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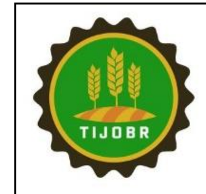

Isolation of SODA gene from *Mycobacterium tuberculosis* and its expression through *Escherichia coli.*

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ABSTRACT

Mycobacterium tuberculosis is the major cause of tuberculosis in humans. It is a non-motile bacteria requiring high level of oxygen for growth. Infection starts when bacteria enters into lungs through inhalation, it grows very slowly, and the infection is mostly caused in people with compromised immune system. Superoxide dismutase (SOD) is a protein present in *Mycobacterium tuberculosis.* SOD is a protective protein present in bacteria, which protects bacteria from poisonous impact of reactive oxygen. SOD fixes radicals of free oxygen molecules present in bacteria. There is an iron co-factored SOD, also known as SODA, which is encoded by Rv3846 gene, and is 624bp long with molecular mass of 23 kDa. SODA is necessary for viability, and it is secreted in large amount. For cloning of SODA 624bp, reverse and forward primers were designed, which were 25bp each, then amplified with annealing temperate of 60°C. Further amplified gene was purified from gel with phenol/chloroform method and geneJet column kit method. Then *E. coli* were grown in LB broth and pet28a^{+} vector was isolated. Then both vector and amplified PCR product were digested with *Hind* III and *Nde* I, and then ligated. The recombinant plasmid having SODA gene was successfully transformed into *E. coli* cells and their presence was conformed through colony PCR of transformed colonies. The work done in this project would provide easy expression of SODA protein. The SODA is potential candidate for rapid detection of tuberculosis through immunoassay.

Keywords: Superoxide dismutase, Cloning, *Mycobacterium Tuberculosis, Escherichia coli,* Polymerase Chain reaction method (PCR).

[I] INTRODUCTION

Tuberculosis has existed for millennia and remains a major health problem. Tuberculosis (TB) is a multisystem affecting chronic infectious disease caused by *Mycobacterium tuberculosis*. It is mostly spread due to inhalation of air droplets, containing the tubercle bacilli, produced during cough or sneeze by an infected individual. Although spread by inhalational route, it not only affects the lungs but other organs as well like abdominal glands, bones and nervous tissue. Tuberculosis is highly life threatening but it can be prevented and cured if the proper necessary measures are taken. Due to its slow and insidious progression, the diagnosis of the disease is usually delayed for a few months after infection of the bacillus (WHO global report, 2016). Unlike healthy people all those having an immunocompromised state are more susceptible to developing this disease. Reservoir of *M. tuberculosis* that causes spreading TB infection is the victim with pulmonary TB, with their chronic respiratory symptoms such as sputum production and cough. They produce aerosol droplets from bronchi when they sneeze, cough or speak (Ait-khaled et al., 2003).

According to an estimate by WHO in 2015, 60% of the global burden of TB is confounded by six countries solely i.e. India, Indonesia, China, Nigeria, Pakistan and South Africa. According to their annual global report an approximate of 10.4 million people contracted the disease out of which 1.8million individuals died in 2015. More than half of the infected people 480,000 developed multi-drug resistant TB. Although the incidence of tuberculosis has dropped by a 1.5% over the last decade, it is nevertheless among the top ten causes of mortality worldwide causing more deaths than HIV/AIDS annually. (Corbett et al., 2005). With a population of more than 189 million people, Pakistan still ranks the fifth alongside other

countries having the highest burden of tuberculosis and fourth highest burden of Drug resistant TB globally. According to the National TB Control Program (NTP) of Pakistan, 231 out of every 100,000 individuals contract TB annually which leads to an approximate of 420,000 new cases every year (Khan et al., 2003).

Tuberculosis is a slowly progressing granulomatous infection transmitted through aerosol droplets containing the causative organism. The bacillus starts infection by invading body's immune system. Most of the bacterial droplets are trapped by the physical defenses like the respiratory mucocilliary system thus preventing the onset of infection in majority of the exposed persons. All those bacterial droplets that are able to evade this system reach the alveoli and get surrounded and are taken up by the macrophages lining these alveoli. These macrophages play an important role in the immune system and help destroy the mycobacterium to further prevent the infection. The complement system also assists in phagocytosing these bacteria (Dye et al., 1999). Most of the individuals infected by TB usually have latent TB that is they have the bacterial strain in them but they are asymptomatic and are not transmitting the disease. The major hindrance in earliest detection of the disease is the delay in appearance of symptoms. By the time the active disease process has started and visible signs appear the disease is already transmitted to people around the contact. This also leads to a delay in seeking proper treatment and attention which may worsen the disease progression (Kleinnijenhuis et al., 2011).

M. tuberculosis is a genuinely extensive non-motile high-impact bacterium. It is aerobic, in this manner it can just survive in an oxygen-containing condition. The bacilli are 2-4 micrometers in length with a width of 0.2-0.5 µm (Todar's textbook of bacteriology, 2011). The cell envelope is made by a core of peptidoglycan, arabinogalactan and mycolic acids, these three macromolecules covalently connected to each other, and a lipopolysaccharide that is believed to be anchored the plasma cell membrane (Crick et al., 2001). The bacterium multiplies slowly, with 16-20 hours between every cell division as compared to other bacteria such as *E. coli* which divide roughly every 20 minutes (Pierce et al., 2010).During the time the bacterium slowly replicates body's defenses wall off the infectious agent by forming a granuloma and thus preventing the spread during this latent period. A granuloma is basically a compact aggregation of various immune activated cells which work in various ways to isolate and inhibit the replication of the bacterium. At the center of this is the weakened bacteria surrounded by cytokines. This creates an unsuitable environment for the bacteria and most of them die (Stanley et al., 2000)**.** There are many methods to differentiate *Mycobacterium tuberculosis* from nontuberculous mycobacteria and one of them is HPLC (high pressure liquid chromatography) method, in this cell wall lipids of bacteria are analyzed, HPLC provides confirmation of disease within 4-14 days *(*Furlanetto et al., 2014)*.* Comparing them for patterns of mycobacterial species, DNA sequencing and nucleic acid probes are also in use. Niacin test, pyrazinamidase test and nitrate reduction test are among conventional methods used. These tests also differentiate *M. tuberculosis* from *M. bovis (*Heifets et al., 2005).

Superoxide dismutase (SOD) is a protein present in aerobic organisms, including pathogenic and non-pathogenic mycobacteria tuberculosis, which catalyze conversion of superoxide anion to hydrogen peroxide (Edwards et al. 2001). Superoxide dismutase is a part of the resistance mechanism which shields cells from the poisonous impacts of reactive oxygen intermediates (Hassett et al., 1993). SOD has been appeared to make a vital commitment to the in-vivo survival of several Intracellular bacterial pathogens (Franzon et al., 1990). SOD is a solid superoxide radical forager, which assumes a critical part in opposing oxidative stress, and SOD mutant strains have been built to characterize the part of this molecule in the immune response to *M. tuberculosis* contamination (Zhang et al., 1991). Among the bacterial culture filtrate proteins growing in mycobacterium tuberculosis cultures SOD is one of the largest secretory proteins. SOD is abundantly present in clinical tuberculosis isolated from tuberculosis patients. The nonappearance or lessening of SOD can propel natural invulnerability and SOD dodge devastation or development hindrance of *M. tuberculosis* (Liao et al., 2013)

Molecular examination for iron co-factored superoxide dismutase (SODA) permits speciation of mycobacteria, Cellular immune responses in infected hosts generated by SODA which is abundantly secreted virulence factor, Mycobacterium species are identified by molecular analysis of SODA gene (Allen et al., 2008). *Mycobacterium tuberculosis* produce SODs of two types, an iron co-factored SOD encoded by SODA and copper-zinc encoded by SODC. *Mycobacterium tuberculosis* secrets large quantities of iron co-factored SOD, also known as SODA. Superoxide anion detoxifies by iron-cofactor (Fe) sod or SODA is necessary for viability, and allelic inactivation of the chromosomal gene and can be achieved if second episomal copy of allele is present (Edwards et al., 2001).

Nonappearance or lessening of sod can propel natural invulnerability and sod dodge devastation or development hindrance of *M. tuberculosis*. SODA protein extensively lessened the generation of NO and oxygen radicals and weakened cell immunologic capacity in early disease. SODA is mostly present in cytoplasmic fraction and lower extent in cell wall fraction. SODA is most effective factor in resisting an oxidative burst. SODA consists 624bp long fragment and molecular mass of 23 Da (Liao et al., 2013) 40 different peptides were generated that collectively span the entire MTB SODA protein, PPD- and PPD^+ subjects to identify immunogenic peptides. Out of the 40 SODA peptides, none were recognized in the PPD-control subjects. Only four of the 40 peptides were most frequently recognized among the PPD+ subjects: SODA 31, 33, 36 and 38. Two or more SODA peptides were recognized in three sarcoidosis subjects, which demonstrated that a Th-1 immune response can be generated by multiple SODA epitopes (Allen et al., 2008).

[II] MATERIALS & METHODS

2.1 Primer designing and preparation of reaction mixture:

The gene of interest SODA has length of 624 base pairs, for PCR amplification forward primer was designed containing 25 base pairs with 44.0% GC content "GAATCATATGGCCGAATACACCTTG" and with restriction site of *Nde*I*.* Reverse primer also with 25 base pairs but with 48.0% GC content "CACAAGCTTCAGCCGAAT ATCAACC" and restriction site of *Hind*III*.* Initial denaturation was at 95°C for 3 minutes, cyclic denaturation at 95°C for 45 seconds and keeping in mind about forward and reverse primer's melting temperature annealing were done at 57°C, 58°C, 59°C and 61°C, but optimized at 60°C. Extension was done at 72°C for 45 seconds and final extension at 72°C for 10 minutes. Total 25 cycles were run.

Reagents	Volume
Reverse-primer	5 ul
Forward-primer	5 ul
Taq buffer	5 ul
MgCl2	5 ul
DNTP's	1 ul
Taq enzyme	1 ul
DNA template	2 ul
Water	22 ul
Total	**50 ul**

Table 1, components of PCR mixture

2.2 Isolation of Plasmid:

Autoclaved 10ml LB broth was taken, inoculated a single colony of bacteria *E. coli* having *pet28a*$^+$ vector, and the medium was supplemented with kanamycin. Then for incubation it was placed in a shaking incubator overnight at 37°C, then added 1.5ml culture to microfuge tube/Eppendorf and Centrifuged it at 12,000 rpm at 4°C for 1 minute, discarded supernatant and transferred 1.5 ml culture to tube again for maximum yield and centrifuged again. To 3ml culture pallet, then 200ul ice-cold alkaline lysis solution I was added, and completely suspended the pellet. Then added 400ul of freshly prepared alkaline lysis solution II, mixed by inverting tube several times and was kept on ice. Without wasting time the addition of 300ul of alkaline lysis solution III was done and mixed all above contents by gently inverting tube, Storage on ice for 5 minutes and centrifugation at 12,000 rpm at 4°C for 10 minutes was done. After that transferred the upper transparent layer/supernatant from white cell debris carefully to a new microfuge tube/Eppendorf. Then added equal volume of cold isopropanol and placed in a freezer (-4°C) for 5 minutes. Centrifuged at 12,000 rpm for 10 minutes, and carefully discarded supernatant. Then added 400ul of 70% ethanol and centrifuged at 12,000 rpm for 10 minutes to wash pallet, Dried pallet for 3 hours and added 50ul of double distilled water or injection water and incubated for 30 minutes at 37°C.

Reagent	Composition
Solution **I**	50mM glucose 25mM tris-Cl pH8.0 (60.5g trisBase, 21.25ml for 500ml) 10mM EDTA pH8.0

Solution **II**	Freshly prepared 0.2m NaoH 1% SDS
Solution **III**	5M potassium acetate 60ml glacial acetic acid 11.5ml water 28.5ml
Isopropanol	100%
Ethanol	70%

Table 2, Reagents for plasmid isolation

2.3 Agarose gel electrophoresis:

Agarose 0.30 grams was taken & dissolved it in 30ml of 1x TAE buffer, provided heat to it until boiling and let it be cool a little at room temperature for 5 minutes. Then added 2.0ul of ethidium bromide, mixed it and poured mixture in gel apparatus with comb in it and left at room temperate to solidify. Poured 250ml of 1x TAE buffer in electrophoresis tank, comb was taken out from solidified gel and then placed the gel in tank, it was made sure that gel was submerged in running buffer. Carefully dispensed DNA ladder, PCR product and plasmid DNA with the help of pipette in wells of gel. Then gel was run on 80 volts for 25 minutes. After completion gel was placed on UV illuminator, ethidium bromide glowed under UV light and identified DNA bands.

Reagents	Weight/volume	Concentration
Tris base	121 grams	0.5M
Glacial acetic acid	23.6 ml	
EDTA	100 ml	500mM (pH 8.0)
water	Till final volume is 500ml	

Table 3, Preparation of 50x TAE buffer.

Reagents	Volume
Bromophenol blue	0.025 g (0.25%)
Glycerol	3ml
Water	7ml
Total	**10ml**

Table 4, Preparation of dye.

2.4 DNA Recovery:

Genejet Kit Method

Running time for gel was 25 minutes at 80 voltage, under UV carefully cut desired band fragment and placed it in pre-weighted eppendorf tube. Added 1:1 volume of binding buffer (100g gel: 100ul buffer) then Incubated at 50-60ºC for 10 mins or until gel completely dissolves. Transferred 800ul of solubilized mixture to GeneJet purification column. Then centrifuged for 1min at 12,000 rpm and discarded flow-through, placed the column back into same collection tube (didn't exceed 1g of agarose per column), then added 700ul of wash buffer diluted with ethanol, and centrifuged for 1 minute at 12,000 rpm, and discarded flow through. Transferred column to new fresh eppendorf and added 15ul elution buffer and left it for 2 minutes, centrifuged for 1 minute at 12,000 rpm. For maximum yield added 10ul elution buffer again and centrifuged.

Reagents/Material	Composition
Binding buffer	
Wash buffer	Diluted with ethanol
Elution buffer	10 mM tris-HCL pH8.5
GeneJet purification column	

Table 5, Components of Genejet kit.

Phenol/Chloroform extraction of DNA from agarose gel

Large combs and low melting agarose 0.7% prepared in 1x TAE buffer was made, then added 40ul of sample and ran apparatus at 80 voltage for 25 minutes, then carefully sliced out desired long agarose fragments under UV light, placed

them in pre-weighted Eppendorf tube, then added equal amount of pre warmed TE buffer and placed them in water bath at 60-67°C till gel fragment is completely dissolved in TE buffer, then added equal volume of PCI, vortexed for 30 seconds and centrifuged at 12,000 rpm for 1 minute. Three layers were visible, clear, cloudy white and agarose in bottom, then removed the top clear layer and cloudy white layer and placed them in a new microfuge tube and repeated the previous step again. Then added equal volume of chloroform and centrifuged, transferred upper layer, now add 2x volume of isopropanol and 1/10th volume of 3M sodium acetate (pH 5.6) and placed tubes in freezer for 20 minutes. Then carefully removed from freezer and centrifuged immediately for 5 minutes at 12,000 rpm. Then took isopropanol off, washed pallet with 300ul of 70% ethanol, centrifuged at 12,000 rpm for 5 minutes and took off supernatant with pippet, dried for some hours and dissolved in 30ul of water.

2.5 Restriction:

Added all reagents in Eppendorf tube and incubated at 37°C for 16 hours.

Reagents	Volume
*Hind*III	2 ul
*Nde*I	2 ul
10x tango buffer	20 ul
Pasmid/PCR sample	20 ul
Water	56 ul

Table 6, Restriction of PCR product and plasmid.

2.6 Ligation:

All components were added in microfuge tube and mixture was kept at 16°C overnight.

Reagents	volume
Restricted insert	3.3 ul
Restricted vector	3.3 ul
Ligase buffer	2.0 ul
Ligase enzyme	1.0 ul
Water	10.4 ul
Total	**20 ul**

Table 7, Ligation components.

2.7 Competent cell preparation:

First the inoculation of 10ml of LB broth with a single colony was done, and then kept it in incubator 37°C for overnight with shaking at 100 rpm. Then the next day 500ul was taken from primary inoculum and transferred to 50ml LB broth and flask was kept in shaking incubator at 37°C. till $O.D_{600}$ reach 0.4 the cells were grown, and then centrifuged at 6000 rom for 10 minutes at 4°C in a sterile tube, the supernatant was discarded in laminar hood, and the pallet was suspended in cold 20ml 50mM $CaCl_2$ solution, centrifuged again at 6000 rpm for 10 minutes and supernatant discarded in laminar hood, then pallet suspended again in 1ml cold $CaCl_2$ solution, Aliquots of 200ul were made in Eppendorf tubes, and kept at 4°C.

2.8 Transformation:

10ul of ligation and 200ul of competent cells were mixed and stored on ice for 40 minutes, then heat shock was given for 2 minutes in a water bath at 42°C, then immediately transferred to ice and kept there for 5 minutes. 0.8ml of sterile LB broth was added and incubated at 37°C for 2 hours. Then centrifuged at 12000 rpm for 1 minutes and discarded 850ul of supernatant. Cells were suspended and spread on LB agar plates containing kanamycin and incubated at 37°C overnight.

2.9 Confirmation of transformation through colony PCR:

A colony was picked up and mixed with 30ul of water. Then heated at 95°C for 5 minutes and 25°C for 5 minutes in PCR. Then centrifuged PCR tube containing our colony at 10,000 rpm for 5 minutes. Then 10ul of supernatant as template was used.

Reagents	Volume
Taq buffer	3 ul
Primer Reverse	3 ul

Primer Forward	3 ul
dNTPs	3 ul
Mgcl2	3 ul
Taq enzyme	0.5 ul
Supernatant/template	10 ul
Water	4.5 ul
Total	**30 ul**

Table 8, Components of colony PCR.[III] RESULTS

3.1 PCR amplification:

Gene of interest was 624bp long, and after PCR reaction ran it with 1kb ladder to check size of gene, the desired DNA band was present near 750bp band of DNA ladder as seen in figure 1. The positive sample was used for further cloning after gel extraction.

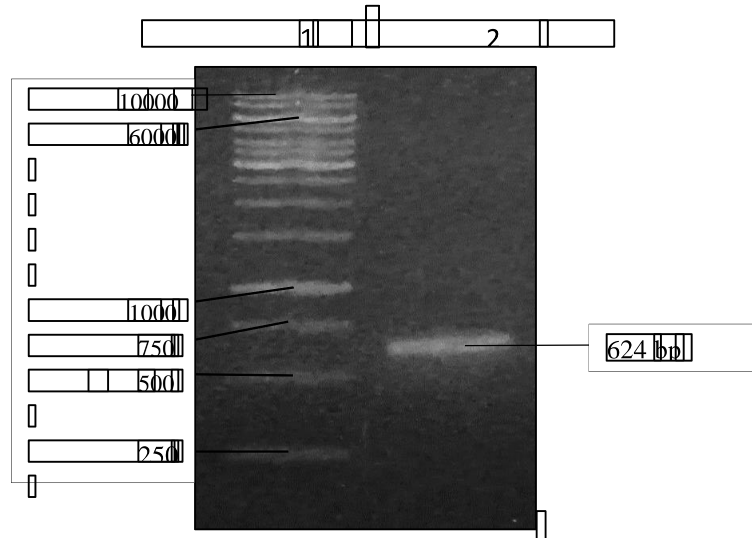

Figure1, 1% gel of PCR product of 624bp. Lane1: DNA marker, Lane 2: PCR

3.2 Double enzyme restriction of PCR product:

In order to insert desired gene into plasmid for cloning, restricted gene with 2 enzymes were seen.

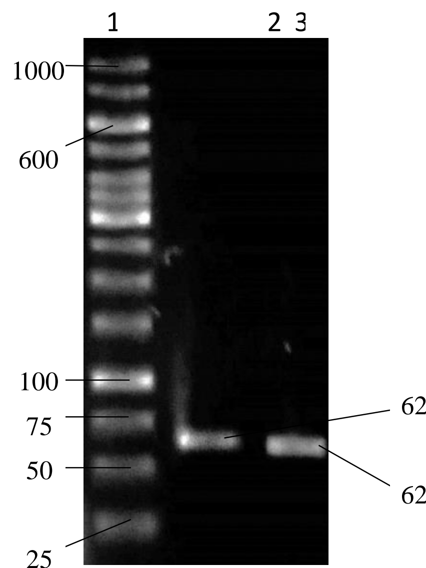

Figure 2, 1% gel of PCR product of 624bp. Lane1: DNA marker, Lane 2: restricted, Lane 3: uncut.

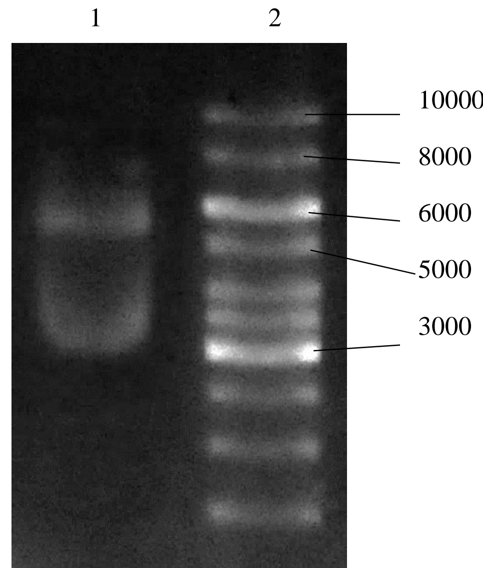

Figure 3, 1% gel of plasmid isolation. Lane1: Uncut plasmid, Lane 2: DNA marker.

3.3 Plasmid isolation:

Pet28a+ vector was successfully isolated from *E. coli.* The plasmid was confirmed through 1% gel electrophoresis.

3.4 Restriction of plasmid:

Plasmid was purified first then restriction was done with the *Hind*III and *Nde*I enzymes to proceed for further steps. Size of plasmid is shown in figure below.The isolated vector was double digested to prepare it for cloning. Two enzyme that used were *Hind*III and *Nde*I. After digestion the restriction was confirmed on 1% agarose gel electrophoresis. Unrestricted plasmid was circular or supercoiled thus showing two bands and restriction the linearized plasmid was observed as single DNA band. The size of linearized plasmid was about 5369bp as seen in figure 4.

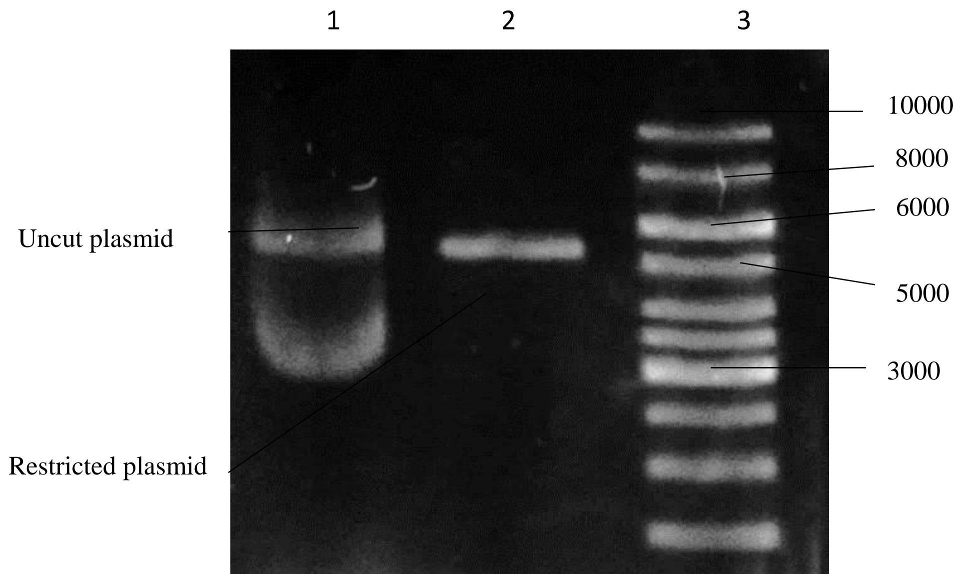

Figure 4, 1% gel of uncut plasmid and restricted plasmid. Lane 1: Uncut plasmid, Lane 2: Restricted plasmid 5369bp, lane 3: DNA marker.

3.5 Colony PCR

After successful growth of bacteria, confirmation was required which was done with colony PCR, to make sure if grown bacteria contained desired gene.

[IV] DISCUSSION

Tuberculosis is a major health problem. TB is a chronic infectious disease caused by *Mycobacterium tuberculosis*. It is mostly spread due to inhalation of air droplets, containing the tubercle bacilli, produced during cough or sneeze by an infected individual. Tuberculosis is highly life threatening but it can be prevented and cured if the proper necessary measures are taken. *M. tuberculosis* mainly affects individuals with a lowered immune system because in healthy individuals, the body's natural defense system kills the bacteria and there are no symptoms.

M. tuberculosis is a non-motile high-impact bacterium. It is aerobic and can just survive in an oxygen-containing condition. The bacilli are 2-4 micrometers in length with a width of 0.2-0.5 µm (Todar's textbook of bacteriology. 2011). Superoxide dismutase are present in bacteria to fix free oxygen radicals which are very dangerous to

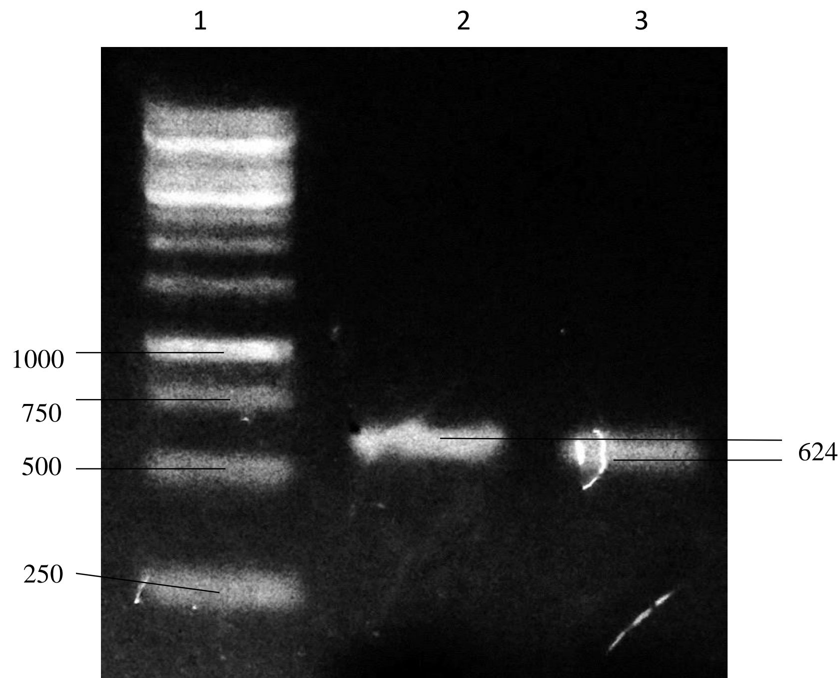

Figure 5, 1% gel of colony PCR. Lane1: DNA marker, Lane 2, 3: PCR of 624bp SODA gene.

bacteria, even oxygen radicals could kill bacteria, in *Mycobacterium tuberculosis* such SODs are present, and there is a type of it which is iron co factored, known as SODA, which is encoded by gene Rv3846, 624bp long. SOD mutant strains have been built to characterize the part of this molecule in the immune response to *M. tuberculosis* contamination (Zhang et al. 1991). SOD has been appeared to make a vital commitment to the in-vivo survival of several intracellular bacterial pathogens (Franzon et al. 1990). Molecular examination for iron co factored superoxide dismutase (SODA) permits speciation of mycobacteria, generation of cellular immune responses in infected hosts by SODA which is abundantly secreted virulence factor, Mycobacterium species are identified by molecular analysis of SODA gene (Allen et al. 2008).

[V] CONCLUSION:

The series of experiment performed showed that *Mycobacterium tuberculosis*, a bacterium that cause Tuberculosis contains a protein named as Superoxide dismutase (SOD). Superoxide dismutase fixes the free oxygen radicals which are very dangerous to *M. tuberculosis* and even kill bacteria. A type of SOD protein in *M. tuberculosis* is present which is iron co factored and known as SODA, which is encoded by gene Rv3846 with length of 624bp. SOD mutant strains have been built to characterize the part of this molecule in the immune response to *M. tuberculosis* contamination. SODA is necessary for the liveliness of the bacteria and is secreted in large amount. The process of cloning was performed for SODA protein, encoded by rv3846 gene which is 624bp long. For this purpose forward and reverse primers were designed that were 25bp long each. There PCR amplification was performed. After that the purification of amplified gene, vector isolation,

ligation, incubation and colony PCR for confirmation of desired gene present in bacteria vector were performed. Cloning SODA could be very helping in future, it could be used for rapid detection of SODA antibodies produced in blood of TB infected patients in immune assay.

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