ORIGINAL ARTICLE

Diagnostic Use of PCR in Carbapenamase-producing *Enterobacteriaceae* (CPE): An Improved Method to Overcome the presence of inhibitors for DNA Extraction from Blood Cultures

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ABSTRACT

Introduction: Carpanenamase-producing *Enterobacteriaceae* (CPE) has emerged as a threat to hospitalized patients. Phenotypic test such as Modified hodge test was less sensitive and specific especially to detect *bla*NDM-1 which is the most predominant genotype in this region. Nucleic acid amplification technology offers improved specificity and sensitivity. Failed amplification due to the presence of inhibitors is a limitation. In this study, we tried to use previous method described by Villumseen et al with some modification using another DNA extraction kit. **Methods:** Ten mls of sterile whole blood taken from nearly expired blood bag from blood bank was spiked with 200 µl of 0.5mcFarland bacterial suspension from thirty-six confirmed isolates of bla_{NDM-1} carbapenamase-producing *Klebsiella pneumoniae* in an aerobic Bactec Plus and incubated until the growth was detected. The blood specimen was subjected to DNA extraction method using Macherey-Nachel, Nucleospin® Blood QuickPure followed with multiplex PCR. **Results:** Out of the 36 isolates, 12 isolates revealed bla_{NDM-1} , 9 isolates revealed bla_{NDM-1} and bla_{OXA-48}, 7 isolates revealed bla_{NDM-1}, bla_{VIM} and bla_{KPC} genotypes that were amplified at cycle threshold of less than 30. Another 8 isolates could not pick up any genotypes possibly due to pipetting error as all the internal control were amplified. Eight true negative gram negative isolates underwent same procedure and none amplified at a cycle threshold less than 30. **Conclusion:** This modified method was proved to give a high yield of CPE genotypes with the cycle threshold was set at less than or equal to 30 and able to overcome the presence of PCR inhibitors.

Keywords: Carbapenamase-producing *Enterobacteriaceae*, PCR inhibitors, Sensitivity, Specificity

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INTRODUCTION

Carbapenem has become a drug of choice for significant infection caused by extended spectrum beta-lactamases (ESBL) *Enterobacteriaceae*. This phenomena has results in the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) as a formidable threat to public health (1). However, limited studies showed that direct PCR can shorten time of detection of CRE compared to culture(2). Direct PCR was proved useful in outbreak settings and high prevalence areas where rapid carbapenamase detection is critical and important for infection control management (3).

In a hospital, blood cultures (BCs) are one of example of sterile specimen usually collected from septic patients. The BCs were send before the antibiotic is given to the patient. BCs act as a close system, therefore contamination is very unlikely. However, the blood culture bottles contain inhibitors of the PCR such as sodiumpolyanetholesulfonate (SPS).

SPS serves as an anti-coagulant in the blood culture bottle. It has become an inhibitor of the PCR as it tends to copurify with the DNA(4). Therefore, there are several studies done to prevent PCR inhibition. Several additional steps is required to overcome the inhibitors because the sensitivity of the assay to yield the CRE genotypes is highly dependent on the DNA-recovery. The aim of this study is to evaluate the Nucleospin DNA extraction kit for recovery of DNA for multiplex qPCR of five carbapenamases gene. Our study is very unique as we are able to use the previous method described by Villumsen et al and apply it with Nucleospin DNA extraction kit with method's modification which is much easier and cheaper . Surprisingly the PCR was not

inhibited and we are able to yield the CRE genotypes implicated in the isolates tested.

MATERIALS AND METHODS

Ten mls of sterile whole blood taken from nearly expired blood bag from blood bank was spiked with 200µl 0.5mcFarland bacterial suspension from thirty-six confirmed isolates of bla_{NDM-1} carbapenamase-producing *Klebsiella pneumoniae* in an aerobic Bactec Plus. These isolates were confirmed phenotypically for the presence of carbapenamases by Modified hodge test and genotypically by using multiplex qPCR by using commercially available LightMix Modular IMP, KPC, OXA-48, VIM and NDM-1 Carbapenamase multiplex kit (by Tib Molbiol, Germany; licensed for local distribution under Roche) . After the blood culture bottle gave the 'beep' sound (flagged positive), 100 µl of the positive blood culture specimen was then used for DNA extraction method using Macherey-Nachel, Nucleospin® Blood QuickPure.

DNA extraction methods

Method 5 (M5) DNA extraction method using DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) that were used for the confirmed isolates were used in this study with some modification done (5).

Modified DNA extraction method using Nucleospin® Blood QuickPure (Macherey-Nachel, Düren, Germany)

Pre-treatment with guanidine hydrochloride and lysis or digestion phase using proteinase K

A 100 µl aliquot of the positive blood culture specimen was mixed with 100 µl lysis buffer [5 M UltraPureTM guanidine hydrochloride] (Invitrogen, CA) in 100 mM UltraPureTM Tris-Hcl (Ph 8.0; Invitrogen, Paisley, UK) and 10 µl proteinase K (20 mg/ml; Qiagen). It was incubated for 10 minutes at room temperature.

Separation phase using 99% benzyl alcohol

A total of 800 ml 99% benzyl alcohol (Reagent Plus H, Sigma-Aldrich, Brondy, Denmark) was added to 600 ml of ultrapure water (Invitrogen) and mixed. In order to separate the phases, the sample was then centrifuged at 20,000 x g for 5 minutes at room temperature.

DNA extraction phase

The 200 µl supernatant (the aqueous phase) from separation phase was then transferred to a new tube. In the procedure stated for this kit, we need to add 25 µl Proteinase K for lysis step. However, Proteinase K was not added as mentioned in the kit protocol as it was added initially in the lysis or digestion phase. The 200 µl lysis buffer BQ1 was added to the supernatant. It was mixed vigorously using vortex to get a higher yield and purity of the DNA. It was incubated at 70°C for 15 minutes and became brownish. Then, 200 µl ethanol (96-100%) was added and vortexed again. Load the lysate into Nucleospin® Blood QuickPure Columns that was placed onto a collection tube and centrifuged for 1 minute at 11,000 x g. The collection tube and flow-through was discarded. Then, the Nucleospin® Blood QuickPure Columns was placed into a new collection tube (2 ml) and 350 µl Buffer BQ2 was added. The column was centrifuged for 3 minutes at 11,000 x g. The collection tube and flow-through was discarded. Lastly, the Nucleospin® Blood QuickPure Columns was placed in a 1.5 ml microcentrifuge tube and 50 µl prewarmed Buffer BE at 70°C was added. It was incubated at room temperature for 1 minute, followed by 1 minute centrifugation at 11,000 x g. The final elution step was done in 50 µl buffer AE.

Multiplex PCR

Multiplex real-time PCR amplification for the simultaneous detection of NDM-1, VIM, IMP, OXA-48 and KPC genes are done using commercially available LightMix Modular IMP, KPC, OXA-48, VIM and NDM-1 Carbapenamase multiplex kit (by Tib Molbiol, Germany; licensed for local distribution under Roche) has been carried out on a CFX-96 Touch Bio-Rad Real-Time PCR machine following the kit protocol.

Each carbapenamase gene will be detected according to target wavelength as described in the kit by the manufacturer.

Data analysis

Sensitivity : A/ (A + C) x 100
Specificity : D/ (D + B) x 100
A = True positive
B = False positive
C = False negative
D = True negative

Ethical considerations

Ethical approval was obtained from the USM Research and Ethics Committee before this study was commenced (Reference number: USM/JEPeM/ 16120537).

RESULTS

The results for this experiment are shown in Table I. Out of the 36 isolates, 12 isolates revealed bla_{NDM-1}, 9 isolates revealed bla_{NDM-1} and bla_{OXA-48} and 7 isolates revealed bla_{NDM-1}, bla_{VIM} and bla_{KPC} that were amplified at cycle threshold less than 30. Another 8 isolates could not pick up any genotypes possibly due to error during the PCR procedure as all the internal control were amplified.

Another 8 random isolates consist of 4 *K.pneumoniae* (sensitive strain), 2 *E.coli* (sensitive strain), 1 *Acinetobacter* spp (sensitive strain) and 1 *P.aeruginosa* (sensitive strain) were also spiked with 10 mls of sterile whole blood and later subjected to DNA extraction and multiplex PCR. Only four out of eight isolates amplified at a cycle threshold more than 30.

Table I: The effectiveness of DNA extraction methods, M1 and M2 in recovering CRE genotypes in carbapenamase-producing *Klebsiella pneumoniae*

Isolates	DNA extraction method						Internal control (IC) amplification	Non-template control (NTC) amplification
	M1 (colony)			M2 (spiked blood)				
	NDM-1	NDM-1 + OXA-48	NDM-1 + VIM + KPC	NDM-1	NDM-1 + OXA-48	NDM-1 + VIM + KPC		
A1	+					+	+	-
A2	+					+	+	-
A3	+					+	+	-
A4	+					+	+	-
A5	+					+	+	-
A6	+					+	+	-
A7	+					+	+	-
A8	+			+			+	-
A9	+				+		+	-
A10	+			+			+	-
A11	+			+			+	-
A12		+			+		+	-
A13	+			+			+	-
A14	+			+			+	-
A15	+			+			+	-
A16	+				+		+	-
A17	+				+		+	-
A18	+			+			+	-
A19	+			+			+	-
A20	+			+			+	-
A21	+			+			+	-
A22	+				+		+	-
A23		+		+			+	-
A24		+		+			+	-
A25	+				+		+	-
A26	+				+		+	-
A27	+				+		+	-
A28	+				+		+	-
A29	+						+	-
A30	+						+	-
A31	+						+	-
A32	+						+	-
A33	+						+	-
A34	+						+	-
A35	+						+	-
A36	+						+	-

M1: Qiagen, DNeasy® Blood & Tissue Kit
M2: Macherey-Nachel, Nucleospin® Blood QuickPure

The internal control were not inhibited in all fourty-four isolates.
Calculation of sensitivity : 28 / (28 + 8) x 100 % = 77.8%
Calculation of specificity: 8 / (8 + 0) x 100% = 100%

According to our result, the sensitivity of the improved method to identify the CPE genotypes was 77.8% and the specificity was 100%. We are able to yield bla_{KPC} genotypes of CPE using this modified DNA extraction method.

DISCUSSION

The first person that effectively solve the problems of inhibitors from blood cultures (BCs) were Fredrick and Relman in which they identified anti-coagulant sodium polyanetholesulfonate (SPS) presence in BCs. (4) This study has its unique feature as we are able to use the method previously described (5) and mixed it with a manufacturer protocol of Nucleospin® Blood QuickPure for DNA extraction from blood culture bottle. To our knowledge, at the moment, there are only two studies found so far that described methods to improve amplification of microbial DNA from blood culture bottle (4, 5).

The M1 column in Table I showed all PCR samples were successful with no inhibition using the DNA extraction method by Qiagen kit but were unsuccessful for isolates A29 up to isolates A36 in the M2 column possibly due to pipetting error that happened during handling the A29-A36 that leads to the absence of the target DNA as the internal control and the positive controls were amplified.

These findings showed 77.8% sensitivity for detection of CPE genotypes from blood culture bottle. However, due to budget constraint we just able to do DNA extraction and multiplex PCR to only eight true negative isolates. Thus, the specificity was 100% as all the isolates was not amplified in less than 30 cycle threshold.

We found out that the modification in the DNA extraction method by pre-treatment with guanidine hydrochloride and addition of early incubation with Proteinase K, increasing the centrifugation speed up to

20000 x g as well as adding extra water in the separation phase has solved the carry-over of inhibitors to the final DNA preparation using the extraction kit by Macherey-Nachel, Nucleospin® Blood QuickPure.

We are able to discover *bla*$_{VIM}$ and *bla*$_{KPC}$ genotypes by using this DNA extraction method in our centre that haven't been found before possibly due to different season of specimen collection.

Currently, the diagnosis of CPE relies on phenotypic method (modified-hodge test) and Carba NP. But with these findings, it suggests that most cases of severe CPE can be diagnosed early with a much cheaper cost on DNA extraction followed by PCR.

However, there are some limitations in this study such as inadequate samplings and inability to evaluate the two DNA extraction kits as stated in Table 1. This study cannot be used as an agreement study as the DNA extraction methods are different and was done to different types of samples which are colony and blood culture broth. We cannot compare the methods by giving the amount of DNA in ng/µl as previously done in one study by Oliveira et al (6) as the value of DNA for this modified method was undetectable possibly due to the amount of DNA obtained after using this modified method was in picogram per microlitre. As this is only a qualitative study, we cannot compare the DNA copies amplified as this study will only shows the presence or absence of carbapenamases gene.

CONCLUSION

In conclusion, this improved method was able to eliminate the effect of inhibitor from blood culture bottle by pre-treatment with guanidine hydrochloride, incubation with proteinase K before separation phase, using pure benzyl alcohol for separation phase, increase speed of centrifugation before the DNA extraction. This modified method has good sensitivity, specificity with a cheaper cost. However, a repeat DNA extraction on the eight false negative isolates are needed to exclude possibility of pipetting error during loading the DNA. Surprisingly, we are able to discover the presence of *bla*$_{KPC}$ which is commonest in the western countries with this modified DNA extraction method using the Macherey-Nachel, Nucleospin® Blood QuickPure kit.

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