CRE-AF MEDIA: AN ALTERNATIVE AGAR FOR SCREENING OF CARBAPENEMASES PRODUCING ENTEROBACTERIALES (CPE)

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ABSTRACT

Carbapenemases Producing Enterobacteriales (CPE) is a significant health threat world-wide. The ability to detect the organism early is of utmost importance to ensure proper delivery of treatment as well as implementation of infection control measure to curb further spread of the infection. CRE-AF media was developed to ease and fasten the screening and detection process of CPE pathogens. The goal of this testing was to evaluate the performance of CRE-AF media as a screening tool for CPE organisms. Hence, a total of 96-Gram negative organisms ranging from CPE, non-CPE, Carbapenemases producing non-Enterobacteriales and *Bukholderia pseudomallei* were selectively chosen to determine the ability of the agar to detect carbapenemases producer and to either inhibit or differentiate the growth of non-CPE organisms. The presence of CPE was specified by the growth of yellow colonies with haze formation around them after 24 hours of incubation. Our finding indicated that CRE-AF was a suitable screening tool for the detection of CPE. The agar was reported to exhibit 92% sensitivity and 93.4% specificity and hence, can be used by laboratory for early detection of CPE.

KEYWORDS: Carbapenemases Producing Enterobacteriales, gram-negative bacteria, screening, laboratory detection

INTRODUCTION

The emergence of Carbapenemases producing Enterobacteriales (CPE) has become a global health threat imposing a significant impact on health care setting. Since its first report 17 years ago, the problem has escalated (Bonomo et al. 2018; Kumarasamy et al. 2010; Naas and Nordmann 1994). In 2013, Centre for Disease Control (CDC) has listed CPE as one of the microorganisms that posed an urgent threat to public health. To date, the prevalence and incidence of CPE have been reported worldwide (European Centre for Disease Prevention and Control 2016; Zhang et al. 2017; Lee et al. 2016; Xu et al. 2015; Uwamino et al. 2017).

The organism has been responsible for many hospital outbreaks and endemic situation, resulting in high rate of morbidity as well as mortality (Sabino et al. 2019). Infection associated with this organism was also linked with prolonged hospital stay as limited treatment option was available for patients. Hence, early detection of the organism is very crucial to ensure the delivery of proper treatment and infection control measure (Nordmann et al. 2012).

Detection of CPE has become an important concern. Various phenotypic and molecular-based techniques that serve as screening and confirmatory methods are available to identify the carbapenemase producers. Screening of CPE organisms was usually performed upon isolation of gram-negative organism from clinical specimens. The process involved identification of the organisms and the use of battery of biochemical tests and sensitivity testing to confirm it. The whole process will require up to 48 to 72 hours. The urgency in the detection of these organisms has produced various methods to enhance and fasten the detection of CPE. These include the commercialisation of various chromogenic agars that selectively allow the growth of CPE, producing colonies with specific colour directly from clinical specimen (Vrioni et al. 2012). In addition, other method such as modified Hodge test (MHT) aimed at detection of carbapenemases enzymes from the culture colonies (Girlich, Poirel, and Nordmann 2012). MHT produces characteristic pattern of tested organism on agar to indicate carbapenemases production. Carba NP, on the other hand, uses bacterial protein extract inoculated in microtube by adopting colorimetric principles which enables detection of carbapenemases hydrolysing process. Molecular method is another available method that is able to identify and confirm CPE. The method examined the genetic entity of bacteria in particularly targeting carbapenem resistant gene that the respective organism carries (Nordmann, Poirel, and Dortet 2012). On average, these methods require an additional of 4 to 24 hours to be performed following isolation of pure isolates from clinical specimen culture. The initial culture of clinical specimen will require an incubation of 24 to 48 hours.

CRE-AF media was developed to ease and shorten the screening process for CPE detection. By using meropenem as its inhibitory component, it would selectively allow only meropenem resistant Enterobacteriales to grow and inhibit the strains that were sensitive to the drug. Like other chrome agars, it would be able to produce presumptive result following 18 to 24 hours of incubation. CPE isolates were indicated by the presence of yellow colonies with surrounding yellowish haze. The growth of gram-positive organisms was inhibited by the presence of crystal violet in the agar. Other organism that was resistant to carbapenem drugs would be able to grow, resulting in purple colonies.

MATERIALS AND METHODS

A total of 96 gram negative isolates were subjected for testing, consisting of CPE resistant to carbapenem drugs (n=50), harbouring various resistance genes as listed in Table 2, Non-CPE strains that exhibited decrease of susceptibility to carbapenems as a consequence of non–carbapenemase-based mechanisms (n=14)(Table 3), strains that were fully susceptible to carbapenems (n=10)(Table 4), multidrug resistant (MDR) *Pseudomonas aeruginosa* (n=10), MDR *Acinetobacter baumannii* (n=7) and *Bukholderia pseudomallei* (n=5) (Table 5). Overnight cultures of quality control ATCC organisms as listed in Table 1, were also included for the study. The plates were incubated at 35 ºC for 18 to 24 hours duration and the colony morphology of the isolates produced was closely inspected by three independent observers. For those producing insufficient growth, the agar plates were further incubated for another 24 hours and the growth morphology were re-examined.

All organisms were identified using

Table 1: List of quality control organisms, MIC reading and culture finding on CRE-AF media

No	Organism	MIC Value (µg/ml)	Expected finding for CRE-AF media	Actual finding for CRE-AF media
1	*E. coli* ATCC 25922	Imipenem: 0.15 Meropenem:0.015	No growth	No growth
2	*K.pneumoniae* ATCC 700603	Imipenem: 0.08	No growth	No growth
3	*Pseudomonas aeruginosa* ATCC 27853	Imipenem: 2.0	No growth	No growth

Table 2: Carbapenemase Producing Enterobacteriales: phenotype, MIC range and CRE-AF finding

No	Species	Phenotype/ Genotype	No	MIC Range, mg/L			CRE-AF-Media Result : Growth Of Yellow With Yellow Haze
				Imipenem	Ertapenem	Meropenem	
1	*Klebsiella pneumoniae*	NDM	11	8->32	>32	8->32	11/11
2	*E. coli*	NDM	4	>32	>32	>32	4/4
3	*Cirobacter freundii*	NDM	1	>32	>32	>32	1/1
4	*Enterobacter gergoviae*	NDM	1	>32	>32	>32	1/1
5	*Enterobacter cloacae*	NDM	1	>32	>32	>32	1/1
6	*Proteus spp*	NDM	1	>32	>32	>32	1/1
7	*Providencia rettgeri*	NDM	1	>32	>32	>32	1/1
8	*Klebsiella pneumoniae*	Oxa-181	5	>32	>32	>32	4/5 1-purple colony
9	*E.coli*	Oxa-181	1	>32	>32	>32	1/1
10	*Enterobacter cloacae*	Oxa-181	1	>32	>32	>32	1/1
11	*Klebsiella pneumoniae*	Oxa-48	7	8->32	8->32	8->32	6/7 1-no growth
12	*Enterobacter cloacae*	Oxa-48	1	>32	>32	>32	1/1
13	*Providencia rettgeri*	VIM	2	>32	>32	>32	2/2
14	*Klebsiella pneumoniae*	VIM	1	>32	>32	>32	1/1
15	*Citrobacter. freundii*	VIM	3	>32	>32	>32	3/3
16	*Enterobacter cloacae*	VIM	3	>32	>32	>32	3/3
17	*Citrobacter freundii*	KPC	4	>32	>32	>32	2/4 2-no growth
18	*Klebsiella pneumoniae*	KPC	2	>32	>32	>32	2

Table 3: Non-CPE with decreased susceptibility to carbapenems: phenotype, MIC range and CRE-AF finding

No	Species	B-Lactamase	No	MIC Range, mg/L			CRE-AF-Media Result
				Imipenem	Ertapenem	Meropenem	
1	*Klebsiella pneumoniae*	ESBL	5	2	8	1	No growth (5/5)
2	*E.coli*	ESBL	1	1	32	0.5	No growth (1/1)

3	*Enterobacter agglomerans*	AmpC	1	0.5	16	1	No growth (1/1)
4	*Enterobacter cloacae*	AmpC	1	1	8	0.5	No growth (1/1)
5	*Citrobacter koseri*	AmpC	1	2	8	1	No growth (1/1)
6	*Citrobacter freundii*	AmpC	1	0.5	32	2	Purple-(insignificant)
7	*Morganella morganii*	AmpC	2	0.25	16	1	1-no growth 1-purple (insignificant)
8	*Proteus mirabilis*	AmpC	2	0.25	8	0.5	1-no growth 1-purple (insignificant)

Table 4: Non-CPE: phenotype, MIC range and CRE-AF finding

No	Species	B-lactamase	No	MIC Range, mg/L			CRE-AF-Media Result
				Imipenem	Ertapenem	Meropenem	
1	*Klebsiella pneumoniae*	non	8	0.125-0.25	0.5-1	0.125-1	No growth (5/8) Slight growth-yellow colony (3/8)
2	*E. coli*	non	1	0.5	0.5	0.5	No growth (1/1)
3	*Enterobacter aerogenes*	non	1	0.25	0.123	0.125	No growth (1/1)

Table 5: MDR non-Enterobacteriales: phenotype, MIC range and CRE-AF finding

No	Species	B-lactamase	No	MIC Range, mg/L			CRE-AF-Media Result
				Imipenem	Ertapenem	Meropenem	
1	*Pseudomonas aeruginosa*	non	10	8->32	16-32	8->32	Growth-purple colonies
2	*Acinetobacter baumannii*	non	7	8->32	16-32	8->32	Growth-purple colonies
3	*Bukholderia pseudomallei*	non	5	8->32	16-32	8->32	No growth (5/5)

API 20NE (Biomerieux, France) and Vitek2 GN (Biomerieux, France). The sensitivity testing was performed using Kirby-Bauer disk diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines (Laboratory Standards Institute 2021). Resistance to carbapenem drugs were further confirmed by measuring the minimum inhibitory concentration (MIC) using commercial Etest® (AB Biodisk, Solna, Sweden) of the carbapenem antibiotics. Modified Hodge test (Girlich, Poirel, and Nordmann 2012) and modified carbapenem inhibition method were used to screen for carbapenemases producing among isolates that were resistant to carbapenem drugs (14). Combined disc method using carbapenemase inhibitors; phenyl boronic acid and EDTA were also used to detect and differentiate *Klebsiella* producing carbapenemase (KPC) and metallo-β-lactamase (MBL) among the isolates (Omair et al. 2012). After incubation at 37 °C overnight, the diameter of the growth inhibitory zones around the meropenem discs with inhibitor added were compared with that around the plain meropenem discs. Finally, all CPE isolates were subjected to molecular testing using specific primers to confirm the presence of carbapenem resistant genes (Queenan and Bush 2007). In total, the whole

process required 72 to 96 hours to be completed.

The CRE-AF agar was modified from Francis media replacing gentamicin with Meropenem. (Francis et al. 2006). In brief, it consisted of nutrient agar (Oxoid Ltd, Basingstoke, Hampshire, United Kingdom), 20 mL glycerol (BDH Laboratory Supplies, Poole, United Kingdom), 2.5 mL aq. 0.1% crystal violet (Sigma Chemical Co., St. Louis, USA) and 15 mL aq. 0.2% bromocresol purple (BDH) as pH indicator in 500 mL distilled water. The media pH was adjusted to 7.1 and sterilised by autoclaving at 15 lbs pressure 110 ºC for 10 minutes and cooled at 45-50 ºC. Later, meropenem was aseptically added at 1 mg/L, mixed well and poured into sterile petri plates. All the selected isolates were sub cultured on this agar, and incubated at 37 ºC for 24 hours. Following 24 hours of incubation, visible growth of yellow colonies with haze formation around them was considered as positive finding, while an isolate either producing colonies other than yellow colour

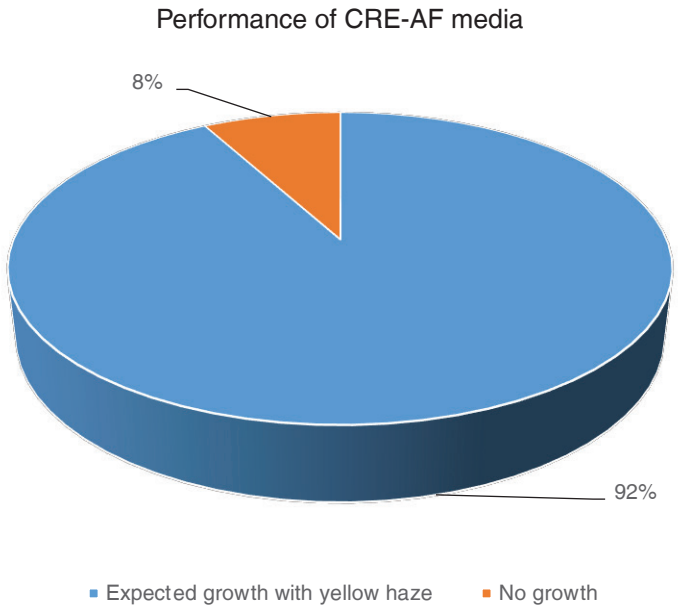

Figure 1: Overall performance of CRE-AF agar in identifying known CPE isolates.

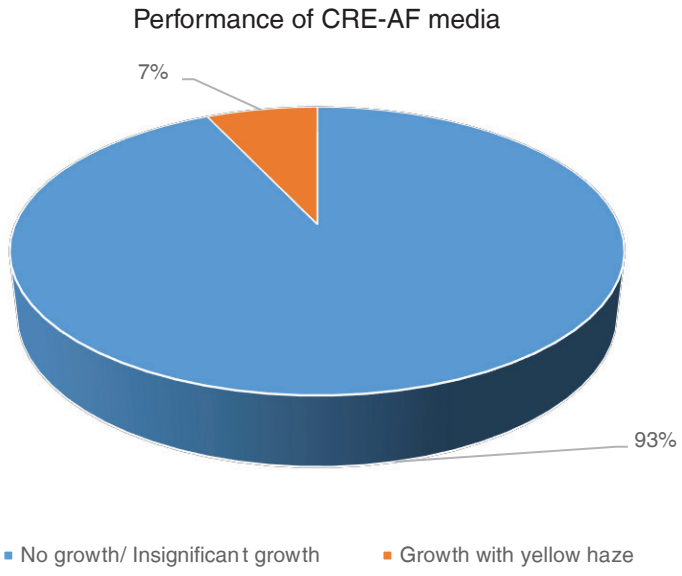

Figure 2: Overall performance of CRE-AF media in identifying non-CPE isolates.

or has not grown on the agar, was considered as negative for carbapenemases production.

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for CRE-AF media in the detection of CPE were calculated. A true positive was defined as growth with phenotypic features compatible with CPE determined by confirmatory testing. A false positive was defined as growth with phenotypic features compatible with CPE and diagnosed as non-CPE by confirmatory testing. The total laboratory processing time from sample sub-culturing to getting a growth on CRE–AF agar was around 48 hours.

RESULTS

Out of 50 CPE tested, 92% (n=46) produced satisfactory results. Four organisms were identified as *Klebsiella pneumoniae, Klebsiella* spp and *Citrobacter freundii* instead did not produce any growth on CRE-AF media. These four organisms were confirmed CPE, harbouring OXA-181, OXA-48 and KPC genes. The CRE-AF media were able to detect NDM and VIM harbouring organism satisfactorily (100%). Results were illustrated in Table 2 and Figure 1.

Three *Klebsiella pneumoniae* (12.5%) isolates that have been previously identified as non-carbapenemases producers and hence were sensitive to carbapenem drugs in contrast produce minimum growth with typical colour on CRE-AF agar, giving rise to false positive results. The others, *Citrobacter feundii* (n=1), *Morganella morganii* (n=1), *Proteus mirabilis* (n=1), although were able to grow on the agar, produced colonies with atypical colour which will enable them to be differentiated with CPE. The rest of the isolates did not produce any growth following 18 to 24 hours of incubation period. Ten isolates with reduced susceptibility to at least one carbapenem antibiotic however were previously confirmed as non-carbapenemases producers, did not produce distinctive growth on CRE-AF media. Results were illustrated in Table 3, Table 4 and Figure 2.

MDR *Pseudomonas aeruginosa* and *Acinetobacter baumannii* selected for the test were able to grow on this agar due to the fact that they were resistant to carbapenem drugs however produced purple-coloured colonies and were able to be distinguished from CPE isolates (Table 5).

Bukholderia pseudomallei isolates selected were sensitive to imipenem hence did not produce any growth on the agar. Similarly, the *Klebsiella pneumoniae* and *E. coli* ATCC isolates tested also did not produce any growth on the agar (Table 5). Based on the above finding, the sensitivity of the CRE-AF media as compared to the PCR method was 92% and specificity was 93.4%. The positive predictive value (PPV) and negative predictive value (NPV) was 93.8% and 91.4%, respectively.

DISCUSSION

Since its first discovery in 2004, there is an increasing report of CPE isolates in the hospital setting. Availability of a simple method is very timely to enable early detection of CPE cases by laboratories. This is particularly important in places where resources are limited and CPE incidence is increasingly reported. Early detection would enable proper treatment being delivered to the patients and ultimately would decrease the mortality rate associated with the infection (Richter and Marchaim 2017).

Early detection of CPE organism is also important to ensure implementation of proper infection control measure to curb the spread of the resistant mechanism (Dik et al. 2016). Due to the fact that it is plasmid-mediated, the spread from one bacterium to other bacterial strains and cross-species is very rapid and effective.

The preparation of CRE-AF agar is considerably easy and indeed very cost effective compared to other screening methods. The estimated cost for each plate was around three Ringgit Malaysia (RM 3). This study indicates that the agar can be used as a secondary subculture medium once purified isolates has been obtained along with the process of identification and sensitivity testing to help fasten the identification of CPE. The agar therefore served as a suitable screening media and may assist in presumptive identification of CPE organisms. In addition, due to the distinctive colour of CPE isolates, it might also be used for screening of CPE carrier using direct inoculation of rectal swab specimen; however further analysis is required for this purpose.

The sensitivity of CRE-AF agar was comparable to the chromID carba agar and was more superior to other screening method discussed in

similar study (Vrioni et al. 2012). Similarly, it also has better performance compared to modified Hodge test (Girlich, Poirel, and Nordmann 2012). The specificity however was lower; hence it was advisable for the personnel's to perform further confirmatory tests as routinely done in their respective laboratories upon isolation of suspected CRE using CRE-AF media.
The performance of CRE-AF agar was slightly inferior compared to Carba NP screening media as the latter detects carbapenem β-lactam ring hydrolysis by CPE bacteria making the detection more definitive and sufficient. CRE-AF media simply allowed the growth of the CPE and inhibited other organisms that were sensitive to meropenem.

With the availability of molecular method in our centre, it enabled the detection of resistant genes harboured by the CRE organisms. Selection of CRE isolates for evaluation of CRE-AF agar was based on the most common genotype reported in our hospital setting. CRE-AF media has successfully detected NDM type as well as VIM metallo betalactamases. NDM beta lactamase was the most prevalent genes among our CPE isolates. Hence this agar proved as a suitable screening media for hospital settings in Malaysia.

CONCLUSION

This study proved that CRE-AF was a suitable agar for screening of CPE and helped in the presumptive identification of CPE. The agar could rapidly identify CPE in laboratory; however additional testing either by phenotypic or genotypic methods were mandatory to confirm the finding. Nevertheless, an infection control measure can be quickly implemented upon presumptive identification result given by CRE-AF media. The limitations of this study were the small sample size selected and lack of suitable clinical specimens to assess its performance in direct sample inoculation. Therefore, a more comprehensive study, using a larger sample size and obtaining suitable clinical specimens with suspect CPE infection was reckoned as important to further evaluate the usefulness of this media. However, we concluded that the agar was indeed a useful alternative tool for detection of CRE organisms. The take home message from this analysis was, CRE-AF media was a cost effective and sensitive method, useful for detection of CPE pathogen and may be used in laboratory testing.

CONFLICT OF INTERESTS

The authors declare that there is not conflict of interest involve in in this study.

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