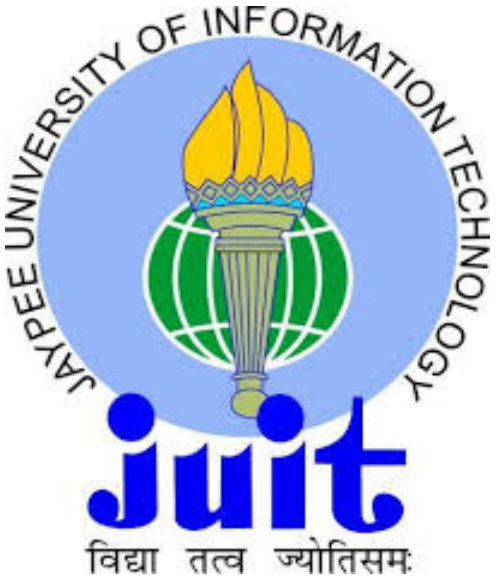
PROJECT TITLE

DEVELOPMENT OF ANTIGEN-ANTIBODY INTERACTION BASED COST EFFECTIVE TEACHING KIT FOR IN-HOUSE APPLICATION



Submitted in partial fulfilment of the award of Degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

Under the supervision of

Dr Rahul Shrivastava

By

Bishal Prasher (131555)

CERTIFICATE

This is to certify that the work entitled "**DEVELOPMENT OF ANTIGEN – ANTIBODY INTERACTION BASED COST EFFECTIVE TEACHING KIT FOR IN-HOUSE APPLICATION"** pursued by **Bishal Prasher** (131555) in partial fulfilment for the award of degree Bachelor of Technology in Biotechnology from Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This part of work has not been submitted partially or wholly to any other University or Institute for the award of any degree or appreciation.

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Date:

Place:

(Bishal Prasher)

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS	ABBREVIATIONS
LB	Luria Broth
LBGT	Lurai Broth Glycerol Tween
UV	Ultraviolet
Ab	Antibody
Ag	Antigen
dH_2O	Distilled water
OD	Optical density
PBS	Phosphate Buffer Saline
pH	Potential of hydrogen
°C	Degree Celsius
g	Gram
µl	Microliter
µg	Microgram
ml	Milliliter
mg	Milligram
rpm	Rotation per minute
%	Percentage
BSA	Bovine Serum Albumin
V	Final Volume
V	Volume of reagent
w	Weight
RID	Radial Immuno Diffusion Assay
DID	Double Immuno Diffusion Assay

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ABSTRACT:

Antigen antibody based teaching kits are majorly used in research and educational activities, apart from its highly standardized and customized version finding diagnostic application too. To achieve the goal to develop a cost effective In-house teaching kit, we utilized *M. fortuitum* culture as a source of antigen in the form of cellular protein lysate and serum from mice model infected with wild type *M. fortuitum* as a source of antigen which is freely available in our laboratory, as it was extracted in earlier studies conducted.

We were able to effectively standardize the protocol for extraction, the use of a suitable buffer combination for interaction of antigen with antibody in a precipitation reaction. We were able to demonstrate the optimum amount of antigen concentration required for carrying out quantitative precipitation and gel based Immuno Diffusion Assay. For future aspects research can be focused on standardizing and refining gel-based antibody-antigen interaction method for utility as in-house teaching kit.

CHAPTER 1:

INTRODUCTION

1.1 INTRODUCTION

Recognition of antigen and further developing a strong immune action of defense and offense is possible due to competent innate and adaptive line of defenses amongst these two line of defence the major action is facilitated by a high affinity, specific binding and interaction between the antigen's epitope and paratope of the antibody via non-covalent bonding like electrostatic interactions, hydrogen bonds, Van der Waal forces and hydrophobic interactions, these weak chemical interactions gets involved on the basis of interaction site. This antigen-antibody interaction leads totheformation of a complex which is transferred to the cellular mechanism for destruction.

Subsequent differentiation of B cells into memory B cells and production of specific antibody againstantigens provides basic forthedevelopment of diagnostic applications for detection of bacterial, fungal, viral and parasitic infections, monitoring infection cause or autoimmune process is also important purpose served by the reaction. The different immunological assays applied for diagnosis are precipitation reaction, agglutination reaction, complement fixation, Immunofluorescent assay, Enzyme-linked immunosorbent assay (ELISA) and Radioimmunoassay.

Antigen-Antibody interaction based test are also performed for research and teaching purposes at research labs, university labs and areanessential part of the curriculum. Antigen – Antibody Interaction based tests are demonstrated in the Lab curriculum of the courses of Immunology, Immunotechnology, Microbiology and Immune System and Diagnostic and Vaccine Manufacturing Technology.

Antigen-antibody interaction based teaching kits are provided by major producers like Sigma, Madox, Genei, etc. The cost incurred by Department of Biotechnology and Bioinformatics, JUIT Waknaghat is approximately around 1.5-2 lakhs per annum. This served the motivation of our study to produce cost effective In-house Teaching kit.

The primary objective was to demonstrate precipitation reactions. Precipitation reactions are based ontheinteraction between the soluble antigen and antibody and their interaction to form the insoluble precipitate. The presence of antigen and antibody in optimal proportions leads to lattice formation; excess of either will fail the cause and no precipitation will occur. The precipitation reaction is performed by placing a constant amount of antibody in a series of tubes and adding increasing amounts of antigen to the tubes. Incubation at suitable temperatures followed by centrifugation gives us precipitates. The curve is plotted between the amounts of precipitate against increasing antigen

concentrations. This graph gives us the zone of equivalence which is the optimal antigen-antibody concentration. Precipitation reaction can also be performed in agar matrix, antigen and antibody diffuse towards one another and yield visible precipitin lines,doubleimmunodiffusion(the Ouchterlony method)or when antigen diffuses into agar matrix incorporated with antibody and a visible precipitate line formation occurs at suitable concentration in the region of equivalence, radial immunodiffusion(the Mancini method), whereas no visible precipitate forms in regions where there is excess or decrease in amount of either antigen and antibody .

In this study, we utilised total protein isolated from *Mycobacterium fortuitum* as antigen and serum obtained from murine sample infected by wild type *M. fortuitum* as a source of antibody. Obtaining this source of antigen and antibody which were utilised and obtained in earlier projects carried out, gave us the cost advantage. The requirement of low-cost material like agar and assay buffer from earlier used kits further strengthened our cost effective goals.

1.2 Mycobacterium fortuitum

M. fortuitum is a non tuberculosis mycobacterium which shows rapid growing capability in lab condition and is classified as a Runyon Group IV [1]. Clinically the most commonly encountered RGM strains are *Mycobacterium abscessus* complex, *Mycobacterium fortuitum*and *Mycobacterium chelonae*complex.

Less than 10% of RGM infections are caused bymycobacterium strains belonging to *M. fortuitum* group, *Mycobacterium mucogenicum* and *Mycobacterium smegmatis* [2, 3]. More than 10 species make the *M. fortuitum* group, which include *M. fortuitum, M. senegalense, M.peregrinum,M. alvei, M. septicum, M. boenickei, M. porcinum, M.houstonense, M. neworleansense, M. conceptionense* and *M. porcinum*.

M.fortuitum grows optimally at a temperature between 30º-37º C and is a chemoheterotroph. It is an obligate anaerobe by oxygen demand. It is a rod-shaped bacillus which stains positively for Acid fast staining owing to the rich content of mycolic acid in its cell wall, further it stains gram-negative when Gram staining is done.

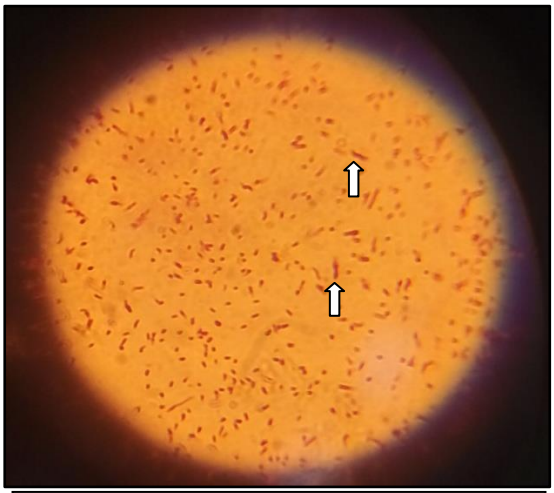


Figure 1: *M. fortuitum* Acid Fast Staining

M.fortuitum rarely infects healthy humans, but exposure to repetitive and large amounts can defeat the immune system and establish disease. Infection is most common in immunocompromised patients. The most general NTM infection is local cutaneous disease, lung diseases, infection site abscesses, surgical

site infection and disseminated disease. Infection at surgical site and abscesses at injection site occurs by contamination through tap water. In immunocompromised patients, death may be extensive inthecase of disseminated diseases or pulmonary infections otherwise death in immune competent patients is rare. Morbidity depends onthesite of infection. Skin lesions can be healed without antibiotics or excision through surgery.

- **Epidemiology of *M. fortuitum*:**

Non-tuberculous mycobacteria (NTM) have a wide distribution in the environment and isolated in water sources [4].Most of these mycobacteria present environmentally are known human pathogens, and the number of cases of infections have been reported to be increased [5; 6],immunocompromised patients are more easy targets for mycobacterial infections [7; 8]. Meta-analysis studied done in Iran showed NTM infections (10.2%) in culture-positive cases of tuberculosis (TB), bringing into focus their impact on human public health [9].

M. fortuitum has been isolated from soil, water, raw milk [10] and from feral buffalo's tissue samples [11] . In soil *M. fortuitum* is known to degrade polyhalogenated compound using polyhalogenated polyphenols, it is beneficial for soil health as it helps to replenish soil nutrient contents by degrading organic waste . *M. fortuitum* are amongst those NTM species capable of growing in vivid water sources like tap water, surface water, wastewater and groundwater [12] .

High concentration of nutrients is not as much a requirement for ***M. fortuitum*** but they prefer high temperatures that enhance their growth [13], resistant to chlorination, biofilm formation and amoeba-associated lifestyle further enhances their ability to survive in vivid water sources [14] . Hospital hot water supply has shown to be a good place for multiplication of NTM's [4].This wide distribution is a reason for worldwide reporting of infections[13] . Exposure to contaminated solutions, medical equipment and surgical materials is also a major source of risk.

M. fortuitum donot have thesame probability of causing infection in the body at the same location. *M. fortuitum* causes majority of infection following bypass cardiac surgery [15] or augmentation mammoplasty [15] . Isolates recovered from infections following bypass surgery shown phenotypic and

genotypic unrelatedness of rapidly growing mycobacterium showed that they originated from local sources rather than contaminated surgical instruments**.** A study of hospital water samples collected in Iran showed that *M. fortuitum* complex like was the most encountered species with 51.9% of total detections, while *M. fortuitum* was the highest detected NTM with a prevalence of 44.1%, *hsp65*-PRA gene was used for this detection [16] .

- **Pathogenesis of *M. fortuitum*:**

Rapidly growing mycobacteria (RGM) are responsible for various human ailments which include skin and soft tissue, bone, lung, catheter-related blood stream infections and also disseminated infections [17] . Immunocompromised patients are primary targets, healthy individuals are generally less reported for infections.

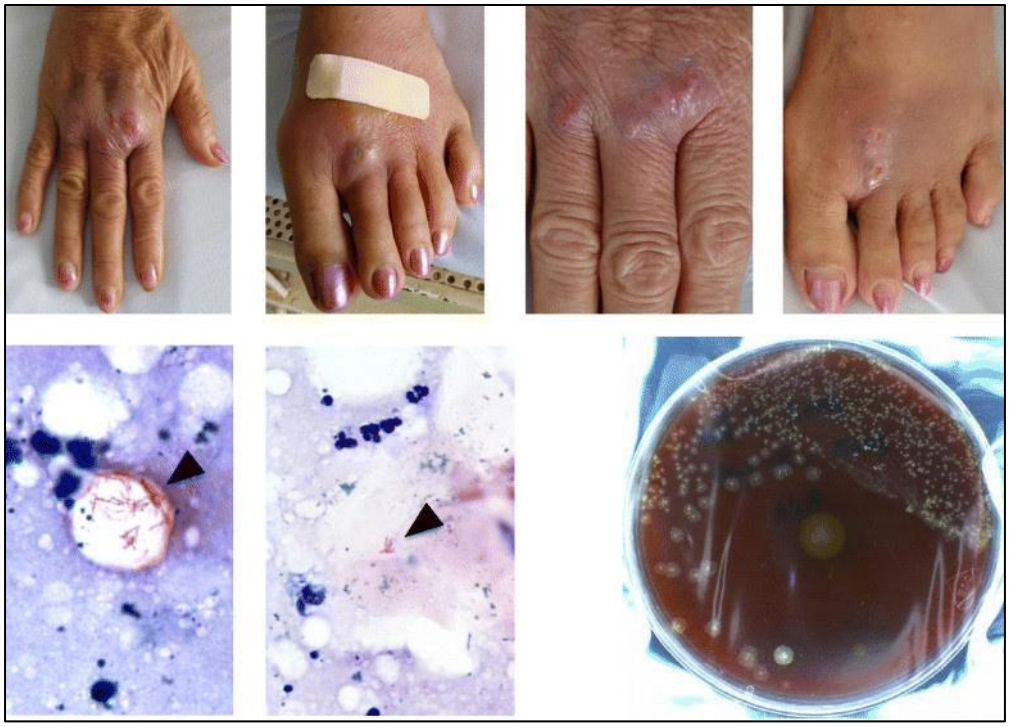


Figure 2.1
***M. fortuitum* infection at various soft tissue sites (above) ; Ziehl-Neelsen stain (1000X) pictures below left and colony growth in blood agar at below right [43]**

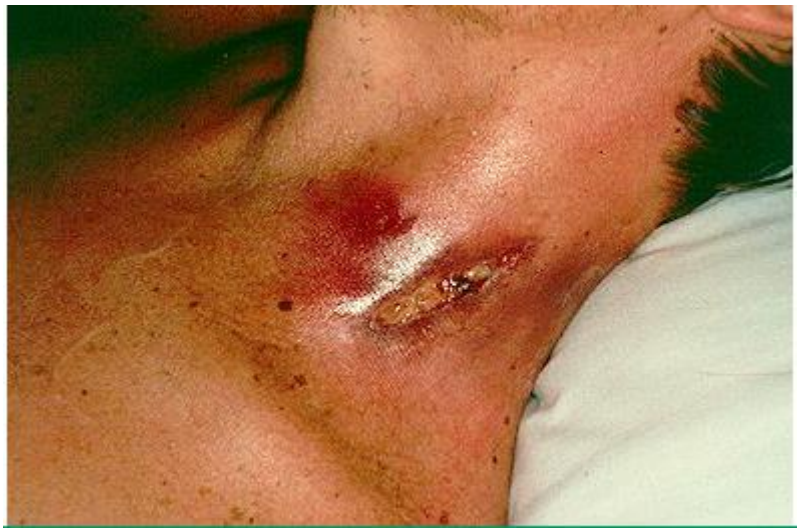


Figure 2.2 M.fortuitum infection [56]

- **Treatment of *M. fortuitum* Infection:**

Antibiotic therapy for treatment of *M. fortuitoum* is required for long term,whereas for disseminated illness intravenous therapy is used. Drug therapy using a single drug like clarithromycin have shown success in certain studies but resistance to single drugs is also a major issue, so preferable therapy utilisesuse of 2 drugs in most patients. Initial isolates are tested for antibiotic sensitivity to determine pathway sensitivity for individual isolates can vary considerably [18] .Correlation of susceptibility testing to that of clinical responses was not assessed and hence no success in treatment can be guaranteed.

The disease being long standing in patients there is not much of urgency and hence sensitivity testing before initiation of treatment can be done to decide the antibiotic regimen. Antituberculous drugs like isoniazid, pyrazinamide and rifampin (first-line drugs) play no role in *M. fortuitum* infection [18] .Amikacin is preferred for *M. fortuitum* infection treatment and most of the isolates are susceptible to this aminoglycoside.Imipenem and cefoxitinshow successful use but susceptibility is variable. Ciprofloxacin, levofloxacin has also shown successful usage while fluoroquinolones have shown good activity. Moxifloxacin has shown good activity in in vitro condition but it is clinically untested [19] . The ocular disease has been successfully treated with ciprofloxacin and topical amikacin, these antibiotics can be used alone or inacombination of parenteral or oral antibiotics. *M. fortuitum* was

isolated along with another atypical mycobacterium which causes port site infection and complication post laparoscopic surgery. This type of infection has shown sporadic outbreak throughout the world. Antimicrobial susceptibility test for the isolated samples showed high sensitivity to clarithromycin (93.3%), imipenem (80%) and amikacin (93.3%) though variability to ciprofloxacin, linezolid and ofloxacin was observed [53] .

Antimicrobial susceptibility study [49] done showed susceptibility of isolates of strains of *M. fortuitum to amikacin,* moxifloxacin, ciprofloxacin and tigecycline, whereas they showed resistance to tobramycin.

Treatment of infection has no standard duration of therapy as treatment last for months and courses longer than 6 months are usual. Administration of drugs is long enough for treatment of a complete resolution of lesions but the regimen of treatment to prevent relapse is unclear. Surgical removal of subcutaneous or cutaneous lesions, mostly if they are extensive, is done [20] .

Surgical removal of bone and ocular lesions is majorly a necessity. Inthecase ofthepulmonarylesion, if therapy is not responding well or the organism has developed resistance to antibiotics, surgical removal may be considered. If the site of infection has an implanted device, the device needs to be removed foracure.

- **Diagnosis OF *M. fortuitum* infections:**

Patients showing symptoms of NTM infection generally undergo the most primitive testing and evaluation of chest radiography, HRCT (high-resolution computed tomography) chest scan ajndatleast 3 sputum specimen are tested by test for acid-fast bacilli (AFB) test. The major concern while doing diagnosis is to make it sure that tuberculosis (TB) is not the causative agent responsible for the symptoms. Other aspects of clinical, microbiological and radiographical examination are also immensely acquisitive and must be confirmative for diagnosis of lung disease caused by NTM [21]

Molecular techniques have also grown quite in significance and are being utilised regularly and quite efficiently for diagnostic purposes.*M. fortuitum* was accurately identified in blood infection ofapretermneonate with a very low weight by 16S rDNA sequencing. Treatment was done using line

removal with a combination of adjuvant treatment applying ciprofloxacin, amikacin and clarithromycin [22] .

For detection, phenotypic studies include colony morphology, growth rate and pigmentation. Molecular strategies like PCR- restriction enzyme analysis (PRA) and *rpoB* gene sequence analysis were also utilised in a study to determinetheprevalence of NTM in hospital water supplies [23] .

Mycobacterial cells were saponified, mycolic acids were extracted and examined by high-performance liquid chromatography(HPLC) supplemented by phenotypic tests for identification of clinical isolates of NTMs [24] .

1.3 Diagnosis of Tuberculosis:

Most of the economically underdeveloped country depend on Ziehl-Neelsen (ZN) staining for diagnosis of Tuberculosis. Ziehl-Neelsen is a cheap and easy to conduct test. Chest X-rays are also used, but they lack in sensitivity and can lead to over or wrong diagnosis, causing the treatment course to be misguided as the symptoms may be due to mycobacterium from other spieces.

Diagnostic Techniques based on molecular aspects are gaining strength A study done [48] to diagnose pulmonary tuberculosis uses AMPLICOR PCR system and compares it with the technique used in underdeveloped countries.

Sputum specimens were analysed for *Mycobacterium tuberculosis* presence by PCR. PCR method showed a sensitivity of 93% and specificity of 84%, respectively. High sensitivity merits its usage inaclinical setting and major commercial testing kit players are utilising these PCR-based methods for their diagnosis kits as they also serve to be cost effective inthecase of mass production.

Various rapid detection tests for tuberculosis has been developed which are based on principles to detect antigen and antibody in the samples. Detection of antibodies via immunodiagnostics tests serves as indirect proof of infections caused by the organism currently or either in the past. All these tests vary in terms of their specificity and sensitivity. Many cellular antigens like cord factor, lipo-arabinomman, A60 etc, have been utilised in antibody detection kit. These tests have not found wide application for diagnosis of chronic tuberculosis infections as they are low in sensitivity and specificity. Antibody detection test showed a variation in sensitivity from 15.7% to 89.2% while specificity for it ranged from

50% to 100 %. This leaves TST (tuberculin skin test) as the only immunodiagnostic test that can be used to detect chronic tuberculosis.

The antigen-based assay has been focused on demonstrating the presence of Mycobacterium cells or its various other breakdown products in fluidic systems. Antigens present in sputum, pleural fluid, ascetic fluid and cerebrospinal fluid have been demonstrated via Enzyme-linked Immunosorbent Assay (EIA), Radioimmunoassay (RIA), latex agglutination and hemagglutination in patients suffering from tuberculosis.

Kadival et al [52] used sonicated antigen (immunoreactive) fractionate of H37 RV which was obtained through sepharose 6B chromatography for standardising RIA and achieving assay sensitivity of 1×10^3 organisms/mL. ELISA techniques (sandwich ELISA and dot-ELISA) are also utilised for antigen detection. Specificity of ELISA has been improved by extraction of more efficiently purified specific recombinant protein [54;55] .

The clinical sample's nature and the test format determine the target antigen. Amongst antigens LAM (lipoarabinomannan) is the target most frequently utilised, sputum detection of LAM has been done through the production of monoclonal and polyclonal antibodies. The presence of LAM in other species mycobacteria is a major disadvantage as it leads to nonspecificity. Other antigens that have been used include whole cells, PPD, 65 kDa, culture filtrate protein (CFP) and 14 kDa.Confirmation of culture using LJ media is time-consuming and delays treatment and even cause dissemination of disease to other organs.

Kits detecting mycobacterial infection by detecting specific antigen can be used as direct evidence and quick remedial action or treatment but it may suffer due to the scarcity of the mycobacterial antigen in clinical samples like blood, urine, CSF and pleural fluid. A study [50] was carried out for evaluation of BioMed Industries produced antigen testing kit in comparison to TB PCR.Their findings showed that specificity and sensitivity of TB PCR were in the range of 60% - 100%. Sensitivity being influenced by specimen type and infecting mycobacterium and thus specificity fortherapid kit was found out to be very low and be around (13.28%). ELISA based techniques fared better for specificity and sensitivity to be around 71% to 91%. Urine detection of lipoarabinomannan antigen was not much encouraging. So it showed that specific antigen based rapid testing kit doesn't fare better in specificity and sensitivity for clinical purpose in comparison of PCR and AFB smear examination and there is a very large scope for improvement in terms of sensitivity and specificity.

1.4 SOURCE OF ANTIBODY AND ANTIGEN:

Model studies are done inthecontext of *M. fortuitum* infections to develop knowledge regarding the internal physiological changes taking place in the host as well as these infections also serves as a source of an antibody which is produced and can be obtained through serum and can be further used in studies.

Carrasius aureus, a goldfish demonstratethetypical characteristic of *Mycobacterial* infection when infected with the *M. fortuitum* [47] . Mice, when given an intravenous injection, develops lesions in kidney and causes cochlear (balancing organ) damage which leads to a state where the mice lose control and it's head spins when it is lifted by its tail, a state known as spinning disease. *M. fortuitum* infection causes lesions in the cochlear which further develop into granulomas [25] .

M. fortuitum has shown to have apreference of localising in thekidney in mice model,thereason for which has remained unexplained [25] .Production of kidney lesion is attributed to lymphocytes causing moderate cellular infiltration. Further investigation into the spinning head phenomenon has shown that along with cochlear damage cellular infiltration causes damage to inner ear collaterally granuloma formation occurs in mice cerebellum and cerebrum[26] .

- ***M. fortuitum* murine infection model:**

To overcome the gaps due to lack of a animal infection model , a project was overtaken in which murine infection models developed (Female BALB/c mice) kept in sterile condition were infected withsuitable microbial loads and the pathophysiological changes were correlated with the advance of infection. A standardized insight into the path as well as the impact of *M. fortuitum* infection onto the host at different organ sites, and the host immunological response throughout the course of infection were developed.Invitro studies done on organs after sacrificing mices showed that the infection was persistent in kidneys even after 25 days of infection whereas it perished in other organs .The IFN-γ production by both T lymphocytes $CD4^+$ and $CD8^+$ remained elevated during the initial phase of infection and declined along with the bacterial load.

Blood drawn from mice infected with *M. fortuitum* (wild type) as part of earlier research work done was utilised to obtain serum. The serum is the source of antibody produced against the *M.fortuitum* (wild type) infection.

- **Source of Antigen**: *M. fortuitum*(ATCC 6841) whole cell lysate protein is to be as antigen.

1.5 ANTIGEN-ANTIBODY INTERACTION:

The crucial reaction of immune defence and immunology is aninteraction between antigens and antibodies. This interaction determines and establishes body's defence against bacterial and viral infections. Foreign substances are recognised by the body's defence as and specific antibodies are raised against them. These antibodies which are raised due to stimulus caused by antigens are generally very specific and donot interact with other antigens [27] . The interaction between antigen and antibody takes place via interaction between complementary molecular markers present on the surface of antigen (epitope) and the antigen binding site present on the antibody (paratope).When in close proximity the interaction between the two takes place via non-covalent forces like Van der Waal's force, hydrophobic force, and hydrogen bonds etc. These forces are very short range forces and are very specific about the interaction and also the direction and orientation of the antigen-antibody couple participating. The binding of theantibody to antigens leads to theformation of large macromolecular complexes. These complexes are formed due to theassociation of each antibody with more than one antigen and similarly interaction of an antigen with more than one antibody [51]. Formation of macromolecular complex leads to precipitation which is cleared away by various mechanisms. Precipitation reaction of antigen and antibody, based onthis immunologic principle of specificity of interaction between the two [27] is useful for various diagnostics and research purposes.

Precipitation was introduced by Kraus in 1897 who observed that enteric bacteria precipitated on mixing with homologous but not with heterologous antiserum. The soluble antigen-antibody interaction takes place within a few minutes but the formation of precipitation line is slow and often takes more than a day, during this time neighbouring antibodies form ionic bonds among each other leading to the loss of charge of the lattice and finally, it becomes insoluble. For precipitation reaction to take place the antibody needs to be bivalent while the antigen should be bivalent or polyvalent.

Immunodiffusion technique involves diffusion of antigen and antibody in solid agarose medium. While diffusing freely through the agarose they come into contact with each other at a particular concentration at which precipitation takes place and white precipitation lines are formed. Precipitation occurs at an optimal concentration of antigen and antibody known as thezone of equivalence. A further requirement for formation of precipitation lines requires the antibody to be bivalent while the antigen can be bivalent or polyvalent. The region at which precipitation occurs can be used for determination of titer concentration of both antibody and antigen. Immunodiffusion principle are important part of

immunology studies and have a wide application in diagnostic purposes as well some of the most important techniques involve Radial ImmunoDiffusion (RID) technique, Ouchtercolony Double Diffusion (ODD) technique, Immunoelectrofocusing electrophoresis and Rocket Immunoelectrophoresis.

- **Radial Immunodiffusion (Mancini 1965):**

is used for quantitative estimation of antibody concentration. Radial immunodiffusion provides for an accurate determination antibody potency of precipitating antisera [28].The antigen is incorporated into molten agarose and thereafter it is pipetted onto aglass slide and left to solidify. Post solidification of agarose gel small wells is punched into which antibody of known concentrations are loaded which serves as standards. Thereafter unknown antibody sample is also loaded which like the antibodies diffuses in a radial or circular pattern. Diffusion continues and precipitation lines are only formed at a point where thezone of equivalence is established between the fixed antigen concentration and the diffusing antibody concentration. It is basically the region where maximum numbers of complexes are formed and there is thegreatest number of cross-linking between antigen and antibody. Correlation between the standard antibody ring diameter and that of the sample antibody ring diameter is done, to determine the concentration of sample antibody.

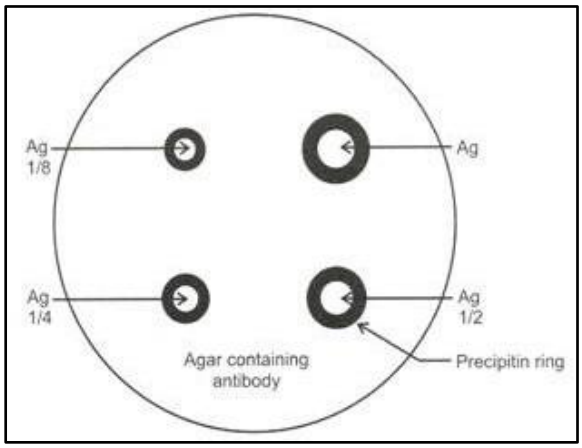


Figure 3: Radial Immunodiffusion [44]

- **The Ouchterlony Double Diffusion [27]:**

technique is also based on the principle of precipitation, but differing from the RID technique it involves the diffusion of both antigen and antibody through the agarose. At the zone of equivalence, linear precipitation occurs where edges of circular diffusion pattern meet. The ODD technique can be utilised to examine the similarity of antigens. For example in evolutionary studies antigens derived from different species are loaded into two wells while a known antibody is loaded into the third well, which is located between and slightly below the other two wells forming a triangle.

On diffusion, there are three possibilities that may occur:

In thecase of non-similarity of antigenic determinants of the two antigens, the precipitation lines will cross each other, in thecase of identity or similarity of antigenic determinants the precipitation line will stop at the point of their intersection. If some antigenic determinants are same or shared, one of the precipitin lines between the antibody well and antigen well halts at the point of interaction while the other progresses past it indicating that the antigens share some of the similar antigenic determinant regions but not all of them [45] .

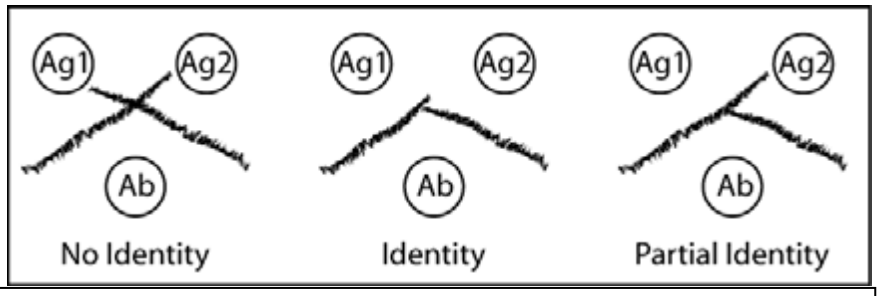


Figure 4.1 Ouchterlony Double Immuno Diffusion [45]

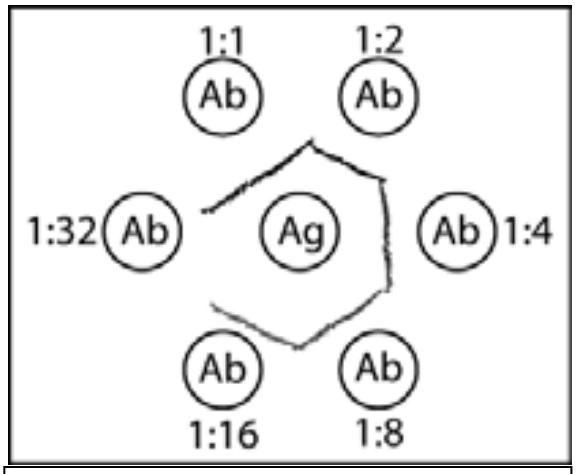


Figure 4.2 Ouchterlony Double Immuno Diffusion [45]

Antibody titration is the second application of ODD technique, which allows visualisation of antibody titer (concentration) in a test solution. In which known amount of antigen concentration is added to the central well and varying titers of antibodies are placed in wells circling the antigen well. Visualisation occurs when one titer has anoptimal ratio of antigen concentration to antibody concentration. It gives a fair idea about the concentration of antigen and antibody.

- **Rocket Immunoelectrophoresis:**

A rapid application of the antigen-antibody interaction, providing a precise reaction involving electrophoresis of antigen in agarose gel containing antibody related to the antigen. A result of this interaction occurring under electrophoresis leads to formation of precipitin line in the form of a rocket, from which it gets it's name. It provides quantitative result, as the length of the precipitate can be correlated directly to the quantity pof antigen. It works with precision even for the smaalest amount of antigen, rapidnees is another advantage as the run time (2-16 hours) depends majorly on the antigen's electrophoretic mobility and the strength of the electric field applied [29] .

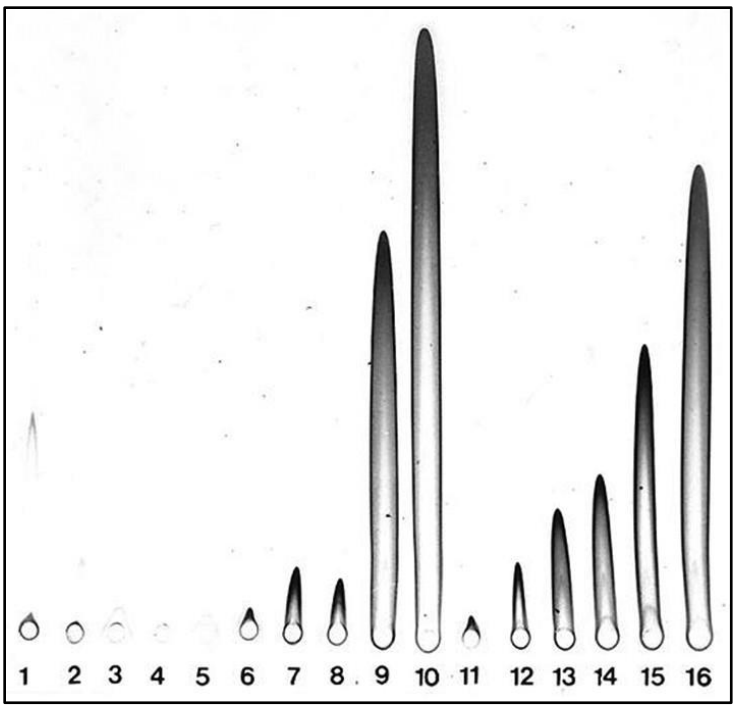


Figure 5: Rocket Immuno- Electrophoresis [29]

- **Immunoelectrofocusing electrophoresis:**

Isoelectric focusing is utilized for separation of antigens in polyacrylamide gel (support medium) is followed by electrophoresis of separated components in a perpendicular direction into a agarose gel containing antibody. This method provides identification for antigens based on the isoelectric point (PI) and thus their immunochemical characterization.This separation helps to provide analysis of antigens at high resolution for complex antigens mixtures and various other protein mixtures of isoenzymes, microheterogeneous forms of single antigens and genetic variants can be resolved for identity, non-identity, and partial identity [30] .

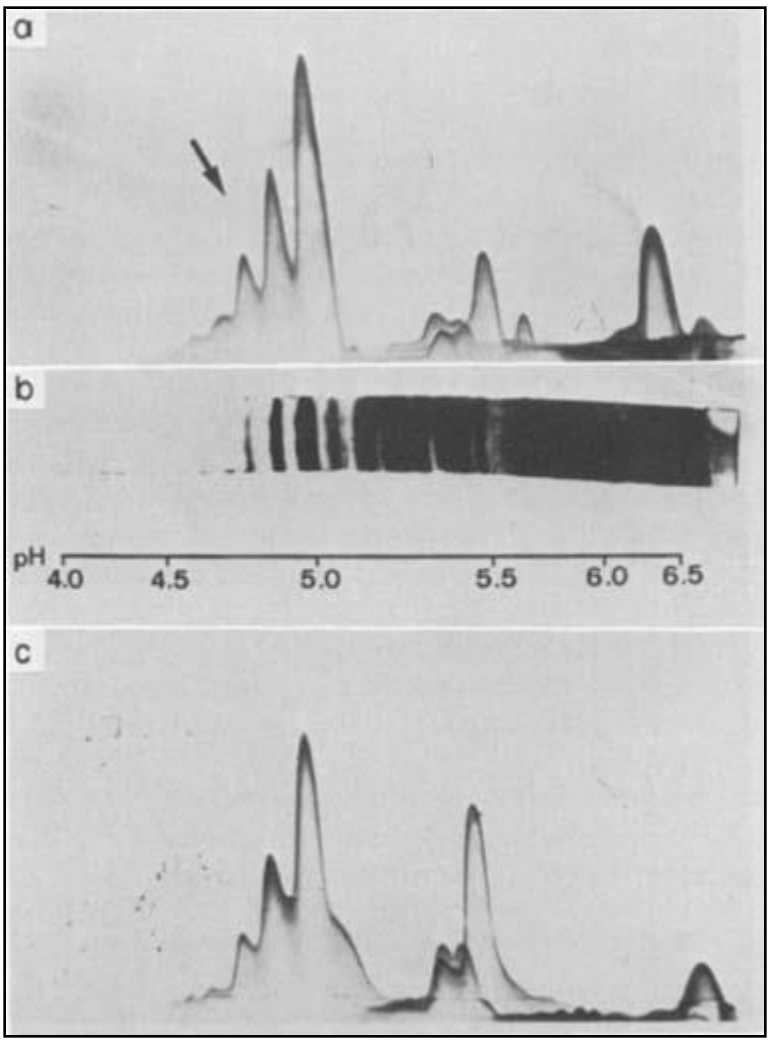


Figure 6: Immunoelectrofocusing electrophoresis: [30]

1.6 LYSIS OF CELL:

Protein extraction strategy involves both mechanical and non-mechanical methods of disruption. The mechanical method focuses basically applying enough stress to cause cell surface membrane while the non-mechanical method involves enzymatic action on the cell membrane causing it to rupture and release cellular organelles and proteins with maximum reproducibility and minimum damage to the extracted protein. Exposure of the target cells to the disruptive force for the minimum time is generally considered better to protect the targeted protein so methods involve short period repetitive exposure of the sample with intervals in between. To address both the condition a suitable homogenization medium

is required which not only helps to extract proteins via effective and reproducible cell membrane disruption, quality cell lysate release but also helps in prevention of cell lysate/protein degradation.

- **Chemical and enzymatic homogenization:**

Homogenization of bacterial culture is generally done in 50mM Tris/PBS buffer containingalow concentration of $Mgcl_2$ and EDTA which maintains pH for the cell lysate. Tris, tricine or hepes are used generally around at pH of 7-7.6 to maintain isotonic conditions. Degradation by protease activity is another critical aspect to take care of, so the homogenization medium is supplemented with protease inhibitor like PMSF (phenylmethylsulfonyl fluoride) which may be added in the concentration of 1mM or 2μg/mL each of antipain, leupeptinoraprotinin may be added to prevent the cells from protease activity. A cocktail of protease inhibitors may also be added to achieve the stated purpose. Certain microbial cells can be stressed osmotically using hypo-osmotic medium using sodium bicarbonate at pH 8 whereas 10mM concentration Tris, Hepes buffer can also serve the same purpose. Selection of a certain homogenization medium depends on the target cell type, its intracellular structure etc.

The cell membrane consists of a lipid double-layer; lysis buffers which contain surfactants can solubilize lipid membranes of cell membranes. Study [31] was done on *Mycobacterium avium* for different protein extractions protocol using various lysis buffer (0.4% v/v Triton X-100, 8 M Urea with 1% w/v CHAPS, 0.4% v/v Tween20, 0.1% v/v Decon90, 0.8 M potassium thiocyanate (KSCN) in 80 mM sodium chloride (NaCl) and 0.5% v/v Decon90) and they were comparatively studied for their effectiveness by using native PAGE SDS-PAGE and SELDI-TOF-MS. The comparison was done on Protein Chip® surfaces with two (EAM) energy absorbing molecules(proprietary formulation by EAM-1 and Ciphergen (sinapinic acid)) for extracts of non-ionic detergent (Triton X-100 and Tween20) and Chaotropic agents (potassium thiocyanate and Urea CHAPS). Detection of Urea CHAPS extracts was seen on all ProteinChip® surfaces and was efficient for protein. Potassium thiocyanateserved showed the greatest detection and was could be inferred to be the most effective. All buffers were found to be suitable for extraction of protein and further work on SDS-PAGE and Tween20 served best for native PAGE.

- **Mechanical Homogenization:**

Lysis of cell is majorly obtained by using mechanical procedures like French Pressure cells, Bead milling, grinding along abrasive agent and ultra sonicationetc, In French Pressure method, hydraulic pressure applied on cell suspension causes cell lysis. Hydraulic pressure (8,000 – 12,000 psi) is applied to the cell suspension following which pressure is suddenly released back to atmospheric pressure. The cell suspension is forced through a narrow orifice using a piston, this causes a sudden and swift change in pressure leading to a liquid shear [32] . It shows efficiency for culture suspension having a volume range 10-30 mL (1:1 to 1:4 g/mL ratio ofthewet weight of cells tothevolume of lysis buffer but has a lack of being time-consuming for larger volume and has shown technical difficulties for smaller volumes.

Sonication suits small-scale protocols for purifications; it exposes cells to mechanical shearing of cells using vibrations. In one set around 1gm of cells or tissues can be lysed. The major concern while doing sonication is the generation of heat which may cause denaturation of the protein. To overcome this time interval of 2-5 minutes is provided between each single run ofthesonicator, in which the sample is suitably stored in ice to cool down.

In nitrogen cavitation devices are made up of durable stainless steel cylinder having a nitrogen gas inlet port to deliver it from a cylinder and an outlet port with the needle valve. The pressure of 5000 kPa is applied for 10-30 mins, nitrogen dissolves into cell buffer as well as into the cellular cytosol. Following pressurisation when the needle valve is opened, the suspension is passed through outlet tube, exposure to normal atmospheric pressure causes swift decompression and leads to cell disruption due to nitrogen gas bubble formation inside cells. The working volume range is 1 to 1000 mL and it is not limited by heat production due to the cooling expanding gas.

Grinding with abrasive material is a very cheap technique to lyse cells, simple as grinding cells with mortar pestle in presence of material like alumina or sand. It can handle a moderate 30 gm ofthesample [33;34] . Working with a pathogenic sample can cause considerable problems and would require precautions, better avoided. Abrasive material can also be used while doing Sonication.

Glass bead vortexing method involves vortexing samples repeatedly with glass beads and suitable for small amount of sample (3 gm weight wet) which can withstand the repeated vortexing. It is generally

utilised for homogenization of yeast cells [35] . Braun MSK Glass Bead mill and Manton-Gaulin homogenizer are commercially available and have upscale the amount of working sample.

Enzymatic breakdown of cells is based on the cleavage of bacterial cell wall using Lysozyme, peptidoglycan layer is made up of alternate N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) which forms the backbone of the cell wall. Lysozyme cleaves these glucosidic linkages between NAM and NAG and leads to lysis of cell wall.

1.7 QUANTITATIVE AND QUALITATIVE ANALYSIS OF PROTEINS:

- **PAGE:**

The qualitative analysis of extracted protein for determining its purity is required to have an affirmation that the extraction was effective. Polyacrylamide Gel Electrophoresis (PAGE) is a steadfast method for determining protein quality and its effective separation fromtheprotein mixture. PAGE also helps to determine relative protein molecular mass and also for protein concentration verification.

Polyacrylamide gels can be casted by two different buffer systems i.e. continuous buffer system [36] which contains a constant concentration of acrylamide (suitable for highly concentrated samples) or the discontinuous buffer system [37] made up of short length "stacking gel" with wide pores below which a long length "resolving gel" which has narrower pore is layered. Both these layers are different with respect to both ionic composition and pH.The stacking gel allows concentration of dilute protein samples by confinement of protein samples to a narrow beginning zone. Kohlrausch studied [38] on starting the electrophoresis that stacking of the protein sample in the stacking gel between the leading chloride ion and the lagging glycine ion is caused by the formation of Kohlrausch boundary (high voltage gradient) limited to the narrow area as the pH of the stacking gel determines the motility of all of them and leads to them being placed in their respective position in the gel. The stacking of protein sample is of high importance to obtain its better resolution in the resolving gel.

SDS is used in PAGE to provide a uniform negative charge on all the protein molecule and it causes a breakdown of protein into individual chains and allows their electrophoretic separation based on their molecular weight only. Thus allows a well resolved qualitative analysis of protein sample possible.

- **Quantification of protein content:**

Quantification of protein content isanimportant component of various proteomics, biochemical, chemical studies etc, and the use of molecular UV and visible spectroscopy has provided with an efficientmeasure to quantify proteins. Some most widely used method for protein quantification is Bradford, Lowry and Biuret methods.

The Bradford Assay is a quick, specific and sensitive method of protein estimation based onashift of absorbance of the Coomassie Brilliant Blue G-250 dye which on binding totheprotein being assayed in acidic condition changes colour from red to blue. The binding of the protein strengthens the blue colour of the dye and gave maximum absorption at a wavelength of 595 nm. A very small volume of sample is required for the assay and also it's a relatively cheap method. The existence of the Bradford protein binding dye in three different forms namely cationic, neutral and anionic causes' problems with non-buffered dye reagents, detergents as there is a shift from one form to another and hencetheoccurrence of interference takes place [39]. The primary target of interaction for this dye is arginine, while the aromatic groups and other basic groups produce slight responses [39].Another sensitive method which can be applied for quantitative protein estimation is Lowry method. The basic reaction is the reduction of Folin-Ciocalteu reagent (phosphomolybdic/phosphotungstic acid blue complex) causingthedevelopment of a blue colour which is measured at 750 nm [40] . Prior totheaddition of Folin-Ciocalteuwith the protein sample is treated with alkaline copper sulphate in presence of tartrate. Thereafter there is a transfer of electrons from tetradentate copper complex toFolin-Ciocalteu, hence leading tothecolour formation by via electronic transition from one valence electron to another [41] . The time required for incubation is around 40 minutes minimum which comes as a major disadvantage along with being a two phase system.

The third assay which can be applied for protein quantification is the Biuret method in which functional group in peptide bond of protein interacts with Cu^{2+} leading to the formation of a complex requiring two peptide bonds and produce a chelate product which gives violet colour [41] .

A study [41] was done to compare Bradford, Lowry and Biuret methods for quantifying protein using hen albumen and egg yolk as samples. The study clearly showed that Bradford was the most sensitive assay (0-0.01mg) followed by Lowry method (0-0.1mg) and then by Biuret method (0-1mg). Biuret method suffered from interference from Amino group [$(NH_4)_2SO_4$] while Bradford suffered interference

from detergents (soap, SDS, Triton X-100) while the Lowry method suffered interference from (Acids, EDTA, DTT, phenol, $[(NH_4)_2SO_4]$.

Table 1 Comparison of protein estimation methods: [41]

Method	Sensitivity	Accuracy	Interference
Lowry	0−0.1mg	Partially dependent on amino acid composition	Acids, EDTA, DTT, phenol, (NH4)2SO4
Biuret	0−1 mg	High, no depends on amino acid composition	Amino-group [(NH4)2SO4]
Bradford	0−0.01 mg	Dependent on amino acid composition	Detergents (soap, SDS, Triton X-100)

CHAPTER 2:

METHOD & PROTOCOL

METHODS AND PROTOCOL

- **Quantification of protein (Bradford Assay):**
  - **a.** BSA (Bovine Serum Albumin) standard concentration ranging from 0µg/mL to 1000µg/mL was prepared in PBS. 20µL of the different concentration stock were added individually to 180µL of Bradford reagent in a 64 well micro titer plate.
  - **b.** Similarly 20µL of the extracted total protein was added to 180µL of Bradford reagent.
  - **c.** The entire experiment was setup in duplicates, after 5 minutes of incubation spectrophotometric readings were obtained at 595nm.
  - **d.** 0µg/mL of BSA served as blank and was subtracted from each reading, the readings were plotted with the different concentration at the X-axis and the spectrophotometric readings obtained at the Y-axis.
  - **e.** MS excel software was used for the purpose of obtaining the scatter plot and Regression value for the data. The spectrophotometric readings for the extracted total protein were estimated using the trend line obtained.

- **SDS PAGE:**
  - **a.** The resolving gel was settled into sealed glass plate's apparatus. Post polymerization after about 15 min, pouring of stacking gel over resolving gel is done and combs were placed.
  - **b.** Post solidification of gel, comb was removed carefully. Unpolymerized acrylamide in the wells were rinsed immediately by using water or running buffer.
  - **c.** Sample was mixed with sample buffer (2X) and boiling was done for 5 minutes.
  - **d.** Gel was placed in running chamber of gel electrophoresis chamber; running buffer was poured into the chamber.
  - **e.** Prepared sample protein was loaded in each well and electrophoresis was done at a voltage of 100V.
  - **f.** Post completion of run, transfer of gel was done from running chamber to a box or chamber filled with staining dye.
  - **g.** Overnight shaking of staining dye chamber was done on a rocker.
  - **h.** Staining dye was removed, and the chamber or box was filled with destaining solution and was subsequently subjected to shaking on a rocker.

i. Distaining solution was replaced repeatedly.

j. Protein bands on gel were visualized on a white background.

- **Protein Precipitation (Acetone Method):**

Acetone method applied for precipitating soluble proteins from samples. Helps to remove salts as well as hydrophilic and lipid soluble contaminants present in the sample solution.

Extracted total protein sample dissolved/present in a suitable buffer/solution was precipitated by adding acetone twice to the amount of protein sample and leaving the solution overnight at -20ºC, following which centrifugation @10,000 rpm for 15 mins was given. Pellet settles down and supernatant was removed carefully.

- **Lyophilization:**

also called "freeze-drying," is a method of desiccation, foremost step of lyophilization is freezing of the sample following which reduction in ambient pressure and temperature is increased slowly inside the lyophilization chamber to cause sublimation of frozen . There is a reversible change caused in the secondary structure of protein upon lyophilization.

Extracted protein sample were taken in cryovials, sealed doubly with parafilm and were transferred to -80ºC and stored there for 2 hours. Post storage at -80ºC, tiny pinholes were made in the parafilm seals and the cryo-vials were swiftly transferred to a Round Bottom Flask which was placed on lyophilizer to freeze under vacuum.

- **Quantitative Precipitation Assay:**

Interaction of antibody with antigen (polyvalent) can lead to the formation of an large complex which precipitates out of solution. Precipitation varies with the ratio of antigen to antibody.

a. 1X assay buffer was prepared; Serum dilution to desired ratio was done using the 1X assay buffer.

b. Extracted total protein (100 μL) was taken in a micro-centrifuge tube.

c. Two fold serial dilutions of extracted total protein was done by transferring half the amount of the total protein into 1X assay buffer of equal amount.

d. Post serial dilution of extracted total protein a fixed quantity of serum was transferred to each tube.

e. Each tube was subjected to mild mixing followed by incubation for 40 minutes at

f. Post incubation at 37ºC, incubation for 20 mins at 4ºC was done. In interval of every 10 minutes, tubes were mixed gently.

g. Centrifugation at 10,000 rpm for 10 to 15 minutes was done to obtain a tight pellet.

h. Aspiration of supernatant was done without disturbing pellet.

i. Pellet suspension was done in 1X assay buffer (volume approximately 1mL).

j. Centrifugation at 10,000 rpm for 10-15 minutes was done.

k. The supernatant was aspirated carefully.

l. Precipitate was dissolved in 0.1N 1X NaOH.

m. Spectrophotometric reading at 280 nm was obtained.

- **Radial Immuno Diffusion:**

a. 10ml Agarose (1.0%) was prepared in 1x Assay Buffer.

b. 1 agarose was added and heated to dissolve.

c. Agarose was allowed to cool to 55º C.

d. 120 µl antiserum of desired concentration was added and swirled to mix.

e. Antiserum containing agarose was poured onto a clean glass plate, left undisturbed to cool.

f. Wells were cut using a gel puncher, once gels solidified.

g. 20 µL of test antigen was added.

h. Gel plates were kept in a moist chamber and incubated overnight at 4º C.

i. Edges of the circle were marked, diameter was measured.

- **Double Immuno Diffusion Assay:**

a. 10ml Agarose (1.0%) was prepared in 1x Assay Buffer.

b. 1% agarose was added and heated to dissolve.

c. Agarose was allowed to cool to 55º C.

d. Agarose was poured onto a clean glass plate, left undisturbed to cool.

e. Wells were cut using a gel puncher, once gels solidified.

f. 20 µL (desired concentration) of test antigen and antibody were added.

g. Gel plates were kept in a moist chamber and incubated overnight at 4º C.

h. Precipitation lines were observed.

- **Acid Fast Staining:**

  Mycobacterium cell walls (acid fast cells) are made up of thick waxy layer of lipid like mycolic acid. Carbol fushin solubilizes the lipid content, and penetrates further to stain the cytoplasm on application of heat, and gives it the typical red colour. Carbol fushin staining is retained by all cells until treatment with decolorizing agent like 3% acid alcohol takes place which causes the smear to move out from the non acid fast stain, while the mycobacterium owing to their mycolic acid content retains the carbol fushin stain as they don't allow any penetration by decolorizing agent. Methylene blue or malachite green is used to counter stain the non acid fast cells, as they now only they can retain the stain. Non acid fast cells appear blue or green in color.

a. Under sterile environment, bacterial smear was prepared on a clean slide.

b. Smear was air dried and then heat fixing was done..

c. Slide was covered with blotting paper carbol fushin stain was added to smear it..

d. Stain was heated until vapors appear to rise. Carbol fushin was added regularly to avoid overheating. Overheating was avoided. Heated stain was allowed to stay on slide for 5 minutes.

e. Rinsing of slide with clean water was done to remove excessive stain.

f. 3% v/v acid alcohol was added onto the slide and allowed to stay for 10 to 15 sec for 5 minutes for decolorizing the smear

g. Rinsing with clean water was done again.

h. Malachite green stain was added and allowed to stay for 3 to 4 minutes. .

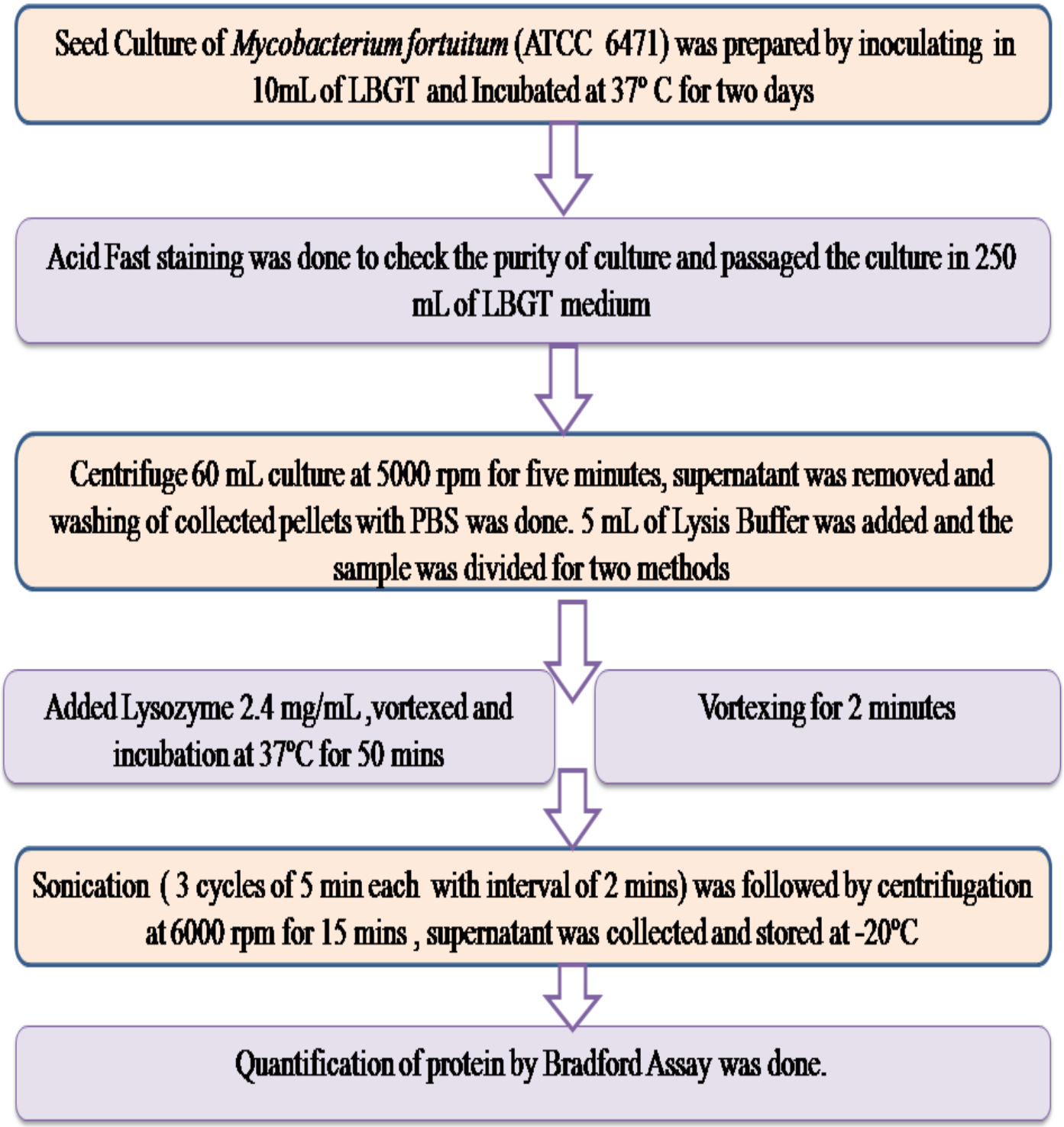
i. Rinsing was done with with clean water.

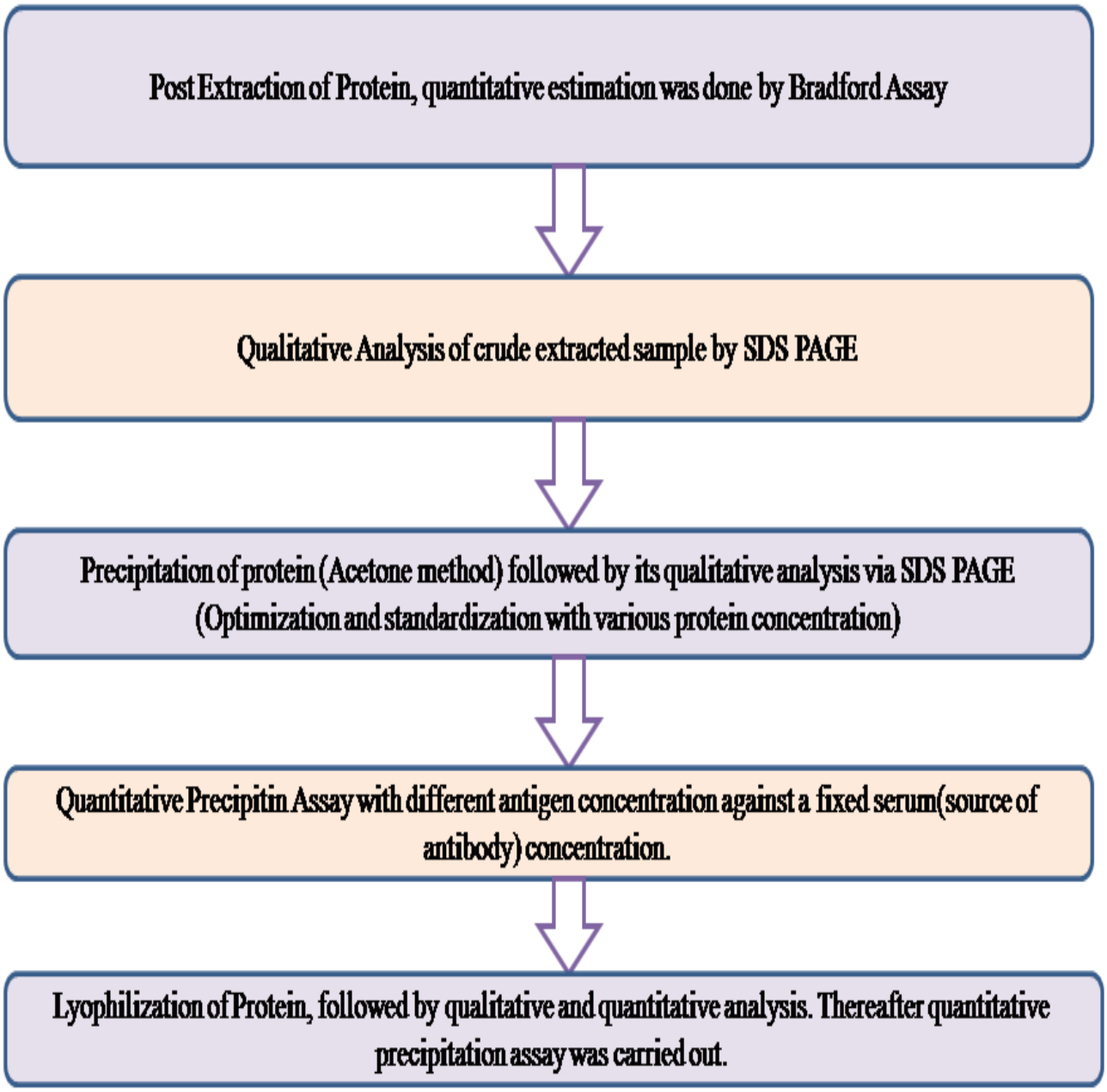
j. Slide was cleaned from backside, and smear was allowed to air dry

k. Using a microscope the smear was visualized and examined at 100X.

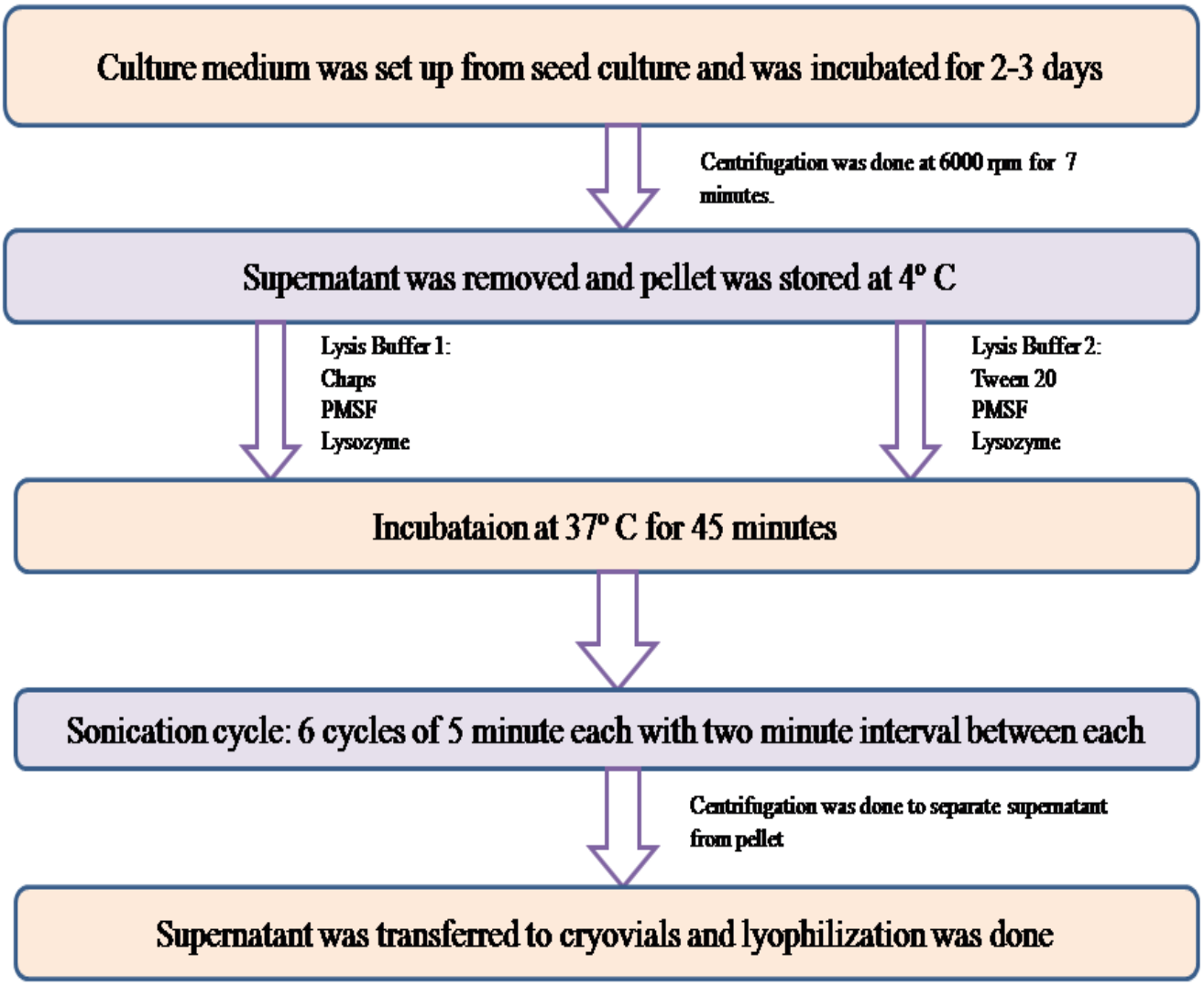
PROCESS FLOW CHART

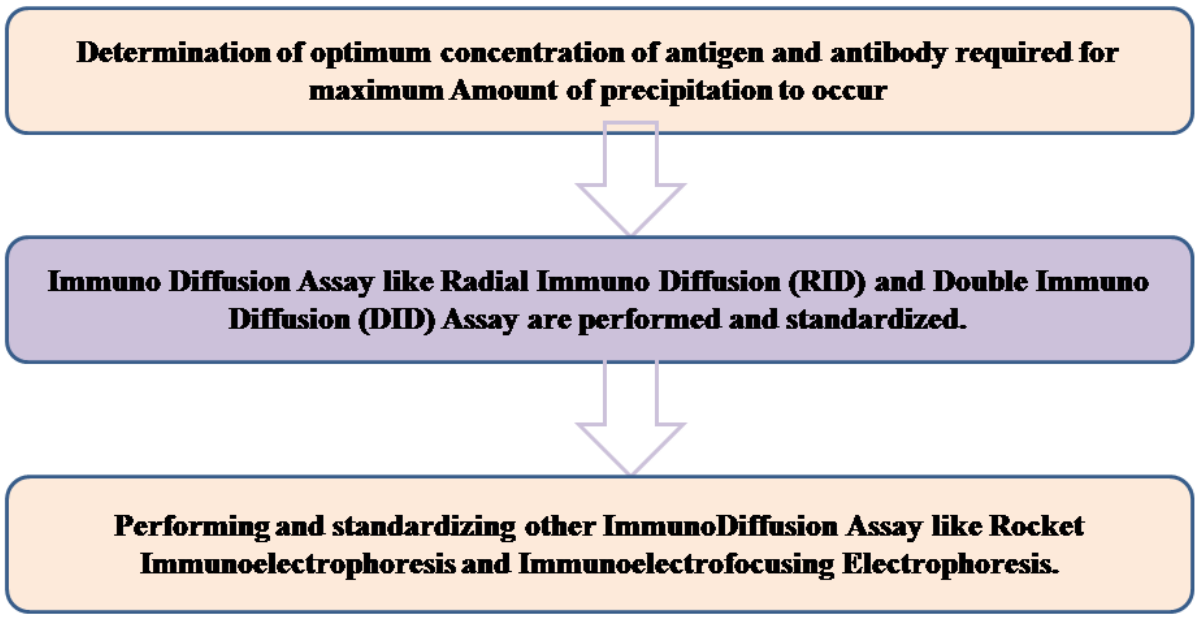
- **PHASE 1**




Post Extraction of Protein, quantitative estimation was done by Bradford Assay
Qualitative Analysis of crude extracted sample by SDS PAGE
Precipitation of protein (Acetone method) followed by its qualitative analysis via SDS PAGE (Optimization and standardization with various protein concentration)
Quantitative Precipitin Assay with different antigen concentration against a fixed serum(source of antibody) concentration.
Lyophilization of Protein, followed by qualitative and quantitative analysis. Thereafter quantitative precipitation assay was carried out.

PHASE 2:


Culture medium was set up from seed culture and was incubated for 2-3 days
Centrifugation was done at 6000 rpm for 7 minutes.
Supernatant was removed and pellet was stored at 4° C
Lysis Buffer 1:
Chaps
PMSF
Lysozyme
Lysis Buffer 2:
Tween 20
PMSF
Lysozyme
Incubataion at 37° C for 45 minutes
Sonication cycle: 6 cycles of 5 minute each with two minute interval between each
Centrifugation was done to separate supernatant from pellet
Supernatant was transferred to cryovials and lyophilization was done


Determination of optimum concentration of antigen and antibody required for maximum Amount of precipitation to occur
Immuno Diffusion Assay like Radial Immuno Diffusion (RID) and Double Immuno Diffusion (DID) Assay are performed and standardized.
Performing and standardizing other ImmunoDiffusion Assay like Rocket Immunoelectrophoresis and Immunoelectrofocusing Electrophoresis.

CHAPTER 3:

RESULT& DISCUSSION

RESULT:

Standardisation of Extraction Method:

The lysis of the cell involved use of chemical action (i.e. lysis buffer), enzymatic lysis and mechanical lysis. Enzymatic lysis involved lysozyme treatment; the first step of the study was the extraction of total protein from *M. fortuitum* for which both the Lysozyme treatment and non Lysozyme treatment method for lysis via Sonication were applied. The results obtained by Bradford Assay estimated the total protein extracted concentration for Lysozyme treated samples to be 567µg/mL and for non lysozyme treated sample total protein extracted concentration was estimated to be 225µg/mL.

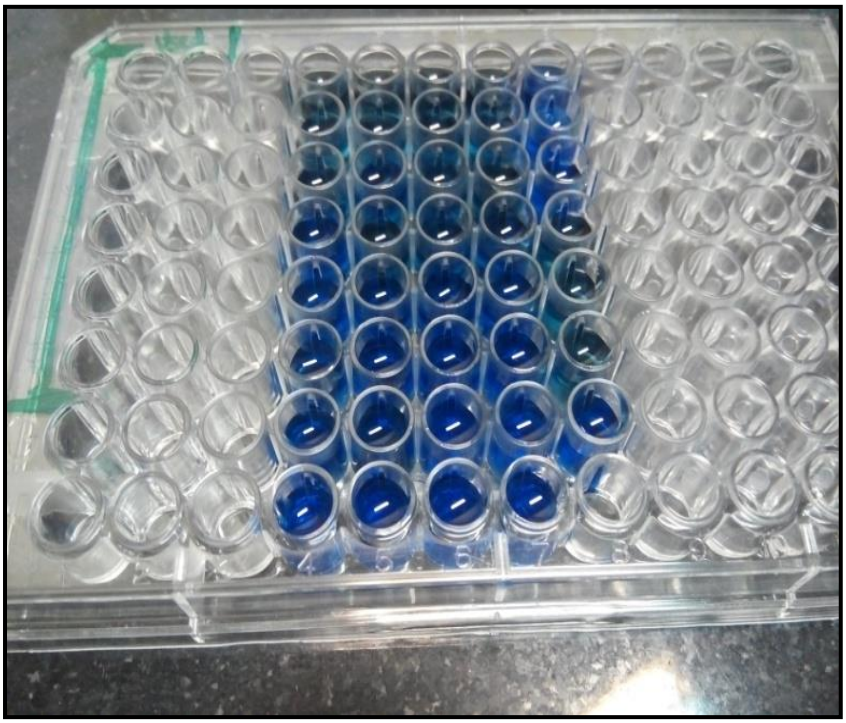
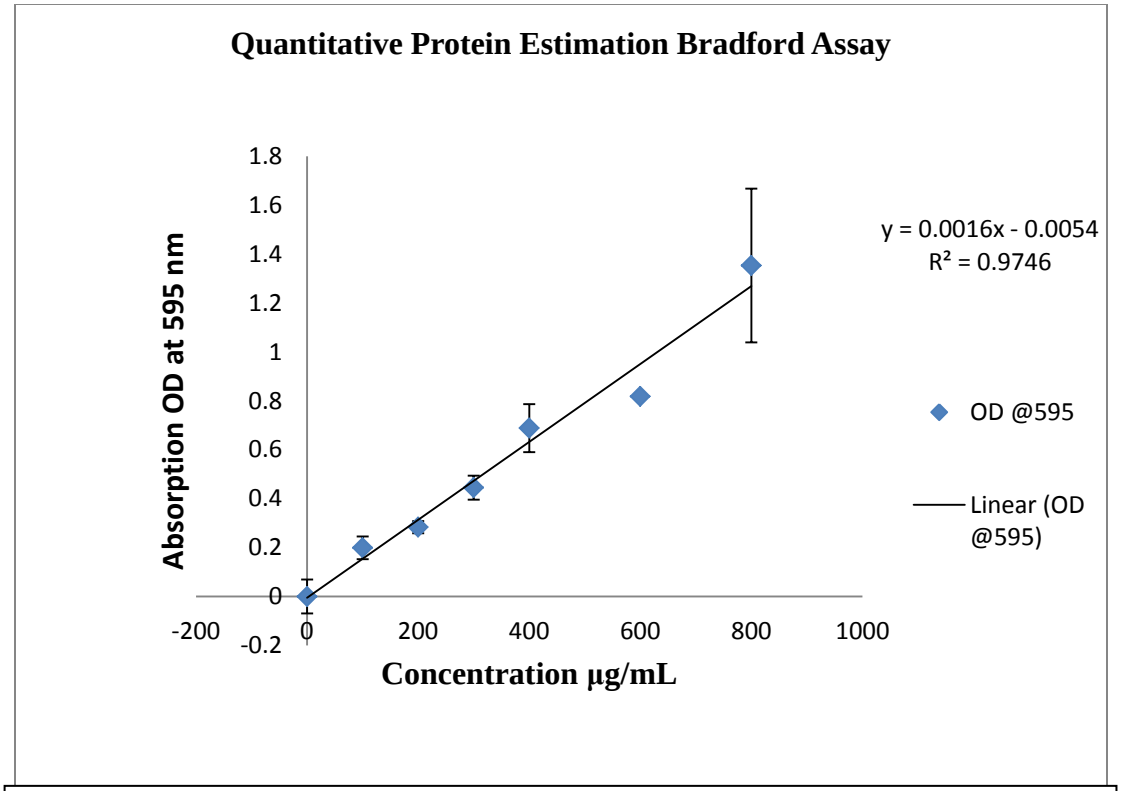


Fig 7. Bradford Assay: BSA was used a standard for protein quantification.



Graph 1: BSA protein as standard for quantitative estimation of protein (Bradford Assay)

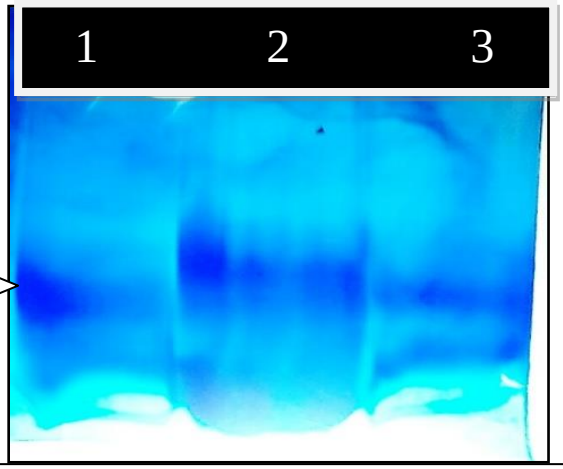
Table 2 : Estimation of extracted protein (Lysozyme treated and non Lysozyme treated) by Bradford Assay	
SAMPLE	**Estimated Concentration (**µg/mL)
Lysozyme treated Sample	567
Non Lysozyme treated sample	225

The results implicated that lysozyme treatment helps to increase the efficiency of the protein extraction as greater amount of protein is extracted in comparison to non Lysozyme treated sample.

Quantitative analysis showed the presence of protein, the use of Lysozyme (2.4 mg/mL) made it necessary to probe the presence of cell lysate protein. We used SDS PAGE as a qualitative and visual aid to view proteins. The first PAGE run was performed on crude sample of both Lysozyme treated and Non-Lysozyme treated sample along with a (biorad low range marker) approx 97 KDa. A clear gel was obtained after destaining with the exception of ladder run which was only visible, the run for sample was completely clear. This indicated towards the very low concentration of extracted protein present in the sample or a complete absence of protein so for verification higher amount of protein was precipitated and was run on SDS PAGE (fig 2).

Precipitation of protein sample was done using acetone precipitation method. These precipitated protein samples were given SDS PAGE run.

The concentrated precipitated protein SDS PAGE run confirmed the presence of protein as high concentration smear was formed. The presence of protein was confirmed next we tried to optimize the PAGE run along correlating with quantification of protein.



Protein Band

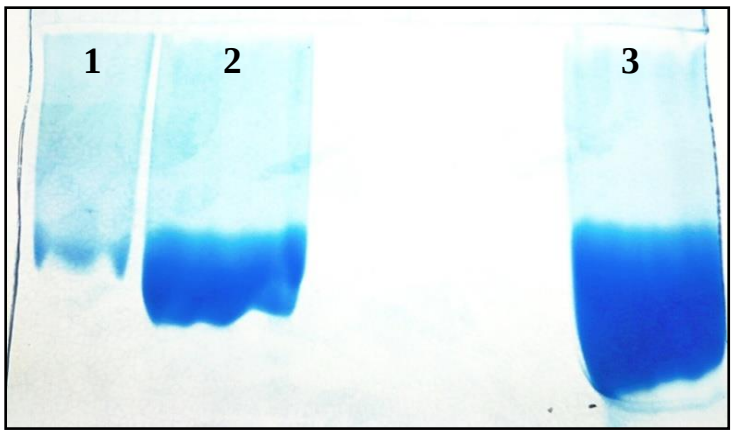
1. Precipitated Lysozyme treated Sample 230.34 ug/mL

2. Precipitated Lysozyme treated Sample 141.75 ug/mL

3. Precipitated Lysozyme treated Sample 141.75 ug/mL

Figure 8. Precipitated Lysozyme treated sample SDS PAGE run:

low concentration of protein, gave no bands earlier, so to concentrate the proteins they were precipitated out using acetone precipitation method.



1. Precipitated Lysozyme treated Sample 26.578 ug/mL

2. Precipitated Lysozyme treated Sample 44.2968 ug/mL

3. Precipitated Lysozyme treated Sample 70.8 ug/mL

Figure 9. Precipitated Lysozyme treated sample SDS PAGE run: Observation of thick smear on SDS gel run(Figure 8), made it necessary to probe lower concentration of protein for which precipitated protein in decreasing order was given a SDS PAGE gel run

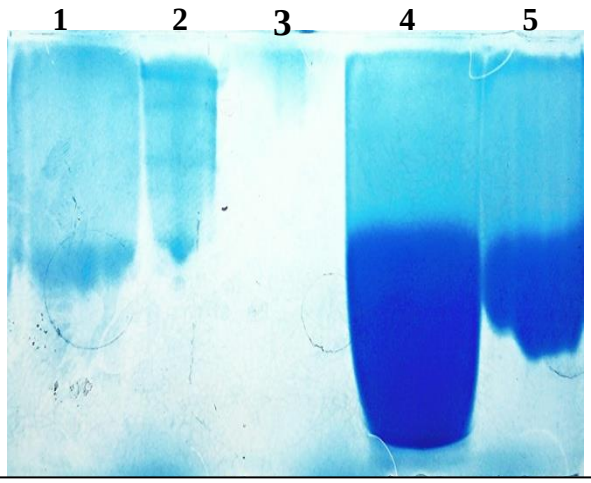
Lower concentration of proteins in ascending order of protein was run (Fig 3) and the gel run was observed to show smears in correlation with the increasing order of protein.

To increase the concentration of protein, lyophilization of crude sample was done. Post lyophilization estimation of lysosome treatment based extracted protein was 720µg/mL and that for non lysozyme treatment based extraction process was 124µg/mL. The significant loss of protein in both the samples from time of extraction was quite evidently noticed.

Table 3: Estimation of extracted protein(Post lyophilization Lysozyme treated and Non lysozyme treated sample)by Bradford Assay

Sample	Estimated Concentration (µg/mL)
Lysozyme treated Sample	720
Non lysozyme treated sample	124

Post lyophilization SDS PAGE run of lyophilized protein samples was done. Lysozyme protein was given a single well run to determine its contribution and its smear or band positioning in the treated sample extracted protein gel run. The low concentration of protein in lyophilized non lysozyme treated samples was also evident by the faint smear it formed.



1. Lysozyme 30 µg/mL

2. Bio rad low range marker 97KDa

3. Lyophilized non lysozyme treated sample 6.2µg/mL

4. Lyophilized lysozyme treated sample 36.05 µg/mL

5. Precipitated lysozyme treated sample 45.36 µg/mL

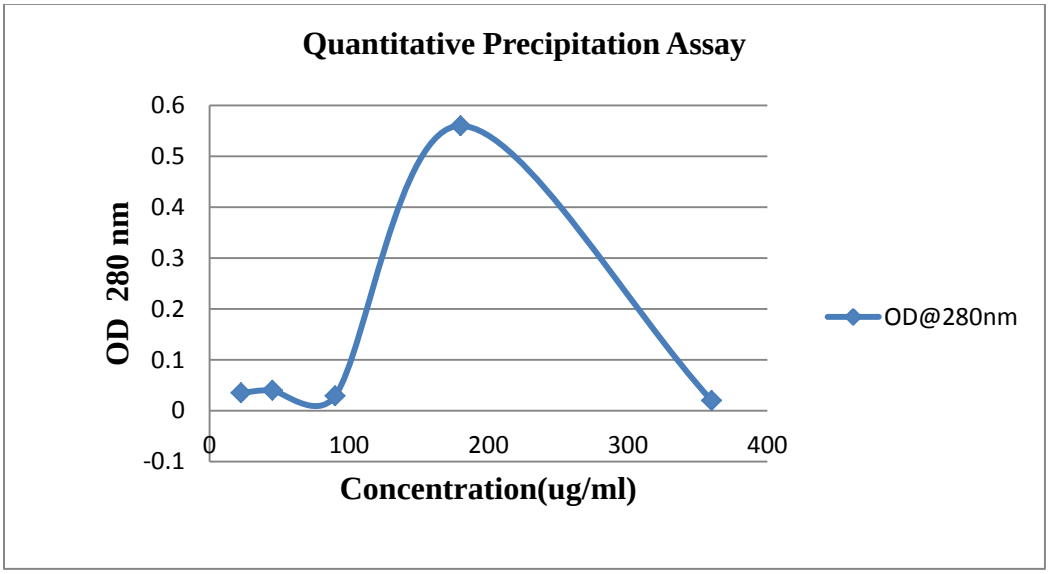
Fig.10: SDS PAGE run on lyophilized protein sample: Use of Lysozyme, provided the buffer with a protein (Lysozyme) that might have been detected and quantified. To remove these doubts, Lysozyme at the same concentration 2.4mg/mL with which it was added in the lysis buffer was also given a SDS PAGE run along with the protein samples.

SDS PAGE run of the lyophilized sample also established the higher quantity protein concentration of the Lysozyme treatment based extraction protein on which further was set up for quantitative precipitation assay.

On setting up quantitative precipitation assay, pellet obtained was very minute in quantity and we went ahead with NaOH solubilization step after solubilization in 0.1N NaOH spectrophotometric readings were taken at 280 nm to determine the amount of precipitation from the values obtained and graph was plotted to find the zone of equivalence.

Table 4: Quantitative Precipitation (Protein Absorption at 280nm)	
Concentration (µg/mL)	**Absorption (OD at 280nm)**
360	0.02
180	0.56
90	0.029
45	0.04
22.5	0.035

Table 4. Quantitative precipitation assay: lyophilized Lysozyme treatment extraction based protein sample: Different concentration of antigen (range) was quantitatively precipitated against Antibody concentration Ab1/5.

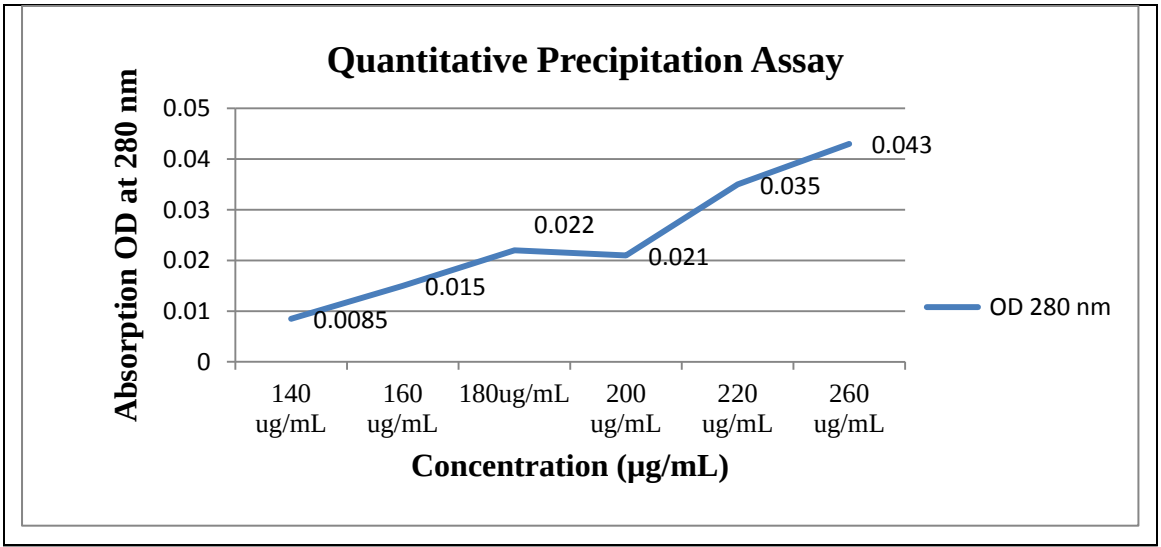


Graph 2: Quantitative Precipitation Assay for CHAPS lysis buffer extracted sample: A major increase in precipitation was observed around a concentration of 180µg/mL, supposed zone of equivalence region, which was taken further as target range for determination of zone of equivalence

Spectrophotometric reading showed a peak around 180 µg/mL; the shape of the graph resembled to that of the zone of equivalence. This was followed up by scanning through different range of concentration for determining optimum concentration at which maximum precipitation can be obtained. The amount of precipitation formed were low at different concentration we scanned, not much visual difference could be made between 220 µg/mL and 260 µg/mL and on moving ahead with dissolution in 0.1 N NaOH (1X) we got an increasing trend in the absorptions.

Table 5: Quantitative Precipitation Assay absorption at 280nm for CHAPS lysis buffer extracted sample

Concentration (µg/mL)	Absorption (OD at 280 nm)
140 µg/mL	0.0085
160 µg/mL	0.015
180 µg/mL	0.022
200 µg/mL	0.021
220 µg/mL	0.035
260 µg/mL	0.043



Graph 3: Quantitative Precipitation Assay at different concentration for CHAPS buffer lysis extracted sample: A regular concentration was observed in the amount of precipitation, 260µg/mL came out to be the concentration with the highest amount of precipitation.

Double Immuno Diffusion Assay was done for antigen ranging for different concentration of extracted protein antigen, with its counterpart antiserum at a dilution of 1/5 and 1/50. Antigen protein concentrations selected for the assay were at concentrations of 100µg/mL, 200µg/mL and 250µg/mL.

After incubation at 4ºC for 48 hrs, there were no precipitin lines observed on the gel, this made us to go for 180µg/mL concentration where we have earlier obtained a significant amount of precipitation. The distance between the wells were also

No precipitation was observed on the gel, indicating that the amount of protein extracted was low for precipitation to actually occur.

To determine if the concentration of extracted protein was suitable for any precipitation reaction to be expected we fixed the antigen concentration at 260µg/mL and took a larger volume from earlier setups and quantitatively precipitated it in broth against antiserum at a concentration of 1/5 of the original antiserum concentration.

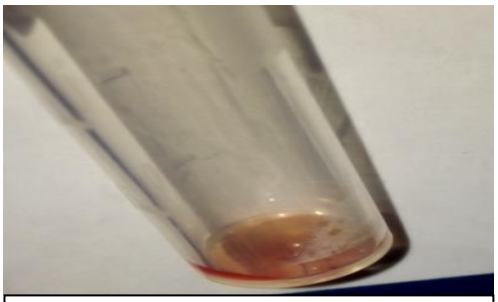


Figure 11: Quantitative Precipitaion Assay (larger volume of antigen 260µg/mL and antibody (1/5Ab) were utilized)

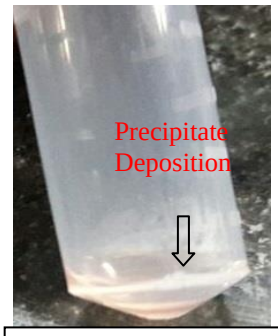
A small tight pellet was formed and was visualized on centrifugation, this was affirmative fact that 260µg/mL is the concentration at which we are obtaining quantitative precipitation.

We tried a second lysis buffer; the mechanical process of Sonication was also run for a greater number of cycles 6 in place of 3 with a time interval of 2 minutes within each cycle. The protein extracted is lyophilized immediately after extraction. The protein sample was quantified using Bradford Assay.

Table 6: Bradford Assay (Protein Quantification) of CHAPS and Tween-20 lysis buffer extracted sample	
Buffer Used	**Concentration (µg/mL)**
CHAPS	879.74
Tween – 20	984.87

Quantitative Precipitation Assay set on 260µg/mL sample of each, showed very small amount of precipitate, which was greater than that observed in the first phase.

CHAPS buffer extracted sample



Tween-20 buffer extracted sample

Figure 12: Precipitation observed upon quantitative precipitation of samples extracted from CHAPS lysis buffer and Tween-20 lysis buffer
(Protein samples extracted by the two lysis buffer were subjected to quantitative Precipitation Assay)

Quantitative precipitation assay showed that the amount of protein precipitated was more in the case of CHAPs buffer extracted sample.

Table 7 : Quantitative Precipitation Assay (Comparison of two buffer extraction sample for antigen-antibody interaction)	
SAMPLE	**Absorption at 280 nm**
CHAPs Buffer extracted Sample	0.06
Tween-20 Buffer extracted Sample	0.024

Double Immuno-Diffusion Assay was set for CHAPS extraction buffer sample and different antigen and antibody concentration were utilized for the purpose. After incubation no precipitation lines were visible.

Second Double Immuno-Diffusion Assay was set up by reducing the distances between the well, yet no precipitin lines were observed. To overcome the issue of low amount of diffusion occurring during DID assay we lyophilized the protein sample again.

Table 8: Concentration of CHAPS and Tween-20 lysis buffer extracted sample post lyophilization	
Sample	**Concentration (µg/mL)**
CHAPS BUFFER EXTRACTION SAMPLE	**1310.33**
Tween-20 BUFFER EXTRACTION SAMPLE	**1312.5**

Double immune diffusion assay was conducted again on different concentration of both antigen (260 µg/mL and 180 µg/mL) and antibody (1/2Ab, 1/5 Ab and 1/10 Ab) and no precipitation line was observed.

Radial Immuno Diffusion Assay (RID), three different concentrations of 260µg/mL, 350µg/mL and 520µg/mL were selected. The antibody concentration selected for the RID assay was 1/5 of the original antibody concentration that was mixed into the agar gel. Diffusion patterns were

visible at all the three wells containing the three concentrations of antigens, while a successful observation of thin precipitin band was observed at 260µg/mL.

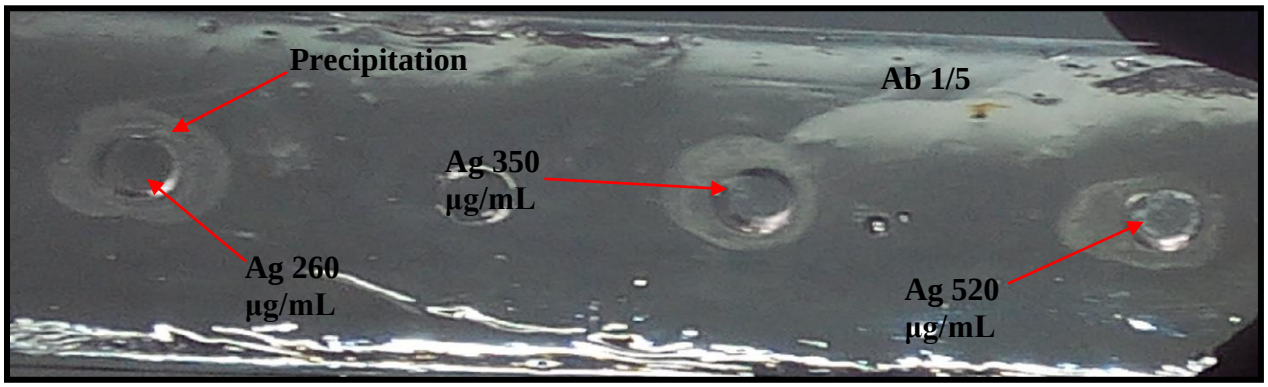


Figure 13: Radial Immuno Diffusion Assay (With a constant amount of antibody 1/5Ab mixed into the agarose gel, three wells were punctured out to add three concentration of antigen 260µg/mL, 350 µg/mL and 520 µg/mL. A precipitation band was obtained surrounding the well containing 260µg/mL of antigen.

CONCLUSION AND DISCUSSION:

Antigen-Antibody based testing kit is used for diagnostic purposes as well as finds major consumption in research and educational based applications. Our university spends around Rupees 1.5-2 lakhs each year on these kits as they are utilized in the lab curriculum for the courses of Immunology, Immunotechnology, Microbiology and Immune System and Diagnostic and Vaccine Manufacturing Technology. This motivated us to develop a In-House testing Kit.

M. fortuitum is a notorious NTM, causing numerous human ailments including skin and soft tissue, bone, lung, catheter-related blood stream infections and also disseminated infections [17] . The lack of major serological or antigen antibody based diagnosis kit causes major complication especially when the suspicion of infection is on M. tuberculosis. This causes a major diagnosis issue and can hamper treatment strategies and delay cure.

Our study is based on developing a cost effective antigen-antibody interaction based teaching kit. Selection of *M. fortuitum* satisfied our cost effective agenda as it was study microrganism for various other studies conducted earlier in our departmental laboratory. The antigen and antibody required for our study was very readily procured. Serum extracted from murine models infected by wild-type *M. fortuitum* served as a source of antibody. M. fortuitum was lysed to extract whole cell lysate protein to serve as antigen. The extraction method was based on three major strategies namely, chemical, enzymatic and mechanical.

Chemical component of the extraction process is based around the use of lysis buffer basically containing a detergent and a protease inhibitor dissolved in a suitable buffer. CHAPS lysis buffer was the first lysis buffer of choice, as CHAPS and Tween 20 has been shown to have good efficiency in protein extraction and further application. Both these buffers were utilized and compared for protein extraction in which Tween-20 lysis buffer fared better on protein extraction with 984.87µg/mL while CHAPS lysis buffer extracted protein was estimated to be 879.74µg/mL. The amount of precipitation on antigen antibody interaction in broth, demonstrated that CHAPS lysis buffer extracted protein showed more precipitation with an absorption greater than that for Tween-20 lysis buffer (Table 8). Thus CHAPS extracted protein was utilized for the immuno diffusion assay set up later.

Enzymatic method revolved around the use of Lysozyme for extraction, we were successful in demonstrating the dramatic increase in protein isolated by its addition in lysis buffer. Lysozyme treatment led to protein isolation to the concentration of 567µg/mL, while that for non Lysozyme treatment was estimated to be 225µg/mL (Table 2). The presence of protein was probed further using SDS PAGE analysis (Figure 8 and Figure 9), which was able to successfully demonstrate the presence of cellular lysate protein. This cleared doubts that the results of quantitative analysis were not solely due to Lysozyme addition. SDS PAGE analysis also revealed a major problem that the amount of protein isolated was of low concentration. The low concentration of protein became evident when the gel run for the samples gave clear gel, troubleshooting was done by acetone precipitation method, which allowed for concentrating protein and we were able to observe gels. Acetone precipitation does cleave away disulphide bonds, causing lose of tertiary structure of protein and hence its capability of specific interaction with antibodies. Lyophilization was utilized as a safer option to concentrate proteins as it causes reversible changes in secondary structure.

Sonicator was used for mechanical lysis, as it is suitable for lysing *M. fortuitum* cells. Optimization of Sonication cycle run was done. It showed an increase in CHAPS lysis buffer extracted protein sample concentration which increased from 720µg/mL to 879.94µg/mL (Table 3, Table 6). The cycle of sonicator was increased from 3 cycles for 5 minutes to 6 cycles of 5 minutes.

Quantitative precipitation assay was set up and various values of antigen were applied (table 4) and on plotting maximum precipitation on the curve were obtained around 180µg/mL. Determination of the optimal concentration was done using various concentrations and maximum precipitation was observed at 260µg/mL (Table 5). The pellet formation was very minute for the various concentrations, which were set up. This problem was sorted by using a higher volume of antigen for Quantitative Precipitation Assay, which gave a thick pellet formation. This also marked the successful completion of standardization of broth precipitation assay. Double Immuno Diffusion assay were set up a few times but due to shortage of time we were not able to satisfactorily refine the assay with our extracted antigens. Refining this assay would be a major aspect of future research. On setting up Radial Immuno Diffusion Assay, precipitin band was observed at 260µg/mL and established this concentration to be the optimum

amount. This was another major affirmation regarding standardization of optimum antigen amount required for quantification assay. On conclusion we have been successfully able to standardize our protein extraction protocol, standardization of precipitation assay and gel based diffusion assay.

FUTURE ASPECTS:

- By further refining gel Diffusion Assay, we can achieve our primary objective to develop In-House Antigen-Antibody based testing kit.
- The successful standardization of precipitation assay along has opened up a scope for development of diagnostic kit specific for *M. Fortuitum*.

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ANNEXURE:

1. Preparation of Luria Broth Glycerol Tween Medium

S.No	Ingredient	Concentration
1	Luria Broth	2% (w/V)
2	distilled water	50% v/V)
3	Glycerol (80%)	0.5% v/V
4	Tween-80	0.15%

2. **Preparation of Carbolfushin stain:**

S.No	Ingredient	Concentration
1	Basic Fushin	1 %(w/v)
2	Ethanol (100%)	10 % (v/v)
3	Phenol	5.5%(v/v)

In a dark reagent bottle ethanol was added to phenol and gentle mixing was given this was followed by adding 100ml of distilled water to dissolve the mixture. Basic Fushin was added and further distilled water was added to dissolve. Filtration was done using Whatmann Filter paper.

3. Preparation of Malachite Green:

0.3% (w/V) of malachite green powder was dissolved in final volume (V) of distilled water. Filtration using Whatmann Filter Paper was done.

4. **Preparation of Sodium Phosphate Buffer**:

0.1M Na_2HPO_4 was prepared by dissolving it in distilled water; 50 0.1M NaH_2PO_4 was prepared by dissolving it in distilled water. 50%v/V of Na_2HPO_4 was taken in a flask and NaH_2PO_4 was added drop by drop to adjust the pH to 7.4. Subsequently Na_2HPO_4 was also

added to raise the buffer volume along with pH balancing done by NaH_2PO_4. Buffer was stored at 4º C.

5. Preparation of Phosphate Buffer Saline :

S.NO	Ingredients	Concentration
1	Nacl	137 mM
2	Kcl	2.7 mM
3	Na_2HPO_4	8 mM
4	KH_2PO_4	2 mM

80% v/V of distilled water was taken in a reagent bottle to which all the listed ingredients were added. 1N Hcl was used to adjust the pH to 7.4, distilled water was used to make up the volume required.

6. **Preparation of Lysis Buffer:**

CHAP's Lysis Bufer:

S.NO	Ingredients	Concentration
1	CHAPs	0.5mM
2	DTTs	0.5mM
3	PMSF	1mM

In a suitable Buffer the above ingredients were dissolved.

7. Tween 20 Lysis Buffer Composition:

S.NO	Ingredients	Concentration
1	Tween 20	0.4% v/V
2	PMSF	1mM

In a suitable buffer the above ingredients were dissolved.

CHAPS: (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) is a zwitterionic detergent useful for membrane protein solubilization when it is important to maintain protein activity.

DTT: 1,4-dithiothreitol is used to confer stability to thiol group.

PMSF: phenylmethylsulfonyl fluoride is a serine protease inhibitor commonly used in the preparation of cell lysates.

8. Preparation of Bradford Reagent:

Bradford Reagent (5X):

S.No	Ingredients	Concentration
1	Coomassie Brilliant Blue G-250	**0.5 %(w/v)**
2	Methanol (10%)	**0.24 % (v/v)**
3	Phosphoric Acid (85%)	**0.5%(v/v)**

In a dark reagent bottle, Coomassie Brilliant Blue G-250 was dissolved in Methanol which was followed by addition of Phosphoric Acid and final volume was made up using Distilled water.

9. Preparation of SDS PAGE Reagents:

SDS-PAGE Buffer (10X)

S. No.	Ingredients	Concentration
1	Glycine	192mM
2	SDS	0.1%W/V
3	Tris pH 8.3	25mM

Final volume was made up using distilled water

10. Staining Solution

S.No	Ingredients	Concentration
1	Methanol	40%V/V
2	Acetic acid	10v/V
3	MQ water	50%v/V
4	CBB R-250	0.25% w/V

11. Destaining Solution

S. No.	Ingredients	Concentration
1	Methanol	40%v/V
2	Acetic acid	10%v/V
3	MQ water	50%V/V

12. Laemmli's buffer or SDS Sample buffer

S. No.	Ingredients	Concentration
1.	Tris (pH 6.8)	0.125M
2.	SDS	4%W/V
3.	Glycerol	20%V/V
4.	β-mercaptoethanol	10%V/V

Final volume was made up using distilled water

13. Preparation of Polyacrylamide Gel Used in SDS-PAGE:

Components	Separating/Resolving gel (12%)(10ml)	Stacking gel (4%)(5ml)
30%acrylamide mixture	4ml	0.66ml
Tris HCl , 1.5 M	2.5ml	0.63ml
Distilled water	3.295ml	3.6ml
10% SDS	100 µl	50µl
10% APS	100µl	50µl
TEMED	5µl	5µl