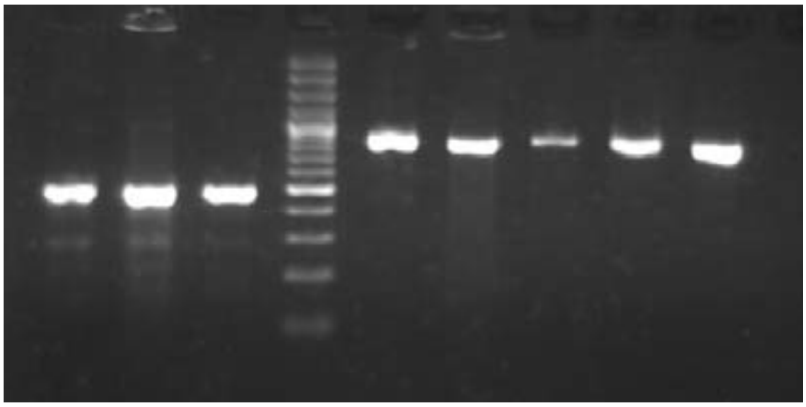
The study sampled a total of 300 patients from all ages ranging from three months and above. Majority of the patients were aged below 5 years (48.3%). Macroscopic examination of stool consistency showed that majority of the stool samples were watery in nature (48%). These findings are summarized in in **Table 1**.

Table 1: Patient age distribution and stool sample appearance

Age range (years)	N (%)
Below 5	145 (48.3%)
6 - 10	17 (5.7%)
11 - 15	11(3.7%)
16 - 20	20(6.7%)
21 - 25	36(12%)
Above 26 years	71 (23.6%)
Stool appearance	**%**
Bloody	6%
Bloody/mucoid	12%
Mucoid	34%
Watery	48%

Figure 1. Phantom phenomena development by cephalosporin resistant *K. pneumonia* and *E. coli* isolates

Figure 2. Multiplex PCR amplification of *K. pneumonia* and *E. coli* TEM and SHV genes.



Extended Spectrum Beta Lactamase testing

Out of the 300 samples from the Machakos District Hospital tested, 18 (6%) were positive for ESBL production by double disk diffusion (DDD).

The 18 DDD and E test-positive strains (3 *Klebsiella* spp and 15 *E. coli*) were retested with VITEK (GNS-532 card), and 17 of these strains (94.4%) were subsequently found to be ESBL positive. One strain tested ESBL negative by VITEK.

The cefotaxime ESBL strip detected the presence of ESBL activity in 18 of the isolates identified as positive for ESBL production by DDD. All the 18 isolates demonstrated phantom phenomena that indicated the presence of ESBL (**Figure 1**).

From genotypic results, TEM was detected in higher percentage compared to SHV resistance genes. Majority of the isolates did not carry any of the two genes (**Figure 2** and **Table 2**).

Table 2. Frequency of TEM and SHV genotypes from *K. pneumonia* and *E. coli*

Organism	No. tested	PCR Results			
		TEM+ve (%)	TEM -ve (%)	SHV+ve (%)	SHV -ve (%)
K . pneumonia	200	8 (4%)	192 (96%)	2 (1%)	198 (99%)
E. coli	100	5 (5%)	95 (95%)	3 (3%)	97 (97(%)

4. Discussion

The correct detection of ESBL producing microorganisms is a challenge for the laboratories, requiring not only phenotypic tests but also genotypic tests for all genes associated with beta-lactamase production. According to the majority of epidemiological studies on ESBL, *K. pneumoniae* and *E. coli* are the most common species implicated in this type of resistance. In this study, *K. pneumonia* and *E. coli* were the only bacteria carrying detectable ESBL (**Table 1**). The results agreed with a study done in Rio Grande do Sul by Freitas and co-workers who observed that these two species were the most prevalent among ESBL producing microorganisms, confirming international multicenter studies (Luzzaro et al, 2006, Sanguinetti et al, 2003; Perilli et al, 2002; Wikler, 2006; CLSI, 2012). These results are important as *K. pneumoniae* is the most frequent gram-negative bacteria involved in nosocomial outbreaks (Tofteland et al, 2007; Freitas et al, 2003; Coque et al, 2002).

TEM was the most prevalent as compared to SHV in the current study (**Table 2**), findings that agree with Freitas et al (2003). The prevalence of SHV was lower compared to other studies done around the world.

The use of three distinct substrates in the combined disk tests increased the sensitivity of the tests (**Figure 2**), and cefotaxime and cefpodoxime performed best, despite the occurrence of non-coincident results. Because of the elevated percentages of ESBL-producing *E. coli* presenting low minimum inhibitory concentrations (MIC) of ceftazidime, Tofteland et al (2002) recommended the use of cefpodoxime alone or a combination of cefotaxime and ceftazidime as preferred substrates for ESBL detection. From the current study, 18 (6%) of the isolated bacterial strains exhibited beta lactamases enzyme activity that is responsible for resistance. Studies in Brazil and Iran reported high prevalence of 61% and 24% respectively (Oliveira et al, 2010, Zaniani et al, 2012).

For the past 40 years, *Klebsiella* spp and pathogenic *E. coli* that are resistant to aminoglycosides have been known to cause outbreaks of hospital-acquired infection (Peterson et al, 2004, Casewell and Phillips, 1981). The discovery of plasmid-mediated ESBL production by *Klebsiella* spp and *E. coli*, together with plasmid-mediated aminoglycoside resistance, in the early 1980s (Vinué et al, 2009) signaled a major new problem with antibiotic resistance. In the current study, 6% of isolates (*Klebsiella* spp and *E. coli*) were found to be ESBL

positive. Many clinicians and other health care providers may be unaware of the problem of ESBL production by gram-negative bacilli resulting in inappropriate medication.

In developed countries, information on resistance to vancomycin especially by *E. faecium* as indicator organism is readily available to clinicians in routine laboratory reports. However, there is no universally accepted applicable marker of the presence of ESBLs. Although resistance of *K. pneumoniae* to ceftazidime is a useful marker for presence of ESBLs, fewer than 50% of *Klebsiella* isolates reported in Kenya undergo testing for susceptibility to ceftazidime, a common practice in developing world (Kariuki et al, 2005). Moreover, some types of ESBL-producing organisms appear susceptible to ceftazidime according to standard methods, and ceftazidime resistance may be due to mechanisms other than ESBL production (Monnet et al, 1997). Unfortunately, many laboratories in Kenya as well as elsewhere in the world do not test for ESBL production (Paterson and Bonomo, 2005, Luzzaro et al, 2006).

Awareness of ESBL production by *K. pneumoniae* and *E. coli* is clinically important. The implications in clinical settings are that in the absence of infection control measures, ESBL-producing organisms can readily pass horizontally from patient to patient. Reliable laboratory methods are now available (DDD, E-strip and VITEK) by which ESBL production can be detected by clinical microbiology laboratories. These methods, which have also been promoted by the CLSI (Monnet et al, 1997; CLSI, 2012), rely on initial screening tests and follow-up confirmatory tests. It is believed therefore that clinicians should not have to specifically request these tests; rather, all *K. pneumoniae* and *E. coli* isolates should undergo routine screening by clinical microbiology laboratories. Isolates suspected of producing ESBLs should not be reported as susceptible to third-generation cephalosporins or cefepime until follow-up confirmatory tests are performed.

From this study, it has been shown that the VITEK system reported one false-positive detection of ESBL activity with one strain of *E. coli*. This strain of *E. coli* had been reported as positive by the DDD and E-strip methods. The best combination by use of E-strip was cefotaxime and ceftazidime. These results were contrary to Vinue et al (2008) findings. Although the percentage of false-positive *E. coli* ESBL strains was only 5.6% it is still a concern that the strain was misreported as ESBL producer by E-strip and DDD antibiotic susceptibility to *ß*-lactam antibiotics. There appears to be no obvious reason for these results. It is, however, unlikely to arise from a technical error, as the VITEK is a highly standardized system.

In reported studies, the DDD test was able to detect 82% and 88 % of ESBL-positive strains, respectively (Thomson and Sanders, 1992) contrary to the findings from this study (100% and 94.4%). The limitations of this test have been described elsewhere (Vinué et al, 2009; Thomson and Sanders, 1992; Bush, 1996). It has been reported that cepodoxime achieves a 100 % sensitivity rate in detecting ESBLs in tested isolates, cefotaxime 92 %, and ceftazidime 82 % (Appleton and Hall, 2000; Oliveira et al, 2010). In contrast, the results on isolates from the current study showed that cefotaxime was the most efficient combination for the detection of ESBLs, with a sensitivity rate of 100%; the values for cepodoxime and ceftazidime were 50 % and 90%, respectively.

The commercially available ESBL E-test strip is a quantitative technique, and is widely regarded as the 'gold standard' for detection in clinical laboratories of ESBL production (Crowley, 2001; M'Zali et al, 2000). In this study, it detected 100 % of the test isolates and this was only possible when both cefotaxime and ceftazidime strips were used in conjunction and not with cefpodoxime. As noted above, the ceftazidime E-test strip was less sensitive when solely used; this could be due to the possibility that there were other ceftazidime-hydrolysing *ß*-lactamases in some strains that were not sensitive to clavulanic acid, which could have reduced the sensitivity of the test. Thus, E- tests with both cephalosporins are recommended; however, this makes the technique expensive, and most clinical laboratories in Kenya would only use it for confirmation rather than routine testing.

It should be noted that in this study one *E. coli* was falsely reported as ESBL positive by the two methods. For all ESBL positive *K. pneumoniae* in this study, the VITEK test was accurate, but it should be noted that this study only included VITEK ESBL - positive strains by the other two methods, and was therefore not an evaluation of the VITEK ESBL test itself per se. To the best of the author's knowledge, this is the first report on ESBL in Kenya reported from isolates in stool samples. The other two documentations available on ESBL were from blood and urine (Kariuki et al, 2005, Kariuki et al, 2000).

5. Conclusion

Due to the 6% prevalence of resistance to cephalosporins used in Machakos County and the confirmation of TEM and SHV resistance genes, their continued use may not be appropriate for the treatment of infections caused by pathogenic *E. coli* and *Klebsiella* spp. This may be one of the reasons for increased morbidity (12.2%). Other effective drugs should be identified and adopted for use on a regular basis. The data underlines the importance of regular antimicrobial surveillance in district hospital settings such as Machakos and other counties. Consequently, the required laboratory infrastructure and protocols for surveillance of resistance must be established, monitored, evaluated and sustained. Since over-the-counter sale of commonly used antibiotics without prescription is inferred as one of the contributing factors for the spread of resistance, the practice must be curtailed if the antibiotic arsenal available to physicians is to continue to be effective.

Conflict of Interest Declaration

The authors declare no conflict of interest.

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