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EVALUATION OF PHENOTYPIC AND MOLECULAR TECHNIQUE IN THE DETECTION OF EXTENDED SPECTRUM BETA-LACTAMASE (ESBL)-PRODUCING GRAM NEGATIVE BACILLI IN OGUN STATE, NIGERIA

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ABSTRACT

Background: **Molecular diagnosis though faster and more sensitive than phenotypic techniques, is more expensive. Resource limited settings are thus limited to using more of phenotypic rather than molecular methods in the routine detection of Extended Spectrum beta lactamases (ESBL)**

Aim: **This study aimed to evaluate the performance of double disc synergy test (DSST) and Epsilometer (E) test with Polymerase Chain Reaction (PCR) and to detect the risk factors associated with ESBL producing organisms among in-patients at Babcock University Teaching Hospital, Ilishan-Remo, Nigeria.**

Methodology: **Hospital-based cross-sectional study in which bacterial isolates of 165 in-patients were collected fromMarch 2018 to September 2019. The isolates were evaluated for ESBL production by the use of DDST, Etest and PCR. The performance evaluation was done. Questionnaire was used to assess the risk factors associated with ESBL, IBM SPSS Version 23 was used to analyze the data.**

Results: **The participants' isolates yielded 50/165 (30.3%) that were ESBL positive by DDST, 47/165 (28.9%) by E-test and 48/165(29.1%) by PCR. Sensitivity and specificity of DSST was 100% and 98.3% while that of E-test was 98% and 100% respectively. Age, antibiotics intake without prescription, being on ventilator, urethral catheterization and nasogastric tubes were all significantly associated with presence of ESBL (p value <0.05).**

Conclusion: **Phenotypic tests remain reliable for the routine detection of ESBL in the absence of molecular methods. Rational use of instrumentation and antibiotics is advocated based on the risk factors detected from this study.**

Keywords: Double disc synergy test, Epsilometer test, Polymerase Chain Reaction (PCR), Extended spectrum beta lactamases.

INTRODUCTION

The continued increase in the prevalence of multi-drug resistant organisms (MDRO) is a known cause of therapeutic failures clinically and this has become a major global concern.[1,2] Among the MDRO, of most importance are Gram-negative bacilli producing Extended Spectrum beta lactamases (ESBL). These organisms cause a large proportion of infections both in the hospital and community but are resistant to most common treatment options including Beta lactam antibiotics.[3-5] Therefore resistance of Gram-negative bacilli to these antibiotics is a public health concern because of limited therapeutic options in infected patients.[1]

ESBL hydrolyzes penicillin, narrow- and extended-spectrum cephalosporins and aztreonam but they are inhibited by beta lactam inhibitors.[5-7] Also, the presence of ESBL in a bacterium can confer resistance to trimethoprim-sulphamethoxazole, aminoglycosides and quinolones because the plasmids carrying ESBL genes are also known to carry resistance genes that encode for resistance to other antibiotics.[5-7] The high transferability of plasmids carrying ESBL genes has increased the risk of resistance transmission in hospital infections leading to prolonged hospital stay, increased medical bills and adverse disease outcomes in patients. All these have made ESBL a serious threat globally.[3,7-8]

ESBLs are prevalent worldwide and the prevalence has continued to escalate over the years.[2,8] In 2017, the Centers for Disease Control and Prevention (CDC) estimated that among hospitalized patients, there were 197,400 cases of ESBL-producing enterobacteriaceae and 9,100 estimated deaths in the United States alone.[9] In Chitwan, South Asia, the prevalence of ESBL was reported to be 64% while in Pakistan the prevalence of ESBL has been increasing over the last decade and is reported to be 79%.[1,10] In Sub-Saharan Africa, ESBL has been reported to be a major public health threat with a prevalence of 62.3% in Mali and 64.3% in Sierra Leone.[11] In Nigeria, the prevalence of ESBL varies from 23.6% in Maiduguri, 11.4% in Enugu to 51.3% in Ile-Ife.[12-14]

Laboratory methods for the detection of ESBL include the phenotypic and molecular methods.[15] Phenotypic methods include the use of double disc synergy test (DDST), Epsilometer (E test) and Combination disc method which are based on the inhibitory activities of beta-lactamase inhibitors.[3,15-16] These methods are the preferred options in the routine medical microbiology laboratory because of the cheaper cost.[3] However, these methods have longer turnaround time, depending on bacterial growth, the results are subjective and there may be the issue of false positivity or false negativity if the ESBL-producing bacteria coexpress AmpC type β-lactamase (ACBL).[17,18]

Molecular diagnosis is faster, more accurate and more sensitive than phenotypic techniques thereby improving treatment outcomes in patients and is also useful in supplying epidemiological data.[17,19] Recently, the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) reduced the susceptibility breakpoints of Cephalosporins therefore making the of use phenotypic ESBL tests routinely unnecessary.[3,16] However, considering the lack of precision that might accompany antimicrobial susceptibility test and also that in-vitro susceptibility may not translate to clinical success in the therapeutic management of patient, confirmatory phenotypic ESBL tests might still be necessary routinely.[3,20] Though rapid and accurate detection of ESBL is needed for appropriate essential antibiotic treatment and infection control activities and the molecular or automated methods fit perfectly for this, however, they are more costly and need trained personnel. Therefore, most laboratories in developing countries cannot afford to employ these methods.[21] This leaves us with the option of using more phenotypic methods than automated or molecular methods in the detection of ESBL. Therefore, it is important to carry out performance evaluation of phenotypic methods as against the molecular methods in our environment. Thus the objective of this study was to evaluate the performance of the double disc synergy test and E-test by comparing with PCR and also to detect the risk factors associated with ESBL among in patients in Babcock University Teaching Hospital, Ilishan-Remo, Nigeria.

MATERIALS AND METHODS

This was a descriptive cross-sectional study conducted from March 2018 to November 2019 among in-patients of Babcock University Teaching Hospital, Ilisan-Remo, Ogun State.

The sample size, 165 was calculated by Leslie fisher's formula[22] and the prevalence used was 11.4% from a study in Enugu, Nigeria.[23] Participants were recruited by simple random sampling and inclusion criteria was the in-patients diagnosed with clinical infections while those unwilling to fill the questionnaire or give permission for specimen collection were excluded. Written informed consent was obtained from each participant after a semi-structured interviewer-administered questionnaire was used to obtain sociodemographic and associated predisposing factors to ESBL infection. Ethical approval with ethical clearance number BUHREC070/18 was obtained from Babcock University Health Research ethics committee Ilishan-Remo, Ogun State, Nigeria. The date of the approval was 28th of February, 2018. Data was collected from March 2018 to September 2019. Specimens such as blood culture, sputum, urine, wound biopsy, swabs, cerebrospinal fluid and/or aspirates were collected from the participants, as indicated, and processed in the Medical Microbiology laboratory by following standard microbiology procedures.[24] The organisms were identified by Microbact TM GNB 24E (Oxoid®, Basingstoke, UK) The Kirby-Bauer disc diffusion method was used for the antimicrobial susceptibility test and the Clinical and Laboratory Standards Institute (CLSI) chart was used for interpretation.[25-26] *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were the positive and negative control strains respectively.[12,25-26] All isolates resistant to one or more third generation Cephalosporin were subjected to phenotypic testing by double disc synergy test and Epsilometric test (E-test) for confirmation of ESBL-production.[27,28]

The double disc synergy test was done according to CLSI guidelines while the E-test (Biomerieux SA) was done with a cefotaxime gradient at one point and cefotaxime plus clavulanate gradient at the other end.[26,27] The procedure and interpretation were done according to the manufacturer's instruction.[27] DNA extraction was done by a DNA extraction kit, quick- DNA fungal/

bacteria miniprep (Zymo research, USA). ESBL genes SHV and TEM were identified and amplified by the conventional PCR method using previously described primers.[12,28] SHV-F-CGCCTGTGTATTATCTCCCT, SHV-R-C, GAGTAGTCCACCAGATCCT at 293bp, TEM-F-TTTCGTGTCGCCCTTATTCC, TEM-R-ATCGTTGTCAGAAGTAAGTTGG at 403bp.

The amplification was carried out by initial denaturation at 94^0C for 5 minutes, followed by 35 cycles of denaturation at 94^0C for 15 seconds, annealing temperature of 55^0C for 20 seconds, followed by extension at 72^0C for 30 seconds and another extension at 72^0C for 7 minutes. Subsequently, the amplified PCR products were separated 1.5% electrophoretic agarose gel.

In comparison to PCR, the performance of DDST was evaluated for true positive (Number of isolates that were DDST positive where PCR was positive), False negative (Number of isolates that are DDST negative but PCR positive), True negative (Number of isolates that are DDST negative where PCR was negative) and False positive (Number of isolates that are DDST positive but PCR negative). The same performance evaluation was done for E-test respectively. Then sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of each test was calculated respectively. The study data was analysed by SPSS version 23 software.

RESULTS

The 165 isolates from various clinical specimens of the participants yielded 50 (30.3%) isolates that were ESBL positive by double disc synergy test, 47 (28.9%) by E test and 48(29.1%) by PCR.

SHV gene was 37/48(77.1%) (Figure 1) while TEM was 24/48(50%) (Figure 2) while 13 were overlapped 13/48(27.1%)

The 47 isolates positive by E test were also positive with DDST. Taking PCR as the standard, the comparison of the result of the double disc synergy test with PCR and E test with PCR is presented in Table 1.

The sensitivity, specificity, positive predictive value (PPV), Negative predictive value (NPV) and accuracy of DDST was 100%, 98.3%, 96%, 100% and 97.6% respectively while that of E test was 98%, 100%, 100%,

Table 1: Comparison of the prevalence of ESBL in participants by using DDST, E test and PCR

Variables	PCR		Total
	Positive (%)	Negative (%)	
DDST			
Positive	48(96.0)	2(4.0)	50
Negative	0(0.0)	115(100)	115
E- test			
Positive	47(100)	0(0.0)	47
Negative	1(0.8)	117(99.2)	118

DDST: Double Disc Synergy Test, E test: Episilometer test

Table 2: Prevalence of ESBL-producing organisms

Organisms Isolated	ESBL		Total (%)
	Positive(%)	Negative(%)	
Hafnia alvei	6(75.0)	2(25.0)	8(4.8)
Escherichia coli	6(16.2)	31(83.8)	37(22.4)
Klebsiella pneumoniae	13(50.0)	13(50.0)	26(15.8)
Serratia liquefaciens	3(37.5)	5(62.5)	8(4.8)
Enterobacter sakazakii	2(33.3)	4(66.7)	6(3.6)
Enterobacter cloacae	2(25.0)	6(75.0)	6(3.6)
Acinetobacter lwoffii	2(33.3)	4(66.7)	6(3.6)
Acinetobacter haemolyticus	1(33.3)	2(66.7)	3(1.8)
Klebsiella oxytoca	3(14.3)	18(85.7)	21(12.7)
Acinetobacter baumanii	4(66.7)	2(33.3)	6(3.6)
Klebsiella ozaenae	2(28.6)	5(71.4)	7(4.2)
Enterobacter gergoviae	0(0.0)	7(100.0)	7(4.2)
serratia marcescens	0(0.0)	7(100.0)	7(4.2)
pseudomonas	4(57.1)	3(42.9)	7(4.2)
Organisms isolated but not significant	0(0.0)	8(100.0)	8(4.8)
Total	**48(29.1)**	**117(70.9)**	**165(100.0)**

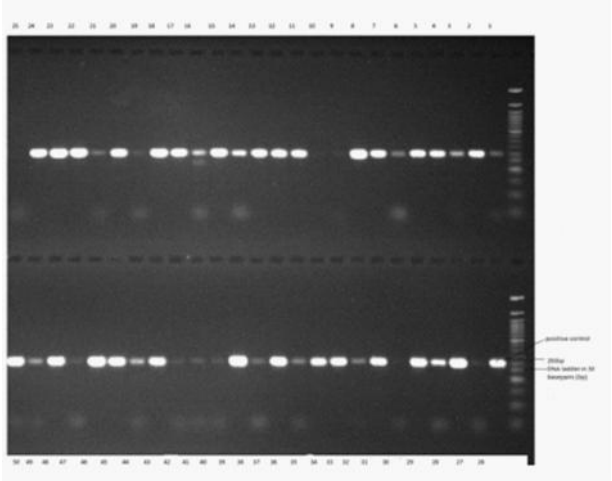


Figure 1: SHV gene amplicon

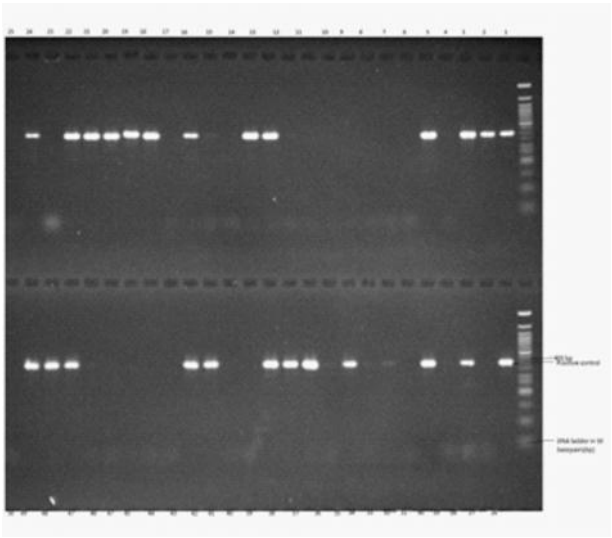


Figure 2: TEM gene amplicon

Table 3: Probability of organism isolated to be ESBL positive

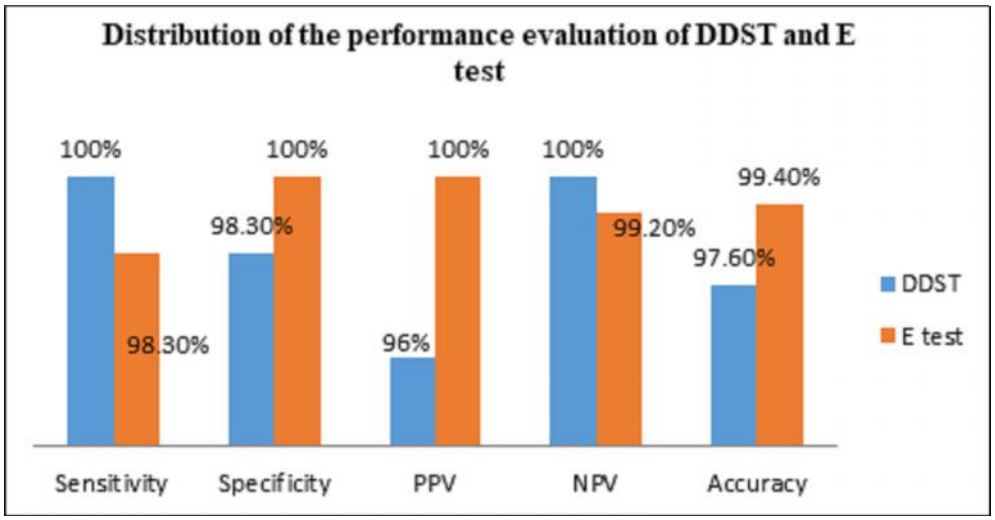
Organism isolated/NO	P-value	OR (95% CI)
Hafnia alvei /8	0.008	17.8(2.13-149.23)
Escherichia coli /37	0.81	1.2(0.24-6.18)
Klebsiella pneumonia /26	0.021	6.3(1.32-30.27)
Serratia liquefaciens / 8	0.17	4.1(0.55-30.87)
Enterobacter sakazakii /6	0.28	3.4(0.37-31.32)
Enterobacter cloacae /8	0.45	2.3(0.27-19.40)
Acinetobacter lwoffii /6	0.28	3.4(0.37-31.32)
Acinetobacter haemolyticus /3	0.39	3.4(0.21-55.70)
Klebsiella oxytoca /20	0.95	0.9(0.15-5.91)
Acinetobacter baumanii /6	0.021	13.7(1.49-125.28)
Klebsiella ozaenae / 7	0.36	2.7(0.31-23.98)
Pseudomonas aeruginosa /7	0.018	9.8(1.48-64.95)

99.2% and 99.4% respectively. This is illustrated in Figure 3

The highest proportion of ESBL producers was found among *Hafnia alvei* and *Klebsiella pnuemoniae*. (Table 2). In contrast, no ESBL producers were found in *Enterobacter gergoviae* and *Serratia marcescens*.

Hafnia alvei, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Acinetobacter baumanii* were more likely to produce ESBL. (p value < 0.05). This is seen in Table 3.

Age, level of Education, history of unconsciousness, recent past hospital admission, recent past ICU admission, history of taking antibiotics without



Keywords
DDST- Double disc synergy test
E test-Epsilometertest
PPV-positive predictive value
NPV- Negative predictive value

Figure 3: Performance evaluation of DDST and E test

prescription, being on ventilator, urethral catheterization and nasogastric tubes were all statistically significantly associated with ESBL.(p value < 0.05). (Table 4).

Table 4: Risk factors associated with ESBL among the participants

Variables	ESBL		P-value	X^2	Df
	Positive (%)	Negative (%)			
Age (Years)					
1-10	3(75.0)	1(25.0)			
11-20	6(75.0)	18(25.0)			
21- 30	9(14.5)	53(85.5)	0.00	27.6	7
31-40	8(24.2)	25(75.8)			
41- 50	7(25.0)	21(75.0)			
51-60	3(20.0)	12(80.0)			
61- 70	4(44.4)	5(55.6)			
71-80	8(100.0)	0(0.0)			
Sex					
Male	28(33.7)	55(66.3)	0.19	1.75	1
Female	20(24.4)	62(75.6)			
Marital status					
Single	15(26.8)	41(73.2)	0.64	0.22	1
Married	33(30.3)	76(69.3)			
Level of Education					
No school	7(58.3)	5(41.7)			
Primary	6(28.6)	15(71.4)	0.07	7.08	3
Secondary	11(20.4)	43(79.6)			
Tertiary	24(30.8)	54(69.2)			
History of unconsciousness			0.00	23.20	1
Yes	9(100.0)	0(0.0)			
No	39(25.0)	117(75.0)			
Recent past hospital admission			0.00	27.09	1
Yes	27(58.7)	19(41.3)			
No	21(17.6)	98(82.4)			
Long ICU stay			0.00	16.5	1
Yes	8(88.9)	1(11.1)			
No	40(25.6)	116(74.4)			
Antibiotics without prescription			0.00	26.29	1
Yes	18(72.0)	7(28.0)			
No	24(20.7)	92(79.3)			
On ventilator			0.00	15.18	1
Yes	6(100.0)	0(0.0)			
No	42(26.4)	117(73.6)			
Urethra Catheter			0.00	52.9	1
Yes	32(71.1)	13(28.9)			
No	16(13.3)	104(86.7)			
On Nasogastric tube					
Yes	15(60.0)	10(40.0)	0.00	13.65	1
No	33(23.6)	107(76.4)			

DISCUSSION

Phenotypic ESBL detection tests have been linked with varying sensitivities and specificities depending on location, species and settings.[20] In our hospital setting, the use of phenotypic tests to detect ESBL is easier and cost effective, hence the need for us to evaluate these tests against the molecular test which is considered the gold standard.[17,19]

In this index study, the sensitivity of DDST was 100% while that of the E test was 98% compared with PCR testing. These high sensitivities indicate that these tests have good utility in identifying ESBL producing organisms. However, it appears that the E test is more sensitive than DDST in detecting ESBL in these isolates. This finding is similar to what was reported by Sedlakova *et al.* in which the sensitivity of DDST was 100% and the E test was 95%.[16] However, Kaur and Aruna reported that there is no difference between

the sensitivities of these two methods.[30] Variations in these reports can be attributed to use of different disks. False positive ESBL tests can result in a falsely high ESBL prevalence rate and create restricted therapeutic options for patients who may have to resort to taking Carbapenems. Minimizing such errors is therefore very important for appropriate patient therapy. One of the ways of reducing false positivity is the use molecular tests, which though are more accurate, are too expensive to perform routinely in developing countries like ours.[17,19,20-21] We found that both DDST and E test had high specificities, with E-test having no false positive isolate, and DDST is only slightly less specific at 98.5%.

The new CLSI guidelines and EUCAST breakpoints for Cephalosporin have reduced the rate of false positivity by the new cephalosporin breakpoints up to a level.[16] The current CLSI guidelines recommends that routine confirmatory phenotypic test for ESBL is not necessary but some authors have argued that a low cephalosporin MIC alone is not a clear predictor of therapeutic clinical success especially in some group of patients with altered antibiotic pharmacokinetics and high risk of therapeutic failure. Thus, the knowledge of confirmatory ESBL status is very important.[30, 31]

Our findings of the positive and negative predictive values (PPV and NPV) of DDST and the E test show that both have high and acceptable values. We also noted based on these results that E test is more reliable when the result is ESBL-positive (100% vs 96%) while an ESBL-negative result is only slightly more reliable when DDST is carried out (100% vs 99.2%). This finding is similar to what was reported by Morrisey *et al.*[30]

Also, in developing countries where routine antimicrobial susceptibility testing may not be done with enough accuracy or precision to stratify isolate into whether they are resistant, intermediate or susceptible, there may still be a need to perform the confirmatory phenotypic ESBL tests.[20]

Overall, our study demonstrates the efficacy of the DDST and the E tests in ESBL detection, especially in resource constrained settings like ours. Although the two tests evaluated showed comparable performance, the E test might not be a feasible method to use routinely because it is more expensive than DDST to carry out. Therefore, DDST might seem to be the most feasible and effective method in developing countries. It should however be borne in mind that there can be false positivity and false negativity, if the isolate co-expresses Amp C.

The prevalence of phenotypic ESBL prevalence varies across the world.[2, 8] In this study, the ESBL prevalence by DDST was 30.3% which is similar to previous reports by Olorunitola and colleagues and Halaji *et al.* with DDST which reported 30% and 31.3% respectively.[32-33] Other prevalence reported by DDST includes 38.18% by Numanovic *et al.*, 51.3% by Bajpai *et al.*, 54% by Ejaz, 5% by Yusuf *et al.* in kano, Nigeria and 34.3% in in Zaria by Giwa *et al.*[15,21,34-35]

The E test ESBL positivity was 28.9% from this index study and its lower than the 61% reported by Abrar *et al.* and Moharty *et al.* respectively.[1,36] Although it is higher than 14% by Chandramohan and Revell by the use of the same methods.[37] Our PCR prevalence is 29.1% which is lower than 52.49% by Sharma *et al.* and higher than 7% by Chandramohan and Revelli by the use of a similar method.[19,38] These discrepancies across the world are most likely caused by geographical locations, type of isolates, diagnostic methods employed, the precision of laboratory procedures, various patients' characteristics, use and misuse of antibiotics.[2,21,33,39] A major conclusion from all these variations is the necessity for the use of molecular methods which will give a definitive result of ESBL. Unfortunately, these might not be realistically carried out routinely in developing countries because it is costly and requires expensive equipment and well-trained personnel.[21]

Klebsiella pneumoniae has been reported in many studies to harbor more ESBL and this report is similar to what was found in this index study.[14,29,40] Although some other literature reported contrary findings.[35, 41] The variations in these reports suggest that many bacteria are now harbouring ESBL and this is a serious threat to patient's management. Some of the Enterobacter species in this study were ESBL positive and this finding is congruent to previous reports by Aibinu *et al.* and Akujobi and Ewuru respectively.[42-43] However, Yusuau and colleagues did not find ESBL in their confirmed Enterobacter isolates.[44] *Hafnia alvei*, though an uncommon pathogen, was observed to harbour a very high proportion of ESBL. It is therefore necessary to closely monitor the emergence of this organism as a pathogen especially in the hospital environment. *Serratia marcescens* did not harbour ESBL in this present study and this finding is consistent with that of Nwankwo *et al.* in Kano.[45]

An understanding of risk factors is important for instituting measures to prevent infections with ESBL-producing organisms. Risk factors found to be associated with ESBL production in this study includes unconsciousness, long ICU stay, recent hospital admission, use of antibiotics without prescription, use

of a urethral catheter, being on ventilator and use of ventilators. Some of these risk factors have been reported previously by some authors to be associated with ESBL.[29,46,47] Age is associated with ESBL in this present study as the risk of ESBL-producing organisms was much higher in children and the elderly. This finding is contrary to previous studies by Maleki *et al.* and Moini *et al.* but it is consistent with reports from Sabrina *et al.* and Jewoola *et al.* in Nigeria. Gender was not an associated risk factor in this study and these findings are congruent with Maleki *et al.* and Moini *et al.* but contrary to Ibrahim *et al.*[39, 48, 49]

The limitation of this study includes the non-differentiation of colonizers from pathogens, limited ESBL genes were evaluated and we did not carry out sequencing to identify probable mechanism of resistance.

CONCLUSION

The prevalence of ESBL by DDST, E test and PCR was 50 (30.3%), 47 (28.9%) by E test and 48(29.1%) respectively. Compared to PCR, which is the standard, the sensitivity and specificity of DDST were 100%, and 98.3% while that of E test was 98% and 100% respectively.

Although the molecular methods are more sensitive and accurate, they are expensive to carry out routinely and based on the findings of specificity and sensitivity from this study, we advocate that the phenotypic tests, DDST and the E test, can still be used for routine detection of ESBL in our environment. They however, need to be evaluated periodically to confirm adequate performance. Based on the identified risk factors, rational use of antibiotics and minimization of instrumentation are advocated in patients.

Conflicts of Interest

There are no conflicts of interest.

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