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Production of Polyclonal and Its Application in Immunoassays: A Systematic Review

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ABSTRACT

According to their antigen-receptor specificity, lymphocytes are believed to generate approximately one thousand clonal populations in the immune systems of the majority mammals. Due to their heterogenous structures, polyclonal antibodies can bind to numerous antigen epitopes. The polyclonal antibody is more likely to successfully attach to the specified antigen under a variety of different test conditions, despite decreasing specificity and possibly increasing non-specific reactions. In a wide range of immunoassays, polyclonal antibodies are essential reagents. Production of polyclonal antibodies involves numerous steps. However, it is possible to achieve the greatest immune responses while minimizing the discomfort of the animal by carefully planning an immunization protocol. The choice of the experimental animals, the immunization protocol, the use of adjuvants, the route of administration, blood sampling, serum collection, and purification of the antibodies collected are all factors that must be taken must be taken into consideration. The crucial steps in the production of polyclonal antibodies are discussed in this article, with a focus on their use in immunoassays.

Keywords: antibodies; immunization; immunoassay; polyclonal; production

INTRODUCTION

Antibodies are useful instruments in the laboratory. Monoclonal antibodies are produced by a single B lymphocyte clone, whereas polyclonal antibodies are produced by a combination of different B lymphocyte clones. Both products have developed into essential tools for basic immunological study, immunohistochemistry, diagnostic testing, and vaccine quality assurance. According to their antigen-receptor specificity, lymphocytes are thought to generate approximately 1000 clonal populations in the immune systems of the majority of mammals. Due to this diversity, the immune system is able to react to a variety of immunogens, such as foreign proteins, polysaccharides, peptides, and bacterial and viral components [1].

There are two main approaches for producing antibodies in animals, and each has advantages and disadvantages depending on the intended use. The monoclonal antibodies produced by such hybridoma cell clones originating from a single B lymphocyte are identical, and specific for a single epitope. The great monospecificity of monoclonal antibodies can be a drawback in some circumstances. The development of assays that can distinguish between two compounds that are quite closely related is made possible by the monoclonal highly specific character. Contrarily, polyclonal antibodies specificity is based on a combination of hundreds or even thousands of clonal products that bind to various antigenic determinants. Because of this, minor alterations in the antigen's structure brought on by genetic polymorphism, heterogeneity in glycosylation, or modest denaturation often have no impact on the binding of polyclonal antibodies. When variation in the target material is known, this lack of specificity may be advantageous and polyclonal antibodies may offer a more reliable test [2].

The desired use of the antibody, as well as the amount of time and money available for its

manufacture, should be accounted for when deciding whether to produce monoclonal or polyclonal antibodies. Monoclonal antibody manufacturing is laborious and takes 3-6 months. In addition to immunizing animals, cell cultures are necessary. The immunostaining of western blots, ELISA, the affinity purification of proteins, and the immunostaining of thin tissue slices viewed by light or electron microscopy are a few applications for the usage of monoclonal antibodies. The immortalized hybridoma provides an endless source of high-quality, standardized antibodies.

Polyclonal antibody induction typically takes 4 to 8 weeks. The serum can be used in a variety of processes, including enzyme-linked immunosorbent assay (ELISA), double diffusion, radial immunodiffusion, western blot, and radioimmunoassays [3]. Polyclonal sera often have high titres and allow for significant dilution, but there may be batch-to-batch variability. A polyclonal antiserum is advantageous for use since it may be acquired quickly and for a low cost. A polyclonal antiserum can be used in research to help with a variety of concerns.

Immunoassay procedures are inexpensive and simple to perform and as a result have been extensively used as an analytical tool in a variety of molecular detection applications. Antibodies could give highly precise recognition of an individual drug or molecule in such tests, and preliminary concentration and extraction procedures are not necessary [4].

We thus propose a review of the literature to assist researchers who will be producing polyclonal antibodies for testing based on immunological reactions. We did not consider data from the literature describing the production of monoclonal antibodies in this study, instead focusing on the production of polyclonal antibodies and their application in immunodetection.

METHODS

A comprehensive search of the PubMed database was carried out. The keywords used in the search is “Polyclonal and Production and Characterization”. We described only data published in the 2013-2023 period to ensure renewal. The inclusion criteria in this study were articles that had titles and contents that matched the keywords, original research articles, full text, year of publication 2013-2023. The exclusion criteria in this study were articles that did not have a complete structure, not full text, and articles reviews.

RESULTS

The results of a literature search were 433 articles, and 35 articles met the predetermined inclusion and exclusion criteria. Some of the literature did not meet the established criteria because literature manuscripts were irrelevant to the topic of discussion, literature could not be found due to errors on the web journal, and literature could not be accessed in full text. A systematic review can be seen in Figure 1.

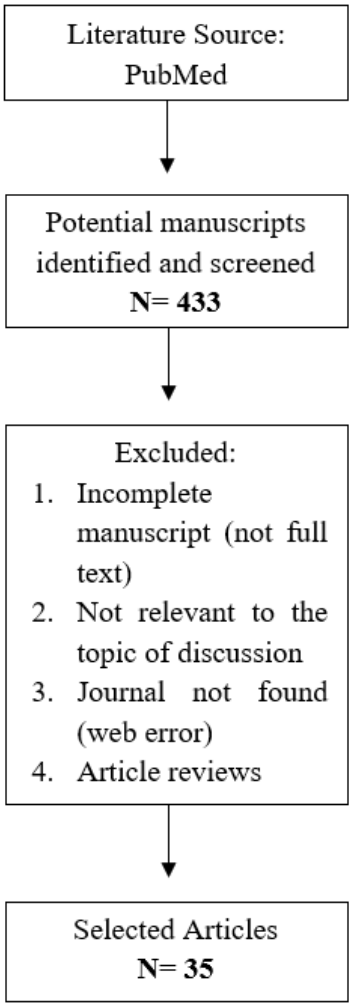

Figure 1. Flow-chart of the study

DISCUSSION

Immunization Protocol

The selection of the animal species, the age at which animals should first be immunized, number of immunizations required, route of injection and maximum volume of injection in the research that has been done are given in Table I.

Table 1. Animals used for the production of polyclonal antibodies

Animal	Age	Immunization frequency	Route of Injection	Maximum volume of injection	References
New Zealand White Rabbit	2-7 months	3	Subcutaneous (s.c) Intramuscular (i.m) Intradermal (i.d)	1- 1.5 mL	(5–7)
		4			(3,4,8–20)
		5			(21–23)
		6			(24)
Shincilla Grey Rabbit	2 months	5	Subcutaneous (s.c)	1 mL	(25)
Sprague-Dawley Rat	6 weeks	3	Subcutaneous (s.c)	2 mL	(26)
Balb/c Mice	5-8 weeks	3	Subcutaneous (s.c) Intraperitoneal (i.p)	0.5 mL (s.c) 1 mL (i.p)	(27–29)
		6			(30)
Swiss Albino Mice	10 weeks	3	Intraperitoneal (i.p)	0.6 mL	(31)
Leghorn laying-eggs Chicken	8 months	4	Subcutaneous (s.c)	4 mL	(11)

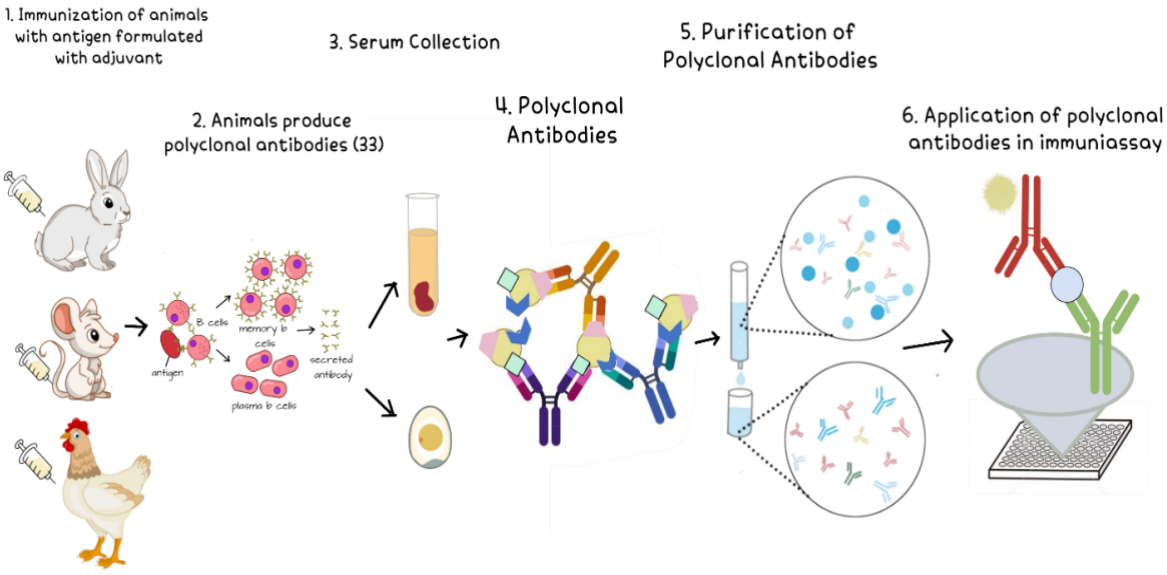

Figure 2. Polyclonal production and its application in immunoassays

Any compound that can trigger an immunological response in an animal when administered is referred to as an immunogen. Since these are the kinds of molecules that are typically found on the surface of pathogens, polypeptides, polysaccharides, glycoproteins, glycolipids, and lipoproteins with molecular weights above 10,000 are considered typical immunogens. Antibodies are made and bind to one or frequently several molecular locations on immunogens called antigenic determinants or epitopes. Antigens are molecules that have an epitope that can be identified and bound by an immunoglobulin or T-cell receptor, in slight contrast to immunogens. All immunogens are antigens, however smaller substances, including peptides with molecular weights below 10,000, could include an epitope but not be able to trigger an immune response when administered alone (haptens). When these haptens are coupled to carrier substances, either in the lab or in vivo, they may develop immunogenic properties [32]. Figure 2 showed an overview of the polyclonal production process and its application in immunoassays.

The animal species chosen for the production of polyclonal antibodies is determined by the amount of antiserum required and the ease of how blood samples may be obtained. The intended usage of the polyclonal antibodies may also play an influence. The antibody that binds to the antigen (the primary antibody) in an ELISA should be from a different species from the conjugated (secondary) antibody that is used in the following phase of the assay [30]. Blood samples from animals that are generally easy to collect (rabbits and mice) should be favored over those that are difficult to bleed. The selection could also be related to the experiment's objective, as each species, strain, stock, or breed of animal may have a different immunological response. For the production of polyclonal antibodies, chickens could be utilized instead of mammals. The synthesis of avian antibodies (IgY) in chickens is regarded as a refinement, as blood collection has been substituted by the extraction of antibodies from egg yolk. Furthermore, chickens may be favored for scientific reasons, such as their evolutionary distance from mammals. Unfortunately, purifying chicken antibodies is more difficult than purifying mammalian antibodies [11].

When utilized as test subjects for the production of antibodies, laboratory animals are typically injected with antigen at least twice. The memory cells that produce class IgG antibodies against the antigen will be activated by the second injection. A pool of antibodies with a greater average affinity is produced as a result of affinity maturation, which also occurs in the memory cells. Mutations in the immunoglobulin gene variable regions cause affinity maturation, resulting in B cells with slightly modified antigen-binding sites. When exposed to the antigen again, B cells with antigen-binding sites that have higher affinity will be driven to proliferate and generate more antibodies than their counterparts with lower affinity [33].

The immunization protocol should reflect the characteristics of the antigen as well as the intended purpose of the antiserum produced. Before beginning the immunization, the researcher should assess the antigen's toxicity. Adjuvants are best defined as substances that boost the immune system's reaction to an immunogen. When injected without an adjuvant, purified proteins are poor immunogens and rarely elicit a strong immune response. Inflammation is one of the requirements for a successful immunological response to an antigen. A critical step in the creation of an immune response, inflammation causes the recruitment of inflammatory cells and the activation of macrophages and dendritic cells. The inflammation has consequences; it can cause different levels of tissue damage and discomfort, which are largely influenced by the kind and intensity of the inflammatory response. The selection of an adjuvant is one of the most crucial and frequently disputed elements in the production of polyclonal antibodies [32]. The ideal adjuvant is a substance that stimulates high and sustainable antibody titer (even with small amounts of antigen) should be effective in a variety of species, applicable to an extensive variety of the antigens, easily and reproducibly prepared in an injection mixture, easily injectable, effective in a small number of injections and low toxicity for the immunized subject. Freund's Complete Adjuvant (FCA), Freund's Incomplete Adjuvant (FIA), and TiterMax™ are commercially available adjuvants used for routine polyclonal antibody production. The choice of adjuvant is, in theory, left to the researcher, however the overall welfare of the laboratory animal to be immunized should take priority. It is suggested that animal care and use committees be involved in the review of immunization procedures in terms of animal welfare. To reduce the risk of contamination, the (antigen and adjuvant) inoculation mixture should be produced

aseptically. It is also essential to determine whether the antigen to be employed requires an adjuvant or has innate adjuvanticity. Adjuvants are typically not required when using whole bacteria, whole cells, or other particulate antigens (for example, cell fractions and bacterial cell walls), but they are frequently required when using soluble antigens (proteins, peptides, polysaccharides). When only a small amount of antigen is available, whenever native antigens are used, or if a specific type of response is desired, an adjuvant may be required.

The frequency of immunization is an essential consideration in the development of polyclonal antibodies. Both the induction of B memory cells and the class switch of B cells (from IgM to other antibody classes and subclasses) can be affected by the interval between two immunization steps. The recommended time between primary and booster immunizations is typically not stated. A booster can typically be thought about once the antibody titer has plateaued or started to drop. This allows for the selection of B-cells that produce antibodies with a higher affinity for the antigen. Timed booster injections encourage the growth of high-affinity memory B-cells, which leads to the production of significant amounts of high-affinity antibody. Boosters that are given too soon after the first vaccination keep levels of immunogen exposure high, put off the selection of high-affinity B cells, and fail to produce the optimal secondary antibody response [32].

In animals with huge muscles, the intramuscular route is routinely employed without issue, allowing for huge amounts of material to be tolerated. For small animals such as mice, this technique was considered neither appropriate nor necessary for adjuvant injections. Although lymphatics can absorb antigen in this region, antigen and adjuvant can spread and reside along interfacial planes between muscle bundles (due to leakage from the muscle bundle or misplacement of the injection mixture) and establish contact with nerve bundles, where serious pathology due to inflammatory processes can occur. Adjuvant mixes should not be injected intraperitoneally due to cause inflammation (macroscopically visible), peritonitis (with the possibility of ascites formation), and behavioral abnormalities (for example, decreased activity and weight loss) [34]. In regard to animal welfare, the subcutaneous route is the best alternative. Researchers should offer scientific explanations for such protocols to ethical committees (for example, the requirement to use exceptionally valuable or unique and irreplaceable antigens, or extremely small amounts of antigen).

To minimize discomfort at the antigen injection location, the injection volume should be kept as low as feasible. These volumes are based on the use of an injectable mixture, such as an immunostimulatory oil emulsion or a viscous gel, which forms a depot at the site of injection. In larger animals, the inoculum should be administered at several injection sites.

Blood Collection

Blood samples should be collected with minimal discomfort to the animal as possible. Animals that will be immunized should be trained to trust the animal care professionals. This is essential not only for the animals' well-being, but also to guarantee that they do not display stress-induced vasoconstriction, which would make blood collecting difficult. Blood collection should take place in conditions that allow the animals to stay warm, which is essential for ensuring adequate blood supply to the periphery. It is also critical that the animals are not subjected to loud noises or other environmental stressors during blood collection or their usual housing. When possible, a non-anesthetic collecting procedure should be used. This may favor the selection of a species where blood sampling in conscious animals is simple for the operator and painless for the animal. Small ruminants and rabbits tend to be advantageous to small rodents. A sedative to facilitate blood collecting is usually unnecessary if an animal is not disturbed during bleeding. However, in large-scale production systems, operators may find that using sedatives to maintain low stress levels and enable quick blood collection is helpful. Because of the difficulties in controlling bleeding from a severed vessel, bleeding rabbits through an ear vein should be done with a needle rather than a scalpel. Exsanguination should be done under general anesthesia and is best accomplished by a cardiac puncture. Following exsanguination, small rodents should be cervical dislocated, and larger animals should be euthanized with an overdose of an adequate anasthetic medication.

When collecting blood for antibody production, it can be beneficial to prepare serum. Since serum remains the gold standard and is necessary for particular assays, laboratories must consider turn-around time, which is an important parameter for

laboratory performance. Some advantages of using serum samples are less cell contamination, the absence of anticoagulants, which prevents any possible interference caused by these substances, and the ability to use the sample for serum protein electrophoresis (the absence of fibrinogen in serum allows for serum protein electrophoresis without any disruption caused by the presence of this protein) [35].

Purification of polyclonal antibodies

Several methods available for purifying antibodies from serum, ascitic fluid, and cell culture supernatant. Antibodies that have been purified can be treated to generate fragments with enhanced properties for use in immunochemistry. The interaction between an antibody and its ligand or antigen target is one of the strongest known in biology. As a result, the specific antibody–ligand interaction will predominate and, in many instances, the presence of other molecules in the antibody manufacturing is of little consequence; therefore, prior purification is not required. In certain instances, however, serum purification may be necessary or preferable because other serum proteins may interfere. Successful protein purification is the result of utilizing one or more of the numerous characteristics responsible for the vast diversity of the protein population, such as charge, size, hydrophobicity, and biological affinity. Immunoglobulins are, fortunately, distinct in a number of aspects, and as a result, an acceptable degree of purity can frequently be attained through relatively simple procedures.

The theory underlying precipitation techniques is complex and poorly understood, whereas the techniques themselves are simple. It is a simplistic but nonetheless useful paradigm to assert that an increase in protein–protein interactions lead to precipitation while protein–solvent interactions promote solubility. Precipitation techniques by themselves do not significantly improve the integrity of a protein solution, but they typically result in an increase in concentration and play an important role in numerous protein purification protocols. Utilizing ammonium sulfate is the most popular method for precipitating proteins. At ammonium sulfate concentrations equivalent to 50% saturation, the majority of immunoglobulins in serum will precipitate. This concentration of ammonium sulfate can be achieved by adding either the required mass of the salt or an equal volume of a saturated solution of the salt. Adiningsih *et al.* [3] showed that seven protein bands produced by the ammonium sulfate precipitation of purified antibodies are likely composed of transferrin, albumin, and other proteins. This result suggests that additional purification steps will result in a purer antibody.

Esparvarinha, *et al.* [13] showed that the IgG was purified from serum through a simple one-step ion-exchange chromatography technique. The immunoglobulin group with elevated isoelectric point (IEP) is an easily exploitable property. In contrast to most proteins, which have values in the pH range of 6.0–7.0, their IEP is in the range of 8.6, whereas that of the predominant serum protein and main "contaminant," serum albumin, is less than 5. Therefore, at most pH values, the charge carried by immunoglobulins will differ from the charge carried by the majority of other serum proteins, allowing the use of high- capacity techniques such as ion-exchange chromatography. Immunoglobulins have a higher isoelectric pH (approximately 8.6) than the majority of other serum proteins. As a result, they will be among the few proteins that do not bind to anion exchange media at neutral pH, providing a rapid and efficient method of purification, especially when combined with precipitation techniques such as ammonium sulphate separation. The most versatile of these is the diethylaminoethyl (DEAE or DE) derivative, which makes agarose-based exchangers the most convenient to use. In addition to having lower flow rates and higher back pressures, it is advisable to precycle cellulose-based alternatives prior to use. The protocol that follows describes a column-based technique, but a batch method using filtration or centrifugation could also be used to separate the antibody-containing supernatant from the ion-exchange medium.

Antiserum was separated from blood using an affinity column containing a Protein A-Sepharose 4 B matrix that binds to the crystallizable fragment (Fc) region of the antibody, as reported by Kokane, *et al* [17]. Several virus-derived proteins with high affinity for the constant regions of immunoglobulins can serve as the basis for affinity purification techniques. For the preparation of affinity chromatography supports, agarose-based chromatography media are a suitable foundation. Protein ligands can be easily affixed to form suitable matrices for affinity chromatography by activating agarose with cyanogen bromide to generate a

derivative that couples to the amino groups of proteins at basic pH. As cyanogen bromide is toxic and forms hydrogen cyanide at acidic pH, it may be beneficial to purchase pre-activated resin. The activation procedure is simple, but an exhaust hood is required due to the toxicity of cyanogen bromide.

Purification of rabbit polyclonal anti-rFgSAP-1 IgG using filtration chromatography was carried out by Kueakhai, et al [5]. The ability of molecules to get through the pores or channels of agarose or dextran beads determines how they should be separated in filtration chromatography, also known as size or molecular exclusion chromatography. The volume that is available for diffusion as a combination of fluid molecules passes through gel beads is dictated by their diameter and the width of the channels they contain. The passage through the column is slower for the smaller molecules since they have more space available to them. Since there is no interaction between the molecules that need to be separated and the column matrix, this delicate method does not require any extreme or possibly denaturing elution conditions. In fact, almost any buffer system can be employed, and because the procedure will separate large protein molecules from small ions and molecules and allow for the transfer of the protein across buffers or the alteration of salt concentration, it is applicable to almost any buffer system. Once the column has been equilibrated, the protein will end up in the buffer. However, the protein will become more diluted, and the capacity (the maximum amount of protein that can be loaded) is low because diffusion is at the center of the separation process. As a result, it is a technique that is typically applied in the last stages of a purification protocol when the protein is in a sufficiently pure condition and the residual components have enough molecular differences to distinguish them.

Application in immunoassays

A wide range of research procedures require the use of prepared antibodies as essential reagents. Since they are heterogeneous, polyclonal antibodies can bind to numerous antigen epitopes. The polyclonal antibody is more likely to successfully attach to the specified antigen under a variety of different test conditions, despite decreasing specificity and perhaps increasing non-specific reactions [32]. The produced antibodies from these processes could be employed in numerous bioassay techniques, including enzyme linked immunosorbent assay (ELISA), western blot, or other techniques where antibodies need to be developed for an immunochemical reaction. Antibody-based immunoassay methods have been essential tools for analysis [3,4,8,24].

The current research showed that polyclonal antibodies can be created in a variety of laboratory animals, and it also indicated that the produced antibodies may be used to detect antigen in an immunodiffusion assay, indicating that they have potential as reagent candidates for an immunodiagnostic kit [3,12,13,22] A potential reagent candidate for an immunodiagnostic test or kit, the specific antibody of the Sumateran wild boar was shown by Adiningsih *et al.* (3) to be feasible to production in rabbit and useful for detecting the antigen of the Sumateran wild boar meat in immunodiffusion test. Esteve-Turrillas *et al*. [12] used two original hapten molecules with a spacer arm attached to different sites of the target molecule, unique polyclonal antibodies have been developed against this contemporary fungicide that enable the sensitive analysis of boscalid. Esparvarinha *et al*. [13] developed a polyclonal antibody against human kappa light chain. Diagnostic kits and biomedical and biochemical experiments benefit from the usage of purified polyclonal anti-kappa. To identify disorders that produce free light chains, polyclonal antibodies against the kappa light chain are utilized. The kappa and lambda light chains are remnants of immunoglobulin synthesis and are normally discharged into the circulation in low amounts in fluids like synovial fluid, serum, and cerebrospinal fluid. A polyclonal antibody (anti-AA) against acrylamide had been discovered by Assaat *et al.* [22] for application in immunochromatographic strip assays for detecting acrylamide in coffee samples.

Enzyme-linked immunosorbent assays (ELISA) based on antibodies are popular due to their robustness and high sensitivity in many common diagnostic procedures for identifying antigen in various samples. The produced polyclonal antibody offers an important substance for the development of an ELISA that is both highly sensitive and selective. Guo *et al.* [4] developed an indirect competitive enzyme immunoassay to identify metallofullerenol. This enzyme immunoassay approach was used successfully to detect metallofullerenol in serum and could lead to a novel method for determining metallofullerenols. Mast *et al.* [14] showed a polyclonal antibody-based sandwich ELISA could

be used as a quick, convenient, and cost-effective method for detecting Alicyclobacillus acidoterrestris spores in various juices in the future, perhaps improving routine analysis. Antispore antibodies have been shown to be very sensitive, even for genetically different strains, as well as species-specific, with minimal cross-reactivity with other bacteria, most likely due to the reduced expression of surface proteins in spores compared to vegetative cells. Mrkvová *et al*. [24] developed a polyclonal antibody for the detection of Tomato Mosaic Virus with adequate criteria (sensitivity, specificity, and cross-reactivity) for serological detection methods (ELISA and Western immunoblotting). This antibody can also be used to screen tomato germplasm, parental genotypes, and breeding lines for Tomato Mosaic Virus, as well as tomato seeds during the seed certification process. Darwish *et al.* (30) produced a polyclonal antibody which is useful for the development of highly sensitive and precise immunoassays for Darunavir total drug monitoring. A very sensitive ELISA was developed using this polyclonal antibody, and the results demonstrated the potential use of this assay to contribute to the safety and efficacy of treatment with Darunavir via its total monitoring drug in patients.

Another application of polyclonal antibodies in immune-based analysis have been developed. Zheng *et al.* [23] developed a polyclonal antibody that could specifically recognize the endogenous and the recombinant HPV58 E7 proteins. This finding provides novel insight into the possible functions of the HPV58 E7 protein and serves as an important first step for the further investigation of the roles of HPV58 E7 in cervical cancer development. Wang *et al*. [26] used immunoblotting, immunoprecipitation, and immunofluorescence testing to assess the sensitivity and specificity of anti-Neuritin antibodies. The results indicate that the produced antibody against Neuritin has excellent immunoreactivity and could be useful for Neuritin detection.

CONCLUSION

A number of crucial processes in the production of polyclonal antibodies can be identified that may influence the outcome in relation to effectiveness antibody responses and minimal pain and suffering for the animals. The following are the steps involved in producing immunoassay-focused polyclonal antibodies: (1) Immunization of animals with antigen formulated with adjuvant, (2) Animals produce polyclonal antibodies, (3) Serum Collection, (4) Polyclonal antibodies were obtained, (5) Purification of Polyclonal Antibodies, and (6) Application of polyclonal antibodies in immunoassays. The ability to produce a good quality of polyclonal antibodies helped us to create various novel immunoreagent combinations, which ultimately led to competitive heterologous immunoassays with higher performance.

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