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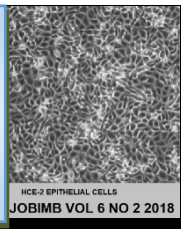

Effectiveness of Double Discs Synergy Test in the Confirmation of Extended Spectrum β-lactamase (ESβL) Production

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ABSTRACT

Extended spectrum beta lactamases (ESβLs) are enzymes which have evolved from bacteria upon exposure to penicillins antibiotics. Such bacteria have developed resistance to Beta lactam antibiotics and their relatives such as cephalosporins (extended spectrum beta lactam antibiotics). The enzymes represent the major source of multidrug resistance in gram negative bacteria. Different methods of confirming ESβL production from bacteria have been described. Most of these methods suggest screening the test isolates in accordance with decrease in susceptibility to extended-spectrum cephalosporins in preliminary susceptibility testing and use any of the available confirmatory tests for ESβL production. However, it is not clear which confirmatory tests are the most sensitive and which extended-spectrum cephalosporins should be tested. In our previous study, disc replacement method was used to detect ESβLs production among Gram negative isolates from Gombe specialist hospital. In this report, the effectiveness of double disc synergy test (DDST) in the confirmation of ESβL- production was studied from the clinical isolates obtained from this hospital for the first time to the best of our knowledge. A total of two hundred and fifty (250) bacteria screened to be suspected ESβL-producers were subjected to confirmatory test using double disc synergy test (DDST). The method effectively confirmed ninety six (96) isolates to be ESβL-producers. *Esherichia coli* had the highest occurrence (26, 27.08%), followed by *Klebsiella pneumonia* (20, 20.83%), *Providencia* spp. (14, 14.58%), *Morganella morganii* (12, 12.5%), *Yersinia enterocolitica* (6, 6.25%), *Pseudomonas aeruginosa* , *Shigella* spp. and *Salmonella paratyphi A* each with a total occurrence of 4(4.17%), *Citrobacter freundii*, *Serratia marcescens*, and *Salmonella typhi* each with an occurrence value of 2 (2.08%) while *Proteus vulgaris* was found to be negative ESβL- producer. In conclusion, the DDST was found to be effective in the confirmation of ESβL-production.

INTRODUCTION

The extended spectrum beta lactamases (ESβLs) are enzymes which have emanated from bacteria upon exposure to penicillins antibiotics. These bacteria have become resistant to penicillins (Beta lactam antibiotics) and their relatives such as cephalosporins (extended spectrum beta lactam antibiotics). The enzymes represent the major source of multidrug resistance in gram negative bacteria. Different classes of ESβLs have been described and the notable ones amongst them are Sulfhydril variable gene (SHV-2) and Temoniera gene (TEM-3) discovered in Germany since 1982 and 1987, respectively. Subsequently, more subtypes were descended from SHV-2 and TEM-3. Other important and widely distributed ESβL phenotype discovered worldwide in *Escherichia coli* are called cefotaximases (CTX-M). Phenotypic study confirmed five major groups of CTX-M with over 100 diverse CTX-M types [1, 2].

Besides *E. coli*, the ESβLs have been reported from other enteric Gram negative rods including *Klebsiella pneumonia, Morganella morganii*, as well as *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Capnocytophagaochracea* [3, 4, 5, 1]. However, the global spread of CTX-M extended-spectrum β-lactamases (ESBL) producing *E. coli* is worrisome due to novel microbiological and epidemiological features [6]. Certainly, the *E. coli* is a commensal organism of the digestive tract of humans and it represents the most frequently isolated bacterium in the community and hospital settings. Hence, an increased multidrug resistant *E. coli* may lead to a heavy use of the little available active antibiotics such as carbapenems, and the likelihood of the emergence of carbapenem- resistant organisms [6, 7]. A report showed that ESβL-producing *Enterobacteriaceae* were found in ambulatory patients with unrecognized risk factors for multidrug-resistant organisms [8]. Therefore, the detection of ESβL-producing bacteria has become a growing concern for general hospitals and private laboratories [6].

There are various methods of confirming ESβL production from *Enterobacteriaceae* [9, 10]. The clinical laboratory standard institute (CLSI) which based in the United States has issued national guidelines for laboratory detection of ESβL production in *E. coli*, *Proteus mirabilis*, and *Klebsiella* spp. only [11]. Later, other guidelines were published by the Health Protection Agency (HPA) in the United Kingdom for detection of ESβL production regardless of the test species [6]. The majority of these guidelines suggest screening the test isolates in accordance with decrease in their susceptibility to extended-spectrum cephalosporins in preliminary susceptibility testing and the use of any available confirmatory tests for ESβL production. However, it is not clear which confirmatory tests are the most sensitive and which extended-spectrum cephalosporins should be tested [6]. A study was conducted by Garba and Yusha'u [12] and found disc replacement method effective in the detection of ESβLs among Gram negative isolates from Gombe specialist hospital. In this report, the effectiveness of double disc synergy test (DDST) in the confirmation of ESβL- production was studied from clinical isolates obtained from the aforementioned hospital.

MATERIALS AND METHODS

Bacterial isolates

A total of two hundred and fifty (250) clinical bacterial isolates of *Klebsiella pneumonae* (40)*, Escherichia coli* (92), *Providencia* spp. (30), *Morganella morganii* (20), *Pseudomonas aeruginosa* (14), *Shigella* spp. (14), *Citrobacter freundii* (12), *Serratia marcescens* (6), *Salmonella paratyphi A* (10), *Yersinia enterocolitica* (6), *Proteus vulgaris* (4), and *Salmonella typhi* (2) were isolated from various samples of stool, urine, sputum, and wound swabs, and screened for ESβL production [12] as shown in **Table 1**. The isolates were collected from the Department of diagnostic services of Gombe State Specialist Hospital in Gombe Metropolis over a period of nine (9) Months. Identity of each isolate was confirmed at the Microbiology Laboratory, department of Microbiology of Gombe State University using standard identification procedures such as Gram staining and biochemical tests. Screening for the ESβL production was performed using Cefotaxime (30µg, Oxoid England) and Cefpodoxime (10µg Oxoid England) discs on prepared Mueller-Hinton agar plates according NCCLS, 1999 [13].

Standardization of Inoculum

The inocula of the bacteria were standardized according to NCCLS, 1999 [13] as described previously [12]. Colonies of the bacteria were mixed in sterile normal saline and compared the turbidity with 0.5 McFarland standards prior to sensitivity test.

Table 1. Clinical Gram negative bacterial isolates.

S/No	Bacteria	Number of Isolates
1	*Klebsiella pneumonae*	40
2	*Escherichia coli*	92
3	*Providencia* spp.	30
4	*Morganella morganii*	20
5	*Pseudomonas aeruginosa*	14
6	*Shigella* spp.	14
7	*Citrobacter freundii*	12
8	*Serratia marcescens*	6
9	*Salmonella paratyphi A*	10
10	*Yersinia enterocolitica*	6
11	*Proteus vulgaris*	4
12	*Salmonella typhi*	2
	Total	250

Double Disc Synergy Test (DDST)

The bacteria screened for ESβL production [12] were confirmed using DDST as demonstrated by Jarlier *et al*., 1988 [9]. Each of the standardized inoculum of the bacteria was swabbed onto a Mueller-Hinton agar plate. A susceptibility disc containing amoxicillin-clavulanate /Augmentin (30µg, Oxoid England) was placed at the centre of the plate. Then, discs containing Cefotaxime (30 µg, Oxoid England) and Cefpodoxime (10 µg, Oxoid England) were placed 15 mm (centre to centre) from the amoxicillin-clavulanate disc. After 30 minutes of pre-incubation time, the plates were incubated aerobically for 24 hrs at 35 ºC.

Statistical Analysis

The results were statistically analysed using Chi-square test.

RESULTS AND DISCUSSION

A total of two hundred and fifty (250) clinical Gram negative bacterial isolates were screened to be potential ESβL-producers based on Clinical Laboratory Standard Institute (CLSI) Breakpoint [12]. Confirmatory test has been performed using DDST which showed ninety six (96) of these isolates to be ESβL-producers. *Escherichia coli* had the highest occurrence (26), followed by *Klebsiella pneumonia* (20), *Providencia* spp. (14), *Morganella morganii* (12), *Yersinia enterocolitica* (6), *Pseudomonas aeruginosa*, *Shigella* spp. and *Salmonella paratyphi A* each with a total occurrence of 4, *Citrobacter freundii*, *Serratia marcescens*, and *Salmonella typhi* each with a total occurrence value of 2 while *Proteus vulgaris* was found to be negative ESβL-producer (**Table 2**).

In terms of percentage occurrence, *E. coli* had the highest value corresponding to 27.08%, followed by *K. pneumonia* with 20.83%, *Providencia* spp. with 14.58%, *M. morganii* with 12.5%, *Y. enterocolitica* with 6.25%, *P. aeruginosa*, *Shigella* spp. and *S. paratyphi A* each with 4.17%, then *C. freundii*, *S. marcescens*, and *S. typhi* with the least percentage occurrence value of 2.08 each (**Fig. 1**). Increase in zones of growth inhibition towards the central Augmentin Disc is recorded as positive DDST as demonstrated in **Fig. 2**.

Table 2. Confirmed Occurrence of ESβLs-producing Gram negative bacteria based on Double Disc Synergy Test (DDST)

S/No	Bacteria	Number positive
1	*K. pneumonae*	20
2	*E. coli*	26
3	*Providencia* spp.	14
4	*M. morganii*	12
5	*P. aeruginosa*	4
6	*Shigella* spp.	4
7	*C. freundii*	2
8	*S. marcescens*	2
9	*S. paratyphi A*	4
10	*Y. enterocolitica*	6
11	*P. vulgaris*	0
12	*S. typhi*	2
	Total	**96**

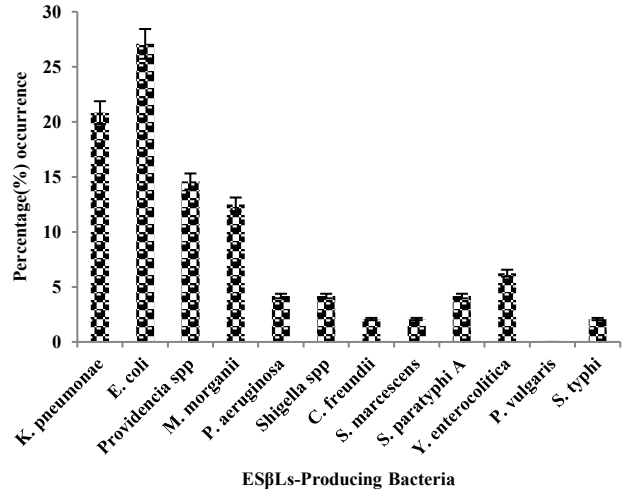

Fig. 1. Percentage (%) occurrence of ESβLs-Producing bacteria. The ESβL production was confirmed using DDST.

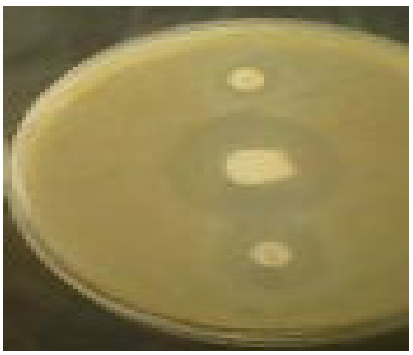

Fig. 2. Positive confirmatory test based on DDST. The test organism was more sensitive to the central Augmentin disc, producing wider zone of growth inhibition more than that produced by the two Cephalosporin discs after an overnight incubation at 37 ºC.

Increase of drugs or antibiotics resistance amongst pathogenic bacteria is quite alarming due largely to versatile microbial genetic system under the stress of various control agent(s) [4]. Several reports showed that resistance to b-lactam antibiotics by Gram-negative bacteria are fundamentally as a result of their ability of extended spectrum beta-lactamases (ESBLs) production which are known to degrade the b-lactam ring [14]. There are various ways by which drug resistance is acquired by bacteria such as mutations and mechanisms of genetic exchange, which involves plasmids or transposons and chromosome that bring about alterations of cell membranes in the target cell. These prevent entry of control agents or developing substitute enzymes, which are not the main drugs target or releasing drug degrading or inactivating enzymes like ESβLs [1,3]. In this report, the suspected ESβL producing bacteria were confirmed to be positive except *P. vulgaris* as shown in **Fig. 1**. The results suggest the effectiveness of DDST in the confirmation of ESβL production. The effectiveness of the DDST observed in this study is comparable to several reports by different researchers [1,4]. However, disc replacement method used in the confirmation of ESβL production was reported with an overall percentage occurrence of 83.33% [12], higher than the total percentage occurrence (32%) observed in this study. Despite the fact that the occurrence of ESβL producers varies from one study area to another, the higher percentage values of occurrence recorded with *E. coli* (27.08%) and *K. pneumonae* (20.83%) in the study generally agree with the established reports that these bacteria are the most prevalent ESβL-producers [12,15- 18].

CONCLUSION

The DDST was found to be effective in the confirmation of ESβL-producing bacteria. The method phenotypically confirmed ESβL production in *Klebsiella pneumonia*, *Providencia* spp., *Morganella morganii*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Shigella* spp., *Salmonella paratyphi A*, *Citrobacter freundii*, *Serratia marcescens*, and *Salmonella typhi* except *Proteus vulgaris.*

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