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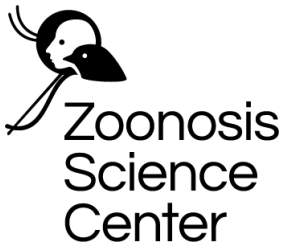

Potential Application of Multiplex Automated Genome Engineering (MAGE) and One-Step Curing Plasmid System for Environmental Cambodian Enterobacterial Isolates

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Abstract

Antimicrobial resistance (AMR) is concerning because it limits antimicrobial drug treatment options. AMR occurs by the overuse and misuse of antimicrobial drugs. In environmental settings, AMR can disseminate from places of high use, which leads to increased exposure to humans and animals. A previous study from our laboratory group showed extended-spectrum cephalosporinase-producing *Escherichia coli/Klebsiella pneumoniae* were isolated from fecal samples obtained in rural Cambodian community settings. Based on these isolates, this study has two aims. The first aim was characterization of selected Cambodian isolates with random amplification polymorphic DNA (RAPD) and antibiotic susceptibility test. From RAPD, the selected six isolates are diverse, except for C61 and C66 bacteria isolates with potential clonality. Additionally, the selected isolates are multidrug resistant (MDR) with reduced susceptibility to beta-lactams and fluoroquinolones. The second aim was to assess two developed methodologies, multiplex automated genome engineering (MAGE) and One-Step Curing Plasmid, by validation in bacteria laboratory strain and development for six Cambodian isolates. To modify AMR genetic elements, MAGE uses pMA7-SacB for homologous recombination with oligos for chromosomal gene disruption. Meanwhile, One-Step Curing Plasmid uses pFREE with the CRISPR/Cas9 system for plasmid and self-curing. Validation showed that MAGE can modify 8% of *E. coli* MG1655 with *lacZ* control screening oligos and almost 90% are cured from pFREE. Selected Cambodian isolates have antibiotic-resistance plasmids of IncR or IncFII replicon. For usage in Cambodian isolates, pFREE was modified to be pCAM-FREE by cloning IncR and IncFII plasmid as gRNA1 and gRNA5, respectively. Sequencing results showed pCAM-FREE have gRNA5. In conclusion, our study managed to characterize selected Cambodian isolates as MDR and diverse. In a laboratory strain, MAGE and One-Step Curing Plasmid are functional methods. Furthermore, pCAM-FREE was constructed to target IncFII and in the future, MAGE and pCAM-FREE could be tested in Cambodian isolates.

Keywords: Antimicrobial-resistance; Cambodian isolates; environmental bacteria; recombineering; plasmid curing; antibiotic susceptibility test; DNA polymorphism

Combating Antimicrobial-resistant Bacteria from Cambodia

Popular Science Summary

Olivia Alexandra

One day, you woke up with a sore throat and decided to go to the doctor. The doctor did some testing and found the bacteria that caused the symptoms. He decided to give you antibiotics and after several days, you are feeling well and healthy again. Imagine, what if the doctor told you that they could not give you any antibiotics since none of them works? Does it sound horrifying?

Well, that is why antimicrobial resistance (AMR) studies are very important. AMR is defined as microorganisms with reduced antibiotic susceptibility. As mentioned before, AMR limits the available antibiotics treatment options. Furthermore, AMR is also present in environmental settings such as wastewater treatment and rivers. Unfortunately, this prevalence will increase dissemination and exposure to humans and animals. In addition, AMR is a complex challenge. There are many factors involved such as the antibiotic resistance genes that could be found in the bacterial chromosome and/or plasmid. Plasmids are extrachromosomal DNA that could be transferred to other bacteria, which enhances AMR dissemination. A previous study in our group found 22% extended spectrum cephalosporinase-producing *Escherichia coli*/*Klebsiella pneumoniae* from 592 fecal samples of humans and livestock in Cambodia. This is concerning because most of the bacterium are multidrug resistant and they are found in rural area of Cambodia community.

Based on the Cambodian isolates, we have two aims in this study. First, characterization of selected Cambodian isolates with DNA polymorphism profile and antibiotic susceptibility. Second, assessment of two developed methodologies: multiplex automated genome engineering (MAGE) and One-Step Curing Plasmid, followed by developing them for a laboratory strain and selected Cambodian isolates. To modify AMR genes, we targeted the bacterial chromosome with MAGE and the plasmid with One-Step Curing Plasmid. One-Step Curing Plasmid uses pFREE plasmid with CRISPR/Cas9 system. CRISPR/Cas9 is an innovative tool for genome editing. In this study, CRISPR/Cas9 is used to eliminate the antibiotic-resistant plasmid while simultaneously eliminating itself (self-curing).

We selected six Cambodian isolates for our study. From the first aim, we found that the selected Cambodian isolates are diverse except for two isolates. In addition, these isolates are multidrug resistant with reduced susceptibility to beta-lactam and fluoroquinolone antibiotics. In our second aim, we assessed the two methodologies mentioned before with validation in *E. coli* laboratory strain. We tested MAGE system by inducing mutation in the chromosome and found that 8% of the bacteria were successfully mutated. Then, we checked the pFREE system for self-curing and amazingly, almost 90% of them were cured. In the next step, pFREE plasmid was modified for usage in Cambodian isolates. The modified pFREE, or pCAM-FREE, sequences were checked and it showed that pCAM-FREE had DNA sequences to target one of the Cambodian plasmid group, the IncFII.

In conclusion, our study managed to characterize selected Cambodian isolates as multidrug resistance and diverse. In a laboratory strain, MAGE and One-Step Curing Plasmid are functional. We have also constructed pCAM-FREE to target the IncFII plasmid group. For future research, MAGE and pCAM-FREE could be tested in these Cambodian isolates. If they are successful, we can even broaden its application for other AMR bacteria!

Abbreviations

AMR	Antimicrobial resistance
ARG	Antibiotic resistance genes
AST	Antibiotic susceptibility testing
Cas	CRISPR-associated proteins
CPE/K	Carbapenemase-producing *E. coli/K. pneumoniae*
crArray	CRISPR locus/array
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNAs	CRISPR-RNAs
EARS-Net	European Antimicrobial Resistance Surveillance Network
E. coli	Escherichia coli
ESBL-E	Extended spectrum β-lactamase enterobacteria
ESBL	Extended spectrum β-lactamase
ESCE/K	Extended-spectrum cephalosporinase-producing *E. coli/K. pneumoniae*
Fw	Forward primer
gRNA	guide RNA
HGT	Horizontal Gene Transfer
K. pneumoniae	Klebsiella pneumoniae
LB	Luria Bertani
MAGE	Multiplex Automated Genome Engineering
MCS	Multiple Cloning Site
Ori	Origin of replication
PAM	Protospacer Adjacent Motif
PCR	Polymerase Chain Reaction
pre-crRNA	preCRISPR-RNA
RADP	Random Amplified Polymorphic DNA
Rv	Reverse primer
TE	Transposable Elements
Tn	Transposons
tracrRNA	Trans-activating crRNA
sgRNA	Single guide RNA
ssDNA	Single-stranded DNAs

1. Introduction

1.1 Antibiotics and antibiotic resistance bacteria

In the history of medicine, antimicrobials are known as one of the most successful forms of chemotherapeutic agent [1]. Antimicrobials are drugs that eliminate or reduce the growth of microorganisms, such as bacteria, that cause infection [2]. The first modern antimicrobial drug used is Salvarsan that was discovered by Paul Ehrlich in the 19th century, which was followed by the discovery of Prontosil and penicillin. As a result, the time period of antibiotics discovery and its widespread usage has been named as the modern antibiotic era. The modern antibiotic era ended when the antibiotics discovery rate declined with an absence of novel antibiotic classes discovered. The end of this era marked the beginning of the post-antibiotic era [1].

The use of antibiotics has increased the survival rate of patients with bacterial infections, and it is indispensable in modern medicine, being a prerequisite for conducting surgical procedures, organ transplantations and cancer treatments [2]. However, antibiotic use enriches resistance as shown in Figure 1, and antibiotic resistance is ever increasing over time. In contrast, the development of novel antibiotic drugs is slowing down [2]. Antimicrobial resistance (AMR) means that microorganisms are fully or partially resistant to antibiotics that are being used against them. In a clinical setting, AMR is of great concern since it limits the available antimicrobial drug treatment options and threaten the use of modern medical procedures [3].

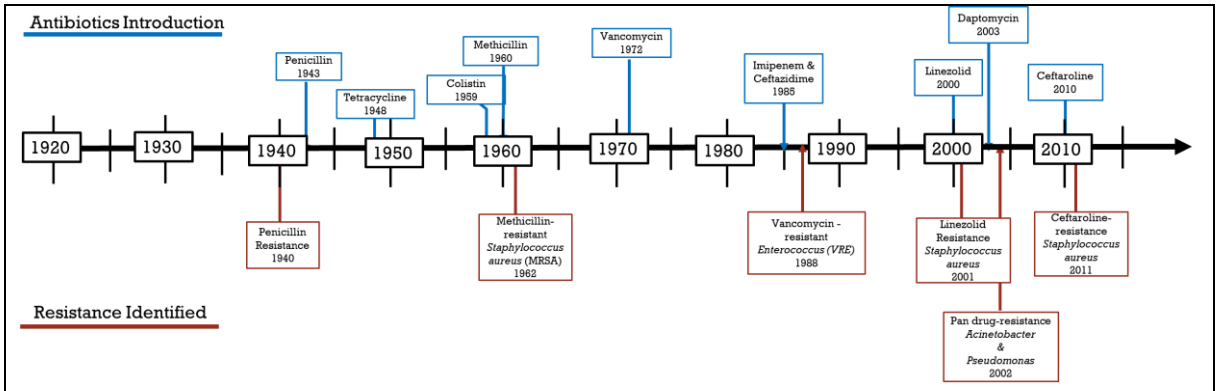


Figure 1. Adapted from [4], [5]. Timeline of antibiotics clinical introduction and antimicrobial resistance identification.

Studies [6]–[9] have also shown the presence of AMR in environmental settings such as fecal samples, wastewater, and rivers. The prevalence of resistant bacteria in environmental settings threaten to accelerate its exposure to humans and dissemination of resistance. Unfortunately, AMR is a complex challenge that involve several elements such as bacterial strain and vectors dissemination due to horizontal gene transfer, interactions between the hosts (human or animals e.g., zoonotic interactions) and the environment, the flow of antibiotic resistance genes (ARGs), antibiotic usage in animal and humans, and lack of sanitation infrastructure. Therefore, there are two useful concepts called One Health and Global Health to combat antibiotic resistance [10], [11].

To provide an overview of antibiotic-resistant bacterial infections, the problem is highlighted in high income countries where data is available such as United States. In the United States,

antibiotic-resistant bacteria caused more than 2,8 million infections each year and more than 35000 deaths. Additionally, infections caused by extended spectrum β-lactamase enterobacteria (ESBL-E) increased by 50% in 2019 [2]. Meanwhile, the occurrence of AMR exists in wide variations across the European Union (EU). Overall, the most common antibiotic-resistant bacterial species found are *Escherichia coli* (44.2%), *Staphylococcus aureus* (20.6%), and *Klebsiella pneumoniae* (11.3%) according to the reported data in 2019 to the European Antimicrobial Resistance Surveillance Network (EARS-Net). Furthermore, resistance to several antibiotics was frequently found in *E. coli* and *K. pneumoniae* with generally higher resistance percentages in *K. pneumoniae* [12]. Unfortunately, there is no or limited data in low to middle income countries.

These numbers are predicted to rise and based on scenarios of six drug-resistance pathogens, the burden of deaths caused by AMR could be up to 10 million each year by 2050 with a cumulative global economic cost of 100 trillion USD. Regardless of the accuracy of the prediction, the lack of interventions and supply of novel antibiotics reasonably suggest the growing problem of AMR. Therefore, the issue must be addressed now before its concerning impact on human health can be felt on a global scale [13].

1.2 Plasmids and antibiotic resistance

Plasmids are extrachromosomal genetic elements with abilities to control and replicate autonomously. Most plasmids are circular double-stranded DNA with non-essential genetic information as opposed to essential housekeeping genes in bacterial chromosomes. Despite that, plasmids have a wide genomic diversity that encode a variety of functions such as xenobiotic resistance properties against antibiotic and heavy metal, bacteriocin production, and toxin production that can increase the survival chance of their host [14].

Albeit their genomic diversity, plasmid genomes generally contain backbone genes, which are considered to be conserved amongst broadly related plasmid families. Backbone genes are associated with key plasmid-specific functions such as plasmid replication, mobility, and stable plasmid inheritance [15]. Besides these genes, plasmids as a vector usually have a multiple cloning site (MCS) to facilitate cloning of the desired genetic material and a selection marker ranging from auxotrophy to antibiotic resistance properties [16].

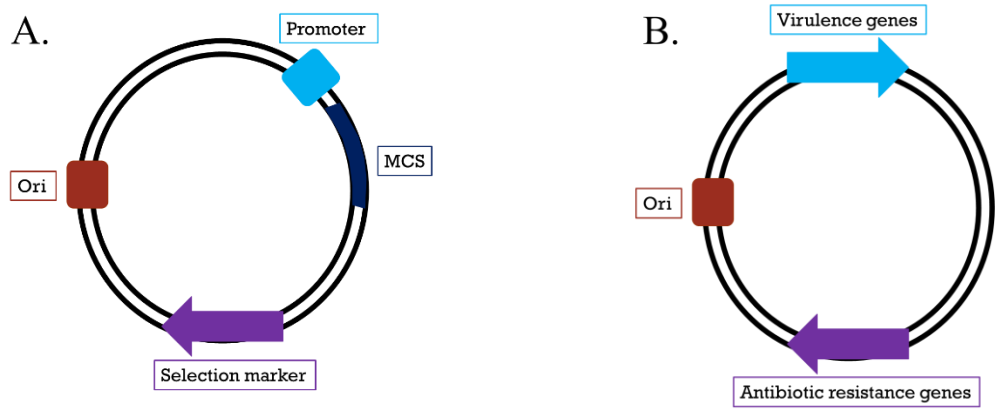


Figure 2. Most common bacterial plasmid components. **(A)** Plasmid vector. **(B)** Antibiotic-resistance plasmids.

On the backbone genes, a clustered 1-3 kb genes of replication control elements i.e., replication inhibitor, and origin of replication (*ori*) can be found. This replication machinery region is known as replicon. The replication inhibitor is important since their concentration could be used to refer to the plasmid copy number. Maintenance of plasmid copy number is important to ensure the plasmid inheritance at the host cell division [14].

Traditionally, plasmids are classified into different incompatibility groups based on the different replication machinery (replicon typing). Incompatibility is the inability of plasmids with similar replicon to coexist stably within the same cell due to the cross-reaction of the replication control systems. When two compatible plasmids are in the same host cell, their different replicons are able to maintain their normal copy numbers separately. In contrast, when two plasmids are incompatible, their replicons cannot act independently for each plasmid thus resulting in a combined copy number that is shared between them. Unfortunately, these plasmids are selected randomly for replication. This will result in imbalance of the plasmid copy number where there will be an excess copy number of one plasmid and less abundance for the other plasmid [14]. However, some plasmids have more than one replicon which initiate alternative classification scheme such as plasmid mobility [15].

Another distinctive characteristic of plasmids are its importance in the exchange of genetic material between bacterial cells through horizontal gene transfer (HGT). Plasmids can disperse to another cell by three HGT mechanisms which are transformation, conjugation, and transduction. Although, conjugation is considered as the most important mechanism. Conjugation requires cell-to-cell contact and the plasmid is transfer from the donor to the recipient cell. Hence, conjugation process will result in an epidemic spread of the plasmid through the population [17].

The successful plasmid transfer to a recipient cell can be followed by integration of transposable elements from the plasmids into the chromosome of the host [17]. Transposable elements (TEs) or transposons (Tns) are DNA elements which are capable to move from the DNA molecule to other places on the same DNA or different DNA molecules. The movement of TEs in bacterial genome could create different phenotypes by causing mutation and carrying additional genes. To add to the flexibility and complexity, bacteria could also acquire genes through integrons. Integrons are genetic elements that can integrate several resistance genes in the form of functional gene cassettes. Although some integrons are not mobile, transposons such as Tn*5053* could incorporate them and act as integrons carrier which aid in their dissemination [18]. Therefore, transmission of resistance is a complex process that involves the combination of interactions between different gene elements and bacterial cells in which, plasmids play a role.

1.3 Utilization of CRISPR/Cas9 system for plasmid curing

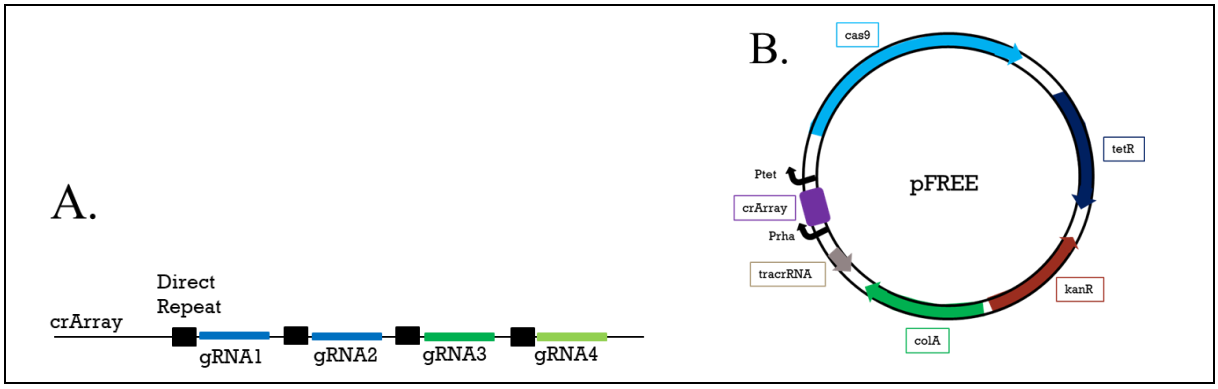
Plasmid curing is defined as the displacement or elimination of plasmid from its host cell. This process remains a challenge and unfortunately, traditional mechanism such as the usage of plasmid curing agents and plasmid incompatibility have variable efficiency and they might promote unwanted mutations [19]. Lauritsen *et al* (2017) developed a novel plasmid curing mechanism with the advent of CRISPR-Cas9 technology. Based on their study, ColE1-like and pSC101 replicon groups are used in more than 90% of the available cloning vectors for biotechnological purposes from the Addgene database. This indicates that a plasmid-curing

system targeting both ColE1 and pSC101 plasmid groups are sufficient to cure most of the common commercial plasmid vectors.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) are parts of the bacteria and archaea immune system against invading phages and plasmids. The CRISPR-Cas are categorized into three types whereof the Type II (CRISPR-Cas9) is used for the development of genome editing technology. Generally, the system consists of CRISPR locus/array (crArray) and a diverse group of Cas genes that encodes the Cas proteins. The crArray contains hypervariable spacers or protospacer that is integrated between two contiguous repeat sequences [20].

The CRISPR-Cas's defense mechanism is a three-step process that begins with acquisition. When phages or plasmids invade a bacteria cell, a part of the foreign DNA is captured and incorporated in the crArray as spacers or protospacer. In addition, the Type II system requires the incorporation of small nucleotides called protospacer adjacent motif (PAM) which is located near the protospacer. The second step of CRISPR-Cas is activated when the same plasmids or phages invade the bacteria for the second time. The crArray will be transcribe by RNA polymerase to produce preCRISPR RNA (pre-crRNA). Afterwards, endonucleases will cleave the pre-crRNA into active CRISPR-RNAs (crRNAs) where combination of crRNAs and a hairpin trans-activating crRNA (tracrRNA) will form a single guide RNA (sgRNA). The endonuclease Cas9 recognize and forms a complex with the sgRNA therefore, guiding the enzyme to the regions of the incoming foreign DNA/RNA for degradation. Cas9 creates a double-strand break (DSB) that usually causes gene knockout by disruption [20].

The study by Lauritsen *et al* (2017) developed the pFREE plasmid which encodes the CRISPR-Cas9 system with four guide RNA (gRNA) in the crArray that recognizes conserved sequences for plasmid replicon groups pSC101 and ColE1-like (Figure 3A). Interestingly, the pFREE vector is based on the colA replicon which can also be recognized by one of the designed gRNAs. This fundamental feature resulting in a one-step workflow as shown in Figure 3C of plasmid curing where targeted plasmids and pFREE plasmid will be cured simultaneously in the host cell. The curing efficiencies of pFREE system range between 40 - 100% with wide applicability and successful application in *Escherichia coli* and *Pseudomonas putida* with pSC101 and ColE1-like plasmids. The variation in plasmid-curing efficiency is presumably caused by the context of the targeted plasmids e.g., common laboratory plasmid vector and natural plasmid, and several factors such as copy-number, fitness cost and plasmid incompatibility [21].



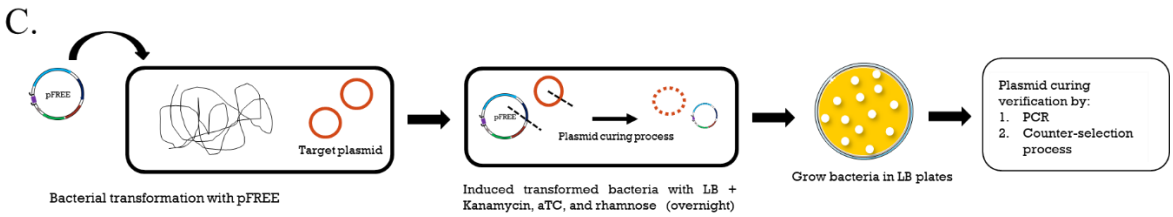


Figure 3. Adapted diagram from Lauritsen *et al* (2017). **(A)** CRISPR locus/array (crArray) in pFREE system. Guide RNA (gRNA) 1 and 2 recognizes pSC101 replicon group. gRNA3 and 4 targeted ColE1-like replicon, only gRNA4 that targeted colA for self-curing of pFREE. **(B)** Plasmid map of pREE. pFREE plasmid consists of colA (*ori*), crArray and Cas9, inducible promoter Ptet (tetracycline) and PrhaBAD (rhamnose), tracrRNA, tetR and kanR. **(C)** One-step workflow of plasmid curing with pFREE system.

Another interesting possibility of pFREE system usage is to combat plasmid-encoding multidrug resistance in pathogenic environmental bacteria. The gRNA could be designed to recognize the conserved region e.g., *ori* of these plasmids thus enable targeted plasmid curing. Previous study in our group showed reports of carbapenemase-producing *E. coli/K. pneumoniae* (CPE/K) in humans or livestocks and community carriage of extended-spectrum cephalosporinase-producing *E. coli/K. pneumoniae* (ESCE/K) in Cambodia from fecal samples [22]. The numbers are concerning especially because the bacteria were found in rural Cambodian community settings and there are multiple ARGs in these bacteria isolates. The usage of pFREE system for these resistant-bacteria is novel since this system could be use for one-step plasmid-curing in environmental/community commensals bacteria, with exciting future possibilities.

1.4 Recombineering the bacterial chromosome

Recombineering or recombination-based genetic engineering is a technique to disrupt chromosomal genes of bacteria by homologous recombination. There have been several developments in recombineering technique that involves mobile plasmids [24]–[26] and prophage [26] to encode λ Red recombinase. Bacteriophage λ Red system encode three genes, γ, β and *exo*, whose products promote recombination with enhanced rate compared to recombination-proficient strain bacteria, *recBC sbcBC* or *recD*, after transformation with short (35 – 40 nucleotides) homologous linear DNA [24]. Additionally, it was discovered that the β subunit from λ Red recombinase with single-stranded DNAs (ssDNA) as short as 30 bp are sufficient for recombineering [27]. The discovery led to the development of more recombineering methods with ssDNA such as multiplex automated genome engineering (MAGE).

Wang *et al.* (2009) created MAGE as a technology that could generate a combinatorial library of genetic diversity by simultaneously and efficiently modify many locations on the target chromosome. They also constructed MAGE automation prototype devices where a mixture of electroporated bacteria with oligonucleotides are cycled multiple times for rapid and continuous generation of mutants. Despite its capability to generate library with high diversity, the MAGE system has an adjustable specificity in which, oligos with well-defined sequences will produce specific modification in the target [28].

As previously mentioned, antibiotic resistance genes could be present in bacterial chromosome due to TEs. Targeted modification on these genes is another interesting technique to reduce AMR bacteria. Plasmid pMA7-SacB as designed [26] are suitable for this purpose since bacteria strains defective in mismatch repair by *mutS* deletion is not required, the system is curable, and there is a reduced off-target mutation rate. pMA7-SacB contains gene encoding arabinose-inducible β subunit of λ Red recombinase and Dam methylase with constitutively expressed *sacB* for plasmid curing by sucrose counterselection. Based on these characteristics, pMA7-SacB and MAGE system could be explored for usage in Cambodian isolates.

2. Aim

This study aimed to characterize selected antibiotic-resistance *Escherichia coli* and *Klebsiella pneumoniae* isolates from Cambodia and to assess two developed methodologies for recombineering, and then develop and use our novel plasmid curing in these Cambodian isolates.

Specific objectives

- To phenotypically characterize six Cambodian isolates of *E. coli* and *K. pneumoniae* by random amplified polymorphic DNA (RADP) and antibiotic susceptibility testing (AST).
- To validate One-Step Curing Plasmid by Lauritsen *et al.* (2017) and Multiplex Automated Genome Engineering (MAGE) by Wang *et al.* (2012) in a control laboratory strain *Escherichia coli* strain K-12 sub-strain MG1655.
- To modify pFREE plasmid in One-Step Curing Plasmid by creating pCAM-FREE to facilitate plasmid curing in Cambodian isolates.
- To study the phenotypic characteristics of pCAM-FREE for plasmid curing and MAGE for recombineering in the Cambodian isolates.

3. Materials and Methods

3.1 Bacteria isolates and plasmids

Escherichia coli and *Klebsiella pneumoniae* bacterial isolates from Cambodia were isolated on ESBL isolation media as described in Atterby *et al.* (2019) and used in the study. The whole genome sequencing and molecular epidemiological reports have been done on these isolates (unpublished data) before the beginning of this study and from this data, isolates for this study were selected. The selected isolates are: C14, C61, C66, C75, C122 and C128. The bacterial isolates C14, C61, C66, and C75 all belong to the bacterial species *Escherichia coli* isolates whilst C122 and C128 belong to the bacterial species *Klebsiella pneumoniae*. In addition, other laboratory bacterial strains have been used: *Escherichia coli* DH5α with pMA7-SacB plasmid from Addgene (Catalog #79967), *Escherichia coli* Top10 with pFREE plasmid from Addgene (Catalog #73950) and *Escherichia coli* strain K-12 sub-strain MG1655 from Dan I. Andersson research group in Uppsala University.

3.2 Plasmid and DNA extraction and purification of Cambodian isolates and *Escherichia coli*

Plasmids harboring antibiotic resistance genes from six Cambodian isolates, pMA7-SacB and pFREE were extracted from bacteria using the Thermo Scientific™ GeneJET Plasmid Midi Prep Kit (Catalog #K0482) [29]. The centrifugation in Protocol A, Step 7 was amended into 1 hour and the heated elution buffer was added into the column in 2 steps, 250µL and 150µL, with 10 minutes of incubation and centrifugation in each step. The total DNA of six Cambodian isolates were extracted and purified with DNeasy® Blood & Tissue Kit (Catalog no. 69506) [30]. The extracted and purified plasmid and DNA are stored in -80°C. The concentration of extracted plasmid and DNA were measured with Qubit Fluorometer using Invitrogen™ Qubit™ dsDNA HS (High Sensitivity) Assay Kit (Catalog no. Q32851) [31].

3.3 RAPD (Random Amplified Polymorphic DNA)

Analysis of RAPD was done with Ready-To-Go RAPD Analysis Beads (Catalog no. 27-9502-01) according to the manufacturer's instruction [32]. *E. coli* BL21(DE3) and *E. coli* C1α are both used as a DNA positive control. Isolated DNA was used as a template for the PCR (Polymerase Chain Reaction). The PCR product was visualized with electrophoresis method on a 1.5% agarose gel using Bio Rad Wide Mini-Sub Cell GT Electrophoresis Cell for 100 volt and 60 minutes with Qiagen GelPilot Mid Range Ladder (Catalog no. 239135). For DNA staining, 1,5 µL GelRed® Nucleic Acid Stain (Catalog no. 41003) from Biotium was added into agarose gel and gel imaging was done with BioRad Gel Doc™ EZ Imager.

3.4 Antimicrobial Susceptibility Test (AST)

The MIC (Minimum Inhibitory Concentration) of the selected isolates were tested using Thermo Scientific™ Sensititre™ Gram Negative GNX3F AST plates (Catalog no. GNX3F). The test used in-house protocol established in Zoonosis Science Center Uppsala University. In brief, bacteria cultures were streaked and incubated at 37°C for 18 hours on Muller Hinton II Agar on the day before testing. Before bacteria samples were measured, 0,5 McFarland standard was used to calibrate Thermo Scientific™ Sensititre™ Nephelometer (Catalog no. V3011). Material from bacterial colonies were then dissolved completely in 5mL saline and adjusted to 0,5 McFarland standard with the nephelometer. From the adjusted bacterial suspension, 50 µL was transferred to 10mL Muller-Hinton II broth as the working inoculum for AST plates. Bacteria inoculum was inoculated 50 µL to each well of the plate and incubated at 37°C for 16-18 hours. A mirror plate reader was used to observe the growth in each well and MIC was determined. In the end, MIC values were compared to the clinical breakpoints determined by European Committee on Antimicrobial Susceptibility Testing, EUCAST, to conclude the susceptibility of the bacteria against a given antibiotic drug [33]. The AST plates assay was repeated on at least two independent occasions for every isolate as independent experimental replication.

3.5 Transformation of pFREE and pMA7-SacB into *Escherichia coli* str. K-12 substr. MG1655

E. coli MG1655 was transformed with isolated plasmid pMA7-SacB and pFREE, to create two strains of *E. coli* MG1655 + pMA7-SacB and *E. coli* MG1655 + pFREE. An overnight culture of the bacteria was inoculated to 2 mL Luria Bertani (LB) broth and grown until logarithmic phase. The bacteria cells were harvested by centrifugation in 2000 x g for 2 minutes. The cells were then washed with 2 mL autoclaved and ice cold MilliQ water three times followed by resuspension and centrifugation in 2000 x g for 2 minutes each step. Afterwards, 50 µL of the respective plasmid was added to the electrocompetent cells into a 1 mm electroporation cuvette. Electroporation process was done using BioRad Gene Pulser Xcell™ for *E. coli* bacterial pre-set protocols. 500 µL LB broth was immediately added to the cuvette after electroporation before the cultures were transferred to another tube with 1,5 mL LB broth. The cultures were then incubated for 2 hours growth in 37°C with 180 rpm in Infors AG CH-4103 Bottmingen shaker. A serial dilution of 10^{-1} to 10^{-5} was done, and bacteria cultures were spread on LB and ampicillin (LB + Amp) or kanamycin (LB + Kan) for selection. Transformed colonies were observed after overnight plates incubation in 37°C and pFREE transformation was verified with colony PCR. Selected colonies were transferred to new LB and antibiotic plates before stored as glycerol stocks in -80°C.

3.6 Colony PCR for verification of transformed *Escherichia coli* str. K-12 substr. MG1655 with pFREE

Colony PCR was used to verify the presence of pFREE plasmid in the transformed colonies. The PCR used Thermo Scientific Phusion Hot Start II High-Fidelity PCR Master Mix (Catalog no. F565L) and primers listed on Table 1. Primers listed in Table 1 were designed and provided by the co-supervisor of the project to recognize pFREE origin of replication (*ori*).

Table 1. Primers used for pFREE amplification

Primer name	**Sequence in 5' – 3' direction**
pFREE_ori_fw	CCCCTGACGAACATCACGAA
pFREE_ori_rv	AGGCGGTTTGATCGAAGGTT

The 20 µL reaction consisted of 10 µL Phusion Master Mix, 2 µL forward primer (fw), 2 µl reverse primer (rv), 6 µl nuclease free water and 1 small colony as template. The program used is described in Table 2 and ran on Bio Rad T100™ Thermo Cycler.

Table 2. PCR colony program for pFREE

Temperature	**Time**	**Cycles**
98°C	2 minutes	1 x
98°C	30 seconds	35 x
65.1°C (Annealing temperature)	30 seconds	

72°C	30 seconds	
72°C	5 minutes	1 x
4°C	Forever	1 x

The PCR products were visualized with gel electrophoresis using 1,5% agarose with conditions of 100 volt for 60 minutes. Qiagen GelPilot Mid Range Ladder (Catalog no. 239135) and GelRed® Nucleic Acid Stain from Biotium was used to stain DNA in agarose gel.

3.7 Multiplex Automated Genome Engineering (MAGE) validation in control laboratory *Escherichia coli* str. K-12 substr. MG1655

The MAGE recombineering process was validated using oligos that mutated the *lacZ* gene in *E. coli* MG1655. The oligos and primers are described in Wang *et al.* (2012) with all listed in Table 3. The protocol was amended from Wang *et al.* (2012).

Table 3. Oligo and primers used for MAGE system

Primer name and function	**Sequence in 5' – 3' direction**
lacZ_oligo oligo to create mutation in *lacZ*	G*GAAACAGCTatgACCATGATTACGGATTCA CTGGCCGTCGTTTGACAACGTCGTGACTGGG AAAACCCTGGCGTTACCCAACTTAATC
lacZ_seq_fprimer fw primer to sequence the mutated *lacZ*	GGCAGTGAGCGCAACG
lacZ_seq_rprimer rv primer to sequence the mutated *lacZ*	TTCTCCGTGGGAACAAACG

Briefly, overnight *E. coli* MG1655 with pMA7-SacB were grown in new LB + Amp medium until logarithmic phase. Plasmid pMA7-SacB was then induced with 0,2% arabinose for 15 minutes for system activation. The cells were then harvested and washed three times with sterile and cold MilliQ water, followed by centrifugation in 2000 x g for 2 minutes in each step. 50 µL oligos 10 µmol were then added to the cells and transferred to electroporation 1 mm cuvette. Electroporation process was done using BioRad Gene Pulser Xcell™ for *E. coli* bacterial pre-set protocols. Cells were left to recover for 3 hours and 24 hours where both cultures were spread on blue-white screening plates for selection. The blue-white screening plates were made with Invitrogen™ imMedia™ Amp Blue. After overnight incubation of the plates, the blue and white colonies were counted in each plate to determine the efficiency. In the replication process, the validation was repeated 3 times for experimental replicate on 3 independent occasions to assess technical error. Afterwards, selected blue and white colonies were sent to Eurofins Genomics for Sanger sequencing genomic verification with sequencing primers on Table 3. Sequences were aligned to determine the mutation in the *lacZ* gene from the *E. coli* MG1655 sub-strain K-12 reference genome.

3.8 One-Step Curing Plasmid validation in control laboratory *Escherichia coli* str. K-12 substr. MG1655

The validation was done to observe the ability of pFREE plasmid to self-cure in *E. coli* MG1655. The protocol was performed as previously described by Lauritsen *et al.* (2017). In addition, counter-selection process was done for further confirmation. Selected cured bacteria colonies from LB agar were streaked on new LB agar for overnight incubation. Afterwards, the colonies were re-streaked on LB + Kan agar plates. The cured bacteria were then used as template for colony PCR with PCR conditions and primers in Table 1 and 2. For replication, the procedure was repeated three times on independent occasions.

3.9 pCAM-FREE construction using USER Cloning recombineering with genomic confirmation

The designed gRNA and primers used in this procedure were designed and provided by the co-supervisor of the project. The crArray was divided into 3 separate parts, fragment 1, 2 and 3, through PCR amplification with different primer sets listed in Table 4.

During pCAM-FREE construction, all PCR products were visualized with gel electrophoresis using 1,5% agarose with conditions of 100 volt for 60 minutes. Thermo Scientific™ GeneRuler 100 bp DNA ladder (Catalog no. SM0243), Thermo Scientific™ GeneRuler 50 bp DNA ladder (Catalog no. SM0373) and GelRed® Nucleic Acid Stain from Biotium were used to size and stain DNA in agarose gel.

Table 4. Primers and gRNA used for pCAM-Free construction

First Set	
Primer name and function	**Sequence in 5' – 3' direction**
crArray_all_fw fw primer to amplify the whole crArray and used as fragment 1AB fwd	CGAGAAGGTCGCGAATTCAG
crArray_all_rv rv primer to amplify the whole crArray	TTATAACCAGACTCGAGGACCA
gRNA1 reverse complement guide RNA to recognize IncR plasmid	CCGTGGCTGGCTGAAGCAGGAAT
crArray_Fragment_1A_rv rv primer to add gRNA1 to fragment 1	(TTAAGTCATA) (CCGTGGCTGGCTGAAGCAGGAAT) GTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACACGACCA
crArray_Fragment_1B_rv rv primer to amplify fragment 1 with gRNA1	TTAAGTCATACCGTGGCTGGCTGAAGCAGGAAT
crArray_Fragment_2AB_fw fw primer to amplify fragment 2 of the crArray	TATGACTTAAGTTTTAGAG

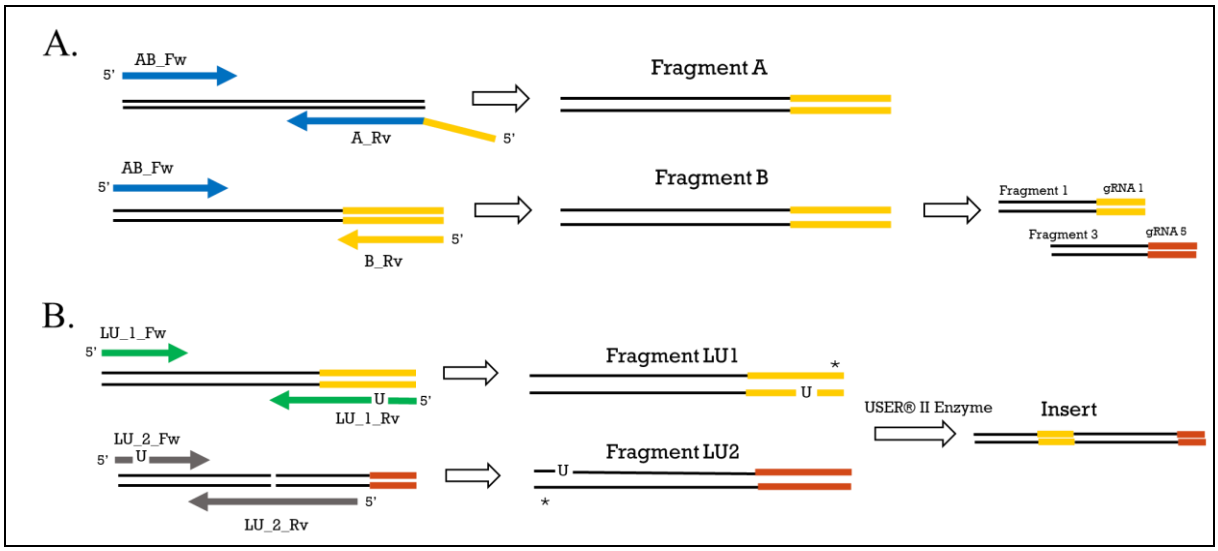
crArray_Fragment_2AB_rv rv primer to amplify fragment 2 of the crArray	CTGTCGGGTTTCGCCGTT
gRNA5 reverse compliment guide RNA to recognize IncFII plasmid	CCCCGTTAATCTTTTCCTTCCGC
crArray_Fragment_3AB_fw fw primer to amplify fragment 3 of the crArray	GACTATAAAGATACCGTT
crArray_Fragment_3A_rv rv primer to add gRNA5 to fragment 3	(CCCCGTTAATCTTTTCCTTCCGC) GTTTTGGGACCATTCAAA
crArray_Fragment_3B_rv rv primer to amplify fragment 3 with gRNA5	CCCCGTTAATCTTTTCCTT
Second Set	
Linear_USER_crArray_Fragment_1_fw fw primer to amplify fragment 1	CGAGAAGGTCGCGAATTC
Linear_USER_crArray_Fragment_1_rv rv primer to amplify fragment 1 and add uracil in 3' end	AAGTGGCUGGCTGAAGCAGGAATGTTT
Linear_USER_crArray_Fragment_2_fw fw primer to amplify fragment 2 and 3, and add uracil in 5' end	AGCCACTUAAGTTTTAGAGCTATGCTGTTTTG
Linear_USER_crArray_Fragment_2_rv rv primer to amplify fragment 2 and 3	CGTTAATCTTTTCCTTCCGCGTTTTGGGACCATTC AAAACAGCATAGCTCTAAAACGGTATCTTTATAG TCCTGTCGGGTTTCGCCGTT
Third Set	
Insert_USER_Seq_1_Fw fw primer to amplify linear insert and add uracil	ACGAGAAGGUCGCGAATTCAGGCGCTTT
Insert_USER_Seq_1_Rv rv primer to amplify linear insert and add uracil	AGGGACGTUAATCTTTTCCTTCCGCGTTTTGG
Vector_USER_Seq_2_Fw fw primer to amplify pFREE backbone and add uracil	AACGTCCCUATCAGTGATAGAGATTGA
Vector_USER_Seq_2_Rv rv primer to amplify pFREE backbone and add uracil	ACCTTCTCGUTACTGACAGGAAAATGGG
pCAM-FREE Confirmation	
224_Fw fw primer to amplify part of the vector and crArray	CCCCTGACGAACATCACGAA

In the first part of cloning, fragment 1 and 3 of crArray were amplified with two different reverse primer and one forward primer. The A reverse primer was used to add new gRNA1 and gRNA5 into fragment 1 and 3, respectively, by PCR as illustrated in Figure 4A. The PCR reaction and program were mentioned in section 3.6 with annealing temperature of 63,8°C and 30 cycles.

PCR products were then purified with Thermo Scientific™ GeneJET PCR Purification Kit (Catalog no. K0702) as protocol [34]. Fragment A was then used as template for PCR with B reverse primer to verify gRNA sequence. For further cloning process, the PCR products were visualized with gel electrophoresis and the desired DNA bands were extracted from agarose gel with Thermo Scientific™ GeneJET Gel Extraction Kit (Catalog no. K0692) as described in the protocol [35]. In contrast, fragment 2 was amplified with AB forward and reverse primer in which the PCR product was purified with kit.

In the second part of the cloning, the fragments were ligated by USER enzyme which required the presence of uracil (U) in the fragments. In Figure 4b, uracil was added into fragment 1 in the 3' end with uracil-containing reverse primer. Fragments 2 and 3 were amplified simultaneously and uracil was added in the 5' end. Both PCR were done with Thermo Scientific™ Phusion U Hot Start PCR Master Mix (Catalog no. F-533S). The PCR program was mentioned in section 3.6 with modification according to the master mix protocol [36]. Amplification of fragment 1 and fragments 2 with 3 were done with annealing temperature of 50,2°C and 53°C, respectively. Thermolabile USER® II Enzyme (Catalog no. M5508S) from New England BioLabs were used according to the protocol (New England Biolabs, 2021) to assemble fragment linear user (LU) 1 and 2, forming modified insert crArray. The insert fragment was amplified with crArray_all_fw and crArray_Fragment_3B_rv before sent to Eurofins Genomics for Sanger sequencing.

The final step of pCAM-FREE construction was PCR with Phusion U Master Mix to add uracil to insert crArray and pFREE plasmid as vector. The annealing temperature to insert crArray and pFREE are 58,6°C and 45,6°C, respectively. PCR products from both were used as template for pCAM-FREE assembly with USER® II Enzyme. Following the construction of pCAM-FREE, the plasmid was transformed into *Escherichia coli* str. K-12 substr. MG1655 with electroporation procedure as mentioned before.



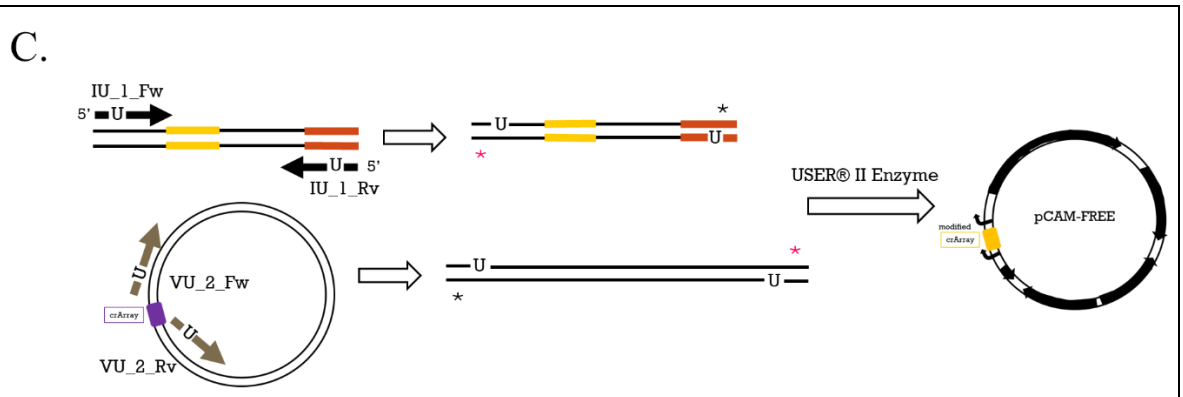


Figure 4. Illustration of pCAM-FREE construction using USER Cloning. Fw = forward; rv = reverse; LU = Linear USER; IU = Insert USER; VU = Vector USER. **(A)** The first cloning step for fragment 1 and 3 amplification. gRNA1 (yellow) and gRNA5 (red) were added to the fragment by primer A_rv. **(B)** The second cloning step where uracil was added to the fragments on different ends. The star indicated complementary sequence on both fragments LU1 and LU2. Addition of USER Enzyme assembled the modified crArray. **(C)** The last cloning step where uracil was added on both ends of the insert and pFREE vector. The star with its respective color indicated complementary sequence on both fragments. USER Enzyme completed the USER cloning by insertion of the linear insert to the pFREE vector which resulted in pCAM-FREE.

For genomic confirmation, 8 colonies with pCAM-FREE were selected and screened by colony PCR with primer 224F and 2AB_Rv (Table 4). From these colonies, 3 colonies were randomly selected for sequencing in Eurofins Genomics.

4. Results

4.1 RAPD shows the diversity of selected Cambodian isolates

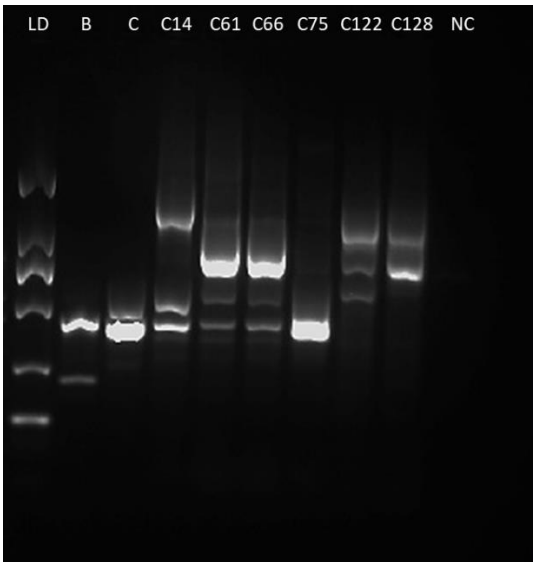


Figure 5. RAPD agarose (1,5 %) gel electrophoresis result of Cambodian isolates with primer 2. Qiagen GelPilot Mid Range Ladder (LD) was used. *Escherichia coli* BL21 (DE3) and *Escherichia coli* C1α DNA were used as control B and C, respectively.

The phenotypic characterization of six Cambodian isolates were firstly done with random amplified polymorphic DNA (RADP). PCR was done using kit with the provided primer 2 which was designed for use in RAPD analysis by Cytiva [32]. Based on the genomic polymorphisms of the samples, amplification with this random primer would result in specific banding pattern for each sample. The method is used to characterize the diversity among strains of the samples. Amplification with the primer resulted in different banding pattern of B and C as controls. In total, there were five banding patterns among six Cambodian isolates in which, C61 and C66 isolates had the same banding pattern indicating potential clonality.

4.2 The selected Cambodian isolates are multidrug resistance

The second phenotypic characterization of six Cambodian isolates were their antibiotic susceptibility or MIC (Minimum Inhibitory Concentration). The MIC values were determined with commercial AST (antimicrobial susceptibility test) plate assay and compared with the clinical breakpoints determined by EUCAST for Enterobacterales bacteria. The EUCAST system categorizes bacterial susceptibility to antibiotics into three categories of the SIR system:

S – Susceptible, standard dosing regimen. This indicates a high likelihood of therapeutic success against a microorganism when a standard dosing regimen of the agent is used.

I - Susceptible, increased exposure. This indicates a high likelihood of therapeutic success against a microorganism when there is an increased exposure to the agent through dosing regimen adjustment or increased concentration.

R – Resistant. This indicates a high likelihood of therapeutic failure against a microorganism even with increased exposure to the agent [33].

Table 5. The MIC values (mg/L) for six Cambodian isolates and the clinical MIC breakpoints indicated by a color-coded SIR system. The SIR system was indicated as green for S, yellow for I and red for R. EUCAST breakpoints are not available for the tested antibiotics doxycycline, minocycline, polymyxin B, and doripenem. Antibiotics within the same class are grouped together.

MIC values (mg/L)							
Antibiotics	Bacteria Isolates						
	MG1655	C14	C61	C66	C75	C122	C128
AMI	4	6	6	4	4	4	4
GEN	1	> 8	1,50	2	2	1,50	1
TOB	1,50	> 8	2	1,50	1,67	1	1
DOX	2	2	> 16	Unclear*	10,67	> 16	16
MIN	2	2	Unclear*	16	3	Unclear*	16
TGC	0,25	0,25	0,25	0,25	0,25	0,25	0,25
CIP	0,06	> 2	> 2	> 2	> 2	0,06	1
LEVO	1	8	> 8	> 8	> 8	1	1
SXT	0,50	5	5	5	5	5	Unclear*
AZT	2	5,33	> 16	> 16	3	2	4
IMI	1	1	1	1	1	1	1
MERO	1	1	1	1	1	1	1
DOR	0,50	0,50	0,50	0,50	0,50	0,50	0,67
A/S2	4	16	24	24	16	8	53,33
P/T4	8	8	8	8	8	8	8
TIM2	16	16	64	64	37,33	16	64
TAZ	1	2	> 16	> 16	1,50	2	1,67
FOT	2	Unclear*	> 32	> 32	24	2	Unclear*
FEP	2	3,33	> 16	> 16	3,33	2	4
COL	0,25	0,25	0,25	0,63	0,25	Unclear*	0,42
POL	0,25	0,25	0,25	0,63	0,42	> 4	0,50

Notes:
* = MIC values are inconclusive from experimental replications results
AMI = Amikacin; GEN = Gentamicin; TOB = Tobramycin; DOX = Doxycycline; MIN = Minocycline; TGC = Tigecycline; CIP = Ciprofloxacin; LEVO = Levofloxacin; SXT = Trimethoprim / sulfamethoxazole; AZT = Aztreonam; IMI = Imipenem; MERO = Meropenem; DOR = Doripenem; A/S2 = Ampicillin / sulbactam 2:1 ratio; P/T4 = Piperacillin / tazobactam constant 4; TIM2 = Ticarcillin / clavulanic acid constant 2; TAZ = Ceftazidime; FOT = Cefotaxime; FEP = Cefepime; COL = Colistin; POL = Polymyxin B.

E. coli MG1655 as negative control is susceptible (S) in most antibiotics and susceptible with increased exposure (I) against levofloxacin (0,5 - 1 mg/L), aztreonam (1 - 4 mg/L), cefepime (1 - 4 mg/L), cefotaxime (1 – 2 mg/L), and ticarcillin / clavulanic acid (8 – 16 mg/L). Along with *E. coli* MG1655, all Cambodian isolates are susceptible (S) against amikacin (≤ 8 mg/L), tigecycline (≤ 0,5 mg/L), imipenem (≤ 2 mg/L), meropenem (≤ 2 mg/L) and piperacillin/tazobactam (≤ 8 mg/L). However, most of the Cambodian isolates showed growth in every concentration of ciprofloxacin and trimethoprim / sulfamethoxazole thus, categorized

as resistant (R). Besides these two antibiotics, all Cambodian isolates, except C122, are resistant against ampicillin / sulbactam (> 8 mg/L). Interestingly, C122 have the least resistant (R) characteristics in comparison with other Cambodian isolates. In addition, among other isolates, C66 and C61 are classified in the same SIR category for all antibiotics. Generally, most Cambodian isolates are less susceptible against fluoroquinolones, cephalosporins, monobactam, ampicillin/ sulbactam 2:1 ratio, ticarcillin / clavulanic acid, and trimethoprim / sulfamethoxazole.

4.3. MAGE system is functional in *E. coli* str. K-12 substr. MG1655

The Multiplex Automated Genome Engineering (MAGE) by Wang *et al.* (2012) methodology was validated in *Escherichia coli* str. K-12 substr. MG1655. After transformation with pMA7-SacB, 90-mer oligonucleotides targeting chromosomal *lacZ* gene were used as mentioned [28]. Then, the mutation was confirmed by screening white colonies as seen in Figure 6. On blue-white screening plates, white colonies indicated mutation in the *lacZ* gene that makes loss of function. In contrast, blue colonies showed unmutated gene.

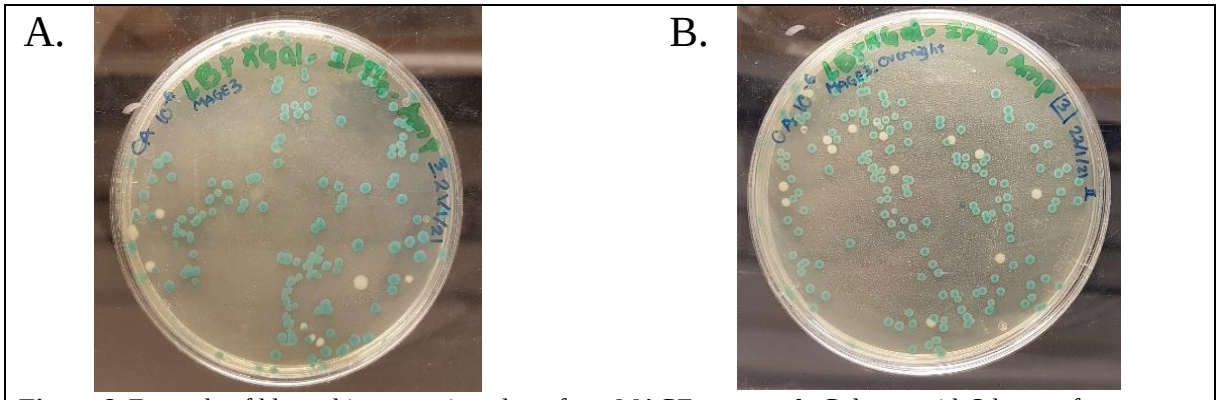


Figure 6. Example of blue-white screening plates from MAGE system. **A.** Cultures with 3 hours of recovery time. **B.** Cultures with 24 hours of recovery time.

The efficiency of the MAGE system was calculated as the ratio of white colonies to total number of bacteria colonies on plates as described in Table 6. The efficiency of the system with 24 hours of recovery time was 1% higher in average compared to the system with 3 hours of recovery time.

Table 6. The average efficiency of MAGE system with comparison of 3 hours and 24 hours recovery time.

Recovery Time	Average	Standard Deviation
3 hr	0,07	0,043
24 hr	0,08	0,033

In addition to the screening method, three blue colonies and six white colonies were sequenced. The sequencing result showed that all blue colonies contained the original *lacZ* gene whereas all the white colonies contained mutation in the gene as shown in Figure 7.

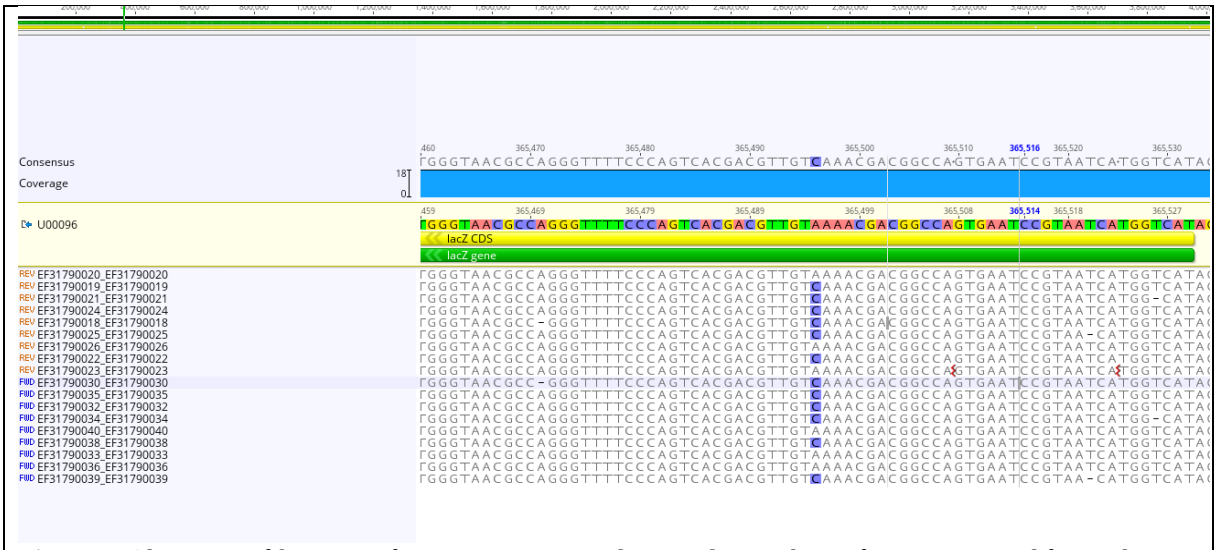
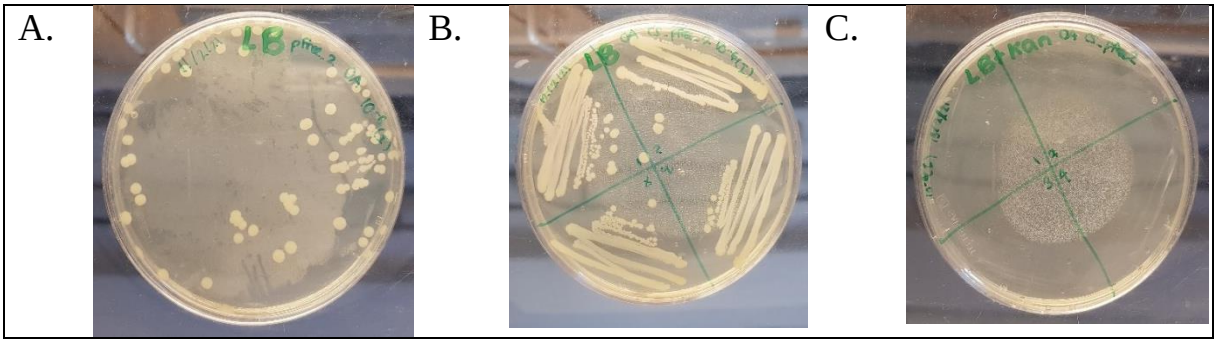


Figure 7. Alignment of *lacZ* gene from sequencing results. Results are shown from reverse and forward sequences. Three sequences showed similar and unmutated gene in the forward and reverse sequencing results while six sequences showed base changes from adenosine (A) to cytosine (C).

4.4 One-Step Curing Plasmid system could eliminate pFREE with high efficiency in *E. coli* str. K-12 substr. MG1655

The One-Step Curing Plasmid by Lauritsen *et al.* (2017) was validated in *E. coli* MG1655. The validation was done by evaluating pFREE ability to self-cure. The protocol [21] was used to transform and induce pFREE self-curing in the bacteria. The cured bacteria were grown in LB agar plates and colonies were counted after overnight incubation (Figure 8A). During the counter-selection process, sixteen colonies were selected from LB agar plates in Figure 8A and streaked on new LB agar plates (Figure 8B). Afterwards, these colonies were re-streaked on LB + Kan plates as in Figure 8C. The validation process was done with three replications and there was absence of growth in antibiotic selection plates for all selected colonies. The colony PCR was done with no amplification product around 500 bp, the expected results of pFREE *ori* replication length, for most of the samples as seen in Figure 8D. The rest of the agarose gel electrophoresis results are mentioned in Appendix. Three independent replications resulted in almost 90% colonies without amplification of pFREE plasmid during colony PCR verification.



D.

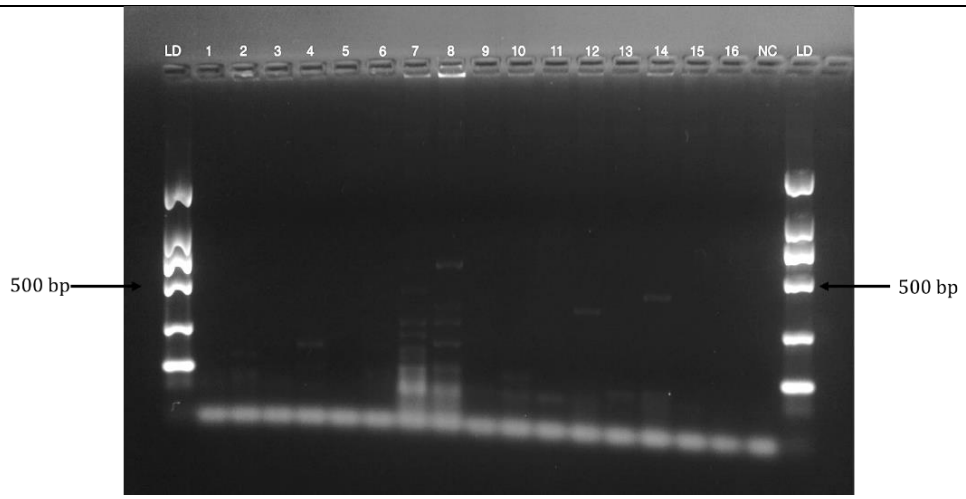


Figure 8. One-Step Curing Plasmid validation result by counter-selection process and colony PCR. (**A)** Bacteria colonies on LB agar plate after pFREE plasmid self-curing. (**B)** Four out of sixteen colonies selected and isolated from plate (A) for counter-selection process showed growth on LB agar plate after overnight incubation. **(C)** Absence of growth on LB + Kan plate from selected four colonies after overnight incubation during counter-selection process. **(D)** Example of agarose (1,5%) gel electrophoresis from pFREE colony PCR. GelPilot Mid Range Ladder (LD) used in the first and last well. Lanes 1 – 16, sixteen colonies from LB agar used in counter-selection process. Negative control (NC), PCR components without template. Bacteria colony in lane 7 showed a thin band of 500 bp.

4.5 pCAM-FREE construction resulted in insertion of gRNA5 in crArray

To accommodate pFREE activity in these Cambodian isolates, the gRNA in the system needs to be modified according to the *ori* of the targeted plasmids. There are two plasmid groups, IncR and IncFII, in selected Cambodian isolates which will be targeted with gRNA1 and gRNA5, respectively. USER cloning was used to modify crArray in pFREE and the modified pFREE is called pCAM-FREE.

The pCAM-FREE construction was done with three parts of cloning as discussed in section 3.9 and Figure 4. In the first part, crArray was divided into 3 fragments. In Figure 4A, the gRNA1 and gRNA5 was added into fragment 1 and fragment 3 using A reverse primer. The insertion was then checked with B reverse primer. In the second part of the cloning, fragment 1 and fragment 2 + 3 were ligated with USER enzyme to create a linear crArray as seen in Figure 4B. Lastly, the new modified crArray was added into the plasmid as seen in Figure 4C. For the first and second part of cloning, the results are reported in Appendix. Meanwhile, the third and the last part of USER cloning result can be seen in Figure 9.

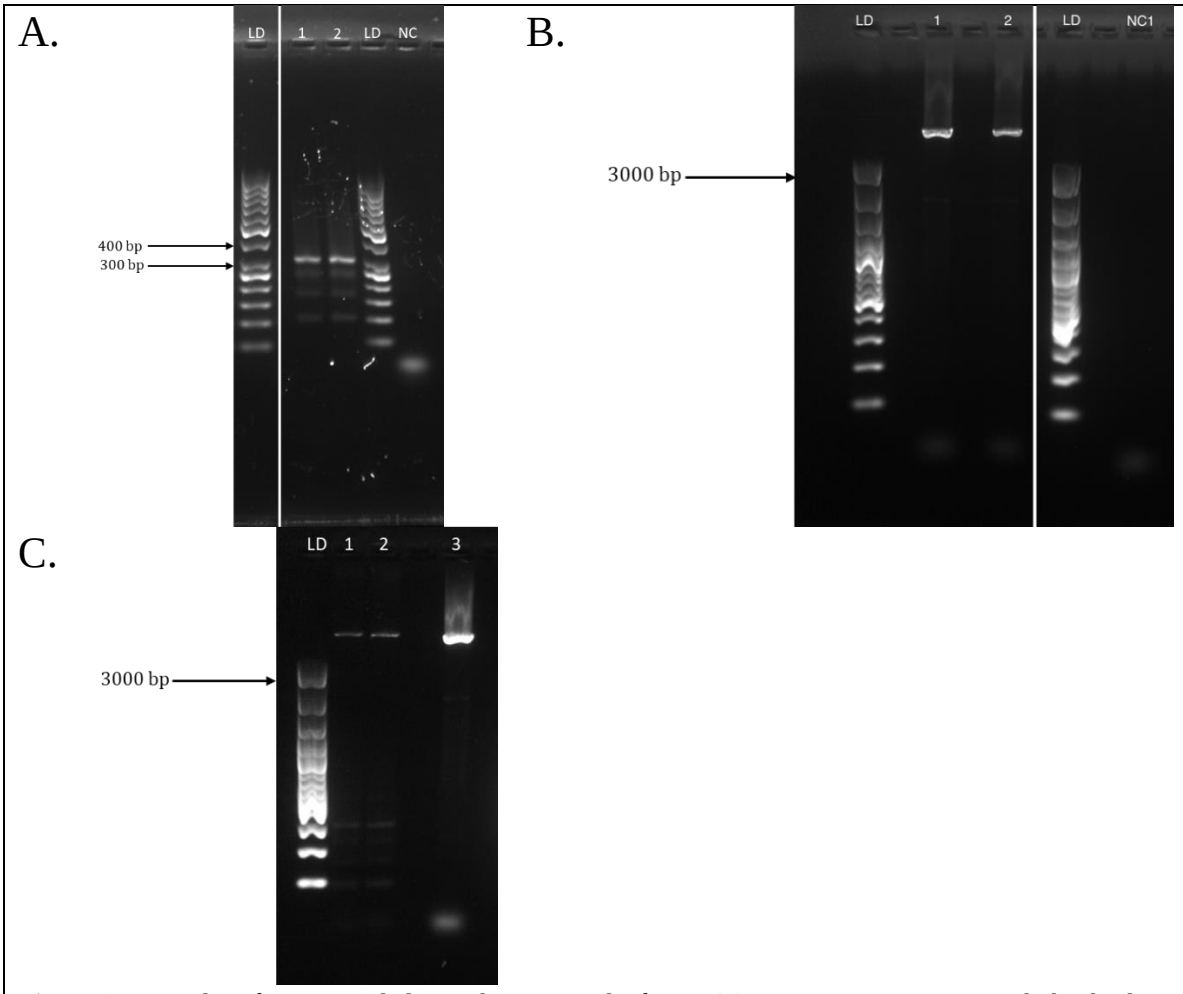


Figure 9. Examples of agarose gel electrophoresis results from pCAM-FREE construction with the third set of primer for the last cloning step. **(A)** Agarose (1,5%) gel showed amplification result of modified crArray or insert fragment with primers-containing uracil. ThermoScientific™ Gene Ruler 50 bp Ladder (LD) was used. Expected band size result of insert is 355 bp. **(B)** Agarose (1,2%) gel showed amplification result of pFREE plasmid as vector with primers-containing uracil. ThermoScientific™ Gene Ruler 100 bp Plus Ladder (LD) was used. Expected band size result of vector is 7282 bp. **(C)** Agarose (1,2%) gel showed USER Enzyme product of pCAM-FREE in comparison with pFREE plasmid vector. ThermoScientific™ Gene Ruler 100 bp Plus Ladder (LD) was used at the first well. Expected band size result of vector is 7637 bp. Lanes 1-2, USER Enzyme product of pCAM-FREE. Lane 3, linear pFREE vector without insert.

In the first step of the pCAM-FREE construction, three separate fragments called fragment 1, 2 and 3 with sizes of 96 bp, 188 bp and 71 bp, respectively, were created as seen in Appendix Figure 2A, 2C, and 2D. Amplification of fragment 1 and 3 to add new gRNA1 and gRNA5, respectively, using the corresponding A reverse primer was successfully done since both fragments could be amplified with their respective B reverse primer (Appendix Figure 2B and 2E). In the next step, fragment 1 (96 bp) and fragment 2 + 3 (259 bp) were amplified with primers-containing uracil for USER cloning. Addition of uracil in these fragments enable USER Enzyme to excise and ligate, creating the modified crArray (355 bp). For further confirmation, primer crArray_all_fw and crArray_Fragment_3B_rv were used to amplify the final modified crArray. Afterwards, the PCR product was sent for sequencing and the new gRNA5 are detected in the modified crArray (Figure 10). From the sequencing result, there are no distinctive sequence signals to interpret sequences upstream of the direct repeat (DR) and thus, gRNA1 sequence could not be detected.

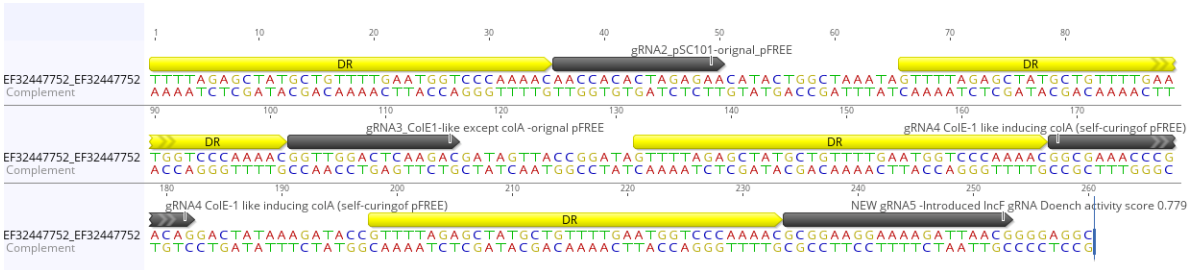


Figure 10. Sanger sequencing result of the modified crArray. The sequences of gRNA2, gRNA3, gRNA4 and the new gRNA5 are detected.

Lastly, the modified crArray or the insert was amplified to add uracil in the fragment (Figure 9). The same procedure was done on pFREE as a vector for USER cloning. Finally, uracil-containing crArray and pFREE were combined with USER Enzyme to create functional pCAM-FREE. In Figure 9C, the circular pCAM-FREE are slightly bigger compared to the linear pFREE vector. The constructed pCAM-FREE was then transformed into laboratory strain *Escherichia coli* str. K-12 substr. MG1655 and spread to LB + Kan plates as seen in Figure 11.

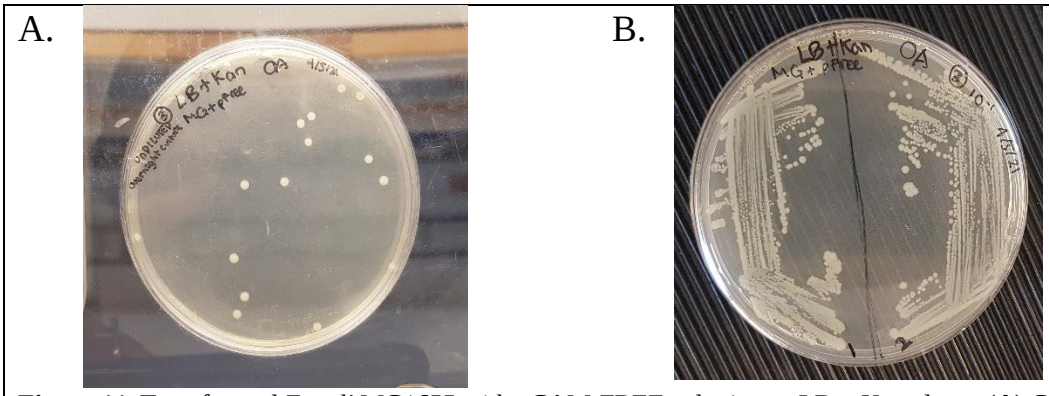


Figure 11. Transformed *E. coli* MG1655 with pCAM-FREE colonies on LB + Kan plates. **(A)** Colonies on LB + Kan plates after overnight incubation. **(B)** Isolated pCAM-FREE colonies streaked on LB + Kan plates.

From the selected colonies, the sequencing results were interpreted as in Figure 12. The presence of gRNA1 and gRNA2 with their direct repeats were detected. Unfortunately, instead of the new gRNA1 as in Table 4, the plasmid contained the previous gRNA1 designed for pSC101 plasmid replicons [21].

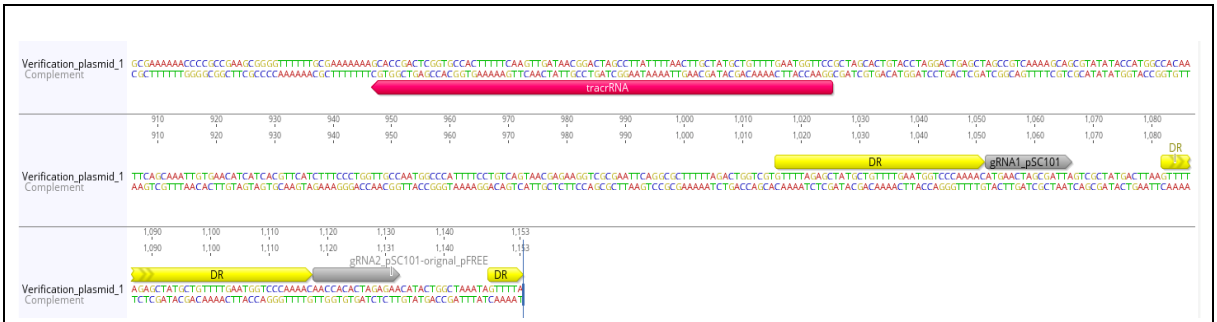


Figure 12. Sanger sequencing result of the pCAM-FREE. The sequences of gRNA1 and gRNA2 are detected. Both are designed for pSC101 plasmid replicon.

5. Discussion

5.1 Phenotypic characterization of six Cambodian isolates

The six Cambodian isolates were discriminated phenotypically by their susceptibility to antibiotic and the banding pattern of their genomic polymorphism through RAPD. The RAPD fingerprinting technique was rapid and accessible for these wild-type bacteria since it does not require the knowledge of the target's DNA sequences. The principle behind RAPD is amplification of random DNA segments with a single primer of arbitrary nucleotide sequences as stated [37]. Since the primer sequences are not specific, they will randomly amplify the DNA template depending on how complement the primer and the templates are. Therefore, when a mutation occurs in a site that was previously complement with the primer, this might prevent the primer to anneal and there will be no amplification. Based on this technique, RAPD could detect polymorphisms in DNA sequences and can be used to characterize the diversity among strains of the samples [37].

Overall, there were five different band patterns that might indicate how related these isolates are with each other. Among all the different cluster, *Escherichia coli* isolates C61 and C66 have similar RAPD band patterns indicating potential clonality. Interestingly, they also have the same sequence type (ST) when analysed with core genome multi-locus sequence typing (MLST) (Hickman *et al*, unpublished data). Since their ST correlates with their RAPD banding pattern, this might indicate that C61 and C66 are members of the same clone. When two bacteria are members of the same clone, they are defined as isolates that are highly similar or indistinguishable when discriminated with a specific molecular typing procedure in this study [38]. However, it is worth to mention that MLST used DNA sequences of seven housekeeping genes of *E. coli* [39] while RAPD analysed the whole genomic [37], [40].This could be seen in *Klebsiella pneumoniae* isolates C122 and C128 which have the same ST (Hickman *et al*, unpublished data) but different banding pattern with RAPD.

Besides RAPD, the six Cambodian isolates were also characterized by their susceptibility to 21 antimicrobial compounds. Based on the study by Atterby *et al* (2019), colistin resistance genes *mcr1* to *mcr5* and carbapenemase bla_{OXA-48} gene were not found on these isolates which is in line with their susceptibility (S) against colistin, imipenem and meropenem. Meanwhile, *E. coli* C66 and C61 isolates have slightly different phenotypes compared with the study by Atterby *et al* (2019) since they are resistant (R) against ciprofloxacin and trimethoprim / sulfamethoxazole.

Among all the Cambodian isolates, *K. pneumoniae* C122 isolate have the least resistance (R) phenotype compared to the rest. This difference might be caused by the variation of ESBL genes among these isolates where C122 have bla_{SHV-28} gene [22]. Besides C122, the rest of the Cambodian isolates are resistant (R) against the combination of ampicillin and beta-lactam inhibitor sulbactam, in 2:1 ratio and ciprofloxacin. In addition to that, there are ≥3 isolates that were resistant (R) against levofloxacin, aztreonam, cefotaxime, trimethoprim / sulfamethoxazole, and combination of ticarcillin / clavulanic acid. Moreover, there are no susceptible (S) isolates against cefepime and ceftazidime, which was expected due to the bacterial isolates' original isolation method.

The selected six Cambodian isolates from the study by Atterby *et al* (2019) are known to harbor extended spectrum β-lactamase (ESBL) genes which are CTX-M and/or SHV. ESBL-producing enterobacteriaceae are gram negative bacteria with β-lactamase enzymes that confer resistance to a wide variety of beta-lactam antibiotics including penicillin, first-, second-, and third - generation cephalosporins, and aztreonam [3]. The presence of these genes is in line with

their resistance (R) or susceptible with increased exposure (I) against the mentioned antibiotics above.

Another interesting characteristic that these ESBL containing bacteria have is co-resistance (R) to fluoroquinolone antibiotics e.g., tested ciprofloxacin and levofloxacin. Fluoroquinolones are broad-spectrum antibiotics with oral usage. It is one of the most widely used antibiotics for the treatment of urinary tract infection, of which *E. coli* is the most common pathogen [41]. Besides that, fluoroquinolones are one of the antibiotics that is used on food animal farms in Cambodia for prevention and treatment of diseases [42]. Based on their antibiotic susceptibility test, these selected Cambodian isolates are categorised as multidrug resistant (MDR) bacteria [43].

Generally, high usage of antibiotics is known to be associated with increased prevalence of resistance bacteria over time [44] and accordingly, it is unsurprising for these isolates to have resistance to multiple antibiotics. There are several factors that could be attributed to the multiple resistance phenotype in these Cambodian bacteria isolates. Studies [45], [42] have shown that antibiotic misuse such as non-prescription antibiotics is a common practice in the Cambodian community. Unrestricted access, antibiotic-seeking behaviour, and poor knowledge of antibiotics are the main factors that has driven inappropriate use of antibiotics. Farmers also believed that antibiotics were necessary for disease prevention and growth promotion in their animals which, leads to high antibiotic usage. Moreover, antibiotic resistance genes are transmissible to another bacteria through plasmids [3], [46] and mobile genetic elements [18]. In the end, these factors lead to the emergence and establishment of multiple resistant bacteria in Cambodia.

5.2. Efficiency of MAGE and One-Step Curing Plasmid system in laboratory strain *E. coli*

The validation of MAGE system in control laboratory strain of *E. coli* str. K-12 substr. MG1655 was done by using specific oligos (Table 3) to induce mutation in *lacZ* gene. The insertion of guanosine (G) instead of adenine (A), replaced the base AAT that encode asparagine to a stop codon. For visualization of this mutated bacteria, the blue-white screening method was used. Blue-white screening is a selection method to distinguish bacterial colonies based on the blue or the white pigment it forms on the plate. The blue pigment forms when β-galactosidase enzyme, encoded by *lacZ* gene, hydrolyze synthetic substrate of X-gal. Hydrolysis of X-gal results in galactose and 5-bromo-4-chloro-4-hydroxyindole of which, the latter will form a blue pigment. When *lacZ* gene is interrupted by a mismatch base as in this study, the bacteria will not produce β-galactosidase and thus, cannot metabolize X-gal which results in white colonies [47].

In the plates of blue-white screening after MAGE recombineering, the white colonies are counted as bacteria that has mutation in the *lacZ* gene. Then, we compare them to the total colonies for efficiency. Generally, the average efficiency after 3 experimental replicates, is less than 10%. Based on Wang *et al* (2009), MAGE system could achieve approximately 1,5 – 16% replacement efficiency when introducing 1 – 30 bp mismatch bases with oligo. However, the number of MAGE cycles also attributed to the system efficiency. For example, the study used 30-bp mismatches oligo for *lacZ* gene with 15 cycles and achieve 21,8% replacement efficiency in the bacterial population. In contrast, although there is only 1-bp mismatch in our primer, we only add the oligos once to the bacteria or one MAGE cycle before plating them.

Furthermore, we found that there is a 1% higher efficiency when we incubate the culture for 24 hours of recovery time instead of 3 hours. Even though we did not study the significance of this difference, we would use a 24 hour of recovery time for future MAGE projects. In the end, we

also sequenced the white and blue colonies. As expected, there are mismatches in the *lacZ* gene of the white colonies when aligned with the *lacZ* gene from blue colonies. This indicates the successful genetic modification with MAGE in a control laboratory strain.

The One-Step Curing Plasmid validation was done by checking the ability of pFREE plasmid to self-curing in a control laboratory strain. A counter-selection process was used to visualize the self-curing ability of pFREE. Among the 3 experimental replicates, there was no growth found in LB + Kan which indicates a high efficiency self-curing mechanism in pFREE. For more precision, colony PCR was done and the efficiencies of pFREE are estimated to be almost 90%. In all the different replications, there are 1-3 colonies that showed a band of 500 bp which correspond to the size of pFREE *ori*. There are also several faint bands that could be seen with different sizes which most likely are unspecific amplification due to the usage of bacteria colony, instead of plasmid, as a template for PCR. As aforementioned, Lauritsen *et al* (2017) obtained pFREE curing efficiency of 40 – 100% depending on the context of the plasmid. Therefore, the curing efficiency of the control laboratory strain is still in line with the previous study.

5.3. The outcome of pCAM-FREE construction

From the sequencing result, we have successfully inserted a new gRNA5 to the crArray. However, we did not manage to change the gRNA1 sequence. Notably, we only sequenced three of our colonies from LB + Kan plates after transformation with pCAM-FREE. We might be able to find more interesting results by screening more colonies and sending them for sequencing. Nevertheless, the gRNA1 in [21] was to recognize pSC101 replicon group plasmid whilst the modified one was for IncR replicon plasmid. From the selected *E. coli* and *K. pneumoniae* Cambodian isolates, we managed to categorize their plasmids into two group based on their replicon, IncFII and IncR (Hickman *et al*, unpublished). Based on the pCAM-FREE that we designed, we would be able to eliminate plasmids with IncFII replicon group however, future research is still needed.

5.4 Limitations

There are several limitations that we experienced within this study. Firstly, it is worth to mention that the crArray was naturally designed to have repeat sequences. Additionally, the foreign DNA or the gRNA was located between two of the repeat sequences [20]. The presence of repeat sequences is one of the main challenges in genomic modification studies using PCR. Furthermore, the primers are a crucial part of the USER cloning method. Consequently, optimization of primers could be an option to improve cloning efficiency for future research purposes.

Secondly, the PCR protocols used in this study require further optimization. Various combinations of primers and templates is an essential part of this study in order to answer research questions. For this reason, it is crucial to conduct a thorough and in-depth optimization. The aim of optimization was to maximize the amplification of the desired product while minimizing the production of unspecific amplicons. This could be achieved through optimization of the denaturation and annealing temperature, concentration of the divalent cations (mostly Mg2+), and number of cycles [48].

Lastly, the lack of a reliable reference method for colistin antibiotic susceptibility testing is a limitation. Colistin is a naturally cationic antibiotic and thus, could adhere to organic or

inorganic materials with a varying degree of adherence such as on polystyrene microplate. Based on this property, disk diffusion and Etest are unreliable to determine colistin MIC. A study [49] showed the possibility of using broth microdilution with polysorbate 80 as the reference method. They also tested Sensititre (GNX3F) plates and showed no false susceptibility in regards with the isolates they tested. Nevertheless, it might be interesting to use other antibiotic susceptibility testing to examine the reliability and reproducibility of this data.

5.5 Future research

For further characterization of these isolates, we could test the constructed pCAM-FREE plasmid in the selected Cambodian isolates with IncFII plasmids. Since the usage of this system in environmental/community bacteria is novel, optimization is required to adjust the conditions for plasmid curing. It should also be noted that these isolates have multiple antibiotics resistance which might complicate the plasmid curing system. Nonetheless, this could provide future opportunities in using pFREE plasmid for a one-step plasmid curing in other environmental/community bacteria isolates. Along with that, the validated MAGE system can also be used to mutate specific antibiotic resistance genes of the isolate i.e., ciprofloxacin, to possibly generate a susceptible bacteria isolate. MAGE with low specificity could also be used to generate a library with high diversity. Then, screening of antibiotic susceptible bacteria could be done with the library. From this result, further characterization of their phenotypical and genomic properties can be done.

5.6 Conclusion

Overall, our study managed to characterize the selected Cambodian isolates, validate, and modify pFREE plasmid in One-Step Curing Plasmid system. The selected Cambodian isolates were successfully characterized with RAPD and AST methods. We discovered that the isolates are diverse and there is a potential clonality between two of the isolates. Additionally, the isolates are categorized as multidrug resistant. Due to the presence of ESBL genes, these isolates are less susceptible or resistant towards beta-lactam antibiotics of penicillin, cephalosporins and aztreonam and towards fluoroquinolone. Developed methodologies of MAGE by Wang *et al.* (2012) and One-Step Curing Plasmid by Lauritsen *et al.* (2017) were assessed in a control laboratory strain of *E. coli* MG1655. From the assessment, we achieved MAGE system's efficiency of 8% and One-Step Curing Plasmid system's efficiency of almost 90%. The plasmid pCAM-FREE was constructed with insertion of gRNA5 for IncFII plasmids although the modified gRNA1 was not inserted. Expression of pCAM-FREE plasmid or MAGE application in Cambodian isolates has not yet been tested in the study.

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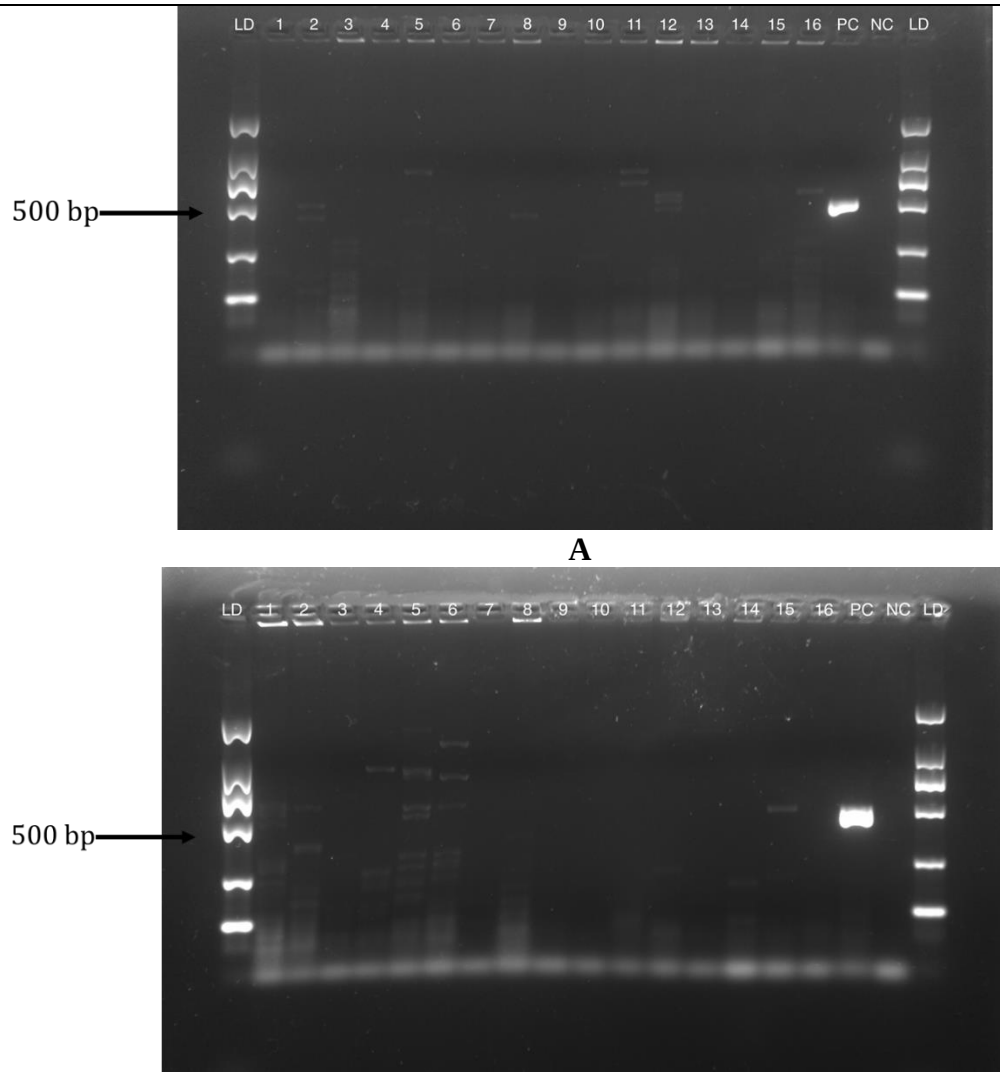
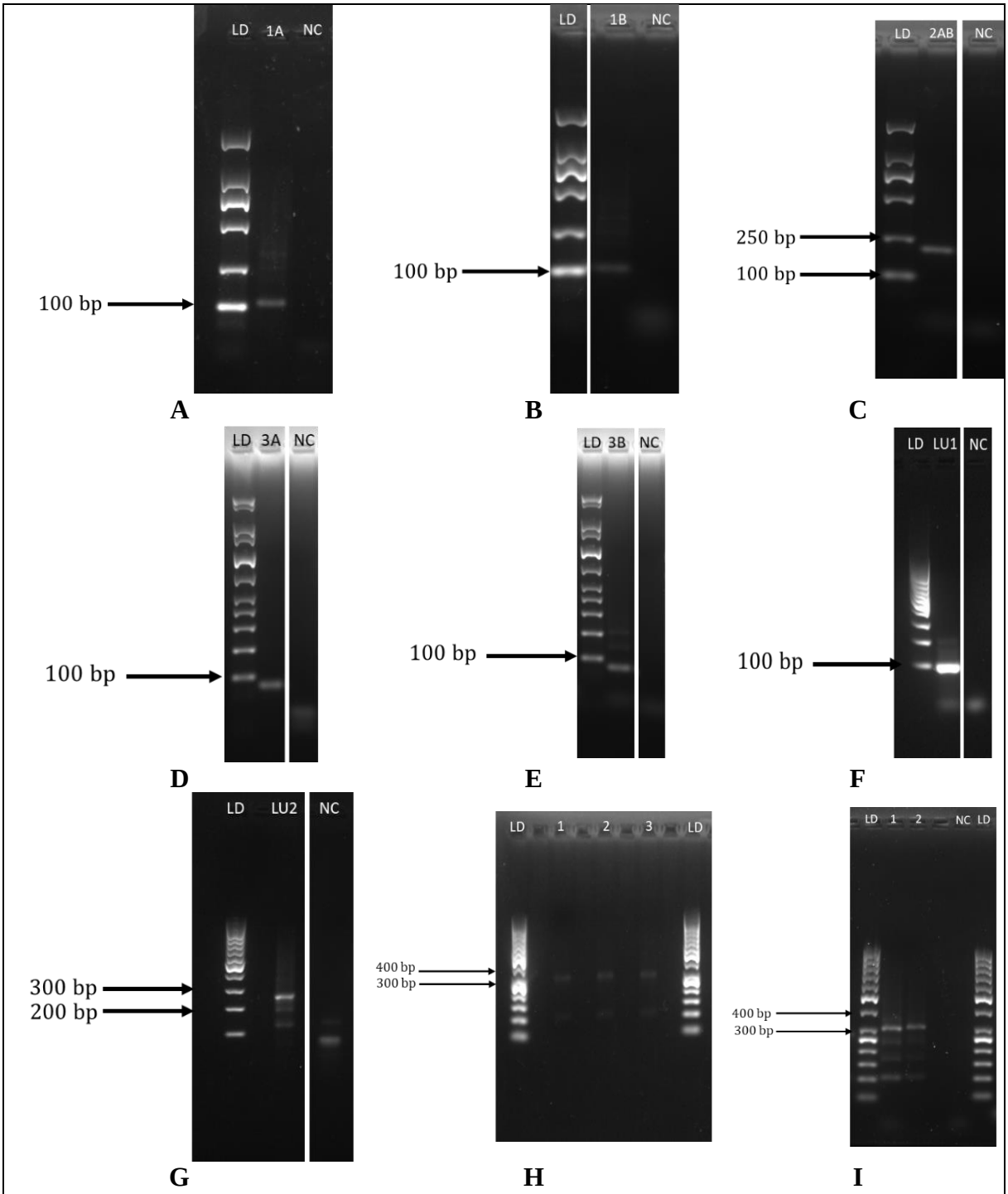
Appendix

Appendix contains additional data including results from the One-Step Curing Plasmid validation (Appendix Figure 1) and pCAM-FREE construction (Appendix Figure 2).



Appendix Figure 1. Agarose (1,5%) gel electrophoresis from pFREE colony PCR. Qiagen GelPilot Mid Range Ladder (LD) used in the first and last well. Lanes 1 – 16, sixteen colonies from LB agar used in counter-selection process. Plasmid pFREE used as Positive Control (PC). Negative control (NC), PCR components without template. **(A)** Bacteria colony in lane 2, 8 and 12 showed a thin band with similar size to PC, approximately 500 bp. **(B)** Bacteria colony in lane 1 showed a thin band with similar size to PC, approximately 500 bp.



Appendix Figure 2. Agarose gel electrophoresis result from the first and second parts of pCAM-FREE construction. Qiagen GelPilot Mid Range Ladder (LD) is used in (A), (B) and (C). Qiagen GelPilot 1 kb Plus Ladder (LD) is used in (D) and (E). Thermo Scientific™ Gene Ruler 100 bp Ladder (LD) is used in (F) and (G). Thermo Scientific™ Gene Ruler 50 bp Ladder (LD) is used in (h) and (i). Negative control (NC) is amplification of PCR components without template. **(A)** Amplification of fragment 1 to add gRNA1. Expected result of band with 96 bp. **(B)** Amplification of fragment 1 to verify the presence of gRNA1. Expected result of band with 96 bp. **(C)** Amplification of fragment 2 crArray that contains gRNA2, gRNA3 and gRNA4. Expected result of band with 188 bp. **(D)** Amplification of fragment 3 to add gRNA5. Expected result of band with 71 bp. **(E)** Amplification of fragment 3 to verify the presence of gRNA5. Expected result of band with 71 bp. **(F)** Amplification of fragment 1 with linear USER (LU) primers-containing uracil. Expected result of band with 96 bp. **(G)** Simultaneous amplification of fragment 2 and 3 as templates with linear USER (LU) primers-containing uracil. Expected result of band with 259 bp. **(H)** USER Enzyme of fragment 1 and fragment 2 + 3 result in agarose gel electrophoresis. Expected result of band with 355 bp. **(I)** PCR confirmation for final modified crArray with crArray_all_fw and crArray_Fragment 3B_R. Expected result of band with 355 bp.