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Evaluation of a Chromogenic Medium for the Detection of ESBL with Comparison to Double Disk Synergy Test

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Authors' contributions

This work was carried out in collaboration between all authors. Author CCE designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors NRA and CEE managed the analyses of the study. Author SIO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Extended Spectrum Beta Lactamase (ESBL) producing bacterial strains are the major causes of nosocomial and community-acquired infections worldwide. The aim of the study was to evaluate the effectiveness of Brilliance ESBL Agar (BEA) (a chromogenic culture medium) for the detection of ESBL in comparison with Double Disc Synergy Test (DDST) and confirm results from both methods by Single-plex Polymerase Chain Reaction (PCR) as gold standard.
Materials and Methods: A total of 75 clinical isolates of *Escherichia coli* were screened for ESBL production using BEA & DDST from various clinical specimens. The antibiotic susceptibility testing

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was done by the Kirby-Bauer disc diffusion method using Cefotaxime (30 µg) and Ceftazidime (30 µg) discs on Mueller Hinton agar. ESBL producing strains were detected phenotypically by DDST and BEA at 24 h and 48 h, respectively. Isolates screened by both methods were confirmed using PCR for the detection of bla_{SHV}, bla_{TEM}, bla_{CTX-M} genes.

Results: The prevalence of ESBL was 61%. The sensitivity and specificity of DDST at 24 h and 48hours incubation time was 91.3% and 89.5%, respectively. BEA showed an increase in sensitivity and specificity at 48 h with 97.8% and 98.0%, respectively. All ESBL producing strains detected by phenotypic tests were also found harboring ESBL genes (bla_{SHV}, bla_{TEM}, bla_{CTX-M}) by PCR.

Conclusion: The use of BEA in the screening of ESBL production was found to give much better results than DDST and can be used where PCR cannot be performed.

Keywords: Brilliance ESBL agar; double disc synergy test; extended spectrum beta lactamase genes; antibiotic resistance.

1. INTRODUCTION

The irrational and inappropriate use of beta lactam antimicrobial drugs has led to the advent of resistant strains worldwide. Beta lactam resistance is mainly attributed to acquiring beta-lactamase genes localized on mobile circular genetic elements such as plasmids or transposons. Most beta-lactamases discovered in *Escherichia coli* fit into ambler class A and can be further grouped into narrow-spectrum beta-lactamases (e.g., TEM-1, TEM-2, and SHV-1) and extended-spectrum beta-lactamases (ESBLs) (e.g., TEM-3, SHV-5, and CTX-M) [1]. ESBL impart resistance to extended-spectrum cephalosporin, generally used to treat infections brought about by Gram negative bacteria.

The phenotypic confirmatory tests rarely identify all ESBLs. Some organisms harboring ESBLs might possess other Beta Lactamases that can mask ESBL production in the phenotypic test thus resulting in a false negative result.

One of the most common phenotypic confirmatory tests used for ESBL detection is Double Disc Synergy Test (DDST). In this test, third generation Cephalosporin and Clavulanated-Amoxicillin disc are kept 30 mm apart center to center, or 20 mm for increased sensitivity, on inoculated Mueller-Hinton agar (MHA) [2]. A clear extension of the edge of the inhibition zone of cephalosporin towards Clavulanated-Amoxicillin disc is interpreted as positive for ESBL production. Here, Cefotaxime and Ceftazidime which are good substrate for CTX-M, TEM and SHV respectively are employed. Cephalosporin/clavulanate synergy is observed in Cephalosporin-resistant isolates [3]. Evaluations of the double disc synergy test revealed sensitivities of method ranging from 79% to 97% and specificities range from 94% to 100% [4-8]. Sensitivity maybe reduced when ESBL activity is very low, leading to wide zones of inhibition around the Cephalosporin and Aztreonam discs, especially for *Proteus mirabilis* [9]. Distance between discs affects the outcome of the result. In isolates which are suspicious for harboring ESBLs are best tested using a closer distance (15- 20 mm) [5-7].

Chromogenic Culture media are the next generation of media which are truly rapid culture-based methods used for detection of ESBL as well as organism identification [10]. Here, there is an incorporation of chromogenic enzyme substrate as a detection system. Chromogenic substrates consist of chromophor linked to an enzyme-recognizing part such as carbohydrate, amino acids or phosphate. Specific enzymes produced by the target micro-organism will cleave to the chromogenic substrate liberating the chromophor which highlight the micro organism by coloration of the grown colony. However, recognition and handling of colored colonies is an advantage in Clinical Microbiology. Furthermore, the recent inclusion of discriminatory antibiotics into chromogenic media has been a revolution for the explicit detection of ESBL from clinical specimens [10]. Currently, Brilliance ESBL Agar is one of the existing mercantile chromogenic media for detection of ESBL-producers. Brilliance ESBL Agar is a ready to use media. Its formulation contains Cefpodoxime (a third generation Cephalosporin). This is a widely recognized marker of ESBL resistance, in combination with additional antibacterial agents, to inhibit most non-ESBL producing organisms, yeast and gram positive organisms. Cefpodoxime is a widely recognized, reliable and selective marker for all forms of ESBL which can be used as a single

substrate [10]. This is in disparity to Ceftazidime which is combined with Cefotaxime to reliably detect ESBL in DDST. Differentiation of the most ubiquitous ESBL producing organisms is achieved through the inclusion of two chromogens- 5-Bromo-3-Indoxyl and N-methylindoxyl. On Brilliance ESBL Agar, *E. coli* expressing β-galactosidase and β-glucuronidase appear as blue colonies (pink if β-galactosidase is negative). The KESC group appears as green colonies due to β-galactosidase and β-glucosidase expression respectively [10]. Few Studies have been done on Brilliance ESBL Agar. But no such research is undertaken and published till date where a comparative evaluation of diagnostic performance of Brilliance ESBL agar with DDST (most common phenotypic test for ESBL) is done.

The presence of an ESBL –producing organism in clinical infection can result in treatment failure. ESBLs can be difficult to detect because they have different levels of activity against various cephalosporins. Reducing the spread of plasmid-mediated resistance such as ESBL in hospitals requires its proper identification in order to control the spread. This study aimed to evaluate Brilliance ESBL Agar in the detection of ESBL with comparison to Double Disc synergy Test.

2. MATERIALS AND METHODS

Seventy five (75) pure cultures of *Escherichia coli* from a number of clinical specimens – Urine (37), Wound Swab (11), Vaginal Swab (18), and Sputum (9) were made in suspension equivalent in turbidity to 0.5 McFarland solutions. Antibiotic susceptibility testing as well as ESBL detection was done using the preparation.

2.1 Study Area

This was a hospital-based study conducted at Nnamdi Azikiwe University Teaching Hospital, a tertiary health care facility in Nnewi, South Eastern, Nigeria, over a 3-month period.

2.2 Antibiotic Susceptibility Testing

Isolates prepared in suspension equivalent to 0.5 McFarland standards were used for antibiotic susceptibility testing with Ceftazidime (30 ug) and Cefotaxime (30 ug) (Oxoid, United Kingdom). The test was conducted in accordance with Kirby-Bauer Disc Diffusion Method. Zones of Inhibition were interpreted as concurring to Clinical Laboratory Standard Institute (CLSI) [11]. Controls were utilized as endorsed by CLSI.

2.3 Detection of Extended- Spectrum Beta-lactamase

Isolates which had diameter of zone of inhibition of ≤17 mm with Ceftazidime and ≤ 22 mm with Cefotaxime were suspicious for producing ESBL and thus subjected to screening.

2.3.1 Double disc synergy test (DDST)

Extended-Spectrum Beta-lactamase (ESBL) was detected by the Double Disc Synergy Test (DDST) [2,12]. The prepared suspension of the isolates to turbidity equivalent to 0.5 McFarland standards was inoculated on Mueller-Hinton agar plate. Clavulanated-Amoxicillin (30 µg) disc was placed at the center of the Mueller-Hinton agar plate. Ceftazidime (30 µg) and Cefotaxime (30 µg) discs were placed 15 mm out from the edge of Clavulanated-Amoxicillin disc. Incubation of inoculated plates at 37°C aerobically for 24h and 48 h was done. Observation of Cephalosporin/ clavulanate synergy was interpreted as positive for ESBL production [13,14]. Controls were used as recommended by CLSI [11].

2.3.2 Brilliance ESBL agar

The presence of Extended-Spectrum Beta-lactamase (ESBL) was also detected by Brilliance ESBL Chromogenic Culture Medium (Oxoid, UK). A prepared suspension of the test organism to turbidity equivalent to 0.5 McFarland standards was inoculated on Brilliance ESBL Chromogenic Culture agar plate. Inoculated plates were incubated at 37°C aerobically for 24 hours and 48 hours; change in color of colonies was observed and interpreted as per Oxoid, UK guidelines. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as negative and positive controls.

2.4 Molecular Detection of Bla_{shv}, Bla_{tem}, Bla_{ctx-M} Genes Using Singlex Polymerase Chain Reaction (PCR)

Plasmid DNA was extracted according to the published method of Johnson and Woodford [15]. PCR experiment was carried out in 25 µl solution consisting of 0.8 mM $MgCl_2$, 0.5U of *Taq* polymerase, 200 µM dNTPs, 1 µl template DNA and 1 µl of each oligonucleotide primer using a master cycler (Eppendorf, Germany). The primers used were: SHV-F-5[1]-AGGATTGACTGCCTTTTTG-3[1], SHV-R-5[1]-

ATTTGCTGATTTCGCTCG-3[1] [16]; TEM-F-5[1]-TTGGGTGCACGAGTGGGTTA-3[1], TEM-R-5[1]-TAATTGTTGCCGGGAAGCTA-3[1] [17]; CTX-M-F-5[1]-ACCGCCGATAATTCGCAGAT-3[1], CTX-M-R-5[1]-GATATCGTTGGTGGTGCCATAA-3[1] [18]. Initial denaturation at 95°C for 4 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, annealing for 1 minute and at 48°C for bla_{TEM} and 60°C for bla_{SHV} and bla_{CTX-M}, primer extension at 72°C for 1 minute. The final extension step was extended to 5 minutes at 72°C for all genes. The amplified genes were separated by gel electrophoresis, in 1% (W/V) agarose gel submerged in TBE 0.5X (Tris/borate/EDTA) buffer. DNA bands were visualized under UV illumination after being stained with ethidium bromide (Merck, Germany) and then photographed. *Klebsiella pneumoniae* ATCC 700603 containing bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes was used as positive control while, *Escherichia coli* ATCC 25922 not containing the bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes was used as negative control.

2.5 Statistical Analysis

Non - Parametric method (Chi-square) was used to determine if a significant difference existed between results from various procedures. Where a significant difference exists, it was interpreted as $P < 0.05$. Sensitivity and specificity was calculated using the conventional formulas:

Sensitivity = (TP/TP + FN) × 100; Specificity = (TN/TN + FP) × 100.

3. RESULTS AND DISCUSSION

The study was done to evaluate the performance of Double Disc Synergy Test (DDST) and Brilliance ESBL Agar (BEA) in screening for ESBL among *Escherichia coli* from a collection of clinical specimens which includes: Urine, Vaginal Swab, Wound Swab and Sputum. Among the clinical specimens, variation in number of ESBL-producing *E. coli* isolates was observed between BEA and DDST (Table 1). The prevalence of ESBL in this research was 61%.

With the two third generations Cephalosporin utilized for susceptibility testing, Cefotaxime exhibited a better specificity and sensitivity than Ceftazidime. Results from the susceptibility analysis showed that Cefotaxime revealed 38 *E. coli* isolates while, Ceftazidime revealed 37 *E. coli* isolates as likely ESBL producers.

Using the DDST and BEA for ESBL screening; ESBL-producing isolates emerged at 24 h and 48h incubation periods (Figs. 1, 2, 3, 4). The sensitivity and specificity of DDST at 24 h incubation time was 91.3% and 89.5% respectively which was the same at 48h incubation time. The sensitivity and specificity of Brilliance ESBL agar was 87.0% and 89.5%, respectively at 24 h with 97.8% and 98.0%, respectively at 48 h. Thus, an increase in sensitivity and specificity was observed using Brilliance ESBL agar (Table 2).

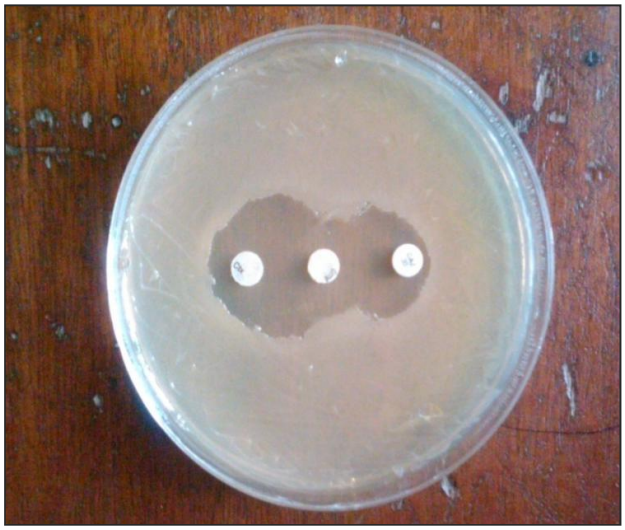

Fig. 1. Culture plate of clinical isolate of *Escherichia coli* from urine showing a clear extension of the edge of the inhibition zone of cephalosporin using Co-amoxiclav disc on Mueller-Hinton agar was interpreted as positive for ESBL production

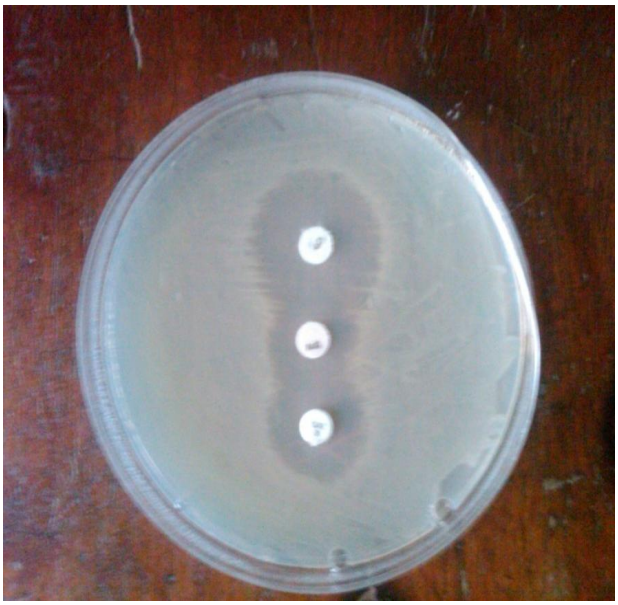

Fig. 2. Culture plate of clinical isolate of *Escherichia coli* from high vaginal swab (HVS) showing a clear extension of the edge of the inhibition zone of cephalosporin using Co-amoxiclav Disc on Mueller-Hinton agar was interpreted as positive for ESBL production

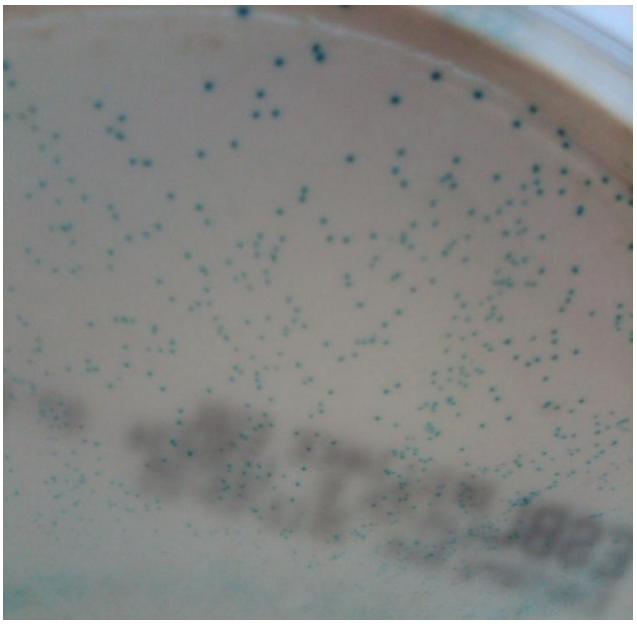

Fig. 3. Culture plate of clinical isolate of *Escherichia coli* from urine showing distinctive blue colony colouration on Brilliance ESBL agar was interpreted as positive for ESBL production

Following confirmation of the screening results, Single-plex Polymerase Chain Reaction (PCR) Amplification of bla_{CTX-M}, bla_{TEM} bla_{SHV} genes (Figs. 5, 6, 7) revealed a prevalence of 17%, 39%, and 43% respectively across the various clinical specimens (Table 3).

The resistance pattern of *Escherichia coli* was observed among Cefotaxime and Ceftazidime. It gave an indication for ESBL production among the isolates. Using the CLSI 2011 breakpoint, it was observed in the study that 38 *E. coli* isolates were resistant to Cefotaxime while 37 *E. coli*

isolates were resistant to Ceftazidime. Studies on ESBL using CLSI guidelines by Ho et al. [14] reported sensitivity of 57.79% for Ceftazidime using Kirby Beaur disc diffusion method. This study's result does not correlate with Ho et al. [14] result as 87.0% sensitivity was observed in Ceftazidime.

Following the outcome of the antibiotic susceptibility result, the *E. coli* isolates were subjected to ESBL screening using Double Disc Synergy Test (DDST) and Brilliance ESBL Agar. DDST is described as a reliable technique for ESBL detection [12]. The sensitivity of DDST from diverse studies ranges from 79% to 96%. The variation in sensitivity results attributes to it being an unstandardized method [19]. DDST is a less technically simple procedure with subjective interpretation of result [20].

The distance between antibiotic discs affects the sensitivity of DDST. Studies by Ho et al. [14] revealed the sensitivity of DDST to be 83.8% at a single interdisc width of 30mm. Their study also showed an increase in sensitivity to 97.9% by decrease in the interdisc width to 20 mm. In this study, sensitivity and specificity of DDST was 91.3% and 89.5% respectively at 24 h which was the same at 48h at a single interdisc width of 15mm. There was no significant difference ($P > 0.05$) among ESBL-producing isolates that emerged between the two incubation periods using DDST. The sensitivity of DDST in this study partially justifies studies by Vercauteren et al. [8] whose study showed DDST having sensitivity of 96.9%. Also, Ravi et al. [20] whose study had 94.89% sensitivity and 75.91% specificity for DDST.

Studies have demonstrated that Brilliance ESBL Agar gives an advantage of easier detection of ESBL-producing *E. coli* as well as other members of the *Enterobacteriaceae* family due to its chromogenic properties. Results are easier to interpret as it employs colony coloration technique. *E. coli* is presently the most predominant ESBL-producing organism in several countries [21-23] thus; this study is of great clinical significance.

In this study was observed that Brilliance ESBL agar had 98.0% specificity at 48 h which is significantly higher than that of DDST with specificity of 89.5% at 48 h. The specificity of Brilliance ESBL Agar in this study was higher than studies by Huang et al. [24] and Brown et al. [25] whose studies revealed a specificity of 95.7% and 93.0% respectively. The sensitivity of Brilliance ESBL agar at 24 h incubation time was 89.5% against 91.3% for DDST. The sensitivity of Brilliance ESBL agar at 24 h incubation time in this study does not correlate with Kjersti et al. [26] in Norway which reported 93%. Grohs et al. [27] from France reported 98.6% sensitivity and 57.9% specificity for Brilliance ESBL after 24 h incubation time. The sensitivity of Brilliance ESBL agar was higher than that observed in this study but vice versa. Our study showed increased sensitivity (97.8%) at 48 h on Brilliance ESBL agar, this correlates with Biane et al. [28] study in Cambridge shire. Biane et al. [28] reported an increase in sensitivity of Brilliance ESBL agar from 59% to 68% after 48 h incubation time. Consequently, there was a significant difference ($P < 0.05$) among ESBL-producing isolates that emerged between the two incubation periods using Brilliance ESBL agar. Fifty *E. coli* isolates which appeared negative by showing no colony coloration (blue colored colonies) at 24 h incubation time turned positive (blue colored) at 48 h. In addition, 20 *E. coli* isolates emerged as false positive with DDST showing Cephalosporin/clavulanate synergy but, negative with Brilliance ESBL agar. These isolates showed high susceptibility to Cefotaxime and Ceftazidime with diameter of zone of inhibition of ≥ 30 mm and ≥21 mm respectively. This justifies the accuracy of Brilliance ESBL agar over DDST. The sensitivity of Brilliance ESBL agar at 24 h in this study does not correlate with studies done by Gazin et al. [10] whose study reported 94.9% sensitivity at 24 h. In our study, extended incubation (48 h incubation) increased the recovery of ESBL producers without the growth of mixed flora unlike reports from previous studies [29,30].

The inclusion of Cefpodoxime in Brilliance ESBL agar rather than Cefotaxime and Ceftazidime (as in DDST) could attribute for its higher sensitivity over DDST. Thus, performance of Brilliance ESBL agar in this study justifies claims that Cefpodoxime is the best substrate for screening all ESBL types in clinical specimens [31]. Furthermore, CTX-M ESBL types which do not confer resistance to Ceftazidime have shown a high prevalence among clinical isolates. Consequently, Ceftazidime proves to be an unsuitable substrate alone for ESBL screening [31].

At the molecular level, the prevalence of bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$ genes was 17%, 39%, 43%,

respectively. CTX-M genes (20 isolates) were the most predominant genes with SHV genes (8 isolates) as the least predominant ESBL gene. In the study, bla_{SHV}, bla_{TEM}, bla_{CTX-M} genes alone were detected. Two or more genes were not detected in a clinical isolate. ESBL-producing *E. coli* harboring bla_{CTX-M} genes were observed to show resistance to Cefotaxime. Cefotaxime is a better marker for ESBL organisms harboring bla_{CTX-M} genes rather than Ceftazidime [32]. The sensitivity and specificity of the PCR method was 96% and 100%, respectively.

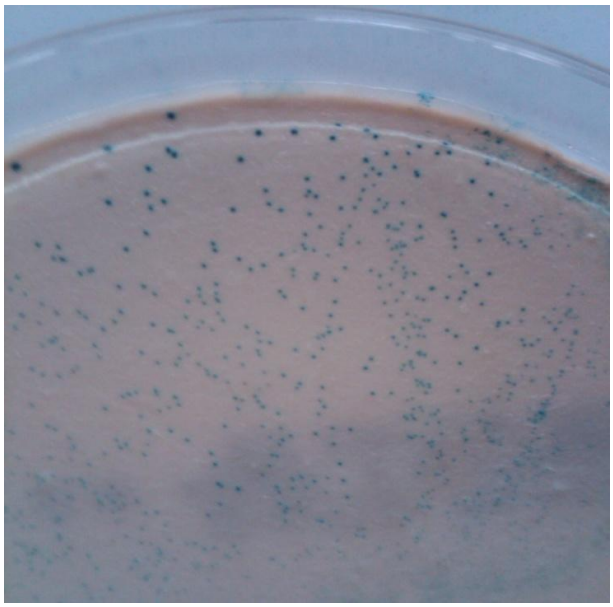

Fig. 4. Culture plate of clinical isolate of *Escherichia coli* from High Vaginal Swab (HVS) showing distinctive blue colony colouration on Brilliance ESBL Chromogenic Culture Medium was interpreted as positive for ESBL production

Table 1. Distribution of ESBL-producing *Escherichia coli* among clinical specimens using Double Disk Synergy Test (DDST) and Brilliance ESBL Agar (BEA)

Clinical specimens	No. of *E. coli* isolates	No. of ESBL-producing isolates	
		DDST	BEA
Urine	37	23	24
Vaginal Swab	18	9	11
Wound Swab	11	4	4
Sputum	9	4	6
Total	**75**	**40**	**45**

Key: No. – Number

Table 2. Distribution of ESBL-producing *Escherichia coli* among clinical specimens using Brillance ESBL chromogenic culture medium

Screening method	No. of ESBL isolates at 24 h (48 h)	Sensitivity (%) at 24 h	Sensitivity (%) at 48 h	Specificity (%) at 24 h	Specificity (%) at 48 h
DDST	42(42)	91.3	91.3	89.5	89.5
BEA	40(45)	87.0	97.8	89.5	98.0
PCR	46	nil	95.0	nil	100.0

Key: No. – Number, % - Percentage

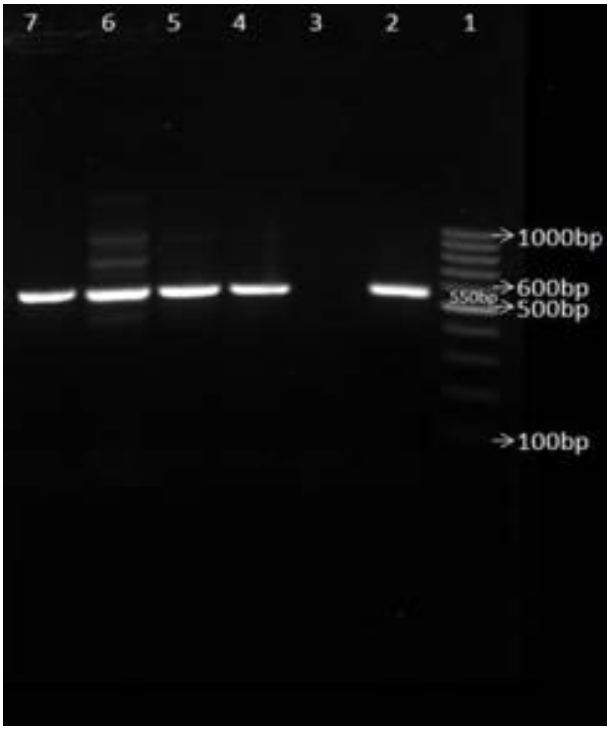

Fig. 5. Electrophoresis of PCR products for specifying CTX-M broad spectrum β-lactamases genes. No.1: DNA Marker (100 bp), No 2: positive control for *bla*$_{CTX-M}$, No. 3: negative control No. 4, 5, 6, 7 isolates with CTX-M (550 bp) gene

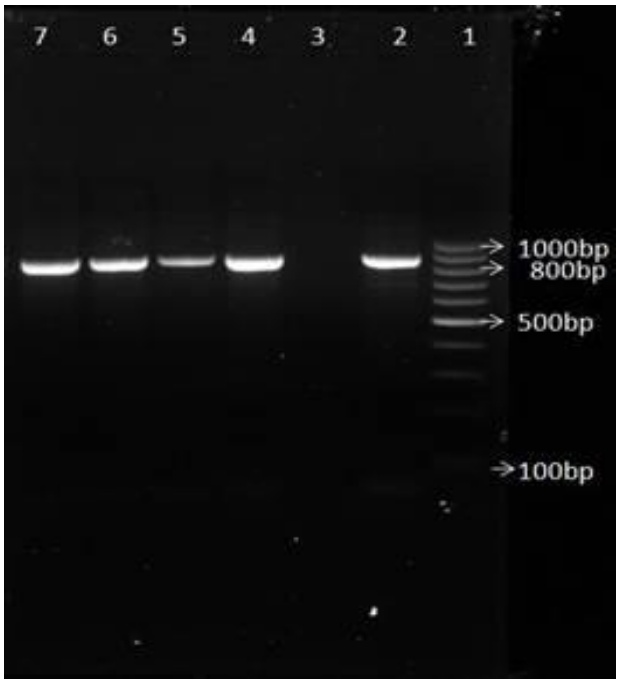

Fig. 6. Electrophoresis of PCR products for specifying TEM broad spectrum β-lactamases genes. No.1: DNA Marker (100 bp), No 2: positive control for *bla*$_{TEM}$, No. 3: negative control, No. 4,5,6,7 isolates with TEM (800 bp) gene

Table 3. Distribution of ESBL genes among clinical specimens

Clinical specimen	No. of *E. coli* isolates	No. of ESBL isolates	No. of ESBL genes		
			bla_{SHV}	bla_{TEM}	bla_{CTX-M}
Urine	37	25	4	9	11
Vaginal Swab	18	11	2	4	5
Wound Swab	11	4	1	2	2
Sputum	9	6	1	3	2
Total	**75**	**46**	**8**	**18**	**20**

Key: No. – Number

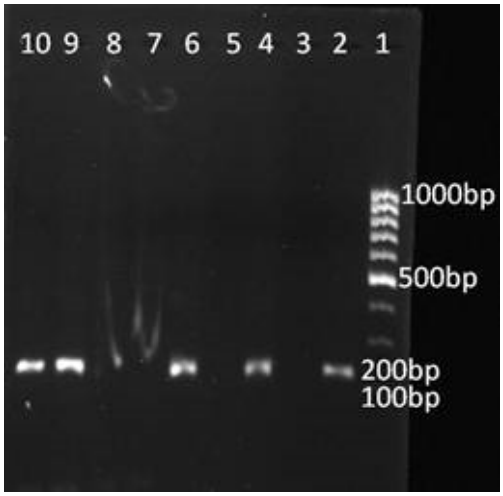

Fig. 7. Electrophoresis of PCR products showing SHV broad spectrum β-lactamases genes. No.1: DNA Marker (100bp), No 2: positive control for *bla*SHV, No. 3: negative control, No. 4, 6,9,10 isolates with SHV (200bp) gene, No.5, 7, 8. Isolates negative for SHV beta-lactamase gene

4. CONCLUSION

ESBL continues to pose a serious public health threat as it receives attention from clinical microbiologists, policy makers as well as the general public. Detection methods for ESBL from clinical specimens should have high specificity and sensitivity as well as short timing in result reporting. Results from our study have revealed that Brilliance ESBL agar had a high specificity and sensitivity making it reliable for ESBL detection among ESBL-producing *E. coli*. This medium which is a ready-to-use medium allows easy differentiation of different bacteria based on colony coloration. Thus, allowing swift implementation of control measures by infection control team. Presently, routine screening for ESBL-producing bacteria using chromogenic culture medium is yet to be adopted in Clinical Microbiology Laboratories in Nigeria and other parts of the world. Although, the media is expensive compared to other microbiological media, it significantly reduce the need for confirmatory test like DDST or molecular test like PCR which is time consuming requiring specialized equipment and expertise.

CONSENT

All authors declare that 'informed consent was obtained from approved parties for publication of this paper and accompanying images'.

ETHICAL APPROVAL

Ethical approval for the study was given by the institutional Research and Ethical Review Committee of the hospital.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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