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In vitro Activity of Tigecycline against Clinical Isolates of Extended Specrtum Beta Lactamase Producing *Enterobacteriaceae* in Tertiary Care Hospital

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Abstract: ***Introduction: In India, the extended-spectrum β-lactamase (ESBL) producing strains of Enterobacteriaceae have emerged as a challenge in the hospitalized as well as the community based patients. Among wide array of antibiotics, β-lactam is most widely used agent of all antibiotics in use. However new β-lactamases emerged against each of the new class of β-lactams that were introduced and caused resistance. So the present study was conducted with an objective to find out the presence of ESBLs among the Enterobacteriaceae isolates and to determine tigecycline activity against it. Aim: To isolate and detect the extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae and to determine in vitro activity of tigecycline among them. Material and methods: Various specimens received to the laboratory within two hours of collection were processed according to the standard microbiological technique. Results: Out of 606 Enterobacteriaceae, 287 isolates were ESBL producers. Urine and pus specimen yielded the highest percentage of ESBL isolates i.e. 55.41% and 47.17% respectively. Majority of ESBL isolates ware seen in age group of above 60 years (61.61%) and we observed male preponderance. Among IPD patients majority of ESBL producers were from ICU followed by Orthopaedic ward i.e. 81.44% and 59.18% respectively. Klebsiella species (74.48%) was most common ESBL producer. 93.73% of the ESBL producers were susceptible and 2.78% isolates were resistant to tigecycline by E Test while 90.6% were susceptible and 4.18% were observed to be resistant by disc diffusion method. Conclusion: Tigecycline can play a key role as therapeutic option in tackling ESBL producing Enterobacteriaceae. However, clinicians need to prescribe tigecycline appropriately, in order to avoid the emergence of resistant strains. From susceptibility testing by E test and disc diffusion, as there was no significant difference for tigecycline susceptibility.***

Keywords: Enterobacteriaceae, Extended spectrum beta Lactamase, E test, Tigecycline.

1. Introduction

The ever increasing bacterial resistance to antibiotics is one of the most challenging tasks of all the medical issues which are being faced today.[1] In India, the extended-spectrum β-lactamase (ESBL) producing strains of *Enterobacteriaceae* have emerged as a challenge in the hospitalized as well as the community based patients.[2] Among wide array of antibiotics, β-lactam is most widely used agent of all antibiotics in use. However new β-lactamases emerged against each of the new class of β-lactams that were introduced and caused resistance. The latest in the arsenal of these enzymes has been the evolution of Extended Spectrum β- Lactamases (ESBLs).[3] The extended-spectrum β-lactamases (ESBLs) are defined as plasmid mediated β-lactamases which confer resistance to all the extended spectrum cephalosporins and aztreonam, except to the cephamycins and the carbapenems.[4] In vitro tigecycline susceptibility can be done by disc diffusion test and minimum inhibitory concentration (MIC) can be determined by agar dilution, broth dilution and Epsilometer (E) test. E test tigecycline gradient strips proved to be robust and reliable that provides accurate and reproducible MIC results when used in daily clinical practice.[5]

2. Materials and Methods

After approval from institutional ethical committee, present cross sectional study was conducted in the department of Microbiology at tertiary care teaching hospital. All clinical samples such as pus, sputum, urine, blood, CSF and other body fluids received in the laboratory were included in the study. All these samples were collected using strict aseptic precautions and immediately transported to the laboratory as per guidelines for transportation of clinical specimens for aerobic bacteriology.[6] Total 606 gram-negative organisms belonging to family *Enterobacteriaceae* were isolated. Detailed clinical history of these patients was recorded in standard format. Only those cases yielding growth of *Enterobacteriaceae* were included in the study and were further tested for ESBL production. Specimens were brought to the laboratory within two hours of collection and further

processing was done. All the specimens were processed according to the standard microbiological technique.

Detection of Extended spectrum β lactamase (ESBL)

Extended-spectrum β-lactamase testing was performed as per the CLSI guidelines.[7, 8]

1) **Initial screening test by disc diffusion method:**
   Procedure:
   The strain to be tested was inoculated into sterile peptone broth. Inoculum was incubated for 4 - 6 hours and turbidity adjusted to 0.5 McFarland. Sterile cotton swab was dipped in above broth and a lawn culture made on Mueller Hinton agar. After drying ceftazidime (30µg), cefotaxime (30µg) discs were applied firmly on the agar. The plate was incubated at 37 ºC for 16 - 18 hours.
   Interpretation:
   If a zone diameter of ≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime was recorded then the strain was considered suspicious for ESBL production.
2) **Phenotypic Confirmatory Test:**
   Suspicious strain for ESBL was confirmed by this test by using disc diffusion method.
   Procedure
   The strain was inoculated into sterile peptone broth and turbidity adjusted to 0.5 McFarland. Sterile cotton swab was dipped in above broth and a lawn culture made on Mueller Hinton agar. After drying **a)** a ceftazidime (CAZ) disc containing 30 µg of the antibiotic and a ceftazidime-clavulanic acid (CAC) disc containing 20+10 µg of the antibiotic were placed at a distance of 30 mm from each other and **b)** a cefotaxime (CTX) disc containing 30 µg of the antibiotic and a cefotaxime-clavulanic acid (CEC) disc containing 20+10 µg of the antibiotics were placed at a distance of 30 mm from each other.[9] The plate was incubated at 37ºC for 16 - 18 hours.
   Interpretation:
   A ≥ 5 mm increase in the zone diameter for CAC versus zone diameter of CAZ and / or a ≥ 5 mm increase in the zone diameter for CEC versus zone diameter of CTX, was confirmed an ESBL- producing strain.
   K. pneumoniae ATCC 700603 and *Escherichia coli* ATCC 25922 were used as positive and negative controls respectively. All ESBL producing isolates were further tested for tigecycline susceptibility.
   1) Disc diffusion method.
   2) MIC by E test

1) Disc diffusion method:

Kirby-Bauer disc diffusion method was performed for all these ESBL isolates using tigecycline disc (15µg) as per the CLSI guidelines.[7,10,11]

Procedure

The confirm ESBL strain was inoculated into sterile peptone broth and turbidity adjusted to 0.5 McFarland. Sterile cotton swab was dipped in above broth and a lawn culture made on Mueller Hinton agar. After drying tigecycline (15µg) disc was applied firmly on the agar. The plate was incubated at 37ºC for 16 - 18 hours.

Interpretation:

Reading of zone diameters was done using the US FDA tigecycline susceptible breakpoints listed for *Enterobacteriaceae.*[12]

Pathogen	Zone diameter in mm		
	S	I	R
Enterobacteriaceae	≥ 19	15 – 18	≤ 14

S=Sensitive I=Intermediate R=Resistant

2) E Test:

MIC of tigecycline was determined by E test.[5]

E test tigecycline (0.016 to 256 µg/ml; HiMedia) was used according to the manufacturer's instructions.

Procedure:

An inoculum was prepared by suspending well isolated colonies in 0.9% saline with a turbidity adjusted to 0.5 McFarland. A sterile cotton swab dipped into the suspension was used to evenly streak the Mueller Hinton agar surface and allowed to dry for approximately 15 min. The test tigecycline gradient strip was applied to the agar surface and the plate was incubated in ambient air at 35°C for 18 - 20 hours.

Interpretation

The MIC endpoint was read where the growth inhibition ellipse intersected on the test gradient strip and breakpoint MIC was interpreted by the following US FDA criteria.[12]

Pathogen	**MIC in µg/mL**		
	S	I	R
Enterobacteriaceae	≤ 2	4	≥ 8

S=Sensitive I=Intermediate R=Resistant

3. Results

A total of 606 non repeated clinical isolates of *Enterobacteriaceae* was obtained from various clinical samples, 287 (47.36%) of these isolates were extended spectrum β-lactamase producer. ESBL producing strains were subjected to the tigecycline susceptibility by disc diffusion and "E Test".

Sample wise distribution of ESBL producing *Enterobacteriaceae.*

Sample	Total Numbers of *Enterobacteriaceae*	ESBL Producers n (%)	Non ESBL producers n (%)
Urine	222	123 (55.41)	131 (44.59)
Pus	248	117 (47.17)	99 (52.83)
Blood	47	21 (44.68)	26 (55.32)
Sputum	53	20 (37.74)	33 (62.26)
CSF	25	03 (12)	22 (88)
Other	11	03 (27.27)	8 (72.73)
Total	**606**	**287 (47.36)**	**319 (52.64)**

Majority of the ESBL producing isolates were obtained from urine sample (55.41%) and from pus sample (47.17%). Very few isolates (12%) were obtained from CSF sample. Other sample includes body fluids (ascitic fluid, peritoneal fluid, pleural fluids, etc). Graph 1 gives distribution of ESBL producing *Enterobacteriaceae* with respect to sample.

Outpatient and Inpatient wise distribution of ESBL producers and non ESBL producers among *Enterobacteriaceae* infection.

	IPD n (%)	OPD n (%)
ESBL producers (n = 287)	284 (98.95%)	3 (1.05%)
Non ESBL producers(n = 319)	302 (94.67%)	17 (5.33%)

Fisher 's exact p value<0.001 Odds Ratio=5.33

Majority of infection caused by ESBL producers among *Enterobacteriaceae* were seen in inpatient isolates i.e. 98.95%.

Ward wise distribution of ESBL producing *Enterobacteriaceae*

Ward	Total number of *Enterobacteriaceae*	ESBL producers n (%)	Non ESBL producers n (%)
ICU	97	79 (81.44)	18 (18.56)
Orthopaedic	49	29 (59.18)	20 (40.82)
Surgery	145	69 (47.58)	76 (52.42)
Paediatrics	26	12(46.15)	14 (53.85)
Burn	85	36 (42.35)	49 (57.65)
Medicine	108	37 (34.25)	71 (65.75)
ENT	13	04 (30.76)	9 (69.24)
OBGY	63	18 (28.57)	45 (71.43)
OPD	20	03 (15)	17 (85)
Total	606	287	319 (52.64)

ESBL producing *Enterobacteriaceae* isolates were more commonly obtained from the ICU (81.44%) followed by orthopaedics ward (59.18%), surgery ward (47.58%), paediatrics ward (46.15%) and burn ward (42.35%).

ESBL producers among different members of family *Enterobacteriaceae.*

Organisms	Total numbers of Enterobacteriaceae	ESBL producers n (%)	Non ESBL producers n (%)
Klebsiella spp	192	143 (74.48)	49(25.52)
Escherichia coli	221	100 (45.24)	121 (54.76)
Salmonella spp	09	04 (44.44)	5 (55.56)
M.morganii	07	03 (42.85)	4 (57.15)
Providencia spp	04	01 (25)	3 (75)
Citrobacter spp	80	18 (22.5)	62 (77.5)
Proteus spp	58	12 (20.68)	46(79.32)
Enterobacter spp	32	06 (18.75)	26 (81.25)
Edwardsiella spp	02	00	100
Serratia spp	01	00	100
Total	**606**	**287**	**287(52.64)**

In the present study *Klebsiella* species (74.48%) was common ESBL producer followed by *E.coli (*45.25%). **Susceptibility pattern of tigecycline in ESBL producing *Enterobacteriaceae* isolates by disc diffusion methods.**

Organisms	*Total*	*S* n (%)	*I* n (%)	*R* n (%)
Escherichia coli	100	96 (96)	2 (2)	2 (2)
Klebsiella spp	143	132 (92.30)	6 (4.2)	5 (3.5)
Citrobacter spp	18	16(88.88)	1 (5.56)	1(5.56)
Enterobacter spp	6	5 (83.33)	1 (16.67)	0
Proteus spp	12	6 (50.00)	4 (33.33)	2(16.67)
Morganella morganii	3	2 (66.67)	0	1 (33.33)
Salmonella spp	4	3(75.0)	1 (25.0)	0
Providencia spp	1	0	1 (100)	0
Serratia spp	0	0	0	0
Edwardsiella spp	0	0	0	0
Total	**287**	**260 (90.6)**	**15(5.22)**	**12 (4.18)**

S – Susceptible I – Intermediate R – resistant

Among members of *Enterobacteriaceae*, *E.coli* (96%) was sensitive to tigecycline followed by *Klebsiella* spp (92.30%). *Proteus* spp (50%) showed least susceptibility to tigecycline.

Susceptibility pattern of tigecycline in ESBL producing *Enterobacteriaceae* isolates by E test.

Organisms	**Total**	**S** n (%)	**I** n (%)	**R** n (%)
Escherichia coli	100	98 (98.00)	1 (1)	1 (1)
Klebsiella spp	143	138 (96.50)	2 (1.40)	3 (2.10)
Citrobacter spp	18	17 (94.44)	1 (5.6)	0
Enterobacter spp	6	5 (83.33)	1 (16.67)	0
Proteus spp	12	6 (50.00)	3 (25)	3 (25)
Morganella morganii	3	2 (66.67)	0	1 (33.33)
Salmonella spp	4	3 (75.0)	1 (25.0)	0
Providencia spp	1	0	1 (100)	0
Serratia spp	0	0	0	0
Edwardsiella spp	0	0	0	0
TOTAL	**287**	**269 (93.73)**	**10 (3.49)**	**8 (2.78)**

S – Susceptible I – Intermediate R – resistant

Among members of *Enterobacteriaceae*, *E.coli* (98%) was highly sensitive to tigecycline followed by *Klebsiella* spp (96.50%). *Proteus* spp (50%) showed least susceptibility to tigecycline.

Comparison E test and disc diffusion test for tigecycline.

		Tigecycline disc diffusion			Total
		S	**I**	**R**	
Tigecycline MIC by E-Test	**S**	257 (95.5)	10 (3.72)	2 (0.74)	**269**
	I	03 (30)	04 (40)	3 (30)	**10**
	R	0	01 (12.5)	7 (87.5)	**08**
Total		**260 (90.6)**	**15 (5.23)**	**12 (4.18)**	**287**

Here MIC by E test was taken as reference method. We observed that 269 isolates were sensitive by E test, of which 257 were sensitive, 10 were intermediate and 2 were resistant by disc diffusion method. Of 10 isolates intermediate by E test, 3 were sensitive, 4 were intermediate and 3 were resistance by disc diffusion method. 8 isolates were found to be resistant by E test, out of which 7 isolates were also

resistant and 1 isolate was intermediate by disc diffusion method.

Therefore there was no significant difference between the two methods in detecting resistance to tigecycline in ESBL producing *Enterobacteriaceae.*

4. Discussion

ESBLs are the one of the most evolving mechanism of antibiotic resistance among the family *Enterobacteriaceae* due to the selective pressure imposed by inappropriate use of third generation cephalosporins.[13] As therapeutic options for ESBL are limited, keeping this view in mind, an attempt was made to know the prevalence of ESBL producing *Enterobacteriaceae* and to determine in vitro activity of newly discovered tigecycline against these organisms.

Total 606 isolates of family *Enterobacteriaceae* were obtained from various clinical samples majority were *Escherichia coli* (36.47%) followed by *Klebsiella* species (31.68%) and *Citrobacter* species. (13.20%). A study carried out by Rudresh et al (2011) also reported similar results. A study from Bijapur by Metri et al[9] isolated 218 *Enterobacteriaceae* and reported 57.8% of *E.coli* and 25.6% of *Klebsiella pneumoniae* in 2011. Also Nema et al in 2014 studied 1044 *Enterobacteriaceae* in their study they reported *E.coli* (65.82%) and *Klebsiella* spp (24.9%) as common members of *Enterobacteriaceae.*[14]

Out of 606 isolates of *Enterobacteriaceae*, 287 were found to be ESBL producers and prevalence of ESBL producing *Enterobacteriaceae* in present study was computed to be 47.36%. Studies by Wadekar et al[15], Kaur et al[16] and Nema et al[14] found prevalence of ESBL to be 43%,45.8% and 48.7% respectively. It was noted that urine specimen yielded highest percentage (55.41%) of ESBL producing *Enterobacteriaceae* followed by pus (47.17%), blood (44.68%), sputum (37.74%), CSF (12%) and other samples (27.27%). A study conducted by Nema et al[14] and Metri et al[9] reflect the similar findings.

When analyzing the isolation of ESBL among *Enterobacteriaceae* from different area, we found that 98.95% of ESBL producing isolates obtained from IPD while 1.05% were from OPD which was found to be highly significant. A similar study by Metri et al[9] and a study conducted in tertiary care hospital on uropathogens by Bajpai et al[17] (2014) showed that more IPD samples (42.1%) were found to be ESBL positive as compared to OPD samples (30%). Among IPD patients highest percentage of ESBL producing *Enterobacteriaceae* were from the ICU and orthopaedic ward i.e. 81.44% and 59.18% respectively, followed by surgery (47.59%), paediatric (46.15%), burn (42.35%), medicine (34.26%) and other wards. This may be due to the more number of invasive procedures carried out in these wards, a longer duration of hospital stay of patients due to chronic illnesses. Also in ICUs patients are referred from the peripheral centres where antibiotic use is extensive. Similarly study conducted by Rastogi et al[18] (2012) and Mehrgan et al[19] (2010) showed the highest percentage of ESBLs were obtained from ICU, studies which were undertaken by Lenhard Vidal et al[20] and Chaudhary et al[21] (2011) revealed same finding.

Escherichia coli and *Klebsiella pneumoniae* are the main ESBL-producing bacteria, even though other members of the *Enterobacteriaceae* family show such resistance. We observed highest percentage of ESBL producers in *Klebsiella* species i.e. 74.48% followed by *E.coli* 45.25%. Also study from Rajasthan in 2013 by Sharma et al[8] reported ESBL production was 67.04% in *Klebsiella pneumoniae* and 56.92% in *E.coli*. The high occurrence of ESBLs in *Klebsiella* species observed in this research is of great concern since infections caused by this bacterium are contagious in nature and resistance of the organism to harsh conditions, which may be due to the presence of capsules that gives some level of protection to the cells.[22] Also *Klebsiella* strains are accompanied by relatively high stability of plasmid encoding ESBLs.[23]

Of all 287 ESBL producing *Enterobacteriaceae* isolates were tested for tigecycline susceptibility by disc diffusion and MIC was determined by E test. In this study MIC by E test was used as reference method since it has been shown good results.[5,24] Using the cut off established by FDA in 2005 for *Enterobacteriaceae*, by disc diffusion method we found that 260 (90.6%) of ESBL isolates were sensitive, 15 (5.22%) were intermediate and 12 (4.18%) were resistant to tigecycline. However by E test 269 (93.73%) ESBL producing *Enterobacteriaceae* isolates were susceptible to tigecycline, 10 (3.49%) isolates were intermediate and 8 (2.78%) isolates were resistant to this potent antibiotic. The MIC_{50} and MIC_{90} for *Enterobacteriaceae* were observed as 0.5µg/mL and 2µg/mL respectively.

In a multi-centric study from India it was observed that, tigecycline exhibit good activity against *Enterobacteriaceae* regardless to the presence or absence of ESBL.[25] Behera et al[26], Taneja et al[27] Shanthi et al (2011)[28] and Singh et al (2014)[29] reported 100% tigecycline susceptibility of ESBL isolates. We observed that 98% *of E.coli* were susceptible to tigecycline.

Behera et al (2009)[26] carried out MIC determination by E test and reported, MIC_{50} and MIC_{90} for ESBL producing *E.coli* and *Klebsiella* isolates as 0.38 and 0.75 µg/mL respectively while Shanthi et al[28] reported lower MIC values by microbroth dilution method (MIC_{50}, 0.12µg/mL and MIC_{90}, 0.25µg/mL) for *E.coli* and (MIC_{50}, 0.25µg/mL and MIC_{90}, 0.5µg/mL) for *Klebsiella* species, thus they concluded that tigecycline was found to be highly effective against ESBL producing *Enterobacteriaceae*. While analyzing tigecycline susceptibility to other species, we observed 94.44%, 83.33%, 75% and 66.67% susceptibility to tigecycline for *Citrobacter* species, *Enterobacter* species, *Salmonella* species and *Morganella morganii* respectively. Ratnam et al[30] reported that six out of seven isolates of ESBL producing *Enterobacter* spp. were susceptible to tigecycline (MIC range 0.5 - 2 µg/mL) which is similar to our findings.

We carried out the in-vitro activity of tigecycline by disc diffusion test and E test. By both methods 257 isolates were found to be sensitive while 4 and 7 isolates were found to be intermediate and resistant respectively. As none of the isolates was found to be sensitive by disc diffusion method and resistant by E test, (very major error, 0%) and only 2 isolates were found to be resistant by disc diffusion method but sensitive by E test (major error 0.74%). Thus we found no significant difference between these 2 methods which was concordant with other studies conducted by Somily et al[24] and Tellis et al.[10]

Disc diffusion method is simple to perform, highly reproducible and inexpensive .While E test is though costly but it can determined MIC easily as compared to agar dilution or microbroth dilution test.[24]

5. Conclusion

ESBLs have already established amongst family *Enterobacteriaceae* and there are limited treatment options against these ESBLs. So facing today's multidrug resistance era, not only the early recognition and spread of these MDR organisms is important but also we should be ready with new antimicrobials which has promising in vitro activity against ESBL-producing *Enterobacteriaceae*. Tigecycline can play a key role as therapeutic option in tackling ESBL producing *Enterobacteriaceae*. However, clinicians need to prescribe tigecycline appropriately, in order to avoid the emergence of resistant strains. From susceptibility testing by E test and disc diffusion, as there was no significant difference for tigecycline susceptibility, we concluded that depending upon availability and cost effectiveness either of the tests can be used.
In the current era of decreased newer antimicrobial development, effective control of risk factors for drug resistance, proper antibiotic policy and judicious use of antimicrobial is important.

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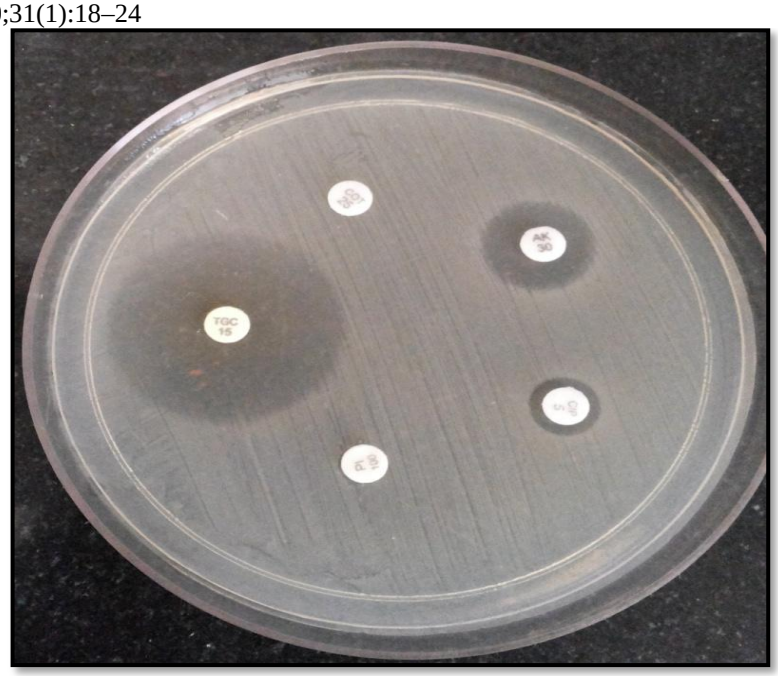
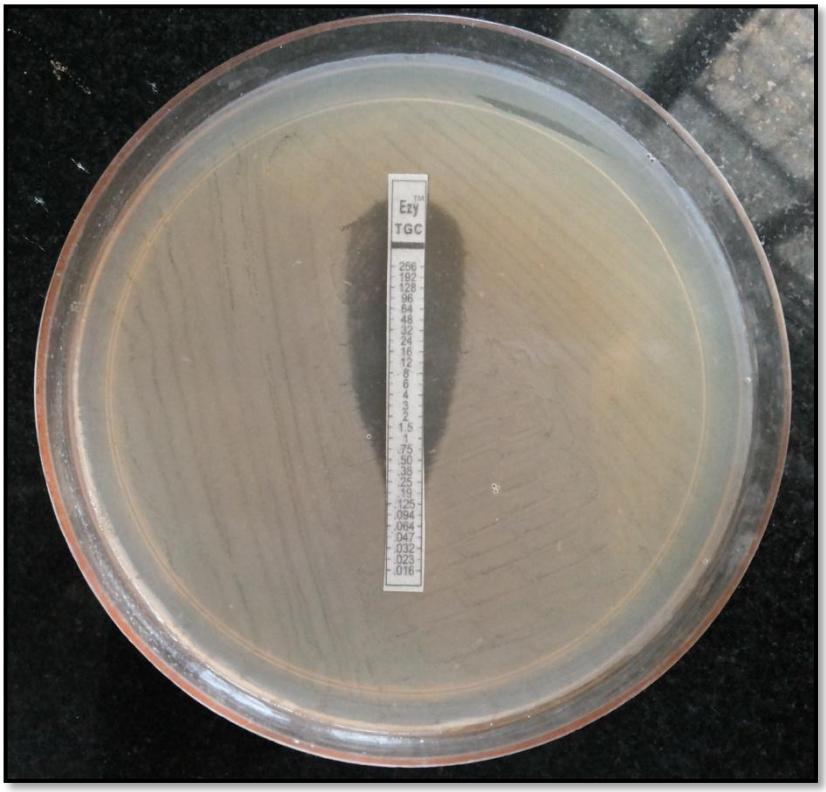


Tigecycline susceptibility by disc diffusion method showing 31 mm inhibition zone diameter.



Tigecycline E test showing MIC 0.5 µg/mL