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Original Research Article

Detection of antibiotic resistance and metallo beta lactamase among Pseudomonas aeruginosa clinical isolates

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Abstract

Introduction: MBL generating *Pseudomonas aeruginosa* (MBL-PA) have a significant clinical problem that poses a significant obstacle to antimicrobial therapy.
Aim: The aim of this study was to detect the metallo-β-lactamase (MBL) production in meropenem (MRP) resistant *P.aeruginosa* isolates.
Materials and Methods: The research was carried out between August 2013 and July 2014. The study includes strains of *Pseudomonas aeruginosa* that were obtained from a variety of clinical specimens at the Coimbatore Medical College's Department of Microbiology. There were around 211 isolates found. In order to demonstrate which phenotypic test was the most dependable for the prompt identification of MBL from meropenem-resistant isolates, the study aimed to ascertain the prevalence of MBL from pseudomonad isolates and compare the Modified Hodge Test (MHT), Combined disc test (CDT), and Double Disc synergy test (DDST) with the E-test.
Results: The prevalence of MBL in *P.aeruginosa* isolates was 7.11%. The majority of meropenem resistant isolates produces MBL. Of the four phenotypic tests, the E test alone had the highest sensitivity (100%) and specificity (100%) as well as the highest positive predictive value (100%) among all four tests.
Conclusions: The E-test was the most effective and trustworthy phenotypic test that allowed for detection of the isolate's MIC (minimum inhibitory concentration) value. The next more dependable tests for detecting MBL after the E-test were CDT and DDST, which were followed by MHT.

Keywords: Modified Hodge, MBL, E-test.

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1. Introduction

Particularly in medical environments, the rise of *Pseudomonas aeruginosa* strains that generate MBL type carbapenemases is a serious worry. Infections brought on by multidrug-resistant bacteria are frequently treated with carbapenems as a last resort.[1-3] Numerous complex infections, including bloodstream infections (BSIs), pneumonia/ventilator-associated pneumonia (VAP), and severe burns in hospitalized patients, particularly those in intensive care units (ICUs), have been linked to MBL-PA infections. These infections have also been linked to increased rates of morbidity and mortality as well as the failure of carbapenem therapy.[4-6]

There are four kinds of β-lactamases: class B needs a bivalent metal zinc ion to function, whereas classes A, C, and D use a serine-based mechanism for MBL.[7] The most concerning of these processes is the appearance of *P.aeruginosa* acquired MBL, which presents a major risk during infection management.[8] Following Japan's 1991 revelation, many other countries reported MBL resistance.[9] MBL-PA was initially discovered in India in 2002[10] and the percentage of MBL-PA varies from 7-65%. The mortality rate from MBL-PA varied from 70-90%.[11]

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MBL have the ability to hydrolyze all β-lactam antibiotics, save monobactams, such as penicillin, cephamycins, cephalosporins, β-lactams with β-lactamase inhibitors, and carbapenems.[12] The MBL genes are presented in mobile genetic elements including integrons, transposons, and plasmids that may move within and across species. Clinical outcomes are poor when people with MBL-PA are treated with β-lactam antibiotics.[13] Therefore, early diagnosis of *P.aeruginosa* that produces MBL is essential. A fundamental tenet of infectious disease management is diagnostic stewardship, which grants physicians and clinical microbiologists the power to choose the best phenotypic assays for MBL identification. The main objective was to separate and validate the *Pseudomonas aeruginosa* isolates from different clinical samples using the proper biochemical responses and recording the microbiological profile of the same. Comparing the E-test findings with those of other phenotypic test techniques, such as the Double Disc Synergy Test (DDST), Combined Disc Test (CDT), and Modified Hodge Test (MHT), was the secondary goal.

2. Materials and Methods

A cross-sectional prospective study was carried out in the Department of Microbiology at the Tertiary care Hospital in Coimbatore, Tamil Nadu, India, between August 2013 and July 2014. The Institutional Review Board (IRB) Committee gave its approval to the study (Number 36/2013/MICRO). It was reasonable to waive consent. About 211 *Pseudomonas aeruginosa* isolates were obtained from a variety of materials, such as sputum, urine, blood, auditory swabs, wound swabs, catheter tips, and pus. Following inoculation onto MacConkey and Blood agar, each sample was incubated at 37ºC for the whole night. Using brain-heart infusion broth, catheter tip culture and blood were carried out and cultured for a whole night. The colonies on MacConkey agar that did not ferment lactose and tested positive for catalase and oxidase were being identified by further processing. Biochemically confirmed isolates of *Pseudomonas aeruginosa* were subjected to Kirby-Bauer susceptibility testing.[14]

2.1. Exclusion and inclusion criteria

The study comprised isolates of *Pseudomonas aeruginosa* that were resistant to meropenem (zone of inhibition <15 mm),[15] and they were tested using phenotypic techniques to identify MBL. The antibiotic discs were acquired from Mumbai's Hi-Media Laboratories Ltd. The control strain was used *P.aeruginosa* ATCC27853. For susceptibility testing, antibiogram discs containing 30 µg of ceftazidime, 10 µg of gentamicin, 30 µg of amikacin, 5 µg of ciprofloxacin, 5 µg of ofloxacin, 75/30 µg of cefaperazone-sulbactam, 10 µg of meropenem, 10 µg of tobramycin, 100/10 µg of piperacillin/tazobactam, 10 µg of cefepime, and 30 µg of aztreonam were used. In accordance with the 2013 CLSI recommendations, the zone's diameter was measured and classified as susceptible, resistant and intermediate.[14] Antibiotic resistance and susceptibility percentages were noted. The study did not include culture-negative samples, isolates of *Pseudomonas aeruginosa* that were susceptible to meropenem, or culture-positive clinical isolates that were not *Pseudomonas aeruginosa*.

2.1.1. The modified Hodge test

Muller-Hinton agar (MHA) was used to cultivate a lawn culture of 0.5 McFarland's standard E. coli ATCC 25922 at a 1:10 dilution. A 10µg Hi-Media meropenem disc was placed in the plate's middle. Meropenem-resistant *P.aeruginosa* test isolates were dispersed from the disc edge to the plate's edge in four different methods. The plates were inspected for a "clover-leaf" shaped zone of inhibition during an overnight incubation period, which signified MHT positive [**Figure 1**a]. This test allows for the testing of four distinct isolates on a single plate. No clover leaf indentation indicates the test isolate was MBL negative [**Figure 1**b].[15]

2.1.2. Imipenem and imipenem with EDTA Combined disc test (CDT)

MBL-producing isolates were phenotypically identified using a combination disc containing 10µg of imipenem, and MBL substrate such as 750µg of ethylenediamine tetra-acetic acid (EDTA), an MBL inhibitor. The imipenem-EDTA combination disc's zone of inhibition is more than 7 mm larger than that of imipenem alone, which determines the phenotypic expression of MBL.[15]

2.1.3. The Meropenem double disc synergy test (DDST) with and without EDTA

Lawn culture was used to inoculate the MHA plate with the organism of investigation. A 10 µg Meropenem disc was placed on the MHA plate 20 mm center to center from the blank disc, which contains 10µL of 0.5M EDTA, in order to get the required 750µg concentration. If the inhibitory zone between the Meropenem and EDTA discs increased by ≥ 5 mm during the overnight incubation, the test was considered positive and the test organism was producing MBL [**Figure 2**a]. If the zone size difference is <5mm indicating that the test organism does not produce MBL [**Figure 2**b].[16]

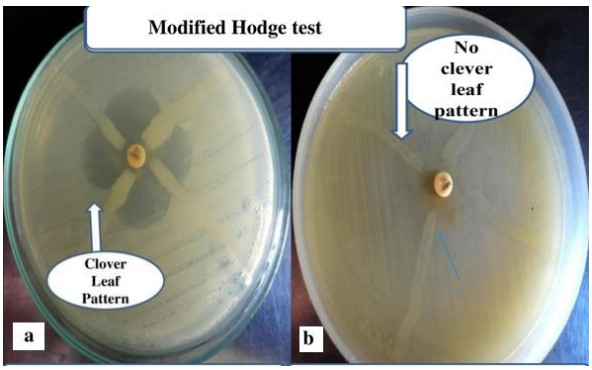

Figure 1: Modified Hodge test; **a:** MBL positive; **b:** MBL negative

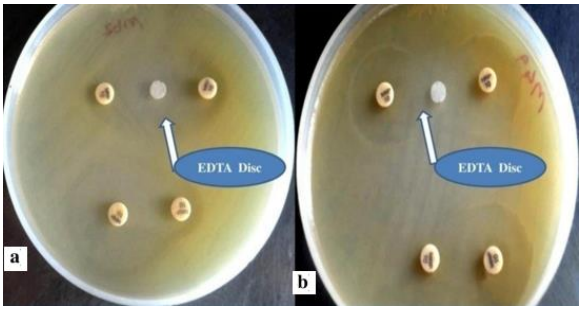

Figure 2: Double disc synergy test; **a:** MBL positive; **b:** MBL negative

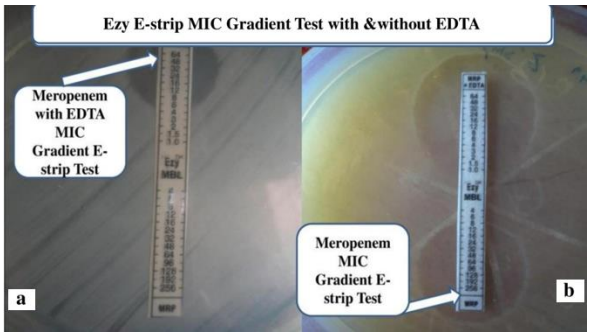

Figure 3: Ezy E-strip MIC gradient test with & without EDTA

2.1.4. Epsilometer or E test (Ezy MIC Strips)

Meropenem with & without EDTA e-strip.

This particular Phenotypic MBL detection test was typical in that it uses a single strip coated with a combination of Meropenem + EDTA with gradient concentrations of 1-64µg/ml and Meropenem with gradient concentrations of 4-256µg/ml, purchased from Mumbai's Hi Media Laboratories. Meropenem + EDTA is present in the top half, with the highest concentration tapering lower, whereas Meropenem was similarly covered in the bottom half in the opposite direction of the concentration gradient.[17] After overnight incubation, MHA plate's MIC values were noted (**Table 3**).

a. MIC of Meropenem strip
b. MIC Concentration of MRP + EDTA strip.
c. Ratio of MRP / MRP + EDTA strip.

2.2. Sample size calculation

Pseudomonas aeruginosa that produces carbapenemase was found in 23.5%of Indian cases.[18] The formula $n=Z^2 p (1-p)/e^2$ was used to determine the sample size, where 'e' is the margin of error (0.05= ± 5%) and "Z" is the value for the associated confidence level (CI), which is 1.96 for 95% CI. and p is the estimated value for the proportion of a sample that have the condition of interest. $n= (1.96)^2 \times 0.297 \times (1 - 0.297) / (0.05)^2 \approx 276$ sample size calculated. As because of it was a time bound study, the sample size was restricted into 211.

2.3. Statistical analysis

The software, IBM Version 28.0 of the SPSS, based in Chicago, USA was utilized to examine the data. A 95% confidence interval was used to determine the odds ratios and p-values for statistical significance. If the p-value was less than 0.05, the proportionate difference was deemed statistically significant, and the findings were interpreted accordingly.

3. Results

About 211 isolates of *Pseudomonas aeruginosa* were obtained for the study from a variety of clinical samples. A maximum of 50 isolates (23.7%) were found in the age group of those over 60. Males made up 143 (67.77%) of the specimens, while females made up 68 (32.23%). 2.1:1 was the male to female sex ratio.(**Table 2**)

The most resistant drug among all Pseudomonal isolates (n=211) was ceftazidime (71.09%), followed by gentamicin (36.02%) and ciprofloxacin (46.45%). In urine isolates (n=21), nitrofurantoin exhibited the greatest resistance (80.95%). All the antibiotics under investigation were statistically significant.

A disc diffusion screening test revealed that 16 (7.58%) of the 211 pseudomonad isolates were meropenem resistant. MIC of Meropenem for the MBL-PA isolates (**Table 3**) that ranged from 24 to 256 µg. As stated in the technique, isolates that tested screening positive for MBL production [16(7.58%)] underwent MIC detection using the Ezy E-strip method in conjunction with EDTA. Approximately 15 isolates (93.75%) were confirmed by the E-strip test as MBL producers. The disc diffusion screening technique revealed that one isolate (6.25%) was an MBL false positive.

The prevalence of MBL-PA was observed as 7.11%. 9(60%) and 6(40%) of the 15 MBL-positive isolates were identified in men and females respectively. The ratio of males to females was 3:2. The majority of isolates that produced MBL (23.69%) were discovered in the over-60 age group.

The majority [14 (93.33%)] of the 15 isolates that produced MBL were taken from inpatients. The majority of MBL-producing isolates were derived from pus sample 11(73.33%), with burn swab cases 2 (13.33%) coming in second.(**Table 4**)

All β-lactam antibiotics were 100% resistant to the isolates that produced MBL. One MBL-producing isolate that was separated from a urine sample was also resistant to nitrofurantoin, norfloxacin, and ofloxacin (**Table 4**). The most efficient antibiotics against MBL producers were determined to be gentamicin and ciprofloxacin, with results showing statistical significance ('p' value <0.05) (**Table 4**).

Table 1: Interpretation of Ezy MIC strip test

MBL Positive isolate[17]	MRP/ MRP + EDTA = >8 MRP/ MRP + EDTA= >256/<64 MRP/ MRP + EDTA = >256/<1	When the ratio of the value obtained for Meropenem (MRP) and the value of Meropenem with EDTA (MRP+EDTA) was more than to 8, **OR** If zone was observed on the side coated with Meropenem + EDTA & no zone is observed on the opposite the side coated with Meropenem, interpret the culture as MBL positive. [**Figure 3**a]
MBL Negative isolate[17]	MRP/ MRP + EDTA ≤ 8	When the ratio of the value obtained for Meropenem (MRP) and the value of Meropenem with EDTA (MRP+EDTA) was less than or equal to 8. (**Figure 3**b).
MIC- Minimum inhibitory concentration, **EDTA**- Ethylene diamine tetra-acetic acid, **MRP**-Meropenem.		

Table 2: *Pseudomonas aeruginosa* - Microbiological profile.

Antibiotics	***Pseudomonas aeruginosa* Isolates (n=211)**				
	Sensitivity Number (%)	**Resistance Number (%)**	**Chi-square test**	**'p' value**	**S/ NS**
Amikacin	143(67.77%)	68(32.23%)	12.16	0.001	S
Gentamicin	135(63.98%)	76(36.02%)	44.78	<0.001	S
Tobramycin	137(64.93%)	74(35.07%)	11.86	0.001	S
Ciprofloxacin	113(53.55%)	98(46.45%)	10.36	0.001	S
Ceftazidime	61(28.91%)	150(71.09%)	43.66	0.001	S
Cefaperazone Sulbactum	137(64.93%)	74(35.07%)	11.82	0.001	S
Cefipime	148(70.14%)	63(29.86%)	42.2	0.001	S
Meropenem	195(92.42%)	16(7.58%)	35.5	0.001	S
Aztreonam	162(76.78%)	49(23.22%)	43.7	<0.001	S
Piperacillin Tazobactum	171(81.04%)	40(18.96%)	45.5	<0.001	S
Urine isolates (n=21)					
Ofloxacin	16 (76.2%)	5 (23.8%)	22.5	<0.001	S
Norfloxacin	6(28.57%)	15(71.43%)	22.25	0.001	S
Nitrofurantoin	4(19.05%)	17(80.95%)	24.46	<0.001	S

S-Significant, NS-Not significant

Table 3: MIC of the isolates resistant to meropenem

Meropenem resistant isolate	**MIC of MRP**	**MIC of MRP + EDTA**	**Ratio of MRP / MRP + EDTA**	**MBL confirmation**
1	>256	4	>8	Yes
2	64	1	>8	Yes
3	24	<1	>8	Yes
4	256	2	>8	Yes
5	96	1.5	>8	Yes
6	256	2	>8	Yes
7	128	4	>8	Yes
8	96	1.5	>8	Yes
9	>256	16	>8	Yes
10	64	16	<8	No
11	24	<1	>8	Yes
12	>256	2	>8	Yes
13	128	4	>8	Yes
14	48	<1	>8	Yes
15	>256	16	>8	Yes
16	128	1.5	>8	Yes
MIC- Minimum inhibitory concentration, **EDTA**- Ethylene diamine tetraacetic acid, **MBL**-Metallo beta-lactamase.				

Table 4: Pattern of antibiotic resistance in isolates that produce MBL

Antibiotics	**MBL producers (n =15)**				**'p' value**	**S/NS**
	Sensitive (Number)	**Percentage (%)**	**Resistance (Number)**	**Percentage (%)**		
Amikacin	5	33.33%	10	66.67%	0.067	NS
Gentamicin	14	93.33%	1	6.66%	<0.05	**S**
Tobramycin	0	NA	15	100%	NA	NA
Ciprofloxacin	14	93.33%	1	6.66%	<0.05	**S**
Ceftazidime	0	NA	15	100%	NA	NA
Cefaperazone Sulbactum	0	NA	15	100%	NA	NA
Cefipime	0	NA	15	100%	NA	NA
Meropenem	0	NA	15	100%	NA	NA
Aztreonam	13	86.67%	2	13.33%	0.002	**S**
Piperacillin Tazobactum	0	NA	15	100%	NA	NA
Urine isolates:						
Ofloxacin	0	NA	1	100%	NA	NA
Norfloxacin	0	NA	1	100%	NA	NA
Nitrofurantoin	0	NA	1	100%	NA	NA
S-Significant, NS-Not Significant, NA-Not Applicable.						

Table 5: Comparing the MHT to the "E" test

MHT	**E test**		**Total**	**Odd's ratio**	**'p' value**	**S/NS**
	Positive	**Negative**				
Positive	12	0	12	3.57	0.044	S
Negative	3	0	3			
Total	15	0	15			
MHT - Sensitivity 80 %, Specificity 80% and positive predictive value 100%,						

Table 6: Comparing the CDT to the 'E' test.

Combined disc test (IMP & IMP + EDTA)	**E test**		**Total**	**Odd's Ratio**	**'p' value**	**S/NS**
	Positive	**Negative**				
Positive	13	0	13	5.4	0.042	S
Negative	2	0	2			
Total	15	0	15			
Combined disc Test method: Sensitivity 86.67 %, specificity 86.67 % and PPV 100%.						

Table 7: Comparing the DDST to the 'E' test.

Combined disc test (MRP & MRP+EDTA)	**E test**		**Total**	**Odd's Ratio**	**'p' value**	**S/NS**
	Positive	**Negative**				
Positive	13	0	13	5.4	0.042	S
Negative	2	0	2			
Total	15	0	15			
Sensitivity of Double disc synergy test 86.67 %, Specificity 86.67 % and PPV 100%.						

Table 8: Comparison of all phenotypic tests

Test	**Sensitivity**	**Specificity**	**Positive predictive value**	**Confidence Interval (C.I.)**
MHT	80 %	80%	100%	0.95
CDT	86.67%	86.67 %	100%	0.95
DDST	86.67%	86.67%.	100%	0.95
E-test	100%	100%	100%	0.95

(**Table 5-7**): MedCalc Software Ltd. Odds ratio calculator was used to detect odds ratio and 'p' value. https://www.medcalc.org/calc/odds_ratio.php (Version23.0.9).

Among all the four phenotypic tests, the maximum sensitivity (100%) and the maximum specificity (100%) and maximum positive predictive value (100%) were observed by E test **(Table 8)** alone. E-test was the most efficient and reliable phenotypic test through which MIC value of the isolate can be detected with the C.I. value of 0.95. The next more dependable tests for detecting MBL after the E-test were CDT and DDST, which were followed by MHT. The sensitivity and specificity of MHT are the lowest.

4. Discussion

MBL-PA has become a major source of potentially deadly Gram-negative hospital-acquired infections because MBL hydrolyzes all classes of β-lactam antibiotics, which has been shown to play a major role in the horizontal transmission of MBL genes to other hospital pathogens.[20] which found to play a significant part in the horizontal transmission of MBL genes to other pathogens in hospitals. These days, the top goals are not just therapy but also detection and containment.[20] In light of the aforementioned viewpoint, the study's objectives were to compare the results of the phenotypic tests, determine the prevalence of MBL in isolates of the same that were resistant to meropenem, and record their antibiogram.

The results of this investigation indicated that hospital-acquired infections are more likely to cause MBL because *P.aeruginosa* isolates were more common among inpatients (94.31%) than outpatients (5.69%).[21] In sex wise distribution of specimens, males were 143 (67.77%) and females were 68 (32.23%). Thus our study correlated with the study conducted by Choudhary V et al[21] and Kothari et al.,[22] where they reported from inpatients 88% and 92% respectively and similar male dominance were reported by the same.

In this study, pus was the predominant specimen in both Non-MBL(62.09%) and MBL(73.33%) producing isolates. Similar results reported by Rani et al[23] and Chowdhury RA et al.[20]

The percentage of *P.aeruginosa* isolates that produced MBL in our investigation was 7.11%. According to reports, the prevalence rate of MBL-PA in India ranges from 11% to 25%.[24] Thus our study correlating with the study conducted by Thaba et al[25] and Chaudhary M et al[26] which showed the prevalence of 5.5%, and 8.7% respectively. Higher prevalence of MBL-PA was detected in countries like Nepal (69.8%)[27] and Egypt (85%).[28] The reported numbers from this study and those from other studies may vary depending on the region, the kind of infection, the substantial use of antibiotics, or the antibiotic treatment regimens at the hospitals selected for the study.[8]

In this study, the MIC (done by Ezy strip test) of Meropenem resistant isolates such as positive for MBL producers were in the range from 24-256µg/ml [Figure 3a]. It was calculated by the formula, MIC ratio of MRP/ MRP + EDTA (**Table 1**). The MIC ratio of MRP/ MRP with EDTA which was <8µg/ml considered as MBL negative, shown in [Figure 3b]. With the highest (100%) sensitivity and specificity, the E test thus produced the most accurate findings. This is consistent with research by Deeba et al.[30] and Rani et al.[23]

Clinical microbiological labs must be able to distinguish between MBL-PA and non-MBL-PA strains since MBL-PA strains have a notably greater risk of treatment resistance. The finding of MBL-PA isolates are linked to a greater hospital case fatality rate and are more likely to produce invasive illness than other meropenem-sensitive isolates.[31]

Ceftazidime (71.09%) was the most resistant medication among all Non-MBL Pseudomonal isolates (n=211), followed by Ciprofloxacin (46.45%) and Gentamicin (36.02%) (**Table 2**). Similar resistance rates were found in studies by Deeba et al[30] and Kumar et al.[29] It was highlighted that greater resistance exhibited by Non MBL-PA strains towards third-generation cephalosporins (ceftazidime), which is frequently used to treat *Pseudomonal* infections. However, poor treatment results have resulted from the increasing occurrence of ceftazidime resistance.[32]

Among MRP resistant isolates, 93.75% (15/16) were MBL producers (**Table 3**). The solitary false-positive result might be the result of hyperproduction of AmpC and co-production of cefotaximase extended spectrum β-lactamase (CTX-M), which could lead to some hydrolysis of carbapenems.[33]

Aztreonam is stable against MBLs. However, 86.67% of the MBL-producing isolates were sensitive to aztreonam and 13.33% isolates were resistant to aztreonem (**Table 4**), pointing to a potential connection with other resistance mechanisms such activation of efflux systems, porin loss, or permeability mutation.[34]

In this study, MHT showed 80% sensitivity and 100% Positive predictive value (**Table 5**). Thus our study correlating with the study conducted by Tellis et al,[35] where they reported with the sensitivity of 74.32% and 98.2% of PPV.

According to **Table 6**, the Combined Disc Test's (CDT) sensitivity, positive predictive value, and specificity were 86.67%, 100%, and 86.67%, respectively. On the other hand, Qu TT et al.[36] reported that the Combined Disc Test had 100% sensitivity, positive predictive value, and specificity. So combined disc diffusion test was one of the most reliable test can be done in all small and medium sized laboratories for the early detection of MBL enzymes, where Gene Xpert/ PCR was not available. Our study's combined disc diffusion

test's decreased sensitivity was most likely caused by the overproduction of AmpC/CTX-M enzymes.[33]

In this investigation, DDST demonstrated 86.67% sensitivity, 100% PPV, and 86.67% specificity (**Table 7**). This study is comparable to those of Tellis et al.[35] and Franklin et al.[37] The most reliable and efficient phenotypic test for determining the isolate's MIC (minimum inhibitory concentration) value was the E-test. Following the E-test, CDT and DDST were the next more reliable tests for identifying MBL, and then MHT (**Table 8**).

5. Limitations of the Study

The lack of a Gene Xpert/PCR analysis for the confirmation of phenotypic methodologies is a drawback of the current study. Furthermore, our study did not use new phenotypic carbapenemase detection techniques such the Carba M and Carba NP test or eCIM/ mCIM, which were not advised during this study period (2013) and are suggested by recent CLSI guidelines.

6. Conclusion

The "E" test, a straightforward and affordable technique, has to be implemented in every clinical microbiological laboratory to support early MBL diagnosis and infection management. Stringent infection control and antibiotic stewardship programs should be employed in all healthcare facility because these programs use the current antibacterial arsenal to restrict the spread of resistance to other microbes that produce MBL.

7. Conflict of Interest

None.

8. Source of Funding

None.

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