**Lec(2) Serology**

**MSc of Zoology**

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**The construction of recombinant antibodies Therapeutic antibodies and their production .Humanized and human antibodies .production of human monoclonal antibodies**

Antibodies are believed to provide protection from infectious diseases and are the only natural host-derived defense mechanism capable of completely preventing infection. Antibodies with the ability to offer both prophylactic and therapeutic protection were first discovered by Behring and Kitasato in the late 19th century. In their seminal work in 1893, they showed that passive transfer of antibody from an infected animal provided immunity against diphtheria, which led to the emergence of the field of antibody production for human therapy. Subsequently, the immune sera from horses, sheep, chickens and humans were collected, pooled and used as therapeutic agents for the treatment of a number of infectious diseases like diphtheria, tetanus, pneumococcal pneumonia, meningococcal meningitis, and toxin-mediated diseases and prompt administration of such polyclonal preparation of immune sera-derived immunoglobulins usually showed an improved survival of the patients

Despite these successes, the discovery of antibiotics rapidly replaced serum therapy during the early 20th century as antibiotics are easier to manufacture and showed more consistent results. The usage of antibiotics is found to be less toxic in nature when compared to serum therapy. However, since 1980, the introduction of new antimicrobial agents for clinical use has been declined because of the cost involved in developing and testing of new drugs. Parallel to this, there has been an alarming increase in bacterial resistance to existing agents . Antibiotic resistance has emerged as one of the pre-eminent public health concerns of the 21st century, in particular to pathogenic organisms like Staphylococcus aureus, Pseudomonas aeruginosa, etc.

To overcome the limitations of antibiotic usage, antibody therapy has gained attention as an alternative and most suitable treatment for several diseases. Moreover, antibiotics are being used for only bacterial infections while the antibody therapies can be used for a wide range of bacterial and viral infections. However, antibody market could thrive under certain situations where it lacked competition, such as in the treatment of diseases which had no other effective therapies. The antibodies have been used to treat snake bite and also as a post-exposure prophylactic agent for rabies, cytomegalovirus, respiratory syncytial virus, hepatitis A virus, hepatitis B virus, vaccinia, and measles .

Monoclonal antibodies are having potential applications in the field of diagnostic, therapeutic and targeted drug delivery systems, not only for infectious diseases caused by bacteria, viruses and protozoa but also for cancer, metabolic and hormonal disorders. Further, they are also used in the diagnosis of lymphoid malignancies, tissue typing, enzyme-linked immunosorbent assay, radio immunoassay and serotyping of microorganisms .

**Monoclonal antibodies**

In the late 70s, Kohler and Milstein pioneered the development of monoclonal antibodies, which later diversified from a laboratory technique of generating antibodies into very important tool for the development of various therapeutic and diagnostic antibodies . Briefly, the technique involves the injection of an antigen into a mouse, which develops antibody-forming cells in the spleen. Single spleen cells will be fused *in vitro* to immortal mouse myeloma (tumor-derived) cells. The fusion products of spleen immune cells and myeloma cells will be placed in culture flasks or wells with liquid selective medium, containing hypoxanthine, aminopterin and thymidine, which promotes the survival, proliferation of hybridoma cells, eliminates nonfused B cells and myeloma cells. Cultures that identify as positive for producing the desired antibody will be subcultured using a limiting dilution approach to ensure that a monoclonal antibody-producing cell line will be obtained. The resultant hybridomas are cloned and monoclonal antibodies are produced by the identical offspring of a single cloned antibody-producing cell, since the original publication of monoclonal antibody generation involves different methods that have been developed to fuse, grow, select and clone hybridomas.

Inspite of rapid progress made in technology, the development of hybridomas still remained unpredictable and, in a number of cases, did not yield the best antibodies. While traditional monoclonal antibodies could be sequenced and manipulated, the process of producing antibodies still remained a complex one with loss of antibody-producing cell lines on long-term storage. In addition, these antibodies induce human antimouse immune reactions in patients, limiting the usage of these antibodies as therapeutics/prophylactics .

**Recombinant antibodies**

Concomitantly, scientists were working on methods that could be used to build these immunoglobulin-based binding sites using various genetic engineering/recombinant approaches. In 1989, antibody genes are directly cloned from lymphocytes of immunized animals and expressed as a single-domain library of antibody heavy or light-chain variable regions or as a combinatorial library of antigen-binding fragment (Fab) in bacteria..Following this technological achievement, a method based on the expression of functional antibody fragments on the surface of bacteriophage (phage) has been described, which provides a way to quickly select antibodies from libraries on the basis of the antigen-binding behavior of individual clones. A few years later, this technique, called phage display which was centered around the use of phages, in combination with polymerase chain reaction (PCR)-based cloning of antibody repertoires, have been successfully used to isolate murine and human antibodies from recombinant antibody libraries. These were built from natural sources, such as from animal or human B lymphocytes, eventually leading to the creation of libraries by *in-vitro* cloning techniques.

**Bacteriophages**

Phages are viruses that infect bacteria and consist of a DNA or RNA genome package within a protein coat . They accommodate segments of “foreign” pieces of DNA and replicate in *Escherichia coli*. Phage display differs from conventional expression systems in that the foreign gene sequence is spliced into one of the phage coat proteins, so that the foreign amino acid sequence is genetically fused to the endogenous amino acids of the coat protein to make a hybrid fusion protein. The hybrid coat protein is incorporated into phage particles or virions as they are released from the cell, so that the foreign peptide or protein domain is displayed on the outer surface. A phage-display library is constructed from diverse variable regions of the immunoglobulin genes (i.e., single chain of joined heavy (VH) and light variable (VL) fragments (scFv), Fab fragments, or single VH or VL domains). Each library is constructed from cDNA derived from immune or naive B cells. The DNA library is ligated into a surface protein gene (gene III) of a phage and will be displayed on their surface as antibody constructs fused with the surface protein *(*[*Fig. 1*](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4029287/figure/F1/)*)*. Phages expressing the required specificities are readily isolated and enriched, using antigen-conjugated affinity binding columns. Eluted phages, similarly reselected, will be used to infect *E. coli* to produce the monoclonal antibody construct. Alternately, the genes of the specific antibody could be excised and cloned into whole human IgG expression vectors and subsequently transfected into appropriate cells to produce fully human monoclonal antibodies. Since this combinatory library randomly matches the V regions of the heavy and light chains, the resulting products include not only combinations that are expressed by B cells *in vivo* but even novel combinations (non-native) which never existed before. The libraries prepared from both naive and immunized human/animal donors enable the selection of high affinity antibodies.

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Fig(1) :Spleen cells from mice (or) peripheral blood lymphocytes from humans**.**

**Antibody libraries**

Libraries are mainly of four types and they include (a) naive antibody libraries, (b) immunized antibody libraries, (c) synthetic antibody libraries and (d) *in-vivo* recombined antibody libraries.

**Naive antibody libraries**

The genomic information coding for antibody variable domains is usually derived from B cells of either “naive” (non-immunized) or immunized donors . An antibody repertoire from immunization is generally restricted to generating antibodies against the antigen of the original immunogenic response, whereas naive libraries have the advantage that they can theoretically be used for different antigens. Construction of these libraries involves relatively straightforward molecular biology techniques such as reverse transcription of mRNA followed by PCR with germline-specific primers to amplify the VH and VL gene segments from the cDNA template, and restriction-based cloning to incorporate the rearranged antibody segments into an appropriate phage display vector. The vectors will be transformed into *E. coli* cells to generate the antibody repertoire. The first naive scFv phagemid library has been constructed using peripheral blood lymphocytes and predicted to have a diversity of 107 . Antibody fragments isolated from this library demonstrated micromolar affinities. The scFv library of 1010 recombinants, derived from tonsil and peripheral blood lymphocytes, enabled the selection of scFvs with affinities in the subnanomolar range and supported theoretical predictions . that the size of the library is directly proportional to the affinities of the isolated antibodies which offers the flexibility of isolating high affinity antibodies.

**Immunized antibody libraries**

Immunized antibody libraries are constructed from heavy and light chains of antibody genes of B cells which are derived from various types of immunized animals like mice, sheep, camel and also from humans . The antibodies derived from immune libraries are biased towards a particular target antigen that has been used for immunization. Though high affinity and specific antibodies can be obtained as a result of *in vivo* affinity maturation by the host’s immune system, new antibody libraries have to be constructed for every individual antigen making it a very laborious process.

**Synthetic antibody libraries**

Construction of synthetic libraries involves rearranging of VH and VL gene segments *in vitro* and introducing artificial complementarity determining region (CDRs) of varying loop lengths using PCR and randomized oligonucleotide primers. One of the earliest synthetic repertoires used a diverse repertoire of human VH gene segments paired with a constant region of light chain. Synthetic libraries contain artificial CDR sequences. Some of the recently constructed synthetic repertoires have opted to restrict the number of frameworks used. The HuCAL library is a fully synthetic antibody library which yields antibodies with nanomolar affinities to a number of antigens and they include granulocyte macrophage colony-stimulating factor, receptor tyrosine kinases, major histocompatibility complex (MHC) molecules, cytokines and proteases, etc. .

***In vivo* recombined antibody libraries**

To circumvent the earlier limits imposed by the transformation step, some libraries have been constructed using *in vivo* recombination or combinatorial infection to generate highly diversified repertoires of either synthetic or naive antibody fragments. A synthetic Fab library was constructed using two vectors, each with a different antibiotic selectable marker. One is a plasmid derived from pUC19, and the second is a phage vector (fdDOG). The heavy and light chain antibody sequences were cloned into this vector . The vectors are then incorporated into the same cell by a simple phage rescue strategy, and cells are subsequently coinfected by the chloramphenicol-resistant phage P1. P1 provides the Cre recombinase which recognizes the loxP sites flanking the heavy and light chain antibody sequences and recombines them within the cell. The constructed library was predicted to contain ~1010 recombinants, and these recombinants were being detected on the basis of their resistance to all three antibiotics. Recently, a single vector system which also used Cre recombinase has been described . A phagemid vector containing both VH and VL segments has been transfected into cells, and the phage population produced from this primary library was then allowed to infect bacteria at a high multiplicity of infection (ratio of phage to cells), which ensures incorporation of more than one vector per cell. The VH and VL segments were then exchanged between vectors generating new multiple VH–VL combinations. Using this method, a naive scFv library with a diversity of 1011 will be created. This library is considered more stable than that previously constructed using *in-vivo* recombination because of the use of the smaller phagemid vector.

**Advantages of production of phage antibodies**

The recombinant antibodies are identical to traditional monoclonal antibodies in their basic functionality. They can be manufactured using fully *in-vitro* processes, thus, offering greater flexibility during their production and greater opportunities for optimization after their creation than typical monoclonal antibodies, as these kinds of technologies also facilitate production, screening and maturation of selected binders, allowing selection on target conformations and formats which are not possible by more traditional routes based on immunization. The easy availability of the gene sequence not only provides a definitive description of the product but also allows electronic sharing and recreation of the binding molecule through gene synthesis.

**Antibodies as therapeutic reagents**

The first approved therapeutic murine monoclonal antibody has been launched in the year 1986 under the trade name Orthoclone OKT3®, which is being used during organ transplantations. A number of other antibodies followed, some of which were chimeric wherein the constant domains of an antibody were human and the variable domains were murine under the trade names Remicade® and Erbitux®, which are being used for the treatment of rheumatoid arthritis and colorectal cancer, respectively. Later, antibodies were completely humanized as in the case of Herceptin®, which is being used for the treatment of breast cancer. In 2002, the first fully human antibody under the trade name Humira® has been isolated using antibody phage display which is directed against TNF-α.

**Antibodies as diagnostic reagents**

Recombinant antibodies offer several advantages over a full-length antibody molecule as diagnostic reagents. These antibodies have become a potential immunological reagent for use in various assays and can be produced in large quantities in bacteria economically without the usage of expensive mammalian cells. ScFvs developed for antigens have been used as immunological reagents in the enzyme-linked immunosorbent assay (ELISA) based assays and they include haptens, proteins, etc. Antibodies can be used in three general ways for the development of diagnostics and they include (a) detection of antigens using antibodies, (b) a reversed way in which the detection of serum antibodies is done using antigens and (c) the competition assay wherein antigens are detected by serum antibodies, which compete with a defined antibody preparation. We have developed an ELISA format for the detection/quantitation of various viral antigens using recombinant antibodies and they are outlined below.

**Development of an assay for quantitation of rabies glycoprotein**

Rabies endemicity in many developing countries is responsible for human deaths. The annual number of human deaths worldwide caused by rabies has been estimated to be 40,000 and 70,000 in Africa and Asia, where rabies is endemic, and India ranks among the highest with 20,000 human deaths. Vaccine manufacturers around the world determine the potency of the rabies vaccines using the *in-vivo* mouse protection test, which is time-consuming, expensive and requires the use of large number of mice and virulent rabies virus for challenge in a biosafety level 3 facility. The test, apart from being labor-intensive, time-consuming and expensive, also suffers from poor intra- and inter-laboratory variations. The rabies virus glycoprotein (RV GP) has been shown to induce neutralizing antibodies and confer protection against lethal rabies virus challenge. Thus, much of the effort has been directed towards the development of *in-vitro* methods like ELISA, which is capable of estimating GP content in rabies vaccine preparations. In an effort to develop a simple and sensitive ELISA using recombinant diabody for quantification of RV GP, the VH and VL domains of an RV GP-specific human monoclonal antibody (MAb) secreted by a human × mouse heterohybridoma (human MAb R16E5) were amplified, linked using splice-overlap extension PCR and expressed as a recombinant diabody (D06) in the pET28a bacterial expression system. The diabody D06 was purified by immobilized metal affinity chromatography on a nickel–nitrilotriacetic acid agarose column and characterized. The purified diabody was used in combination with a well-characterized RV GP-specific mouse MAb-M5B4, to develop an immunocapture ELISA (IC-ELISA) for the quantification of RV GP in human rabies vaccine preparations. The IC-ELISA format using the M5B4-D06 combination showed a sensitivity of up to 31.25 ng/ml of RV GP. The specificity of the diabody has been established by its nonreactivity towards other human viral antigens as determined by ELISA and towards RV GP as determined by immunoblot transfer assay and competitive ELISA with the parent human MAb R16E5 and MAb M5B4. The IC-ELISA can be readily adapted to measure the RV GP content in purified antigen and a vaccine can be formulated based on the estimated GP. The diabody provides a simple, novel and efficient option for quantification of RV GP antigen in vaccine preparations without loss of antigen and aids in the manufacture of good-quality vaccines, which can reduce the cost and make the vaccine affordable in developing countries, where rabies prevention and control are a challenge .

**Development of an assay for quantitation of hepatitis A virus**

Hepatitis A virus (HAV) is the major pathogen responsible for acute infectious hepatitis A, a disease that is prevalent worldwide . Protection against HAV infections is brought about by the use of a conventional inactivated vaccine. Blending of the vaccine is done on the basis of antigen concentration, which is determined by the number of international units present in the antigen preparation in comparison to the National Institute for Biological Standards and Control, UK. Though the kit used for the determination of the concentration is also available commercially, regular use of the kit is very difficult because of its prohibitive cost.

Using phage display technology, a human monoclonal scFv was selected from a human immunized antibody library against hepatitis A virus. The binding activity and specificity of the scFv were established by its nonreactivity towards other human viral antigens as determined by ELISA and immunoblot analysis. The scFv was further used in the development of an in-house IC-ELISA format in combination with a commercially available mouse monoclonal antibody for the quantification of hepatitis A virus antigen in human vaccine preparations. The scFv showed very high specificity and a sensitivity of as low as 0.5 IU. Quantitation of hepatitis A antigen in vaccine preparations using scFv by IC-ELISA therefore offers a cheap, simple and convenient way of estimation of virus by immunocapture ELISA without the loss of antigen.

**Development of an assay for bovine IgA**

Immunoglobulin A (IgA) is a predominant class of antibody present on the mucosal surfaces, which constitutes the first line of defense against various infectious diseases. It is one of the primary determinants that would indicate enhanced mucosal immune response/protection . Secretory IgA is transported into mucosal secretions and is resistant to proteases; it prevents adhesion of bacteria/toxins to target cells, neutralizes viruses and toxins, among other characteristics .

We constructed the recombinant scFv from hybridoma expressing a monoclonal antibody against bovine IgA by splice-overlap PCR using a 15 amino acid peptide linker system. The scFv against bovine IgA was expressed, purified in *E. coli* and used as a potential reagent for the detection of foot-and-mouth disease virus specific IgA in salivary samples by ELISA. The in-house kit showed higher sensitivity in comparison to the commercial kit. This scFv holds promise in the detection of foot-and-mouth disease virus specific IgA in salivary samples by IC-ELISA and offers a simple, novel, cheap and efficient option for detection of IgA, which could replace the usage of commercial IgA due to better sensitivity, reagent stability and ease of production .

**Development of an assay for the detection of human IFN-γ**

Human interferon-gamma (IFN-γ) plays a major role in the regulation of immune response and inflammation in several diseases. It acts as a hormone and exerts pleiotropic effects such as antiproliferative, antiviral and antimicrobial activities. It activates the macrophages in killing intracellular organisms. IFN-γ is a dimeric protein and is a member of type II interferons which is secreted by T helper cells, cytotoxic T cells and natural killer cells with the ability to inhibit the replication of virus by its immunostimulatory and immunomodulatory effects. Further, IFN-γ is also known to induce the MHC class I and II antigens .

Using phage display technology, a human Fab fragment was selected from a human naive library against human IFN-γ. The binding activity and specificity of the Fab were established by its nonreactivity towards bovine IFN-γ as determined by ELISA. The Fab fragment will further be used in the development of an in-house IC-ELISA format in combination with commercially available human monoclonal antibodies for the detection of IFN-γ in infected PBMC preparations.