Purification and Characterization of the 64kDa Surface Protein from *Mycobacterium bovis* BCG

M. Phil Thesis

DEPARTMENT OF MICROBIOLOGY
FACULTY OF BIOLOGICAL SCIENCES
UNIVERSITY OF DHAKA
DHAKA-1000, BANGLADESH
JANUARY, 2017.

SUBMITTED BY
REGISTRATION NO.: **132**
SESSION: **2012-2013**

Purification and Characterization of the 64kDa Surface Protein from *Mycobacterium bovis* BCG

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF DHAKA FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

IN

MICROBIOLOGY

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Dedicated to…

My respected supervisor, with whom, my dreams are still alive after a long period of hibernation

Certification

It is hereby certified that student bearing Reg. No. 132, Session 2012-2013 has carried out the research work entitled "**Purification and Characterization of the 64kDa Surface Protein from *Mycobacterium bovis* BCG**" for the fulfillment of M. Phil Degree from University of Dhaka, Bangladesh, under my academic supervision in the Department of Microbiology, University of Dhaka.

..……

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Abstract

Cancer is one of the leading causes of death now-a-days. There are various treatment options for cancer and each have their own side effects because most of the therapeutic agents of cancer are non-selective and causes cancer cell destruction as well as normal cell destruction. So, search for a highly specific therapeutic molecule is still going on. As it is known that, immune molecules are highly specific in terms of antigen-antibody reaction, immunotherapy should be a better and more specific choice for cancer therapy compared to conventional molecules in use. It was suggested by previous literatures that, a 64kDa *Mycobacterium bovis* BCG surface protein has antigenic similarity (as it shares common antigenic determinants) with various mouse and guinea pig cancer cells and is cross reacting to one of these cancer cell antigens (64kDa). The anti-BCG 64kDa antibody also has anti-cancer effects against various solid tumors of experimental animals due to same reason. But there was no report of similar experiment on malignant ascites cell line. Considering the above facts, we got interested in this study to assess the effects of anti-64kDa on Ehrlich's Ascites Carcinoma (EAC) cells. In our study, we found that, mouse anti-64kDa has cytotoxic effect on HeLa cell line *in vitro* when compared to control and has anticancer effect on EAC cells. Pre-immunized animals (immunized with BCG 64kDa) showed 50% increase in life span compared to control and a plummeted rate of weight gain than control after challenge with EAC cells. Moreover, when Whole cell extracts of EAC cells were immunoblotted with BCG 64kDa immunized mice sera, a 64kDa band was observed. All these data suggest us that, water soluble BCG 64kDa surface antigen shares common antigenic determinants with malignant ascites cells and has anti-cancer activity in mice in terms of survival and rate of weight gain. Combining these results, we present BCG 64kDa surface antigen as a potent immunotherapeutic agent.

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Abbreviations

%	Percentage
et al.	And others
bp	Base pair
BCG	Bacillus Calmette–Guérin
TB	Tuberculosis
rpm	Rotation per minute
nm	Nanometer
WHO	World Health Organization
NIH	National Institute of Health
AIDS	Acquired Immune Deficiency Syndrome
kDa	Kilodalton
µg	Microgram
LJ	Lowenstein-Jensen medium
mA	Mili ampere
Rf	Retention frequency
v/v	Volume in volume
°C	Degree Celsius
FBS	Fetal Bovine Serum
µl	Micro liter
ml	Milliliter
mm	Millimeter
M	Molar
mM	Milli molar
mg	Milligram
gm	Gram
UV	Ultra violet
X	Times
M	Molar
P^H	Negative logarithm of hydrogen ion concentration
OD	Optical density
BSA	Bovine Serum Albumin
DMSO	Dimethyl sulfoxide
EAC	Ehrlich's ascites Carcinoma
CO_2	Carbon dioxide
HCl	Hydrochloric acid
NaCl	Sodium chloride
KCl	Potassium chloride
MST	Mean Survival Time
ILS	Increase in Life Span
Na_2HPO_4	Disodium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate

$MgCl_2$	Magnesium chloride
PBS	Phosphate buffer saline
EDTA	Ethylene di amine tetra acetic acid
TE	Tris EDTA
TBE	Tris borate EDTA
pg	Pico gram
µM	Micro mole
pmol	Pico mole
dNTP	Deoxyribonucleoside triphosphate
AFB	Acid Fast Bacilli
WCE	Whole Cell Extract
CAIE	Crossed affinoImmunoelectrophoresis
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
Taq	*Thermusaquaticus*
HeLa	Henrietta Lacks
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
ID	Intradermal
ASI	Active Specific Immunotherapy
TAA	Tumor Associated Antigen
WEM	Water Extracted Materials

1.1 Introduction

1.1.1 History of *Mycobacterium bovis* BCG

Humans have been infected with M. tuberculosis for millennia. The introduction of Bacille Calmette-Guérin (BCG) and chemotherapy in the past century marks an important advance in the history of tuberculosis (TB), which accounted for optimism to fight the disease especially in endemic area. To date, BCG remains as the most widely used vaccine worldwide with astonishing safety records (McShane, 2011; Ottenhoff and Kaufmann, 2012). Next to BCG, no other vaccines are available for treating TB and of the many new candidates in the pipeline none is close to market use.

In 1900, Albert Calmette and Camille Guérin began their research for an anti-tuberculosis vaccine at the Pasteur Institute in Lille. They cultivated tubercle bacilli on a glycerin and potato medium but found it difficult to produce a homogeneous suspension of the bacilli. In an attempt to counteract their tendency to clump they tried the effect of adding ox bile to the medium and, to their surprise, they noted that subculture led to a lowering of the virulence of the organism. It was this fortuitous observation that led them to undertake heir long term project of producing a vaccine from this attenuated tubercle bacillus (Calmette, 1922). In 1908, starting with a virulent bovine strain of tubercle bacillus supplied by Nocard (originally isolated by him in 1902 from the udder of a tuberculous cow), they cultured it on their bile, glycerin and potato medium and then proceeded to subculture at roughly three weekly intervals. By 1913 they were prepared to initiate a vaccination trial in cattle which was interrupted by outbreak of World War I. Sub-culturing was continued throughout the German occupation of Lille, despite the greatly increased cost of potatoes and the difficulty of obtaining suitable ox bile from the abattoir. Yet, they managed to obtain this by grace of the veterinary surgeons of the German occupying force. By 1919, after about 230 subcultures carried out during the previous 11 years, they had a tubercle bacillus which failed to produce progressive tuberculosis when injected into guinea pigs, rabbits, cattle, or horses. At Guerin's suggestion, they named it Bacille Calmette-Guerin; later they omitted "Bilie" and so BCG was born (Calmette, 1922).

In 1921, Calmette decided that the time was ripe for a trial of the vaccine in man. The first human administration of BCG was by Benjamin Weill-Halle (1875-1958) assisted by Raymond Turpin (1895-1988) at the Charité Hospital, Paris. A woman had died of tuberculosis a few hours after giving birth to a healthy infant. On 18 July 1921, Weill-Halle and Turpin gave a dose of BCG by the oral route to the infant. There were no undesirable sequels. The oral route was chosen since Calmette considered the gastrointestinal tract to be the usual route of natural infection by the tubercle bacillus. Weill-Halle then tried the subcutaneous and cutaneous routes on other infants but local reactions were objected to by the parents, and so the oral method was continued, an emulsion of BCG prepared by Boquet and Negre being used. By 1924 they were able to report a series of 664 oral BCG vaccinations of infants (Calmette et al., 1924). The Pasteur Institute at Lille began the mass production of BCG vaccine for use by the medical profession. From 1924 to 1928, 114 000 infants were vaccinated without serious complications (Calmette et al., 1927). In 1928, Calmette called Guerin to join him in Paris, since he did not feel it necessary for Guerin to continue the BCG experiments on animals in Lille. By 1931, there was a special laboratory for the preparation of BCG and Guerin was placed in charge. The method of BCG vaccination was therefore proved to be safe.

The strain was determined to be attenuated and then concurrently maintained on three media: potato bile, potato with glycerin beef broth, and potato Sauton media (Fomukong et al., 1992). The early BCG sub strains (ATCC 35736, TMC 1009, TMC 1019, and TMC 1022) were distributed between 1925 and 1928. In 1932, the potato bile and potato with glycerin beef broth lines were discontinued. Other BCG sub strains were subsequently distributed. BCG has since been used predominately as a vaccine against tuberculosis. It is also an effective cancer immunotherapy (Brosman, 1992). Most recently, BCG has been developed as a recombinant vehicle for multivalent vaccines against other diseases (Lugosi, 1992). The ability to differentiate BCG from other members of the *M. tuberculosis* complex is important for at least two reasons. First, although less virulent than its parent strain, BCG can cause disease in humans (Lotte et al., 1988; Lotte et al., 1983). Approximately 5% of patients undergoing intravesical BCG immunotherapy for bladder cancer experienced adverse reactions (Lamm, 1992; Lamm et al., 1992). Accurate identification of BCG will give

health care providers important epidemiological and treatment information. Second, the ability to identify BCG in areas where BCG is used and other *M. tuberculosis* complex organisms are prevalent is required to establish the rate of BCG complications. This is especially relevant in the era of AIDS (Edwards and Kernodle, 1996; Talbot et al., 1997).

1.1.2 Molecular biology of *Mycobacterium bovis* BCG

BCG sub strains retain certain genetic characteristics of the parent *M. bovis* strain. Both have a deletion of the MTP40 genetic fragment (Parra et al., 1991), a specific mutation in the *pncA* gene, conferring pyrazinamide resistance (Scorpio and Zhang, 1996), and a specific mutation in the *oxyR* gene (Sreevatsan et al., 1996). Some of these genetic characteristics have been exploited to distinguish *M. bovis* (including BCG) from other species of the *M. tuberculosis* complex (Sreevatsan et al., 1996; Liébana et al., 1996).

Culture conditions between 1908 and 1921 probably allowed the selection of multiple mutations in the parent *M. bovis* strain, leading to attenuation. Identification and characterization of these mutations are important because all or some may have contributed to the loss of virulence of BCG. Understanding the mechanisms of attenuation may yield insight into virulence mechanisms of *M. tuberculosis*, which may in turn facilitate improvement of our ability to prevent and treat tuberculosis. Recently, regions of BCG's genetic uniqueness were sought by using subtractive hybridization and DNA sequence comparisons between the BCG and *M. bovis* genomes (Mahairas et al., 1996). One region of difference, RD1, encodes a 9.5-kb fragment which contains at least eight open reading frames in *M. bovis*. This region was found to be absent in all BCG strains tested (Figure 1.1). When the RD1fragment was cloned into BCG, the resultant recombinant clones expressed many of the encoded proteins, some of which appeared to down-regulate expression of other proteins in this recombinant strain (Mahairas et al., 1996). Although a direct link between this deletion and the attenuation of BCG cannot be established because the virulent parent strain has been lost (Fomukong et al., 1992), preliminary data indicate that reintroducing RD1 into BCG may enable BCG to elicit pathology in a manner

similar to that of *M. bovis* (Mahairas et al., 1996).Therefore, it seems likely that RD1 is associated with the attenuation of BCG. Further characterization of RD1 may facilitate our understanding of BCG attenuation.

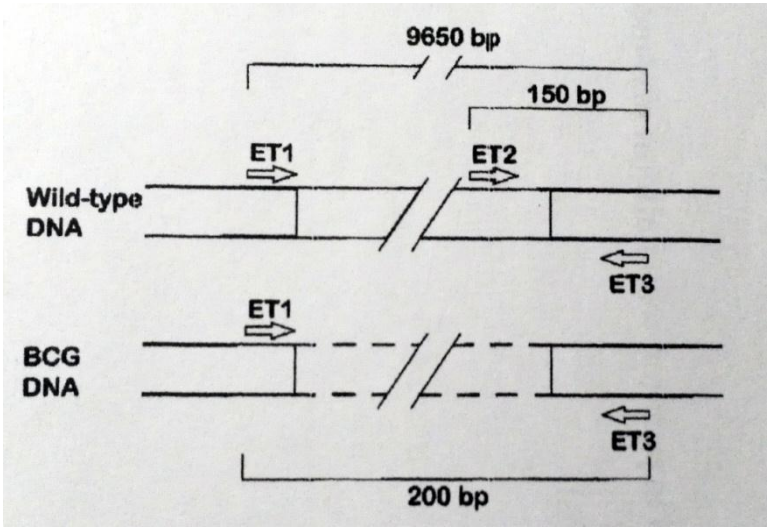

Figure 1.1: Mutation in BCG. Wild-type *M. tuberculosis* complex DNA is represented by the parallel lines in the top diagram. The shaded region contains the 9.5kb sequence RD1. RD1 is deleted from *M. bovis* BCG DNA, represented by the dashed lines in the lower diagram. The PCR primers ET1, ET2 and ET3 are shown as arrows oriented in the direction of amplification (Talbot et al., 1997).

1.1.3 Cancer statistics

Cancer can simply be defined as uncontrolled growth of cells of one or more tissues. Cancer is one of the most common, in fact one of the leading causes of death worldwide. The most common cancers are projected to be cancers of breasts, lung and bronchus, prostate, colon and rectum, bladder, skin, lymph nodes, thyroid, kidney and renal pelvis, blood, endometrium and pancreas. The number of new cases of cancer (cancer incidence) is 454.8 per 100,000 men and women per year (based on 2008-2012 cases) (National Cancer Institute, 2016). The number of cancer deaths (cancer mortality) is 171.2 per 100,000 men and women per year (based on 2008-

2012 deaths) (National Cancer Institute, 2016). Cancer mortality is higher among men than women (207.9 per 100,000 men and 145.4 per 100,000 women) (National Cancer Institute, 2016). It is highest in African American men (261.5 per 100,000) and lowest in Asian/Pacific Islander women (91.2 per 100,000) (National Cancer Institute, 2016). (Based on 2008-2012 deaths) The number of people living beyond a cancer diagnosis reached nearly 14.5 million in 2014 and is expected to rise to almost 19 million by 2024 (National Cancer Institute, 2016). Approximately 39.6 percent of men and women will be diagnosed with cancer at some point during their lifetimes (based on 2010-2012 data) (National Cancer Institute, 2016). In 2014, an estimated 15,780 children and adolescents aged 0 to 19 years were diagnosed with cancer and 1,960 died of the disease (National Cancer Institute, 2016). National expenditures for cancer care in the United States totaled nearly $125 billion in 2010 and could reach $156 billion in 2020 (National Cancer Institute, 2016).

Cancers figure among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012 (WHO, 2014). It is expected that annual cancer cases will rise from 14 million in 2012 to 22 million within the next 2 decades (WHO, 2014). Among men, the 5 most common sites of cancer diagnosed in 2012 were lung, prostate, colorectal, stomach, and liver cancer (WHO, 2014). Among women the 5 most common sites diagnosed were breast, colorectal, lung, cervix, and stomach cancer (WHO, 2014). Around one third of cancer deaths are due to the 5 leading behavioral and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use and alcohol use (WHO, 2014). Tobacco use is the most important risk factor for cancer causing around 20% of global cancer deaths and around 70% of global lung cancer deaths (WHO, 2014). Cancer causing viral infections such as HBV/HCV and HPV are responsible for up to 20% of cancer deaths in low- and middle-income countries (de Martel et al., 2012). More than 60% of world's total new annual cases occur in Africa, Asia and Central and South America. These regions account for 70% of the world's cancer deaths (WHO, 2014). All these statistics suggest that, cancer is a global, social and economic burden.

1.1.4 Treatment options for cancer

Treatment of cancer has following protocols: Radiotherapy, chemotherapy and surgery. Each of these treatment modes has its own drawbacks. Radio and chemotherapeutic agents are not highly specific and as a result they destroy cancer cells as well as normal cells. Surgery has its own side effects along with anesthetic hazards. A more specific and effective mode to cancer treatment should be immunotherapy in which, body's natural defense is boosted (Both actively and passively) to fight against the cancer cells. As immune molecules have high specificity regarding binding, immunotherapy is expected to be one of the most specific treatment options and to have fewer side effects.

1.2 Review of literature

1.2.1 Use of BCG as immunotherapeutic agent

Mycobacterium bovis BCG is widely and effectively used in live attenuated form to immunize individuals against tuberculosis. But it has other therapeutic uses. There are a number of research works regarding treatment of cancer using BCG. Clinical studies on prevention of acute leukemia by neonatal vaccination with BCG reports that BCG inhibits the development of experimental neoplasms, including spontaneous murine leukemia, have prompted several groups to consider the possibility of lowering the incidence of leukemia in a human population by means of BCG vaccination. By comparing records of leukemia deaths with records of children receiving BCG vaccination at less than 15 years of age in the Province of Quebec, it was found that death from leukemia was approximately half as common among children who had been vaccinated against BCG as among those who had not (Bast et al., 1974).

BCG invoked immunological reactivity, inhibiting tumor growth in experimental animals, led to clinical trials showing that intravesical BCG eradicated and prevented recurrences of superficial bladder tumors. Since then, for the last 3 decades, BCG therapy has remained the most effective local therapy for superficial bladder cancer, an outstanding example of success in urology (Herr and Morales, 2008).

1.2.2 Further researches to establish BCG as an immunotherapeutic agent

Intradermal (ID) inoculation of cells of a syngeneic transplantable ascites guinea pig hepatocarcinoma led to a progressively growing Line 10 tumor (Zbar at al., 1972). Tumor cells metastasized to the regional lymph node by 6–7 days after tumor implantation (Zbar at al., 1972). Excision or irradiation of the tumor prevented tumor growth locally but did not prevent metastases from growing and killing the host (Zbar at al., 1972). Specific immunization did not impair growth of established Line10 tumors or growth of tumor in the draining lymph node (Zbar at al., 1972). Inoculation of living BCG into established Line10 tumors caused local tumor regression and

prevented growth of tumor cells present in the regional lymph node (Zbar at al., 1972). Intralesional injection of BCG led to complete tumor regression in 63% of guinea pigs with tumor nodules weighing 95 mg and in 16% of guinea pigs with tumor nodules weighing 425 mg (Zbar at al., 1972). Guinea pigs treated by BCG developed systemic tumor immunity; some animals rejected distant artificial metastases (Zbar at al., 1972).

Randomized controlled phase III clinical trial in Stages II and III colon cancer patients with active specific immunotherapy (ASI) using autologous tumor cells with an immunomodulating adjuvant BCG vaccine (OncoVAX) in an adjuvant setting was evaluated (Uyl-de Groot et al., 2005). In this study, patients were randomized to receive either OncoVAX therapy or no therapy after surgical resection of the primary tumor and stratified by stage of disease (Uyl-de Groot et al., 2005). Since the biologic essence of the effective tumor immunotherapy is the presence in the vaccine of a minimum number of viable, metabolically active, autologous tumor cells, the processing of the vaccine product, occurred within 48 h after surgery (Uyl-de Groot et al., 2005). Analysis of prognostic benefit in the pivotal phase III trial, with a 5.8 year median follow-up, showed that a beneficial effect of OncoVAX is statistically significant for all endpoints including recurrence-free interval, overall survival, and recurrence-free survival in Stage II colon cancer patients (Uyl-de Groot et al., 2005). Surgery alone cures approximately 65% of Stage II (Dukes B2, B3) colon cancer patients (Uyl-de Groot et al., 2005). In the remaining patients, OncoVAX in an adjuvant setting, significantly prolongs recurrence-free interval (57.1% relative risk reduction) and significantly improves 5-year overall survival and recurrence-free survival (Uyl-de Groot et al., 2005). No statistically significant prognostic benefits were achieved in Stage III (Duke's C1-C3) patients (Uyl-de Groot et al., 2005). A health economics assessment was performed on these results in Stage II colon cancer patients using disease-free survival and overall survival (for the entire intent-to-treat population). Cost-effectiveness, cost-utility and sensitivity analysis were applied with, cost of life years, recurrence-free life years and quality adjusted life years (QALYs) as the primary endpoints to this analysis (Uyl-de Groot et al., 2005). The perspective of the economic analysis was the current direct medical cost established by the health care providers. The introduction of new technologies often leads to additional costs.

This report verified that the use of OncoVAX for patients with Stage II colon cancer not only has significant prognostic benefit and positive clinical outcomes, but also showed that OncoVAX therapy yields impressive health economics benefits (Uyl-de Groot et al., 2005).

1.2.3 Attempts to isolate an immunotherapeutic subunit from BCG

There are other studies diving deep into the mechanisms behind these beneficial effects of BCG. Presence of Tumor Associated Antigens (TAA) in the membrane of neoplastic cells is a preferential subject in the field of immunotherapy. Many attempts have been made to isolate these antigens (Baldwin and Moore, 1969; Baldwin et al., 1973; Mann et al., 1969; Meltze et al., 1971; Natori et al., 1978; Ahsan and Sasaki, 1989 and Gillis et al., 1985), but most of the isolations reported have been performed by using the whole cells but not from the surface membrane of tumor cells. In a study, Water Extraction Method (WEM) was applied to Line 10 tumor cells of strain 2 guinea pig and a high concentration of TAA containing solution was obtained which were not detected in normal liver cells. These materials were further analyzed by different immunological techniques to demonstrate their major components and antigenicities. These materials were identified to be glycoproteins of different molecular weights (44, 46, 62, 64 and 68kDa) which were not common to molecular weight of the same materials from normal liver cells which contained (Ahsan and Sasaki, 1989).

1.2.4 The 64kilodalton surface protein of *Mycobacterium bovis* BCG

A number of studies have attempted to purify and characterize species-specific immunologically active mycobacterial antigens associated with the host cellular immune responses (Gillis et al., 1985; Buchanan et al., 1987; Shinnick, 1987; Shinnick et al., 1988). However, one particular antigen from *Mycobacterium bovis* BCG, designated the 64kilodalton (kDa) antigen, has received much attention because it appears to be one of the major soluble, immunologically active protein antigens (Emmrich et al., 1986; Harboe et al., 1986; De Bruyn et al., 1987; Thofe et al., 1987). Purified 64kDa antigen also elicited a strong delayed type hypersensitivity reaction in

experimental animals and promoted a significant level of IgG antibody in sensitized guinea pigs (De Bruyn et al., 1987). *M. bovis* BCG has also been investigated widely within the scope of cancer immunotherapy of experimental animals and humans (Reif and Kim, 1971; Baldwin and Pimm, 1973; Pinsky et al., 1973; Baldwin and Pimm, 1974). Much effort has also been made to isolate the specific components possessing antitumor activity from BCG (Weiss et al., 1966; Zbar et al., 1973; Meyer et al., 1975), and there are reports on the antigenic relationships between BCG and different animal tumor and human cancer cells (Minden et al., 1974; Minden et al., 1976; Sasaki et al., 1989). Uses of BCG monoclonal antibodies have shown that there are common antigenic determinants between surface extracts of Line 10 hepatoma cells and BCG (Ahsan and Sasaki, 1989). It has also been demonstrated that 64kDa protein from BCG has anti-line 10 activity in immunized experimental animals (Ahsan and Sasaki, 1991). But there are no reports on *in-vitro* cytotoxic effect of this protein or its corresponding antibody and *in-vivo* study on ascitic cells (all the studies were performed on solid tumors).

1.3 Aims and objectives

As all the previous studies were based on solid tumors and no reports on cytotoxic assays and on ascitic cells exist, our study was based upon following objectives:

- Purification of 64kDa surface protein from *M. bovis* BCG WEM
- Performing *in-vitro* cytotoxic assay on HeLa cells
- Executing *in-vivo* anti-cancer experiments on Ehrlich Ascites Carcinoma (EAC) cells.

2. Materials and Methods

The materials and methods of this study can be shortly described in the flow chart below (Figure 2.1):

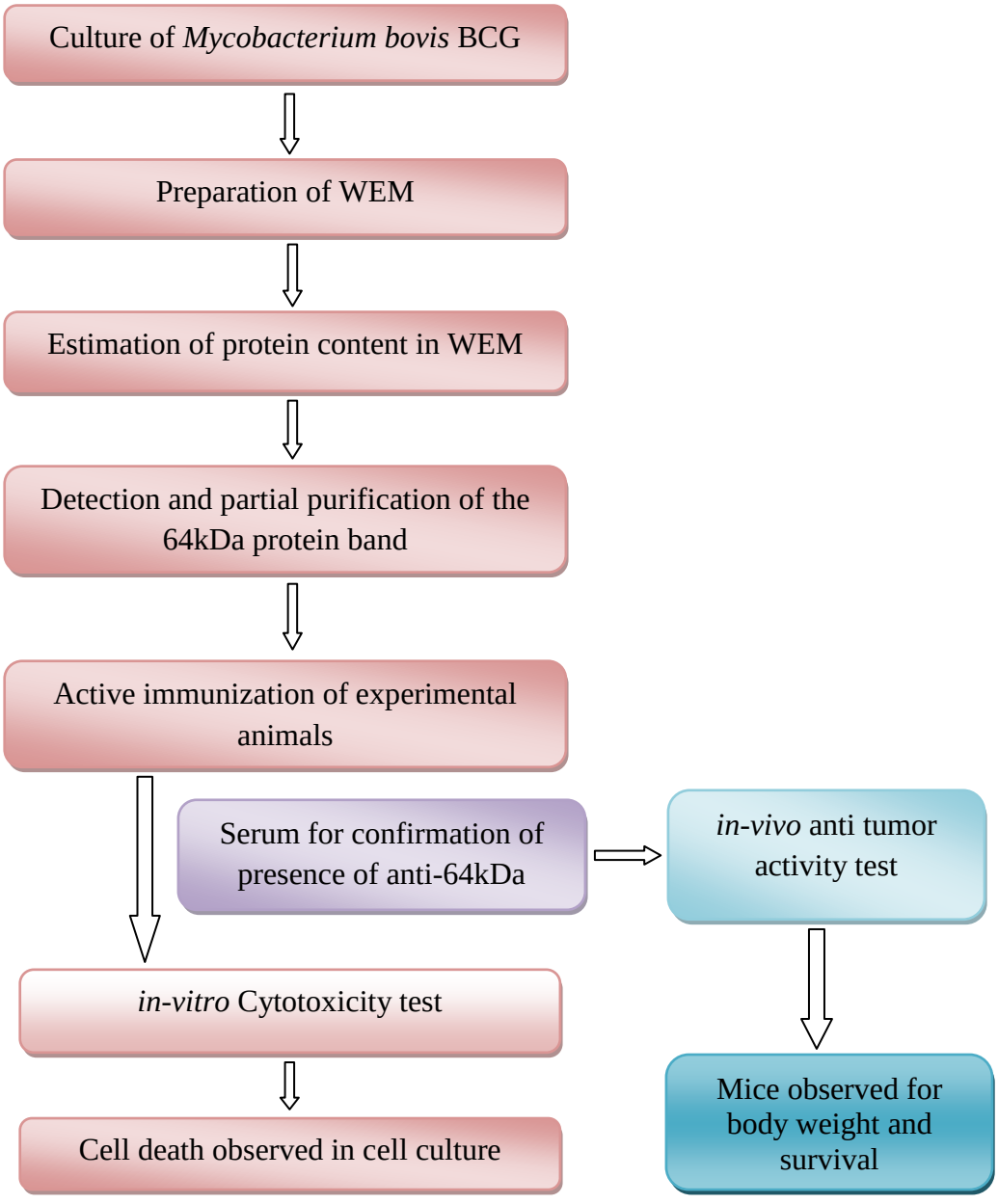

Figure 2.1: Methodology of the study.

2.1 Culturing *M. bovis* BCG

2.1.1 Culture in Lowenstein - Jensen (LJ) medium

Lyophilized *M. bovis* BCG (Bulgarian strain SL 222 Sofia) was cultured at first on Lowenstein - Jensen (LJ) medium, which is selective for culture of *Mycobacteria* due to the presence of malachite green that inhibits growth of other bacteria and consequently gives the medium a light green color.

Each of the lyophilized ampoules of *M. bovis* BCG is provided with an ampoule of 1 ml sterile normal saline. The procedure of inoculation was performed in laminar airflow and maintaining additional aseptic precautions.

The inoculation was done in zigzag pattern using inoculating loop, which was sterilized by heating red hot followed by cooling. The cap of the vial was kept loose to permit access of oxygen into the vial as *M. bovis* BCG is an aerobic bacteria. After proper labeling, the vial was kept in incubator, where incubation temperature was previously adjusted to 37ºC. Incubation was done for 2-3 weeks. After getting sufficient growth of the bacteria (indicated by change of color of the medium and appearance of distinct colonies of BCG), further culture was done in 10% Dubos broth.

2.1.2 Culture in Dubos broth

At first, Dubos broth base (BD Difco™, USA) was dissolved in deionized water as required and then distributed in equal volumes in different conical flasks taking into consideration the fact that, the ratio of left over volume in the flask to the total volume of medium in that particular flask should be approximately 4:1. Cotton plugs were placed at the inlet of the flasks, covered with aluminum foil and the flasks were ready to be autoclaved (at 121ºC and 15 psi pressure for 15 minutes) after proper labeling and sticking autoclave indicator tape.

Once autoclaved, the flasks were allowed to cool down to normal temperature. Fetal Bovine Serum (FBS) was allowed to melt previously at normal temperature. Then everything was transferred to a biosafety cabinet II. The cabinet was sterilized by wiping its working area with 70% alcohol and then by turning on the UV light for 45 minutes. To avoid risk, the room UV was also left on for the same duration. After proper

sterilization, UV lights were turned off and the airflow was turned on before the hood was lifted. Sterile pipette (10 ml), pipette filler, FBS bottle and all the flasks were transferred into the cabinet after wiping with 70% alcohol.

Maintaining all aseptic precautions, 10% (v/v) FBS was added to each flask, covered with cotton plug and then incubated at 37ºC overnight. On the next morning, each flask was checked for contamination. All the contents of the contaminated flasks were discarded after autoclaving. The flasks containing clear solutions were taken in the laminar airflow and maintaining above aseptic precautions, BCG was inoculated into Dubos broth, rest of the bacterial colonies in LJ medium were used for identification and the flasks containing inoculated Dubos broth were incubated at 37ºC. After 16 hours, all the flasks were checked again for contamination (as BCG has a generation time of 20 - 22 hours). All the media containing turbid solutions were discarded after sterilization. Then incubation was continued for 2 weeks.

2.2 Identification of *Mycobacterium bovis* BCG

2.2.1 Acid fast staining

A clean, grease free slide was taken and labeled. A drop of normal saline was placed on the slide and then a suspension was made by mixing part of bacterial colony in the saline. The suspension was spread widely and heated to prepare a fixed smear. The slide was flooded with heated carbol fuchsin for 5 minutes (primary staining). After removing carbol fuchsin, the smear was covered with 3% (v/v) acid alcohol mixture for 5 minutes (Destaining) or washed alternatively with acid and alcohol till the smear appears colorless or light pink and then washed with deionized water. Counter staining was done by pouring malachite green on the smear and allowed to stand for 2 minutes. The smear was air dried and examined under 100X objective.

2.2.2 Polymerase Chain Reaction (PCR)

2.2.2.1 DNA preparation

Bacterial DNA was extracted from culture by using phenol - chloroform DNA extraction method. *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* (wild type) grown on LJ medium was transferred to micro centrifuge tubes (1.5 ml) and tubes were wrapped by parafilm at the level of the lids to avoid popping up during the heating process. 1 or 2 colonies were scrapped from the media slant in 1 ml deionized distilled water. Heat was applied to the sample at a temperature of 80ºC for at least 1 hour to kill organisms. 500µl of culture suspension was taken into another tube and spinned at 4000 rpm for 5 minutes. Soup was discarded and pellet resuspended in 500µl TE buffer. 50µl of lysozyme (10mg/ml) and 50µl of RNase A were added to the resuspended pellet and later incubated at 37ºC for 1 hour. 55µl 10% SDS and 6µl of proteinase-k (10mg/ml) were added (Current volume is 661µl) and incubated at 65ºC for 1 hour. Two micro centrifuge tubes (1.5 ml) were taken and the whole mixture was divided equally into those tubes. Equal volume (330.50µl in each tube) of phenol - chloroform - isoamyl alcohol (25:24:1, v/v) mixture was added to each tube. The mixture was shaken gently on a flatbed shaker for 10 minutes. Centrifugation was done at 13000 rpm for 5 minutes and the aqueous layer of soup was transferred to another tube. Equal volume of chloroform was added to the aqueous phase and shaken by hand. Centrifugation was performed again at 12000 rpm for 5 minutes and the aqueous phase was transferred to another tube. 2 - 2.5X volume of ice cold 95% ethanol and 1/10 volume of 5M NaCl were added to the aqueous phase. Then the samples were kept at -20ºC overnight. On the next morning, samples were centrifuged again at 12000 rpm for 15 minutes and the supernatants were discarded. Samples were washed with 1 ml of ice cold 70% ethanol and centrifuged at 13000 rpm for 5 minutes and the soup was discarded. Pellets were allowed to air dry for 1 - 2 hours. Pellets were resuspended in 20µl nuclease free water.

2.2.2.2 PCR methods

The PCR primers (Talbot et al., 1997) those were used have been listed in Table 2.1. PCR was performed with 5 µl of each coded DNA sample in a total volume of 50 µl of PCR mix. The PCR mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001%

(w/v) gelatin, 2 mM $MgCl_2$, 200 µM of deoxynucleoside triphosphate, 1.25 U of *Taq* DNA polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk, Conn.), 5 pmol of each of primers ET1 and ET3, and 25 pmol of ET2. One interspersed negative control (Reagents only; no DNA) was included. The positive control was 200 pg of chromosomal DNA from *M. tuberculosis*. After a brief spin, the PCR tubes were placed in a thermal cycler (Bio-Rad, USA).

Target region	Nucleotide sequence (5´-3´)
ET1	AAGCGGTTGCCGCCGACCGACC
ET2	CTGGCTATATTCCTGGGCCCGG
ET3	GAGGCGATCTGGCGGTTTGGGG

Table 2.1: Primers used for PCR identification of BCG

2.2.2.3 Thermal cycling Parameters

The PCR reaction was performed according to the following program:

95°C…….3 min ……..initial denaturation

94°C…….... 30 sec

65°C…… 60 sec } 40 cycles

72°C…….....10 min……final extension

After this, PCR tubes were stored at -20ºC until further analysis.

2.2.2.4 Preparation of Agarose Gel

A 3% agarose gel was prepared by melting 3.0 gm agarose (Sigma, USA) in 100 ml 1× TBE buffer (Appendix II). The melted agarose was allowed to cool to about 50°C and was poured into gel electrophoresis unit (Sigma, USA) with spacers and comb. After solidification of the gel, the comb was removed and wells were formed. Then the gel was submerged in 1× TBE buffer in a running gel tank.

2.2.2.5 Loading and Running the Sample

Five µl of PCR product was mixed with 1µl of 6×gel loading dye (Appendix-II). The mixture was slowly loaded into the well using disposable micro-pipette tips. Marker DNA of known size was loaded in one well to determine the size of the PCR products. Electrophoresis was carried out at 90 volts.

2.2.2.6 Staining and Visualization of the Gel

After electrophoresis, the gel was submerged in staining solution containing ethidium bromide (Et-Br) (Appendix-II), for 15 minutes. Destaining was performed by submerging the gel in distilled water for about 15 minutes. The Et-Br stained DNA bands were observed on a UV transilluminator (Vilber Lourmet Doc Print VX05, France). Photographs were taken using a gel documentation system and bands were analyzed.

2.3 Identification of 64kDa surface protein from BCG

2.3.1 Harvesting BCG cells from Dubos broth

After observing sufficient turbidity of the Dubos medium, a suspension was made by gently shaking the flasks. These suspensions were transferred in sterile 50 ml centrifuge tubes (40 – 45 ml in each tube). Each tube was placed in the centrifuge machine in a manner that the weight of a single tube was countered by the equal weight of the tube in the opposite slot to prevent damage to rotor. Centrifugation was done at 16000g for 25 minutes (Ahsan and Sasaki, 1991). After centrifugation, supernatant containing medium was discarded after sterilization by autoclaving. The cells were suspended in sterile normal saline (0.85% NaCl) and then collected in a single 50 ml centrifuge tube for convenience. Centrifugation was done again at 10000g for 10 minutes and then supernatant was discarded. This process of suspending the cells in normal saline, centrifugation and then discarding supernatant was repeated for 2 - 3 times for proper media wash out.

2.3.2 Preparation of WEM

After washing out media, the cells were finally suspended in sterile deionized distilled water (3 - 5 ml depending upon approximate cell volume observed in naked eye) and the suspension was transferred in a sterile flat bottomed (100 ml) conical flask. A flat surface is required to gain speed for the low volume of suspension to hit the flask wall during horizontal shaking as this collision force will extract the surface proteins. A horizontal shaker was set at a speed of 100 - 120 oscillations per minute and turned on after placing the flask on it. Shaking was continued overnight for 16 hours at room temperature.

On the next morning, the suspension was collected in a sterile 15 ml falcon tube and centrifuged at 16000g for 30 minutes. The supernatant was collected in a sterile 5cc syringe. This fluid contains the water soluble surface proteins and designated as WEM. WEM was aliquot into sterile 1.5 ml centrifuge tubes after filtration through 0.2µm milipore filter. The filtered WEM was preserved at -20ºC. Cells were discarded after sterilization.

2.3.3 Estimation of protein in WEM

Protein in the WEM solution was estimated by Bradford protein assay protocol by using Bio Rad protein estimation dye concentrate. To reconstitute the Bovine Serum Albumin (BSA) standards, a stock solution of 10mg/ml was prepared by mixing deionized water and BSA at required amounts until dissolved. If the standard was not to be reused within 60 days, it could be aliquoted and frozen at -20°C.

Required volume of dye reagent was prepared by diluting 1 (one) part Dye Reagent Concentrate with 4 (four) parts distilled deionized water and filtering through Whatman #1filter (or equivalent) to remove particulates. This diluted reagent maybe used for approximately 2 (two) weeks when kept at room temperature. Four dilutions of BSA (0.2, 0.4, 0.6 and 0.8 mg/ml) was prepared keeping into consideration the fact that the linear range of the assay for BSA is 0.2 to 0.9 mg/ml. 100 µl of each standard and sample was pipette into a clean, dry test tube. Protein solutions were assayed in duplicate. 5 ml of diluted dye reagent was added to each tube and mixed well using vortex mixer.

A mixture of dye reagent and deionized distilled water served as blank. All mixtures were incubated at room temperature for at least 5 minutes. As absorbance increases over time; samples were incubated for no more than 1 hour. Absorbance was measured at 595 nm using a spectrophotometer (Genesys 5, Canada). All the absorbance values of standard were plotted to produce a standard curve and the absorbance of sample was plotted on the standard curve to estimate protein concentration.

2.3.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS - PAGE)

Preparation of all the buffers and solutions for SDS - PAGE has been described in Appendix II

At first, the large glass with the 1mm spacer and the small glass were clipped together and the space between the glasses at the bottom was sealed by applying pressure on the top of the large glass and placing the whole setting on a rubber strap. This setting was done in a manner so that the small glass was faced towards the investigator. After introducing the comb in the gel chamber, a mark was put on the exterior of the small glass 1cm below the lower end of the comb. Leak check was done and the 12.5% separating gel was prepared in the allotted beaker.

As soon as TEMED was added, the preparation for transferring the mixture to the gel chamber was complete. The gel mixture was added up to a bit higher than the mark, so that, when the gel solidifies and retracts, the upper border of the gel reaches down to the marking. After loading the gel, the upper border of the gel was protected from oxygen mediated decay, by adding butanol saturated water. Solidification of the gel was confirmed by visualization of a refraction gradient line at the junction between butanol saturated water and the solidified gel near the marking.

After solidification of separating gel, stacking gel was prepared in the same beaker. After adding TEMED, the butanol saturated water was discarded and the chamber was soaked with a filter paper to confirm absence of any butanol saturated water. Then, the stacking gel was transferred to the chamber and the comb was introduced in the stacking gel solution, keeping the fact in mind that, there was no air bubble at the bottom of the comb.

Solidification of the stacking gel was confirmed by observation of the solidification of the solution that remained in the beaker.

Sample was mixed with 2X sample buffer at a ratio of 1:1 and heated in boiling water for 3 minutes. Then after cooling the mixture, 5µl of tracking dye (Bromophenol blue) was added to each sample, mixed well using a vortex mixer and then short spinned for final preparation of the sample. When samples were prepared, and the stacking gel was solidified, the gel cassette was placed into the electrophoresis tank in a manner that the large glass facing towards the investigator. Running buffer was poured into the both chambers (outer and inner). Then the comb was removed for visualization of the wells. Samples were loaded with long tips and the tank was closed with the lid and being cautious about the connections of wires. The other ends of the wires were connected to the power pack and the electrophoresis was started at 16-18mA current flow and freeing the volts. Sample descent was monitored and when samples reached the separating gel, the current flow was increased to 20-22mA. If there was any form of curving of the samples, those were managed accordingly. When the samples reached the bottom of the gel, the power pack was turned off and the gel was separated from the gel chamber cautiously, stacking gel was cut off using a gel cutter and the separating gel was subject to staining or blotting.

Staining was done using the staining solution. After flooding the gel with staining solution and gently shaking for 1 hour, the staining solution was restored for reuse and destaining solution was added. Destaining was done overnight. The protein band was visualized in naked eye against a bright background and the molecular weight was determined by Rf value method.

2.4 Isolation of 64kDa protein by blotting

To perform blotting, the unstained separating gel was used. A sandwich was made using Whatman filter paper, gel and nitrocellulose membrane. The sequence was as follows from negative (black) to positive (red): Filter paper, gel, nitrocellulose membrane and filter paper. Air bubbles were not allowed to stay within the layers of the sandwich. The sandwich was then placed in the blotting chamber and transfer buffer (Appendix II) was added to the chamber.

The process of transfer was carried out at 12 to 15 volts overnight and freeing the current flow. On the next day, the membrane was separated from the sandwich and stained with ponceau stain and then destained with water to confirm the attachment of the proteins to the nitrocellulose membrane. The desired band was identified by calculating Rf value, cut and sonicated in normal saline (0.85% w/v sodium chloride) to fine enough particles that can pass through 25G hypodermic needle; so that, this suspension can be used for animal immunization.

2.5 Confirmation of anti-64kDa in immunized animal sera by Western blotting

Blotted nitrocellulose membrane strips were blocked with 2% skimmed milk in PBS for 1 hour. Strips were then shaken in primary antibody diluted (Immunized animal sera) at a ratio of 1:100 in 2% skimmed milk in PBS for 1 hour. Washing was performed by shaking the membrane strips in 0.1% Tween 20 in PBS for 3 times (5 minutes each time) and in PBS once for 5 minutes. Then the strips were shaken in secondary antibody (anti mouse IgG alkaline phosphatase conjugate) diluted at a ratio of 1:10,000 in 2% skimmed milk in PBS for 1 hour. Strips were washed again as above. Substrate solution (Appendix II) was prepared and added to the washed strips in dark room.

2.6 *In-vitro* cytotoxicity study by cell culture technique

2.6.1 Cell Thawing

Cell thawing was done as rapidly as possible to avoid damage to the cells due to temperature fluctuation. The water bath was adjusted to 37ºC and allowed time to get heated. The cryovial was removed from the liquid nitrogen cylinder and rapidly transferred to -80ºC refrigerator (Nuaire, USA). From refrigerator, the vial was removed after 5 minutes and transferred to the water bath rapidly. After proper thawing, the vial was taken to the cabinet and disinfected with 70% ethanol. 5 ml of 10% Dulbecco Modified Egales Medium (DMEM) was taken in a 15 ml Falcon tube and the thawed materials were transferred into the tube. The tube was then centrifuged at 1000 rpm for 1 minute. After centrifugation, the supernatant was discarded to get rid of DMSO. Another 5 ml of 10% DMEM was added to the pellets and mixed thoroughly to form a

suspension. This suspension was transferred to a flask and then the flask was labeled with passage number (Same passage number used during freezing as no proteolysis by trypsin took place before thawing).The flask was examined under the microscope for viable cells and then incubated.

2.6.2 Cell passage

Sub culturing, also referred to as passaging, is the removal of the medium and transfer of cells from a previous culture into fresh growth medium, a procedure that enables the further propagation of the cell line.

The growth of cells in culture proceeds from the lag phase following seeding to the log phase, where the cells proliferate exponentially. When the cells in adherent cultures occupy all the available substrate and have no room left for expansion, or when the cells in suspension cultures exceed the capacity of the medium to support further growth, cell proliferation is greatly reduced or ceases entirely. To keep them at an optimal density for continued growth and to stimulate further proliferation, the culture has to be divided and fresh medium supplied.

Flask was examined under microscope to confirm that the cells were 90%-100% confluent. All materials were disinfected including the cabinet (As general guidance, ethanol was not sprayed on the flask) and hands and flask was quickly placed in the cabinet. Previous medium was discarded and 4-5 ml of PBS (Free of Ca^{2+} and Mg^{2+}) was added to flask. After slight agitation, PBS was discarded. 800µl trypsin (0.125%) was added to flask and the flask was gently tapped with finger for detachment of cells. If tapping was unsuccessful, the flask was placed in incubator until cells were detached. Close observation was kept with an interval of 1 minute. Detachment of cells was observed either by naked eye or under the microscope. 5 ml of medium was added to dilute trypsin and thorough pipetting was done to mix the medium and cell to form a suspension. 1 ml of suspension (Inoculum) was taken into another flask and 4 ml fresh medium was added to the suspension and then the flask was shaken gently. The flask was labeled with cell type, passage number and date and was examined under microscope for living cells. Incubation was done at 37ºC with 5% CO_2, and appropriate humidity to prevent evaporation of the medium.

2.6.3 Cell counting

2.6.3.1 Procedure

Cells were counted by using improved Neubauer counting chamber. Each of the 4 (four) large squares at the 4 (four) corners of the chamber has a length of 1mm and width of 1mm. When the cover slip is placed over the chambers the depth becomes 0.1mm. So the volume of each large square stands $0.1mm^3$. An average cell number is calculated from the total calculated cells in 4 (four) large squares and the cellular concentration in the suspension is expressed as no. of cells/ml. Cell suspension in medium was taken in a 15 ml centrifuge tube. 10µl of the suspension was added to a hemocytometer covered with slide. Number of cells was counted under inverted microscope for the 4 (four) cornered square marked A, B, C, D in Figure 2.2.

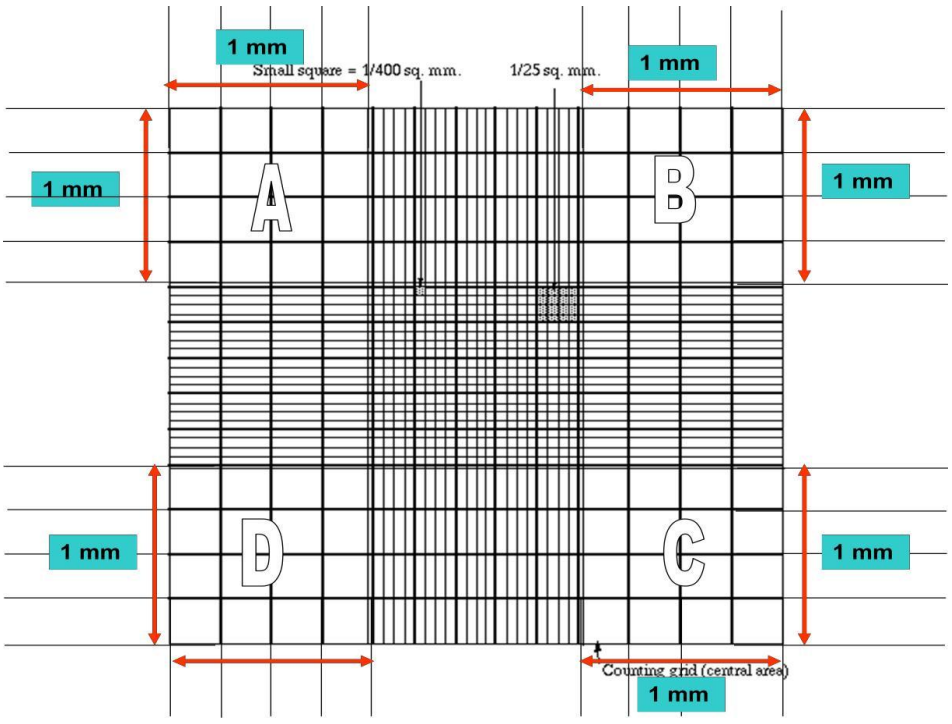

Figure 2.2: **Neubauer counting chamber showing the graduation**. Squares A, B, C, D are designated as large squares and cells were counted in each large square (Having area of $1mm^2$ and volume of $0.1mm^3$) including cells overlying any 2 adjacent margins of the square and excluding those overlying the opposite 2 margins.

2.6.3.2 Calculation

We got following data from the counting chamber:

A = 15cells, B = 18cells, C = 14cells, D = 17cells

Average = (A+B+C+D)/4 = 16cells

So, cell concentration = 16 cells per 0.1 mm^3

= 160 cells per mm^3

= 160×10^3cells per ml

= 1.6×10^5 cells per ml (Stock suspension)

For seeding, we needed 2 final concentrations of 1.25×10^5 cells per ml (Suspension-1) and 1.1×10^5 cells per ml (Suspension-2) so that we could seed 5×10^4 cells/400µl/well and 5×10^4 cells/450µl/well respectively and 5 ml of each of these suspensions. So, for suspension - 1, $S_1 = 1.6\times10^5$ cells per ml, $S_2 = 1.25\times10^5$ cells per ml, V_2 = 5ml then V_1=?

We know, $V_1S_1 = V_2S_2$

Or, $V_1 \times 1.6\times10^5 = 5 \times 1.25\times10^5$

Or, V_1 = 3.9 ml.

So we took 3.9 ml of the stock suspension and added 1.1 ml of medium to obtain 5 ml of suspension - 1 (1.25×10^5 cells per ml). Same calculation was done for preparation of suspension - 2 (1.1×10^5cells per ml) by taking 3.44 ml of stock suspension and adding 1.56 ml medium.

2.6.4 Procedure of cytotoxicity assay using immunized animal sera

In our experiment, we used HeLa cell line and the cytotoxic effect of control (raw and heat inactivated) and experimental (raw and heat inactivated) mice sera was observed at different concentrations. Heat inactivation of sera was done by heating the sera in water bath at 56ºC for 1hour. All sera were syringe filtered using 0.2µm milipore filter. A 24 well microtiter plate was taken and on day-0, 8 wells were seeded with 5×10^4 cells/400µl/well and labeled as 20% and another 8 wells were seeded with 5×10^4 cells/450µl/well and labeled as 10%. On day-1, each of the seeded well was examined

under inverted microscope to confirm adherence of the cells to the respective well floor. Then 100µl of each control sera (raw and heat inactivated) and each experimental serum (raw and heat inactivated) were added in duplicate to each 20% labeled wells. On the other hand, 50µl of each control sera (raw and heat inactivated) and each experimental serum (raw and heat inactivated) were added in duplicate to each 10% labeled wells. Then the microtiter plate was incubated and cytotoxic effect was observed up to 72 hours.

2.6.5 Cell Preservation

Cells were preserved in cryovials. Fully confluent cells in one culture flask were used to preserve in one cryovial. It requires high concentration of cells with as low passage number as possible and there must be at least 90% viable cells before freezing. Cells were cryopreserved at a temperature below -80ºC. Temperature rise above -60ºC deteriorates the cells. The best method for cryopreserving cultured cells is storing them in liquid nitrogen (-196ºC to -210ºC) in complete medium in the presence of a cryoprotective agent such as dimethylsulfoxide (DMSO). Cryoprotective agents reduce the freezing point of the medium and also allow a slower cooling rate, greatly reducing the risk of ice crystal formation, which can damage cells and cause cell death. DMSO is known to facilitate the entry of organic molecules into tissues.

Old culture medium was discarded from the flask. Cell monolayer was then rinsed with 4 ml of PBS to remove all traces of FBS. After addition of 0.8 ml of 0.125% trypsin, flask was tapped or incubated for short duration for detachment of the cells. After successful detachment, an idea about number of viable cells was taken. After addition of 5 ml 10% DMEM, the cells were washed from the flask and transferred to a 15 ml Falcon tube and then centrifuged at 1000 rpm for 1 minute. While cells were being centrifuged, 2 ml of 10% DMSO preservation media was prepared. After centrifugation, supernatant was discarded and cells were re-suspended in 1 ml of the preservation media and then the whole suspension was transferred to a cryovial. The cryovial was labeled with passage number and date and kept at -80ºC refrigerator for overnight cooling. On the next day, the vial was moved to liquid nitrogen cylinder.

2.7 Animal experiment design

2.7.1 Immunization and collection of immunized sera

Swiss albino mice were included in this study. 4 (four) groups of mice were taken including 6 (six) mice in each group (Figure 2.3). The groups were designated as control group 1, control group 2, experimental group 1 and experimental group 2. Immunization was done in 4 intramuscular doses given at day-0, day-14, day-28 and day-42 at different sites. Control group 1 and control group 2 received sonicated nitrocellulose membrane only whereas, experimental group 1 and experimental group 2 received 64kDa protein conjugated to nitrocellulose membrane. On day-49, all mice in control group 1 and experimental group 1 were sacrificed after collection of blood by cardiac puncture. On the same day, blood was collected from tail veins of mice in control group 2 and experimental group 2. Sera were collected from each sample and were preserved for further study. After collection of sera from blood, a western blot analysis was done to confirm the presence of anti-64kDa in the sera.

2.7.2 *In-vivo* anti-tumor assay

After confirmation of presence of anti-64kDa in mice in experimental group 2, they were challenged with EAC cells on day-50. Each mouse was seeded with $4.5x10^5$ cells by intraperitoneal injection using 100IU insulin syringe. Cell counting technique has been described in the cell culture section. After seeding of EAC cells, survival was recorded. Mean Survival Time (MST) and percent Increase in Life Span (ILS) after challenge were calculated using following formulae:

$$\text{Mean Survival Time (MST)} = \frac{\sum \text{survival time (Days) of each mouse in a group}}{\text{Total number of mice in that group}}$$

$$\text{Percent Increase in Life Span (ILS)\%} = \left(\frac{\text{MST of treated group}}{\text{MST of control group}} - 1\right) \times 100$$

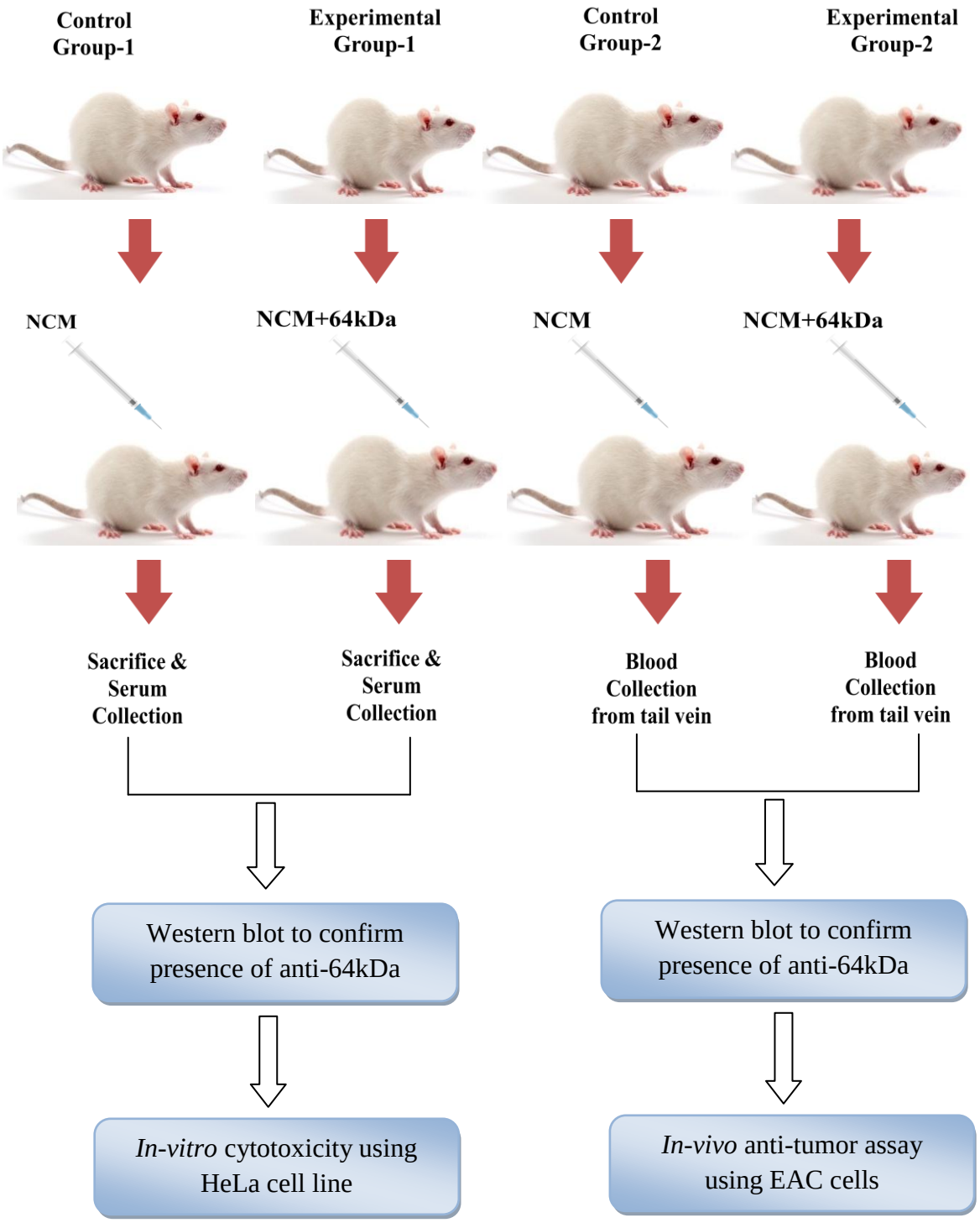

Figure 2.3: Figure showing flow chart of the animal experiment design

Another parameter that was included in the anti-tumor assay was rate of weight gain in different groups of mice with slight modification of a previously described method (Gupta et al., 2004; Islam et al., 2012). In this part of the assay, weight of individual mouse was recorded on day-50and at 5 days interval starting from day-60 through to the survival of control group. After recording weight of individual mouse, an average of eights was determined and this value was compared with the value on day-50 (Just before challenge) to gain a percentage increase in the weight using the following formula:

$$Percent\ increase\ in\ weight = \left(\frac{Average\ present\ weight}{Average\ initial\ weight\ (Before\ challenge)} - 1\right) \times 100$$

3. Results

3.1 Identification of *Mycobacterium bovis* BCG

3.1.1 Culture in LJ medium and identification by AFB staining

Culture of *Mycobacterium bovis* BCG in LJ medium revealed yellowish white colonies on the slope which were stained by Acid Fast Bacilli (AFB) staining and observed under 100X objective to see red rods (Beaded appearance) against a greenish background (Figure 3.1).

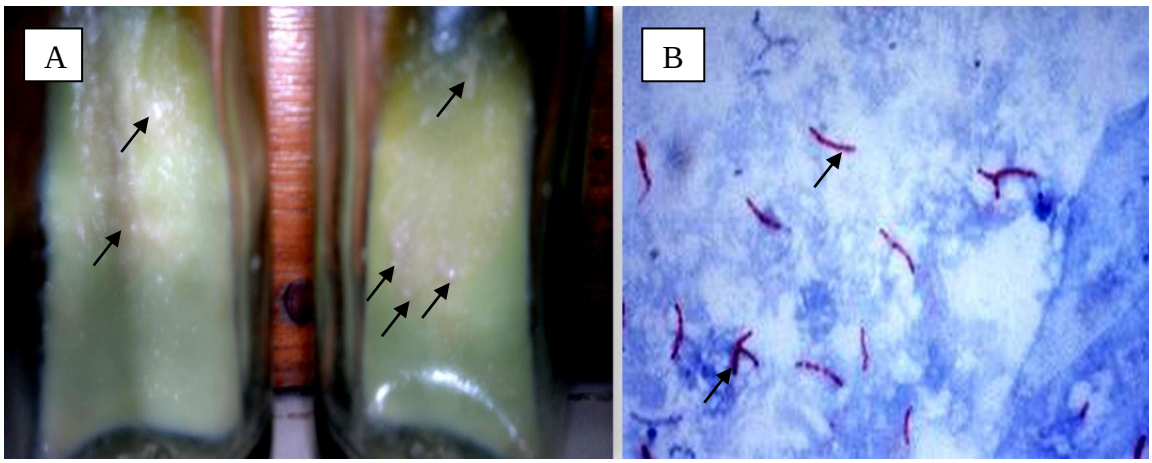

Figure 3.1: Culture colonies and AFB staining appearances of BCG. A. Yellowish white colonies (Marked by arrows) of *Mycobacterium bovis* BCG on LJ medium (Green sloped surface) inside McCartney bottles. **B.** Red rods with beaded appearance (Marked by arrows) during microscopy.

3.1.2 PCR identification

After separating the cells from Dubos broth, most of the cells were used for WEM preparation, some for AFB staining and some for PCR based identification. The presence of *Mycobacterium bovis* BCG was confirmed by observing a 200bp band in ethidium bromide stained 3% agarose gel, where *Mycobacterium tuberculosis* showed a 150bp band (Figure 3.2)

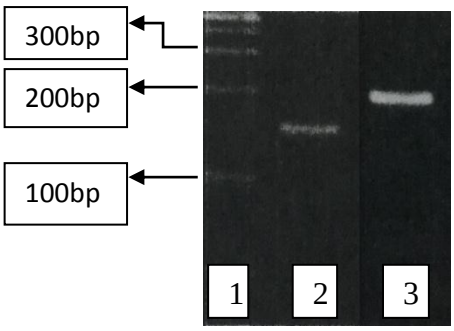

Figure 3.2: Figure showing differences in PCR identification characteristics between *M. tuberculosis* and *M. bovis* BCG. *M. tuberculosis* shows a 150bp band (Lane 2) and *M. bovis* BCG shows a 200bp band (Lane 3). Lane 1 contains marker.

3.2 Protein estimation in WEM

The concentration of protein was determined by Bio-Rad protein assay based on Bradford method. From this standard curve of BSA (Figure 3.3), protein content of the WEM was measured, which was estimated to be approximately 1.1 mg/ml.

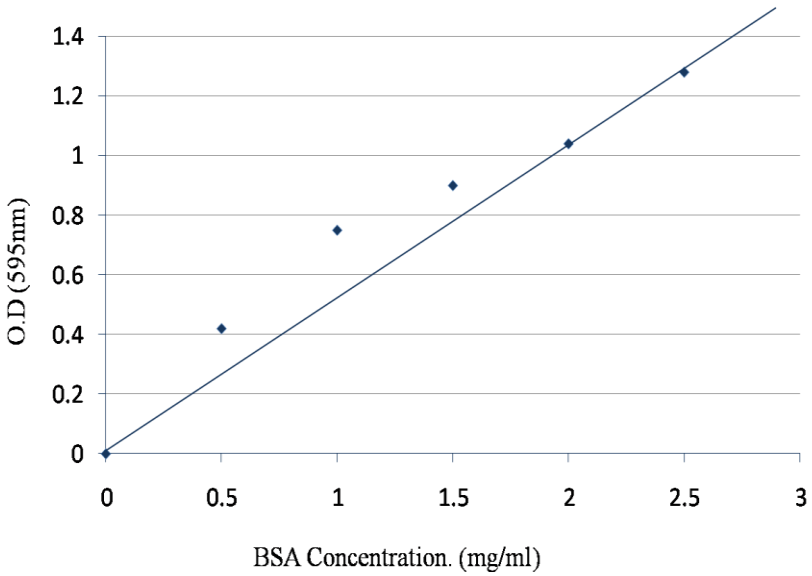

Figure 3.3: Preparation of standard curve for protein estimation

3.3 Determination of Molecular Weight

The SDS-PAGE analysis of the WEM showed 3 distinct bands of proteins (Figure 3.4). The molecular weight of the desired protein band was determined by Rf value (Figure 3.5), which was 64kDa. After detection of 64kDa band in WEM, the band was cut along with nitrocellulose membrane, sonicated and mice were immunized.

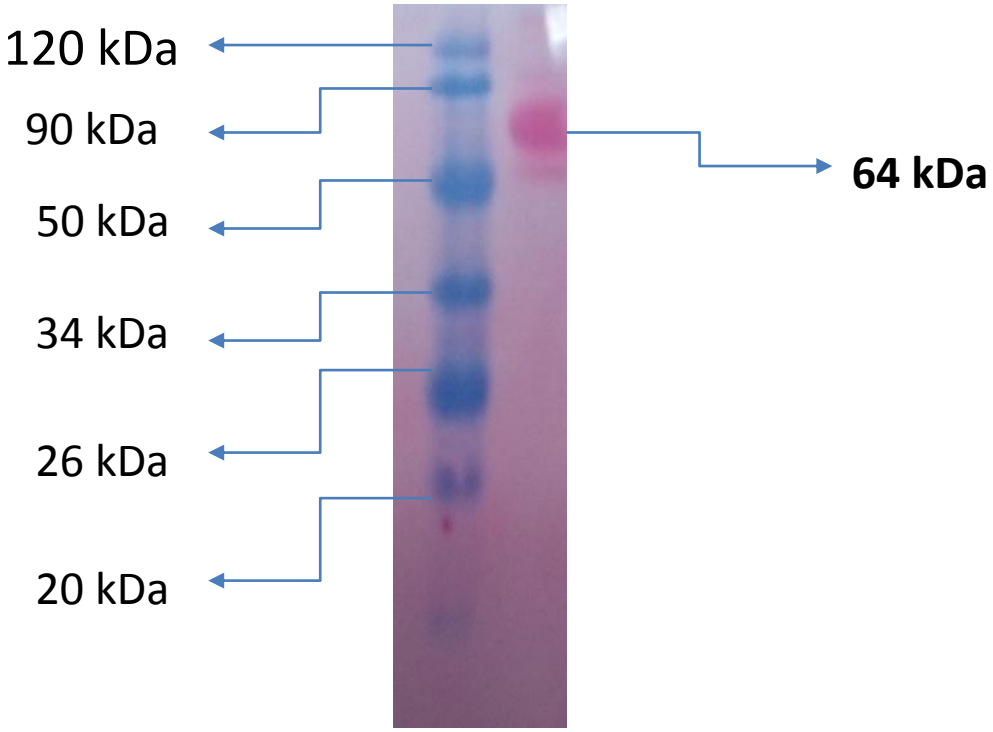

Figure 3.4: Figure showing results of SDS-PAGE analysis. Protein markers are the blue bands on left side and 3 distinct red bands on the right side are the protein bands in WEM after performing SDS-PAGE using 12.5% separating gel and 5% stacking gel and then transferring the proteins to a nitrocellulose membrane followed by staining and destaining with 0.2% ponceau stain and water respectively. The molecular weight of the wide band on the right side was calculated to be 64kDa by Rf value method. This band along with the nitrocellulose membrane was cut and used for animal immunization.

For the detection of molecular weight, a calibration curve (Figure 3.5) was prepared by using molecular weight standards where Rf values of marker bands were plotted (x-axis) against the known molecular weight (y-axis).

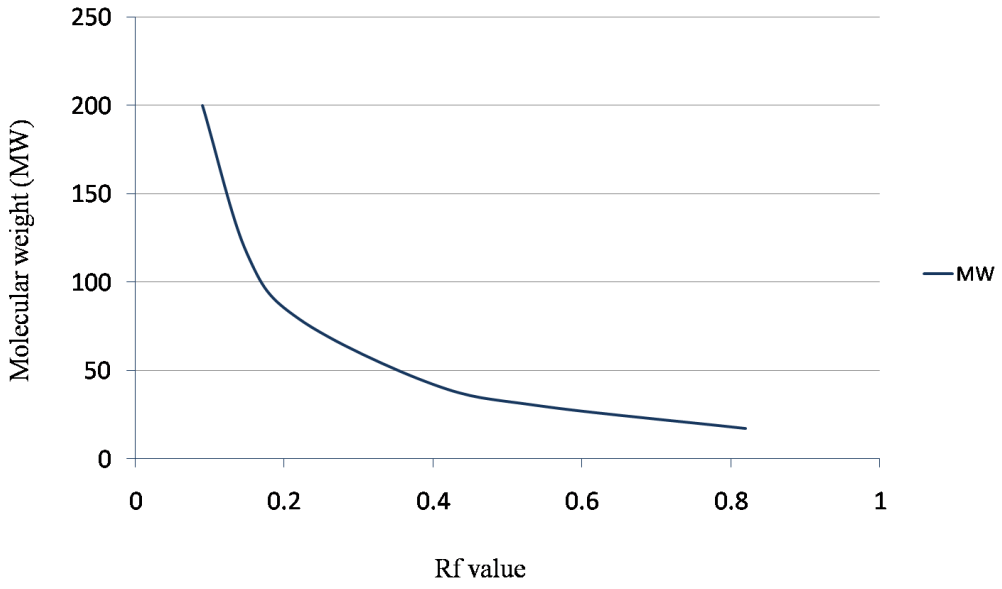

Figure 3.5: Preparation of standard curve for determination of molecular weight

3.4 Immunization of mice

Successful immunization in mice was tested by performing Western blot analysis by using WEM as antigen and immunized mice sera as primary antibody. Appearance of a 64kDa band (Determined by Rf value method) after addition of substrate was the confirmation of development of anti-64kDa antibody in immunized mice (Figure 3.6).

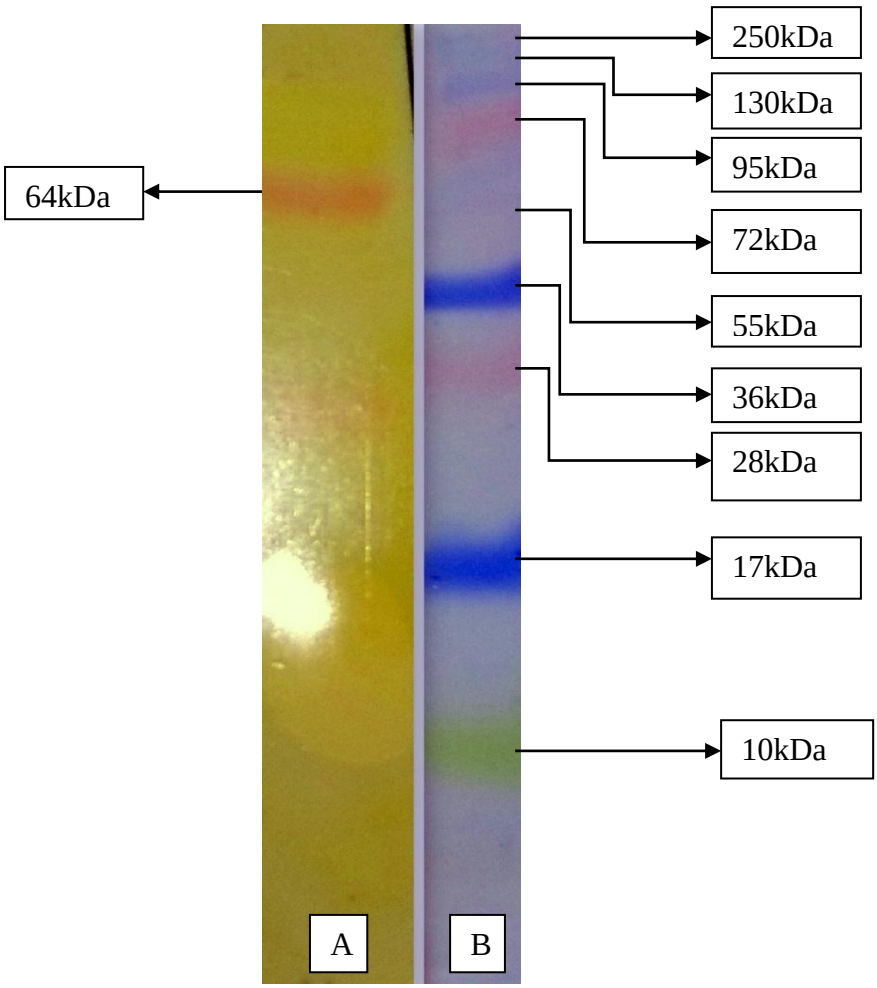

Figure 3.6: Figure showing result of western blot analysis. Western blotting was performed using WEM as antigen and immunized mice sera as primary antibody. The red band on the nitrocellulose membrane strip **A** has a molecular weight of 64kDa as determined by Rf value indicating successful immunization. Strip **B** contains protein marker.

3.5 *In-vitro* cytotoxic effect of anti-64kDa antibody

Cytotoxic effect of mouse sera containing anti-64kDa was observed on HeLa cells. Different concentrations of control and immunized mice sera (10% and 20% per well) were used on a specific number of cells (5×10^4 cells per well). Cytotoxic effect of experimental sera was more marked with increased concentration of serum and with increased incubation time. Cytotoxic effect was determined by comparing the effect of

experimental sera with that of control sera. Cell death was characterized by detachment of cells from the well floor and clumping.

Effect of 10% mice sera after 48 hours incubation has been shown in figure 3.7. The figure on the left side shows effect of control sera, i.e., serum from the group of mice that received sonicated nitrocellulose membrane only. Here cells are showing confluence and no sign of cell death (Figure 3.7).

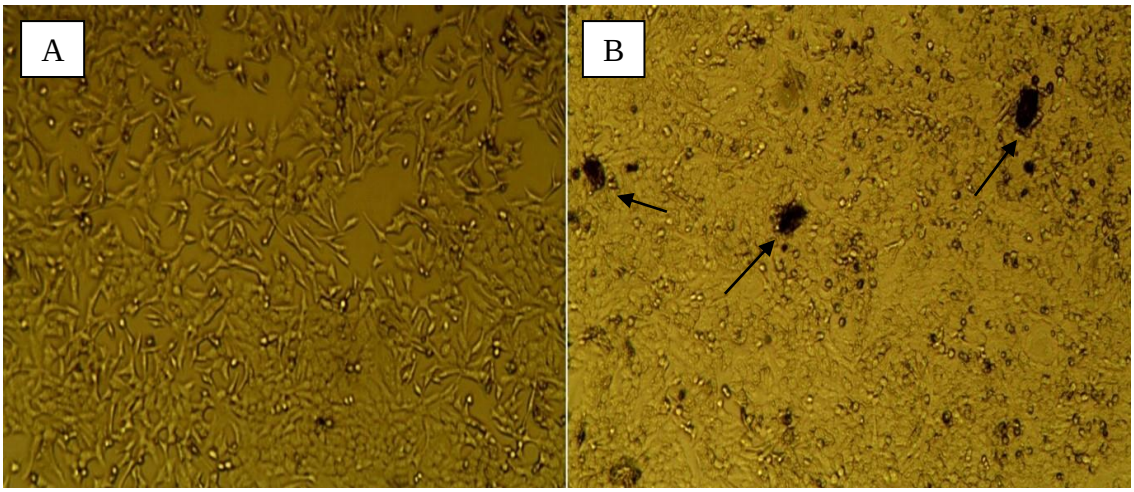

Figure 3.7: Figure showing cytotoxic effect of 10% mice sera on HeLa cells after 48 hours incubation. A. Represents the effects of 10% control mice serum, where cells are almost 80% confluent and there is no sign of cell death and **B.** shows effects of 10% experimental serum, where cell death is characterized by detachment and clumping (Marked by arrows).

After extension of the duration of incubation for another 24 hours with 10% mice sera, the cell death in the well containing experimental serum was more marked than that after 48 hours incubation, characterized by detachment of cells from the well floor and more prominent clumping. Incubation for the same duration with same concentration of control mouse serum had no sign of cell death, but cells were fully confluent there (Figure 3.8).

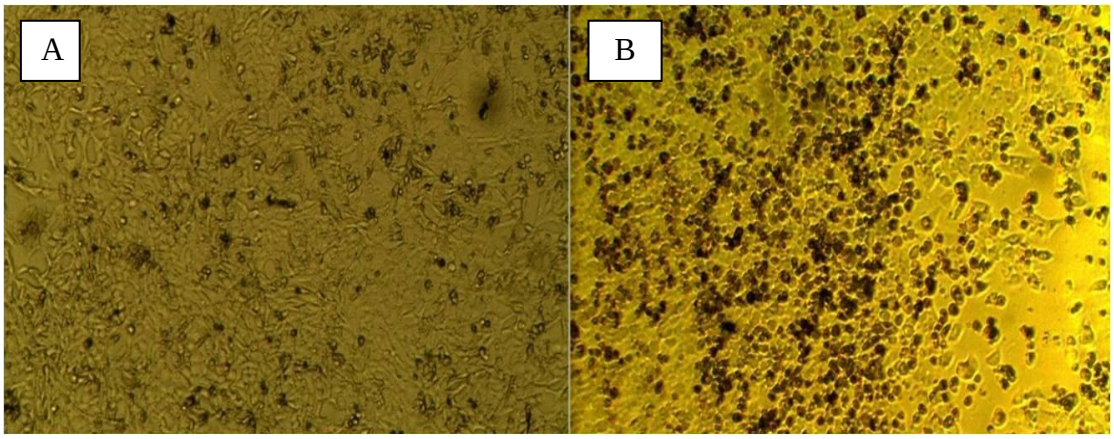

Figure 3.8: Figure showing cytotoxic effect of 10% mice sera on HeLa cells after 72 hours incubation. A. Control mouse serum shows 100% confluence without any sign of cell death and **B**. Experimental mouse serum shows marked cell death characterized by detachment and clumping which are more marked than those in figure 3.7.

When serum concentration was increased to 20% per well, the cytotoxic effects were more marked, compared to control. After 48 hours of incubation with 20% control serum, cells showed only confluence, but no cell death. Same duration of incubation with same concentration of experimental serum showed characteristics of cell death, which are more marked than those observed after 48 hours incubation with 10% experimental serum (Figure 3.9).

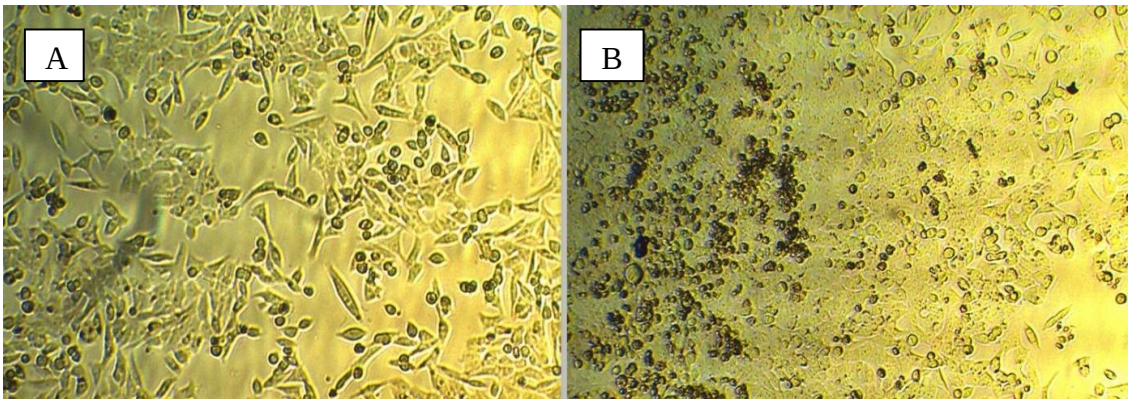

Figure 3.9: Figure showing cytotoxic effect of 20% mouse sera on HeLa cells after 48 hours incubation. A. Shows effect of control serum, where cells are 90% confluent with no sign of cell death. **B.** Shows effect of experimental serum, where cell death is characterized by detachment and clumping of cells which is more marked than those when 10% experimental serum was used.

After 24 hours extension of the incubation period with 20% sera; i.e., after 72 hours of incubation, the well containing control mice serum showed full confluence and the well containing experimental mice serum showed profound cell death, more remarkable than any other previous observation (Figure 3.10).

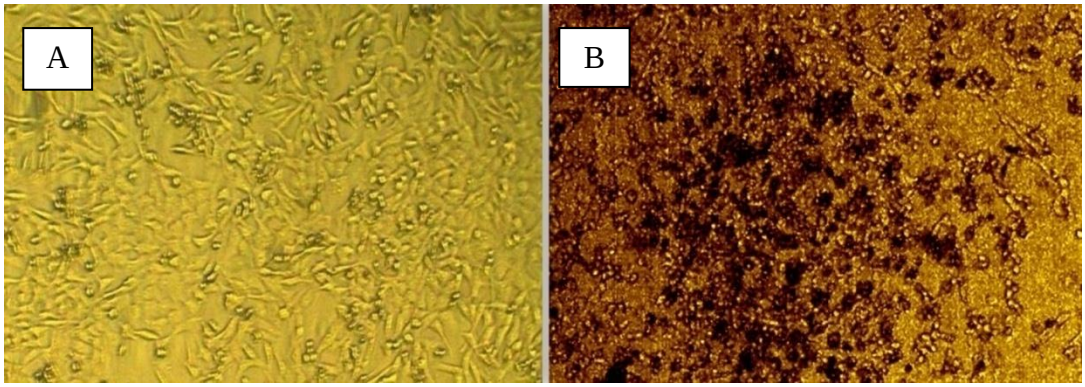

Figure 3.10: Figure showing effect of 20% mice sera on HeLa cells after 72 hours incubation. A. shows effect of 20% control mouse serum where cells are 100% confluent and **B.** shows cellular clumps and detachments which are more marked compared to that after 48 hours of incubation.

From above results, we got interested in investigating the mechanism of HeLa cell death. So, another cytotoxic assay was conveyed using heat inactivated control and experimental mice sera at same concentration and duration of incubation against approximately same number of HeLa cells per well. This experiment showed no cell death in either well even at the highest concentration and highest duration of incubation (Figure 3.11).

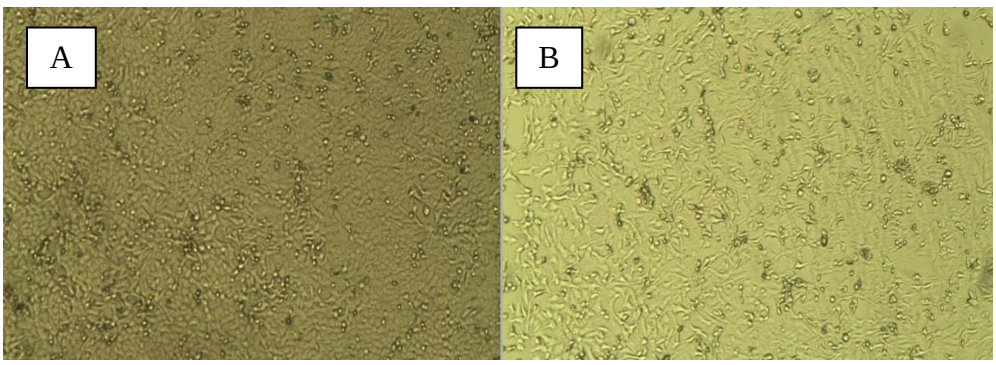

Figure 3.11: Figure showing effect of heat inactivated 20% mice sera on HeLa cells after 72 hours incubation. A. shows effect of 20% control serum where cells are fully confluent and **B.** shows effect of 20% experimental serum where characteristics of cell death are absent.

3.6 *In-vivo* anticancer activity

3.6.1 Mice survival (%ILS)

Both control and experimental mice were observed for survival. Time of survival (in days) of each mouse is given in Table 3.1.

Name of groups	Survival of each mouse in days (After challenge with EAC cells)
Control group	19
	20
	20
	20
	21
	22
Experimental group	27
	29
	30
	30
	31
	33

Table 3.1: Table showing results of survival of mice of different groups in days

3.6.2 Calculation (%ILS)

We can see from the table that, mean survival time (MST) of the control group is 20 (±1) days and that of experimental group is 30 (±1) days (According to the formula provided in section 2.7.2). So the percent increase in life span (%ILS) will be 50%. A graphical representation of this finding has been given in Figure 3.12.

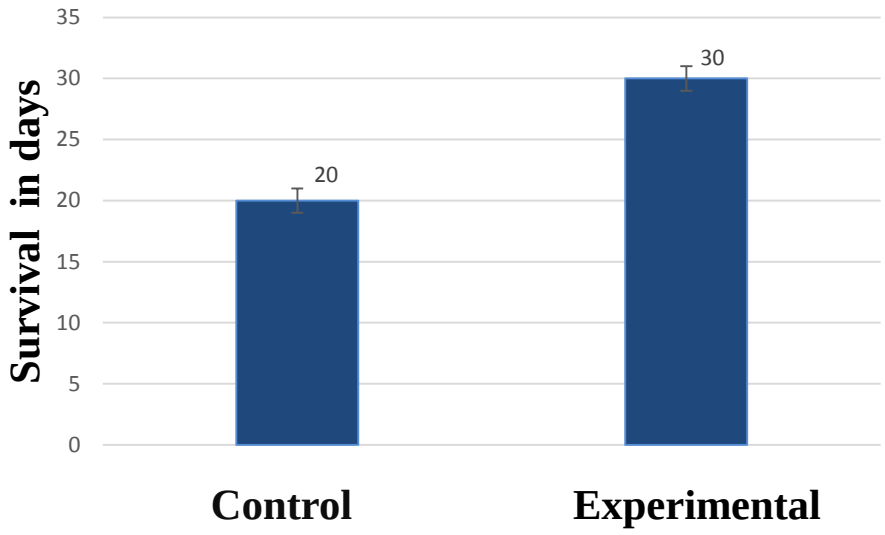

Figure 3.12: Bar diagram showing increased survival of experimental group of mice after being immunized with mice anti-64kDa. The control group (Survived for 20±1days) received mice sera without anti-64kDa. There was a 50% increase in life span of the experimental group (Survived for 30±1 days), who received sera from BCG 64kDa immunized mice.

3.6.3 Percent weight gain of mice

Weight of individual mouse was recorded on day-50, day-60, day-65 and day-70. Table 3.2 shows the weight of individual mouse in a group and the average weight of the group at different time durations of the study. Then calculations were done for percent weight gain of mice in each group.

Name of groups	Wt. on day-50 (gm)	Avg. wt. on day-50 (gm)	Wt. on day-60 (gm)	Avg wt. on day-60 (gm)	Wt. on day-65 (gm)	Avg. wt. on day-65 (gm)	Wt. on day-70 (gm)	Avg. wt. on day-70 (gm)
Control group (Mice receiving control serum)	29.5	30.25 (±0.69)	34.6	35.3 (±0.52)	43.2	44.0 (±0.62)	47.8	48.48 (±0.57)
	29.5		35		43.9		48	
	30		35.4		44		48.2	
	30.5		35.2		44		48.7	
	31		35.8		44.6		49	
	31		36		45		49.2	
Experimental group (Mice receiving serum containing anti-64kDa)	29	30 (±0.71)	33.2	33.9 (±0.46)	36.8	37.45 (±0.61)	41.6	42.9 (±1.04)
	29.5		33.8		37		42.1	
	30		34		37		42.6	
	30		34		37.6		43.1	
	30.5		34.2		38		43.9	
	31		34.6		38.3		44.3	

Table 3.2: Weight of mice in different groups at different durations

3.6.4 Calculation (Percent weight gain)

From above data (Table 3.2), we can predict using the formula provided in section 2.7.2 that, the percent weight gain in control group of mice on day- 60, day-65 and day-70 were 16.7%, 45.45% and 60.3% respectively. On the other hand, percent weight gain in experimental group of mice on day-60, day-65 and day-70 were 13%, 24.83% and 43% respectively. From these results, we can clearly predict that, percent weight gain of experimental mice (Mice receiving anti-64kDa containing serum) was always slower than that of control mice (Mice receiving control serum). After day-70, further comparison could not be done as control mice didn't survive longer. A graphical representation of this data has been given in figure 3.13.

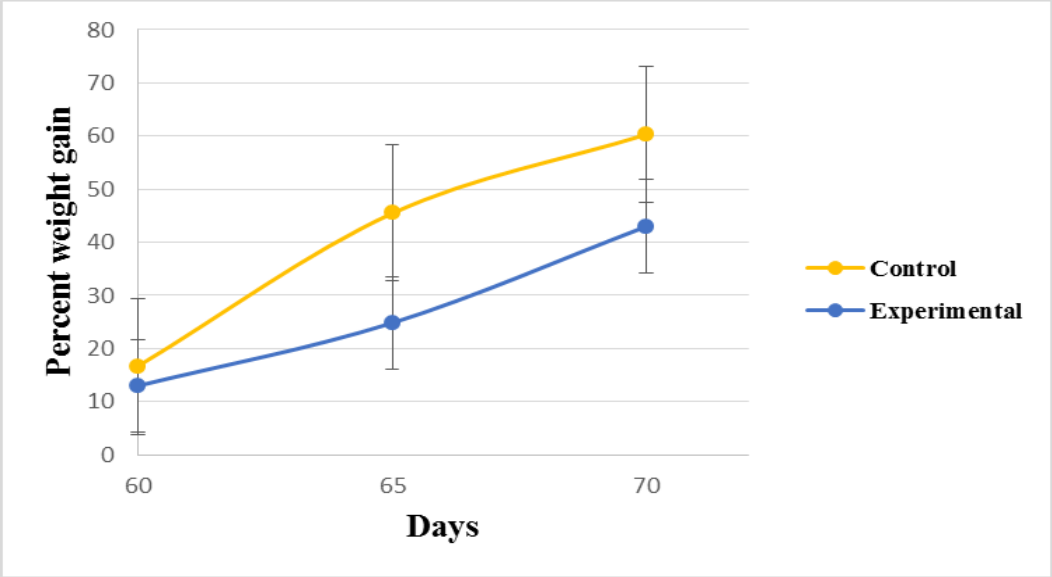

Figure 3.13: Figure showing percent weight gain of control and experimental group of mice against days. All mice were transplanted with EAC cells (4.5×10^5 cells/mouse) on day-50 after immunization and weight was recorded from day-60 at a regular interval of 5 days up to day-70. All the later weight records were compared with the weights recorded on day-50 and a percent weight gain was calculated. Further comparison was not possible after day-70, because control mice didn't survive longer after day-70. Throughout the observation period, we can see from the figure that, percent weight gain of the mice in experimental group was always lower than that of the control group.

3.7 Western blot

A western blot analysis was performed using freshly prepared Whole Cell Extract (WCE) of EAC cells as antigen and various mice sera as antibody. The mice sera included serum from healthy mouse, serum from tumor mouse and serum from immunized mouse. When WCE of EAC cells was blotted with healthy mouse serum, the strip showed non-specific bands of various molecular weights. Western blotting of WCE of EAC cells with tumor mouse serum showed a 64kDa band and blotting with immunized (*M. bovis* BCG 64kDa surface protein) mouse serum also showed a 64kDa band (Figure 3.14).

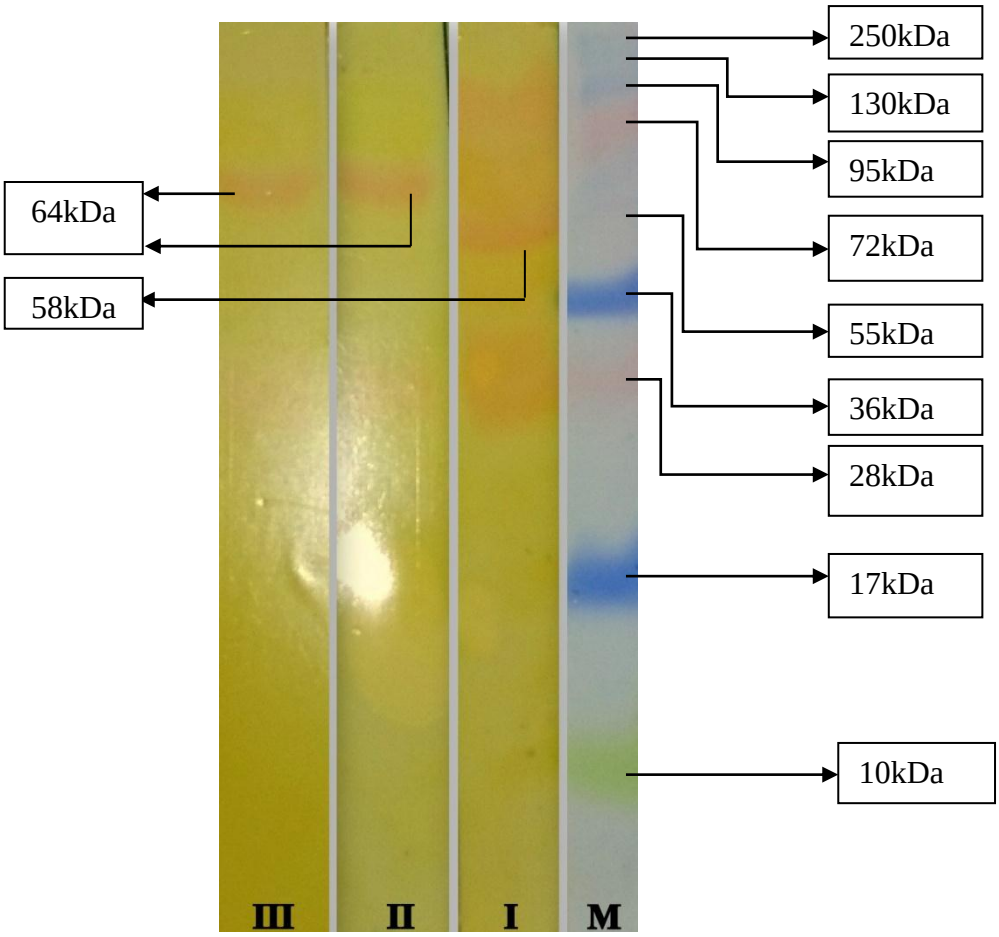

Figure 3.14: Figure showing results of western blot analysis. The strip designated as 'M' contains markers of specific molecular weights. Strip labeled as 'I' was blotted with serum from control mouse and shows a 58kDa band which is non-specific. Strip 'II' and 'III' were blotted with tumor mice serum and immunized (BCG 64kDa) mouse serum respectively and show 64kDa bands.

4. Discussion

In our study, we detected a 200bp band from *M. bovis* BCG after PCR with ET1, ET2 and ET3 primers compared to a 150bp band from wild type *M. tuberculosis* using the same primers. This finding validates our identification of *M. bovis* BCG. It was found that RD1 was deleted in all BCG substrains, including recent clinical isolates and early and late reference strains (Talbot et al., 1997). RD1 was present in all human and bovine *M. bovis* strains from various parts of the world and in other strains of the *M. tuberculosis* complex. These data suggest that this region of the BCG genome is a suitable target for the specific identification of BCG.

There were various studies involving methods of isolation and purification of 64kDa surface protein of *Mycobacteria*. One of these studies (Thole et al., 1987) attempted purification from fermentor-grown clonned *E. coli* cells. Cells were disrupted by lysozyme treatment and sonication, and a mixture of proteins was obtained from the soluble fraction. The protein antigen was purified by ammonium sulfate precipitation and anion-exchange chromatography. About 80% of the purified protein was composed of the 64kDa protein. Minor components were also observed on the Coomassie blue-stained SDS-PAGE. The purified 64kDa protein reacted with monoclonal antibodies and did not degrade further on storage at -20°C for at least 3 months. Moreover, it was found that, monoclonal antibodies to various *Mycobacterium* species reacted with an approximately 64kDa protein (Kolk et al., 1984).

Several extraction methods for tumor-associated antigens have been reported. However, most of them involved some chemical treatment for the isolation. Among the procedures, the 3 M KCl extraction (Meltze et al., 1971) has been used extensively, which results in a recovery of 15%-40% of the antigenic activity present on live tumor cells. A distinct drawback of this method is that the cells and their nuclei are also disrupted, and this interferes with the subsequent purification of the antigen (Reisfeld and Kahan, 1971). A study (Ahsan and Sasaki, 1989) demonstrates that soluble tumor surface components can be isolated from the tumor cells by the water extraction method without using any chemicals. Removal of the surface materials from the tumor cells have been confirmed by both microscopic and immunochemical techniques. Under the electron microscope

the surface-removed cells were found to contain no microvilli structures, indicating the shedding of surface structures during the water extraction. Distilled water easily shed surface structures by repeated pipetting without bursting of cells by osmotic shock (Sasaki et al., 1987). It is known that the lectin like concanavalin A exert a direct activating effect on T lymphocytes by merely binding to the cell surface glycoproteins (Andersson et al., 1972) and the surface components of some types of cells including tumor cells have the specific binding site for the FITC-ConA (Sasaki et al., 1987). The results of FITC-ConA binding test and the gel diffusion analysis clearly indicate that the surface components were completely removed by the water extraction method. Antigenic activity of the surface structures isolated was demonstrated by the crossed immunoelectrophoresis technique.

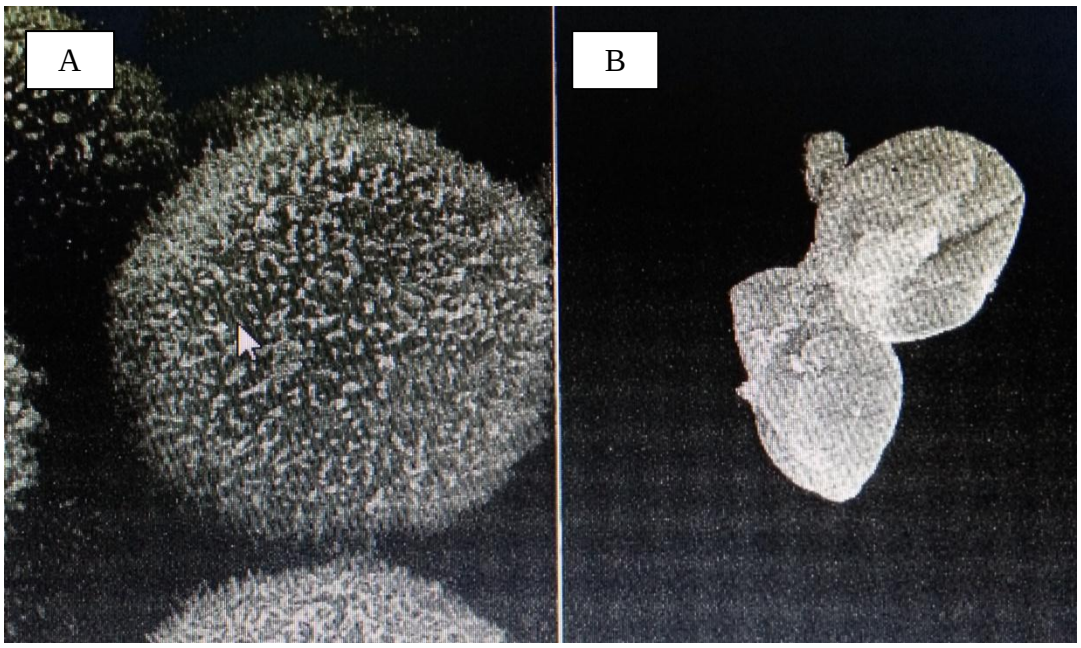

Figure 4.1: Scanning electron micrograph of the Line 10 control tumor cells (A) and surface removed cells (B) after water extraction. The surface structures containing the microvilli projections were prominent in control cells, whereas, after the water extraction, the microvilli projections were completely removed (Ahsan and Sasaki, 1989).

Sixteen antigens were detected in the surface materials of the Line 10 tumor cells when tested against the homologous rabbit antiserum, suggesting presence of many protein

components in the surface materials after the water extraction. In a previous report, three lines of precipitation were detected when concentrated Line 10 ascites fluid was reacted with anti-Line 10 rabbit serum (Detrick-Hooks et al., 1976), which was demonstrated to be membrane surface protein substances. The SDS-PAGE demonstrated about 25 protein components in the Line 10 WEM and among them five major bands were prominent having apparent molecular weights of 44, 46, 62, 64, and 68kDa which possessed carbohydrate moieties examined by FITC-ConA binding test and Crossed Affino Immunoelectrophoresis (CAIE) analysis. In marked contrast, these major protein bands were completely lacking in the normal liver WEM, although the same extraction procedure was used for both of them, indicating these major bands to be only specific for the Line 10 tumor cells and probably associated with TAA activity. Normal liver WEM showed one prominent band of apparent molecular weight of 82kDa and the corresponding band to this in Line 10 WEM was very faint, suggesting that the 82kDa protein is a representative major band in the normal liver cells. Many investigators used nonionic detergents (Bjerrum and Lundahl, 1974; Bjerrumand BogHansen, 1976; Gurd et al., 1973; Natori et al., 1978) in aqueous solutions to dissolve the membranes. However, in the CAIE, no significant differences in the number of precipitin lines were observed between the detergent-free and detergent containing gels except an additional line. A part of an antigen with more than one determinant appeared in the detergent-containing gel, indicating very little effect of the detergent to further solubulize the membrane proteins; this suggests that the water extraction itself is sufficient to solubilize a large number of protein components on cell surface with high antigenic activities. Four precipitin lines showed reactions of partial identity, which probably suggests that these membrane proteins might have a portion of their amino acid sequence in common, or these proteins might appear in separate complexes, containing a common subunit. Glycoprotein nature of some of the antigens was confirmed by the CAIE where four of the antigenic bands interacted with the free ConA and were retainedin the intermediate gel. As ConA has four binding sites (specific for a-n-mannopyranosyl and α-D glucopyranosyl groups) and acts as an antibody by joining several molecules in a complex and builds up a macromolecular lattice by the lectin and glycoprotein molecules, the interacted glycoprotein components probably have two or more binding sites. The simple low ionic strength extraction by the use of distilled water has been used previously to release several protein components from other sources (Harris, 1971). As such extraction procedure does not contain any chemical treatment or drastic conditions,

there is less possibility of altered immunologic specificity of the product and the proteins may remain in their native undissociated conformation which has been shown by the high antigenic activity of the Line 10 WEM. The major components in the Line 10 WEM provide a new approach to analyze the TAA activity and further purification of the protein components and their immunotherapeutic value.

The simple low ionic strength extraction by the use of distilled water has been used previously to release several protein components from bacteria and other sources (Ahsan and Sasaki 1989; Harris, 1971; Oaks et al., 1986). One main advantage of the water extraction, as has been revealed by electron microscopy, is that it only removed the surface components of the cells without significantly affecting the morphological structures of the cells, thus making subsequent purification of the surface proteins easier. Isolation of the soluble 64kDa protein from the water extracted materials also suggested that the BCG 64kDa is a surface protein. The findings and explanation from previous studies mentioned above suggest that water extraction method is an authentic and established method of water soluble surface protein extraction from cells.

In further studies (Ahsan and Sasaki 1991), 64kDa protein of *M. bovis* BCG was extracted by water extraction method and identified. The water-extracted materials showed one major protein band of 64kDa and some other low molecular weight protein bands in SDS-PAGE after Coomassie brilliant blue R250 staining. The major band was also faintly stainable in periodic acid Schiff's reagent, suggesting the nature of the 64kDa band to be a protein with carbohydrate moieties. Isolation of BCG 64kDa with less or no contamination of other proteins was demonstrated by immunoblot analysis, where only a single band was developed on the nitrocellulose membrane.

In our study, we also found a 64kDa band and two other non-specific bands after performing an SDS-PAGE of the WEM and then staining with Coomassie brilliant blue R250. This band was used for animal immunization and after immunization, only a 64kDa band was visualized on nitrocellulose membrane after western blot analysis, indicating isolation of 64kDa band from WEM without contamination of other proteins.

It was also described that the major surface components of Line 10 hepatoma cells and their antigenicities, where 5 proteins with carbohydrate moieties of apparent molecular

weights of 44, 46, 62, 64 and 68kDa were prominent (Ahsan and Sasaki 1989), and suggested common antigenic determinants between the surface extracts of Line 10 tumor cells and BCG using BCG-monoclonal antibodies (Ahsan and Sasaki 1991). The indirect imrnunofluorescence analysis showed sharp fluorescences from Line 10 tumor cells treated with rabbit anti-BCG 64kDa, indicating that the BCG 64kDa protein and Line 10 tumor cells have antigenic determinant(s) in common, which are probably present on the surfaces of the tumor cells. This is again supported by the immunoblot analysis where a faint but clear band from Line 10 tumor water-extracted materials, corresponding to the BCG 64kDa band, was obtained on nitrocellulose membranes. All these data combined, confirm each other and clearly indicate that the antigenic determinants between the BCG 64kDa and the 64kDa major surface antigenic component of Line 10 tumor cells are identical. Strong antitumor activity of BCG 64kDa was clearly demonstrated in the test animals when compared with the control group. The experiment was repeated to check the reproducibility of the results, and the test animals which received BCG 64kDa showed either complete rejection or partial inhibition of the tumor growth when observed for 40 days after tumor transplantation. However, the control animals which were injected with fresh sonicated membrane only, developed big tumors without much difference in size, suggesting that the nitrocellulose membrane only acted as an adjuvant (Diano et al., 1987) and did not affect the results of the experiments. Evidence that the isolated BCG 64kDa can induce an immune response in normal guinea pigs was suggested by the fact that, all immunized animals demonstrated significantly increased titres of anti-BCG 64kDa antibody. However, the most relevant and simultaneously the most stringent criterion for the functional integrity of the isolated BCG 64kDa antigen, is the ability to induce protective tumor immunity in normal animals either by activating lymphocytes or by inducing Natural Killer (NK) cells by acting as an adjuvant, which eventually may inhibit the tumor cells. This criterion demands not only that BCG 64kDa be immunogenic but also that the level and type of immunity that is developed provide the immunized animals with the ability to reject or inhibit a viable tumor cell challenge. The anti-Line 10 tumor activity of BCG 64kDa in the pre-immunized animals also suggests that BCG 64kDa is probably identical with the tumor specific antigen (Ahsan and Sasaki, 1991).

Sharing of antigenic determinants between BCG and Line 10 tumor cells has also been suggested by others. A study showed that sera from guinea pigs immunized with BCG

reacted with Line 10 cells (Minden et al., 1974). Another study showed that the Line 10 cells shared some determinants with BCG (Minden et al., 1976) and immuno electron microscopy demonstrated binding of anti-BCG to the surface of line10 and human melanoma cells (Ahsan and Sasaki, 1989). These studies additionally suggest that antigenic determinants between the two 64kDa surface proteins of BCG and Line 10 hepatoma cells are common and that the 64kDa protein from BCG is probably identical with the tumor specific antigen.

Antigenic cross reactivity between the BCG and different mouse tumor cells, including Meth A and CT-26, was also demonstrated using BCG polyclonal and monoclonal antibodies (Sasaki et al., 1986; Sasaki et al., 1989), where faint but clear bands, corresponding to the 64kDa proteins, were developed from each of the water extracted materials, which indicated that the mouse anti-BCG 64kDa could recognize the corresponding 64kDa antigenic determinants from Meth A and CT-26 tumor cells. All the test mice which were immunized with BCG 64kDa and later challenged with Meth A or CT-26 tumor cells showed either complete rejection or partial inhibition of tumor growth, when observed for 28 days after tumor transplantation. The size of the tumor growth varies from mouse to mouse, however, 37% of the Meth A-challenged animals and 50% of the CT-26-challenged animals completely rejected further tumor growth and other test mice showed much reduction of size of tumors when compared with the control animals without any BCG 64kDa immunization. The control mice for both Meth A and CT-26 groups, which were injected with the fresh sonicated membrane only, either died (12.5%) or developed big tumor masses (Ahsan and Sasaki, 1993). It was also found that antiserum developed in rabbit against the Line 10 water extracted materials and later absorbed in guinea pig could detect the 64kDa protein antigen from other mouse tumor cells and BCG (Ahsan and Sasaki, 1993). All these data strongly suggest that the BCG 64kDa surface protein, which shares common antigenic determinants with the 64kDa surface proteins of guinea pig and different mouse tumor cells and has strong anti-tumor activity in different immunized animals, is probably identical with the TAA (Ahsan and Sasaki, 1991).

In our study, we found that, mouse serum containing anti-BCG 64kDa antibody causes cell death *in-vitro* against HeLa cells, which is a cancer cell line. But there was no cell death when HeLa cells were exposed to control mouse serum. This confirms the cross-

reactive property of the 64kDa protein antigen of BCG. As there was no evidence of cell death when HeLa cells were treated with heat inactivated control and experimental sera (serum containing anti-BCG 64kDa antibody), this cross reaction is clearly a complement mediated antigen-antibody reaction.

Antigen- antibody cross reaction may be simply defined as a reaction that takes place between an antibody that was developed in response to a specific antigen and an antigen that is similar in many ways to the antigen in response to which, the antibody was developed. Figure 4.2 shows a diagrammatic representation of the antigen-antibody cross reaction.

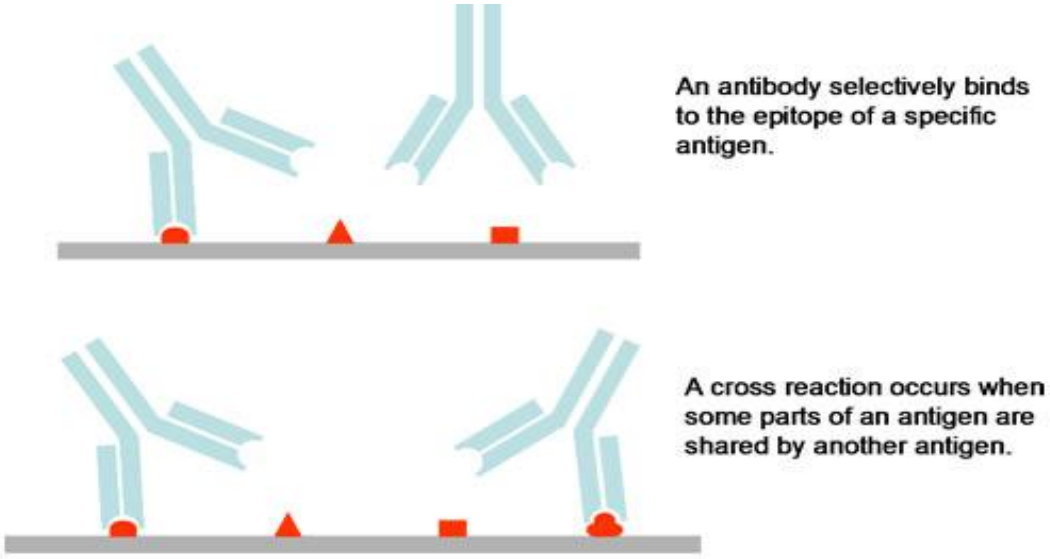

Figure 4.2: Figure showing a diagrammatic representation of antigen-antibody cross reaction

Another finding that confirms the cross reactive property of the BCG 64kDa antigen is that, when WCE of EAC cells was immunoblotted with mouse anti-BCG 64kDa containing serum, a 64kDa band was visible (Figure 3.14 strip III). This cross reactive property of this protein may have contributed by turns to the *in*-vivo anti-cancer property of the anti-BCG 64kDa antibody in pre-immunized mice in terms of 50% increased survival and lower rate of weight gain throughout the survival duration when compared to control.

The most interesting finding of the immunoblot analysis was that, when WCE of EAC cells were blotted with serum from a tumor mouse, a 64kDa band was visible (Figure 3.14 strip II). This finding suggests formation of an anti-64kDa antibody in tumor mice, which should be another clue for further study. This band was not visible in strip I, in which, WCE of EAC was blotted with serum from control mouse. This finding suggests us that, 64kDa antigen could probably be a protective antigen from tumor cells studied so far.

From all the findings of our study, we can conclude that, BCG 64kDa surface protein shares common antigenic determinants with 64kDa protein from ascitic tumor cell (EAC cells), and as a result, mouse anti-BCG 64kDa cross reacts with WCE of EAC cells, animals pre-immunized with BCG 64kDa show anti-tumor activity when challenged with EAC cells and mouse anti-BCG 64kDa shows some extent of cell death against HeLa cells (Increased cell death with increased concentrations). These data confirms that, BCG 64kDa antigen is probably identical also with TAA from ascitic cancer cells and should have better anti-tumor effects.

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Appendix-I

Media Composition

Unless otherwise mentioned, all media were sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure. Distilled water was used for preparation of all media. The media used in this thesis have been given below:

1. Lowenstein Jensen medium

Ingredients	Amount (gm/L)
Malachite green	0.4 gm
Magnesium sulphate	0.24gm
Glycerol	12ml
Potato Flour (Potato Starch)	30gm
L-Asparagine	3.6gm
Monopotassium Phosphate	2.4 gm
Magnesium Citrate	0.6 gm
Egg suspension	1000 ml
Distilled Water	600 ml

2. DMEM (Dulbecco Modified Egales) Media

Ingredients	Amount (gm/L)
DMEM	6.73 gm
$NaHCO_3$	1.85 gm
HEPES	2.38 gm
Glutamine	0.58 gm
Deionized water (18Ω)	Up to 500 ml
P^H	7.2-7.4

* Media was not autoclaved. It was filtered through Millipore membrane of 0.20 µm average pore diameter.

3. **Dubos broth** (Approximate formula for 900ml purified water)

Ingredients	Amount (gm/L)
Pancreatic Digest of Casein	0.5 g
L-Asparagine	2.0 g
Monopotassium Phosphate	1.0 g
Disodium Phosphate	2.5 g
Ferric Ammonium Citrate	0.05 g
Magnesium Sulfate	0.01 g
Polysorbate 80	0.2 g
Calcium Chloride	0.5 mg
Zinc Sulfate	0.1 mg
Copper Sulfate	0.1 mg
FBS	100.0 mL

Appendix-II

Solutions and Reagents

A. 10x-TBE (Tris-borate –EDTA pH 8.0)

108 gm of Tris-base, 55 gm of boric acid and 40 m1 of 0.5 M EDTA (pH 8.0) were taken and distilled water was added to the mixture to make 1000 ml. The buffer was stored at room temperature.

B. 0.5 M EDTA

18.61 gm of $Na_2EDTA.2H_2O$ (disodium ethylene diamine tetra-acetic acid) was dissolved in 80 ml of distilled water and the pH was adjusted to 8.0 with pellets of NaOH. The final volume was made up to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

C. 1x-TE buffer (Tris-EDTA)

TE buffer (10 mM Tris-Cl/1mM EDTA, pH 8.0) was prepared by diluting concentrated stocks of 1 M Tris-Cl and 0.5 M EDTA in distilled water. For making 1000ml or 1 L of TE buffer, 10 ml of 1 M Tris-HCl (pH 8.0) and 2 ml EDTA (0.5 M) and distilled water added up to 1000 ml. The buffer was autoclaved and stored at room temperature.

D. EtBr (0.5 µg/ml)

10 µl of 10 mg/ml Ethidium bromide solution was added to 200 ml distilled water. This solution was stored at room temperature and covered with aluminum foil.

E. 6×-Gel loading buffer (Glycerol & bromophenol blue)

Glycerol (30%)	3 ml
Bromophenol blue (0.25%)	25 mg
Distilled H_2O	Up to 10ml

F. Normal saline (0.85% NaCl)

0.85 gm of NaCl, upto 100 ml by distilled water.

G. Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na_2HPO_4 and 2.0 gm of KH_2PO_4 in 800 ml distilled water. pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 L by distilled water.

The solution was sterilized by autoclaving and was stored at room temperature.

H. SDS-PAGE Analysis stock solution

i. 30% acrylamide-bis acrylamide solution

Acrylamide	14.5 gm
Bis-acrylamide	0.5 gm
Distilled water	Up to 50 ml

The solution was stored at 4^{o}C. The powder is neurotoxic. Mask was used during handling.

ii. Upper gel buffer (0.5 M Tris HCl, pH 6.8)

Tris-base	6.57 gm [0.5×MW (121.14)/10]
Distilled water	up to 100 ml
pH	adjusted to 6.8 with concentrated HCl

The solution was stored at 4^{o}C.

iii. Lower gel buffer (1.5 M Tris HCl, pH 8.8)

Tris-base	36.34 gm [1.5×MW (121.14)/20]
Distilled water	up to 200 ml
pH	adjusted to 8.8 with concentrated HCl

The solution was stored at 4^{o}C.

iv. 10% SDS

SDS	5.0 gm
Distilled water	Up to 50 ml

The solution was stored at room temperature.

v. 10% ammonium per sulfate (APS)

Ammonium per sulfate (APS)	5.0 gm
Distilled water	Up to 50 ml

The solution was aliquot into 0.5 ml in each eppendrof tube and stored at -20^{o}C.

vi. TEMED

Readymade and was stored at room temperature.

vii. Butanol saturated water

Fifty ml of distilled water was taken in a beaker with a magnetic stirrer. Butanol was added to water provided the machine was turned on. Addition of butanol was stopped when the solution got saturated. The saturated solution was collected using a dropper and was stored at 4^{o}C.

viii. 0.1% BPB (Bromophenol blue solution) or Tracking dye

At first, 2 ml of 50% glycerol solution was made by mixing 1 ml glycerol with 1 ml dH_2O. Then 2 mg of Bromophemol blue was weighed and 50% glycerol was added up to 2 ml mark. It was stored at 4^{o}C.

ix. 2× Sample loading buffer

0.5 M Tris- Cl (pH 6.8)	0.4 ml
10% SDS	0.4 ml
2-mercaptoethanol	0.04 ml
Glycerol	0.4 m
Distilled water	0.76 ml

The solution was stored at 4^{o}C.

x. Running/Electrophoresis buffer (pH 8.3)

Tris base	3.0 gm
Glycine	14.4 gm
10% SDS	10 ml
Distilled water	1000 ml

The pH of the solution should be 8.3, which may range from 8.1 to 8.5. The solution was to be stored at 4 ^{o}C. The bottle was to be marked after every usage. It should not be used more than 8-10 times.

xi. Staining solution

Coommassie brilliant blue R-250	200 mg
Acetic acid	10 ml
Methanol	10ml
Water	80ml

The solution was stored at room temperature.

xii. Destaining solution

Acetic acid	10 ml
Methanol	10ml
Water	80ml

The solution was stored at room temperature.

xiii. Composition of 12.5% lower gel

Distilled water	1.60ml
1.5M Tris HCl pH 8.8	1.25ml
30% acrylamide	2.1ml
10% SDS	0.10ml
10% APS	30µl
TEMED (Added last)	8.5 µl

xiv. Composition of upper gel

Distilled water	2.137ml
0.5M Tris HCl pH 6.8	0.937ml
30% acrylamide	0.625ml
10% SDS	37µl
10% APS	22µl
TEMED (Added last)	8.5 µl

I. Western blot solutions

i. Transfer buffer

Glycine	14.4gm
Tris base	3.03gm
Methanol	200ml
Water	800ml

ii. Substrate solution (For alkaline phosphatase conjugated secondary antibody)

50mM Tris HCl pH 9.14	10ml
AS-MX Napthol phosphate	10mg
Fast Red TR	20mg

Appendix-III

Apparatus used

Autoclave	HL-42AE, Hirayama corp, Japan
Balance	Adventurer AR1140, Mettler Toledo EL202
Centrifuge	Eppendorf Centrifuge 5804, Germany
Class II Biosafety Cabinet	Lab Caire, USA
CO_2 Incubator	Shel Lab
Electrophoresis Unit	Sigma E0638, Consort N.V.
Freezer (-30^{o}C)	Liebherr comfort, Germany, Siemens
Gel Documentation System	Vilber Lourmat Doc Print VX05
Heater/Magnetic Stirrer	Spinot
Incubator	Japan
Inverted Microscope	KYOW Optilab TR-T
Laminar Air Flow	HF – 48 Flow laboratories, Japan
Microcentrifuge	Hettich, Tarsons Spinwin MC-02
Microwave Oven	Butterfly, China
pH Meter	Hanna HI2211
Power Pack	Biometra Standard Power Pack
Refrigerator	Royal Frestech, Vestfrost,
SDS-PAGE Unit	BioRad Mini-Protean II cell
Horizontal shaker	Electro Plus, Schuttelmaschine LS10
Spectrophotometer	Genesys 5
Thermomixer	Eppendorf
Thermocycler	Biorad C1000, Biorad MJ Mini, USA
Vortex mixer	IKA MS3 Basic
Vacuum Pump	Sartorius GMBH
Waterbath	Grant SUB6, England