Process Design Report
BE 3340

Idealized Process for the Production of Snake Antivenom

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Abstract – Antivenom has been able to neutralize venomous bites for over a century. The process for antivenom production follows the standard procedure of removal of insolubles, isolation of product, purification, and polishing. This general method is constantly modified to incorporate more efficient methods for venom milking and animal injection, plasma separation and fractionation, and quality control. For the purposes of the paper, the Mojave Rattlesnake has been chosen as an example for its toxic venom and wide geographic distribution. This paper selects the appropriate methods to produce 120 mL of Mojave snake antivenom (enough for 1 to 3 doses) from one blood withdrawal (6 L blood) from a Belgian Draft horse. After the snake and animal to be immunized to produce the immunoglobulins are chosen, plasma is separated and collected from red blood cells then stored individually, pooled together, and checked for contamination before fractionation. The IgG molecules are isolated from plasma then fractionated to produce Fab fragments, which are the active ingredient of the serum. Several quality control tests are then used to check the consistency of each batch and compare them to regional standards. Finally, the antivenom is dispensed into vials to be distributed to the United States. These several process considerations are analyzed for production of effective and safe antivenom for Mojave snakes.

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1. Objectives

The purpose of this paper is to specifically describe the steps in converting the raw product of Mojave snake venom to the final product of antivenom. 120 mL of antivenom will be produced, based on the amount of blood drawn (6 L) from one Belgian Draft horse. This will produce 12 vials of antivenom, which is enough for 1 to 3 doses.

2. Introduction

Venomous snakes and their potentially fatal bites are a hazard in many parts of the world. Fortunately, modern medicine has made venomous bites easily survivable through the use of antivenom. In short, antivenom is generally derived from the antibodies produced by select animals that have been exposed to one of many venoms that pose a danger to humans. These antibodies are carefully processed and packaged to be injected into an envenomed person, stopping any adverse affects brought about by the venom. Despite having effective treatments for most dangerous venoms, there are still many obstacles to overcome before venomous animals are no longer a threat.

The first antivenom to be used against venomous snakes was developed by Albert Calmette in 1895. Calmette worked as a toxicologist and bacteriologist for the Pasteur Institute in modern day Vietnam, researching many different toxic and venomous specimens. Through his research, Calmette realized that many animals could be hyper-immunized against venomous serums, including a study where a horse, after months of immunization treatment, was able to withstand an injection 80 times the normally lethal dose of cobra venom. He also theorized that serum extracted from the blood of these animals could be used to effectively treat humans that had been envenomed by the same or closely related species (Calmette, 1908).

The core components of the antibodies useful in antivenom production can be separated into three groups: IgG molecules, $F(ab')_2$ and Fab fragments. Most early antiserums were mostly comprised of Immunoglobulin G molecules (IgG), which were intact antibodies. However, serums derived from these were also contaminated with many proteins not needed to inhibit the venom. The process known as fractionation was eventually applied to break down these antibodies even further by removing the Fc (crystallizable) region from the $F(ab')_2$ and Fab (antigen binding) fragments.

The key difference between these is that IgG and $F(ab')_2$ molecules tend to be much be larger than Fab fragments, meaning that they act at different rates when injected into an envenomed person. When injected, Fab fragments circulate throughout the body quickly, allowing for a faster response time (Meyer, 1997). However, they are also excreted just

as quickly. This can lead to an envenomed person only being partially treated, with the adverse affects of the venom returning once the antivenom has been excreted from the body. In contrast, IgG and F(ab')$_2$ spread throughout the body slowly, and are retained more effectively, allowing for the affects of the antivenom to act over a longer period of time (Scherrmann, 1994).

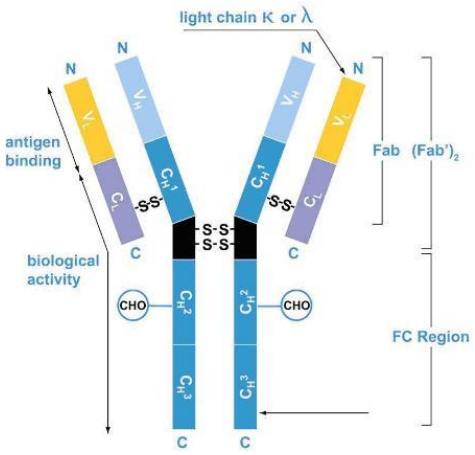


Fig 1. Image: Shows antibody structure, Fc (crystallizable) region is removed, F(ab')$_2$ fragments can be broken down into Fab fragments (source: www.abcam.com)

Most early antivenoms were classified as monovalent, in that they could only be used against one specific type of venom. However, there are still many relevant monovalent antivenoms used against venomous species that are geographically isolated, and cannot be used in conjunction with other types of antivenom effectively. In contrast, there are also polyvalent antivenoms, which are used to treat more than one type of venom. They are often produced to treat a wide range of venomous species of similar geographic distribution.

Polyvalent antivenoms can be produced through two different methods. The first method is to inject the antibody producing animal with several types of venom, so that the blood produced will already contain a mixture of the desired antibodies. One advantage to this procedure is that by injecting the host animal with more than one venom, the hyper-immunization process will often be exaggerated, producing higher levels of the desired antibodies than when using monovalent techniques (Raweetith and Ratanabanangkoon, 2005). The second method is to mix several different monovalent antivenoms, producing a polyvalent antivenom with the desired concentrations. The procedure allows for the production of antivenoms that can treat multiple types of venom from species that are not closely related to one another.

The procedures introduced in this report represent an idealized process for the production of antivenoms that can be used in the treatment of venomous snake bites. For the purposes of this report *Crotalus scutulatus,* also known as the Mojave Rattlesnake, has been chosen due to its wide distribution and the general threat that it poses to the North American human population. The Mojave Rattlesnake is a pit viper located mainly in the southwestern United States and northern Mexico. It is known for the deadly neurotoxin that it produces, nicknamed the "Mojave toxin" (Massey, 2012). Being one of the most common venomous snakes, there is a high demand for effective antivenom. Also, with the consumption of the product being primarily in North America, many of the complications that arise, such as the need to keep the serum at a low temperature, do not need to be considered.

3. Process Considerations

3.1 Venom Selection and Extraction

3.1.1 Milking

Snake venom is collected from the desired snake through milking, and can either be performed by the company or bought from a snake farm. Any snake bought or captured in the wild must be immediately checked by a veterinarian for proper identification and to ensure the snake's health. The snake should be kept in quarantine until assimilated into the herd of snakes kept for milking. The milking process can either be performed by mechanical stimulation of the venom gland or by applying an electrical current to the striated muscles surrounding the gland (Chippaux, 1991). The collected venom may be centrifuged to remove any foreign particles. Fresh venom must be immediately lyophilized or dried to preserve toxicity and stored in a cool, dark area.

3.2 Preparation of Venom Mixture

3.2.1 Detoxification of Venom

Unmodified venom can cause severe adverse reactions in animals at the injection site, so venoms must be detoxified before inoculation. Formalin, tannin, and glutaraldehyde may be added to the venom and later removed by dialysis. These chemicals essentially denature the toxin proteins which cause necrosis of tissue. Ionizing radiation detoxification is a common practice and also efficiently detoxifies venom (Chippaux, 1991). However, addition of an adjuvant that is an emulsifier (such as Freund's Adjuvant) has been shown to properly detoxify venom and eliminates the need for any other chemical detoxifiers (WHO, 2010).

3.2.2 Dilution

The snake venom should be diluted in a solution of either phosphate-buffered saline solution (PBS) or distilled water and filtered through a .22μm membrane (WHO, 2010). Saline solution provides a closer resemblance to the animal's blood and so does not pose a hypotonic threat to blood cells, however, distilled water is cheaper and more readily available than PBS.

3.2.3 Selection of adjuvant

Adjuvants are substances that are added to the venom mixture in order to increase the body's immune response to the venom toxins (CDC, 2010). Five common adjuvants added are Freund's Complete Adjuvant (FCA), Freund's Incomplete Adjuvant (FIA), aluminum salts, bentonite, and liposomes. Freund's adjuvant is a water-in-oil emulsion containing heat-inactivated mycobacterium tuberculosis. FCA has been known to cause infected wounds from granuloma accumulation at injection sites, but can be avoided by using multiple injections of small doses of venom mixtures. Follow-up injections after the initial injection using FIA can also help minimize animal discomfort (WHO, 2010).

3.2.4 Addition of adjuvant

The adjuvant should be added to the venom mixture and mixed or emulsified according to the manufacturer's directions (WHO, 2010).

3.3 Preparation of Immunizing Doses of Venoms

The process of filling syringes for inoculation and dosage formulation depends on the animal used, the size and age of the animal, and the adjuvant added. Manufacturer directions should be consulted when an adjuvant is used (WHO, 2010).

3.4 Selection of Animals

The following considerations for selecting an animal species to be used in antivenom production are: cost of the maintenance, locally prevalent diseases, species availability in the region, age of the animal, and the animal's ability to adapt in a local environment (WHO, 2010). The most preferred choices of species are horses, goats, rabbits, donkeys, and sheep. However, species such as sharks, chickens, camels, llamas, rodents and dogs have all been experimentally used for antivenom production.

For commercial production, equine has been the most preferred of all species also due to the large volume of plasma acquired and their ability to adapt in most climates. The disadvantages of using equine as a donor are due to higher expenses (for raising and maintaining) over other species (WHO, 2010).

3.5 Quarantine and Vaccinations

The quarantine period lasts for 6-12 weeks and allows the chosen animal to adapt to a local environment for 3 months if it is not already an existing regional animal. If it is already an existing regional animal, the quarantine period is decreased. The animal is also given a form of identification (branding, ear clipping, or microchipped) for means of traceability throughout the production process (WHO, 2010).

Depending on the location, the animal is tested for prevalent infectious diseases by using its blood serum and can only continue to the next step of vaccinations if free of disease. Necessary vaccinations are given to the animal. In the case of horses, the vaccinations can include tetanus, rabies, equine influenza, anthrax, brucellosis, glanders, African horse sickness, and equine encephalitis, which ensures its health before entering the immunization program (WHO, 2010).

3.6 Inclusion in the Herd

After a successful quarantine period, the animal is finally released into the herd. Although health surveillance begins prior to immunization, veterinarians should monitor the animal's health and administer vaccination regimes throughout the entire process. An animal should be pulled from the program if it becomes ill or negatively responds to any venom or immunogen injections. If the animal is temporarily eliminated from the immunization program, the time cannot exceed for a month or the animal will be permanently withdrawn.

Proper nutrition should also be followed throughout the program with the feed including appropriate amounts of mineral supplements and vitamins.

3.7 Immunization Program

Each manufacture is responsible for choosing its own amounts of injections, adjuvants, sites of injection, and bleeding schedule. The variations are considered with each type of animal and chosen to achieve the highest antibody response from an animal (WHO, 2010).

The sites of injection usually remain close to the animal's major lymph nodes, which is usually the dorsal neck or back. To prevent the animal from causing irritation to the injected site, the selected areas should remain in places the animal cannot easily scratch. The sites should also be thoroughly scrubbed with a disinfectant, shaved, and rubbed with 70% ethanol. The immunogen containing FCA is subcutaneously injected using a small volume at each site for the primary immunization. The small amounts of volume at each site prevent local tissue damage. The animal is to be injected after 2 weeks with FCI (instead of FCA). Booster shots should be given in 2-week intervals until the desired titer is achieved.

According to the WHO guidelines, "the details of each immunization step should be recorded precisely. The details to be recorded include:
— date of immunization;
— batch(es) of venom(s) used with its (their) reference number(s) (see section 8);
— venom dose(s);
— adjuvant and/or salt used;
— names of the veterinary and supporting staff in charge of the immunization;
—eventual reaction and/or sickness.

The antivenom titre of the immunized animals should be followed throughout the immunization procedure either in vitro, using EIA, during the immunization phase, or in vivo, by neutralization potency assays of lethality when the immunization plateau is reached or before each blood collection."

3.8 Collection of Blood or Plasma

The animal is isolated from the herd and brought to the bleeding room for collection of blood. This process begins with the proper cleansing applied to a jugular vein so that venipuncture can occur. Depending on the size of the animal and desired volume, the time to take blood can range from 30-45 minutes. After drawing blood, the animal is still kept under careful surveillance for any signs of discomfort or distress. The blood should be retrieved and collected in a plastic bag or glass container with proper labeling. The collection container should also be sterilized with an anticoagulant (WHO, 2010).

3.9 Storage and Pooling of Plasma

After collection of whole blood, the next step is to prepare components of the blood that is separated from blood cells for fractionation. In past years, serum was used, but recently plasma is the common component of the blood to use in the process because it is more

advantageous over serum (WHO, 2010). This is because red blood cells (erythrocytes) can be returned to the animal source through reinfusion. Specifically in the past, "antisera from immunized animals" was common in clinical practice to use as immunoglobins against infections (Stiehm, 2008). However, according to the World Health Organization, because plasma is preferred over serum, the term used should be "antivenom immunoglobin" to prevent confusion, since serum is used for therapy purposes (WHO, 2010). Returning red blood cells to the animal source is done to prevent a deficiency of red blood cells (WHO, 2010). Other advantages include an increase collection of antibodies, less contamination, and rapidity compared to the separation of serum from blood (WHO, 2010). The plasma to be separated is hyper-immune plasma, which is pathogen-specific, and is one of the most commonly used antibodies for clinical practices (Stiehm, 2008). The separated components, plasma or serum, are a necessity because they isolate antibodies or immunoglobulins (IGs) for the use of antivenom treatment (Stiehm, 2008).

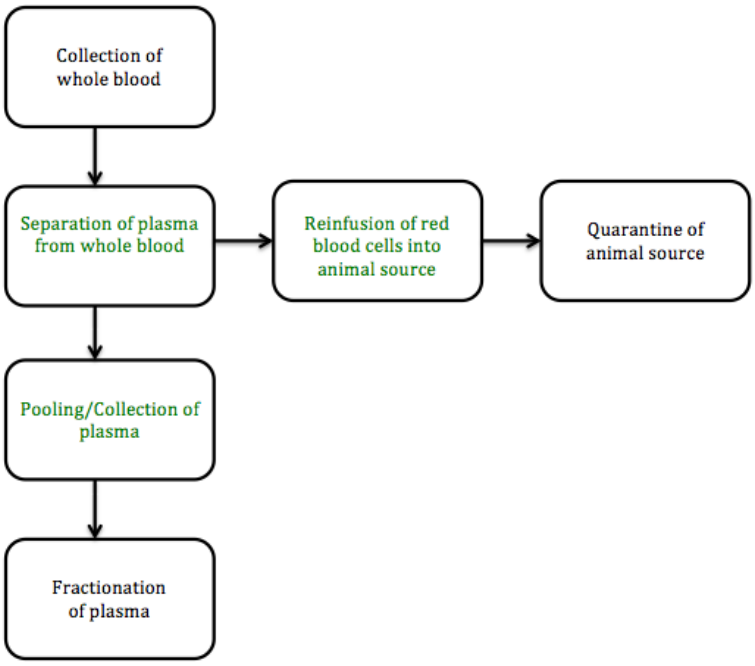


Fig 2. This flowchart shows a visualization of where the separation of plasma takes place and what comes before and after this process in the production of antivenom. The highlighted part is what will be described in this section.

3.9.1 Separation of Blood Components

The methods for separating plasma from the blood of a chosen animal are either by collection of whole blood or by apheresis procedure. The collection process takes from one to four hours (WHO, 2010). Separation through centrifugation by the whole blood

method is performed in clean rooms under aseptic conditions with sterile tools and equipment. The separation of plasma and red blood cells can also be done by gravity sedimentation (Guidlolin, 2010). In gravity sedimentation, the blood-plasma mixture sit overnight and the suspended material separate by the force of gravity. Suspended material are any particles floating in the "source water," which in this case, would be plasma (Sedimentation, 2013). The blood separates from the plasma by settling to the bottom. Sterile containers such as plastic bags or bottles and stainless steel containers are used for plasma storage (WHO, 2010). A laminar flow cabinet is used in the separation of plasma from blood when using plastic bottles (WHO, 2010).

Through the method of separation by apheresis, plasma is separated from the blood and red blood cells are returned back into the body of the animal in order to replenish what they have lost. The red blood cells should be placed in sterile saline solution from a temperature that is between 32 and 37°C before it is reinjected into the animal . This should not take place more than twenty-four hours after blood collection (WHO, 2010). Sterile saline solution is used to keep the red blood cells clean, free of infection, and to keep the cells from drying out.

There are two types of apheresis: As described in the previous paragraph, one is manual plasma apheresis, and the other is automatic plasma apheresis. In automatic apheresis, a plasmapheresis machine is used (WHO, 2010). Automatic apheresis is more advantageous over manual apheresis. Automatic apheresis for the separation of plasma gives more control over the process and is cleaner to use (Gutiérrez, 2011). This method also prevents contamination by other blood cells, produces higher yields, and it makes it easier for the fractionation step (WHO, 2010).

In the step of automatic apheresis, whole blood mixed with anticoagulant is sent through an automatic cell separator, where the plasma is separated from the blood through centrifugation, filtration, or centrifugal filtration (WHO, 2010). At the same time, the red blood cells are returned to the animal source when the plasma is separated out of the blood. The red blood cells are sent back into the animal through the jugular vein (Guidlolin, 2010). The volume of plasma taken out is dependent on the animal. For a horse, six liters of plasma can be pulled out per bleeding period, which is often times between thirty and fourty-five minutes, depending on how much an animal weighs and how much blood is to be collected. After apheresis, plasma is stored in sterile containers in a dark, refrigerated room at 2-8°C until it is needed for fractionation (WHO, 2010).

The anticoagulant solution that is mixed with the blood is composed of AB16 and anticoagulant citrate dextrose formula. AB16 is a mixture of chemicals which includes 35.6 grams of sodium citrate, 12.6 grams of citric acid monohydrate, and 51.0 grams of

glucose monohydrate per one liter of water used for injection. The anticoagulant citrate dextrose formula per one liter of water includes 22.0 grams of sodium citrate, 8.0 grams of citric acid, 24.5 grams of dextrose monohydrate (WHO, 2010).

3.9.2 Pooling of Individual Plasma

The next step before fractionation is the pooling of individual plasma together. Plasma is pooled into sterilized and sanitized containers, and the number of individual plasma gathered for the pool is recorded. It is critical that the room designated for pooling is to be kept clean, sanitized, and free of contamination. It is recommended to add 3 grams per liter of diluted phenol or cresol preservatives to the plasma to prevent microbial contamination (WHO, 2010). Also, 0.25 % -0.35 % of phenol and cresol can be used (Gutiérrez, 2011). Pooled plasma should still be stored at 2-8°C, but can also be stored at -20°C if no preservatives were added. Storage of plasma should not be stored for very long periods of time, such as six months, to prevent contamination before the fractionation step. Any storage below six months is okay, but will not be as effective as storing the plasma for a short period of time, such as a week. The longer the plasma is stored, the less effective it will be, and therefore, the less effective the antivenom product. In fact, the pooling should be fractionated as soon as possible after the pooling step. If prolonged storage should happen, careful measures are made to ensure that the plasma will not badly affect the fractionation process (WHO, 2010).

3.10 Quality Control of Plasma

The final step right before fractionation is to check the plasma pool for any contamination, precipitates, total protein content, pyrogenic substances, and neutralizing ability. Bioburdent tests are completed for bacterial contamination to make sure they do not go below or exceed the stated limit. Pools that go above the limit get discarded, while ones that are under can still be used as long as steps are taken to ensure that they do not affect fractionation. Pools that are below the stated neutralizing limit and hemolyzed plasma are thrown away. The neutralizing limit is a number set by the producer (WHO, 2010).

3.10.1 Neutralizing Ability

One special characteristic of plasma is its neutralizing ability. The neutralizing ability of plasma is a critical important part of the process of the production of antivenom. According to Gutiérrez and Burnouf's *Antivenoms for the Treatment of Snakebite Envenomings: The Road Ahead*, "All quality factors, i.e. efficacy, safety, availability and

cost, largely depend on the neutralizing activity reached by the plasma of immunized animals" (2011).

There are three advantages to a high neutralizing ability. If a high neutralizing ability is attained, the results could be a higher product volume, a higher potency, a lower protein formulation, and affordability (Gutiérrez, 2011). In that case, the optimization of the process of attaining hyper-immune plasma is a necessity in order to have a great quality product. Gutierrez and Burnouf state that in order to achieve this, the animal used, the preparation and control of venom used as immunogens, and the validation of the antibody response assays have to be taken into consideration (2011). The table below, taken from a study on antivenom purity in physiochemical and immunochemical methods, shows that the high titres of plasma give higher results compared to the lower titres of plasma. According to the article, "These observations indicate that the selection of plasma from animals having a high antibody titre is a key parameter for increasing the purity of antivenom formulations" (Segura, 2012).

Table 2
Effect of the titre of anti-snake venom antibodies in initial plasma, on the purity of antivenom.[a]

Groups	High titre		Low titre	
	Plasma	Purified IgG	Plasma	Purified IgG
FPLC analysis (% Igs)	63	93	63	93
Electrophoretic analysis (% Igs)	63	99	58	100
Total protein (mg/mL)[b]	88 ± 2	44 ± 10	86 ± 3	40 ± 3
Anti-*B. asper* antibodies (mg/mL)[b]	18 ± 3	16 ± 4	2 ± 2	5 ± 2
Antibodies/protein ratio	0.204	0.364	0.023	0.125
Potency (mg/mL)[c]	3.98 (2.78–5.71)	3.98 (2.72–5.82)	1.08 (0.79–1.48)	1.63 (1.10–2.42)
Potency/protein ratio	0.045	0.090	0.013	0.041

Fig 3. Source: SciVerse Science Direct, Biologicals xxx (2012)1-5

3.10.2 Sterilization

Pooling and quality control of plasma, as well as storage, are critical and precise processes in the production of antivenom. In each step, everything- all equipment, rooms, people, animals need to be cleaned and sterilized daily. Handlers keep track of records daily and are very precise. Each step requires carefulness, observation, details, precision, accuracy, attentiveness, and slowness to attain great quality. Special attention is needed for the traceability between animal donors and plasma pools. Finally, before manufacturers of antivenom go onto the next process of fractionation, a certification from a veterinarian is needed to document and ensure that the animal is healthy during the time of plasma collection and afterwards (WHO, 2010).

3.11 Purification of Immunoglobulin Molecules

The plasma separated from the equine blood samples must undergo processing to isolate the active molecules used in antivenom. The plasma sample is first mixed with ammonium sulfate to cause the immunoglobulin and other proteins to precipitate. This mechanism operates by increasing the salt content of the plasma solution, thereby reducing the solution's ability to dissolve the contained proteins (Genck, 2000). In this case, the salt concentration is increased to roughly 45%, which is the amount required to precipitate immunoglobulin molecules (WHO, 2010). By doing this, it is possible to limit the amount of precipitate that isn't the desired product. The solution is then filtered or centrifuged to collect the precipitate. In order to increase the yield of this process, it is possible to repeat the precipitation steps.

3.12 Isolation of Fab Fragments

3.12.1 Papain Digestion of Immunoglobulin Molecules

Once collected, the immunoglobulin precipitate are dissolved in a phosphate buffer saline solution, bringing it to a pH 8.4. Papain is then added to this solution to begin digestion of the immunoglobulin molecules (WHO, 2010). Papain is a cysteine protease enzyme that operates by cleaving the peptide bonds in proteins. In this process, it can be used to separate the Fab (antigen binding) fragments from the Fc (crystallizable) fragments in antibodies (Rowe and Fahey, 1965). This digestion is carried out at 37°C for 18-20 hours in a jacketed tank. This reaction is stopped by adding iodoacetamide, which is an irreversible inhibitor of cysteine peptidases (WHO, 2010).

3.12.2 Diafiltration of Fab Solution

The solution is then applied to a tangential flow diafiltration (TFF) system. This is a membrane filtration system that operates by allowing the solution to flow parallel to a membrane, preventing a cake from accumulating while allowing for smaller impurities to be filtered out of the solution (Schwartz and Seeley, 2013). In this case, the system removes iodoacetamide, salts, and peptides with a lower molecular weight than the desired products. The solution is circulated through the system until most of the impurities have been removed, and then is brought to equilibrium with an isotonic sodium chloride solution (WHO, 2010).

3.12.3 Anion-Exchange Chromatography Separation

The prepared solution is then applied to a diethylaminoethyl (DEAE)-based chromatograph. DEAE-based chromatographs are anion-exchangers, meaning that the column will bind negatively charged molecules in the solution. Initially, the solution is buffered to a specific basic pH. The desired pH will leave the Fab fragments at a net zero charge, but most of the other proteins present in solution will have a net negative charge. This will result in column binding of the unwanted proteins, while the Fab fragments flow past with the solution (WHO, 2010).

3.13 Purification of Fab Solution

Once again, the solution is applied to the TFF diafiltration system to remove any excess proteins, salts, and undesired solvents that the chromatography separation may have missed. The solution is adjusted through the addition of sodium chloride, antimicrobial agents, and any other additives depending on the desired uses of the final product (WHO, 2010).

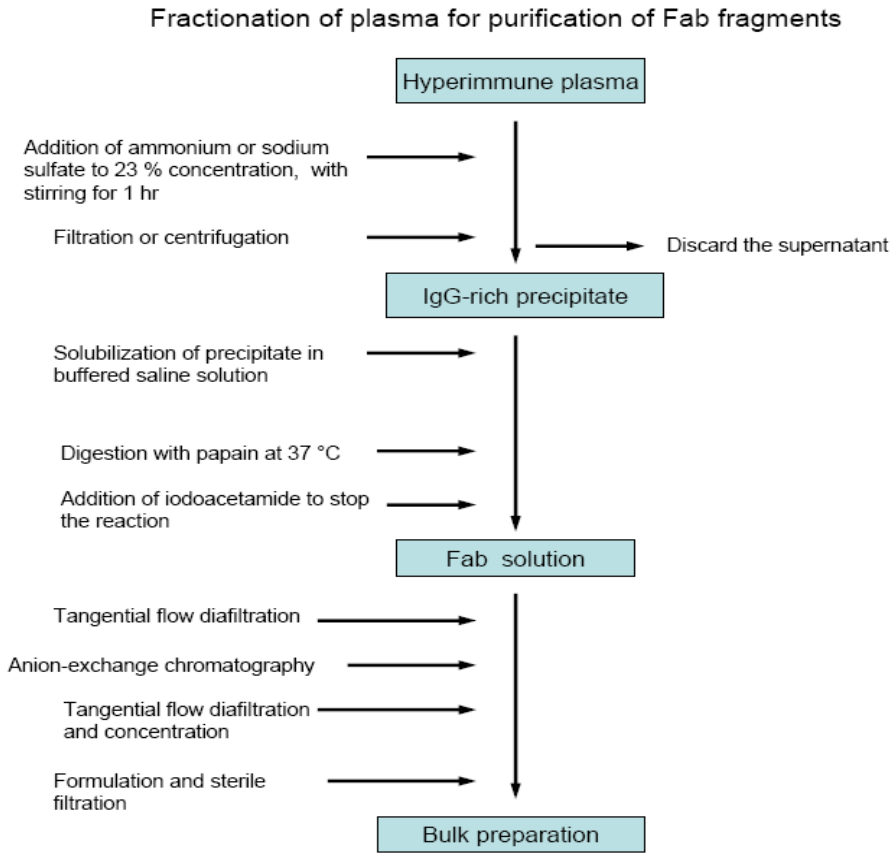


Fig 4. Summarizes the process of separating and digesting IgG to yield Fab antibody fragments (WHO, 2010).

3.14 Quality Control

Quality control is a flexible process consideration for antivenom depending on the demand and funds available for production. Thus the main issue with quality control is to balance the profit margin between cost and quality. Since production quality heavily relies on government funded infrastructure, quality control highly depends on the government's desire for antivenom (Simpson, 2008). If a government is experiencing financial strain keeping their regular health care services running then quality control for antivenom will not be a priority.

Quality control for antivenom concerns the amount of adverse reactions people will have to the product. Reports of high reaction levels around 80% are common in antivenom, but can range as low as 5% (Theakston, 2003). If these reactions are easily treatable and not life-threatening then such a low quality is not frowned upon (Simpson, 2008). There has been no difference in the amount of adverse reactions when using caprylic acid precipitation versus pepsin and papain digestion during production (Simpson, 2008).

Like all products, reference standards have to be used to ensure consistent quality. National or regional reference venoms cover the variability of geographic snakes. Snake species are classified usually by physical features instead of DNA analysis for timeliness and cost reasons. Geographical origin should also be noted to prevent venom composition differences between populations. These documentation steps allow venom traceability (WHO, 2010). In many cases, national standards are not used because of the high variability of snakes within the nation. Instead, national regulatory agencies will determine tests for manufacturers to use to analyze their own quality and consistency. Such tests include potency, pH, and visual inspection (WHO, 2010) and are examined below. Classification of snakes used for these references must be thoroughly documented. Reference standards must be made from a pool of at least 50 same species snakes. After characterization, the pool should be lyophilized and stored at -20°C in aliquots (Theakston, 2003).

To achieve the appropriate protein concentration for a high quality product, ion-exchange chromatography can be used to remove Fc then affinity chromatography can be used to concentrate the antivenom. The potency and residual reagent detection tests can be used on the final bulk whereas the other tests must be performed on the final product. Venom consistency is also quality tested by verifying biochemical characteristics. There are several additional tests to be run for quality control. These tests are normally performed in a quality control laboratory and are further described below (WHO, 2010). They include appearance, solubility, potency, median lethal dose assay, median effective dose assay, osmolality, biological assay, immunological identification, gel electrophoresis,

size exclusion liquid chromatography (HPLC), pyrogens test, sterility test, pH, removal of chemical reagents, and residual moisture.

3.14.1 Solubility Test

Appearance is analyzed visually to determine if the batch is the correct color. The cloudiness of the antivenom is also noted. A room temperature solubility test is run for freeze-dried antivenoms by adding solvent and timing the dissolution. A poor quality product does not dissolve within 10 minutes (WHO, 2010).

3.14.2 Potency Test

A potency test is the most important because if the antivenom cannot neutralize the designated venom then the product is useless. This test consists of a median lethal dose assay (LD50) in at least five mice of 18-20 grams. A range of acceptable potencies is created be intravenously injecting a solution of venom doses in saline solution then marking the number of deaths after 24 hours. In this way, the lethality of each dose is determined by seeing what venom amount kills 50% of the mice. The median effective dose (ED50) assay further tests these potencies by incubating a venom amount with antivenom at 37°C for 30 minutes. The mixture should not be centrifuged in case the precipitant includes toxicity. Again, number of deaths after 24 hours is recorded and the median effective dose is determined from the antivenom amount that saves 50% of the mice. Of course, this dose can be designated by regulatory agencies to ensure consistent quality in laboratories. These assays, though, must be performed before human use (WHO, 2010).

3.14.3 Concentration Tests

Other quality control tests include an osmolality test and additional identification assays. Under the osmolality test for tonicity, an acceptable antivenom is at least 240 mosmol/kg. This information can be used to determine the salts added during production. Identification of each batch can be done by biological assays and even immunological identity tests to check the production source of the antivenom. Protein concentration is determined via nitrogen determination with the Kjeldahl method and must yield a protein concentration of 10 g/dl or less (WHO, 2010).

3.14.4 Purity Test

Many other quality control tests ensure a reliable product purity. Absorbance can also be measured to check quality. Purity of immunoglobulins can be verified by gel

electrophoresis which would detect IgG, F(ab')2, Fab, non-immunoglobulin plasma protein contaminants and degradation products. Specifically, under 1% of the total protein should be albumin. The composition purity can be determined under SDS-PAGE non-reducing or reducing conditions. Then HPLC can be used to determine the molecular size distribution and densitometric analyses of chromatographic profiles can be used for protein quantification (WHO, 2010).

Concentrations in liquid antivenom are verified using small-scale affinity chromatography, while size-exclusion gel filtration can be used to verify the purity and stability of the batch (Al-Abdulla, 2013). The small scale affinity chromatography is used to set reference standards of concentrations by regulatory agencies.

3.14.5 Endotoxin Limits Test

The antivenom must also meet a test for pyrogens in rabbits to meet established endotoxin limits. An additional abnormal toxicity test is sometimes used. A sterility test then ensures the antivenom is not contaminated by bacteria or fungi. This test first requires neutralization of preservatives by filtering the antivenom through a 0.45-μm pore membrane. Then the antivenom is added to culture media, incubated for 14 days at 20–25 ºC for trypticase soy broth and 30–35 ºC for thioglycolate while the bacterial growth is monitored. A potentiometer is used to determine if the antivenom is at an appropriate pH. Preservatives quantification must present phenol concentration less than 2.5 g/l via spectrophometrics and cresol concentration less than 3.5 g/l via HPLC (WHO, 2010).

3.14.6 Removal of Chemical Reagents

Chemical reagents used in the production process must be removed from the antivenom for a quality product. This can be achieved via diafiltration or dialysis until the established acceptable residual amount is achieved. This step is crucial for the chemical reagents pepsin or papain so that proteolytic activity is ceased the prevent harm to the antivenom. Removing the chemical reagents can also be achieved during the manufacturing process instead of the quality control. Freeze-dried preparations require residual moisture tests such as gravimetric methods or the more common Karl-Fisher titration. The amount of stability and shelf-life required determines the level of residual moisture allowed, usually under 3%. Venoms that need long-term storage should be dessicated often to ensure stability (WHO, 2010). Instead of removing chemical reagents as a quality control step, they may be removed during the Purification of the Fab Solution (above section 3.13).

3.14.7 Sterilization

Another consideration in quality control is to prevent transmissible agents. Such agents include HIV from chimpanzees, Marburg and Ebola from monkeys, Hantaan and Lassa from rodents, Nipah and paramyxoviruses from pigs, and equine morbilli virus (aka Hendra) in horses (Theakston, 2003). Alternatively, the virus may be removed during the production of the antivenom with the processes of low pH pepsin digestion, caprylic acid, or pasteurization (Theakston, 2003), all of which would inactivate lipid-containing viruses. Heat treatment at 58-60°C can also be used during production to inactivate viruses (Gutiérrez, 2011). For more robust viruses, nanofiltration is used. However, nanofiltration is inefficient when whole IgG is used (Gutiérrez, 2011). A more recent, dependable method to rid the antivenom of viruses is to use dedicated solvent-detergent treatment on the horse plasma before caprylic acid fractionation (Mpandi 2007). These process steps can be performed under the Collection of Blood or Plasma (above section 3.8) rather than the quality control tests.

3.15 Labeling, Packaging, and Release

3.15.1 Storage

After quality control tests, the antivenom is either kept in liquid form or lyophilized. Liquid antivenom has shown long term stability at ~25°C but short term stability at a temperature of 37°C (Al-Abdulla, 2013). The WHO (2010), however, recommends storage at 2°C-8°C to prevent product deterioration. If the cold chain cannot be ensured during transportation or storage then the antivenoms should be lyophilized. Thus, freeze-drying would be favorable in undeveloped countries. Freeze-drying also extends shelf life from 3 years to about 5 years (WHO, 2010).

3.14.2 Packaging for Sale

The final product is dispensed into labeled vials then packaged into cardboard boxes which are also subsequently labeled (WHO, 2010). The antivenom must be kept at 2°C-8°C during transportation and can then be distributed to its respective region (WHO, 2010).

4. Chosen Methods

4.1 Venom Selection and Extraction

4.1.1 Venom Selection

Lyophilized venom will be purchased from Sigma Aldrich (Table 1).

4.2 Preparation of Venom Mixture

4.2.1 Detoxification of Venom

The venom will be heated to 100 degrees Celsius for 5 minutes, then allowed to cool for 1 hour at 25 degrees Celsius.

4.2.2 Dilution

The venom will be diluted with PBS, as it elicits a better response in the animal for innoculation. For each dose the amount of lyophilized venom will be diluted with an equal amount of mL of PBS, then filtered through a .22 micron membrane to prevent any granulated venom particles from moving further through the inoculation process.

4.2.3 Selection of adjuvant

Freund's Complete Adjuvant was chosen for the first injection and only the first. The second adjuvant added will be Freund's Incomplete Adjuvant, and additional injections following will be adjuvanated with sodium alginate. Freund's Complete and Incomplete Adjuvants are very effective in eliciting an immune response, but several uses can cause pain and infection in the animal. Sodium alginate will be used in order to prevent granulation in the animal's skin.

4.2.4 Addition of adjuvant

Each dose of venom will be adjuvanated with an equal amount of adjuvant, as per manufacturer's directions. The sodium alginate will be rehydrated with PBS prior to addition to the diluted venom.

4.3 Preparation of Immunizing Doses of Venoms

Table 1. Price of Immunization Doses

	Item	Amount	Price
Dose 1	Venom	1.5 mg	$ 0.38
	PBS	1.5 mL	$ 0.05
	Adjuvant	1.5 mL	$ 4.05
Dose 2	Venom	3.0 mg	$ 0.76
	PBS	3.0 mL	$ 0.10
	Adjuvant	3.0 mL	$ 7.44
Dose 3	Venom	9 mg	$ 2.28
	PBS	18 mL	$ 0.60
	Adjuvant	9 mg	$ 1.15
Dose 4	Venom	18 mg	$ 4.55
	PBS	36 mL	$ 1.20
	Adjuvant	18 mg	$ 2.30
Dose 5	Venom	36 mg	$ 9.11
	PBS	72 mL	$ 2.41
	Adjuvant	36 mg	$ 4.61
Dose 6	Venom	45 mg	$11.39
	PBS	90 mL	$ 3.01
	Adjuvant	45 mg	$ 5.76
Dose 7	Venom	50 mg	$12.65
	PBS	100 mL	$ 3.34
	Adjuvant	50 mg	$ 6.40
Total Price	$		83.54

4.4 Selection of Animals

The Belgian Draft was chosen due to its high population in the United States and ability to produce a large volume of plasma at minimal cost. It is a powerful horse breed that weighs approximately 2,000 pounds on average and is easily manageable due to its calm

temperament. They are expected to live anywhere between 20-25 years long (HBL, 2009).

A 6-year old gelding "Rock" was chosen from a New Hampshire breeding farm with a weight of 2,350 pounds. A gelding is a castrated horse, which lowers any male aggression that could possibly occur throughout the program. Possible minor complications (edema, peritonitis, hemorrhage) due to neutering the male occur shortly after the procedure, so buying a horse that was neutered at a young age was most ideal. "Rock" has a temperament of 2/10, which identifies his usual calm state. A 10/10 on the temperament scale would indicate difficulty with handling (not wanted for antivenom production). A veterinarian checked the horse's health and performed physical exams to ensure proper health needed for entering the immunization program.

Fig 5. A Belgian Draft Mare. (http://www.benmarfarm.com/irishdraughtsporthorsessold.html)

4.5 Quarantine and Vaccinations

After branding the Belgian to give it proper identification, the horse is to be secluded for only 9 weeks since it was raised in the country and already has prior exposure to the local ecology. It is withheld inside a barn and kept away from any contact other than the caretaker or veterinarian. Bedding, water and food containers are changed every day and kept sanitary. Any cleaning or handling equipment is kept separate from the barn since any contamination from used equipment could transfer disease.

The horse receives the following annual vaccinations throughout the entire program: tetanus, Equine Encephalomyelitis, west nile virus, rabies, influenza, and Potomac horse fever (AAEP, 2008).

4.6 Inclusion in the Herd

Due to the selection of only one horse, there is no herd.

4.7 Immunization Program

The first set of injections included FCA and was administered at 10 sites (0.2 ml at each site). After two weeks, the second set of injections was given at 10 sites of the horse, which allowed 0.2 ml at each site. The proceeding injection days were given every two weeks apart and included Sodium Alginate as the adjuvant in the immunogen. The dosage increased with each injection period and ended at day 84 (week 12). The total amount of volume administered to the horse at the end of the immunization program was 24 ml, and the total there were a total of 7 injection periods.

The injections were used with 3-ml syringes (x40), 18G needle (x40), and given subcutaneously at each site. 70% ethanol was also used to properly clean before injecting the immunogen into the horse. The areas for injection sites were the upper part of the neck, outer hip, and thighs.

Table 2. Immunization Protocol

Week	Venom Dosage (mg) (PBS + Adjuvant + Venom)	Immunogen Composition	Total Volume (ml) and No. of Injection Sites
0	**1.5 + 1.5 + 1.5**	**PBS + FCA + Venom**	**2 ml (10 sites x 0.2 ml)**
2	**3 + 3 + 3**	**PBS + FIA + Venom**	**2 ml (10 sites x 0.2 ml)**
4	**18 + 9 + 9**	**PBS + Sodium Alginate + Venom**	**4 ml (4 sites x 1 ml)**
6	**36 +18 + 18**	**PBS + Sodium Alginate + Venom**	**4 ml (4 sites x 1 ml)**
8	**72 + 36 + 36**	**PBS + Sodium Alginate + Venom**	**4 ml (4 sites x 1 ml)**

10	**90 + 45 + 45**	**PBS + Sodium Alginate + Venom**	**4 ml (4 sites x 1 ml)**
12	**100 + 50 + 100**	**PBS + Sodium Alginate + Venom**	**4 ml (4 sites x 1 ml)**

4.8 Collection of Blood or Plasma

The horse is bled for a volume of 6 liters from the jugular vein. The session lasts for 30-45 minutes, and the blood is collected in plastic bags and labeled.

4.9 Storage and Pooling of Plasma

Some of the factors that are taken into consideration when choosing a specific method for the quality, control, pooling, and storage of plasma are time, cost, efficiency, and neutralizing activity. Time for each step are key factors that effect the quality of the plasma for the antivenom product by assuring that batches of plasma are fresh for the next step. The cost of the equipment and materials used need to be affordable, but not cheap so that the materials and equipment are of good quality and can be used effectively. All chosen procedures need special attention and care so that very few mistakes are made. The neutralizing activity needs be high in order to have a better antivenom production outcome.

The parameters in dealing with the collection of plasma are the volume of plasma needed to be collected, the type or breed of horse, the separation method, the type of preservatives added, storage, refrigeration temperature, neutralizing limit, and bioburden limit. The known parameters are the desired amount of blood, the cresol preservative, sterilized saline solution, anticoagulant, refrigeration temperature, neutralization limit, and biodurden limit, which all are parameters that WHO recommends to use. The unknown parameters are the volume of plasma that will be collected, the quality of the plasma, the amount of microbial growth, and the velocity at which the centrifuge bowl spins, the flow rate, area of the centrifuge bowl, and time it takes for centrifugation. However, the unknown parameters can be calculated or tested. The amount of plasma to be collected can be calculated using the size, weight, and known breed of the horse (Shelanski 2012). The quality of the plasma and amount of microbial growth can be tested using the neutralization assay and biodurden assay.

Using the known volume of blood, the volume of plasma can be calculated by using the simple mass-volume-density equation: $V_T = \frac{m_T}{r_T}$

Where V_b = volume of blood and plasma (total volume) =6 L= V_b (Volume of blood) plus V_p (Volume of plasma).
m_T = mass of blood and plasma (total mass) =?
ρ_t = density of blood and plasma (total density) = ρ_b (density of blood) plus ρ_p (density of plasma)
ρ_b = 1125 kg/m^3
ρ_p = 1025 kg/m^3

The total mass of the blood and plasma needs to be found:

$$m_T = V_T r_T$$
$$m_T = V_T(r_b + r_p) = 6L(1125+1025)kg/m^3 = 12.9kg$$
$$m_T = r_b V_b + r_p V_b$$
$$12.9kg = (1125kg/m^3)V_b + (1025kg/m^3)V_p$$

Divide by 1125 kg/m^3 to get a second equation for the system of equations:

$$\frac{12.9kg = (1125kg/m^3)V_b + (1025kg/m^3)V_p}{1125kg/m^3}$$

System of equations:

$$12.9 = 1125V_b + 1025V_p \text{ ----1}$$
$$0.011467 = V_b + 0.91V_p \text{ ----2}$$

Multiply equation 1 by -1025 and subtract the two equations to cancel out the variable V_p and solve for V_b :

$$-11.75 = -1025V_b + (-932.75V_p)$$
$$\frac{11.739 = 1023.75V_b + 932.75V_p}{-0.011 = -1.25V_b}$$
$$V_b = 0.0088m^3$$

Substitute V_b into the equation below to solve for the mass of blood:

$$V_b = \frac{m_b}{r_b}$$
$$m_b = (0.0088m^3)(1125kg/m^3)$$
$$m_b = 9.9kg$$

Substitute m_b into the below equation to solve for the mass of plasma:

$$m_T = m_b + m_p$$
$$12.9 = 9.9kg + m_p$$
$$12.9 - 9.9 = m_p$$
$$3kg = m_p$$

Substitute m_p:

$$V_p = \frac{m_p}{r_p}$$
$$V_p = \frac{3kg}{1025\frac{kg}{m^3}} = 0.003m^3$$
$$V_p = 0.003m^3 x \frac{1000L}{1m^3} = 3L$$

The velocity can be calculated using the equation: $V_g = \frac{d^2}{18\mu}(\rho_b - \rho_p)g$

Where V_g = velocity due to rotation = ?

d = diameter = 0.27m

μ = viscosity = 0.003 Pa s

ρ_b = density of blood = $1125\ \frac{kg}{m^3}$

ρ_p = density of plasma = $1025\ \frac{kg}{m^3}$

g = acceleration due to gravity = $9.81\frac{m}{s^2}$

Substitute the values and solve for V_g:

$$V_g = \frac{0.27^2}{18(0.003)}(1125-1025)(9.81)$$
$$V_g = 1{,}324.35\frac{m}{s}$$

Substitute V_g into the below equation to solve for the flow rate:

$$Q = V_g(\frac{2\pi dR^2\omega^2}{g})$$

$$Q = 132435[\frac{2\pi(0.52)(0.135^2)(1666077^2)}{9.81}]$$

$$Q = 22x10^6\ \frac{m^3}{s}$$

Where: Q = flow rate = ?

l = length of centrifuge bowl = 0.52m

R = radius = 0.135m

ω = angular velocity = $1666.077\ \frac{rad}{s}$

4.9.1 Separation of Blood Components

The chosen method of separation of plasma from blood is through automatic apheresis, where an apheresis machine is used. Specifically, a plasmapheresis machine is used. A replacement fluid for the plasma is chosen to not be returned. Plasmapheresis separates and removes plasma from the apheresis machine while returning blood into the animal's body without a replacement solution in which some other solution replaces the removed plasma. Plasma is chosen for antivenom production over serum because it is more advantageous in that erythrocytes can be returned back to the animal to prevent deficiency of erythrocytes, as stated before. The other advantages, also stated previously, is that plasma produces more antibodies, has less contamination, and takes less time when being separated from the blood. Six liters of blood is what is desired out of the Belgium horse for one bleeding period. This part of the process will take around thirty to forty-five minutes.

Apheresis contains a centrifuge inside the machine for separation of plasma. It contains a spinning centrifuge bowl (Shelanski 2012). This is compared to the tubular bowl centrifuge. Whole blood from the Belgium horse is pumped using the apheresis machine into the spinning centrifuge bowl and separates from the plasma (Figure 1). High-density fluid is separated from low-density fluid through gravitational forces. Plasma being the lower density separates from the blood to the top of the bowl, while blood components having the higher density separate towards the bottom.

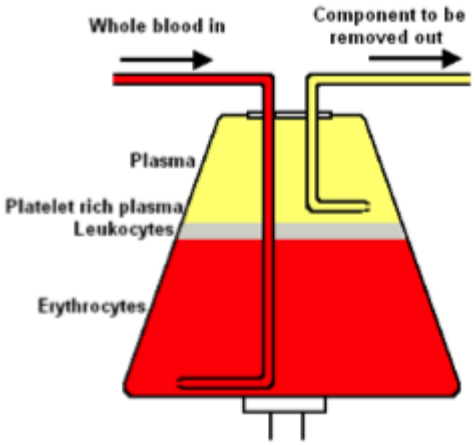


Fig 6. Plasma separates from blood components in a spinning centrifuge bowl as part of the apheresis machine. (http://dragonmommie.wordpress.com/tag/apheresis/)

The preservatives added to the plasma are 0.1 % cresol and sterilized saline solution, which are what WHO recommended to use. According to Masuci's results, in the figure below, 0.1% cresol is the better concentration of cresol preservative ot use with plasma.

TABLE 2
CITRATED PLASMA

Percentage of Preservative	Color	Appearance	Fibrin
0 cresol (control)	Yellow	Coagulated	Completely formed
0.1 cresol	Yellow	Coagulated	Completely formed
0.2 cresol	Greenish-yellow	Coagulated	Completely formed
0.3 cresol	Greenish-yellow	Coagulated	Completely formed
0.4 cresol	Greenish-yellow	Turbid and coagulated	Completely formed
0.5 cresol	Greenish-yellow	Turbid and coagulated	Completely formed
0.2 ether-cresol	Greenish-yellow	Clear and coagulated	Completely formed
0.3 ether-cresol	Greenish-yellow	Clear and coagulated	Completely formed
0.4 ether-cresol	Greenish-yellow	Clear and coagulated	Completely formed
0.5 ether-cresol	Greenish-yellow	Clear and coagulated	Completely formed
0.6 ether-cresol	Greenish-yellow	Clear and coagulated	Completely formed
0.7 ether-cresol	Greenish-yellow	Turbid and coagulated	Completely formed
0.8 ether-cresol	Greenish-yellow	Turbid and coagulated	Completely formed
0.9 ether-cresol	Greenish-yellow	Turbid and coagulated	Completely formed
1.0 ether-cresol	Greenish-yellow	Turbid and coagulated	Completely formed

Fig 7: Table shows that 0.1 % Cresol is a good choice to use as a preservative of plasma. (http://www.jstor.org/stable/30082211)

4.9.2 Pooling of Individual Plasma

The material used to collect and store the plasma from the plasmapheresis machine is a disposable plastic bag, which WHO recommends. The plasma will be stored in a refrigerator at a constant chosen temperature of 5°C, since it is halfway between the recommended temperatures of 2° to 8°C. The refrigerator with plasma will be located in a

sterilized and clean storage room away from everything else. Plasma should be stored not long at all, and therefore, the plasma to be stored will not exceed over a week.

When ready for fractionation, a bioburden assay will be used on the plasma using the zone of inhibition. In this procedure, plasma is in direct contact with the inoculum of a specified organism and analyzed for the zone of inhibition. The procedure usually takes three to five days (WuXi AppTec 2013). Zone of inhibition was chosen to give enough time to analyze whether there are any pathogenic microbes on the plasma that would damage the plasma, but also does not take a long amount of time like some other bioburden assays. The procedure is also familiar and simple. A mathematical distribution is fitted to the bioburden set of data collected from the assay, and a 95% probability limit is set as the biourden limit, which follow the standards from the International Organization for Standardization provided in the Sterilization of Medical Devices-Microbial Methods Part 3: Guidance on evaluation and interpretation of bioburden data (ISO 2004).

4.10 Quality Control of Plasma

4.10.1 Neutralizing Ability

Since the neutralizing limit is a key factor in determining the quality of the antivenom product, a neutralizing assay in the plasma will give a high titre number, and thus, will have a high neutralizing activity. Plasma serves as the neutralizing agent/antibody/immunoglobulin that "cancels out" the effect of the venom. The neutralizing assay for the neutralizing limit or potency, should be around 2.78-5.71, according to Figure 3 on page 15. The chosen neutralizing limit that will be tested for in the plasma is the average of the ranges, which is 4.245, giving a high neutralizing potency, which will result in a high quality of antivenom. Toxin Neutralization Assay is the specific test that will be performed based on the protocol of abcam®, which is a biological sciences company that provides the protocol documents. Toxin Neutralization Assay shows how well antibodies protect biological cells from a given sample of toxin (BioReliance 2012).

4.11 Purification of Immunoglobulin Molecules

The plasma that was separated from the blood must be further processed to isolate the immunoglobulin g (IgG) fragments used as the active ingredient in the antivenom. The first step in this process is through ammonium sulfate precipitation. Ammonium sulfate precipitation first requires that the serum being used is free of all free floating solids, meaning that centrifugation for 20 minutes at 10,000g_{av} may be required (Table 3).

Table 3. Centrifuge parameters for IgG isolation.

Centrifuge Capacity	Amount Required	Time Per Cycle	Time Required
4 L	3 L	20 minutes	20 minutes

Solid ammonium sulfate can be added to a batch system at 2.97 grams per 10 mL to achieve the desired 45% saturation. This solution must then be centrifuged for 20 minutes at 20,000g_{av} to obtain the desired immunoglobulin pellet (Table 4). The pellet can then be dissolved in Phosphate Buffer Saline (PBS) to 20% of the original volume of the fluid sample. The PBS used in this process can be prepared by mixing 0.14M NaC1, 2.7 mM KC1, 1.5 mM $KHEPO_4$, 8.1 mM Na_2HPO_4.

Table 4. Centrifuge parameters for immunoglobulin pellet produced.

Centrifuge Capacity	Amount Required	Time Per Cycle	Time Required
3 L	3 L	20 minutes	20 minutes

When properly mixed, continue adding PBS until the solution reaches 50% of the original volume (Table 5), resulting in an IgG concentration of 2 grams/L (Kent and Blackmore, 1985). This procedure must be performed at 5 °C to preserve the immunoglobulin solution. The procedure used in this process was adapted from *The Protein Protocols Handbook* (Page and Thorpe, 2002).

Table 5. Final volume calculated from volume increase.

Initial Volume	Increase in Volume	Final Volume
3 L	100%	6 L

4.12 Isolation of Fab Fragments

4.12.1 Papain Digestion of Immunoglobulin Molecules

When the 2 gram/L solution of IgG has been prepared, the solution is digested with a 2.5% (w/w) solution of papain that is of equal volume to the solution being digested, resulting in a 100% increase in volume (Creswell et al, 2005). This papain solution can be produced within the lab, but it is more economical to buy a pre-prepared solution. In order to have the digestion go to completion, the mixed solutions must be kept at 37°C for 18-20 hours in a jacketed tank (WHO, 2010). The jacketed tank is large enough to contain the entirety of the produced volume from the previous step, streamlining the

process. The papain digestion can be stopped by adding 0.03 M iodoacetamide to the solution (Andrew and Titus, 2000).

4.12.2 Diafiltration of Fab Solution

The digested solution is next applied to a tangential flow diafiltration system. The system used in this process will be the LabScale^{TM} TFF System using the Pellicon 2 mini cassettes produced by Millipore. This system has a volume capacity of 80 mL to 10 liters and has a micro-filter membrane that selects for protein of 5 to 1000 kD, so it is ideal for the filtration of the immunoglobulin fragment solution produced through the previous steps. The TFF system filters the solution from a stock and feeds it through system at 0.4 L/min. The system produces filtrate at an average of 0.1 L/min (Table 6).

Table 6. Filtration parameters to calculate time required.

Filtration Rate	Volume to be Filtered	Time Required
0.1 L/min	6 L	60 minutes

The system removes most of the salts and peptides used fragment the IgG, but retains the proteins with higher kD values. The specifications of the TFF machine were acquired from the *Protein Concentration and Diafiltration by Tangential Flow Filtration* guide published byMillipore.

4.12.3 Anion-Exchange Chromatography Separation

The solution will be separated into the different immunoglobulin fragments through the use of anion-exchange chromatography. The solution is dialyzed with roughly 20 times the volume of the sample of 0.01 Tris-Cl resulting in a pH 8.4. The 1.5x50 cm chromatography columns are loaded using Macro Prep DEAE and equilibrated with the 0.01 Tris-Cl solution. The loaded column will initially bind the fragments in the solution, while eluting out any additional contents that will be discarded as waste. The column is then eluted using a 250 mL linear gradient of the Tris-Cl solution to an equal volume of 0.5 M NaCl/0.01 Tris-Cl. The flow rate of the elution process will be adjusted to 20 mL/min (Table 7).

Table 7. Flow rate parameters to calculate time required.

Flow Rate of Column	Number of Columns	Volume to be Filtered	Time Required
20 mL/min	5	6 L	60 minutes

The Fab immunoglobulin fragments will be collected upon completion. The remaining contents of the column are discarded, and the column is regenerated. The procedure used in this process was adapted from *Current Protocols in Cellular Biology* (Andrew and Titus, 2000).

4.13 Purification of Fab Solution

The solution is reapplied to the TFF system in order to remove any excess proteins, salts, or solvents not desired in the final product. Ideally, the recovered volume of the solution should be equal to what it was prior to the first diafiltration process (Table 8).

Table 8. Filtration parameters to calculate time required.

Filtration Rate	Volume to be Filtered	Time Required
0.1 L/min	6 L	60 minutes

The concentration of the Fab solution will be less than that of the intact IgG molecule due to the differing kD values. The size of an IgG molecule is roughly 150 kD, and the digestion process results in 2 50 kD Fab fragments (Freitas, 2003). As a result, the final concentration will be 2/3 of the concentration of the papin digestion step, resulting in a 0.33 grams/L of the Fab solution (Table 9).

Table 9. Parameters to calculate final concentration.

Initial Concentration	Size of Intact IgG	Size of 2 Fab Molecules	Ratio	Final Concentration
1 grams/L	150 kD	50 kD	2/3	0.33 grams/L

The solution must be further concentration before entering quality control. This can be done by centrifuging the sample for 20 minutes at 10,000g_{av}, then reusing the supernatant to adjust the concentration to 50 grams/L (Meyer et al, 1997) (Table 10). The sample is then kept at 5 °C to maintain the stability of the Fab fragments.

Table 10. Calculation of final volume from initial concentration.

Initial Concentration	Initial Volume	Final Concentration	Final Volume
1 grams/L	6 L	50 grams/L	**0.12 L**

4.14 Quality Control

Quality control tests for the final product are performed on samples of the batch and thus will not affect the concentration of the overall product. The necessary tests below will be performed by sending a sample of the batch to the central Abcam center in Cambridge, MA (www.abcam.com). These tests will ensure the product is safe and effective for human use. Since the quality control tests will be done at an off-site location from our actual antivenom production, just the overall cost of the tests will be accounted for and not the equipment and reagents for these tests.

4.14.1 Solubility Test

The batch will be visually analyzed to ensure correct color and cloudiness level. Since the batch is not freeze-dried, the solubility test is not necessary.

4.14.2 Potency Test

Since the potency test has already determined that 50 grams/L is the appropriate potency concentration (Meyer et al, 1997), the median lethal dose assay and median effective dose assay for the potency test will not have to be done. The Kjeldahl method will be used to ensure this proper concentration (see Concentration Tests below).

4.14.3 Concentration Tests

Under the osmolality test for tonicity, an acceptable antivenom is at least 240 mosmol/kg. Protein concentration is determined via nitrogen determination with the Kjeldahl method and must yield a protein concentration of 10 g/dl or less (WHO, 2010). The three steps of this method are digestion, distillation, and titration (Blamire 2003) (Figure 8).

The Kjeldahl method digestion begins by dissolving a 1 gram product sample in 12-15 mL H_2SO_4 and 7 grams potassium sulfate. The solution is then heated at 370^{o}C to 400^{o}C for 60-90 minutes before distillation (Table 11).

Table 11. Kjeldahl method digestion parameters. (Adapted from Blamire 2003)

Initial Sample Amount	Amt H_2SO_4	Amt potassium sulfate	Heating Temperature	Heating time
1 gram	12-15 ml	7 grams	370^{o}C to 400^{o}C	60-90 minutes

The Kjeldahl method distillation raises the pH until ammonium ions are changed to ammonia in 45% NaOH solution. Distillation traps the solution with a solution of 15 mL HCl in 70 mL of water (Table 12).

Table 12. Kjeldahl method distillation parameters. (Adapted from Blamire 2003)

Raise pH	Distillation trapping solution
In 45% NaOH solution	15 ml HCl (hydrochloric acid) in 70 ml of water.

For the Kjeldahl method titration, an indicator dye is added and then the solution is titrated with NaOH until the dye turns orange. The neutralizing base volume is then used to calculate the amount of ammonia originally (Table 13).

Table 13. Kjeldahl method titration parameters. (Adapted from Blamire 2003)

Titration indicator	Titration base	Amount base added
Dye	NaOH	Until dye turns orange

The protein value calculated from the amount of base required to titrate the distilled solution in the Kjeldahl method should be 10 g/dL or less (WHO 2010). If it is not this amount, then the product should be repurified.

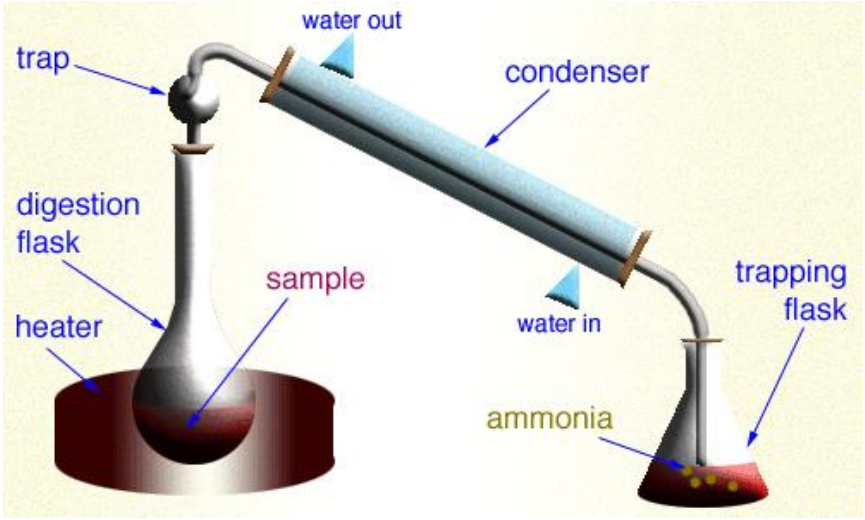


Fig. 8. Kjeldahl method apparatus. The sample is first digested, then heated and distilled after conversion to ammonia gas, which dissolves back into ammonium ions, then titration traps the ammonia in a flask for calculations (McClements 2003).

4.14.4 Purity Test

Absorbance will be measured by a spectrophotometer. HPLC will then be used to determine the molecular size distribution. HPLC will also be used for immunoglobulin purity verification and will substitute for gel electrophoresis, small-scale affinity chromatography, and size-exlusion gel filtration, thus reducing the number of tests we will have Abcam perform on our sample. Also, since the small-scale affinity chromatography is used by regulatory agencies to determine the reference standard concentration, it will not be required for our batch. If the purity does not result in under 1% of the total protein as albumin, then the product will have to repurified. The densitometric analyses of chromatographic profiles for protein quantification will not be used because the Kjeldahl method (Concentration Tests above) will determine the protein concentration.

4.14.5 Endotoxin Limits Test

If the product has the proper concentrations verified above (Concentration Tests), then endotoxin limits will not be exceeded. A sterility test will be performed by passing through a 0.45-μm pore membrane to ensure no contamination. The antivenom is then added to culture media for further testing. Trypticase soy broth will be used and incubated for 14 days at 20–25 ºC while the bacterial growth is monitored. The pH will then be checked with a potentiometer to ensure it is at pH 8.4. Spectrophotometrics done under Purity Test will also test for preservatives quantification and will present a quality product if phenol concentration is less than 2.5 g/L. HPLC done under Purity Test will additionally test that cresol concentration is less than 3.5 g/L.

4.15 Labeling, Packaging, and Release

4.15.1 Storage

After the quality of the product is assured, then the antivenom is ready for
Since production and distribution will all occur within the United States, the cold chain can be guaranteed during transportation and storage so the final product will be stored as a liquid at 5°C to prevent product deterioration.

4.14.2 Packaging for Sale

The final product will be dispensed into labeled 10 mL vials then packaged into cardboard boxes which are also subsequently labeled.

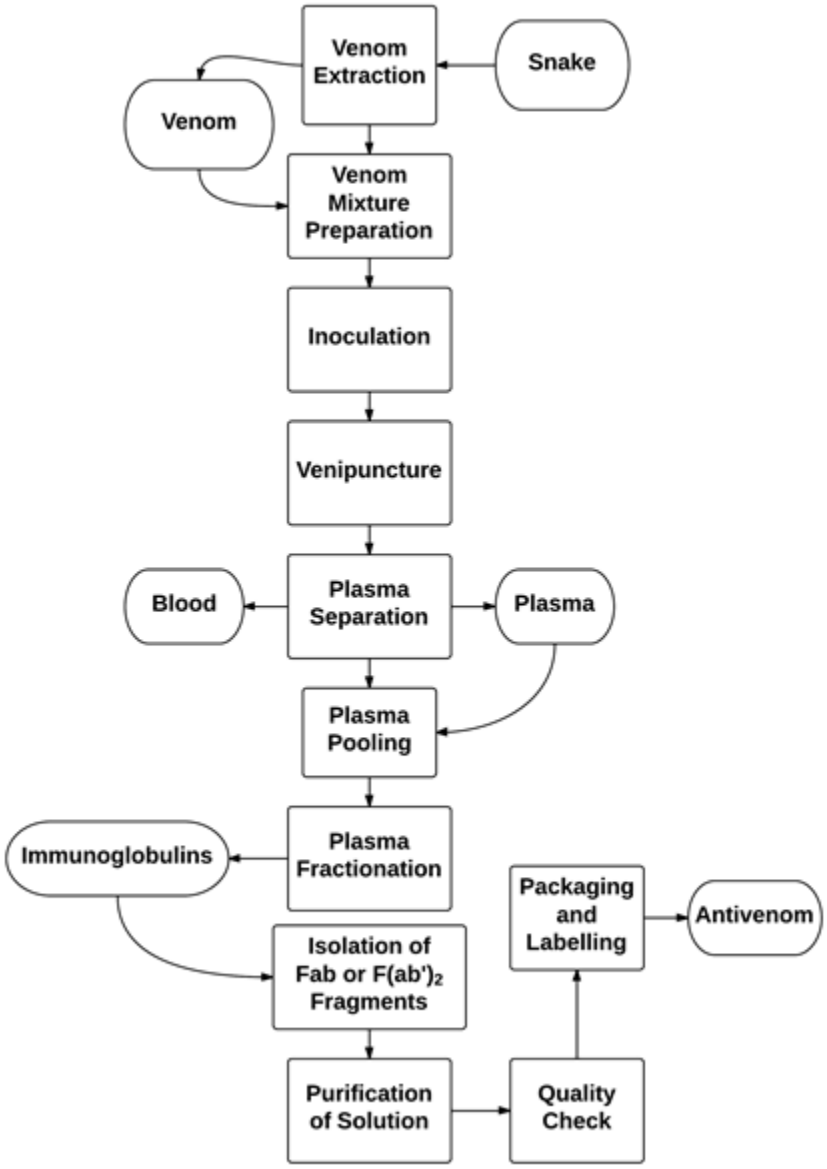
Since, 120 mL of antivenom will be produced, 12 vials are needed for dispensing the final product:

$$120\ mL\ product * \left(\frac{1\ vial}{10\ mL}\right) = 12\ vials$$

10 mL vials come in packages of 100 vials, so one package will be purchased at $28.70 from Sigma Aldrich, resulting in a cost of $3.44 for each vial:

$$\frac{\$28.70}{1\ pkg} * \left(\frac{1\ pkg}{100\ vials}\right) * 12\ vials = \$3.44/vial$$

5. Flow Chart


Venom Extraction
Snake
Venom
Venom Mixture Preparation
Inoculation
Venipuncture
Blood
Plasma Separation
Plasma
Plasma Pooling
Immunoglobulins
Plasma Fractionation
Packaging and Labelling
Antivenom
Isolation of Fab or F(ab')2 Fragments
Purification of Solution
Quality Check

6. Cost Analysis

6.1 Equipment Used in Production

Table 14. Existing Capital

Item	Quantity	Cost ($)	Source
Horse	1	15,000	www.equinenow.com
Vaccinations	1	227.00	www.horsevaccines4less.com
Plasmapheresis machine	1	20,000.00	www.medwow.com
Beckman Coulter Avanti J-E Centrifuge	1	9,350.00	www.beckmancoulter.com
LabScale™ TFF	1	6,211.80	www.fishersci.com
Pellicon Mini Cassette	2	400.00	www.millipore.com
Flex-Column 1.5X50 Cm, 5/PK	1	153.10	www.pgcscientifics.com
3-ml Syringe with 18G Needle	1 (100/box)	15.51	www.drugsupplystore.com
10 mL Storage Vials	1 pkg (100 vials)	28.70	www.sigmaaldrich.com
0.5 L disposable plastic storage bags	1 (24 units/case)	135.00	www.cenmedonline.com
Agar plates	4	24.00	www.mansionschools.com
Total Cost of Equipment		**52017.11**	

6.2 Chemicals and Reagents Used in Production

Table 15. Cost of chemical and reagents required.

Chemical	**Cost ($)**
Ethanol	37.10
Phosphate Buffer Saline	308.00
Ammonium Sulfate	15.70
Papain Solution	33.90
Iodoacetamide	50.10
Macro Prep DEAE	140.00
Tris-Cl	44.00
NaCl	52.20
0.1% Cresol	27.10
Total cost of chemicals and reagents	**$708.10**

Table 16. Cost of inoculation

Inoculation		
Item	Price	Amount
Venom	$126.50	500 mg
PBS	$16.72	500 mL
FCA	$27.00	10 mL
FIA	$24.80	10 mL
Sodium Alginate	$128.00	1 kg

$$\frac{\$708.10}{12\ vials} = \$59.01/vial$$

7. Conclusion

Although the general guidelines of antivenom production are known, the production is low in many developing countries mainly due to high cost. There is only one FDA approved snake antivenom (CroFab) in the United States' market – with each vial costing approximately $2,000. The administered number of vials per patient can vary and even add up to 30 vials/person depending upon the patient's immunity and type of snakebite. By redefining the ways of antivenom production and improving the overall market and distribution, a victim of a poisonous snakebite could easily recover.

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