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Monoclonal antibodies in immunodiagnostic assays: a review of recent applications

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Abstract

Monoclonal antibodies (mAbs) have proven to be effective biological reagents in the immunodiagnostic assays. This is due to their binding accuracy to many pathogens, thus, making them valuable research tools. Since the discovery of hybridoma technology by Kohler and Milstein, the use of monoclonal antibodies produced by the hybrid cells have been employed in diagnosis of several diseases. Monoclonal antibody production has several procedures with considerable variations, but the principles remain the same. Improvements in the field of cell culture technology have led to the production of improved qualities of monoclonal antibodies. In general, these antibodies are important biomedical reagents used in research, especially in the field of laboratory diagnostics for a number of different types of diseases in humans and animals. Some of the areas where application of monoclonal antibodies triumph are herein discussed. This review is aimed to assess various diagnostic assay techniques where monoclonal antibodies are applied in order to provide a first-hand information, especially for beginners in monoclonal antibody production, characterisation, evaluation and /or its applications in research and diagnosis.

Keywords: Monoclonal antibodies, immunodiagnostic, assays, recent applications
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Introduction

Monoclonal antibody production is an old immunological technique with great applications in the fields of biochemistry, immunology, biotechnology and applied biology among others (Deb *et al*., 2013). Monoclonal antibody production using hybridoma technology was first discovered by Georges Kohler and Cesar Milstein (Pandey, 2010). One unique advantage of hybridoma production is that mixture of antigens can be used to generate specific antibodies. This also enables screening of antibodies of choice from a mixture of antibody population generated by purified antigen where single cell clones can be isolated (Buss *et al*., 2012). Monoclonal antibodies can be used in immunodiagnostic techniques as reagents to demonstrate the antigen of the causative agents or

indirectly for serological detection of antibodies against the causative agents (Wilson & Walker, 2010). They have also been reported to be used in experimental purposes ranging from molecular detection of antigenic epitopes to monoclonal anti-idiotype antibody for utilization as a vaccine to induce protective immunity (Pandey, 2010). Medium scale quantities (0.1 - 1.0 g) are used for development of detection kits and for testing of new mAbs in animals. In this context, large scale production of mAbs is defined as over 1.0 g which are mostly considered for both diagnostic and therapeutic procedures (Shukla & Thommes, 2010; Wilson & Walker, 2010).

Interestingly, mAbs application in competitive ELISA has come to the forefront as a technique to detect the presence of anti-organism antibody. Since after its inception, the prominent advantage of its specificity of a single clone preparation allows the use of even crude antigen preparation for its production (Zumaquero-Ríos *et al*., 2012). Advancement in biotechnology has contributed in the large scale production of mAbs which forms an integral part of many diagnostic techniques. These assays are frequently employed either for detection of infectious agents or any of its structural components (antigen) or even the antibodies generated against the infectious agents (Deb *et al*., 2013).

Additionally, the role of monoclonal antibodies in disease prediction and detection is promising. Monoclonal antibody technology plays a significant role in the development of specific serologic reagent towards antigen in limited amounts. They provide both highly specific and reproducible immunological assay for rapid and accurate diagnosis of different types of infectious diseases (Smith, 2012). The merits and demerits of the use of monoclonal antibodies in a number of immunoassays need to be evaluated. This will help in specific diagnosis of infectious diseases in various laboratories (Marra *et al*., 2010).

Monoclonal antibodies in diagnosis of infectious diseases

The robust specificity of mAbs has made them become one of the most promising fields of research in biomedicine. They have also played significant role in laboratory diagnosis of parasitic and tropical diseases (Shukla & Thommes, 2010; Gupta & Singla, 2012). However, their diagnostic applications in various livestock and human diseases is an important area for consideration as these diseases form a major and increasingly important factor affecting health and productivity in various parts of the world (USDA, 2008). The available diagnostic techniques have relatively low sensitivities and specificities which necessitates replacement with new diagnostic tools with high sensitivity to detect infections agents in individuals and to assess chemotherapeutic efficacy.

Since after the discovery of mAbs, they have been used in diagnosis of many important parasitic helminths (Zumaquero-Ríos *et al*., 2012) and protozoan (Srinivasan *et al*., 2014) diseases (*Plasmodium* spp, dirofilariosis, trichinellosis, trypanosomosis, leishmanosis, anaplasmosis, etc), bacterial (Tamborrini *et al*., 2010) diseases (anthrax, brucellosis, paratuberculosis, leptospirosis, listeriosis, clostridial infections, mycoplasmosis, etc), fungal diseases (zygomycosis, cryptococcosis, histoplasmosis, etc) viral diseases (foot-and-mouth disease, infectious bovine rhinotracheitis, bovine viral diarrhoea, blue tongue, classical swine fever and rabies, etc). Similarly mAbs are used to evaluate emerging viral diseases like Hendra (Xu *et al*., 2013) and Nipah viral infections. The specifications of each diagnostic assay is selected based on convenience of the diagnosticians, scientists, researchers for use in field and laboratories (Deb *et al*., 2013).

Diagnostic techniques using monoclonal antibodies

The application of monoclonal antibodies in diagnosis is by far the most advanced, especially for tests that are performed on body fluids such as blood and urine (Ghosh & Ansar, 2013). Monoclonal antibodies can be used to detect the presence of antigens. They can be used in different technologies which include ELISA, western blot, immunodot blot, flow cytometry, immunohistochemistry, radioimmuno assay (RIA), microscopy (electron, fluorescence, confocal) and other biotechnology applications (Gupta & Singla 2012; Lelli *et al*., 2012).

Immunohistochemistry

Immunohistochemistry (IHC) is a powerful diagnostic tool for antigen detection in tissue sections. The method has been used in the diagnosis of cancer in humans and animal species (Casartelli-Alves *et al*., 2014). The use of mAbs in IHC is most preferred than the use of polyclonal antibodies, this is because mAbs are more specific and typically results in less background staining (Zafra *et al*., 2015). Immunohistochemistry is still subjected to variable factors (pre-analytic, analytic and post-analytic) that decrease its reproducibility, including scoring systems, reagents, staining methods, tissue preparation, fixation and definition of results.

Although, a considerable variability has been attributed to selection and validation of primary antibodies, quality controls and lack of proper assay optimization (O Leary *et al*., 2013).

The public health significance of most zoonotic parasites especially those transmitted through the consumption of infective agents in tissues of infected animals such as *Toxoplasma gondii* and *Trichinella spirali s*is alarming (Chharba & Singla 2009). Modified agglutination test (MAT) has been used for the detection of antibodies against *T. gondii* in many animal species (Alvarado-Esquivel *et al*., 2011). Examination by histopathology is widely employed for the diagnosis of *T. gondii* with only few studies describing the immunohistochemical detection of the protozoan parasite (Pereira-Bueno *et al*., 2004). However, in a study to determine the association between serology (MAT) and presence of cysts in tissues (IHC) of infected animals, conclusion was drawn that MAT positive animals could serve as potential for human infection because bradyzoites were found in different tissues, regardless of the MAT titration (Silva *et al*., 2013).

Zoonotic visceral leshmaniases for which the domestic dog represents the main reservoir host in urban environments is also one of the important protozoan diseases of humans (WHO, 2010; Chhabra & Singla, 2014). Lack of reliable laboratory diagnostic standard is a significant challenge and a major problem. This necessitates establishment of an accurate diagnostic protocol for detection of the parasite in tissues other than the use of serological tests (ELISA, IFAT) for detection of antibodies whose accuracy is limited (Peixoto *et al.*, 2015). More recently, results from a comparative study on the various methods and techniques for accurate diagnosis of visceral leshmaniasis revealed that immunohistochemistry is more sensitive and offers advantage of diagnosing different *Leshmania* species compared to others and was recommended for a reference standard test for the condition (Furtado *et al*., 2015).

There is no doubt that alveolar ecchinococcosis (AE) is a life threatening disease. It is caused by the metacestode stage of *Ecchinococcus multilocularis* while cystic ecchinococcosis (CE) is caused by the larva stage of *E. granulosus* (Stojkovic & Junghanss, 2013). The differential diagnosis of alveolar ecchinococcosis (AE) with the cystic ecchinococcosis (CE) is usually challenging. This has led to the improvements in diagnosis of AE on sections of infected human tissues by immunohistochemical testing of a specific monoclonal antibody (Em2G11). Staining infected tissue with this monoclonal antibody that is highly specific to the metacestode stage of *E. multilocularis* revealed a highly sensitive and specific diagnostic tool for alveolar ecchinococcosis (Barth *et al*., 2012).

In another study, Foxp3 antibody was used to identify regulatory T cells, a subset of lymphocytes which play role in the maintenance of immune homeostasis. These lymphocytes are able to suppress immune responses to self-antigens, prevent autoimmune diseases and control the immune response to different pathogenic agents, including parasites (Zafra *et al*., 2015). Another identified mAb (M92/20) was used in immunochemistry (IHC) for confirmation of suspected contagious bovine pleuro pneumonia (CBPP). Other monoclonal antibodies have also been evaluated in this test for diagnosis of CBPP-affected lungs from Portuguese cattle. All cases were detected by immunohistochemistry which illustrates that M92/20 mAb-based immunohistochemistry is a sensitive and robust test for contagious bovine pleura pneumonia (Deb *et al*., 2013). With the help of monoclonal antibodies, immunohistochemical procedures such as immunoperoxidase have been improved and used as important tool for the detection of viral diseases notably the cutaneous viral infections such as the herpes viruses and papillomaviruses which are difficult to diagnose (Molina-Ruiz *et al*., 2015). Likewise, the use of monoclonal antibodies based IHC provide a more sensitive and specific detection of rabies virus induced inclusion bodies in tissue and reduces false positive reactions associated with H and E as well as Sellers stains (USDA, 2008).

Enzyme-linked Immunosorbent Assay (ELISA)

Several immunological assays have been developed for the diagnosis of infectious diseases which were either through detection of antigen or anti-antibodies in sera of infected animal or individuals (Moreno *et al*., 2013). For example, hybridoma secreting monoclonal antibodies (mAbs) specific to *Strongyloides stercoralis* protein (antigen) have been produced to aid the development of specific and sensitive ELISA. In this regards, specific epitopes targeted by the produced mAbs were protein in nature and are located in the content of the infective stage larva of the parasite. This approach uses mAbs which were IgG1 isotype known to have high affinity to this epitope, so they were used in a blocking ELISA to detect the antigen of the parasite. The mAbs have shown some reactions to the homologous antigen in an indirect ELISA but did not reveal positivity with the SDS-PAGE separated homologous antigen in a

western blot analysis, suggesting conformational epitope specificity. The mAbs were filariform stage-specific and thus could not also detect the antigen of the rhabditiform larvae contained in the patient's stool (Taweethavonsawat *et al*., 2002).

Due to predominantly zoonotic importance of most nematode parasites, efficient control measures can only be achieved if accurate diagnosis of these conditions can be made (Chowdhury *et al.,* 2013). Monoclonal antibody specific for *Toxocara cati* was prepared and was experimentally used to increase the sensitivity of a capture ELISA for antigen detection. IgG3 isotype was produced which has shown a good reaction against the excretory-secretory (ES) antigen of larval stage of *T. cati*. The antibody shows no cross-reaction with the antigens of other nematode parasites and had enough sensitivity to detect circulating antigens in serum. The results indicated that this antigen detection system provides a useful method for the diagnosis of both visceral and ocular larva migrans caused by *T. cati* and fulfils the requirement of a serological assay for the diagnosis of toxocariasis (Zibaei *et al*., 2010).

Similarly, specific monoclonal antibodies have been produced to further characterise the suppressive components of the extract of *Ascaris suum*. The immunosuppressive fractions isolated from the adult worms were used to stimulate antibody production. The proteins (PAS-1, PAS-2 and PAS-3) were purified from the crude extract and were prepared as antigen with ovalbumin for the immunization of Balb/C mice. Three monoclonal antibodies (MAIP-1, MAIP-2 and MAIP-3) obtained from the cloned hybrid cells were screened to determine their specificities in ELISA using coated plates with each fraction of the *A. suum* extract isolated by gel filtration. They were shown to react with different antigenic epitopes of high molecular weight proteins. These antibodies have recognized the antigen with different affinity constants (Oshiro *et al*., 2004).

Cryptococcosis is a mycotic disease of major concern and a potentially fatal disease that is the cause of the most common life-threatening meningitis in patients with acquired immunodefficiency syndrome (Andama *et al*., 2013). Due to the difficulties and lack of sensitivity and specificity associated with the conventional microbiological and histopathological diagnostic techniques of fungal diseases, interest in consideration of developing non-culture approaches arose. The recent birth of use of monoclonal antibody diagnosis plays an important role in early diagnosis for guide to appropriate treatment and to prevent mortality (Yeo & Wong, 2002). The capsular polysaccharide antigen has been considered as one of the most valuable and rapid tests for fungi. Murex Cryptococcus test incorporated with a mouse monoclonal IgM-based latex agglutination assay have been reported to effectively eliminate false positive reactions (Kiska *et al*., 1994).

In order to reduce cross-reaction with other fungal organisms in detection of histoplasmosis, a mAb was produced that recognises a species-specific antigenic epitope of *Histoplasma* spp by inhibition ELISA. Thus, a more specific detection technique was achieved (Gomez *et al*., 1999). In the same vein, a mAb (P1B) was also applied in an inhibition ELISA for the detection of circulating antigen of *Paracoccidiodomyces brasiliensis* that revealed a more promising method (Gomez *et al*., 1998). Competitive ELISA assays based on mAbs not only allow specific detection but also aid accurate quantification of antibodies to human and animal viruses. For example, Singh *et al*. (2004) evaluated the use mAbs base on the neutralizing epitope of haemagglutinin protein in the titration of antibodies specific to peste des petits ruminants (PPR) virus. They concluded that the c-ELISA test showed potentials in replacing virus neutralization test which have been widely used for sero-surveillance, sero-monitoring, diagnosis and end-point titration of PPR virus antibodies. Similar c-ELISA assay was found useful for the evaluation of H5 type influenza virus in samples that are difficult to be evaluated by haemaglutination inhibition test. In this regards, the assay was found to be very sensitive with excellent diagnostic performance and discriminatory power with high sensitivity and specificity values of 99.6-95% (CI 98.0-100) and 99.4-95% (CI 98.5-99.8) respectively (Pal *et al*., 2013). Compared with the indirect ELISA, monoclonal antibody-based sandwich direct ELISA (MSD-ELISA) was reported to be 7 times more sensitive in detection of FMD virus when RT-PCR was used as gold standard (Morioka *et al*., 2014).

Western immunoblotting

Since the inception of the protocol for protein transfer from an electrophoresed gel to a membrane, protein blotting has evolved greatly. Western blotting analysis can detect one protein in a solution that contains any number of proteins and giving the protein information, and it is widely used in protein detection. It is a method in molecular biology/biochemistry/immunogenetics to detect protein in a given sample of tissue homogenate or extract (Yang & Ma, 2009), which is normally used with high antibody directed against a desired protein (antigen).

In a report of a study for evaluating the detergent and aqueous phases of total saline (TS) and alkaline extracts of *Strongyloides venezuelensis* for immunodiagnosis of human disease, each extract presented a totally different antigenic profile as recognised by immunoglobulin (IgG) in western immunoblotting. The total extracts of detergent and aqueous antigenic fractions were separated by Triton X-114 and were analysed to detect immunoglobulin G (IgG). The results indicated that saline detergent fraction purified from *S. venezuelensis* yielded the most valid results for the immunodiagnosis of strongyloidosis and could be employed as a useful alternative antigen source of specific polypeptides (Feliciano *et al*., 2010).

No doubt, WB has been an invaluable tool used in the detection of viruses. A novel WB was developed based on mAbs for the detection of cytomegalovirus in latently infected blood donors, pregnant women, and transplant recipients with ongoing human cytomegalovirus (HCMV). Evaluation of this assay revealed high sensitivity and specificity compared to the conventional IgM based ELISA (Lazzarotto *et al*., 1997)

One of the major bacterial pathogens that causes morbidity and mortality in young, elderly and immunocompomised persons worldwide is pneumococcal infections (Saha *et al*., 2012). It is caused by *Streptococcus pneumoniae* that is composed of about 90 serotypes, based on their carbohydrate capsule (Henrichsen, 1995). Although early detection of these bacteria species in blood is of clinical importance, as culture of blood is the only widely accepted definitive technique of pneumococcal diagnosis (Pozzi *et al*., 1995). This has led to continued search by investigators for a rapid, sensitive and specific diagnostic test for pneumococcal infections. In an earlier study, five mAbs were produced against pneumococcal surface adhesion A (Psa A) that is common in the cell wall protein of *S. pneumonia.* These mAbs were used in a dot immunoblot and western blot study of clinical isolates of *S. pneumonia* to detect the presence of the protein. All the five mAbs reacted with five multidrug-resistant strains which indicated that they may be useful for detection of pneumococcal antigen and diagnosis of pneumococcal diseases (Crook *et al*., 1998).

Additionally, reports about people with cystic fibrosis being more susceptible to infection by several *Burkholderia* species, including *Burkholderia cepacia* complex (Bcc) which has a potential to cause life-threatening human infections have been documented (Lipuma, 2005). In an attempt to develop a mAb that binds to the exopolysaccharide produced by *B. pseudomallei* a mAb (5D8) was produced against *B. cepacia* strain (BTS13) isolated from a cystic fibrotic patient. This mAb was found to be specific for the polysaccharide antigen on the surface of the bacterium. The mAb reacted with the lipopolysaccharide (LPS) of BTS13 in a ladder pattern on western blot but was only reactive with a 22 kDa antigen of *B. pseudomallei*. Conclusion was drawn that since the mAb (5D8) reacted with the 22 kDa antigen in all *B. cepacia* complex (Bcc) species tested, it may be helpful in diagnosis of *B. cepacia* complex (Bcc) infections in cystic fibrotic patients (AuCoin *et al*., 2010).

Immunofluorescent antibody test (IFAT)

The immunofluorescence test is described as a useful technique in the immunological diagnosis of strongyloidosis. The method is used to detect antibody present in the serum of patients through binding to surface antigens or within the parasite. The reaction is read in a fluorescence microscope after adding an anti-human Ig antibody conjugated to a fluorochrome. This technique (IFAT) has the advantage of providing a quantitative result by the accurate determination of antibody titer (Carrera *et al*., 2010). This factor is particularly useful for therapeutic evaluation. However, it is a more complex technique in relation to other serological methods and requires skilled and trained technical personnel for both the antigen preparation and reading the slides. This technique has demonstrated high sensitivity and specificity, with minimal cross-reactivity with sera from patients that were positive for other helminthic infections (Feliciano *et al*., 2010). In an attempt to update the immunological and molecular diagnostic methods for the diagnosis of human strongyloidosis, the different methods currently employed were analyzed. It demonstrates the necessity of developing innovative methodologies capable of maintaining diagnostic accuracy, particularly for regions with limited technological resources (Levenhagen & Costa-Cruz, 2014).

To test the binding properties of the generated antibodies on cells from cultures and seawater samples, an indirect immunofluorescent labeling assay was used, where the specific antibodies were bound with secondary antibodies containing a fluorescent molecule that can be detected by fluorescent microscopy. Immunofluorescence of cultured whole algal cells was carried out by an epi-fluorescence microscope and confocal laser scanning microscope to identify the recognition by these

mouse antisera, and their degree of specificity. All sera tested were able to recognize the toxic dinoflagellate. The antibodies specifically labeled the two tested strains (AL1V and AMP13) of *Alexandrium minutum* and did not show any cross-reaction with other species (MDQ1096, SZN19 and SZN12) or screened members of other phylogenetic classes (Carrera *et al*., 2010). Interestingly, some other studies have reported that etiological diagnosis of bovine aspergillosis can be accomplished by indirect immunofluorescence staining and indirect immunohistochemical techniques. A rat IgM monoclonal antibody (EB-A1) against the galactomannan of *Aspergillus fumigates* (Stynen *et al*., 1992) and a murine IgG1 monoclonal antibody ($1A_7B_4$) reacting specifically with somatic antigen of *A. corymbejeru* (Jensen *et al*., 1996) were applied in immunofluorescent assay and were found more sensitive than the latex agglutination assay (LAT) techniques.

Flow cytometry

Flow cytometry is a method that principally measure optical and fluorescence characteristics of a single cell or particle of a nuclei, microorganism or chromosome preparation (Brown & Wittwer, 2000). It gives more information about infectious agents than any other available method. While cytometry equipment remains comparatively expensive, there still exist expanding understandings that use of these machines is necessary in endemic populations at or near points of care. The important signals provided about cells by cytometers cannot be neglected even because of the speed at which results are obtained. This has also made cytometry to become of particular significance because it overcomes the shortcomings associated with the non-cytometric methods (Kaushansky *et al*., 2012).

Historically, there have been reports of well-known shortcomings during examinations of the inhibition of growth/invasion of the malaria parasite for measuring the effectiveness of anti-malarial treatments. This is no different, whether by using drugs or antibodies through the use of microscopy or radioactive uptake which were both considered as gold standards (Grimberg, 2011). With the advent of flow cytometry and use of fluorescent DNA stains, speed, ease of reproduction and estimated quality of the effectiveness of drugs and antibodies have been increased to limit parasite growth as oppose the challenges of traditional techniques (Grimberg, 2011). The development of a simple flow cytometric assay to quantitatively assess *Plasmodium falciparum* infection *in vitro* in both low and medium throughput has been described. Utility of this assay in drug inhibition of infection and measuring the efficacy of antibodies in blocking the parasite has shown that this method will help in assessing functional antibody responses to vaccination and novel drugs that prevent mosquito-to-man transmission of malaria (Kaushansky *et al*., 2012).

In a study, where flow cytometry and light microscopy were used to estimate the infectivity potential of *Leishmania infantum* isolates infecting THP-1 cells *in vitro*, results revealed that the number of the parasite per cell in culture and infected cell percentages matched in the two different methods. It was suggested that the survival and rate of multiplication of an isolate within the macrophage environment is an important factor to determine the infectivity potential of the isolate and disease manifestation. Conclusion was drawn that flow cytometry can be used as a rapid, easy, reproducible and cost effective method either in biological, epidemiological or clinical tests to study the infectivity potential of the Leshmania isolates, particularly for the evaluation of drug efficiency (Kanellopoulos *et al*., 2014).

In conclusion, advancements in biotechnology have led to a large scale production of monoclonal antibodies for use in many diagnostic assays. These assays are frequently employed either for the detection of an immunogenic agent or any of its antigenic component. Future modifications of these assays and their application will no doubt help to ease early detection and diagnosis of emerging infections for proper control and preventions.

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