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Immunisation – Choice of host, adjuvants and boosting schedules with emphasis on polyclonal antibody production

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ABSTRACT

Polyclonal antibodies are frequently used as immunodiagnostic tools in fundamental research. They are also used for routine diagnostic purposes in human and veterinary medicine and for quality control procedures in the food-processing industry.

The antibody is a major component of the detection system. It binds with the molecule to be identified. This conjugate is subsequently revealed by means of binding the antibody with a radio-isotope, a fluorescent substance, an enzyme inducing a color change, or a biosensor based analytical system.

Polyclonal antibodies are also used for treatment purposes in various pathologies. They might have immunomodulating or anti-inflammatory properties. Snake venom, rabies and tetanus antisera are examples of a therapeutic application; immunosuppressive antithymocyte serum used in order to avoid rejection in organ transplantation is another example from human medicine.

These therapeutic aids need hyperimmunisation of animals. Since these are subject to a certain number of interventions such as injections and blood samplings, animal welfare prescriptions have to be taken into account.

The optimisation of the immunisation protocol allows for reducing the numbers of animals used as well as reducing stress and pain while obtaining high quality antibodies.

This article describes the critical steps in polyclonal antibody production with a particular focus on the choice of the animal species, the age of the subjects, the injection protocol and the sampling times.

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1. Introduction

Antibodies are produced by the immune system in response to an immunogen. It is essential to make sure that the target antigen – the molecule capable of binding with an antibody or a cellular receptor, whatever its nature (protein, peptide or else) – is immunogenic. The target antigen should be capable to elicit an antibody response. The immunogenicity depends not solely upon the antigen, but also on the immunisation protocol and on the choice of the host animal.

The immune system functions by means of two main types of mechanisms. Immune responses can be either humoral and antibody mediated or cellular and not involving antibodies.

A polyclonal humoral response is comprised of multiple antibodies having each a different specificity. Most antigens are highly complex and they present numerous epitopes that are recognised by a large number of lymphocytes. Polyclonal antibodies are produced by multiple B lymphocyte clones to the contrary of monoclonal antibodies which are secreted by a single clone of B lymphocytes. Each lymphocyte is activated to proliferate and differentiate into plasma cells and the resulting antibody response is polyclonal. The aim of polyclonal antibody production is to obtain high titer, high affinity and highly specific antisera consistent with current animal welfare guidelines.

The antibody titer is a measure of the quantity of an antibody recognising a specific antigen. Whereas the affinity is characterised by the strength of the antigen-antibody interaction for one epitope, the avidity represents the overall antigen-antibody binding strength. This is because the antigen may be bound by several antibodies, each recognising a different antigen epitope. The polyclonal humoral antibody response cumulates the affinity constants of all binding antibodies. The affinity of a monoclonal antibody to the contrary is selective for one single epitope.

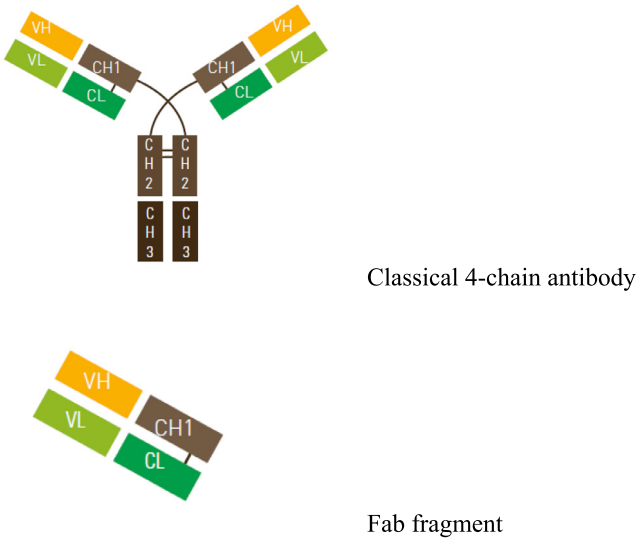

Fig. 2–1. Mammalian four-chain antibody structure and Fab fragment.

The specificity of an antibody refers to its ability to recognise a specific epitope in the presence of other epitopes.

The humoral immune responses following injection of an immunogen to the host animal consists in the production of different types of immunoglobulins which are species-specific. Common mammals such as rabbits for instance, chicken and llamas have different structures and types of antibodies.

Immunisation protocols for polyclonal antibody production comprise an initial immunisation for priming followed by one or more booster injections of an immunogen, according to a predefined schedule.

The best polyclonal antibody results are obtained from antigens that present the most number of desirable epitopes.

2. The immune system of the main animal species used for polyclonal antibody production

Differences exist between the various animal species regularly used for the production of polyclonal antibodies.

Polyclonal antibodies for diagnostic tests use or for therapeutic purposes can usually be obtained in sufficient volumes within reasonable time periods (1 to 3 months).

The humoral immune system – which is the subject of our concern – produces antibodies in the body fluids. The so-called polyclonal antibodies with different specificities and epitope affinities are secreted by multiple B lymphocytes which are produced by the bone marrow in mammals and by the bursa of Fabricius in poultry. However for protein antigens, the activation of the B lymphocytes requires the participation of T-helper cells on the one hand and the participation of dendritic cells and macrophages (antigen presenting cells or APCs) on the other hand. Non-protein antigens are T-helper independent [1].

2.1. Mammals

Mammals produce 5 classes of immunoglobulins (IgG, IgM, IgA, IgD and IgE). They are all made of four polypeptide chainstwo identical heavy chains and two identical light chains, they have thus a 4-chain structure composed of two identical halves, which together form the Y-like shape. Each end of the fork contains an identical antigen binding site. Each arm of the antibody is composed of two variable regions (V_L and V_H) and two constant regions (C_L and C_H). The variable region, composed of 110–130 amino acids, gives the antibody its specificity for binding antigen. The variable region includes the ends of the light and heavy chains. Treating the antibody with a protease can cleave this region, producing the fragment antigen binding (Fab) which consists of the variable ends of an antibody as shown in Fig. 2–1. The antigen specificity is associated with the Fab fragment.

IgGs, IgDs and IgEs exist as 4-chain monomers only, while IgMs have a pentameric or hexameric 4-chain structure. IgAs exist as 4-chain monomers or as dimers [2].

Each antibody molecule recognises a unique antigenic epitope (5–6 amino acids or monosaccharide units) and is able to bind to the immunogen [3].

The primary response is mainly of IgM type while the booster response is of IgG type and presents with a high affinity and a high titer. The IgM response develops within one week after a first challenge. The elicited IgM antibodies are usually of low affinity

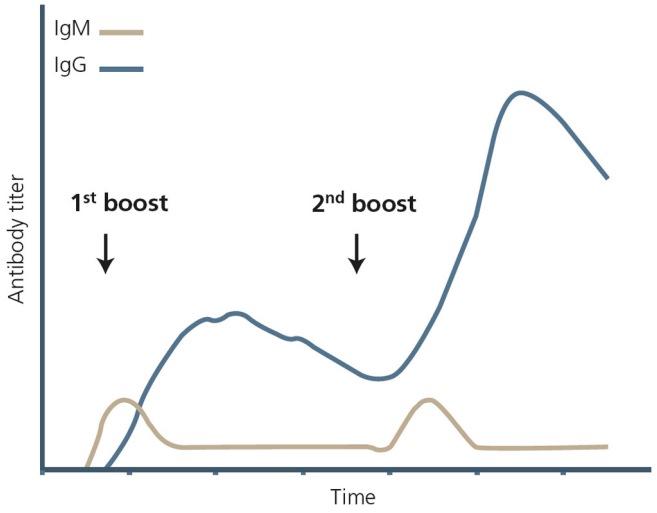

Fig. 2–2. Sample kinetics of mammal IgM and IgG antibodies following initial and booster immunisations. Source [2].

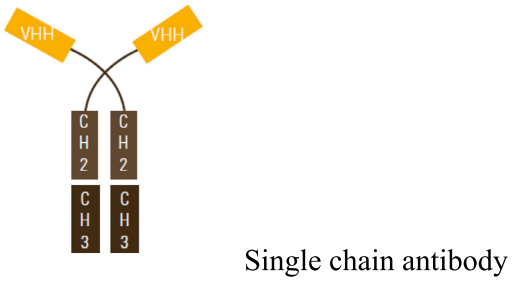

Fig. 2–3. Single heavy-chain only llama IgG2 and IgG3 antibody structure.

and are present at a low titer. IgG antibodies are produced a few days later. The secondary response is quicker, triggers IgG antibodies, peaks 10–14 days following contact with the antigen and lasts longer than the primary response. The production of IgM antibodies is often lower following the booster injections. IgG antibodies have higher titer, higher specificity and higher binding affinity to the antigen than IgMs [2,4].

Each immune response is different. However, sample kinetics curves of IgM and IgG titers following immunisations with a protein antigen administered without adjuvant are shown in Fig. 2–2.

The booster injection elicits a faster immune response with high titers of high affinity IgG antibodies.

The booster response – also called anamnestic response – is mainly due to the stimulation of memory cells.

The addition of an adjuvant to the antigen enables to increase antibody titers and to provide a prolonged response. See Section 4.

2.2. Laying hen

Eggs contain 3 types of antibodies (IgAs, IgMs and IgYs). IgAs and IgMs are mainly present in egg whites and IgY is present in huge quantities in egg yolk [2,5].

Egg yolk IgYs and mammal IgGs have similar structures with two heavy and two light chains. The main difference lies in the constant regions of the heavy chain [2,5] .

2.3. Camelids

Camelids are members of the *Camelidae* family. The IgG2s and IgG3s produced by camels, alpacas and llamas do not possess light chains. These heavy-chain only antibodies contain a single variable domain (VHH) and two constant domains (CH_2 and CH_3) as shown in Fig. 2–3. These single variable domains, with their unique structural and functional properties, form the basis of a new generation of therapeutic molecules known as Nanobodies® [2,6].

3. Preparation of immunogens

Antigens can be customer-provided proteins, lyophilised to be dissolved prior to injection or formulated as a solution. The huge number of epitopes present at the protein surface provides for an efficient way of producing antibodies. Antigens can be more complex and consist of whole cells (bacteria, tissue cells, viruses), cell lysates or mixtures of proteins. In such cases the use of a proper formulation is critical for the success of the immunisation.

Production of high-quality antibodies requires high quality and pure immunogens. The antigen should not be toxic *e.g.* presented as solutions containing agents that are toxic to the host animal such as heavy metals, guanidine hydrochloride, urea at too high concentrations and the preparation should not contain toxic contaminants or pyrogens. Purification of the antigen results in an increased number of specific antibodies, avoiding the need to proceed to removing unwanted antibodies. The quantity of immunogen to be injected depends on the animal species, the adjuvant, the immunisation protocol and on the nature of the antigen [2,7,8].

Small molecules of low molecular weight (less than 8–12 kDa) – so called haptens – require to be coupled to a large carrier protein in order to become immunogenic. Upon immunisation, antibodies are produced against the hapten and against the carrier. The critical step resides in the covalent binding of the hapten to the carrier protein.

Most used carrier proteins are bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH) and thyroglobulin (THY). By choosing the carrier it is important to avoid phylogenic homology between the carrier and source of the protein against which antibodies are to be produced. Linkers and spacers influence the immune response. By definition, the hapten's molecular structure should remain unchanged in order to provide for an immune response which is specific for the hapten upon immunisation with the coupled immunogen.

The synthesis of the hapten-carrier complex is of utmost importance prior to initiating any immunisation protocol [2].

Protein antigens can be injected upon SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The standard Coomassie staining procedure can be used taking into account that the band must be washed thoroughly in water in order to remove acetic acid and methanol residues. The advantage of antibody production with SDS-PAGE gel fragments resides in the fact that the antigen diffuses slowly out of the gel and ensures antigen presentation for a long duration. The main disadvantage relates to the possible injection site reactions. Another disadvantage of using SDS-PAGE gel slices is that the antibodies may not (well) recognise the antigen in its native form due to the fact that the antigen is presented in a denatured form (conformational epitopes are mostly lost) [2].

In most instances a protein antigen is used in its native form or coupled to a carrier. However, the use of denatured protein antigens – by heat or by the use of a reducing agent such as sodium docedyl sulfate (SDS) – rather than the native protein antigen might be interesting. This is particularly the case for identifying allergens in processed food. Antibodies to native proteins react best with native proteins and antibodies to denatured proteins

Table 5–1
Minimum recommended age for immunisation of animals for polyclonal antibody production.

Species	Age
Mouse	6 weeks*
Rat	6 weeks*
Guinea pig	3 months*
Rabbit	3 months*
Chicken	3–5 months*
Goat	6–7 months*
Sheep	7–9 months*
Llama	2 years**
Horse	18 months

* Source: [1].
** Source: [14].

react best with denatured proteins. If elicited antibodies are to be used on membrane blots (proteins subjected to denaturing conditions) then antibodies should be made against denatured proteins. On the other hand, if antibodies are to be used to react with a native protein or block a protein active site, then antibodies should be made against the native protein [7].

Specific peptide sequences – judiciously chosen into the immunogenic part of the protein -might be synthesised and used for immunisations when the protein of interest is not available. This method provides for a highly specific antibody [2].

4. Adjuvants

Experimental polyclonal antibody production is subject to the constraint to obtain – in a cost-effective way – high volumes of high titer and high affinity antisera taking into account the welfare of the host animals.

Some antigens are weak immunogens and some antigens are scarce or expensive. In such cases the concomitant use of an adjuvant is required in order to produce strong and long lasting immune responses.

An adjuvant is defined as a substance that accelerates, enhances or prolongs the immune response when administered with the antigen [1,4]. In a broader sense, adjuvants might also include immunostimulatory substances administered separately from the antigen [1].

Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) are known for their general immunostimulatory properties and are mostly used in experimental antibody production. They are water-in-oil (W/O) emulsions of mineral oil and the complete adjuvant contains also a *Mycobacterium tuberculosis* or a *M. butyricum* component. These adjuvants act by depot effect to generate prolonged and sustained high antibody titers. Freund's adjuvants are considered as the gold standards despite their association with possible injection site and distant site reactions [9]. However recent FCA formulations cause less adverse reactions due to changes in oil refining procedures and improvement in purification techniques [9]. Also, to date FCA is being used better *i.e.* at lower volumes and avoiding improper injection sites [8]. The Canadian Council on Animal Care (CCAC) [7] recommends using FCA only for initial subcutaneous immunisations.

There are numerous alternative commercially available adjuvants [10].

They are oil-in-water (O/W) emulsions such as *e.g.* the RIBI Adjuvant System® and the Syntex Adjuvant Formulation® or W/O emulsions such as *e.g.* Specol, Montanide™ adjuvants and TiterMax®. Both types of emulsions have an antigen depot effect – however more pronounced for the W/O emulsions – and provide a prolonged exposure of the immune system to a low level of antigen [9]. Oil-in-water emulsions are recommended with hydrophobic antigens while W/O emulsions work better with hydrophilic antigens or with antigens bearing both properties [1].

Saponin based adjuvants such as Quil A elicit the formation of immune stimulating complexes (ISCOMs). The use of ISCOMs as adjuvants for experimental polyclonal antibody production is limited by their complicated and labor-intensive preparation [11].

Mineral-based adjuvants such as aluminum salt adjuvants are known to increase responses against weak immunogens. Aluminium salts have effects, in addition to their depot effect, that account for their adjuvant properties *i.e.* pro-phagocytic effect, activation of the pro-inflammatory NLRP3 pathway and enhancement of both T helper 1 as well as T helper 2 cellular responses [12].

Adjuvants containing the immunomodulatory N-acetylglucosaminyl-(b1-4)-N-acetylmuramyl-L-alanyl-D-isoglutamine and the immune enhancer cimetidine (such as the Gerbu® adjuvants) are well tolerated but appear not to produce adequate polyclonal antibody responses [9,13].

The choice of the adjuvant is dependent upon its effect on the antigen. The adjuvant should preserve the conformational integrity of an antigen and present the antigen to the appropriate effector cells [9]. Selecting the best adjuvant might need to perform a preliminary study comparing different adjuvants in combination with the antigen of interest.

When preparing the antigen/adjuvant aseptic mixture, the mixing process of the immunogen with the adjuvant is of utmost importance in order to ensure stability of the injectable product. Inappropriate emulsification resulting in a non-homogenous mixture is a major cause of immunisation failure [7,8].

Publications on adjuvant comparisons with regard to their safety profile have led to variable conclusions. A variety of factors impact on the lesions produced by the adjuvant; they include the antigen, the host species, and the administration route [9].

5. Choice of the host animal species

The choice of the animal host depends on the quantity of antiserum needed, the ease of sampling the animal and the homology with the antigen.

Prior to initiating an immunisation protocol, particular attention should be paid to the choice of the animal species. The investigator should take the following into account:

- the amount of antiserum needed and the ease of sampling,
- the phylogenic relation between the source of the antigen and the animal species used for producing the antibodies. Highly conserved mammalian antigens may not induce good antibody responses in rabbits because the rabbit might not recognise the protein as being foreign. In such a case it is being advised to choose for the chicken as host in order to overcome the homology issue,
- the usage made of the antibody: for an ELISA application for example the primary antibody should be produced in a different animal species than the secondary antibody (since the secondary antibody is directed against the species of the primary antibody),
- the need to check whether the non-immunised host's serum does not cross-react with the antigen.

The recommended minimum age for immunising animals for polyclonal antibody production is presented below in Table 5–1.

5.1. Rabbits

The rabbit belongs to the family of the *Leporidae* and to the order of the *Lagomorpha*. The rabbit is a mammal of choice for

producing polyclonal antibodies due to the ease of housing the species, the ease of manipulation, the lifespan (5–8 years) and to the important volume of serum produced in comparison to rodents.

They produce high-affinity antibodies at a high titer. Blood sampling is relatively easy at the ear (marginal ear veins) or by means of cardiac puncture for terminal sampling under anaesthesia. The age of the animal is another important element. Young adults (beyond 14 weeks) are preferably used because of their better immune response.

Does are preferred to males since they are more docile and easier to manipulate. In addition, current animal welfare standards recommending group housing are favoring aggressive behaviors amongst males with sometimes major resulting injuries.

5.2. Rodents

Rodents are interesting when only small amounts of blood are needed. Most of the time blood is sampled in anaesthetised animals by means of retro-orbital puncture for small samplings or by cardiac puncture for final bleeding. Guinea pigs are used less frequently now than in the past for production of antibodies since they do not offer significant advantages over the rabbit [1].

However, guinea pigs were found to provide superior responses to porcine insulin as compared to rabbits, presumably because of a significant difference in amino acid sequence of their own insulin [1,7].

5.3. Laying hen

The interest in producing antibodies in chickens has increased over the last 25 years. The reason thereof is that antibodies are present in the yolk. This avoids the blood sampling procedure and fits with the Replacement, Reduction and Refinement (3R) approach [3,5]. The egg yolk contains high amounts of antibodies (5–6 times more than in an equivalent volume of rabbit serum).

The production of polyclonal egg yolk antibodies is recommended when there is a homology concern between the origin of the antigen and the host species [1,3].

The main difficulty in producing chicken antibodies is linked to the need for extracting the IgYs from the yolk. The choice of the chicken as antibody producer is subject to the effectiveness of the extraction (preservation of the activity, purity and level of recovery of the antibody) [5].

When choosing for the chicken model the cost of the equipment needed for the purification steps of the IgY antibodies is also an element to be taken into account [3,5].

5.4. Camelids

With a molecular weight of only 12–15 kDa, single-domain antibodies are much smaller than common antibodies. The size reduction of an antibody into a nanobody (in the nm range) provides for valuable applications since enabling to access “hidden” epitopes, that larger antibodies such as a traditional IgG antibody may not be able to bind to *e.g.* the active sites of enzymes. The nanobodies are also able to penetrate into difficult to access tissues, such as solid tumor masses, and can cross the blood-brain barrier without the aid of any transporters. The llama antibodies have high tolerance to extremes in pH and temperature meaning that they retain native structure and biologically active conformation making them ideal for biosensor applications [6].

Table 7–1
Minimum antigen amount to be injected per host species.

Species	Minimum amount of antigen per injection (Molecular weight < 18 kDa)	Minimum amount of antigen per injection (Molecular weight > 18 kDa)
Mouse	40 μg	15 μg
Rat	50 μg	30 μg
Guinea pig	50 μg	30 μg
Rabbit	200 μg	100 μg
Chicken	200 μg	100 μg
Goat	400 μg	200 μg
Sheep	400 μg	200 μg
Llama	400 μg	200 μg
Horse	400 μg	200 μg

Source: [2].

5.5. Farm animals: goats, sheep and horses

Despite they are more expensive and require specific housing facilities, they are preferred when large volumes of antisera are needed and are commonly used for the production of commercial productions such as antitoxins and antivenoms. They are also quite easy to handle, can be bled without anaesthesia and live relatively long. It should be noted however that the horse does not tolerate oil-based adjuvants [1].

6. Administration route

The administration route of the immunogen should be selected with the aim of causing the least adverse effect in the host animal while providing the best immune response. The choice of the administration route is based on the nature of the antigen, the nature of the adjuvant, the animal species and the immunisation sequence course (primary or booster). The effectiveness of the immune response is relying upon the distribution of the antigen to lymphoid tissues.

Routinely the subcutaneous route is being favored, certainly for oily or viscous adjuvanted products while the intramuscular route with this type of products should be avoided in small animal species due to possible local inflammatory adverse reactions. Intraperitoneal injections of adjuvanted products and injections into the foot pad, into lymph nodes or into the spleen are to be avoided if possible. The use of the intradermal route should be restricted to small animals and volumes should be small.

The intravenous route might be the route of choice for small immunogens but adjuvanted antigen preparations should not be injected intravenously.

Chickens are routinely being injected intramuscularly in the breast muscle or subcutaneously, not in the leg due to possible subsequent lameness [5].

7. Amount of antigen and injection volume

The amount of antigen to be injected in order to obtain a satisfactory immune response depends upon numerous factors: the intrinsic properties of the antigen, the animal species and the immunisation protocol.

When associated to the use of an adjuvant, usual doses are in the range of micrograms to milligrams.

Typically, average volume for each individual injection accounts for 500 μL antigen plus 500 μL of adjuvant. Minimum antigen amounts to be injected in each species are tentatively shown in Table 7–1.

When large volumes of immunogen have to be administered it might be useful to multiply the number of injection sites, both for effectiveness (the antigen reaching more lymphoid tissue) as well as for host animal safety reasons.

Table 8–1
Typical blood volumes per host species at the various sampling times.

Species	Blood volumes			
	Pre-immunisation	Small bleed	Large bleed	Final bleed
Mouse	50–70 µL			±300 µL
Rat	1 mL	1 mL	2–3 mL	7–10 mL
Guinea pig	1 mL	1 mL	2–3 mL	7–10 mL
Rabbit	2 mL	2 mL	20–25 mL	50–70 mL
Goat	2 mL	2 mL	250 mL	1 000 mL
Sheep	2 mL	2 mL	250 mL	1 000 mL
Llama	2 mL	2 mL	250 mL	1 000 mL
Horse	2 mL	2 mL	250 mL	1 000 mL

Source: [2].

Table 8–2
Typical sampling of eggs in chicken at the various sampling times.

Species	Eggs*			
	Pre-immunisation	Small sample	Large sample	Final sample
Chicken	1 egg	1 egg	± 10 eggs	± 10–20 eggs

Source: [2].
* Four eggs contain as much antibodies as 50 mL of rabbit serum.

Table 8–3
Sodium pentobarbital (54.7 mg/mL injectable solution) anaesthesia. Dosages in different host species.

Species	Average weight	Sodium pentobarbital 54.7 mg/mL solution:dosage per kg BW	
Mouse	30 g	0.4–0.6 mL/kg	Intraperitoneal*
Hamster	100 g	0.4–0.6 mL/kg	Intravenous or Intraperitoneal*
Rat	300 g	0.4–0.6 mL/kg	Intravenous or Intraperitoneal
Guinea pig	400 g	0.4–0.6 mL/kg	Intravenous or Intraperitoneal
Rabbit	4 kg	0.33**–0.6 mL/kg	Intravenous
Poultry	2 kg	0.25 mL/kg	Intravenous
Small ruminants	40 kg	0.4–0.6 mL/kg	Intravenous

* The 54.7 mg/mL pentobarbital solution administered to rodents are to be diluted 10 times in a NaCl 0.9% solution
** Minimum dose recommended by the Institutional Animal Care and Use Committee (IACUC) [15].

Table 8–4
Xylazine 20 mg/mL injectable solution/ ketamine 100 mg/mL injectable solution anaesthesia. Dosages in rodents and rabbits.

Species	Average weight	Mix	
Mouse	30 g	5 mL xylazine 20 mg/mL inj. sol. 10 mL ketamine 100 mg/mL inj. sol. 85 mL NaCl 0.9%	0.15–0.3 mL Intraperitoneal
Hamster	100 g	5 mL xylazine 20 mg/mL inj. sol. 10 mL ketamine 100 mg/mL inj. sol. 85 mL NaCl 0.9%	1 mL Intraperitoneal
Rat	300 g	5 mL xylazine 20 mg/mL inj. sol. 10 mL ketamine 100 mg/mL inj. sol. 85 mL NaCl 0.9%	0.75–1.5 mL Intraperitoneal
Guinea pig	400 g	12.8 mL xylazine 20 mg/mL inj. sol. 42 mL ketamine 100 mg/mL inj. sol. 3 mL atropine 0.1% 42.2 mL NaCl 0.9%	0.2 mL Intramuscular
Rabbit	4 kg	50 mL xylazine 20 mg/mL inj. sol. 50 mL ketamine 100 mg/mL inj. sol.	0.2 mL Intravenous or 2 mL Intramuscular

8. Sampling of blood and eggs

Depending on the species, blood can be tapped from veins (jugular vein, saphenous vein, cephalic vein, subclavian vein), from arteries (femoral artery, ear artery, tail artery) or by means of cardiac puncture. In rabbits marginal ear veins are used for small volumes and the central ear artery might be used for larger volumes.

Pre-immunisation samples are being taken as negative controls. Small blood samples are being taken at specified time periods in order to monitor the titer evolution. Larger samples are being taken after a 2-month period or so in order to further monitor the antibody response. Final sampling provides with the optimal amount of antibodies.

Typical blood volumes taken per sampling are presented in Table 8–1. Egg sampling is presented in Table 8–2. The frequency of sampling might impact the blood volumes drawn.

Venipuncture requires prior shaving and cleaning and the use of needles in function of the animal size.

Heart puncture is the most practical method for terminal procedure.

Table 9–1
Example of a 87-day immunisation protocol 4 injections/4 blood samplings.

Day	Procedure
0	Pre-immunisation blood sampling Primary immunisation
14	Booster
28	Booster
38	Blood sampling for testing
56	Booster
66	Blood sampling for testing
87	Production/final blood sampling

Source: [2].

Blood samples should not exceed 1–1.5% of the animals' weight. For a 3 kg rabbit sampled on a 2–4 weeks' interval basis this means that 12–30 mL blood is drawn on each sampling.

In rabbits, intermediate samplings are carried out without anaesthesia and usually by venipuncture with a vacutainer blood

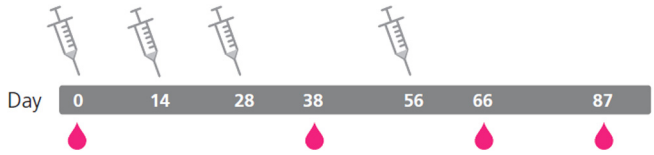

Fig. 9–1. Schematic representation of the 87-day immunisation protocol 4 injections/4 blood samplings

Table 9–2
Example of a 28-day immunisation protocol 4 injections/3 blood samplings.

Day	Procedure
0	Pre-immunisation blood sampling Primary immunisation
7	Booster
10	Booster
18	Booster
21	Blood sampling for testing
28	Production/ final blood sampling

Source: [2].

collection tube with safety-engineered closure. The vacutainers used for obtaining the serum do not contain any additives (red stoppers). Butterfly needles are recommended because they minimise discomfort and allow for the animals to move freely their head during the procedure. The animals should be suitably restrained in a stressless and quiet environment.

In large production units it might be useful investing to sedate the animal in order to lower the stress level and shorten the sampling time.

Blood collection via cardiac puncture in rabbits requires heavy sedation with an analgesic or general anaesthesia and is limited to terminal collections.

Commonly used anaesthetic agents include pentobarbital and the xylazine/ketamine combination [15].

Intravenous (IV) sodium pentobarbital (54.7 mg/mL solution) can be used at the dose of 20–30 mg/kg BW as shown in Table 8–3. The injection should be slow in order to avoid apnea.

A combination of xylazine 20 mg/mL injectable solution and ketamine 100 mg/mL injectable solution can also be administered to the rabbit via IV administration of 0.2 mL of the volume per volume mix as shown in Table 8–4.

9. Immunisation protocol

An optimal immune response requires a carefully designed immunisation protocol. Size and composition of the antigen, choice of the host species, age of the animals, volume/route of injection, schedule of administration, and choice of the adjuvantation are to be considered when designing an immunisation protocol destined to provide the best effectiveness/side effect balance.

9.1. Classical protocol for polyclonal antibody production

Polyclonal antibodies for diagnostic tests use or for therapeutic purposes can usually be obtained in sufficient volumes within reasonable time periods (1–3 months).

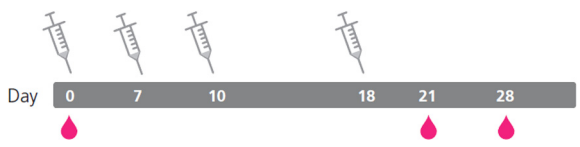

Fig. 9–2. Schematic representation of the 28-day immunisation protocol 4 injections/3 blood samplings.

The typical *in vivo* part of an immunisation program consists of taking pre-immunisation sampling of blood/eggs, initial and booster immunisations, intermediate samplings (for intermediate testing) and final samplings of production batches of blood/eggs destined for further extraction of the targeted immunoglobulins.

An example of a classical 87-day protocol is provided below in Table 9–1 and is represented schematically in Fig. 9–1.

9.2. Speedy 28-day protocol for polyclonal antibody production

Faster immunisation programs – based on the use of a proprietary adjuvant and immunostimulatory compounds – are on the market since 2007. A example thereof is given in Table 9–2 and is represented schematically in Fig. 9–2.

10. Regulatory aspects

Many sets of guidelines on the production of polyclonal antibodies have been issued worldwide over time by national regulatory bodies as well as by various organisations and institutions. They provide guidance on practical aspects of polyclonal antibody production.

Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes was adopted on 22 September 2010. The Directive is firmly based on the principle of the three Rs, to replace, reduce and refine the use of animals used for scientific purposes. It lays down minimum standards for housing and care, regulates the use of animals through a systematic project evaluation requiring *inter alia* assessment of pain, suffering distress and lasting harm caused to the animals. It requires regular risk-based inspections and improves transparency through measures such as publication of non-technical project summaries and retrospective assessment. The development, validation and implementation of alternative methods is promoted through measures such as establishment of a Union reference laboratory for the validation of alternative methods supported by laboratories within Member States and requiring Member States to promote alternative methods at national level.

11. Conclusions

The aim of polyclonal antibody production is to obtain high titer, high affinity and highly specific antisera in sufficient volumes and in a cost effective way taking into account current applicable animal welfare guidelines. The purpose is to minimise pain and distress in the animal while maximising the host's antibody response.

The success of polyclonal antibody production is a complex issue to manage and relies on:

- the choice of the best host for immunisation: species-specific immune system offering distinct advantages in antibody production, age, ease of manipulation and cost of housing
- the nature of the antigen: its size, intrinsic immunogenicity and phylogenic relation to the host animal
- the choice of the most suitable adjuvant: its safety and adjuvantation properties in relation with the specific antigen
- the administration route: knowing that in mammals immunogens are administered in most instances subcutaneously at multiple injection sites but that other routes might in some cases provide better responses
- the immunisation protocol: schedule of administration, immunisation intervals and time to final antibody collection.

Lack of immune response following immunisation can be due to:

- homology between the antigen and the host: the host should recognise the antigen as being foreign
- individual response of the host: status qualified as immunotolerance or unresponsiveness of the immune system
- low intrinsic immunogenicity of the antigen because *e.g.* of its low molecular weight or its composition.

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