A comparative study of M.I.C evaluator test with the broth microdilution method for antimicrobial susceptibility testing of *Enterobacter cloacae* isolated from cooked food

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Abstract: Agar dilution and broth microdilution are widely recommended quantitative antimicrobial susceptibility test methods, but they are tedious and time consuming to implement as routine tests in many clinical laboratories. Therefore, this study aimed at comparing the broth microdilution and the M.I.C Evaluator method which has been validated for its high accuracy and easy performance for routine diagnostic use. Twenty *Enterobacter cloacae* strains were isolated following microbiological procedures and confirmation of the isolates used the API 20E test. The strains were evaluated for their susceptibility to seven antimicrobials using the broth microdilution and MIC Evaluator methods. The doubling dilution difference (essential agreement) in the MIC result was derived from: $\log_2$ (MIC by BMD) -$\log_2$ (MIC by M.I.C Evaluator method). The categorical agreement, interpreted as breakpoints of sensitive and resistance strains was also noted. Categorical agreement between M.I.C Evaluator strip and broth microdilution for amoxicillin, metronidazole and erythromycin was 100%: while categorical agreement for ciprofloxacin was 90%. The essential agreement for erythromycin, ciprofloxacin and tetracycline were 90%, 70% and 15% respectively. Results indicate a high efficiency of the M.I.C Evaluator strip method in determination of minimum inhibitory concentration as compared to broth microdilution method. However, further analysis regarding the suitability of the M.I.C Evaluator for testing *Enterobacter cloacae* is warranted considering that no consensus guidelines exist for the use of this method with the organism.

Keywords: M.I.C Evaluator, broth microdilution, *Enterobacter cloacae,* antimicrobial susceptibility.

INTRODUCTION

Enterobacter cloacae is a Gram-negative rod that belongs to the family *Enterobacteriaceae*. It is ubiquitous, and a commensal microflora in the intestinal tracts of humans and animals (Krzyminsk *et al*., 2010). The bacteria may be transmitted by direct or indirect contact with infectious agent, or can also be spread feco-orally through contaminated water or food. Like most members of the family *Enterobacteriaceae*, these organisms are capable of causing opportunistic infections including bacteraemia, skin and soft-tissue infections, intra-abdominal infections, urinary tract infections, among others in hospitalized or debilitated patients (Gosh *et al*., 2007). Infections due to this organism have the highest mortality rate compared to other *Enterobacter* infections due to their multiple antibiotic resistances (Fraser, 2007).

Antibiotics including piperacillin-tazobactam, cefepime, aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole are some of the drugs used to treat *Enterobacter cloacae* infections (Fraser, 2007). carbapenems are mostly used against complicated infections, such as pyelonephritis or bacteraemia caused by multidrug-resistant strains. However, resistance of these organisms to carbapenems, ampicillin and cephalosporins has been reported (Vaux *et al*., 2011).

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During therapy, antimicrobial agents act both on pathogenic and commensal bacteria of animals and humans especially inhabitants of the intestinal tract, subjecting them to high exposure to these drugs; these organisms develop resistance in order to survive hence creating a repository of resistance genes which can be disseminated in the environment through faeces. Resistance genes are spread by vertical gene transfer to other bacteria; including pathogenic species by these organisms albeit being opportunistic (Knezevic *et al.,* 2008). Therefore, monitoring antibiotic resistance of pathogenic and commensal bacteria is highly imperative. DeFrancesco *et al*., (2004) found that commensal *E. coli* had higher rates of resistance than pathogenic multi-drug resistant *Salmonella* from the same herds, suggesting that commensal bacteria could be used to survey the prevalence of antibiotic resistant bacteria.

Emergence of antimicrobial resistance by these resistant organisms is associated with high morbidity and great economic loss due to treatment failure which might even lead to mortality (Vaux *et al.*, 2011). These emerging resistance patterns necessitates quantitative determinations of antimicrobial susceptibility (minimum inhibitory concentration (MIC) determination), that may identify significant changes in antimicrobial susceptibility hence, providing early warning of emerging resistance (Giovanni *et al*., 2002). Worthy of note is the fact that most of the

recommended test methods are tedious and time consuming to implement as routine tests in many clinical laboratories (Gales *et al*., 2001; Arroyo *et al*., 2005). The M.I.C Evaluator strip operates on a principle similar to that of the E-test device. Antimicrobial susceptibilities of various microorganisms; including fastidious bacteria could be determined using this rapid and accurate test (Arroyo *et al*., 2005). Therefore, this study aimed at comparing the broth microdilution and the M.I.C Evaluator antimicrobial susceptibility methods on *Enterobacter cloacae* strains as a guide for clinico-epidemiological investigations.

MATERIALS AND METHODS

Bacterial strains

Twenty *Enterobacter cloacae* strains were isolated from various food samples which comprised of beef and chicken stew, potatoes, rice, vegetables and pies purchased from cafeterias in Alice. The organisms were identified as previously reported (Akoachere *et al*., 2009). Briefly, 25 grams of each sample was homogenized under aseptic conditions in 225 mL of buffered peptone water and 1mL of the homogenate was transferred into a tube containing 9 mL of sterile buffered peptone water before incubating aerobically at 37^{o}C for 12-24 hours. A loopful of enrichment broth was plated on Mac Conkey and Eosin-Methylene Blue (EMB) agar (Oxoid, Basingstoke, England); plates were incubated aerobically for 24-48 hours at 37^{o}C. Isolates were characterised based on colony pigmentation, Gram staining appearance and cytochrome oxidase reaction; confirmation of the isolates was done using API 20E kit (Biomerieux, Marcy-l'Etoile, France) as per manufacturer's instruction for use.

Minimum inhibitory concentration determination by broth microdilution (BMD) method

BMD technique was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines (2010). A total of 5 antibiotics were used in this assay; Inocula matching turbidity of 0.5 McFarland standard in 5 mL of sterile saline were prepared from overnight growth cultures. Antibiotic concentrations from 0.001-256 µg/mL were tested in microtiter plates. An automatic ELISA microplate reader (SynergyMx, Biotek^{R} USA) was used to measure the absorbance of the plates at 570nm before and after incubation at 37^{o}C for 24 hours. The absorbencies were compared and the values plotted against concentration; MIC was determined. The following reference strains *Staphylococcus aureus* NCTC 6571 and *Pseudomonas aeruginosa* ATCC 15442 were used as control organisms.

Minimum inhibitory concentration determination by the M.I.C Evaluator

The M.I.C Evaluator test was performed following the manufacturer's guidelines (Oxoid, Basingstoke, England). A suspension equivalent to 0.5 McFarland standard turbidity was swabbed on Mueller-Hinton agar plates. M.I.C Evaluator strips were placed aseptically and plates were incubated at 37°C for 18 to 20 h. The MIC was read where inhibition of growth intersected the strip. *Staphylococcus aureus* NCTC 6571 and *Pseudomonas aeruginosa* ATCC 15442 reference strains were used to test the efficacy of the strips.

ANALYSIS

All MICs were interpreted using CLSI breakpoints. M.I.C Evaluator and broth microdilution methods were compared using categorical and essential agreement, a method commonly used to compare different antimicrobial susceptibility testing methods. Categorical agreement is based on interpretive breakpoints of sensitive and resistant strains. The doubling dilution difference (essential agreement) in the MIC was calculated as: $\log_2$ (MIC by BMD) -$\log_2$ (MIC by M.I.C Evaluator method) whereby the agreement of the two methods within ±1 was the essential agreement (Rennie *et al*., 2012).

RESULTS

Categorical agreement for M.I.C Evaluator compared to broth microdilution for 20 strains of *Enterobacter cloacae* tested against five antibiotics are shown in table 1. All the 20 (100%) strains demonstrated categorical agreement to amoxicillin and metronidazole and erythromycin. On the other hand, 18/20 (90%) and 12/20 (60%) strains were susceptible to ciprofloxacin and tetracycline using the BMD method while with the M.I.C Evaluator test, all strains (100%) and 11/20 (55%) were susceptible to ciprofloxacin and tetracycline respectively.

The MIC results of the 2 methods (BMD and M.I.C Evaluator) were compared; agreement within ±1 twofold dilution was 90% (18 strains), 70% (14 strains) and 15% (3 strains) for erythromycin, ciprofloxacin and tetracycline respectively (table 2).

DISCUSSION

Antimicrobial susceptibility testing of infectious pathogens is essential for effective patient management especially in critical care settings. MIC results help clinicians to monitor development of resistance, and are also relevant for clinical pharmacists in the determination of optimal pharmacodynamic dosage (Rennie *et al*., 2012). Nosocomial infections due to *Enterobacter cloacae* are often treated with quinolones and aminoglycosides (Fraser, 2007). Interestingly, the study reported marked susceptibility of these organisms to ciprofloxacin, (table 1) suggesting that ciprofloxacin may still be the best therapy in the event of these infections in

Table 1: Antimicrobial susceptibility patterns of *Enterobacter cloacae* using broth microdilution and M.I.C Evaluator test method

Drug	MICs (μg/mL)						
	Broth micro dilution method			M.I.C Evaluator test method			Break point S, R
	Range	S (%)	R (%)	Range	S (%)	R (%)	
T	0.014->30	12 (60)	8 (40)	2->265	11 (55)	9 (45)	<2, >2
CIP	0.001-2.5	18 (90)	2 (10)	<0.002-0.06	20 (100)	0 (0)	≤1, ≥2
A	>256	0 (0)	20 (100)	16->256	0 (0)	20 (100)	≤8, >8
MET	>256	0 (0)	20 (100)	>256	0 (0)	20 (100)	≤32, >32
E	32->256	0 (0)	20 (100)	32->256	0 (0)	20 (100)	≤16, >16

T, tetracycline: CIP, ciprofloxacin: A, amoxicillin: MET, metronidazole: E, erythromycin. All break points derived from CLSI standards.

Table 2: Distribution of differences in MICS antimicrobials by 20 strains of *Enterobacter cloacae* by the M.I.C Evaluator test versus the broth microdilution method

Antimicrobial	Mean tubes difference [a]	No. of isolates with a MIC difference [b] of:							% agreement within ±1 $\log_2$ dilution
		<-2	-2	-1	0	+1	+2	>+2	
Tetracycline	2.314	0	0	0	1	2	10	7	15
Ciprofloxacin	-1.446	0	0	8	3	3	6	0	70
Amoxicillin	0.00	0	0	0	20	0	0	0	100
Metronidazole	0.00	0	0	0	20	0	0	0	100
Erythromycin	0.15	2	0	2	12	4	0	0	90

[a] mean difference in $\log_2$ MICs (broth microdilution-M.I.C Evaluator); [b] expressed in $\log_2$ dilutions.

our environment. The findings are in agreement with those of Dekitsch *et al*. (1999) who demonstrated marked susceptibility of clinical isolates of *Enterobacter cloacae* from Vienna to ciprofloxacin and nalidixic acid.

The study compared the BMD and the M.I.C Evaluator methods, seeking the more suitable of the two for application to *Enterobacter cloacae* antimicrobial susceptibility determination (Arroyo *et al*., 2005). For comparison of the MICs, the doubling dilution difference in the MIC was calculated as: $\log_2$ (MIC by BMD) -$\log_2$ (MIC by E-test method). Thus, a MIC tube difference of one tube indicates that the BMD MIC was 1 doubling dilution higher, that is, double the E-test MIC. The percentage of isolates with MICs measured by the two methods agreeing to within ±1 and ±2 doubling dilutions was calculated; essential agreement was reported as the percentage of isolates for which the E-test MIC was the same or one doubling dilution apart from the BMD MIC (Reynolds *et al*., 2003).

According to Rennie *et al.* (2012), the United States Food and Drug Administration requires >90% essential and category agreement when two methods are compared. Interestingly, all the 20 strains demonstrated 100% categorical agreement to amoxicillin, metronidazole and erythromycin (table 1), while erythromycin, ciprofloxacin and tetracycline registered 90%, 70% and 15% essential agreement respectively (table 2). Although erythromycin registered 100% categorical agreement, when the doubling dilution difference of the two methods was compared, discrepancies were recorded surmounting to 10% hence the 90% essential agreement. Our findings are in agreement with those of Rennie *et al.* (2012) who reported essential agreements between 40 to 90% when broth microdilution was compared with M.I.C Evaluator against clinical strains of anaerobes and other fastidious bacteria. Similarly, Bilal *et al*. (2007) demonstrated that all β-lactamase negative *H. influenzae* isolates which were resistant to ampicillin registered high MICs on E-test than on BMD, with essential agreement varying from 42-68%. In another study, Arroyo *et al*. (2005) reported 16.5% (19/115 strains) essential agreement of *Acinetobacter baumannii* strains to colistin. Poor diffusion of polymyxins into the agar was associated with the discordance.

Seeking factors that impinge on optimum performance of the E-test against a range of organisms might be daunting; however, other studies reiterated the remarkable and convenient usage of the E-test and M.I.C Evaluator especially in clinical management (Tristram *et al*., 2008; Rennie *et al*., 2012). Differences in antibiotics and microorganisms utilised may enhance some of the differences observed with the use of these methods. We speculate that similar factors could be the possible cause of poor concordance in the present study. In spite the fact that BMD is standardised and recommended method for use, it is prone to human errors through general manipulations of the assay compared to M.I.C Evaluator

which has clear breakpoints scale and easy to manipulate. Therefore, standardisation regarding the use of the M.I.C Evaluator for testing *Enterobacter cloacae* is of importance.

CONCLUSION

The M.I.C Evaluator method could be a dependable, quick and definite alternative method for antimicrobial susceptibility testing. Errors due to manipulation of the BMD method could render it less suitable and might have accounted for the discordance observed. Standardisation and further analysis regarding the suitability of the M.I.C Evaluator for testing *Enterobacter cloacae* is warranted.

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