

The Effectiveness of Yeast Protein Extraction Reagent (Y-Per) in Releasing Genomic DNA of Nosocomial Pathogens for Rapid Detection

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Abstract: Although, molecular detection has been found to be sensitive and specific but DNA extraction step is still essential, which is known to be time-consuming, costly and a high risk of contamination. Y-PER reagent had previously demonstrated its effectiveness in disruption of some bacterial cells for releasing genomic DNA with an extremely simple and rapid procedure. We therefore want to investigate if this reagent and technique could be applied on six common nosocomial pathogens including *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp. Two extraction methods were performed using boiling and Y-PER reagent technique and the resulting genomic DNA extractions were then subjected to PCR analysis by real-time multiplex PCR assay. DNA templates extracted by boiling method has shown a successful amplification for all of the six bacteria, while Y-PER technique only shows a positive amplification for *Escherichia coli* and *Streptococcus pneumoniae*. Although, Y-PER method has a potential to be rapid and simple molecular diagnostic tool for releasing genomic DNA for PCR analysis, further optimization need to be done for many bacteria in order to fully utilize Y-PER as replacement for boiling method.

Key words: Yeast Protein Extraction Reagent (Y-PER), Nosocomial pathogens, SYBR green real-time PCR, diagnostic, molecular

INTRODUCTION

Hospital is a place that responsible for providing a medical and health care to treat sick people, however, treating with a large number of sick people under the a single roof could easily facilitate the transmission of infectious disease from one patient to another. The existence of these problems has been existed since the very inception of hospitals themselves and it has become increasingly obvious that infections acquired in the hospital become the major clinical threats and lead to the important source of morbidity and mortality to hospitalized patients. The infection that is being acquired from the hospital is term as "Nosocomial infection". According to Lindsay, it is expected that at least 5-8% of patients admitted in the hospital will get these infections and the most likely to get infected are patients in the Intensive Care Unit (ICU).

A large number of organisms are involved in nosocomial infections. Even though viruses, fungi and parasites are one of the sources of this infection, bacterial

agents remain as the most commonly recognized cause of infections in the hospitalized patients (Singh *et al.*, 2006). Although, bacterial agents remain as the common causes of this infection, rapid and sensitive diagnosis is difficult. Although PCR offers a better approach for rapid and sensitive detection of microorganisms but, it still required a DNA extraction step, which is known to be time and cost consuming. By simplifying this step it will reduce detection time, cost and risk of contamination and moreover it may decrease Turn-Around Time (TAT) which is good for patient's management.

Based on previous study that have been done, Y-PER reagent has demonstrated its effectiveness in the disruption of yeast cells, microalgae and some gram-negative (*Escherichia coli*) and gram-positive bacteria (*Enterococcus faecalis*, *Bacillus subtilis* and *Bacillus coagulans*) by simply resuspending the cells in it and vortexing a few seconds at room temperature (Packeise *et al.*, 2013). Based on these findings, we want to examine its effectiveness in lysis of other bacteria (*Escherichia coli*, *Staphylococcus aureus*,

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Table 1: Target genes with the primers pairs and its size (bp) for the specific detection of pathogens in qPCR assay

Pathogens	Gene	Primer sequence	Product size (bp)
<i>Escherichia coli</i>	uidA	F -5'- AGC GAC GTC GGT GTA GTA AC -3' R -5'- ATT TCG CCG ATT TTG CGA CC -3'	277
<i>Staphylococcus aureus</i>	nuc	F - 5'- ATG GAC GTG GCT TAG CGT AT -3' R - 5'- GCG TTG TCT TCG CTC CAA AT -3'	181
<i>Streptococcus pneumoniae</i>	ply	F - 5'- GCC TCT TTT TGT CGC AAG CA -3' R - 5'- CTA GTG CGT AAG CGG ACG AT -3'	108
<i>Klebsiella pneumoniae</i>	16S rRNA	F - 5'- AGT ACG GCC GCA AGG TTA AA -3' R 5'- ACC GCT GGC AAC AAA GGA TA -3'	247
<i>Pseudomonas aeruginosa</i>	lasA	F - 5'- CCG TTC CTC TTC GTC TTG CT -3'	257
<i>Acinetobacter</i> spp.	efp	F - 5'- CGA AGT TCG GTG GGC TTA CT -3' R -5'- CTG TAG TAG CCG CTC CAG TG -3'	133

Streptococcus pneumoniae, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp.) for releasing PCR-quality genomic DNA.

MATERIALS AND METHODS

Experimental

Sample preparation: A total of six common nosocomial pathogens which are *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp. were used in this study (Anbazhagan *et al.*, 2011). The bacterial strains were obtained from the stock culture of Microbiology Laboratory of Faculty of Health Sciences, UiTM Puncak Alam, Selangor. These pathogens were subcultured and grown on the Blood Agar (BA) and Nutrient Agar (NA) which then incubated at 37°C overnight in incubator except for *Streptococcus pneumoniae* which was grown under anaerobic condition (CO₂ incubator).

Genomic DNA extraction

Boiling lysis method: Five hundreds microliter of distilled water was dispensed into six labelled 1.5 mL microtubes. About five bacterial colonies from each agar plates were transferred into each microtube by using a sterile wire loop and mixed to homogenize. All tubes were spun at 10,000 rpm for 1 min and supernatant were discarded. Fifty microliter of distilled water was then added into each tube and the mixtures were resuspended. The mixture was boiled for 10 min in the waterbath with temperature of 95°C. After that, the mixture was immediately placed on ice for 2-5 min. The tubes were spun at 10,000 rpm for 1 min and supernatant (DNA template) was collected for amplification and it were kept at -20°C until further use.

Yeast protein extraction reagent technique: The genomic DNA were extracted from the bacterial cultured by using DNA lysis buffer Y-PER (Cat# 78991, Thermo Scientific) as described by Packeiser *et al.* (2013), About 4-5 colonies of bacteria on the agar plate were picked up by using a sterile wire loop and resuspended in 20 µL of Y-PER

lysis buffer. The mixture was vortexed for 10 sec for lysing the cells. The lysate was then microcentrifuged and the resulting supernatant were collected. Finally, the extracted DNA were diluted with distilled water at a 1:5-1:20 dilution ratio and subjected to PCR analysis or kept for frozen at -20°C until further used.

Primer selection: The primer-specific target genes for each organism were designed by using primer design application in NCBI (National Center for Biotechnology Information) website. The primer pairs and their sizes are shown in Table 1. The designed primers were BLAST (Basic Alignment Search Tool) and analyzed their specificity by using In silico online software (Millan *et al.*, 2013) before they were synthesized by AITbiotech PTE Ltd., Singapore.

SYBR green real-time PCR assay: The SYBR Green Master Mix kit (Qiagen, Germany) was used for the multiplex real-time PCR assay. The reaction was optimized and was carried out in a set of six pathogens that consisted of *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Acinetobacter* sp., *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, which each set of primers comes with a close melting Temperature (T_m) (±2°C). The PCR amplification reactions were performed in a volume of 25 µL, consisting of 12.5 µL SYBR Green PCR Master mix, 0.5 µL of forward primer, 0.5 µL of reverse primer, 10 µL of DNA template and 1.5 µL of RNase-free water. The Real Time Thermal Cycler (BioRad) was programmed for initial denaturation at 95°C for 5 min followed by 40 cycles amplification program consisting of 10 sec at 95°C (denaturation) and 30 sec at 46.7°C (combined annealing and extension). This step was followed by a dissociation stage (40 sec at 95°C) and melting curves analysis from 65-95°C in 10 sec and afterwards cooling to room temperature.

RESULTS AND DISCUSSION

Optimization of bacterial genomic DNA extraction: In order to get an optimum condition for isolation of bacterial

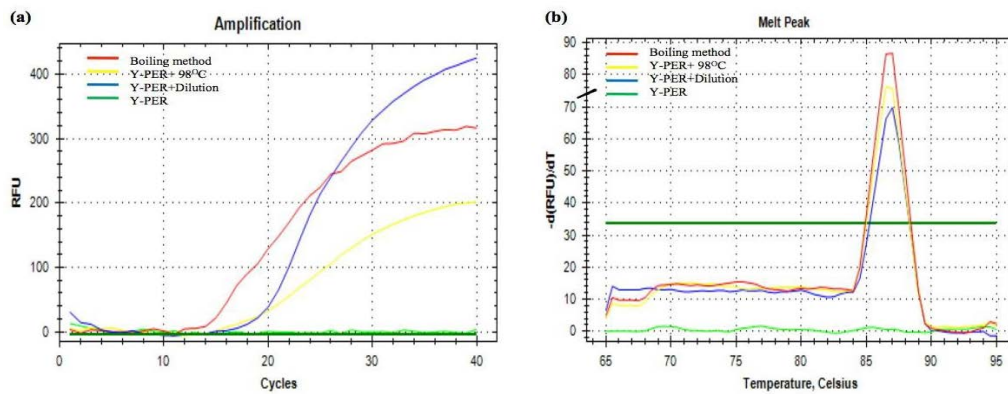


Fig. 1: Amplification curve and melt peak of *Escherichia coli* by different genomic DNA extraction conditions: a) Amplification curve of *Escherichia coli* extracted by conventional boiling method and Y-PER reagent with different conditions. The bacteria were successfully amplified by conventional boiling method, Y-PER with additional boiling at 98°C and Y-PER with 1:10 dilution. However, the flat line shows no amplification occurs at Y-PER without boiling and dilution condition; b) A typical result of melting curve analysis (T_m) of *Escherichia coli* in various extraction conditions. There is only single peak was generated in the melting curve analysis with a melting temperature of 87°C

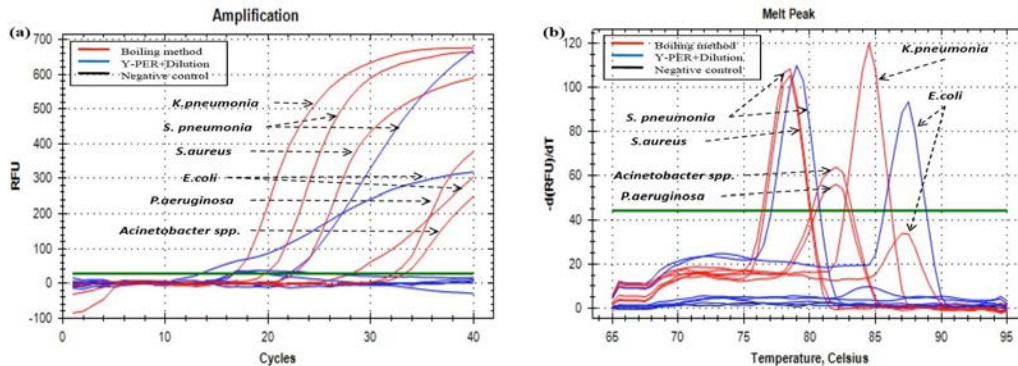


Fig. 2: Amplification and melting curves analysis for the selected genes of six nosocomial pathogens in a multiplex real-time PCR reaction: a) Amplification curve of the six bacteria with two different extraction methods. The amplification plot shows that boiling method has shown a positive amplification curve for all of six bacteria while only two positive amplification could be seen in Y-PER reagent method; b) Melting peak of the six bacteria between two methods of extractions

genomic DNA, various methods of DNA extraction were applied on *Escherichia coli* colony by using Yeast Protein Extraction (Y-PER) reagent and also by a standard boiling method for PCR analysis. Among those different extraction conditions used for the isolation of genomic DNA, three of the conditions which were conventional boiling method, Y-PER with additional boiling at 98°C and Y-PER with further dilution 1:10 ratio have shown successfully amplification without any non-specific amplification product by real-time PCR using BioRad CFX96 real-time PCR system (Fig. 1). Thus, from the results achieved we selected Y-PER with 1:10 dilution method as the optimal method to extract the bacterial DNA together with standard boiling method as our

control extraction method. As the conditions of PCR and primers have been optimized, these bacteria are then subjected to analysed by a real-time multiplex PCR assay. The results were compared between these two extraction methods: standard boiling method and Y-PER reagent technique. The optimum temperature 46.7°C was selected as the annealing temperature in this multiplex reaction. From the test conducted, we can see that the positive amplification curve of all six genes were successfully amplified by a standard boiling method, however for Y-PER reagent method only two target genes which are uidA (*Escherichia coli*) and ply (*Streptococcus pneumoniae*) had shown a positive amplification curves (Fig. 2). As the results shown in this

study, it revealed that Y-PER reagent could not be applied on *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Acinetobacter baumannii* strain; however it is clear that this method is effective on *Escherichia coli* and *Streptococcus pneumonia*. Although, culture technique is a standard method used for bacterial detection and identification, however, in some specific situation especially when diagnosing critically ill patients such as patients in ICU unit more rapid tests are needed

Eventhough, real-time PCR offers a rapid and sensitive detection but, it still requires a DNA extraction step that is known to be time and cost consuming. Due to that reason, recent study has reported on the effectiveness and extremely simple procedure of Yeast Protein Extraction (Y-PER) reagent in releasing genomic DNA of bacteria, yeast and microalgae (Packeiser *et al.*, 2013). However, in their study there are only some bacteria have been tested which are *Escherichia coli*, *Enterococcus faecalis*, *Bacillus subtilis* and *Bacillus coagulans*. Therefore, in our study, we examined the effectiveness of Y-PER reagent in releasing PCR quality genomic DNA of other bacteria, precisely six pathogens that commonly causes nosocomial infections (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp.) and resulting DNA templates are subjected to multiplex real-time PCR assay.

In this study, two methods of DNA extraction were used, that are standard boiling method and Y-PER reagent technique. In order to optimized the best condition for disrupting the bacterial cell, the specificity and sensitivity of the Y-PER technique was first determined by applying different conditions of extraction on the bacterial colony. The control strain that is used in this study was *Escherichia coli* (ATCC 25922) which it had demonstrated its effectiveness on Y-PER reagent. This control strain was extracted in three different conditions that are Y-PER without dilution, Y-PER with 1:10 dilution and Y-PER with boiling at 95°C and the reliability of the test is compared between this technique with the existing boiling method (Queipo *et al.*, 2008). Then, the resulting genomic DNA templates were subjected to real-time PCR analysis.

The results show that the real-time PCR yielded an amplification curve on DNA templates that were extracted by boiling method, Y-PER with 1:10 dilution and Y-PER with additional boiling step. However, there is no amplification curve were observed for the genomic DNA extracted by Y-PER without dilution condition. This result may be explained by the fact that the present of high concentration of detergents or other unknown

components in the Y-PER might inhibit the PCR performance (Packeiser *et al.*, 2013). This effect could be minimized by the dilution of the extracted template where it can reduce the concentration of inhibitors present and simultaneously it may improve the performance of PCR analysis. The results of this study indicate that, the genomic DNA of *Escherichia coli* was successfully extracted by using Y-PER technique with 1:10 dilution which consistent with previous study done by Packeiser *et al.* (2013). Therefore, Y-PER with 1:10 dilution was selected as the optimal method for the bacterial extraction.

Real-time polymerase chain reaction assay is used to detect the presence of bacterial DNA templates that were extracted by boiling and Y-PER techniques. Six common nosocomial pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter* sp.) were tested in this study in order to investigate the effectiveness of Y-PER reagent over a wide range of bacteria. The main concern of this study also wants to detect all of the six bacteria simultaneously in one reaction by a multiplex real-time PCR assay method. By using our designed primers, the target genes (*uidA*, *nuc*, *ply*, 16S rRNA, *lasA* and *efp*) of each bacterium were amplified individually in order to optimize the optimum annealing temperature for a multiplex assay. Annealing temperature has been identified as a crucial parameter during the optimization of PCR protocol. Furthermore, by optimizing the annealing temperature it could reduce a nonspecific amplification (Jeshveen *et al.*, 2012). Based on the optimization, the annealing temperature for the multiplex PCR was found to be 46.7°C

CONCLUSION

From the results obtained, it revealed that all of the six bacteria were successfully amplified with the genomic DNA templates extracted by a standard boiling method, however only *Escherichia coli* and *Streptococcus pneumoniae* showed a positive amplification by using Y-PER technique. Therefore, in accordance with the present results, Y-PER reagent may only be effective on some of bacteria strains and it may not be effective on others (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp.). In conclusion, Y-PER technique is an extremely simple and rapid as compared to boiling method by simply resuspending cells in it and vortexing a few seconds at room temperature. However, there are some limitations in this study due to delayed reagent received and insufficient amount of reagent which it leads to

restricted time for optimizing the condition of PCR as well as the extraction condition, hence, further optimization of extraction condition on each bacteria may required since this study only used the optimum condition tested on *E. coli* which it may be affected by the different properties of bacterial structures, this is because different morphological structure present different physical features, and these features may help each bacteria to cope and adapt to external condition (Young, 2007).

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