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Evaluation of Brilliance CRE Agar for the detection of carbapenem-resistant Gram-negative bacteria

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SUMMARY

The aim of this work was to evaluate the performance of the new chromogenic medium Brilliance^{TM} CRE Agar (Thermo Fisher Scientific) for determining the limit of detection of carbapenem-resistant enterobacteria (CRE). A total of 70 clinical isolates were studied. Of these, 30 were well-characterized CRE, including *Klebsiella pneumoniae* strains producing KPC-, VIM-, and OXA-type enzymes, VIM-positive *Enterobacter cloacae* and *Escherichia coli*, NDM-positive *E. coli*, and enterobacterial isolates characterized by porin loss associated with ESBL production or AmpC hyperproduction. Ten carbapenem-resistant non-fermentative isolates were also included as well as 30 carbapenem-susceptible isolates. Carbapenem-resistant strains were inoculated at three different concentrations onto Brilliance CRE Agar (from $1.5x10^1$ CFU/ml up to $1.5x10^4$ CFU/ml) whereas carbapenem-susceptible isolates were inoculated at a concentration of $1.5x10^2$ CFU/ml. The medium sustained the growth of carbapenem-resistant isolates, showing detection limits from $1.5x10^1$ CFU/ml (in 31/40 cases) to $1.5x10^4$ CFU/ml. No growth was observed with carbapenem-sensitive control strains. Our results indicate that the Brilliance CRE Agar allows the growth of carbapenem-resistant isolates with low detection limits and could represent a useful screening medium for both enterobacteria and non-fermentative Gram-negative strains resistant to carbapenems.

KEY WORDS: Surveillance, Antimicrobial resistance, Carbapenemases, Chromogenic medium, Detection limit.

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INTRODUCTION

Infections caused by carbapenem-resistant enterobacteria are an emerging problem worldwide and a serious danger, especially in the case of patients admitted to ICUs (Nordmann *et al.*, 2011). Since the use of carbapenems has become predominant as second- or third-line drugs against multidrug-resistant Gram-negative bacteria, resistance to these antibiotics poses a serious problem in the management of nosocomial infections (Gupta *et al.*, 2011).

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Resistance to carbapenems in Gram-negative bacteria may be caused by different mechanisms, including production of enzymes able to hydrolyze carbapenems (i.e., carbapenemases) and porin loss associated with production of extended-spectrum beta-lactamases (ESBLs) or AmpC hyperproduction (Jacoby *et al.*, 2004). Carbapenemases belong to different classes of beta-lactamases. The Ambler class A includes the recently emerged *Klebsiella pneumoniae* carbapenemases (KPC), mostly reported in *K. pneumoniae* strains, that have been frequently found on mobile genetic elements with the consequent potential to spread widely (Nordmann *et al.*, 2009). Class B enzymes, also named metallo-beta-lactamases (MBLs), include different enzymes such as IMP-, and VIM-type carbapenemases, that historically were widespread in *Pseudomonas aeruginosa* and

Acinetobacter baumannii but more recently have emerged in Enterobacteriaceae (Cornaglia *et al.*, 2011). Finally, class D includes the OXA-type carbapenemases, commonly found in *A. baumannii*, with the OXA-48 enzyme rapidly spreading in *K. pneumoniae* (Poirel *et al.*, 2012).

Due to the rapid spread of these Gram-negative carbapenem-resistant bacteria and the scarcity of treatment options, it is essential to adopt adequate surveillance methods to implement appropriate infection control policies (CDC, 2009b; Stuart *et al.*, 2010). In this regard, the gastrointestinal tract may serve as an important reservoir for enterobacterial strains allowing the transmission and rapid spread of carbapenem-resistant strains, especially in high-risk wards (Gijon *et al.*, 2012). Thus, it appears useful also in clinical practice to detect carbapenem-resistant Gram-negative bacteria from surveillance rectal swabs of colonized patients (Calfee and Jenkins, 2008). In previous years, various protocols based on selective agar media have been developed for preliminary screening allowing different carbapenem-resistant enterobacterial species to be recognized easily (Adler *et al.*, 2011; CDC, 2009a; Gazin *et al.*, 2012; Landman *et al.*, 2005; Lolans *et al.*, 2010). Brilliance^{TM} CRE Agar (Thermo Fisher Scientific) is a new commercially prepared chromogenic solid medium supplemented with a modified carbapenem that inhibits the growth of carbapenem-sensitive bacteria. The aim of this work was to evaluate the performance of this medium to determine the limit of detection of the medium (expressed in CFU/ml). Size and color of growing colonies were also evaluated.

MATERIALS AND METHODS

Bacterial isolates

A total of 70 clinical isolates were studied. Of these, 30 were well-characterized carbapenem-resistant enterobacterial strains from our collection (Table 1), including *K. pneumoniae* producing KPC-type (n=12), VIM-1 (n=3), and OXA-48 (n=2) enzymes, VIM-positive *Enterobacter cloacae* (n=3), *Escherichia coli* producing VIM-1 (n=1) and NDM-1 (n=1) enzymes, and isolates characterized by porin loss associated with ESBL production (*K. pneumoniae*, n=3), or AmpC hyperproduction (*E. coli*, n=2; *Serratia marcescens*, n=2; *E. cloacae*, n=1). In the case of OXA-48-positive *K. pneumoniae*, both isolates co-produced the CTX-M-15 ESBL enzyme. Ten additional carbapenem-resistant non-fermentative isolates were also included in the study: *P. aeruginosa* producing IMP- (n=2), and VIM-type (n=5) enzymes, and *A. baumannii* (n=3) producing OXA-23, -24, and -58 enzymes, respectively. Of note, a VIM-positive isolate of *P. aeruginosa* co-produced the PER-1 ESBL enzyme. All the strains had previously been characterized at the molecular level. The following 30 carbapenem-susceptible Gram-negative isolates were also tested: *E. coli* (n=15), *E. cloacae* (n=3), *K. pneumoniae* (n=4), *S. marcescens* (n=3), and *P. aeruginosa* (n=5).

The study

For each isolate, MICs for ertapenem (ERT), imipenem (IMP), and meropenem (MEM) were determined using the Etest strips (bioMérieux, Marcy l'Etoile, France). Starting from frozen isolates, strains were first inoculated on Columbia blood agar (bioMérieux) to resume their metabolic activity.

After 18 h incubation at 36°C, the colonies were inoculated on MacConkey agar (bioMérieux) with a 10 µg ertapenem disc (Oxoid, Basingstoke, UK) and plates were incubated aerobically at 36°C for 18 h. Then, a 0.5 McFarland suspension of each isolate was prepared in sterile 0.9% saline (corresponding to a cell density of $1.5x10^8$ CFU/ml) followed by further seven 10-fold serial dilutions (from $1.5x10^7$ CFU/ml to $1.5x10^1$ CFU/ml). Finally, 100 µl of each suspension from carbapenem-resistant strains were inoculated at three different concentrations onto Brilliance CRE Agar ($1.5x10^1$ CFU/ml, $1.5x10^2$ CFU/ml, $1.5x10^4$ CFU/ml), whereas 100 µl of each suspension from carbapenem-susceptible isolates were inoculated at the fixed dilution of $1.5x10^2$ CFU/ml. After 24 h incubation at 36°C, the plates were evaluated to verify number, size and color of colonies, and to determine the level of detection.

Overall, results were interpreted as recommended by the manufacturer. Colony size was defined as follows: large colonies (4-5 mm diameter), medium colonies (3 mm diameter), and small colonies (1-2 mm diameter).

The level of detection was determined based on the minimal colony count allowing detection on the screening plate.

TABLE 1 - *Detection sensitivity of Brilliance CRE Agar, MIC values, and resistance mechanism(s) for the 40 carbapenem-resistant strains.*

Species	*Lowest limit of detection (CFU/ml)*	*MIC (mg/L)*			*Resistance mechanism(s)*
		ERT[a]	*IMP*[a]	*MEM*[a]	
K. pneumoniae	$1.5x10^1$	8	8	4	KPC
K. pneumoniae	$1.5x10^1$	8	16	8	KPC
K. pneumoniae	$1.5x10^1$	>32	24	4	KPC
K. pneumoniae	$1.5x10^2$	8	16	8	KPC
K. pneumoniae	$1.5x10^1$	>32	8	16	KPC
K. pneumoniae	$1.5x10^2$	16	8	8	KPC
K. pneumoniae	$1.5x10^1$	>32	>32	>32	KPC
K. pneumoniae	$1.5x10^1$	>32	8	2	KPC
K. pneumoniae	$1.5x10^1$	>32	8	4	KPC
K. pneumoniae	$1.5x10^2$	>32	16	>32	KPC
K. pneumoniae	$1.5x10^1$	>32	2	4	KPC
K. pneumoniae	$1.5x10^1$	>32	1	2	KPC
K. pneumoniae	$1.5x10^1$	>32	>32	>32	VIM-1
K. pneumoniae	$1.5x10^2$	>32	>32	>32	VIM-1
K. pneumoniae	$1.5x10^1$	4	2	1	VIM-1
K. pneumoniae	$1.5x10^1$	8	0.19	1	CTX-M-15 + porin loss
K. pneumoniae	$1.5x10^2$	8	0.19	2	CTX-M-15 + porin loss
K. pneumoniae	$1.5x10^1$	16	0.125	1	CTX-M-15 + porin loss
K. pneumoniae	$1.5x10^2$	16	1	1	CTX-M-15 + OXA-48
K. pneumoniae	$1.5x10^2$	16	0.5	1	CTX-M-15 + OXA-48
E. cloacae	$1.5x10^1$	8	4	2	VIM-1
E. cloacae	$1.5x10^1$	8	16	4	VIM-1
E. cloacae	$1.5x10^1$	8	32	2	VIM-4
E. cloacae	$1.5x10^1$	2	0.25	0.19	AmpC + porin loss
E. coli	$1.5x10^1$	2	4	0.75	VIM-1
E. coli	$1.5x10^1$	2	0.25	0.19	AmpC + porin loss
E. coli	$1.5x10^1$	2	0.25	0.25	AmpC + porin loss
E. coli	$1.5x10^4$	>32	8	16	NDM-1
S. marcescens	$1.5x10^1$	0.5	4	0.125	AmpC + porin loss
S. marcescens	$1.5x10^1$	0.5	2	0.125	AmpC + porin loss
P. aeruginosa	$1.5x10^1$	>32	>32	>32	VIM-1
P. aeruginosa	$1.5x10^1$	>32	>32	>32	VIM-2 + PER-1
P. aeruginosa	$1.5x10^1$	>32	>32	>32	VIM-1
P. aeruginosa	$1.5x10^1$	>32	>32	>32	VIM-1
P. aeruginosa	$1.5x10^1$	>32	>32	>32	VIM-1
P. aeruginosa	$1.5x10^2$	>32	>32	>32	IMP-2
P. aeruginosa	$1.5x10^1$	>32	>32	>32	IMP-13
A. baumannii	$1.5x10^1$	>32	>32	>32	OXA-23
A. baumannii	$1.5x10^1$	>32	>32	>32	OXA-24
A. baumannii	$1.5x10^1$	>32	>32	>32	OXA-58

[a]ERT, Ertapenem; IMP, Imipenem; MEM, Meropenem

RESULTS

Brilliance CRE Agar was consistently able to sustain the growth of carbapenem-resistant isolates, showing a detection limit of $1.5x10^1$ CFU/ml in 31/40 cases (including isolates carrying different resistance determinants). Different resistance mechanisms were also present in isolates growing at $1.5x10^2$ CFU/ml (including the two isolates producing both OXA-48 and CTX-M-15 enzymes). Of note, a detection limit of $1.5x10^4$ CFU/ml was found in the case of the NDM-1-producing *E. coli*. Overall, carbapenem MICs ranged from 0.5 mg/L to >32 mg/L for ertapenem, and

from 0.125 mg/L to >32 mg/L for both imipenem and meropenem. The above data are summarized in Table 1. No growth was observed with carbapenem-sensitive control strains.

Regarding color and size of carbapenem-resistant strains, steel blue colonies of different sizes were obtained in the case of *K. pneumoniae* (Figure 1A), with the majority of them being large (n=13), and the remaining medium (n=5) or small (n=2). Blue colonies were also observed in the case of *E. cloacae* and *S. marcescens* strains, even though most of them were characterized by a sea green color, clearly distinguishable from the steel blue of *K. pneumoniae*.

A pale pink color was observed for *E. coli* (Figure 1B). Notably, *E. coli* strains grew with both medium and large sizes whereas a single type of colonies was observed in the case of *E. cloacae*

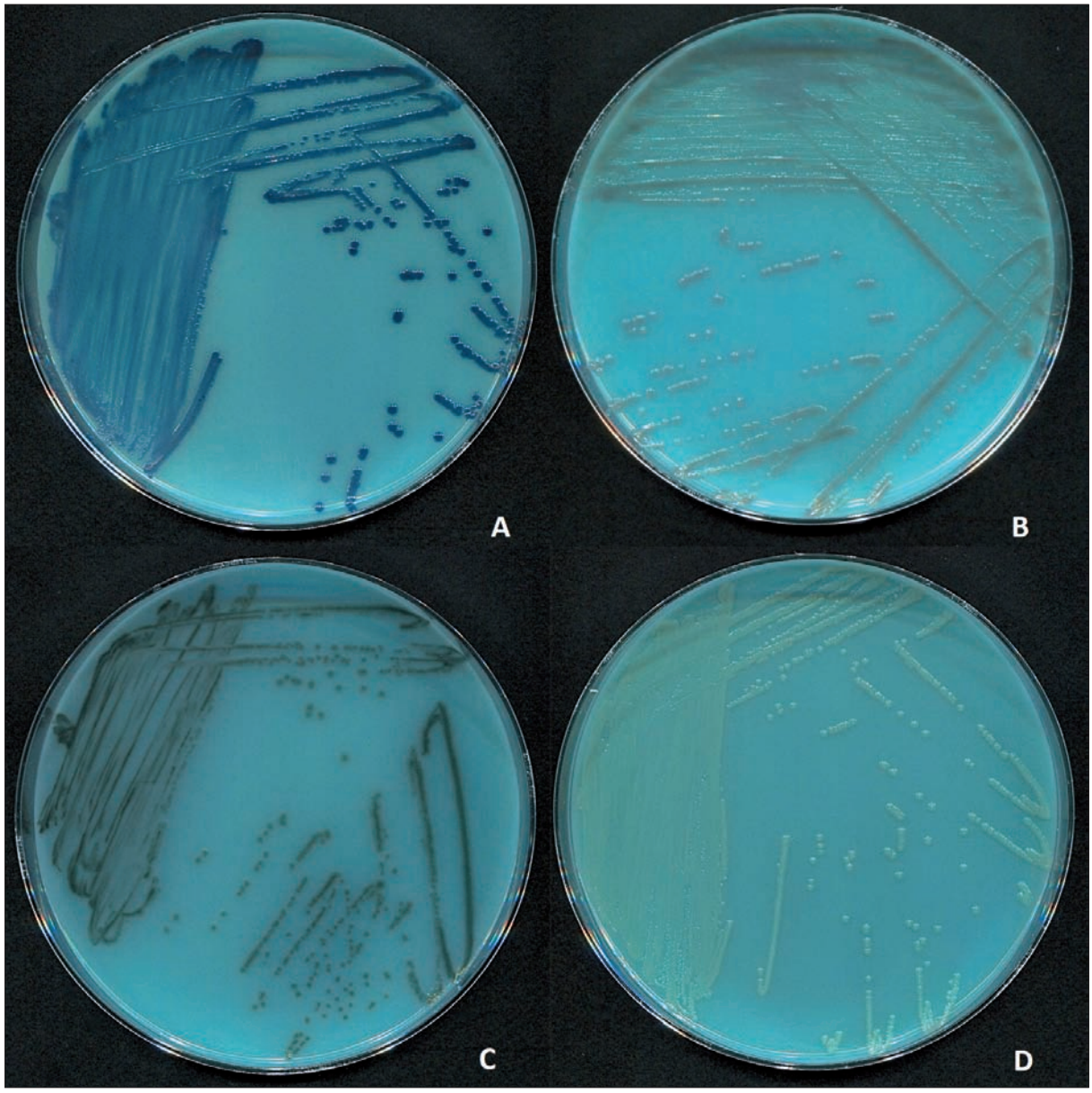

FIGURE 1 - *Features of colonies growing on Brilliance CRE Agar: A: Klebsiella pneumoniae; B: Escherichia coli; C: Pseudomonas aeruginosa; and D: Acinetobacter baumannii.*

and *S. marcescens* (medium and small, respectively).
Concerning non-fermentative Gram-negative strains, *P. aeruginosa* strains showed a light brown color (Figure 1C) and different sizes (large, n=4; medium, n=2; small, n=1), whereas *A. baumannii* consistently grew with small colorless colonies (Figure 1D).

DISCUSSION

Rapid detection of carbapenem-resistant Gram-negative bacteria from surveillance rectal swabs of colonized or infected patients is extremely important in clinical practice to implement adequate infection control policies and to prevent difficult-to-eradicate hospital outbreaks, especially when considering the ability of KPC-producing *K. pneumoniae* to spread quickly in the hospital environment (CDC, 2009b; Nordmann *et al.*, 2009; Migliavacca *et al.*, 2013).
Molecular methods have great potential for further development of rapid diagnostic tests to be utilized on clinical samples to enable direct detection of carbapenemase genes. However, current methods are still not available for daily use in many laboratories (Gazin *et al.*, 2012; Naas *et al.*, 2011). Thus, several culture-based techniques have been developed, including methods that use in-house-prepared selective media such as carbapenem supplemented MacConkey agar or Trypticase soy broth containing a 10 µg carbapenem disk (Adler *et al.*, 2011; Birgy *et al.*, 2012; CDC, 2009a; Lolans *et al.*, 2010), or commercial chromogenic agar media (Carrër *et al.*, 2010; Nordmann *et al.*, 2012; Panagea *et al.*, 2011; Samra *et al.*, 2008; Vrioni *et al.*, 2012; Wilkinson *et al.*, 2012).
We evaluated the performance of the new chromogenic medium Brilliance CRE Agar for determining the lowest limit of detection of both carbapenem-resistant enterobacteria and non-fermentative Gram-negative strains using isolates carrying resistance determinants previously characterized at the molecular level.
In our experience, Brilliance CRE Agar was able to sustain the growth of carbapenem-resistant isolates even when they had very low carbapenem MICs, whereas it did not allow the growth of sensitive control strains. In particular, isolates producing KPC, VIM and IMP enzymes were efficiently identified with detection limits from $1.x10^1$ CFU/ml to $1.5x10^2$ CFU/ml. Similarly, Brilliance CRE Agar was able to sustain the growth of *K. pneumoniae* producing OXA-48 at $1.5x10^2$ CFU/ml level.
In this case, however, the associated production of a CTX-M-15 ESBL enzyme could have played a role since detection of isolates producing OXA-48 alone is regarded as particularly critical (Carrër *et al.*, 2010).
Another crucial point is commonly represented by isolates producing the NDM-1 enzyme since they have usually low carbapenem MICs, so these strains may not be efficiently detected on chromogenic media (Nordmann *et al.*, 2012). In our study, Brilliance CRE Agar was able to detect NDM-1-producing *E. coli* at a detection limit of $1.5x10^4$ CFU/ml. However, the above isolate was characterized by high carbapenem MICs (≥8 mg/L) thus partially reducing the value of this finding.
Different sizes and colors of colonies growing on the Brilliance CRE Agar represent an additional advantage when performing surveillance cultures for screening of carbapenem-resistant isolates. Although the manufacturer's instructions report that strains belonging to the KESC group (*Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia*) grow with the same blue color, we found that colonies of *E. cloacae* and *S. marcescens* showed a sea green color, well distinguishable in most cases from the steel blue of *K. pneumoniae*. Of note, colonies of *E. coli* and *P. aeruginosa* showed different colors (pink pale and light brown, respectively) whereas *A. baumannii* grew with colorless colonies.
Taken together these features appear very useful for screening purposes when considering the emergence and spread of carbapenemase-producing strains including NDM-1-positive *E. coli* and non-fermentative Gram-negative bacteria (e.g., *A. baumannii* strains carrying transferable OXA-type carbapenemases) (Nordmann *et al.*, 2011; Roca *et al.*, 2012).
Overall, our results indicate that Brilliance CRE Agar allows the growth of carbapenem-resistant isolates with low detection limits and could represent a useful screening medium for both enterobacteria and non-fermentative Gram-negative strains resistant to carbapenems.

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