Beginning Steps in Construction of a Plasmid Free from Antibiotic Resistant Genes for DNA Vaccine

Evan L. Khaleef 1, Farhad Abdukarim 2

1Assist. Lecturer (M.Sc.Med.Microbiol.), Ph.D Student, Medical Research Center, HMU, 2 Assist. Professor, (Ph.D. Molecular Biology) Kurdistan Institute of strategic studies and scientific research

Abstract

Objectives: Bacterial cell cultures containing antibiotic resistant genes represent a problem of growing concern. Spreading of bacterial genes encoding antibiotics resistance provides an environmental hazard, which is worsened by the release of antibiotics from the fermentation facility as well. This study has been undertaken to construct ,a new plasmid free from antibiotics to be used for DNA vaccine in the next future.

Subject and Methods: DNA vaccine has initiated a new era of vaccine research. Restriction enzyme and T4 DNA ligation from NEB were used for Restriction/Ligation of DNA and two PCR methods were used in this study long and short PCR. All plasmid and PCR products were analysed on 1% agarose gel.

Results: A plasmid free from antibiotic resistant genes was constructed from pBR322 by delete the bla gene by long PCR and tet^r gene was deleted by direct repeat homologous recombination the final constructed plasmid is confirmed by gel electrophoresis in compare with two control, origin pBR322 and pBR322-Δamp in addition to specific lambda DNA marker.

Conclusion: It is concluded that the vector used as DNA vaccine which be free from antibiotic can play the same role as those contain antibiotic and eventually prevent horizontal separation of antibiotic resistant gene.

Keywords: Plasmid, Antibiotics resistant genes, pBR322, PCR

Corresponding author: Evan L. Khaleef, E-Mail: evan-latif@yahoo.com

Introduction

DNA vaccination is a technique for protecting an organism against disease by injecting it with genetically engineered DNA to produce an immunological response. Nucleic acid vaccines are still experimental, and have been applied to a number of viral, bacterial and parasitic models of disease, as well as to several tumors models. DNA vaccines have a number of advantages over conventional vaccines, including the ability to induce a wider range of immune response types. Vaccines are among the greatest achievements of modern medicine – in industrial nations, they have eliminated naturally-occurring cases of smallpox, and nearly eliminated polio, while other diseases, such as typhus, rotavirus, hepatitis A and B and others are well controlled(1).

Conventional vaccines, however, only cover a small number of diseases, and infections that lack effective vaccines kill millions of people every year, with AIDS, hepatitis C and malaria being particularly common.

First generation vaccines are whole-organism vaccines – either live and weakened, or killed forms. Live, attenuated vaccines, such as smallpox and polio vaccines, are able to induce killer T-cell (T_C or CTL) responses, helper T-cell (T_H) responses and antibody immunity. However, there is a small risk that attenuated forms of a pathogen can revert to a dangerous form, and may still be able to cause disease in immunocompromised people (such as those with AIDS). While killed vaccines do not have this risk, they cannot generate specific killer T cell responses, and may not work at all for some diseases. In order to minimize these risks, so-called *second generation vaccines* were developed.

These are subunit vaccines, consisting of defined protein antigens (such as tetanus or diphtheria toxoid) or recombinant protein components (such as the hepatitis B surface antigen). These, too, are able to generate T_H and antibody responses, but not killer T cell responses [(2)].

DNA vaccines are *third generation vaccines*, and are made up of a small, circular piece of bacterial DNA (called a plasmid) that has been genetically engineered to produce one or two specific proteins (antigens) from a micro-organism. The vaccine DNA is injected into the cells of the body, where the "inner machinery" of the host cells "reads" the DNA and converts it into pathogenic proteins. Because these proteins are recognized as foreign, they are processed by the host cells and displayed on their surface, to alert the immune system, which then triggers a range of immune responses [(1, 2)].These DNA vaccines developed from “failed” gene therapy experiments. The first demonstration of a plasmid-induced immune response was when mice inoculated with a plasmid expressing human growth hormone elicited antibodies instead of altering growth[(3)].Thus far, few experimental trials have evoked a response sufficiently strong enough to protect against disease, and the usefulness of the technique, while tantalizing, remains to be conclusively proven in human trials. However, in June 2006 positive results were announced for a bird flu DNA vaccine[(4)]and a veterinary DNA vaccine to protect horses from West Nile virus has been approved (2005)[(5)]. In August 2007, a preliminary study in DNA vaccination against multiple sclerosis was reported as being effective [(6)]. Therefore, this study has been undertaken for construction a new plasmid.

Materials and Methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this work are listed in Table 1. Competent cells from *E. coli*-strain DH5α were used as transformant cells for all plasmid constructs.

2.2. Media and growth conditions

Liquid and solid media were based on LB medium (1% Bactotrypton, 0.5% yeast extract, 1% NaCl and 1.6% Agar). Antibiotics were added to the medium when required to give the following final concentrations: ampicillin 200mg / Tetracycline 25mg /L and chloramphenicol 20mg/ L [(7)].

2.3. DNA manipulations and Transformation

Restriction enzymes and T4 DNA ligation (New England Biolabs) were used for restriction/ligation of DNA. The DNA manipulations were performed, according to conventional methods [(8)] or following manufacture recommendation. QIAGEN II extraction kit (QIAGEN) was used for gel purification of DNA-fragment from 0.8% agarose gel. The exonuclease I enzyme (New England Biolabs) was used to create blunt ended PCR products by cleaving the non-template nucleotide overhangs. Gen Elute plasmid Mini prep kit (Sigma-Aldrich) was used for all plasmid extractions. The plasmid transformations

were done following the $CaCl_2$-procedure [9].QIAGEN II extraction kit (QIAGEN) was used for gel purification of DNA-fragments. The primers used for PCR amplifications are listed in (Table2).Two PCR methods were used in this study: Short template PCR amplifications were performed Phusion High-Fidelity PCR Kit (New England Biolabs). Preparation of the PCR cocktail was performed as kit manufacture. b) Long template PCR amplifications were performed using the Long Amp PCR Kit (New England Biolabs). Preparation of PCR cocktail was performed, according to the manufactures description. All plasmids and PCR products were analyzed on 1% agarosegels [8].

Table 1: Bacterial strain and plasmids.

Genotypes and Comments		Reference
plasmids		
pBR322	*bla*$^+$,*tet*$^+$	Fermentas
pBR-Δamp	*tet*$^+$	This work
pBR-Δtet	*bla*$^+$	This work
pTrc99A	*bla*$^+$,*lacI*^{q},*trc-promotor*	Dr. Farhad
pTrc600	*bla*$^+$,*lacI*^{q},*trc-sinfA*	This work
pTrc2000	*bla*$^+$,*lacI*^{q},*trc-infA*	This work
pTrc-ΔsinfA	*bla*$^+$,*lacI*^{q},*trc-ΔsinfA*	This work
pKO3	*cat*, M13 ori, *sacB, repA(ts)*	Dr. Farhad
pKO3-ΔsinfA	*cat*, M13 ori, *sacB, repA(ts)*plus the flanking regions of infA	This work
strains		
DH5α	*sup*E44 D*lac*U169(ϕ80*lac*ZDM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Dr. Farhad
W3110	Wild type	Dr. Farhad

Table 2: Primers and their relevant sequences

Primers	Sequence	T(a)
AMPus	CTGTCGGGCCCAGTTTACTCATATATAC	65
PSGS1	GTATCACGGGGCCCTTTCGTCTTCAAGA	65
PHIA2	CATGTTCCCGGGATTACAGACAGA	61
PHIA3	CCAGAGGATTCCATGGCCAAAGAA	61
InfA3	AAAAGGCCGGTTAAACCGACCT	61
Amp1	GAGTAAAGTTGATATGACAG	54
Amp2	AAAGGAAGCATATGAGTTATT	54
Rek1	TGAATGGAATTCGGCGGCAC	56
Rek3	CAGGACCGAATTCTGCCCGA	56
Inf+1	TTTCTCCAGTCGACCCCACCTCT	61
Inf2-R	ACATCTGGATCCGACGTGACCCAT	61
Deli 1	TTTGGCCATCTAATCCTCTGGGGT	61
Deli 2	GTCGCTCATTCTTTACCGCCTGA	61
Pko3-F	AGGGCAGGGTCGTTAAATAGC	58
Pko3-R	TTAATGCGCCGCTACAGGGCG	58

Results

3.1 Construction of pBR322-ΔAmp lacking the *bla* gene

As first step the plasmid pBR322 was modified to carry the tetracycline-resistance gene (*tet*^{r}) as sole antibiotic resistant gene. In order to delete the *bla* gene (ampicillin resistance gene) from pBR322, the long template PCR technique was used to amplify whole plasmid except *bla* gene. Primers AMpus and PSGS1, complementary to regions upstream and downstream in an outward direction from the *bla* gene, were used (Table 2). The resulting linear DNA-fragment was treated with exonucleas I, blunt end ligated and transformed in to competent DH5α-cells. To confirm deletion of *bla* gene in pBR322-ΔAmp, *tet*^{r} colonies were screened for their Amp^S phenotype on LB-plates with ampicillin, followed by restriction enzyme analyses of the pBR322-ΔAmp and PCR amplification by two primers Amp1 and Amp2 which pBR-Δamp gave no result while the control pBR322 gave 861bp (*bla* gene size)(Fig.1a & 1b).

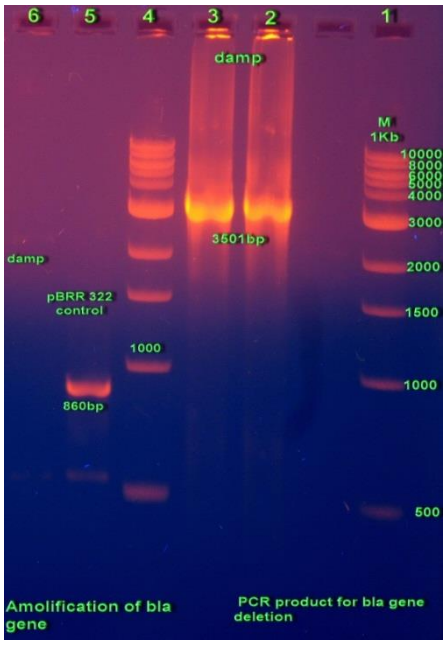

Figure (1a):Shows the deletion of bla gene from pBR322 (lane2&3) and lane 3&4 show the amplification of *bla* gene in pBR322 as control and for damp.

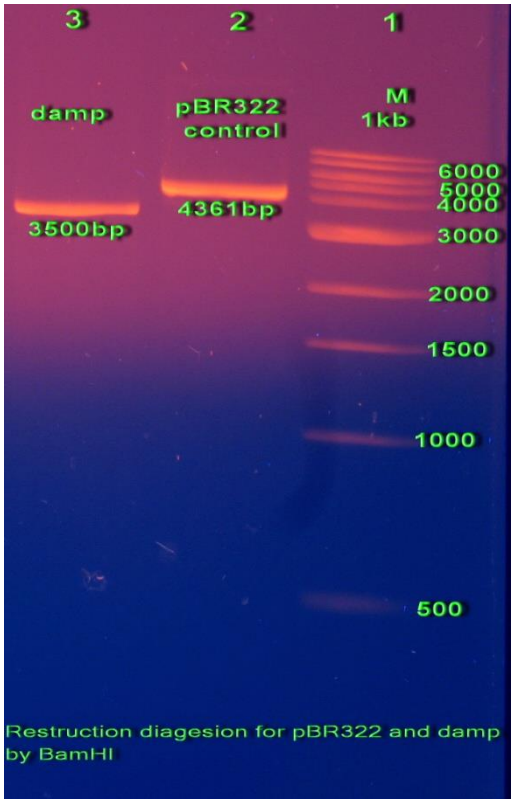

Figure (1b): Shows restriction digestion by *Bam*HI for pBR322 (control) and damp.

3.2.Construction of A5(pBR322-dRek) plasmid with two direct repeats (Rek) for spontaneous homologous recombination

A 150bp long sequence downstream of the tetracycline-resistance gene, was PCR amplified. Primers Rek1 and Rek3, complementary to regions downstream of the *tet*^{r}gene, were used for this purpose (Table 2). The obtained sequence was then cloned into the *Eco*RI-site upstream of the *tet*^{r} gene on plasmid pBR322, giving rise to plasmidA5 (pBR322-dRek). In this plasmid the *tet*^{r} gene is thus flanked by two identical sequences in same orientation. These identical sequences will serve as spontaneous homologous recombination targets in order to later promote deletion of the entire plasmid–borne *tet*^{r}gene (Fig. 2a & 2b).

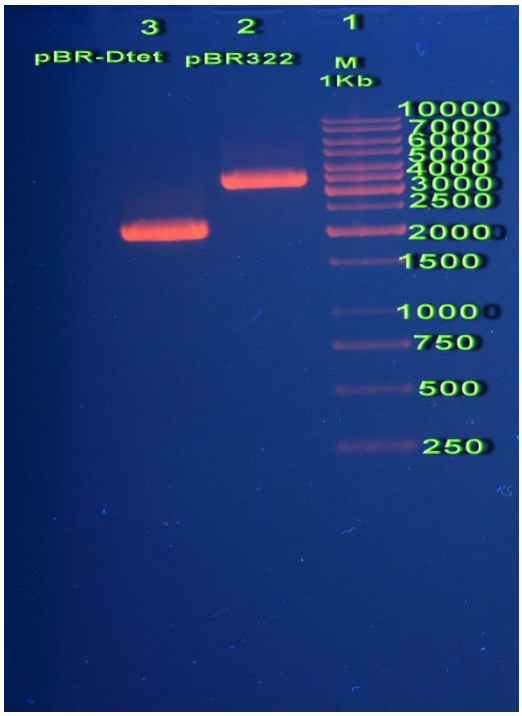

Figure 2a:Shows the differences in size between pBR322 and pBRdtet after deletion of *tet*^{r}gene from pBR322 by homologous Recombination.

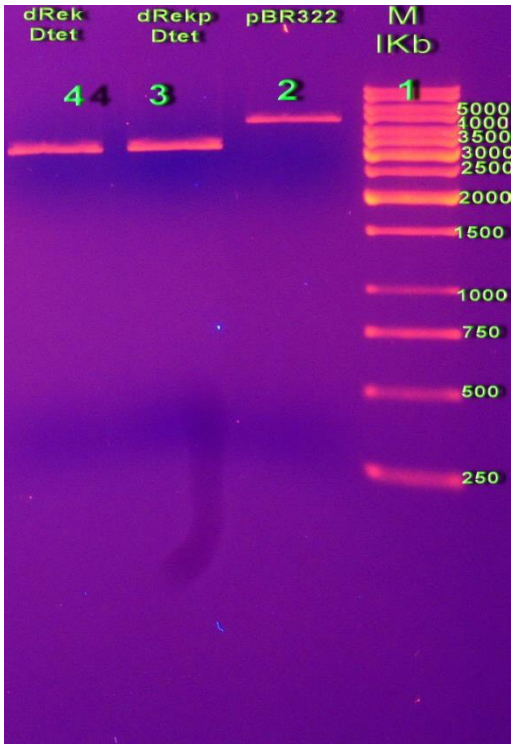

Figure 2b: Shows restriction digestion by *Eco*RI for pBR322 as control and pBR-dtet. See the difference.

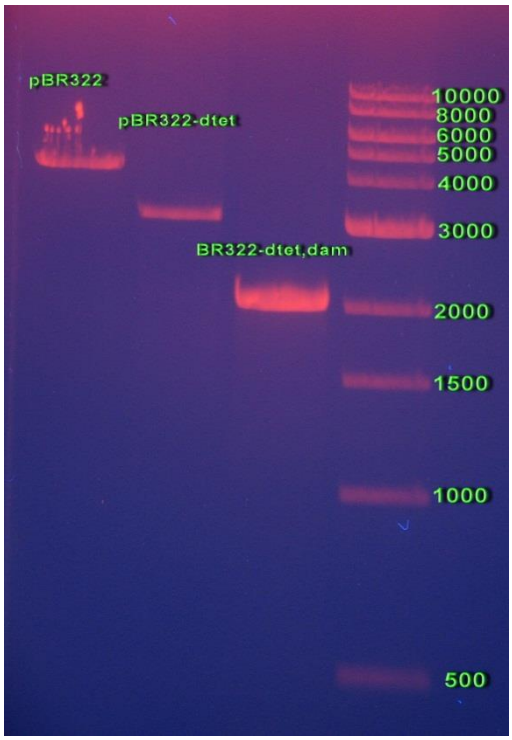

Figure (3): Shows the deletion of two antibiotic resistant genes from pBR322 as pBR322-dtet, damp plasmid in compare with two controls pBR322 and pBR322-dtet.

3.3. Deletion of bla gene from pBR322-Δtet by long PCR amplification

After deletion of tet^R gene from pBR322 by spontaneous homologous recombination, we get plasmid with single antibiotic resistant gene (*bla* gene) pBR322-Δtet, long PCR amplification then used to delete the *bla* gene. Two primersAMpus and PSGS1 were used for deletion of *bla* gene from pBR322-Δtet. After PCR amplification, gel document system showed the result in compare with two controls, one intact pBR322 other was pBR322-Δtet (contain just *bla* gene) and our PCR product pBR322-ΔtetΔamp which are named EB5 plasmid (Fig.3).

3.4. Construction of pTrc600 with the *infA* gene

The *infA* gene was cloned into plasmid pTrc99A. To do this *infA*gene was PCR amplified using chromosomal DNA of wild type *E. coli* strain W3110 as template. Primers PHIA3 & InfA3 were used (Table 2) and (Fig. 4a). Next, the *infA* gene was sub cloned into the (MCS) site of pTrc99A, using the sites *Nco*Iand*Sma*I, to place it under control of IPTG-inducibletrc promoter and give pTrc600 plasmid (Fig.4b).

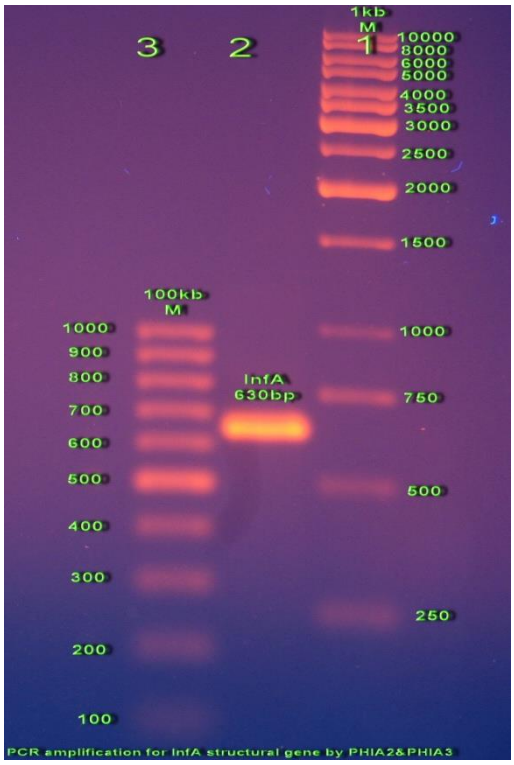

Figure 4a: Shows PCR amplification of 630bp (lane 2) structural *Inf*A from W3110 by two primers PHIA2&PHIA3. Lane 1 is 1kb marker and lane 3 is 100bp maker.

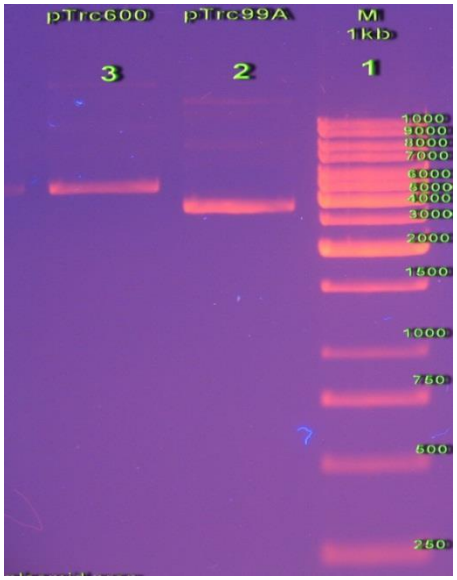

Figure 4b: Shows linearized pTrc99A (as control) and cloned plasmid pTrc600. See the difference.

3.5. Construction of plasmidpTrc2000

In order to give dependence on the *infA* gene in plasmids, a chromosomal deletion of this gene must be created. For this purpose, a 2000bp (*inf*A gene operon) fragment including the *infA* gene with surrounding regions, was PCR amplified using DNA from W3110 and primers inF+1&inF2-R (Table 2) and (Fig. 5a). The resulting fragment was inserted into pTrc99A cloning site to give pTrc2000 (Table 1) (Fig. 5b).

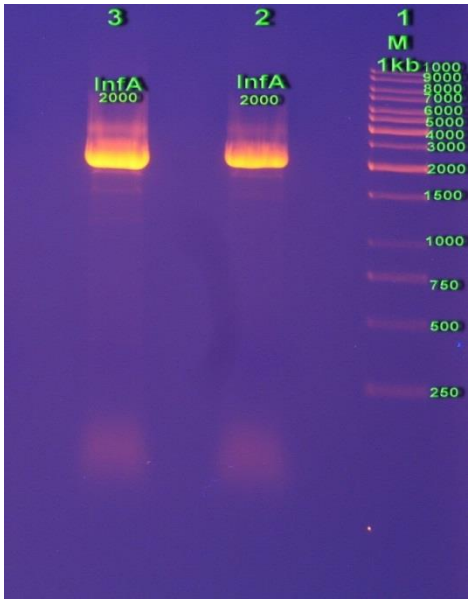

Figure 5a:Shows PCR amplification of InfA operon (lane2&3) by two primers inf+1 and inf2-R. Lane 1 shows 1kb marker.

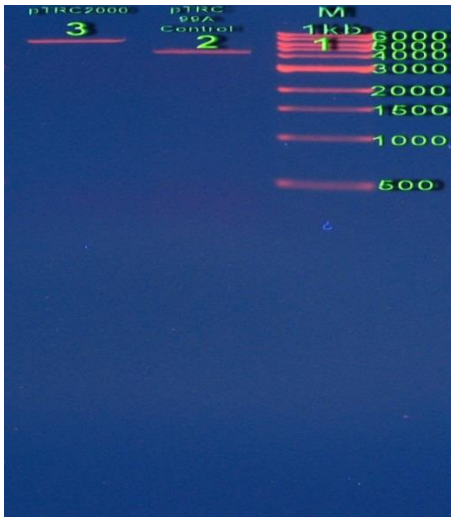

Figure 5b:Shows pTrc99A cloned with infA operon (2000bp). Lane 2 Shows pTrc99A as control & lane 3 is cloned plasmid (pTrc2000). Lane 1 shows 1kb marker.

3.6. Construction of plasmid pKO3-ΔinfA for deletion of the chromosomal infA gene

This plasmid was used as template for a second round of PCR amplification, by

using the two primers Deli1 and Deli2 (Table 2) and (Fig. 6). These primers are complementary to regions surrounding the structural *InfA* in an outward direction. They were used to amplify the whole pTrc2000-Δ*infA*, plasmid including the upstream and downstream regions of *infA*, but excluding the structural gene itself. The PCR product was religated giving plasmid pTrc2000-Δ*infA* that was transformed into DH5α competent cells. The result confirmed by PCR amplification for structural infA gene by two primers PHIA3 & InfA3 (Table 2) and the result showed complete deletion of infA open reading frame (ORF) in compare with positive control without any deletion pTrc2000 (Fig. 7). After plasmid preparation and digestion with *Sal*I&*BamH*I, the ΔinfA fragment from pTrc2000-Δ*infA* was finally sub-cloned into pKO3 to generate pKO3-Δ*infA* (Fig. 8).

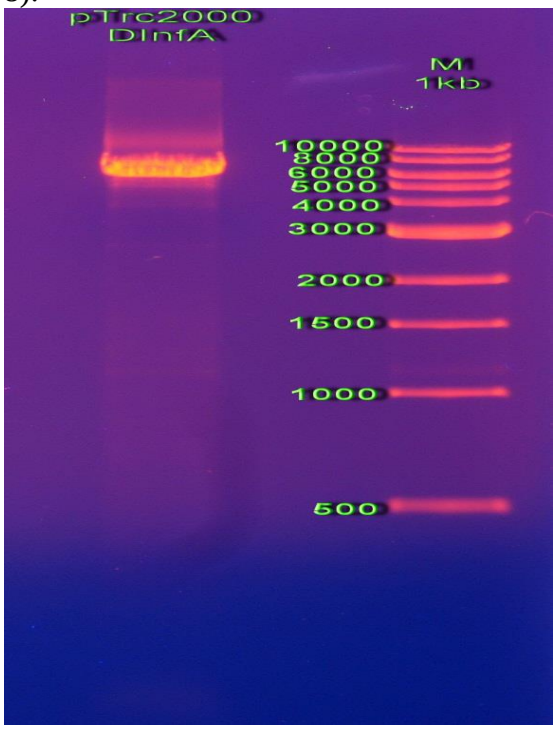

Figure 6: Shows the PCR amplification for deletion of *inf*A structural gene from pTrc2000.

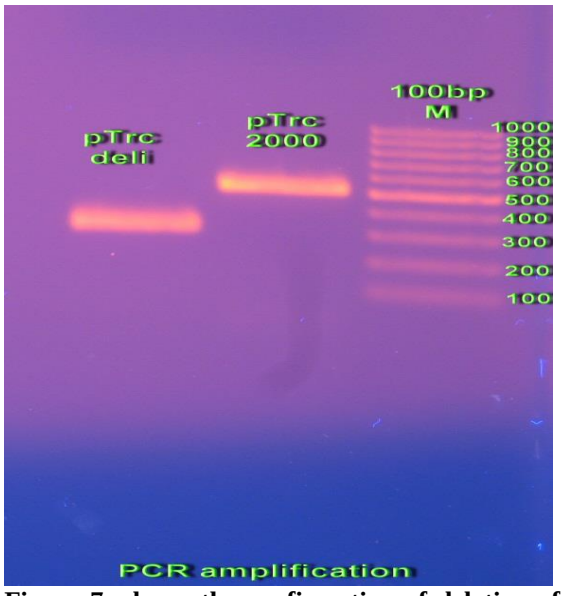

Figure 7: shows the confirmation of deletion of structural *inf*A by PCR amplification to deleted region by two primers PHIA3 & InfA3 in compare to positive control pTrc2000.

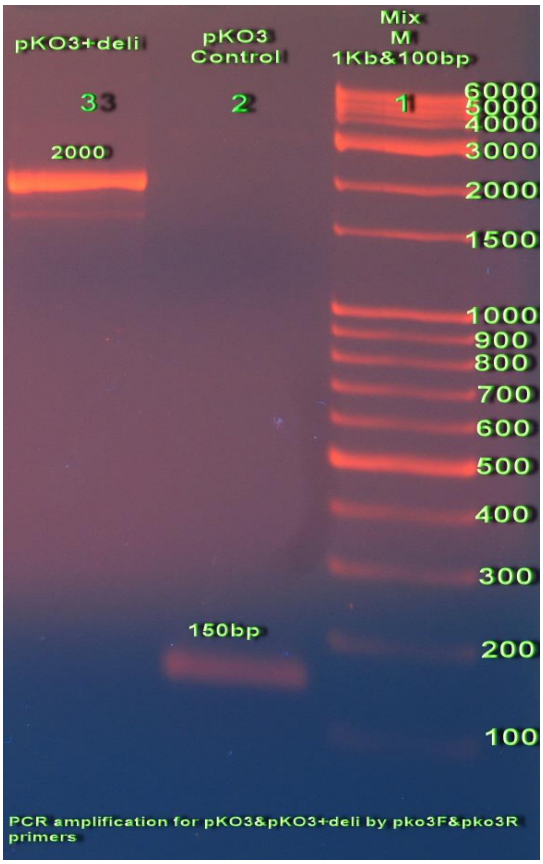

Figure 8: Shows PCR amplification for pKO3 MCS after cloning of ΔinfA by two primers pKO3-F and pKO3-R. Lane 1 is 1kb marker, lane 2 is pKO3 without cloning as control and lane 3 shows pKO3 with cloned fragment.

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Discussion

Construction of plasmid free from antibiotic resistance gene for DNA vaccine and other biotechnology process is very important point and require wide study and evaluation. In our study, we tried to construct plasmid free from any antibiotic resistance gene and we start with pBR322 which contain two antibiotic resistant genes ampicillin (*bla)* and tetracycline (*tet*^{r}*)*. We choose pBR322 because:
I. It is stable. II. It is high copy number. III. It has good number of restriction enzymes. IV. It is small in size (4361bp). Our study started with construction of pBR322-Δamp. The plasmid pBR322 contain two antibiotic resistance genes (*bla*gene about 860bp, and *tet*^{r} gene about 1190bp). It was necessary to delete *bla* gene from pBR322 and construct pBR322-Δamp to use it as control in compare with the plasmid free from any antibiotic resistance gene (pBR322-Δtet-Δamp). Construction of A5 plasmid (pBR322-dRek) done by clone 150bp long sequence upper to *tet*^{r} in pBR322 homologous with with downstream to *tet*^{r} gene helped to yield plasmid free from *tet*^{r} gene by spontaneous homologous recombination. The deletion of *tet*^{r} gene from pBR322 by direct repeat spontaneous homologous recombination is supported by Lurie and Delbruck theory (1943)[10], they observed that the occurrence of spontaneous mutations would result in much greater fluctuations in the observed number of Ton^r colonies per culture than random induction of resistance following contact with the phage (the induction) on assay plate. When we applied the experiment of Lurie and Delbruck to our experiment, we found that in the absence of induction the spontaneous homologous recombination will occur (the induction here is tetracycline antibiotic) and lead to loss of tetracycline resistant gene (*tet*^{r}*)* from pBR322 plasmid by spontaneous homologous recombination. Hagg and co-workers (2004)[11] menshed that the plasmid born *tet*^{r} was deleted by taking advantage of *invivo*homologous recombination between two homologous regions of DNA flanking the *tet*^{r} gene using fusaric acid plate that selects for *tet*^{s} cells. That is mean they didn't get spontaneous homologous recombination but they induct the deletion of *tet*^{r} gene by fusaric acid. Bochner (1980)[12] and Maloy (1981)[13] used simple technique that allows direct plate selection of tetracycline-sensitive clones from predominantly tetracycline resistant population. The technique is based on their finding that tetracycline-resistant cells are hypersensitive to lipophilic chelating agents, such as fusaricscid. This finding supports the contention that certain metal ions critically facilitate tetracycline uptake and leads us to suggest possible molecular mechanisms for tetracycline resistance. Since tetracycline resistance is an inducible trait [14, 15], chlortetracycline could be used to generate the membrane modifications associated with tetracycline resistance. When chlortetracycline is autoclaved in broth, its denatured so as to lose its toxicity toward *tet*^{s} cells while retaining its inducing ability for *tet*^{r}cells [16].

The main goal of our study was construct plasmid free from any antibiotic resistant gene, the result of this PCR amplification was plasmid contain ColE1 origin of replication, rop protein and several restriction enzymes without any antibiotic resistant gene. Antibiotic resistance genes are commonly used to select and maintain recombinant bacteria in the presence of antibiotic. However, the use of antibiotics is undesirable for manufacturing gene therapy products [17]. Antimicrobial drug resistant bacteria present a significant risk to public health, and consequently there is great interest in reducing the prevalence of antibiotic resistance genes in both commensal and pathogenic bacteria. One strategy to decrease the prevalence of antibiotic

resistance in bacteria is discontinuous using antimicrobial drugs [18, 19, 20, 21, and 22]. Endogenous essential gene marker (*inf*A) tried to use in the plasmid pBr322-Δtet-Δamp instate of antibiotic resistant genes. We started to study IF1 to be used as selective marker and we established the fundementals for this process by cloning infA to pTrc99A (pTrc600) under $LacI^q$ and trcpromotor to be used as essential gene in the pBr322-Δtet-Δamp. Initiation factor IF1 has molecular weight of 8.2KDa and its smallest of three protein factor required for optimal translation in *E col*[23]. In *E coli*IF1 contains 71a.a residues of known sequence [24] and its structural gene has been cloned and mapped at 19.9 mim on *E coli*chromosome [25]. Two promotors (P1&P2) are control the transcription of the *E coli* gene as monocistronic mRNA both ending at one termination [26]. The *inf*A gene production of *E coli* is an intracellular and essential protein for cell viability [27] and therefore can be used as selective marker instate of antibiotic resistance marker. Overexpression of IF1 can be cell inhibitory [28], therefore *inf*A gene was cloned into pTrc99A plasmid under $LacI^q$ repressor with Trcpromotor. If the *inf*A gene cloned into high copy number plasmid under its normal promotor it will be overexpressed. The resulting amount of IF1 protein inside the cell might rise to level that kill the cell. Promotor of pTrc plus $LacI^q$ repressor which induce by IPTG making *inf*A gene expression under control, therefore the level of IF1 protein remains at its normal level inside the cell.

The vector pKO3 is about 5681bp, it has a temperature sensitive pSC101-ts replication origin. Strain harbouring the plasmid must be grown at 30°C. It contain*Sac*B gene which give ability to degrade sucrose and also contain chloramphenicol resistance gene (*cat* gene). This vector has ability to integrate into chromosome by homologous recombination [29]. In our study, the 1400bp of *inf*A gene contain flanked region of *inf*A gene excluded structural gene was successfully cloned into pKO3 plasmid and its ready to gene replacement and allele exchange.

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