Production of DNA vaccine coding for MCE4A gene of *Mycobacterium bovis*

[2]El-Shazly, M.A.[2] Soliman, Y.A.; [1]Mahmoud, A.A., [1]Khadr, A.M; [1]Elshemey, T.M.; [1]Abd-Elrahman, A.H. and Nasr, E.A.[3]

[1] Department of Animal Medicine, Faculty of Veterinary Medicine, Alexandria University
[2]Central Laboratory for Evaluation of Veterinary Biologics, Abassia, Cairo, Egypt
[3]Vet serum and vaccine institute, Abassia, Cairo, Egypt.
amirhamed22@yahoo.com

Abstract: One of the major aims to overcome tuberculosis infection is developing a vaccine that could prevent mycobacterial cells from entering macrophages and hence will be subjected to the immune defense mechanisms. MCE4A protein antigen is one of the secretory antigens that enables Mycobacteria to enter macrophages thus neutralization of such antigen will aid in the restriction of disease development. In the current study the 1200bp full length MCE4A gene was amplified and cloned in gateway entry cloning vector (pEnter\SD\topo), then homologous recombination with the destination vector (pDEST 40) was done in order to develop the DNA vaccine. Vaccination of guinea pigs with this DNA vaccine and subsequent challenging with *M. bovis* revealed comparable results with that of BCG showing that the vaccine could be promising approach to be tested on the cattle.
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1. Introduction:

Mycobacteria survives phagocytosis and replicates within macrophages. Following infection with *M. tuberculosis*, both healthy subjects and patients with active tuberculosis develop T-cell responses against mycobacterial antigens.

Arruda et al., 1993, Identified a DNA fragment of 450 bp from *M. tuberculosis* H37Ra that conferred the ability on nonpathogenic *E.coli* to enter mammalian cells and survive, they designated this gene mce1. Subsequently, (***Parker et al., 1995***) showed the presence of a similar gene in *M. avium*, *M. intracellulare* and *M. scrofulaceum* by PCR approach.

Since the delineation of the complete genome sequence of *M. tuberculosis* H37Rv, four *mce* operons, designated mce1 to mce4, having similar organization and containing the 450-bp core sequence were identified (***Cole et al., 1998***). Subsequently, the presence of *mce1* was also detected in *M. leprae*, while all the *mce* operons except *mce3* were detected in *M. bovis* (***Wiker et al., 1999*** and ***Zuma´rraga et al., 1999***). Furthermore, (***Chitale et al., 2001***) demonstrated that *mce1*encodes a surface protein and that polystyrene latex microspheres coated with purified recombinant *mce1* protein can enter HeLa cells, while *mce2*, which has 67% similarity with mce1, does not display this property. This suggests that in addition to cell entry, *mce* operons perhaps have other functions. Later, the presence of *mce* operons in *M. smegmatis*, which is a saprophytic species, was also reported.

The emerging reports on the role of mce4 operon in survival of mycobacteria in host tissue indicates that Mce4 proteins may be involved in maintaining the mycobacteria in a nutrient deficient environment for long term survival, also it has been suggested that the proteins of the *mce4* operon may operate as a major cholesterol import system because strains lacking *mce4* operon exhibit drastically reduced ability to take up and metabolize cholesterol in vitro and hence grow poorly when cholesterol is the primary source of carbon (***Pandey and Sassetti 2008***). It was reported earlier that mutation in *mce1* operon produces growth defect in early phase of infection in mice and mutation in *mce4* operon produces growth defect after 3–4 weeks post infection, indicating thereby that *mce4* operon is required in later phase of infection (***Sassetti and Rubin 2003***).

The current study aimed to production of the mammalian expression DNA vaccine that encode for the Mce4A protein antigen and preliminary evaluation of its protective efficacy against subsequent challenge with the *M. bovis* virulent strains as a control measure for the tuberculosis infection.

2. Material and Methods:

Mycobacterium bovis isolation:

Mycobacterium bovis field strain was isolated from cervical lymph nodes of tuberculin positive cattle showed visible lesions in the PM examination according to the method adopted by ***Marks 1972***.

After 2 months of incubation on pyruvated Lowenstein – Jensen media at 37°C, there were clear visible colonies growing onto the surface of the slants. These colonies were then propagated on Middlebrook 7H9 medium for 21 days as a surface pellicle. A loop of the cells were then used for inculcation of 20 mL of modified sauton media and incubated for 15 days as shaked couture to obtain cell suspension. The cells were harvested by centrifugation at 13000 rpm/20 min at 4°C, washed three times with cold TE buffer pH 8 and kept at -20°C till used.

Identification of the isolate:

Species level identification of growth of acid fast bacilli (AFB) positive mycobacterial isolates was done by standard biochemical tests [niacin production, nitrate reduction, catalase activity at 68°C and at room temperature, tween hydrolysis, arylsulphatase and thiophen-2 carboxylic acid hydrazide (TCH) sensitivity and urease test] as per CDC Manual, a combination of positive urease and negative activity for niacin, nitrate reduction, catalase at 68°C, tween hydrolysis, arylsulphatase and TCH were considered as characteristics of *M. bovis* (***Vestal, 1977***).

DNA Extraction:

The DNA was extracted from the mycobacterial strain by Triazol method (***Soliman et al., 2011***) according to the manufacture instructions with some modification. Briefly, the pellets were reconstituted in 1 ml TE buffer pH 8 and incubated at 37^{o}C for 2 hours with 20 ul lysozymes (final concentration 100µg/ml). Proteinase-K 100µl/1ml (final concentration 100µg/ml) and SDS (final concentration 1%) were added and incubated for further 3 hours at 56^{o}C with shaking then 1 ml of Triazol was added. After vortexing for 30 sec, 0.5 ml of chloroform was added and centrifuged for 10 min at 14000 rpm. The DNA in the interphase was precipitated with 0.5 ml of absolute ethanol, washed twice with 0.1M sodium citrate in absolute ethanol and finally redissolved in 50ul of 8 mM NaOH. The pH was then adjusted at 8 by adding 115 µl/ml HEPES (0.1 M). Two µl of RNAase were then added and incubated at 37^{o}C for 1 hour. Purification of the genomic DNA was done using Wizard DNA clean up system (Promega).

PCR amplification:

The full length *mce4A* gene was amplified using the cloning primer that enables the gene to be cloned in pEnter SD/D topo cloning vector (invitrogene cat # K2420-20). These primers were designed using DNASTARE version 10, mce-F (5'-CACCATGTCCGGCGGCGGATCT-3') and mce-R (5'-GAAGTCGTCCCGTTCCGCGAAC-3'). PCR was performed in 50-µl reaction mixtures containing 50 mM KCl, 10mM Tris-HCl (pH8.8), 3 mM MgCl2, 200 mM (each) deoxynucleoside triphosphate, 10µl Q solution (Qiagene) and 2.5 U of thermostable *pfu* DNA polymerase and 100 pmol of each oligonucleotide primer. DNA samples (1 µg) was pipetted through into the mix true. Thermal cycling was performed using T professional thermal cycler (Biometra, Germany), the parameters for amplification were denaturation at 95 ^{o}C for 3 min for one cycle and then 40 cycles at 95^{o}C for 1 min (denature), 62 ^{o}C for 45 sec, and 72^{o}C for 1.5 min (extension). A final extension at 72 ^{o}C for 10 min was also included. The amplicon was then visualized under U.V. transelumination after electrophoresis on 1% agarose. The size of the amplicons was analyzed in comparison to gene ruler 100pb plus DNA ladder (Fremantase cat # SM0323).

Cloning of mce4a gene in the entry vector:

The mce4a gene was first cloned in pENTR/SD/D-TOPO Cloning vector (Invitrogen cat # K2420-20) according to the manufacture instruction. Briefly, two µl of the purified gene were mixed with one µl of the cloning vector and 1µl of the salt solution. The volume was adjusted to 6µl using nuclease free water and the directional cloning was done by incubation of the mixture at 22°C/30min. one µl of the cloning mixture was added to a vial containing the Topo 10 chemically competent *E. coli* and transformation was done at 42°C/45 sec followed by rapid incubation of the cells at ice /5min then 100µl of these transformed cells were spread onto the surface of LB agar plates containing ampicillin (100µg/ml) and incubated at 37°C overnight. The growing colonies were picked up and inoculated in LB broth containing ampicillin and incubated over night at 37°C. The recombinant plasmid was then purified using plasmid miniprep kit (BioFlux #K0502). Amplification of the full mce4A gene was done using the M13 forward sequencing primer and the mce-R reverse primer in order to verify the sense orientation of the gene in the donor vector.

Cloning of mce4a gene in the destination vector:

Plasmids that shows cloning of the mce4A gene in sense orientation was selected and subjected to homologues recombination with the pDEST40 destination vector (Invitrogen cat # 12274-015) as per manufacture instruction to produce the DNA vaccine. In order to perform the homologous recombination, both donor vector containing the mce4a gene insert and the destination vector were mixed in ratio of 1:1 with 1µl of the LR clonase II enzyme mix and the reaction was incubated at 25°C for 30 min, the reaction was then used for transformation of ***E. coli*** competent cells. The transformants were analyzed for the correct orientation as before.

Evaluation of the vaccinal potential of the pDEST 40 mammalian expression vector that express the mce4a gene:

A total number of 15 healthy female guinea pigs were divided equally into 3 groups 5 animal each and vaccinated as shown in table (1), guinea pigs were then challenged 5 weeks after the last dose of the vaccine with I/M inoculation of $5x10^4$ CFU of *M. bovis* in the inner aspect of the thigh.

Two weeks post challenge the animals were sacrificed and lungs, spleen, liver and lymph nodes were aseptically taken from each animal and processed for bacteriological count (Leal *et al.,* 1999) the organs were homogenized with PBS and serial dilution of the homogenate plated out on Middle brook 7H10 agar plates and incubated for one month at 37°C. The bacterial load was then enumerated and result values were presented as mean of Log $_{10}$ CFU per organ ± one standard deviation or as Log $_{10}$ unit of resistance, corresponding to the difference between Log $_{10}$ CFU in control non vaccinated animals and Log $_{10}$ CFU in immunized animals. Other parameters were used for evaluation of the efficacy of the vaccinal potential of the DNA vaccine secluding Percentage mortality and survival (alive versus total) in the immunized animals as well as control were noted for 30 days post challenge, Lung density determination (**Crowle, 1958**) and evaluation of root specific lung weight ***(Oiozco et al., 1986).*** The root specific lung weight (RSLW) of both control and immunized animals post challenge were determined at varying periods after challenge and calculated using the following equation:

$$RSLW = \frac{\text{lung weight in grams}}{\text{Body weight in grams}} \times 10$$

Table (1): Vaccination regimen of the guinea pigs used in this study.

Group number	Route and the type of the vaccine/ animal	Boostering
1	DNA vaccine expressing the mce4a gene	2 doses 2 weeks intervals I/M injection (10µg/dose)
2	BCG vaccine	Once I/D injection 10^3 bacilli / animal
3	Negative control	-

3. Results:

Results of DNA isolation and purification:

The genomic DNA of *M. bovis* was isolated by simple mechanical disruption of the cells followed by triazole. The OD_{260} was 0.196 and OD_{280} was 0.326, the DNA concentration was 0.49 µg/µl and the purity was calculated as 1.66.

After purification, the OD changed markedly. In triazole extraction, the OD_{260} was 0.185 and OD_{280} was 0.310, the DNA concentration was 0.46 µg/µl and the purity was 1.67

Results of mce4a gene amplification:

The *mce4a* gene was amplified by PCR, when DNA concentration was 0.5µg/reaction a clear visible band migrate at 1200 bp was visualized under the UV illumination whereas using lower DNA concentration or absence of Q-solution gave no bands but only primer dimmers. Fig. (1) Shows the electrophoretic mobility of *mce4a* gene on 1% agarose.

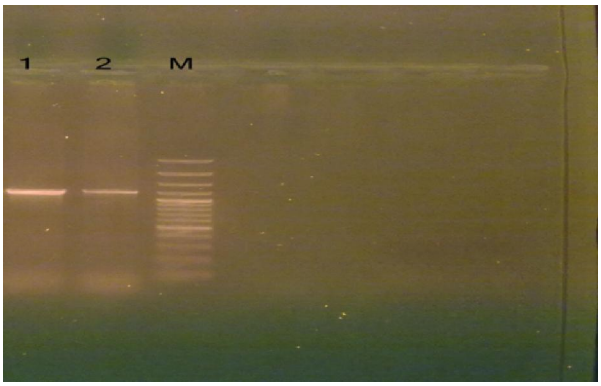


Fig (1): Agarose gel electrophoresis of amplified *mce4a* from the genomic DNA of *M. bovis* (lane 1) or from the miniprep of the recombinant donor vector (lane 2). (M) DNA size marker.

Results of mce4a gene cloning:

The full length *mce4a* gene of *M. bovis* was cloned first in the pENTR/SD/D-TOPO entry vector. After recombination the vector was transformed into E.coli Topo competent cells and plated onto LB agar plates containing the selective antibiotic kanamycin. After 24h incubation at 37 °C, more than 200 colonies were grown as seen in fig(2). Most of the colonies were homogenous in term of shape, size and appearance. Some of these colonies were selected and subjected to further analysis using PCR.

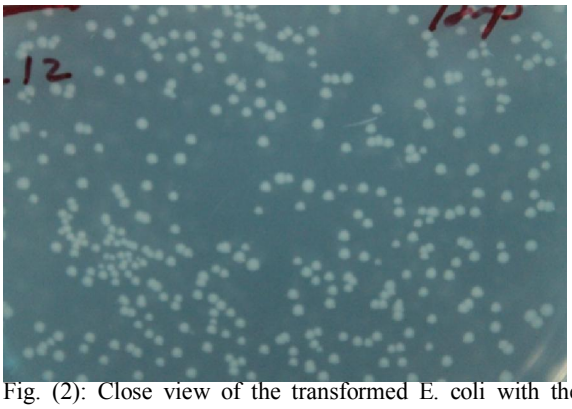


Fig. (2): Close view of the transformed E. coli with the donor vector containing the mce4a gene of *M. bovis*

Results of miniprep of the donor vector:

As seen in fig (3) the agarose gel electrophoresis of the recombinant donor vector prepared from overnight shaked culture showed a clear band migrating about 3.8 kb corresponding to the 2.6 kb plasmid and the 1.2kb mce4a insert. It was noticed that there were some co-migrating bands with much small size that represent the recombinant plasmid with some degree of supercoiling which made it much faster in migration.

Results of mce4a gene amplification of miniprep of the donor vector:

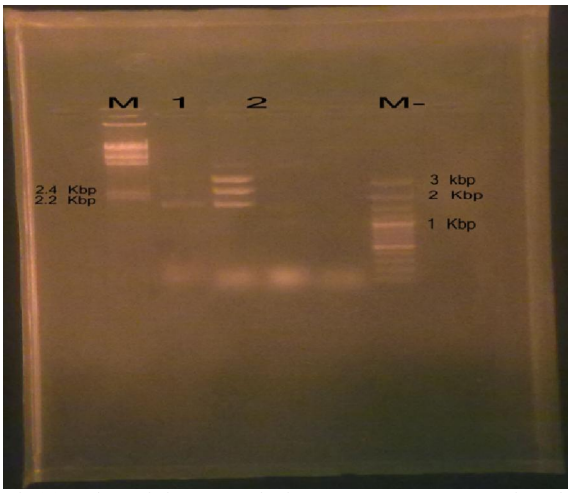
In order to verify the positive orientation of the cloned gene within the vector, miniprep of the plasmid was done from the overnight shacked culture of the selected colonies and PCR amplification was done to the mce4a gene using one primer within the gene (the reverse primer) and the other one lies within the vector itself (the M13 forward primer). As showed in fig 1 lane 2, the full length mce4a could be amplified from the recombinant donor vector indication positive orientation of the gene within the vector.

Results of cloning of the mce4a gene in the distention vector:

Homologues recombination between the donor vector and destination vector was done and the resulted vector is the final mailman expression DNA vaccine. After homologous recombination the reaction mix was used to transform *E. coli* topo 10 competent cells to propagate the plasmid. It was noticed that the number of the transformants was much less than in the previous step, yet all the colonies were harboring the plasmid when subjected to downstream analysis.

Results of miniprep of the destination vector:

As seen in fig 8 the agarose gel electrophoresis of the recombinant destination vector (pDNA DEST 40) prepared from overnight shaked culture showed a clear band migrating about 8.3 kb corresponding to the 7.1 kb plasmid and the 1.2kb mce4a insert. It was noticed that there were some co-migrating bands with much small size that represent the recombinant plasmid with some degree of supercoiling which made it much faster in migration.



Fig(3): The miniprep analysis on 1% agarose. Notice the presence of a clear band migrating about 3.8 kb representing the recombinant plasmid along with some other more shorter bands representing the recombinant plasmid with some degree of supercoiling. M HindIII digested λ phage marker and M- 100 bp plus ladder

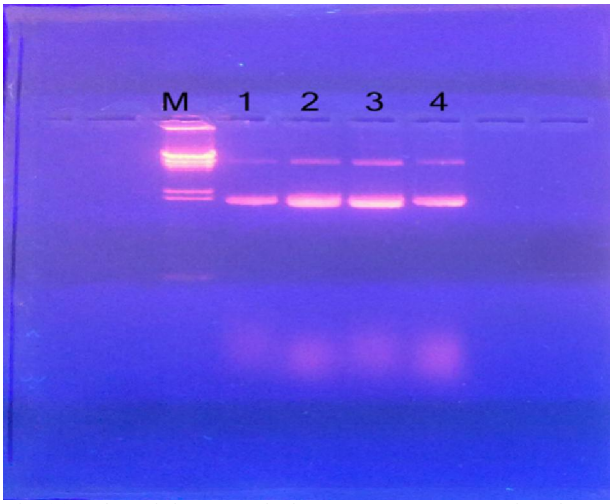


Fig (4): The miniprep analysis on 1% agarose. Notice the presence of a clear band migrating about 8.3 kb representing the recombinant plasmid along with some other more shorter bands representing the recombinant plasmid with some degree of supercoiling. M HindIII digested λ phage marker.

Results of evaluation of the vaccinal potential of the mce4a gene expressed by the pDEST 40 mammalian expression vaccines:

Different parameters were undertaken in order to perform preliminary evaluation of the vaccinal potential of the DNA vaccine expressing the mce4a gene of *M.*

bovis. All the used parameters directed towards the measurements of the protective efficacy of the DNA vaccine in comparison with the standard BCG vaccine.

Results of the mean of Root specific lung weight of guinea pigs groups vaccinated with the mce4a DNA vaccine :

Table (2) shows the results of mean root specific lung Wight of guinea pigs vaccinated with mce4a DNA vaccine or BCG

No.	Groups vaccinated with	Log_{10} mean viable count + SEM
1	Mce4a DNA vaccine	4.06 + 0.34
2	BCG	3.98 + 0.06
3	Control	4.67 + 0.02

Results of Protective effects of vaccination of guinea pigs measured by lung density

Table (3) shows the results of lung density of guinea pigs vaccinated with Mce4a DNA vaccine or BCG

Results of viable bacterial count in lung of vaccinated guinea pigs

Table (4) shows the results of viable bacterial count in lung of vaccinated guinea pigs vaccinated with Mce4a DNA vaccine or BCG

Results of viable bacterial count in lung of vaccinated guinea pigs

Table (5) shows the results of viable bacterial count in spleen of vaccinated guinea pigs vaccinated with Mce4a DNA vaccine or BCG.

Results of Results of survival rate of vaccinated guinea pigs

Table (6) shows the results of survival rate as measured by the following equation

$$\text{Survival rate} = \frac{\text{No. of alive}}{\text{Total No.}}$$

Table (2): Result of the mean of Root specific lung weight of guinea pigs groups

	Mce4a DNA vaccine	BCG	Control
RSLW	13	11	17.7

Table (3): Protective effects of vaccination of guinea pigs measured by lung density

	Mce4a DNA vaccine	BCG	Control
Lung density according to degree of sinking	+	-	++++

Table (4): Result of viable bacterial count in lung of vaccinated guinea pigs.

No.	Groups vaccinated with	Log_{10} mean viable count + SEM
1	Mce4a DNA vaccine	4.58 + 0.92
2	BCG	4.36 + 0.93
3	Control	5.66 + 0.89

Table (5): Result of viable bacterial count in spleen of vaccinated guinea pigs

No.	**Groups vaccinated with**	**Log_{10} mean viable count ± SEM**
1	**Mce4a DNA vaccine**	**4.06 ± 0.34**
2	**BCG**	**3.98 ± 0.06**
3	**Control**	**4.67 ± 0.02**

Table (6) shows the survival rate of vaccinated and non-vaccinated groups

	Mce4a DNA vaccine	BCG	Control
After 14 days	10/10	10/10	6/10
After 1 month	8/10	8/10	4/10
After 2 months	7/10	8/10	0/10

4. Discussion:

Deciphering the biology of M. tuberculosis from the complete genome sequence ***(Cole et al., 1998 and Tekaia et al., 1999)*** revealed the presence of a large operon coding for a putative virulence genes called mammalian cell entry genes enable the mycobacterium to invade and/or intracellular survive within macrophages. This Mce was subsequently shown to be preceded by two integral membrane proteins (YrbE) and followed by five other Mce proteins arranged in a mce operon. The genome sequencing also revealed four copies of mce, arranged in four homologous mce Operons (mce1–4). The mce genes encode invasin/adhesin-like proteins with putative signal sequences at the N-terminal end and are most likely located at the mycobacterial cell surface ***(Tekaia et al., 1999 and, Chitale et al., 2001)*** The antipeptide antibodies corresponding to a predicted B-cell epitope in each of the Mce1A-F showed that these Mce are expressed during the in vitro growth of M. tuberculosis ***(Harboe et al., 1999 and Joshi et al., 2006)***. Mce1A and Mce1E are also expressed and elicit antibody production during natural infection with M. tuberculosis ***(Ahmad et al., 1999)***.

The first step in the current protocol is to isolate highly purified whole genomic DNA of the *Mycobacterium bovis* strain which will be used for the

amplification of the mce4a gene that will be used for the cloning procedures. A hindrance in the development of efficient and rapid procedures for isolation of mycobacterial genomic DNA has been the mycobacterial cell wall. Mycobacterium species are endowed with a unique cell wall composed of a covalently attached complex of peptidoglycan, arabinogalatan, and mycolic acid (***Brennan and Nikadio, 1995***). In addition, an array of glycolipids, lipoglycan and a unique polar lipid form an outer leaflet that is closely associated with the cell-wall mycolic acid. This structure composed of tightly packed lipophilic and highly branched polysaccharides is largely responsible for the low permeability of the mycobacterial cell envelope and results in a formidable protective barrier. Owing to this unique cellular envelope the standard methods for isolating DNA from Gram -ve (***Sambrook et al., 1989***), and Gram +ve bacteria (***Caparon and Scott, 1991 and Hoch, 1991***) are not optimal for mycobacteria. Several methods, with varying approaches to achieve efficient cell lysis are reported for isolation of genomic DNA from mycobacterial species including enzymatic (***Patel et al., 1986 and Shoemaker et al., 1986***) mechanical (***Yandell and McCarthy 1980***, ***Jacobs et al., 1991 and Barrera et al., 1993)*** and/or chemical methods (***Gongalez et al., 1996***).

The mycobacterium cells were treated with a simple mechanical management in the form of repeated freezing and thawing followed by homogenization. After enzymatic digestion with Proteinase-K and lysozymes (***Whipple et al., 1987 and Soliman et al., 2011)*** the organic extraction was done by triazole extraction. Despite the low yield of DNA extracted by triazole, still gave a high purity than traditional phenol extraction. DNA purification was then done to eliminate not only protein contaminant but also salts and polysaccharides that portioned with the aqueous soluble DNA and may interfere with the subsequent PCR or endonuclease digestion analysis (***Hurley et al., 1988 and Brennan and Nikadio, 1995***).

Amplification of the whole mce4A gene was carried out using one set of primer pair that targets the full length gene. The design of the primer was carried out using DNA*STARE* version 10 software based on the previously published *mce4A* gene sequence in the gene bank data base (***Soliman et al., 2011***). The full length 1200bp gene was successfully amplified without any nonspecific or fragment amplifications this full length gene was sliced off the gel for the sequencing analysis and cloning in the pENTR/SD/D-TOPOentry vector. The full length mce4a gene was cloned in the mammalian expression vector pDEST 40. Gate way cloning strategy was conducted in this study, this technology consists of cloning of the gene of interest in a donor vector and analysis of the recombinants for the correct cloning orientation then using homologous recombination between the donor vector containing the gene of interest with the destination vector which will be amplified and used as mammalian DNA vaccine.

The first step in the current study was done by TA ligation of the amplified full length mce4a gene in the donor vector (entry clone) and the recombinant vector was transformed in the Topo10 ***E.coli*** competent cells by heat shock transformation. These procedures gave more than 200 transformants when the cells were plated onto ampicillin LB plates. These transformants were tested for the presence of the recombinant vector and for the correct orientation of the cloned gene within the vector by PCR amplification using the m13 forward primer and the reverse primer that target the 3' end of the gene, the expected size of the amplicon would be ~ 1200 bp. As shown in fig 2 lane 2 the amplicon was in the correct size denoting the correct orientation of the gene in the donor vector.

Miniprep analysis of the transformants revealed also the presence of the donor recombinant plasmid with migration size of 3800bp corresponding to the size of the donor vector plus the cloned gene. As shown in fig 3, lane 2 there were about 3 visible bands with migration rate 1800, 2400, 3800 bp corresponding to the recombinant donor vector either coiled format (3800bp) or in slightly supercoiled format (1800 and 2400 bp) which migrates more faster than its true size (***Sousa et al., 2009***).

The recombinant donor vector which contains the mce4a gene in correct orientation was subjected to homologous recombination with the destination vector pDEST40 which will be used as DNA vaccine. This recombination is done using the *LR* colonase enzyme II mix which enables the recombination between the *attR* sit in the donor vector and the *attB* sit in the destination vector resulting in directional insertion of the mce4a gene in the destination vector and this recombination is done within 5 min at room temperature (***Landy, 1989***).

As seen in fig 4 the recombinant destination vector purified from the *E.coli* and electrophoresed on 1% agarose gel and the 8.3Kbp fragment was clearly seen which corresponding to the size of the destination vector plus the mce4a gene insert.

The last step in the current study was aimed to perform preliminary study on the protective efficacy of the mammalian expression vector (destination vector) that carry the mce4a gene against the *M. bovis* infection in the experimental infection model of the guinea pigs. First the guinea pigs were vaccinated with the DNA vaccine and BCG vaccine was used as a positive control, the guinea pigs were later

challenged with virulent *M. bovis* strain used originally in amplification of the mce4a gene.

Different parameters were used to assess the protective efficacy of the DNA vaccine. as shown in table 2, the RSLW revealed that the BCG vaccination gave better results while the DNA vaccine coding for the mce4a gene gave much more index but still far away less than the control (17.7) this index measure the degree of the developing of the tubercle nodules in the lung. More nodules gave more weight which reflects the inability of the immune system to localize and prevent the spread of the infection. These results gave much hope about the DNA vaccine as it could gave much restriction to the spread of the infection. Because BCG is an attenuated form of *M. bovis*, it gave better results than mce4a DNA vaccine as it contains a complex mixture of the antigens that could potentiate the immune system much more than the single antigen. The same results were obtained using different parameters such as lung density.

Bacterial count in the lung and spleen reflects the ability of the bacteria to overcome the immune system of the animals, low counts means high immune reaction that restrict the ability of mycobacteria to multiply, as seen in table 5 and 6, bacterial count in lung and spleen in both vaccinated animals with either BCG or mce4a DNA vaccines where much lower than the negative control challenged guinea pigs.

In conclusion, the DNA vaccine coding for mce4a gene gave a promising protective efficacy which could be used for the control of the tuberculosis infection in animals.

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