PREPARATION AND PURIFICATION OF MONOCLONAL ANTIBODY

AGAINST HEPATITIS B SURFACE ANTIGEN (HBsAg)

Dissertation submitted in partial fulfillment of the requirement for the

Degree of

MASTERS OF SCIENCE

IN

BIOTECHNOLOGY

BY

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2024

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DECLARATION

I hereby declare that the work presented in this report entitled **"Preparation and Purification of Monoclonal Antibody against Hepatitis B Surface Antigen (HBsAg)"** in partial fulfillment of the requirements for the award of the degree of **Master of Science** in **Biotechnology** submitted in the Department of Biotechnology & Bioinformatics**,** Jaypee University of Information Technology, Waknaghat is an authentic record of my own work carried out during academic year 2024-2025 under the supervision of **Mr. Kailash Singh** J. Mitra & Co. Pvt. Ltd.A-180-181, Okhla Industrial Area, Phase-1, New Delhi-110020, INDIA, **Dr. Rahul Shrivastava** Department of Biotechnology and Bioinformatics, and **Prof. Shruti Jain,** Dean (Innovation) Department of Electronics and Communication Engineering, Jaypee University of Information Technology, Solan, Himachal Pradesh.

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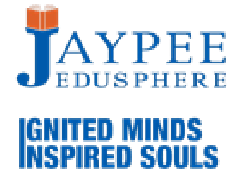

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This is to certify that the work reported in the dissertation entitled **"Preparation and Purification of Monoclonal Antibody against Hepatitis B Surface Antigen (HBsAg)"** which is being submitted by **Prajjwal Jagwan (225111001)** in fulfillment for the award of **Master of Science in Biotechnology** by the **Jaypee University of Information Technology, Waknaghat Solan** is the record of candidate's own work carried out by him under our supervision. This work is original and has not been submitted partially or fully anywhere else for any other degree or diploma.

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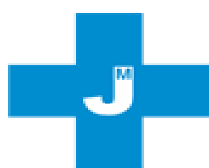

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LIST OF ABBREVIATIONS

LAFH	Laminar Air Flow Hood
mAb	Monoclonal antibody
HAT	Hypoxanthine-aminopterin-thymidine medium
FBS	Fetal Bovine Serum
PBS	Phosphate Buffer Saline
EDTA	Ethylene Diamine Tetra-acetic acid
TAE	Tris-acetate-EDTA
µg	Micro gram
µl	Microliter
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's modified eagle medium
HBsAg	Hepatitis B surface antigen
BSL	Biosafety level
HBV	Hepatitis B virus
ELISA	Enzyme linked immunosorbent assay
FDA	Food and Drug Administration

Abstract

Multiple tests can be performed diagnosis for Hepatitis B infection. One of the keys focuses for these tests is HBsAg, which is crucial for detecting hepatitis B, a viral illness that can lead to long-term liver infection and poses a significant global health problem. The development and purification of HBsAg monoclonal antibodies against Hepatitis B surface antigen (HBsAg) are critical for enhancing diagnostic and therapeutic applications for Hepatitis B infection. In this work, myeloma cells were prepared and the human monoclonal anti-HBsAg specific neutralizing agent was generated through traditional hybridoma development technique by the fusion of antigen HBsAg and myeloma cell lines, offering increased accuracy and specificity. These HBsAg cell lines were cultured and revived to support antibody production. The specificity of the antibodies was confirmed through Hepatitis B surface antibody (HBs Ab) ELISA, which indicates the amount of antibody present in cell culture samples of infected persons. Results demonstrated successful culture and production of HBsAg antibodies from the samples, and were successfully purified using Protein A affinity chromatography.

Key words: Hepatitis B surface antigen, ELISA, Monoclonal antibody, Hepatitis B virus.

CHAPTER 1

INTRODUCTION

Introduction

Hepatitis B surface antigen (HBsAg) also known as an Australia antigen is a protein that is found on the outer surface of hepatitis B virus. It is present in the blood as a sign of ongoing hepatitis B infection. Hepatitis B is considered a global health problem and HBsAg has proven to be the most immunogenic of all Hepatitis B virus (HBV) proteins.

HBsAg is an antigen that is related to hepatitis B:

It is a present on the bases of hepatitis B virus that is present at high amount in the blood during hepatitis B infections. The presence of this antigen in the blood means that the person is infected, although they may be temporarily. The body normally produces antibodies against HBsAg in response to an infection as part of the natural immunological response. This antigen is used in the development of the hepatitis B vaccine.

Antibodies against the surface of the hepatitis B virus:

They are also known as anti-HBs, and their presence is usually interpreted as a sign of recovery and immunity from the hepatitis B virus. Anti-HBs are also found in people who have been vaccinated against hepatitis B, although their levels may decrease with time.

Counteracting substance against hepatitis B core (HBc) antigen:

Anti-HBc antibody targets the surface antigen (HBsAg) of the virus, a counteractive agent towards the HBc antigen. In severe hepatitis B, the side effects often start with the presence of both IgM or IgG antibodies that last for an indeterminate amount of time. Antibody anti-HBc, appears early during acute infection and usually disappears within several months. Its presence suggests a recent or ongoing hepatitis B infection. A positive anti-HBc test indicates exposure to HBV at some point, but doesn't specify if the infection is current or past. People who are immune to anti-HBs antibodies typically do not produce anti-HBc IgM.

Different approaches to detect Hepatitis B virus (HBV) infection:

Currently, it is possible to design genetically engineered antigen-proteins (such as using transgenic *E. coli*) to create a simple antigen test that identifies the existence of HBV. This suggests ongoing research on developing new and potentially rapid diagnostic tools.

Hepatitis B infection (HBV) markers are detectable in the blood of people with the infection, regardless of symptoms. Antibodies against HBsAg (anti-HBs) indicates patients has either recovered from past infection or is immune due to vaccination. These individuals are generally considered non-contagious.

Immunoassays are group of tests that utilize antibodies to detect the HBsAg antigen in the blood. Detecting HBsAg helps, diagnose HBV infection and monitor the effectiveness of antiviral treatment.

In histopathology, HBsAg is regularly identified utilizing the Shikata Orcein Stain strategy, which utilizes a particular dye to bind to the HBsAg antigen in infected liver cells.

A positive HBsAg test can occur shortly after a recent hepatitis B vaccination, this is because the vaccine stimulates the immune system to produce HBsAg initially. This is a temporary effect, and HBsAg levels typically disappear within 14 days after vaccination.

Aims and objectives

a. Preparation of monoclonal antibody against HBsAg (Hepatitis B surface antigen).

b. To check titer of Monoclonal antibody and its purification.

CHAPTER-2

REVIEW OF LITERATURE

Review of Literature

In the field of diagnosing infectious diseases, accuracy and specificity are essential. HBsAg surface antigen is crucial for detecting hepatitis B, a viral illness that can lead to long-term liver infection and poses a significant global health problem [1]. The development of Human monoclonal anti-HBsAg for detecting the hepatitis B virus, in which mAbs neutralize the effect of a pathogen by binding to HBsAg and prevents it from infecting cells, is crucial. This technology is offers increased specificity and reliability [2]. This advancement does not just represent a significant improvement in hepatitis B testing, it also reflects a larger trend towards more precise, user-friendly, and effective disease management methods [3].

This work will include a range of important perspectives, including explanation of HBsAg and its importance in the early detection of hepatitis B infection. Monoclonal antibodies in HBsAg testing enhance the accuracy of the HBsAg test, followed by the purification of monoclonal antibodies for HBsAg testing.

2.1 HBsAg and Its Importance in Detecting Hepatitis B

Hepatitis B surface antigen (HBsAg) is a protein particle found on the outer envelope of the Hepatitis B virus (HBV). It is most abundant protein produced by the HBV and is secreted into the bloodstream of an infected individual [4]. Detection of HBsAg through serologic testing is considered vital in identifying individuals with long-term hepatitis B virus (HBV) infection. For precise results, it is recommended to test for HBV infection using an FDA-approved serologic test for HBsAg with a sensitivity and specificity above 98% as stated by the manufacturer [5].

A positive HBsAg test which can be detected by Hepalisa or through rapid testing for detection of HBsAg antigen indicates a developing sickness that could be either severe or ongoing. Differentiating between these phases of infection involves additional serological indicators of HBV infection, such as the existence of hepatitis B core IgM antibody. Screening methods focusing only on HBsAg, are crucial for identifying individuals at risk for chronic infection [6].

Understanding HBV screening serology is essential for making clinical decisions. Individuals diagnosed with chronic HBV infection receive treatment, if necessary, according to home guidelines that take into account the initial test results and the progression of the disease stage. Additionally, they are advised on, how to prevent the spread of the infection to avoid promoting transmission of the infection [4].

In countries with limited resources, where hepatitis B infection (HBV) is mostly prevalent, they often lack specialized facilities, leading to delays in diagnosis and treatment. In order to improve access to diagnostics and treatment, it is essential to assess advance the options and techniques, including using dried blood samples. Studies have shown the effectiveness of dried blood spots (DBS) in detecting and evaluating HBsAg, which could help with hepatitis B diagnosis in areas with limited access to advanced diagnostic tools. Automated systems such as the Planner Analyzer are frequently used in manufacturing sites and have been authorized for use with plasma or serum [5]. However, the lack of these specialized platforms in resource-constrained areas hinders access to automated diagnostic methods, especially in remote areas. Similarly to HIV testing, the use of dried blood spots (DBS) appears to improve access for the detection of hepatitis B for people in rural areas.

Screening for HBsAg is essential to determine a patient's hepatitis B status, which is the initial step in identifying other markers, including hepatitis B virus DNA (HBV DNA) testing. This assessment is fundamental for monitoring the virological status of infected individuals. Furthermore, the evaluation of HBsAg levels is crucial for determining the response to treatment, its duration, and the clearance of HBsAg, all of which are essential aspects of managing the illness [7].

There are mainly three prime serologic indicators used to identify HBV infection status which are hepatitis B surface antigen (HBsAg), anti-HBs, and anti-HBc. These signs alter as an acute sickness progresses into a chronic infection. The presence of HBsAg implies an acute or chronic HBV infection, although it can also be positive immediately following the Hep B vaccination. Chronic infection is defined by the American Association for the Study of Liver Diseases (AASLD) as the presence of HBsAg for at least 6 months [8].

The blood tests for hepatitis B consist of three tests, and all three results must be known to confirm a person's status. Hepatitis B virus (HBV) acute infections are first identified by the serologic marker HBsAg, which can be seen as early as one week and as late as nine weeks after exposure. Anti-HBs (Hepatitis B surface antibody) is the second marker that detects antibodies against the Hepatitis B surface antigen. If test is positive for anti-HBs test it indicates you gained immunity to Hepatitis B, either from vaccination or past infection that the body successfully fought off. Anti-HBc (Hepatitis B core antibody) is the third marker detects antibodies against the core protein of the Hepatitis B virus. A positive Anti-HBc test can indicate past or current infection with Hepatitis B [9]. HBsAg can be detected for a different period of time along with HBV DNA, although approximately half of individuals will have negative results for both HBsAg and HBV DNA 7 weeks after symptoms appear. Anyone who experiences a sudden recovery from an illness will show negative results for HBsAg and HBV DNA around 15 weeks after symptoms first appear [7].

2.2 Monoclonal Antibodies in HBsAg Testing

Advancements have been made in the development and identification of specific Human Monoclonal Antibodies (mAb) targeting main Hepatitis B Virus (HBV) S protein. The B-cell clones producing three mAbs, including two IgG1κ and one IgG1λ, were isolated from the Peripheral Blood Mononuclear Cells (PBMC) of individuals who have recovered from severe hepatitis B or been vaccinated against it [8]. A denaturation-sensitive, conformational epitope inside the HBsAg common "a" determinant is the target of mAbs produced from vaccinated individuals, while those originating from patients with severe hepatitis B infection aim at a denaturation-insensitive epitope in the p24 protein. In an in vitro model of HBV infection using primary hepatocytes from *Tupaia belangeri* as the target cells, a particular mAb, ADRI-2F3, showed a remarkable protective titer of more than 43,000 IU/mg mAb and revealed significant neutralizing action. [9]. Recombinant versions of the ADRI-2F3 mAb, with cloned variable heavy and light chain configurations, were produced in eukaryotic systems and exhibited identical fine specificity and a 1 $\log_{10}$ titer higher than the original IgG1λ [10]. This suggests that these mAbs could effectively predict HBV reinfect after transplantation of liver for end-stage chronic HBV infection, promoting a vast supply of valuable protective anti-HBs antibodies [11].

Research from DZIF (Deutsches Zentrum für Infektionsforschung, which translates to German Center for Infection Research**.** It's a national research center in Germany dedicated to studying infectious diseases) in Heidelberg and Hamburg has supported the preclinical advancement of VIR-3434, a monoclonal antibody developed by Vir Biotechnology, Inc., that specifically targets the (HBsAg) located in the viral envelope [10]. Several monoclonal antibodies were isolated and examined from memory B cells of individuals vaccinated against HBV, targeting a conformational epitope located within the antigenic loop of the small HBsAg. Out of over 30 antibodies created, a monoclonal antibody called HBC34 demonstrated strong neutralizing activity against both HBV and HDV. Changes in the configuration of HBC34 mAb lead to the development of VIR-3434, which shows potential candidate for clinical advancement by effectively removing both viral and subviral particles from the bloodstream [12].

These findings underscore the fundamental role that monoclonal antibodies play in HBsAg testing. By targeting specific epitopes on the HBsAg, these monoclonal antibodies provide a high level of specificity and sensitivity in detecting HBV infections. Their progress not only improves the accuracy of HBsAg testing but also creates new possibilities for preventing and treating HBV infections, particularly in situations like liver transplantation and post-exposure prophylaxis.

2.3 Purification of Monoclonal antibody for HBsAg testing

Purification of monoclonal antibody is an essential step to ensure the accuracy and consistent quality of the test. There are four strategies for filtering HBsAg particles are identified these are:

1) Isotropic Ultracentrifugation
2) anti-HBs-coated Microparticles
3) Sucrose Cushion Sedimentation
4) Isocratic Column Gel Filtration

Among these, the strategy of using microparticles coated with anti-HBs has proven to be suitable, achieving a decontamination level of more than 98% [13].

❖ **Monoclonal antibody filtering methods**

1. **Isotropic Ultracentrifugation**: Isotropic ultracentrifugation is a powerful technique used to separate particles suspended in a liquid based on their size and density. It spins a rotor containing samples at high speeds, this creates a strong centrifugal force that pushes particles outward from the centre of the rotor. Denser particles experience a greater force and sediment faster towards the bottom of the tube. Lighter particles are less affected by the force and sediment slower or remain suspended in the supernatant [9].

2. **Sucrose Cushion Sedimentation**: Sucrose cushion sedimentation is a centrifugation technique commonly used to separate biological samples, particularly those sensitive to shear stress, based on their size and density.
   A density gradient is created within a centrifuge tube using a sucrose solution. Sucrose is a sugar molecule that readily dissolves in water and forms solutions with increasing density as the concentration increases the sample is layered on top of the pre-formed sucrose gradient. During centrifugation, particles experience both the centrifugal force pushing them outwards and the opposing buoyant force due to the increasing density of the sucrose solution as they move down the tube. Particles will eventually reach an equilibrium point within the gradient where the opposing forces balance, separating the particles based on their size and density. Denser particles will sediment further through the gradient [12].
   These are conventional strategies, although effective, but gives result in lower levels of efficiency. They are labor intensive and require extensive optimization to achieve the desired level of performance.

3. **Isocratic column gel filtration**: It is also known as size exclusion chromatography (SEC), a powerful technique used to separate biomolecules based on their size and shape.
   The technique utilizes a chromatography column packed with microscopic beads with defined pore sizes. A liquid buffer, called the mobile phase, continuously flows through the column. A sample containing a mixture of biomolecules is injected into the mobile phase. Larger molecules are excluded from entering the smaller pores within the beads and travel through the column faster through larger channels between the beads. They elute (exit the column) first. Smaller molecules can access the inner pores of the beads, taking a longer, more tortuous path through the column and elute later [13].

This strategy provides a more affordable approach than ultracentrifugation, but still does not achieve the same efficiency as with anti-HBs antibody-coated microparticles.

4. **Anti-HBs-coated microparticles**: Anti-HBs-coated microparticles are a powerful tool used in Hepatitis B testing for the specific capture and separation of patient samples' of HBsAg (hepatitis B surface antigen) particles. A highly specific antibodies bind to HBsAg, attached (coated) onto the surface of the microparticles. Microparticles are tiny, insoluble beads typically made up of materials like polystyrene or magnetic polymers. Then the patient's blood or serum sample suspected to contain HBsAg is mixed with the anti-HBs-coated microparticles. HBsAg particles present in the sample will specifically bind to the anti-HBs antibodies on the surface of the microparticles through an antigen-antibody interaction. Unbound components from the sample are washed away.
   This approach successfully removed contaminants, which bind tightly to HBsAg particles and are difficult to separate using other methods. Essentially, this strategy preserves pre-S components, important for improving the body's immune response against HBV infection [14].

Advantages of anti-HBs coated microparticle:

1) Achieving purity above 98%, this strategy ensures the removal of contaminants while protecting the basic components of HBsAg particles.
2) Compared to other decontamination methodologies such as precipitation, partiality chromatography, and ultracentrifugation, which are both time-consuming and costly, the anti-HBs-coated microparticles strategy stands out for its straightforwardness, speed, and cost-effectiveness [12].

Execution of Anti-HBs Coated Microparticles for HBsAg Filtration

HBsAg particles obtained from HuH-7 cells (HuH-7 is a human liver cell line) [13]. These HBsAg particles were likely produced using a vaccinia system, a method that utilizes a weakened vaccinia virus to express the HBsAg protein within the HuH-7 cells. The use of anti-HBs coated microparticles as a strategy for filtering these HBsAg particles, shows both the forms of HBsAg glycosylated (sugar molecule attached) and non-glycosylated, after filtration. This method preserves the natural heterogeneity of HBsAg, which might be important for detection in diagnostic tests. Some methods like silver-stained SDS-PAGE (Sodium Dodecyl Sulphate) are used to confirmed the purity, size, and antigenicity (ability to bind antibodies) of the filtered HBsAg particles.

In conclusion, the filtration of monoclonal antibodies for HBsAg testing using anti-HBs coated microparticles offers several advantages over conventional methods. It achieves high purity, preserves the structure of HBsAg particles, and ensures the adequacy of HBsAg testing [15].

Steps involved the preparation of Anti-HBs Coated Microparticles:

1) **Well Coating**: The procedure begins by coating the microwell plates with a mouse monoclonal antibody specific for HBsAg (anti-HBs). If HBsAg is present in the patient's serum or plasma sample, it will bind specifically to the anti-HBs antibodies coating the well. This initial binding step ensures that any HBsAg present in the sample gets captured in the well and remains bound to the well for further detection steps [16].
2) **Additional Tests and Controls**: Serum or plasma tests, along with appropriate controls, are included in coated wells. A negative control in which well containing all the reagents except the patient's sample. This ensures the absence of non-specific binding. A positive control in which the well containing a known amount of HBsAg, this verifies the functionality of the antibodies and overall test performance. After incubation, the strips are washed to remove unconnected tissue.
3) **Washing step:** After adding the sample and control and allow the HBsAg-anti-HBs binding, the wells are washed to remove unbound components from the sample. This washing step is crucial to eliminate any substances in the sample that might interfere with the specificity of the HBsAg detection assay in later steps. These could be other proteins, cellular debris, or various molecules present in blood [17].
4) **Conjugate addition**: A conjugate solution is added to the wells after washing. This conjugate is essentially an antibody specific HBsAg (mouse monoclonal antibody) linked (conjugated) to an enzyme called horseradish peroxidase (HRP).

5) **Color Development:** After washing, a substrate solution is added to the wells containing a colorless chromogen (a molecule that can be converted to a colored product). The HRP enzyme acts on the chromogen in the substrate, converting it into a colored product (typically blue or green). The intensity of the color produced is directly proportional to the amount of HBsAg present in the sample. More HBsAg leads to more HRP-conjugated antibodies bound, leading to a stronger color signal.
6) **Stopping the Reaction:** The chemical reaction ends by adding an "acidic solution" which stops the enzymatic reaction. This solution changes the color of the product to yellow for easier measurement. The color intensity (absorbance) in each well is measured using a spectrophotometer, and the results are used to determine the quantity of HBsAg present in the patient's sample compared to controls [18].
7) **Measurement:** Spectrophotometer is used to measure the absorbance of samples and controls at a wavelength of 450 nm. This wavelength is likely specific for the colored product generated by the HRP enzyme. Higher absorbance values indicate a more intense color, which correlates with a higher concentration of HBsAg in the sample [19].
8) **Interpretation:** Based on the measured absorbance values and a predefined cut-off value, samples are categorized as either HBsAg reactive (positive) or non-reactive (negative) according to the specific HBsAg EIA 3.0 test. The HBsAg EIA 3.0 test is an enzyme immunoassay (EIA) used to detect Hepatitis B surface antigen (HBsAg) in human serum or plasma samples [20]. Received samples are rechecked in duplicate for confirmation. HBsAg EIA 3.0 used for confirming presence of HBsAg, demonstrate severe or persistent HBV infection. The distinction between severe and persistent disease depends on the proximity of other HBV serological markers and the clinical setting.

2.4 Pathogen Antigen Antibody Techniques

Various immunological methods depend on the specific interaction between pathogens (such as viruses, bacteria, fungi and protozoa) and antibodies. Some of which techniques are explained below:

1) **ELISA (Enzyme-Linked Immunosorbent Assay)**:

This technique is widely employed to detect antibodies or antigens in a sample. In the case of HBsAg detection, a known HBsAg is coated onto a plate, followed by the addition of the patient's serum. If antibodies against HBsAg are present, they will bind to the antigen. A secondary antibody linked to an enzyme is utilized to identify the bound antibodies, resulting in a colorimetric change when a substrate for the enzyme is added [21].

Types of ELISA are:

1. Direct ELISA (Ag--Ab**Enz + S) – Detecting antibody.
2. Indirect ELISA (Ag--Ab—Anti Ab**Enz + S) – Detecting antigen/antibody.
3. Sandwich ELISA (Ag—Ab—Ag** Enz +S) – Detecting antigen.
4. Competitive ELISA – Detecting antibody.

1) **Direct ELISA:**

The principle of direct ELISA involves the direct detection of an antigen using a specific enzyme-conjugated antibody. In this method, the antigen of interest is immobilized onto the surface of a microplate well. To prevent non-specific binding a blocking agent is added after immobilization [22]. Following this, an enzyme-linked antibody that is specific to the target antigen is added. This antibody binds directly to the immobilized antigen. Subsequently, any unattached antibodies are removed through washing. An enzyme substrate is then introduced, which the enzyme transforms into a visible signal, typically a change in color. The strength of the signal corresponds to the quantity of antigen in the sample, enabling quantitative analysis [23]. Direct ELISA is known for its simplicity and speed, as it requires only a single antibody, but it can be less sensitive compared to other types of ELISA due to the lack of signal amplification.

2) **Indirect ELISA**:

The principle of indirect ELISA involves two-step process for the detection of an antigen through a using both antibodies primary and secondary. First, immobilized the antigen onto the surface of a microplate. To prevent the non-specific binding, a blocking agent is added after immobilization. Next, an antigen specific primary antibody is added and allowed to interact to the antigen [24]. Subsequently, all unbound primary antibodies are removed through washing. A secondary antibody conjugated with an enzyme, designed to target the primary antibody, is introduced. This secondary antibody attaches to the primary antibody. Once any unbound secondary antibodies are washed away, a substrate for the enzyme is introduced. The enzyme transforms the substrate into a visible signal, usually causing a change in color. The strength of this signal corresponds to the quantity of antigen found in the sample [24].

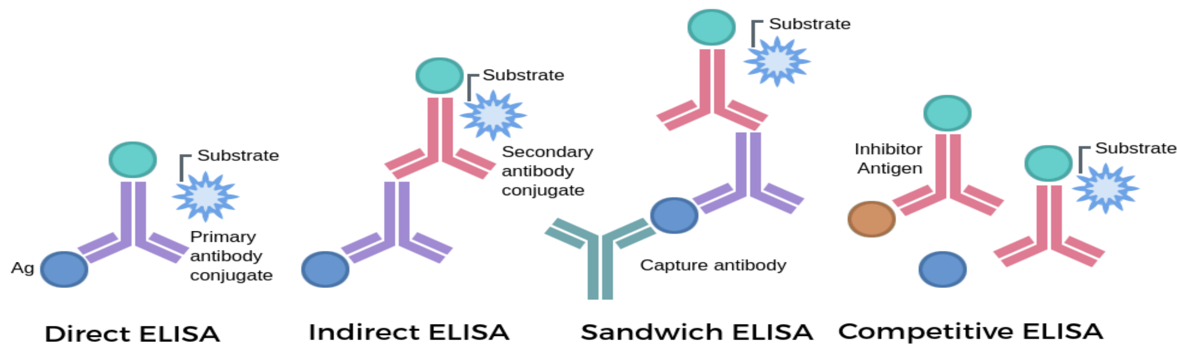

Fig.1 Different types of ELISA (i) Direct ELISA (ii) Indirect ELISA (iii) Sandwich ELISA (iv) Competitive ELISA [25]

3) **Sandwich ELISA**:

As the name suggests it is called sandwich because Ag that needs to be detected is sandwiched between primary Ab (capture Ab) and secondary Ab (detection Ab). In this ELISA ab are passively adsorbed to solid surface of plate these Ab are known as capture Ab. After that serum having the Ag is added if specific Ag is present in that it will go and bind to the Ab. The Ag is diluted in sample diluent which is a buffer used to blocking the nonspecific attachment to solid surface of plate. Ag and Ab forms complex on attachment to solid surface after that secondary Ab conjugated with Enzyme is added which can be same as that of primary Ab. The sandwich is formed as Ab-Ag-Ab. Enzyme followed by addition of substrate and stop solution after particular time frame. The OD it taken at 450nm [26].

4) **Competitive ELISA**

In this ELISA competition occurs between Ab in the sample and enzyme labelled Ab against the Ag coated onto the microtiter plate. These 2 Ab will compete against each other for binding Ag when added into wells. If the color changes mean enzyme labelled Ab has bound to the Ag and the test will be declared negative while the absence in the development of color means Ab are present in the sample and test is declared positive. Thus, higher the Ab in the sample, lower will be the binding of enzyme labelled Ab to Ag and vice versa. This is a simple method just like direct ELISA [27].

Applications of ELISA

a) **Diagnosis of Infectious Diseases:** ELISA plays a crucial role in diagnosing various infectious diseases by detecting antibodies produced by the body in response to a specific pathogen, Such as:

a. HIV/AIDS
b. Hepatitis B and C
c. Lyme disease
d. Syphilis [28]

b) **Food Allergen Detection:** ELISA is a valuable tool in the food industry for ensuring food safety. It helps detect the presence of potential food allergens and contaminations in food products, aiding in accurate labeling and preventing allergic reactions in consumers [29].

c) **Hormone Level Measurement:** Various hormone concentration is determined using ELISA. This information is crucial for diagnosing and monitoring hormonal imbalances associated with various medical conditions [30].

d) **Drug Abuse Detection:** Detection of drugs or their metabolites in urine samples can be achieved using ELISA. This has applications in drug screening programs and monitoring treatment progress [31].

e) **Environmental Monitoring:** ELISA can be used to detect the presence of harmful substances in environmental samples, such as pesticides, herbicides, and heavy metals in water.

f) **Cancer Research:** ELISA plays a role in cancer research by detecting tumor markers, proteins associated with specific types of cancer. This can be helpful in diagnosis, treatment monitoring, and identifying patients at risk [29].

2. **Western Blot:** This method separates proteins in a sample based on their size and employs antibodies to detect specific proteins of interest. In this case, it could be used to determine the presence and size of HBsAg in a given sample.

Applications of Western Blot

a) **Protein Expression Analysis:** Western blot is a crucial tool for verifying protein expression in a biological sample. It gives the presence, size, and relative abundance of a specific protein of interest [32].
b) **Post-translational Modification Detection:** Western blotting can detect modifications on proteins after their translation, such as phosphorylation, glycosylation, or ubiquitination. This information provides insights into protein function and regulation [33].
c) **Protein-Protein Interaction Studies:** Western blotting can be used in co-immunoprecipitation experiments to predict the proteins which interact with a specific protein of interest [34].
d) **Antibody Specificity Testing:** Western blotting is used to assess the specificity of newly developed antibodies by verifying their ability to bind only to the intended protein target.
e) **Disease Diagnosis:** Western blotting can be used to diagnose certain diseases by detecting specific protein markers associated with the disease state [34].
f) **Monitoring Gene Expression:** Western blotting can be used alongside techniques like gene expression analysis to confirm changes in protein expression levels upon gene manipulation.
g) **Protein Localization Studies:** Western blotting can be used in conjunction with techniques like cell fractionation to determine the cellular location of a specific protein [35].

3. **Immunofluorescence**: This technique involves the use of antibodies labeled with fluorescent molecules to visualize the location of antigens in cells or tissues. It could be utilized to identify HBsAg on the surface of infected hepatocytes (liver cells).

Applications of Immunofluorescence

a) **Cellular and Subcellular Localization of Antigens:** Location of antigen can be determined within a cell. This can include visualizing proteins in the nucleus, cytoplasm, or on the cell membrane [36].
b) **Identifying Antigen Expression Levels in Different Cell Types:** It can be used to compare the relative abundance of antigens expressed by different cell populations within a tissue sample [37].
c) **Multiplex Immunoassays:** By using different fluorophore-conjugated antibodies (antibodies with fluorescent tags), It can detect multiple antigens simultaneously within the same sample. This allows researchers to study interactions between different proteins or analyse the expression of multiple markers in a cell [38].
d) **Autoantibody Detection:** It can be used to diagnose autoimmune diseases by detecting the presence of autoantibodies (antibodies that target the body's own tissues) in a patient's serum. Specific antigens are immobilized on a slide, and the patient's serum is incubated with the slide. The presence of autoantibodies binding to the antigens is then detected using fluorescently labelled secondary antibodies [39].
e) **High-Throughput Screening:** It is adaptable to high-throughput screening (HTS) platforms, allowing researchers to rapidly analyse protein expression patterns in large numbers of samples. This is valuable for drug discovery and biomarker identification [40].

CHAPTER-3

MATERIAL AND METHODS

Materials and Methods-

3.1 Materials Used

Table 1- Instruments used in the preparation of monoclonal antibody (HBsAg)

S.no	Instrument
1	Laminar Air Flow Hood
2	Autoclave
3	Sterilized pipette and tips 10 to 1000µl
4	Water bath
5	Weighing balance
6	pH meter
7	Microscope
8	Centrifuge
9	Incubator

PRINCIPLES

1. Laminar Air Flow Hood

Laminar air flow, also known as a laminar flow cabinet or tissue culture hood, is an enclosed work station designed to create a contamination-free work environment. Which is achieved by using HEPA filters they are filters that trap tiny particles from the air that pass through them. A HEPA filter can traps at least 99.97% of airborne particles 0.3 microns in diameter and by maintaining a constant flow of clean air across the work surface.

2. Autoclave

An autoclave is a device that used to sterilize the equipment and materials. It is a pressure cooker that kills microorganisms (bacteria, viruses, fungi, and spores) by exposing them to high temperatures and pressure. The sterilization process typically occurs at temperatures 121°C and at 15 psi pressure.

3. <u>Water Bath</u>

A water bath is an apparatus used to incubate the samples at a constant temperature, in water for a particular time interva. It is usually used in biological, chemical, and medical labs for a variety of applications, including heating reagents, melting substrates, or incubating cell cultures.

4. <u>Weighing Balance</u>

A weighing balance, also known as a laboratory balance or scale, is an essential piece of equipment in scientific, medical, and industrial settings used to measure the mass of objects with high precision and accuracy. There are various types of weighing balances designed for different applications and accuracy requirements. Electronic balances are more common because they are more accurate and easier to use. They can also be connected to computers or printers to record weight measurements.

5. <u>pH Meter</u>

A pH meter is a device utilized for measuring the activity of hydrogen ions in water-based solutions, indicating their pH level as either acidic or alkaline. By measuring the variance in electrical potential between a pH electrode and a reference electrode, the pH meter is commonly known as a "potentiometric pH meter." This variance in electrical potential is directly related to the acidity or pH level of the solution. pH meters are extensively employed in various applications, including laboratory experimentation and quality control, to test the pH levels.

6. <u>Microscope</u>

A microscope is an instrument used to magnify and resolve the fine details of small objects that are invisible to the naked eye. Microscopes use lenses to magnify an object, creating a larger image that we can view. Microscopes are fundamental tools in many fields, including biology, materials science and medicine. There are different types of microscopes such as Light microscope, Electron microscope and Fluorescence Microscope.

7. Centrifuge

A centrifuge is a device used to separate the components of a mixture based on their density by spinning them at high speeds. When a sample is spun at high speeds, centrifugal force acts on particles with in the sample, causing denser components of the mixture to move at the bottom of the tube, while less dense components remain closer to the center.

8. Incubator

It provides a sterile and controlled environment. Maintains a constant temperature, humidity, and CO_2 level to support the growth of microorganisms such as bacteria, fungi, and cell cultures.

Table 2-Chemicals Used

Sno.	**Description**	**Quantity**	**unit**
1.	DMEM	200	ml
2.	Gentamycin	2	g
3.	Sodium Bicarbonate.	3.7	g
4.	Fetal bovines' serum (FBS)	45	ml
5.	Sodium chloride	3	g
6.	Sodium Azide	76	g
7.	Anti HBsAg frozen cell	1	ml

1. DMEM (Dulbecco's Modified Eagle's Medium)

DMEM, or Dulbecco's Modified Eagle Medium, is a widely used, nutrient-rich cell culture medium designed to support the growth and maintenance of various types of cells in vitro. It is a modification of the original Eagle's Minimal Essential Medium (MEM) and is particularly favored for its ability to support a wide range of mammalian cells.

2. Gentamicin

Gentamicin is an antibiotic medication used to treat a variety of serious bacterial infections. The typical recommended concentration for eukaryotic cell culture is 50µg/ml, while for prokaryotic cells it is 15µg/ml. Gentamicin sulfate is an antibiotic with a broad spectrum, effectively inhibiting the growth of various Gram-positive and Gram-negative microorganisms, including those resistant to tetracycline, chloramphenicol, kanamycin, and colistin. This includes strains of *Pseudomonas, Proteus, Staphylococcus, and Streptococcus*. Its mechanism of action involves binding to the 30S subunit of the ribosome, thereby inhibiting bacterial protein biosynthesis.

3. Sodium bicarbonate

Sodium bicarbonate is commonly used as a buffer to maintain the pH of cell culture media within the physiological range (approximately pH 7.2-7.4). It works in conjunction with carbon dioxide (CO_2) in the incubator to create a bicarbonate-CO_2 buffering system. When CO_2 gas comes into contact with water, a small portion reacts with water to form carbonic acid (H_2CO_3). Carbonic acid is a weak acid, it partially dissociates in water to form bicarbonate ions (HCO_3^-) and hydrogen ions (H^+). Sodium bicarbonate readily dissolves in water, releasing sodium ions (Na^+) and bicarbonate ions (HCO_3^-). If there is excess H^+ ions from the acid reacts with the readily available bicarbonate ions (HCO_3^-) to form carbonic acid (H_2CO_3). This reduces the free H^+ ion concentration, preventing a significant decrease in pH. The carbonic acid in the system acts as a weak acid and releases H^+ ions. These additional H^+ ions can neutralize the excess OH^- ions from the alkaline solution.

4. **Fetal bovine serum (FBS)**

Fetal bovine serum (FBS) is a highly utilized supplement in cell culture media. It is derived from the blood of fetal calves and contains a complex mixture of proteins, growth factors, vitamins, and other essential nutrients that support the growth and maintenance of various cell types in vitro.

5. Sodium chloride (NaCl)

Cella have a natural tendency to maintain an internal environment with a specific concentration of solutes (dissolved particles) compared to their surroundings. This balance is called osmotic pressure. When cells are frozen, ice crystals form outside the cell, can ruptures the cells. NaCl, when added in a stepwise manner during thawing, helps the osmotic pressure around the cell. It causes a gradual increase in the external salt concentration, permitting water to slowly enter the cells without causing them to burst due to rapid influx of water. This controlled rehydration component is critical for cell viability.

5. **Sodium azide**

Sodium azide is a common preservative in biological samples such as proteins and buffers. It inhibits the growth of bacteria and other microbes by interfering with their cellular metabolism. However, some bacteria are resistant to its effects. Due to its ability to bind to specific molecules, sodium azide can be used as a research tool to study protein function and interactions.

6. **Anti-HBsAg frozen cells**

Anti-HBsAg frozen cells are typically derived from a cell line that has been infected with HBV. These cells produce large quantities of HBsAg, which can then be purified and used in diagnostic tests.

There are two types of symptomatic tests that utilize anti-HBsAg frozen cells:

1) HBsAg ELISA: This is a blood test that recognizes the presence of HBsAg surface antigen in the blood. A positive test result shows a current or future HBV contamination.
2) HBsAg neutralization test: This test is utilized to measure the level of antibodies against HBsAg in the blood. High antibody levels show that a person has been immunized against HBV or has recovered from a past HBV infection.

3.2 Methodology

3.2.1 Preparation of myeloma cells

The selection of myeloma cell lines with the fusion of immune spleen cells was based on several factors. The Sp2/0 cell line's exhibits a high growth rate which is beneficial for generating hybridomas after fusion. Furthermore, this cell line was selected due to its lack of natural production or release of Ig (immunoglobulin) heavy chain and light chains. The Sp2/0 myeloma cell line was originally created by Schulman et al. in 1978. Other commonly used cell lines, such as P3X63-Ag8.653 and NS-1, exclusively produce k light chains.

To ensure the sensitivity of myeloma cells to the Hypoxanthine-aminopterin-thymidine medium (HAT) selection medium, following cell fusion, they are cultured with 8-azaguanine. Earlier, to cell fusion, myeloma cells are cultivated in a medium that does not contain 8-azaguanine for a week. The conditions of the cell culture are carefully adjusted to ensure that the Sp2/0 cells are in the logarithmic growth phase and have a high viability when collected for fusion.

Materials

1) Sp2/0 murine, myeloma cell lin.
2) Culture medium.
3) 20μg/ml 8-azaguanine
4) Animal tissue culture vessel (T-25 cm^2 or T-75 cm^2)
5) Gas mixture of 8% CO_2 in air
6) Humidity at 37°C with 8% CO_2
7) Inverted microscope.

Steps

1. Sp2/0 frozen cells were recovered from liquid N_2.
2. Sp2/0 cells grown in tissue culture flasks overnight at 37°C with complete medium in a $C0_2$ incubator with 98% relative humidity.
3. Cell growth was assessed by monitoring the cell culture flasks through an inverted microscope and subsequently placing the flask back into the CO_2 incubator to allow for further cell growth.
4. In order to maintained the aminopterin sensitivity of the Sp2/0 cells during the fusion selection process, it is necessary to add 8-azaguanine at a concentration of 20µg/ml to the complete culture medium for one week. Subsequently, the cultured cells can be maintained on a medium without 8-azaguanine.
5. An optimal seeding cell density for Sp2/0 cells ranges from 2.5 to 5 x 10^4 cells/ml, resulting in maximum density of 6 to 9 x 10^5 cells/ml. Once this cell density is reached, there is a sharp decrease in cell viability. The Sp2/0 culture was split every 2 to 3 days by removing a suitable volume from the old flask and adding fresh medium, or by transferring an appropriate cell volume to a new flask along with fresh medium.
6. A quantity of 10 million Sp2/0 cells (1:10 ratio to immune spleen cells) were utilized for fusion. The feasibility of the cell's during collection was ensured to be above 95%. To ensure that the cells were collected during the logarithmic growth phase, the cell density was adjusted to 200,000 cells/ml one day prior to fusion by adding fresh medium. The trypan blue exclusion method is used to determination of cell viability.

Important parameters

The growth of myeloma cells is influenced by their bulkiness. To ensure optimal growth, it is necessary to split the cultures again and again, aiming to continue the viability of over 95%. It is important to avoid culturing Sp2/0 cells for more than a month to prevent gene variation and the emergence of antibiotic-resistant contaminants. To preserve the cells, multiple vials of Sp2/0 cells storage in liquid nitrogen.

3.2.2 Human monoclonal anti-HBsAg generation and purification

The human monoclonal anti-HBsAg specific neutralizing agent was generated through traditional hybridoma development technique. The HBsAg antigen fused with Sp2/0 murine, myeloma cell lines. The fused cells were cultured overnight in media with hybridoma growth factors and drugs. Clones of anti-HBs-positive cultures were generated through restriction enzyme digestion, resulting in several monoclonal hybridoma lines that were screened using commercial anti-HBsAg ELISA.

The Hu-mAbs (Human monoclonal Antibody) was purified using standard Protein A columns, such as Hitrap Protein A HP. These are chromatography columns packed with a resin material called Protein A. Protein A, a protein derived from bacteria, has a high affinity for the Fc region (crystallizable fragment) of most antibodies, including human IgG (Immunoglobulin G). This is a commonly used and efficient method for the purification of antibodies. The supernatant of cell culture, containing the Hu mAbs is passed through affinity column having protein A beads. The Hu mAbs bind specifically to the protein A, while other proteins and cellular debris flow through the column. The bound Hu mAbs are then eluted using a low pH solution or a competing ligand that disrupts the antibody-Protein A interaction.

3.2.3 Cell viability test by Trypan Blue.

This technique is employed to quantify the number of viable cells within the cell culture. A viable cell will display a clear cytoplasm, while a non-viable cell will exhibit a blue cytoplasm.

<u>Supplemental Materials</u>

1. Phosphate-buffered saline (PBS) or trypan blue used in serum-free culture media at a concentration of 0.4%.

2. Binocular magnifiers

3. A Hemocytometer

4. Centrifuge

Procedure

1. Centrifuged 1ml of cell suspension.
2. Suspended the cellular pellets in 1ml of PBS or Serum-free culture medium. Trypan blue can dye serum proteins, leading to inaccurate outcomes; therefore, opting for a serum-free solution is advisable.
3. Made attenuation by mixing the trypan blue solution and cell suspension in equal volume (made dilution factor 2).
4. Using a magnifier, independently examined viable (unstained) and dead (stained) cells in the hemocytometer. The hemocytometer consists of four corner squares, each consisting 16 small squares. Each square has a side of 1mm and a depth of 0.1mm. Determined the total count of cells within the four corner squares, containing the cells on the bottom and left border, while excluding those on the top and right border. Computed the average number of cells. Multiplied the result by 10^4 to derive the number of cells/ml.

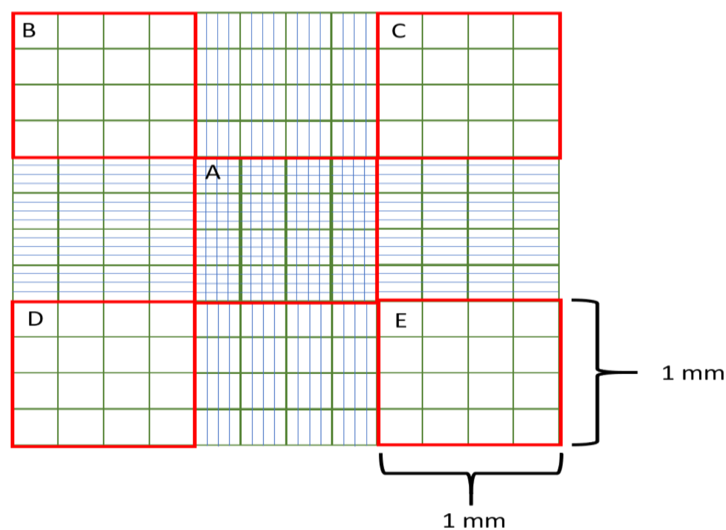

Figure 2: -Hemocytometer

Calculations for cell counting:

Cells at the bottom and left edge were considered and cells at top and right edge were ignored. Four boxes at the corners were considered for checking and center one was ignored.

Total volume of cell suspension was 200µl (100 µl cell suspension + 100µl trypan blue).

The rate of cells is calculated by:

$$\text{Viable cells (\%)} = \frac{\text{Number of viable cells in sample}}{\text{Total number of cells in sample}} \times 100$$

3.2.4 Cell Culture of HBsAg

Roller Vessel Cell Culture

Roller vessel cell culture is a technique used to grow large quantities of adherent cells in a laboratory setting. A roller vessel is essentially a cylindrical bottle made of autoclavable glass or plastic. It lays horizontally on a motorized roller apparatus that continuously rotates the vessel at a slow speed.

Steps involved in roller vessel cell culture:

1. Standard container (Cleanliness): The cleanliness of the bottle was essential because normal tissue culture plate cleaning strategies were inadequate because as compared to culture plates, roller bottles had significantly larger internal surface area. Standard methods were not that effective to reach and clean all areas. Standard cleaning solutions might not be strong enough to remove these residues effectively.
2. Sterilization
   Autoclave, was used to sterilize the roller bottles and caps. After autoclaving, it was crucial to exhaust the autoclave slowly and let the roller bottle cool down completely before removing it. Roller cell generation may require a few experimentations with respect to the optimal rotation speed of the culture in the bottle, number of cells in the inoculum, etc. because different cell lines have varying growth requirements and sensitivities.
3. Seeding
   $1\text{-}3 \times 10^6$ cells per 20 millilitres were used straight from the bottle. Mixed the cells in the bottle containing culture medium, shaken the bottle completely, and then placed it on the roller. The continuous rolling motion gently bathes the cells with fresh medium and promoted their growth.
4. Medium

   The total culture medium comprises of Dulbecco Modified Eagle's Medium (DMEM) with a high-glucose condition supplemented with sodium bicarbonate, FBS, L-glutamine, sodium pyruvate, penicillin and streptomycin.

5. Bottle speed

The speed was 0.1-0.3 rpm at the begin of the 24-hour association and maximum 2 rpm thereafter. The ideal speed for a specific cell type may change. The course of action of well-dispersed homogeneous cultures depends on the rate at which cells settle and fuse with the glass. The quicker they combine, the more prominent the scattering of cells on the glass surface. The association speed depends on the temperature of the environment, the turn speed of the bottle and of the cells involved.

Advantages of the Roller Vessel Cell Culture

Cell developed in rolling flask cultures varies from developed in static jars in two aspects. The first concerns the distribution of cells on the glass surface, an immobilized culture can be produced quickly and uniformly. Cells in a rolling flask culture can support roughly twice the thickness of a fixed culture.

3.2.5 Revival of HBsAg cell line

Day-1

1. Took out a frozen vials of HBsAg from the liquid nitrogen tank.
2. Washed the vials with 70% IPA (Isopropyl Alcohol).
3. Kept the vials into water bath at 37ºC for thawing.
4. Took a T-25cm^2 flasks, added 10 ml media followed by addition of 1ml HBsAg frozen cells, made the total volume 11ml in the flask.
5. Took a sample for cell count.

Cell count-

Table 3 - Cell viability- 0.47X10^{6}cell/ml (11ml)

Live	Dead
22	14
25	20
23	18
24	18

Day-2

1. HBsAg cells were observed under microscope.
2. 45% cells were live & 60% cell was dead.
3. Used sterile pipette to transfer the viable cell from T-25cm^2 flask to 50ml falcon.
4. Centrifuged the tube at 1200 rpm for 7 min.
5. Took sample for the cell count.

Cell count-

Table 4 - Cell viability- 0.21X10^6cell/ml, live cell was 30% and dead cell was70%

Live	Dead
12	28
11	18
8	16
11	19

6. Took a new sterile T-25cm^2 flask and added 5ml media in it.
7. After centrifugation discarded the supernatant (centrifugation is done to concentrated the cell) and in the remaining pellet (present in 50ml falcon) added 5ml media from the new T-25cm^2 flask and named it as N2.
8. Took sample for the cell count.

Cell count-

Table 5 - Cell viability- 0.4X10^6 cells/ml

Live	**Dead**
24	45
16	32
16	38
22	40

9. The old T-25cm^2 (master flask) flask in which 10ml media was added was named std-1 M1. Removed the culture sample from the flask, added 10ml fresh media without any disturbance and named it std-2 M2.
10. Now there were three flasks named std-1 M1, N2 and std-2 M2.
11. Std-1 M1→First old master flask.
12. Std-2 M2→Replica of old master flask.
13. N2→ First running flask staring volume was 5ml.

Day 3

1. HBsAg cells were observed under microscope.
2. 60% cells were live and 40% cells were observed dead.
3. The cells were healthier as compared to previous cell growth.
4. Mixed the sample in the T-25cm^2 flask gradually with the sterile pipette by rinsing the flask from inside with culture sample.
5. Took sample for the cell count.

Cell count-

Table 6 - Cell viability- 0.6 X 10^6 cells/ml

Live	Dead
30	32
28	38
40	31
26	38

6. From the T-25cm^2 flask transferred the entire volume to 50ml falcon.
7. Then washed the T-25cm^2 flask with the medium 2-3 times, so that no bubbles were present in the flask.
8. Added 10ml medium into the T-25cm^2 flask.
9. In the old T-25cm^2 flask discarded the entire volume and added 10ml fresh medium.

Day 4

1. Both the flasks were observed under microscope.
2. No medium was changed.
3. Hold for 24h.

Day 5

1. HBsAg flasks were observed under microscope.
2. In the both flask T-25(std-1 M1) and (N2) the cells were alive and healthy when observed under the microscope.
3. In T-25cm^2 std-1 M1 which is master flask, the cell growth was good.
4. In T-25cm^2 N2 flask the cell growth was much better than the previous growth.
5. In the T-25cm^2 N2 flask, discarded the entire culture cell with the help of pipette.
6. Then added 10ml fresh medium in the T-25cm^2 N2 flask.
7. Split the T-25cm^2 std-1 (old master flask) flask with the help of scraper.
8. Took a new T-25cm^2 (std-2 M2) flask and added 10ml fresh medium (DMEM + 10%FBS).
9. From the T-25cm^2 (std-1 M1) flask after scraping, transferred 1ml to new flask T- 25cm^2.
10. In the master flask (std-1 M1) discarded the entire culture sample apart from 1ml culture sample and then added 10ml fresh media (DMEM 10% FBS) to the flask.
11. Took a sample from T-25cm^2 flask (std-1 M1) for cell count before discarding culture sample.

Cell count-

Table 7 - Cell viability- 0.18 X 10^6 cells/ml

Live	Dead
10	00
10	00
9	00
7	00

The total volume of all flasks –

1. T-25cm^2 (std-1 M1) master flask → 11ml
2. T-25cm^2 (std-2 M2) replica flask → 11ml
3. T-25cm^2 (N2) → 10ml

Day 6

1. Both the flasks were observed under microscope.
2. No media was changed.
3. Observed good growth in (N2) flask, % of dead cells was very low.

Day 7

1. There was different T-25cm^2 flasks.
2. Std-1 M1 (old master flask which is M1)
3. Std-2 M2(Replica of mater flask M2)
4. N2 (Running flask form staring N2)
5. (M1) flask showed clumping under the microscope.
6. (M2) flask showed more adherent cell.
7. (N2) flask cell growth was less.
8. (N2 and M2) flask rinsed 2-3 time with their own culture sample.
9. (M1) observed under microscope.
10. After rinsing with culture sample (M2).
11. Took sample for the cell count.

Cell count-

Table 8 - Cell viability- 0.1 X 10^6 cells/ml

Live	Dead
6	00
6	00
4	00
5	00

12. (M2) first detached the cell from their surface and rinsed with their culture sample.
13. Took (M1 std-1) T-75cm^2 new flask having total volume 20ml.
14. In (M2 std-2) old flask, 9ml culture sample was transfer to T-75cm^2 flask, in which 1ml was already present and added 10ml medium and made total volume 11ml.
15. In N2 flask discarded all the culture sample except 1ml culture sample and added 9ml media made total volume 10ml.
16. Took a sample before discarding the culture sample for cell count.

Cell count-

Table 9 - Cell viability- 0.12 X 10^6cells/ml

Live	Dead
5	00
5	00
6	00
9	00

Day 8

1. Observed flasks under microscope.
2. No media was changed.

Day 9

1. Observed HBsAg flask under the microscope.
2. First, took the (M1a Std-1a) flask T-75cm^2 and rinsed it 2-3 times with culture sample.
3. Took a sample for the cell count.

Cell count-

Table 10 - Cell viability- 0.18 X 10^6cells/ml

Live	Dead
10	00
7	00
9	00
11	00

4. (M2 Std2) T-25cm^2 flask was rinsed with its own culture sample 2-3 times.
5. Took a sample for the cell count-

Cell count-

Table 11 - Cell viability- 0.36 X 10^6 cells/ml

Live	**Dead**
18	00
21	00
16	00
18	00

6. (Std1M1) old master flask was rinsed.
7. Took sample for cell count.

Cell count-

Table 12 - Cell viability- 0.38 X 10^6 cells/ml

Live	**Dead**
20	00
9	00
30	00
17	00

8. (N2) flask was rinsed.
9. Took sample for the cell count.

Cell count-

Table 13 - Cell viability- 0.39 X 10^6 cell/ml

Live	**Dead**
26	00
13	00
11	00
28	00

10. Took culture sample and transferred it to falcons.
11. Took sample for the cell count.

Cell count-

Table 14 - Cell viability- 0.17 X 10^6 cells/ml (total volume of this tube was 40ml)

Live	Dead
16	01
20	00
7	00
14	00

12. Mixed culture sample of flask (M1a Std-1a) T-75cm^2, M1 Std-1(T-25cm^2) and M2 Std-2 (T- 25cm^2).
13. Discard the supernatant and dissolved the pellet (concentrated the cells) in 5ml medium (DMEM, 10% FBS).
14. Took a fresh sterile T-75cm^2 filter flask and added 15ml medium and 5 ml dissolved pellet.
15. Took sample for the cell count.

Cell count-

Table 15 - Cell viability- 0.68X10^6cells/ml

Live	Dead
32	00
31	00
35	00
38	00

Day 10

1. No media was change.

Day 11

1. HBsAg flasks were observed under microscope.
2. (Std-1 M1) flask showed clumping in some area and in some area the cells were floating.
3. (Std-2 M2) flask showed differential growth and the number of cells were very less.
4. Mixed std-1 M1, std-2 M2 and M1a Std-1 flask.
5. N2 flask was (which was running) observed under the microscope which showed 90% confluent growth.
6. From the Std-1 M1 flask took 0.5ml sample for cell count.

Cell count-

Table 16- Cell viability- $1.3X10^6$cells/ml

Live	Dead
54	10
71	28
86	16
63	16

7. (Mixed std-1 M1) flask was tapped 2-3 times from outer side so that the cells detach from the bottle than rinsed 2-3 times with its own culture sample.
8. Took 1ml sample for the cell count.

Cell count-

Table 17 - Cell viability- $0.68X10^6$ cells/ml

Live	Dead
21	00
24	03
25	04
22	02

9. From the Std-2 M2 T-25cm^2 flask 4ml culture sample was transferred into (Std-2a M2a) 20ml T- 75cm^2 flask.
10. Now in the Std-2a M2a total volume was 24ml and added 10ml medium.
11. Std-2 M2 contained 0.5ml culture sample and added 10ml medium, making total volume 10.5ml.
12. Mixed std-1 M1a T-75cm^2 flask made total volume 20ml.
13. Discarded 10ml culture sample.
14. Added 10ml fresh medium (DMEM 10% FBS).
15. Total volume was 20ml (Mixed std-1 M1a T-75cm^2 flask).
16. N2 flask (Running flask) was tapped 2-3 times than rinsed 3-4 time with their culture sample.
17. Discard all culture sample.
18. 1ml remained in the flask and added 10ml fresh medium (DMEM 10% FBS).
19. Making total volume of N2 flask is 11ml.
20. Std-1 M1 flask T-25cm^2 rinsed with its own culture sample.
21. Discarded all culture sample apart from 1ml.
22. 1ml remained in the flask and added 10ml fresh medium (DMEM 10% FBS).
23. Made total volume of Std-1 M1 flask was 11ml.

3.2.6 Hepatitis B Surface Antibody (HBs Ab) ELISA

HBs Ab ELISA is a qualitative enzyme immunoassay designed to identify antibodies to HBsAg in human serum or plasma samples.

❖ **Principle of the HBs Ab ELISA Test**

The determination of Anti-HBsAg antibody titer is essential for monitoring the recovery progress of persons infected with the hepatitis-B virus. Additionally, it also serves as an indicator of previous exposure to the virus. The HBs Ab ELISA is a simultaneous immunoassay performed on a solid-phase plate to identify the presence of antibodies against HBsAg. Microwells of the plate are coated with HBsAg, and a serum sample is added along with Horseradish Peroxidase (HRP) conjugated HBsAg. After incubation, the formation of the antigen-antibody-antigen complex (consisting of HRP-conjugated HBsAg, anti-HBsAg antibody, and HBsAg on the wells) take place. Consequently, the amount of HRP-HBsAg bound to the well is directly proportional to the anti-HBsAg antibody concentration in the sample. The unbound enzyme conjugates are subsequently washed away after washing, and a chromogen substrate solution containing hydrogen peroxide is added to the wells resulting a blue color correlates with the quantity of anti-HBsAg antibody present in the sample. The enzyme-substrate reaction is stopped with addition of hydrochloric acid. Finally, the absorbance of both control and specimen is measured using an ELISA reader, with the wavelength at 450nm.

❖ **Materials used**

1. Specific inactivated HBsAg antigen applied to a 96-well microtiter plate. The plate consists of 8 removable strips, each containing 12 wells and stored at a temperature between 2-8°C.
2. A mixture of HRP-conjugated HBsAg with a volume of 7.5 ml and stored at temperatures between 2-8°C.
3. HBsAg negative control samples with a volume of 1 ml. It contains HBs Ab-negative human serum and stored at temperature 2-8°C.
4. HBsAg positive control samples with a volume of 1 ml. It contains HBs Ab-positive human serum and stored at temperature 2-8°C.
5. The substrate arrangement with a volume of 7.5 ml. It contains hydrogen peroxide and stored at temperatures between 2-8°C.
6. Colorant arrangement with a volume of 7.5 ml. It contains stable 3, 3-5, 5' Tetramethylbenzidine (TMB) and stored at 2-8°C.

7. The stop reagent with a volume of 7.5 ml. It contains 1M H_2SO_4.
8. The cleaning reagent with a volume of 30 ml. This is 20X concentrated phosphate buffered saline.

❖ **Equipment's Used**

1. Micropipette and pipette tips.

2. Incubator

3. Automated dish washing machine.

4. Microtiter plate reader

5. Absorbent paper

6. Vortex shaker

❖ **HBsAg titer ELISA plate procedure -**

Step 1. Made a serial dilution of sample in sample diluent (SD).

1:10 added 900(SD) +100 (sample)
1:100 added 900(SD) +100(sample)
1:200 added 200(SD) +200(sample)
1:400 added 200(SD) +200(sample)
1:8000 added 200(SD) +200(sample)
1:1600 added 200(SD) +200(sample)
1:3200 added 200(SD) +200(sample)
1:6400 added 200(SD) +200(sample)
1:12800 added 200(SD) +200(sample)

Step 2. Added 100µl prepared dilutions in ELISA plate (96 well plates) (1:100-1:12800 dilution).

Step 3. Added blank (only sample diluent) in one or two wells in ELISA plate. Followed by incubation at 37°C for 30 min.

Step 4. After incubation, washed the plate 5 times with Hepalisa 1X buffer.

a. First removed the sample directly from the plate.
b. Washed with Hepalisa 1X buffer by adding and removing the buffer.
c. Repeated the process for 5-times.

Step 5. Added 100µl HRP to the wells and incubated it for 30 min at 37°C.

Step 6. After incubation, washed 5 times with Hepalisa 1X buffer.

a. First removed the sample directly from the plate.
b. Washed with Hepalisa 1X buffer by adding and removing the buffer.
c. Repeated the process for 5-times.

Step 7. Added substrate (TMB) and TMB diluent in 1:1 ratio concentration (100µl) and

Incubated at room temp. for 30 min.

Step 8. Added stop solution (H_2SO_4). Took absorbance at 450nm in ELISA plate reader.

❖ **Purification of HBsAg**

1. Affinity column was regenerate with 0.1 M NaOH (50 ml).
2. Filled the column with 1X PBS buffer after the NaOH.
3. The column was washed with 1X PBS buffer until the pH reached 7 (verified by adding a drop to the pH strip). So, the pH of column and Ab is uniform. Titer the culture sample before loading the column at a ratio of 1:100 to 1:25600 dilution. The greater the dilution, the greater the amount of Ab present in the sample.
4. After washing, equilibrated the column by adding buffer and distilled water in 1:1 ratio, using 0.1M tris + 2.5M NaOH of pH 8.3.
5. Loaded the column with fresh culture sample in 1:1 ratio with binding buffer (0.1M tris + 2.5M NaOH of pH 8.3). Left the column for overnight loading.
6. Titer the loading samples after 24 hours from flow through 1 (samples that passed the column and eluted in the beaker), flow through 2 (drop of sample passing through the column) to check whether the column is underload or overloaded. If titer of loading sample is equal to flow through 2 means column is overloaded.
7. Column was washed with washing buffer (50 mM Tris + 1.2 M NaCl + 5 mM EDTA + 2M Urea + 50 mM PO_4^{3-} having pH 8.3).

8. Washed the column and check the OD of the eluted volume. When the optical density reaches zero, the nonspecific bonds are removed and eluted.
9. Took the column to the AKTA filter. In AKTA there were buffers. Buffer 'X' (0.1 M Tris + 0.15 NaCl) pH 8.3 and buffer 'Y' (0.1 M citric acid) pH 3. At pH 3, the bond was broken between the protein A beads and antibody present in the column.
10. Collected the eluted antibody and added neutralizing buffer (100µl/ml) to the antibody which is 2 M tris pH 8.
11. Dialysis the recovered antibody in 1 X PBS buffer pH 7.
12. Checked the Ab concentration and yield.

Formula to check concentration and yield

1. Conc. (mg/ml) = $\frac{\text{OD x 25}}{1.4}$

Where 1.4 is coefficient factor and 25 is the dilution factor (1000/25)

2. Yield (mg) = volume x conc.

CHAPTER-4

RESULTS

Results

1. Culture of HBsAg antibody

The Production process of 1-liter of HBsAg cell culture from a roller bottle, yielded 24 liters of HBsAg antibody. From the 1-liter roller bottle two roller bottles were obtained having equal proportion of HBsAg culture sample and media (500 ml each). Which was done by transferring 500 ml of cell culture sample from this bottle to autoclaved empty bottle and added 500 ml of media (DMEM + 10 % FBS) to each flask. These bottles were kept at 37 °C for 24 hours. From these 2 bottles 1 was kept for termination (bottle kept at 37°C for 15 days without any disturbance) and 1 acted as running bottle (used for further subculturing after 24 hours). One roller bottle was terminated every day and the other acted as running bottle for the next Sub-culturing for 15-20 days.

Sample were tittered to check the amount and quality of antibody.

2. Titer of HBsAg antibody

Samples were diluted form 1:100 to 1:12800 dilutions. Taken the absorbance at 450 nm after 1.5 hour of testing as follows.

Table 18– Absorbance of HBsAg samples

Dilutions	Sample1	Sample2	Sample3	Sample4	Blank
1:100	2.367	2.352	2.356	2.24	0.015
1:200	2.348	2.032	2.136	1.864	0.011
1:400	2.246	1.562	1.57	1.287	0.012
1:800	1.782	1.028	1.055	0.754	
1:1600	1.204	0.577	0.612	0.54	
1:3200	0.698	0.32	0.376	0.49	
1:6400	0.412	0.166	0.167	0.32	
1:12800	0.242	0.081	0.081	0.231	

Cut-off Value (COV): The cut-off value was established as the mean of the blank wells + 0.2, resulting in a COV of 0.23. Titer of the following results were:

Table 19– Titer of HBsAg antibody

Samples	OD	COV/OD	Titer
1	0.242	1.05	1:12800
2	0.32	1.39	1:3200
3	0.376	1.63	1:3200
4	0.231	1.0	1:12800

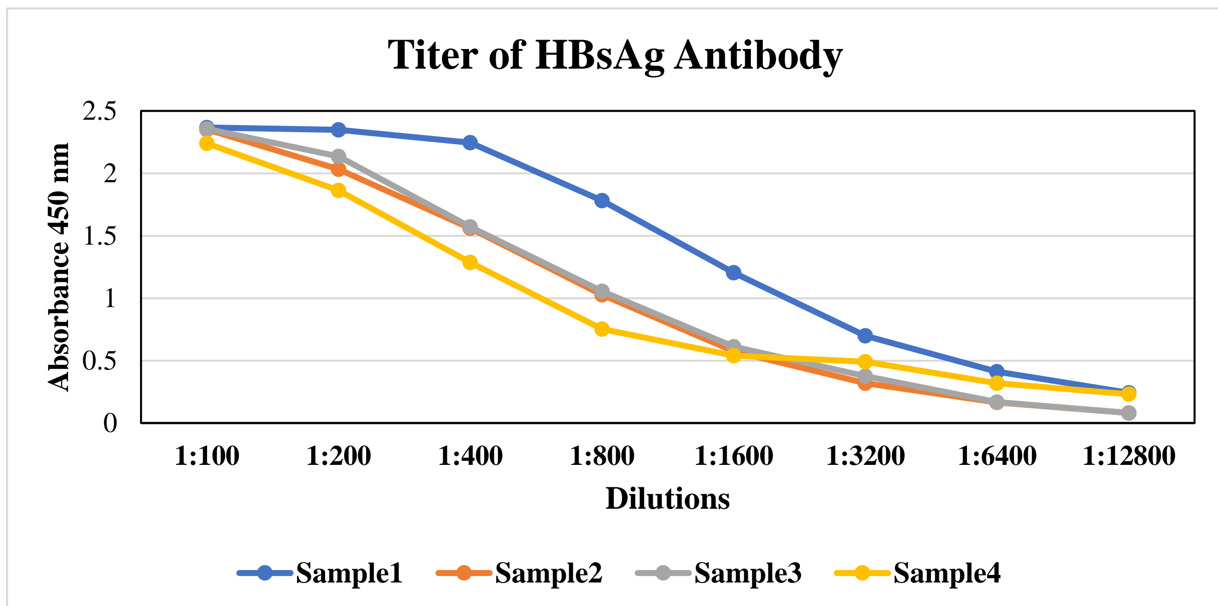

Fig.3 - Titer of HBsAg

The above graph represents various dilution of 4 HBsAg antibody samples ranging from 1:100 to 1:12800. The more the dilution of the sample, means if antibody is diluted to that much dilution it will still perform actively which is confirmed using indirect ELISA. **Sample 1** (blue color) showed titer of antibody at 1:12800 dilution as optical density (OD) of sample 1 was 0.242 and cut off value (COV) was 0.23. **Sample 2** (Orange color) showed titer of antibody at 1:3200 dilution as optical density (OD) of sample 2 was 0.320 and cut off value (COV) was 0.23. **Sample 3** (grey color) showed titer of antibody at 1:3200 dilution as optical density (OD) of sample 3 was 0.376 and cut off value (COV) was 0.23. **Sample 4** (yellow color) showed titer of antibody at 1:12800 dilution as optical density (OD) of sample 4 was 0.231 and cut off value (COV) was 0.23.

3. Result of Purification of HBsAg antibody

The sample that has titer 1:12800 dilution (sample 1 and sample 4 as per this project) and above were first concentrated using ammonium sulphate precipitation to 350ml which was further purified using affinity column having protein A beads specific for monoclonal antibody (HBsAg antibody). After one week antibody was recovered, concentrated and checked the yield.

Sample 1 of HBsAg antibody was concentrated from 24L to 350ml which showed following results after affinity chromatography

i. **OD_{280}:** 0.655
ii. **Concentration:** 11.69mg/ml (calculated using formula: Concentration = (OD_{280} x dilution factor) / coefficient factor)
iii. **Volume:** 34ml
iv. **Yield:** 397mg (calculated using formula: Yield = Concentration x Volume)

Sample 4 of HBsAg antibody was concentrated from 24L to 350ml which showed following results after affinity chromatography

v. **OD_{280}:** 0.562
vi. **Concentration:** 10.03mg/ml (calculated using formula: Concentration = (OD_{280} x dilution factor) / coefficient factor)
vii. **Volume:** 28ml
viii. **Yield:** 280.84mg (calculated using formula: Yield = Concentration x Volume)

Stocks of 0.1mg/ml were prepared of the recovered antibody by using formula 100/concentration. Titer of the antibody was done from dilution 1:100 to 1:12800.

Table 20– Titer of HBsAg antibody (0.1 mg/ml after purification)

Dilutions	**HBsAg Ab-1**	**HBsAg Ab-2**	**Standard**	**Blank**
1:100	2.8	2.54	2.43	0.017
1:200	2.548	2.324	2.21	0.013
1:400	2.446	1.643	1.75	
1:800	1.21	1.023	1.01	
1:1600	1.097	0.678	0.712	
1:3200	0.69	0.502	0.456	
1:6400	0.399	0.389	0.343	
1:12800	0.225	0.243	0.298	

Titer of the recovered antibody was 1:12800 as COV was 0.22. Which means if HBsAg antibody was diluted to 1:12800 dilution still it will show it activity

Interpretation: Titer of final recovered antibody

Total 62ml of antibody was recovered after dialysis of samples in 1X PBS + Azide which gave yield of 677.84mg. The antibody was stored at 2°C to 8°C refrigerator. Now this antibody is ideal to be used for coating purpose in ELISA.

CHAPTER 5

DISCUSSION & CONCLUSION

Discussion and Conclusion

This work investigated production, quantification and the purification of specific monoclonal antibodies against HBsAg. ELISA testing was used to determine HBsAg antibody titer, which reflects the amount of antibody present in samples. Serial dilutions were performed to establish this titer. After determining the titer, protein A chromatography was used to successfully purify the HBsAg specific monoclonal antibody from the sample, yielding 397mg of antibody at a concentration of 11.69mg/ml.

The cell culture techniques and fusion process with myeloma cells resulted in viable hybridomas capable of producing the desired antibodies. The purification of these antibodies through Protein A chromatography was efficient, yielding highly pure monoclonal antibodies.

The viability of the cells throughout the process, as confirmed by the trypan blue exclusion test, ensured that the monoclonal antibody production was consistent and reliable. The use of the Hepatitis B surface antibody ELISA further validated the functionality and specificity of the produced antibodies, confirming their potential utility in HBsAg detection.

The successful preparation and purification of monoclonal antibodies against HBsAg provide a valuable tool for improving Hepatitis B diagnostics. These antibodies can enhance the sensitivity and specificity of HBsAg detection assays, contributing to better disease monitoring and management.

Future aspects

The yield of HBsAg antibody titers may be improved by incorporating a standard curve to determine the exact titer, Characterization of the purified antibody using techniques such as SDS-PAGE and Western Blot is important to confirm its size, purity, and specificity for HBsAg. Additionally, optimization of Protein A chromatography conditions, such as elution buffer and flow rate, can improve antibody performance and purity. Ultimately, optimization of these methods could significantly improve HBsAg detection and pave the way for advances in hepatitis B diagnosis.

Exploring the use of these monoclonal antibodies in vaccine development is another exciting prospect. They could serve as critical components in the creation of more effective vaccines against Hepatitis B, potentially leading to longer-lasting immunity and better protection.

Studying the stability and storage conditions of these monoclonal antibodies is crucial for their practical application in clinical settings. Long-term stability studies would ensure that the antibodies remain effective over extended periods, making them more viable for widespread use.

CHAPTER-6

REFERENCES

REFERENCES

[1] M. H. Nguyen, G. Wong, E. Gane, J.-H. Kao, and G. Dusheiko, "Hepatitis B Virus: Advances in Prevention, diagnosis, and therapy," Clinical Microbiology Reviews, vol. 33, no. 2, Mar. 2020, doi: 10.1128/cmr.00046-19.

[2] A. J. S. Ndiaye et al., "Detection and quantitation of hepatitis B surface antigen (HBsAg) on dried blood spots: a solution for easy access for hepatitis B diagnosis and elimination in remote areas," the Pan African Medical Journal, vol. 42, Jan. 2022, doi: 10.11604/pamj.2022.42.100.30531.

[3] R. Burm et al., "A human monoclonal antibody against HBsAg for the prevention and treatment of chronic HBV and HDV infection," JHEP Reports, vol. 5, no. 3, p. 100646, Mar. 2023, doi: 10.1016/j.jhepr.2022.100646.

[4] Q.-Q. Yao et al., "Hepatitis B Virus Surface Antigen (HBsAg)-Positive and HBsAg-Negative Hepatitis B Virus Infection among Mother-Teenager Pairs 13 Years after Neonatal Hepatitis B Virus Vaccination," Clinical and Vaccine Immunology, vol. 20, no. 2, pp. 269–275, Feb. 2013, doi: 10.1128/cvi.00539-12.

[5] A. Cerino, C. M. Bremer, D. Glebe, and M. U. Mondelli, "A Human Monoclonal Antibody against Hepatitis B Surface Antigen with Potent Neutralizing Activity," PloS One, vol. 10, no. 4, p. e0125704, Apr. 2015, doi: 10.1371/journal.pone.0125704.

[6] A. Cerino, S. Mantovani, D. Mele, B. Oliviero, S. Varchetta, and M. U. Mondelli, "Human monoclonal antibodies as adjuvant treatment of chronic hepatitis B virus infection," Frontiers in Immunology, vol. 10, Sep. 2019, doi: 10.3389/fimmu.2019.02290.

[7] M. C. P. Soares, G. Bensabath, P. R. B. Cartágenes, M. M. Alves, F. A. A. Silveira, and A. C. Linhares, "Hepatitis B virus surface antigen (HBsAg) and antibody (anti-HBs) forming immune complexes in fulminant hepatitis," Revista Da Sociedade Brasileira De Medicina Tropical, vol. 32, no. 3, pp. 295–297, Jun. 1999, doi: 10.1590/s0037-86821999000300012.

[8] I. V. Chernikov et al., "Cholesterol-Modified Anti-IL6 SIRNA reduces the severity of acute lung injury in mice," Cells, vol. 13, no. 9, p. 767, Apr. 2024, doi: 10.3390/cells13090767.

[9] E. Reuschel, W. Jilg, B. Seelbach-Goebel, and L. Deml, "Comparative purification and characterization of hepatitis B virus-like particles produced by recombinant

vaccinia viruses in human hepatoma cells and human primary hepatocytes," PloS One, vol. 14, no. 2, p. e0212800, Feb. 2019, doi: 10.1371/journal.pone.0212800.

[10] D. A. Asandem, S. P. Segbefia, K. A. Kusi, and J. H. K. Bonney, "Hepatitis B virus infection: A mini review," Viruses, vol. 16, no. 5, p. 724, May 2024, doi: 10.3390/v16050724.

[11] J. Lee, S. Y. Lee, Y. G. Cho, D. S. Kim, and J. Park, "Accuracy validation of the ELECSYS HBSAG II quant assay and its utility in resolving equivocal qualitative HBSAG results," Medicina, vol. 59, no. 3, p. 443, Feb. 2023, doi: 10.3390/medicina59030443.

[12] Y.-L. Fu et al., "Metabolic interventions improve HBV envelope-specific T-cell responses in patients with chronic hepatitis B," Hepatology International, vol. 17, no. 5, pp. 1125–1138, Mar. 2023, doi: 10.1007/s12072-023-10490-4.

[13] Z.-L. Wang, J.-R. Zheng, R.-F. Yang, L.-X. Huang, H.-S. Chen, and B. Feng, "An ideal hallmark closest to complete cure of chronic hepatitis B patients: high-sensitivity quantitative HBSAG loss," Journal of Clinical and Translational Hepatology, vol. 000, no. 000, p. 000, Aug. 2022, doi: 10.14218/jcth.2022.00289.

[14] M. R. Brunetto, "A new role for an old marker, HBsAg," Journal of Hepatology, vol. 52, no. 4, pp. 475–477, Apr. 2010, doi: 10.1016/j.jhep.2009.12.020.

[15] Y. H. Yeo et al., "Factors Associated with Rates of HBSAG seroclearance in Adults with Chronic HBV infection: A systematic review and meta-analysis," Gastroenterology, vol. 156, no. 3, pp. 635-646.e9, Feb. 2019, doi: 10.1053/j.gastro.2018.10.027.

[16] M. C. Kuhns, S. H. Kleinman, A. L. McNamara, B. Rawal, S. Glynn, and M. P. Busch, "Lack of correlation between HBsAg and HBV DNA levels in blood donors who test positive for HBsAg and anti-HBc: implications for future HBV screening policy," Transfusion, vol. 44, no. 9, pp. 1332–1339, Aug. 2004, doi: 10.1111/j.1537-2995.2004.04055. x.

[17] R. Marabini et al., "The Electron Microscopy eXchange (EMX) initiative," Journal of Structural Biology, vol. 194, no. 2, pp. 156–163, May 2016, doi: 10.1016/j.jsb.2016.02.008.

[18] J. J. Lander, J. P. Giles, R. H. Purcell, and S. Krugman, "Viral hepatitis, Type B (MS-2 strain)," New England Journal of Medicine/˜ the œNew England Journal of Medicine, vol. 285, no. 6, pp. 303–307, Aug. 1971, doi: 10.1056/nejm197108052850601.

[19] N. A. D'Amato, "AUSTRALIA ANTIGEN IN BLOOD-DONORS," Lancet, vol. 301, no. 7808, p. 885, Apr. 1973, doi: 10.1016/s0140-6736(73)91454-2.

[20] L. Romanò, S. Paladini, C. Galli, G. Raimondo, T. Pollicino, and A. R. Zanetti, "Hepatitis B vaccination," Human Vaccines & Immunotherapeutics, vol. 11, no. 1, pp. 53–57, Nov. 2014, doi: 10.4161/hv.34306.

[21] N. G. Allam, M. L. Salem, H. Elbataa, and M. M. Nabieh, "Bacterial pathogens associated with severity of HCV infection," Delta Journal of Science, vol. 37, no. 2, pp. 207–214, Dec. 2016, doi: 10.21608/djs.2016.139954.

[22] B. K. Van Weemen and A. H. W. M. Schuurs, "Immunoassay using antigen—enzyme conjugates," FEBS Letters, vol. 15, no. 3, pp. 232–236, Jun. 1971, doi: 10.1016/0014-5793(71)80319-8.

[23] J. A. C. Nascimento Junior et al., "Trends in MERS-COV, SARS-COV, and SARS-COV-2 (COVID-19) diagnosis Strategies: A Patent review," Frontiers in Public Health, vol. 8, Oct. 2020, doi: 10.3389/fpubh.2020.563095.

[24] S. D. Gan and K. R. Patel, "Enzyme immunoassay and Enzyme-Linked immunosorbent assay," the Journal of Investigative Dermatology/Journal of Investigative Dermatology, vol. 133, no. 9, pp. 1–3, Sep. 2013, doi: 10.1038/jid.2013.287.

[25] S.-H. Kim, "ELISA for Quantitative Determination of Hepatitis B Virus Surface Antigen," Immune Network/Immune Network, vol. 17, no. 6, p. 451, Jan. 2017, doi: 10.4110/in.2017.17.6.451.

[26] A. Civera et al., "Development of sandwich ELISA and lateral flow immunoassay to detect almond in processed food," Food Chemistry, vol. 371, p. 131338, Mar. 2022, doi: 10.1016/j.foodchem.2021.131338.

[27] S. Sakamoto et al., "Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites," Natural Medicines/Journal of Natural Medicines, vol. 72, no. 1, pp. 32–42, Nov. 2017, doi: 10.1007/s11418-017-1144-z.

[28] M. K. Grötsch, D. M. Wietor, T. Hettich, and U. Ehlert, "Validation of a commercial Enzyme-Linked immunosorbent assay for allopregnanolone in the saliva of healthy pregnant women," Biomolecules, vol. 12, no. 10, p. 1381, Sep. 2022, doi: 10.3390/biom12101381.

[29] L.-N. Ma, J. Zhang, H.-T. Chen, J.-H. Zhou, Y.-Z. Ding, and Y.-S. Liu, "An overview on ELISA techniques for FMD," Virology Journal, vol. 8, no. 1, Sep. 2011, doi: 10.1186/1743-422x-8-419.

[30] M. J. Sue, S. K. Yeap, A. R. Omar, and S. W. Tan, "Application of PCR-ELISA in molecular diagnosis," BioMed Research International, vol. 2014, pp. 1–6, Jan. 2014, doi: 10.1155/2014/653014.

[31] B. Xinyu, F. Sun, and Z. Xu, "How protein labeling applying," E3S Web of Conferences, vol. 290, p. 01020, Jan. 2021, doi: 10.1051/e3sconf/202129001020.

[32] H. Towbin, T. Staehelin, and J. Gordon, "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.," Proceedings of the National Academy of Sciences of the United States of America, vol. 76, no. 9, pp. 4350–4354, Sep. 1979, doi: 10.1073/pnas.76.9.4350.

[33] R. Sule, G. Rivera, and A. V. Gomes, "Western blotting (immunoblotting): history, theory, uses, protocol and problems," Biotechniques/BioTechniques, vol. 75, no. 3, pp. 99–114, Sep. 2023, doi: 10.2144/btn-2022-0034.

[34] L. Björck and G. Kronvall, "Purification and some properties of streptococcal protein G, a novel IgG-binding reagent.," the Journal of Immunology/˜ the œJournal of Immunology, vol. 133, no. 2, pp. 969–974, Aug. 1984, doi: 10.4049/jimmunol.133.2.969.

[35] E. B. Speth, L. Imboden, P. Hauck, and S. Y. He, "Subcellular Localization and Functional Analysis of the Arabidopsis GTPASE RABE," Plant Physiology, vol. 149, no. 4, pp. 1824–1837, Feb. 2009, doi: 10.1104/pp.108.132092.

[36] J. R. Lakowicz, Principles of fluorescence spectroscopy. 2006. doi: 10.1007/978-0-387-46312-4.

[37] M. Yan, M. Schwaederle, D. Arguello, S. Z. Millis, Z. Gatalica, and R. Kurzrock, "HER2 expression status in diverse cancers: review of results from 37,992 patients," Cancer and Metastasis Reviews/Cancer Metastasis Reviews, vol. 34, no. 1, pp. 157–164, Feb. 2015, doi: 10.1007/s10555-015-9552-6.

[38] Y. Kim, P. Ganesan, and H. Ihee, "High-throughput instant quantification of protein expression and purity based on photoactive yellow protein turn off/on label," Protein Science, vol. 22, no. 8, pp. 1109–1117, Jun. 2013, doi: 10.1002/pro.2286.

[39] The autoimmune diseases. 2006. doi: 10.1016/b978-0-12-595961-2.x5000-8.

[40] F. X. R. Sutandy, J. Qian, C. Chen, and H. Zhu, "Overview of protein microarrays," Current Protocols in Protein Science, vol. 72, no. 1, Apr. 2013, doi: 10.1002/0471140864.ps2701s72.

CHAPTER 7

LIST OF PUBLICATIONS

LIST OF PUBLICATIONS

CONFERENCES:

1. Shan Ghai, Prajjwal Jagwan, Rahul Shrivastava, Shruti Jain, Analysis of differentially expressed *M. fortuitum* proteins for biomarker prediction using Support Vector Machine, 2023 Seventh International Conference on Image Information Processing (ICIIP -2023), November 22 - 24, 2023 at Jaypee University of Information Technology, Waknaghat, Himachal Pradesh, INDIA.

2. Prajjwal Jagwan, Shan Ghai**,** Rahul Shrivastava and Shruti Jain, "Prediction of Protein Biomarkers for *Mycobacterium fortuitum* using Machine Learning Technique," 2023 9th International Conference on Signal Processing and Communication (ICSC), NOIDA, India, 2023, pp. 416-421, doi: 10.1109/ICSC60394.2023.10440976. December 21-23, 2023 https://ieeexplore.ieee.org/document/10440976