Int.J.Curr.Microbiol.App.Sci (2016) *5*(8): 176-181

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 8 (2016) pp. 176-181**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.508.019

Screening Agars for Detection of *Klebsiella pneumoniae* Carbapenemase - Carbapenem Resistant *Enterobacteriaceae* from Rectal Swabs of ICU Patients

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Keywords

Carbapnem Resistant Enterobacteriaece (CRE), KPC, Carbapenemase, PCR, blaKPC gene, Carbapenem.

Article Info

Accepted:
12 July 2016
Available Online:
10 August 2016

A B S T R A C T

Patients colonized with cabapenem resistant *Enterobacteriaece*(CRE) especially gastrointestinal carriers are source of transmission in healthcare setting . This emphasizes the need for a sensitive screening procedure to identify these micro-organisms to the success of infection control measures. Our aim was to assess different agar-based screening methods for detection of colonization with *Klebsiella Pneumoniae* Carbapenemase (KPC) producing Carbapenem- Resistant *Enterobacteriaece* (CRE) in rectal swabs of geriatric intensive care unit patients. Rectal swabs from 100 patients were collected. All 100 swabs were subcultered on MacConkey agar supplemented with 1µg/ml imipenem (Mac IPM), Mac. agar supplemented with 1µg/ml ertapenem (Mac ETP), and MacConkey agar with 10µg discs of imipenem, meropenem and ertapenem (Mac disks) and detection of bla_{KPC} gene by polymerase chain reaction (PCR). 74% of rectal swabs were positive by PCR. Mac ETP showed the highest sensitivity for detection of KPC producing CRE 71% followed by Mac. Disks 66%, and Mac.IPM 65%. In conclusion the Mac ETP is the most reliable, sensitive, rapid and cost-effective screening media for detection of KPC – producing CRE from rectal swabs for good implementation of infection control measures.

Introduction

The global spread of Carbapenem Resistant *Enterobacteriaceae* (CRE) especially *Klebsiella pneumonia* carbapenemases (KPC) in *Enterobacteriaceae* in the last decade represents a serious public health threat, since it leads to variable levels of carbapenem resistance and few therapeutic options remain available for treating such infections. Moreover, KPC codifying genes are harbored in genetically mobile elements allowing their rapid spread among gram-negative rods (Riberio *et al.*, 2014).

Exposure to health care and antimicrobials are among the most prominent risks for Carbapenem- Resistant *Enterobacteriaece* (CRE) colonization or infection. Other risk factors include poor functional status and

intensive care unit (ICU) stay (Wiener-Well *et al.,* 2010).

Patients colonized with CRE especially gastrointestinal carriers are thought to be a source of transmission in the healthcare setting (Calfee and Jenkins, 2008). This emphasizes the need for a sensitive screening method to identify these micro-organisms to improve the infection control measures. Although Polymerase Chain Reaction (PCR)-based methods have been proven to be highly sensitive and reliable for rapid diagnosis, however, these methods require expertise that is not readily available in many laboratories, costly and unable to detect novel unidentified genes (Nordmann and Poirel, 2013). So, culture-based methods are still essential for the initial detection of these strains, directly from rectal swabs like CHROM agar KPC; MacConkey agar with imipenem; and MacConkey plates with imipenem, meropenem, and ertapenem disks (Adler *et al.,* 2011).

Our aim was to assess different agar-based screening methods for detection of colonization with *Klebsiella Pneumoniae* Carbapenemase (KPC) producing Carbapenem- Resistant *Enterobacteriaece* (CRE) in rectal swabs of geriatric intensive care unit patients.

Patients and Methods

Specimen collection

Rectal swabs from 100 patients (3 swabs from each patient) were collected from Geriatric Intensive Care Unit at Ain Shams University Hospitals, Cairo , Egypt, over the period from April to September 2014. The ages of the patients were ranged from 23-94 years old (with a mean of 55.4±26) The patients or his/her family were informed about the study and consent form was signed. One swab for real time PCR, stored in 5 ml trypticase soya broth at -20°C, and the other two swabs were used immediately for phenotypic methods.

Specimen processing

The microbiological work and PCR were carried out at the Central Microbiology Laboratory, Ain Shams University Hospitals. The swabs from 100 patients were subjected to:

Culture on different screening agars for KPC producing CRE [MacConkey agar supplemented with 1µg/ml ertapenem (Mac ETP), MacConkey agar supplemented with 1µg/ml imipenem (Mac IPM), and MacConkey agar directly streaked completely in two perpendicular directions and a third diagonal one, then 10µg antibiotic discs of imipenem ,meropenem and ertapenem (Mac disks) were placed at 12, 4, 8 O'clock for detection of Carbapenemase production.

Real time PCR for detection of bla_{kpc} gene as a reference method.

MacConkey agar supplemented with 1µg/ml ertapenem (Mac ETP) and MacConkey agar supplemented with 1µg/ml imipenem (Mac IPM)

Each rectal swab was subcultued on Mac ETP and Mac IPM. Growth of colonies on the agar plates suspected the presence of carbapenemase producing organism.

MacConkey agar with 10µg antibiotic discs of imipenem, meropenem and ertapenem (Mac disks).

Presence of growth within 21mm diameter of the inhibition zone of at least one carbapenem disk suspected the presence of carbapenemase producing organism (Adler *et al.,* 2011).

Real time PCR

Polymerase chain reaction was used for detection of bla_{kpc} gene using PCR instrument: (Stratagene Mx3000P). Bacterial DNA was extracted using DNA extraction kit (Thermo Scientific, EU Lithuania). The reaction mixture included Maxima® SYBR Green qPCR Master Mix (2X), (Thermo Scientific, EU Lithuania), Oligonucleotide primers; KPC forward (5'- GGC CGC CGT GCA ATA C-3') and KPC reverse (5'- GCC GCC CAA CTC CTT CA -3') (Sigma, Germany) in a final volume of 25µL. *E.coli* ATCC 25922 and *bla*KPC carrying *K. pneumoniae* ATCC BAA-1705 were used as negative and positive controls, respectively.

The amplification was done as described by manufacture instructions. The thermal cycler was programmed with initial denaturation step at 95°C for 10 min and 40 cycles for amplifications consisting of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension for 30 seconds. A positive result is considered when a cycle threshold (Ct) of 40 or less and a negative result for a Ct of more than 40. The amplification program was followed immediately by a melting program (to detect primer dimer) consisting of 1 minute at 95°C, 30 sec at 55°C then again to 95°C for 30 sec.

Statistical analysis

Data was presented and suitable analysis was done according to the type of data obtained for each parameter.

Descriptive statistics: Frequency and percentage of non numerical data.

Analytical statistics

Mc nemar test: was used to examine the difference between two investigational methods.

Cochrane test: was used to examine the difference between more than two investigational methods.

The diagnostic test evaluation; sensitivity, specificity, the positive and negative predictive values of different tests were calculated for determining the diagnostic validity of the test.

P- value: level of significance; $P>0.05$: Non significant (NS), $P< 0.05$: Significant (S), $P<0.01$: Highly significant (HS). All the analyses were performed with commercially available software (SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, USA, 2001).

Results and Discussion

Performance of screening agar plates in recovery of CRE from rectal swabs

Rectal swabs were collected from 100 ICU patients. CRE were detected in 71% of rectal swabs by Mac.ETP, 66% by Mac.disks, and 65% by Mac.IPM and 62% of rectal swabs were positive for CRE by all media. bla_{KPC} was detected in 74% of rectal swabs and KPC producing CRE were detected in 57% of rectal swabs by all media.

This increase in the percentage detected by PCR might be explained by the fact that, the gene could be present but not expressed phenotypically or PCR detected non-viable bla_{KPC}-positive isolates present in any specimen, thus decreasing the sensitivity of direct plating methods. The diagnostic performances of the different screening agar plates for detection of KPC producing CRE are shown in (table 1).

MacConkey agar supplemented with 1µg/ml ertapenem (Mac ETP)

Mac.ETP detected 64/74 of positive PCR with the highest sensitivity (86.5%) in detection of KPC producing CRE compared to PCR. The false positive results may be due to the presence of other types of carbapenemases rather than KPC, or the presence of other carbapenem resistance mechanisms such as decreased outer membrane permeability or over expression of efflux pumps associated with hyperproduction of AmpC β-lactamases and extended spectrum β lactamases (ESBLs) (Nordmann *et al*., 2009), or the presence of other KPC variants that were not detected by the primers used in our study (Al Sehlawi *et al*, 2013).

MacConkey agar supplemented with 1µg/ml imipenem (Mac IPM) and MacConkey disks (Mac. Disks)

Both Mac.IPM and Mac.Disks detected 58/74 and 59/74 respectively of the positive PCR swabs with a sensitivity of (78.4% and 79.7%) respectively. This may be due to the low sensitivity of IPM antibiotic for detection of KPC producing CRE in both tests. Among the positive results by Mac.Disks, ETP disk showed the highest sensitivity (79.7%) as it detected KPC producing CRE in (59/74) + ve PCR swabs, followed by MEM disk which detected it in 53/74 (71.6%) PCR +ve swabs. While, imipenem disk had the least sensitivity in detection of KPC producing CRE, as it detected it in 50/74 (67.6%) PCR +ve swabs. Although, ETP disk supposed to improve the sensitivity of the test, however, the sensitivity doesn't improved significantly. This might be due to the use of non-standardized inhibition zone diameter (≤ 21 mm) for all antibiotic that might render resistant isolates to fall in the sensitive zone. Also, the inoculums concentration in rectal swabs is not known.

On the other hand, Blackburn *et al*., (2013) who used Mac.Disks method using (ertapenem, imipenem and meropenem disks) on 39 simulated stool specimens showed that MEM disk had the best sensitivity (88.7%) followed by ertapenem disk (86.2%), while, imipenem disk had the least sensitivity of (85.0%). Although, they established the optimal zone diameters for carbapenem resistant gram negative rods (CRGNR) ≤ 24mm for ETP, ≤ 34mm for MEM, ≤ 32mm for IPM, by using diversity of the carbapenemase enzymes produced by selected strains, using standardized inocula, and a wide range of carbapenem MICs., however, they had some limitations; First, the species selected for their study may not correspond to those that would be isolated from clinical specimens. Second, they used standardized dilutions of collection strains (10^1, 10^2, 10^3, and 10^4CFU/ml) and the correlation with real specimen inoculum concentrations in patient stools or rectal swabs remained unknown.

Comparable to our results, Lolans *et al*., (2010) compared the detection of KPC-producing lactose fermenting colonies in 149 surveillance rectal swabs by 2 methods in relation to PCR from the recovered isolates. One of these two methods was direct streaking of the swabs on MacConkey agar plate by the quadrant technique, then, one 10µg ertapenem disk was placed at the junction of quadrants 1 and 2, a second was placed at the junction of quadrants 3and 4. They established a zone diameter of inhibition of ≤ 27 mm as a discriminating cutoff between positive and negative KPC producing isolates. The sensitivity and specificity in relation to PCR were 97.0% and 90.5% respectively for detection of KPC producing CRE.

Table.1 Diagnostic performances of screening agar plates compared to PCR in detection of KPC producing CRE

Method*	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Mac.ETP	86.5%	73.1%	90,1%	65.5%
Mac.disks	79.7%	73.1%	89.3%	55.8%
Mac.IPM	78.4%	73.1%	89.2%	54.3%

* Mac.ETP, Macconkey ertapenem; Mac.disks, Macconkey disks; Mac.IPM, Macconkey imipenem

While, Adler *et al*., (2011) evaluated the performance of Mac.IPM at 1 µg/ml as the main screening agar plate for the detection of CRE from 139 rectal swabs and compared this method to two other culture-based methods, namely, CHROMagar-KPC (Chrom) and Mac.Disks (ETP, IPM and MEM). They found that Mac.IPM was the most sensitive and specific method for detection of CRE carriage as it showed a sensitivity and specificity of 84.9% & 94.3% respectively based on PCR for detection of bla_{KPC}. While, Mac.Disks showed the least sensitivity of 75.8% with a specificity of 89.6% using the cutoff inhibition zone diameter of ($\leq$ 21 mm).

In our study, there was no significant difference ($p > 0.05$) between different tests as regard their sensitivity compared to PCR. However, Mac.ETP showed significant difference in its sensitivity with Mac.IPM ($P < 0.05$) compared to PCR in detection of KPC producing CRE. This can be explained by the fact that ertapenem is more sensitive than imipenem in detection of KPC producing CRE (Nordmann *et al*., 2012).

Also, there was no significant difference between different tests as regard their specificity with PCR. All tests had a specificity of (73.1%).

In Conclusion, the MacConkey agar supplemented with 1 µg/ml ertapenem is the most reliable, sensitive, simple, rapid and cost-effective screening culture media for detection of *Klebsiella Pneumoniae* Carbapenemase (KPC) – producing Carbapenem- Resistant *Enterobacteriaceae* (CRE) from rectal swabs of colonized patients, particularly in poor resource laboratories where PCR technique is expensive or not available. The MacConkey agar supplemented with imipenem is the least sensitive screening culture media for detection of KPC producing CRE. In MacConkey agar with carbapenem disks, ertapenem disk has a higher sensitivity in detection of KPC producing CRE than imipenem and meropenem. PCR is a rapid sensitive method for detection of bla_{KPC} gene, however, it can not detect the newly emerging carbapenemase genes. So we recommend the use of MacConkey agar supplemented with 1ug/ml ertapenem as a reliable, simple and cost effective method for screening of GIT colonization with CRE. Standardizing the cut off of the inhibition zone around different carbapenem disks when using MacConkey agar with carbapenem disks for detection of KPC producing CRE.

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How to cite this article:

Hala B. Othman, Dalia H. Abd El Hamid, Hoda Ezz El –arab Abdul-wahab, Enjy Alaa Eldin and Walaa W. Aly. 2016. Screening Agars for Detection of *Klebsiella pneumoniae* Carbapenemase -Carbapenem Resistant *Enterobacteriaceae* from Rectal Swabs of ICU Patients. *Int.J.Curr.Microbiol.App.Sci.* 5(8): 176-181.
doi: http://dx.doi.org/10.20546/ijcmas.2016.508.019