ORIGINAL RESEARCH PAPER

Microbiology

KEY WORDS: Extended-spectrum β-lactamases, Double Disk Synergy Test, Inhibitory Potentiated Disk Diffusion Test, Zone of inhibition

COMPARISON BETWEEN DOUBLE DISK SYNERGY TEST (DDST) AND INHIBITORY POTENTIATED DISK DIFFUSION TEST (IPDDT) FOR DETECTION OF ESBL ISOLATES.

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ABSTRACT

Objective: To establish effectiveness between Double Disk Synergy Test (DDST) and Inhibitory Potentiated Disk Diffusion Test (IPDDT) for detection of ESBL isolates. **Material and Method:** Total two hundred screened isolation of *E. coli* and *Klebsiella* species obtained from 3146 various clinical samples such as pus, urine, blood, CSF, ear discharge, pleural fluid & sputum were included in the study. ESBL production where detected by Double Disk Synergy Test (DDST) and Inhibitory Potentiated Disk Diffusion Test (IPDDT) as per CLSI guidelines. **Results:** 200 isolates 117(58.5 %) *E.coli* and 83(41.5%) *Klebsiella* were identified. By DDST test 92.6% & 89.1%, 66.7% & 71.7%, 88.8% & 71.7% showed synergy between Ceftazidime, Cefotaxime, Ceftriaxone and Amoxicillin with Clavulanic acid by *Klebsiella* species and *E.coli* respectively. By IPDDT test 27.7% & 40.1%, 28.9% & 43.4% and 36.1% & 44.4% showed synergy between Ca Vs Ca/C , Ce Vs Ce/C , Ca Vs Ca/C + Ce Vs Ce/C by *Klebsiella* species and *E.coli* respectively. The ESBL isolate detected by DDST was 73(36.5%) and by IPDDT was 82 (41%). **Conclusion:** Additional 4.5% (9) ESBL isolates detected by IPDDT than DDST. ESBL detection have a significant impact on several important clinical outcomes. ESBLs outbreaks require accurate and precise detection.

INTRODUCTION

Antibiotics are useful in treating infectious diseases, both in outdoor and indoor patients. Initial excitement was quickly decrease by the emergence of pathogens that became resistant to these agents. The favorable outcome of common infections in community and hospital settings is tempered due to emerge of resistant bacteria [1]. Production of β-*lactamases* is the most common mechanism of the bacterial resistant for these antibiotics. These enzymes are numerous and are plasmid mediated, capable of hydrolyzing and inactivating a wide variety of β -*lactam* antibiotics [2]. ESBL producing Gram Negative bacteria are increasingly being associated with hospital infections. Majority of ESBL producing strains are *E.coli and K.pneumoniae, K.oxytoca* [3]. The continued emergence of ESBLs presents diagnostic challenges to the clinical microbiology laboratories, which should be aware of the need for their detection by accurately identifying the enzymes in clinical isolates. It appears that there is a difference in the ability of various susceptibility testing methods used for detecting cephalosporin resistance in an ESBL producing strain. This lack of sensitivity and specificity in traditional susceptibility tests to detect ESBLs, has prompted the search for an accurate test to detect the presence of ESBLs in clinical isolates. In the years since ESBLs were first described a number of different testing methods have been suggested. Various methods are implemented for detection of ESBL like phenotypic, molecular and epidemiological. Considering the above facts, ESBL detection should reach the clinician accurate and as early as possible to enable him to treat the critical patients favorably.

MATERIALS AND METHOD

The present study was carried out on two hundred screened isolates of *E.coli and Klebsiella* species from 3146 various clinical samples such as pus, urine, sputum, blood, CSF, ear discharge and pleural fluid, were included in the study. The clinical samples were processed by plating on Nutrient agar, Blood agar and MacConkey agar. All the isolates were identified using standard biochemical tests [4]. Total 200 screened isolates of *E.coli* and *Klebsiella* species were tested for antimicrobial susceptibility by Disc Diffusion technique (*Kirby- Bauer* Method) according to CLSI guidelines [5]. Various antibiotic discs manufactured by Hi- Media were used.

Muller Hinton agar plates were used for the test & plates were stored at 4°C and used within one week of preparation. Before inoculation, plates were dried so that there were no droplets of moisture on the agar surface. Direct colony suspension, equivalent to 0.5 McFarland standard was used. A Sterile cotton swab was dipped in suspension and surplus removed by rotation of swab against the Side of tube. The plate was inoculated by even streaking of swab over the entire surface. The results were interpreted as per the Criteria of CLSI guidelines. Isolates with resistance or with decreased susceptibility (intermediate by CLSI (criteria) to third generation cephalosporin were selected for ESBL production [5].

ESBL detection was done by Double Disk Synergy test & Inhibitor Potentiated Disk Diffusion test. [6]

Double Disk Synergy Test (DDST)

Muller-Hinton agar plates were prepared and inoculated with standardized inoculums (corresponding to 0.5 McFar lands standard) to form a lawn culture. With a sterile forceps, a disk of Amoxycillin/Clavulanic acid (20 μg /10μg) was placed in the centre of the plate. Then giving a centre to centre distance of 20 mm around the Amoxycillin/Clavulanic acid disk the Cefotaxime (30μg), Ceftriaxone (30μg) and Ceftazidime (30μg) disks were applied. This distance was found to give the best results for detection of ESBLs in our laboratory. The plates were incubated at 37°C in incubator for overnight incubation [7,8,9].

Observation and interpretation

Strain interpreted as ESBL producer when –

A- Irrespective of the individual zone size, the zone size around the test antibiotics Third Generation Cephalosporins (3GC) was extended on the side nearest the Amoxicillin / Clavulanic acid disk. An increase in zone of inhibition of > 2.5 mm on one side was considering/

considered significant.

B- Antibiotic disk was not inhibitory alone but bacterial growth was also inhibited between the two disks.

C. Bulging or broadening of the inhibitory zone between the 3 GC disk and the disk of Amoxycillin/ Clavulanic acid.

Non- ESBL producing strain did not show synergy as demonstrated by any of these patterns. *E.coli* ATCC 25922 was used as the negative control and *Klebsiella pneumoniae* ATCC 700603 obtained from JIPMER Pondicherry were used as the positive control.

Inhibitor Potentiated Disk Diffusion Test

Muller- Hinton agar plates were prepared and inoculated with a standardized inoculum (corresponding to 0.5 Mc Farland's standard) to from a lawn culture. Using sterile forcep disks of Cefotaxime (Ca) & Cefotaxime/ Clavulanic acid (Ca/C) , disks of Ceftazidime (Ce) & Ceftazidime/ Clavulanic acid (Ce/C) and Cefotaxime/ Clavulanic acid(Ca/C) & Ceftazidime/ Clavulanic acid (Ce/C) were placed, by keeping a 15 mm center to center distance. Plates were incubated at 37°C in incubator for overnight [9].

Interpretation

A > 5 mm increase in the Zone diameter for either of the (3 GC) antimicrobial agent tested in combination with Clavulanic acid versus its zone when tested alone indicated ESBL production.

***Quality* Control**

The negative control i.e. *E.coli* ATCC 25922 shows a < 2 mm increase in zone diameter for antimicrobial agent tested alone versus its zone when tested in combination with Clavulanic acid. The positive control i.e. *Klebsiella pneumoniae* ATCC 700603 shows a > 5 mm increase in Ceftazidime zone diameter and a > 3 mm increase in Cefotaxime zone diameter. [9].

RESULTS AND OBSERVATION

The present study was conducted on 200 Screened isolates of *E.coli & Klebsiella* species to know the comparison of two different detection method of ESBL, obtained from various clinical samples. Of the 200 screened isolates of *E.coli & Klebsiella* species, 58.5% (117) were identified as *E.coli* and 41.5 % (83) *Klebsiella* species. The incidence of *K. pneumoniae* isolates was 27 % (54) & *K. oxytoca* was 14.5% (29) [Table 1]. In screening, the maximum resistance among *E. Coli and Klebsiella* species isolates was observed against Cefpodoxime 100% & Cefotoxime 73.5% for both followed by other antimicrobial agents as Ceftazidime 77.0% & 69.9% , Ceftriaxone 74.4.% & 72.3 % & Aztreonam 65.0% & 63.9% respectively. Overall 200 isolates showed resistance to at least one of the 3GC or Aztreonam [Table 2].

The incidence of ESBLs among *E.coli* isolates is 48.7% (57) and among *Klebsiella* species is 34.9% (29) [Table 1]. ESBL positivity among 117 *E.coli* isolates was 39.3% (46) & among 83 *Klebsiella* species was 32.5% (27) by DDST[Table 3]. Among the *Klebsiella* species, *Klebsiella pneumoniae* and *Klebsiella oxytoca* isolates shows ESBL positivity rate was 35.1% (19) & 34.4% (10) [Table 1]. 92.6 % of *Klebsiella* spp. and 89.1 % of *E.coli* showed synergy between Ceftazidime and Amoxicillin/Clavulanate in the DDST. Whereas Cefotaxime showed synergy 66.7% & 71.7% and Ceftriaxone showed 88.8 % & 71.7 % *Klebsiella* species and *E.coli* isolates respectively in DDST [Table 3].

By the IPDDT, Overall incidence of ESBLs among *E.coli and Klebsiella* species isolates was 44.4 % (52) and 36.1 % (30) respectively, when tested with Ca Vs Ca/C & Ce Vs Ce/C by IPDDT. The total incidence of ESBLs was 41.0% (82) with Ca/ Ca/C & Ce/Ce/C [Table 4].

The incidence of ESBLs among *E.coli and Klebsiella* species isolates was 40.1 % (47) & 27.7 % (23) when Ceftazidime and combination Ceftazidime with Clavulanic (Ca/Ca/C) acid tested respectively, and total incidence of ESBLs was 35% (70) with Ca/Ca/C where as Cefotaxime and combination of Cefotaxime with Clavulanic acid (Ce/ Ce/C) was tested, the percentage of ESBLs positive *E.coli* were 43.5 % (51) & that of *Klebsiella* spp. is 28.9 % (24). The Total incidence of ESBLs was 37.5% (75) with Ce/ Ce/C [Table 4].

The ESBL isolate detected by DDST was 73(36.5%) and by IPDDT was 82 (41%). Thus, Inhibitory Potentiated Disk Diffusion test detected an additional 4.5% (9) ESBL isolates than the DDST [Table 5].

Table No: 1

Incidence of ESBLs: in E.coli & Klebsiella species isolates.

Isolates	ESBL	Non- ESBL
E.coli (n=117)	57(48.7%)	60(51.3%)
Klebsiella pneumoniae (n=54)	19 (35.1%)	35(64.9%)
Klebsiella oxytoca (n=29)	10 (34.4%)	19(65.6%)
Total (n=200)	86 (43%)	114 (57%)

Table No: 2

Antimicrobial sensitivity pattern of E.coli and Klebsiella species to 3rd generation cephalosporins and aztreonam: Initial screen test for detection of possible ESBLs produces

Antibiotic Disks	*E.coli* (n=117)				*Klebsiella species* n (83)			
	Resistant		Sensitive		Resistant		Sensitive	
	No	%	No	%	No	%	No	%
Ceftazidime	90	77	27	23	58	69.9	25	30
Cefotaxime	86	73.5	31	26.5	61	73.5	22	26.5
Ceftriaxone	87	74.4	30	25.6	60	72.3	23	27.7
Cefpodoxime	117	100	0	0	83	100	0	0
Aztreonam	76	65	41	35	53	63.9	30	36.1

Table No: 3 Percentage of ESBL positive isolates showing synergy between Clavulanic acid and individual Third Generation Cephalosporin antibiotics in the Double Disk Synergy Test (DDST).

Organism	No. of ESBL Positive by DDST	Third Generation Cephalosporin					
		Ceftazidime		Cefotaxime		Ceftriaxone	
		No	%	No	%	No	%
E.coli	46	41	89.1	33	71.7	33	71.7
Klebsiella	27	25	92.6	18	66.7	24	88.8
Total	73	66	90.4	51	69.8	57	78.1

Table No: 4 Percentage of ESBLs by Inhibitory Potentiated Disk Diffusion Test (IPDDT).

Organism	Total Tested	Inhibitor Potentiated Disk Diffusion Test				Ca Vs Ca/C + Ce Vs Ce/C	
		Ca Vs Ca/C *		Ce Vs Ce/C †			
		No	%	No	%	No	%
E.coli	117	47	40.1	51	43.5	52	44.4
Klebsiella Species	83	23	27.7	24	28.9	30	36.1
Total	200	70	35.0	75	37.5	82	41.0

* Ceftazidime and combination Ceftazidime with Clavulanic (Ca/Ca/C)

† Cefotaxime and combination of Cefotaxime with Clavulanic acid (Ce/ Ce/C)

Table No: 5 Comparative study of different methods for detection of ESBL positive isolates.

Organism	Total Number Tested	Double Disk Synergy Test (DDST)		Inhibitor Potentiated Disk Diffusion Test (IPDDT)	
		No (+ve)	%	No. (+ve)	%
E.coli	117	46	39.3	52	44.4
Klebsiella species	83	27	32.5	30	36.1
Total	200	73	36.5	82	41

DISCUSSION

As their occurrence of ESBL has been increasing, it becomes essential to evaluate their occurrence in the population. The detection of ESBLs screening and confirmatory methods have been routinely used to investigate the production of these enzymes. The present study was conducted on 200 screened isolates of *E. Coli and Klebsiella* species obtained from 3146 various clinical samples received at the Department of Microbiology, R.N.T. Medical College, Udaipur, Rajasthan. In the present study out of 200 screened isolates 58.5% (117) were identified as *E.coli* & 41.5% (83) were *Klebsiella* species. Most of studies as S.abigail et al (1995), Hansotia et al (1997), Thomson et al (1997), S. Mehta et al (1998), A. Subha et al (2002), YK Kin et al (2002) and Xiong Z et al (2002) has emphasized *K. Pneumoniae* and *E. Coli* as the organism most likely to harbor ESBLs.Hence detection of ESBLs in the present study was restricted to *Klebsialla and E. Coli* isolates. The maximum resistance among *E.coli and Klebsiella* species isolates was observed against cefpodoxime 100% and cefotoxime 73.5% respectively in screening. Overall 200 isolates showed resistance to at least one of the third generation Cephalosporin or Aztreonam. Isolates with resistance or decreased susceptibility (intermediate by CLIS criteria) to third generation Cephalosporin were selected for ESBL production & this showed the incidence of ESBLs among *E.coli* isolates is 48.7% and among *Klebsiella pneumoniae* (35.1%) & *Klebsiella oxytoca* (34.4%).

Percentage of ESBLs positive isolates showing synergy between Amoxicillin/Clavulanic acid and Ceftazidime, Cefotaxime & Ceftriaxone individually antibiotics in Double disk synergy test were 92.6%, 66.7% and 88.8% for Klebsiella species & 89.1%, 71.7% and 71.7% for *E.coli* respectively. This suggests that Ceftazidime and Ceftriaxone are superior to Cefotaxime for demonstrating the synergistic effect in DDST.

Sensitivity and specificity of DDST in our study is 84.9% and 100% respectively and this correlates well with the findings of other studies. The various sensitivities as reported previously are 76.5% by Hansotia et al (1997), 97% by Vercauteren et al (1997), 96% by HoPL et al (1998) and 100 % sensitivity by Thomsom et al (1997) [9,10,12,13].

In the present study, from the Inhibitory Potentiated Disk Diffusion Test (IPDDT), the incidence of ESBLs among *E.coli and Klebsiella* species isolates was 40.1% (47) & (27.7%) (23), when Ceftazidime and combination Ceftazidime with Clavulanic acid tested respectively. Total incidence of ESBLs was 35% (70) with Ca/CaC where as Cefotaxime and combination of Cefotaxime with Clavulanic acid was tested, the percentage of ESBLs positive *E. coli* were 43.5% (51) & that of *Klebsiella* species is 28.9% (24).The total incidence of ESBLs was 37.5(75) with Ce/CeC. Thus, overall incidence of ESBLs among *E.coli and Klebsiella* species isolates was 44.4% (52) and 36.1% (30) respectively when tested with CaVs Ca/C & Ce Vs Ce/C. The total incidence of ESBLs was 41.0% (82) with Ca/ CaC & Ce/CeC. Sensitivity and specificity of IPDDT in our study is 95.4% and 100% respectively. This correlates well with the findings of other studies done by M'Zali et al (2000) in which 93% of ESBLs producers were detected both cefotaxime and Ceftazidime and in combination(CaVs Ca/C & Ce Vs Ce/C) were used as compare to 86% detection rate with Ceftazidime alone and in combination (CaVs Ca/C) and 66% detection rate with Cefotaxime alone and in combination (CeVs Ce/C) [11].

Navon Venezia et al (2003) and Mehta et al (2003) has also reported that the sensitivity is 100% when both cefotaxime and ceftazidime and in combination (CaVs Ca/C & Ce Vs Ce/C) were used.The ESBL isolate detected by DDST was 73(36.5%) and by IPPDT was 82 (41%) [16,17].

Comparative study of this two method shows that, Inhibitory Potentiated disk diffusion test detected an additional 4.5% (9) ESBL isolates.

CONCLUSIONS

The present study is preliminary attempt to evaluate comparison between two method DDST and IPDDT to detect the ESBLs in *E.coli & Klebsiella* species from various clinical samples. ESBL production has been observed in large percentage of *E.coli and Klebsiella* isolates. The inhibitor based phenotypic confirmatory test using both cefotaxime and Ceftazidime is reproducible enough, easy to perform, cost effective and highly sensitive and also does not required rigorous standardization.

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