

THE MANUFACTURE OF VETERINARY CLOSTRIDIAL VACCINE

A vaccine is an antigenic preparation used to stimulate the production of antibodies and procure immunity from one or several diseases.

Clostridium bacteria are found in soil and in the guts of animals. They are anaerobic (grow in the absence of oxygen), and produce a wide range of toxins leading to disease. Vaccines are required to give animals protection against these diseases.

Production of clostridial vaccines involves growing the organism (*clostridial* bacteria) in a growth medium to produce large amounts of toxins, and altering the toxins so that they are no longer toxic, toxoids. These toxoids become antigens which the immune system recognises and hence produces antibodies to act against genuine toxins. After cultivation the live bacteria in the culture must be deactivated, and the resulting mixture is diluted and mixed with an adjuvant, a substance which improves the immune response to the toxoids.

The final vaccine product must pass stringent tests before being released for use on animals.

This article is based around the methods used by Mallinckrodt Veterinary Ltd in New Zealand.

INTRODUCTION

The *Clostridium* bacteria are **anaerobic** (requiring the absence or very low partial pressure of oxygen for growth). In nature, they are often found in the soil and in the gut of humans and animals. They grow in culture under relatively basic conditions and will produce **toxins** (molecules that can cause damage to living tissue) in culture and in living hosts under the right conditions.

Diseases caused by bacteria of the genus *Clostridium* have been diagnosed in humans and animals since the early part of the century. The diseases tend to be opportunistic in nature as the bacteria do not require a living host in order to survive. The gas gangrenes caused by the *C. perfringens* species caused a large number of deaths in humans in World War One and continue to be a problem in Third World countries. Tetanus is the most well known of the clostridial diseases and continues to be a problem in people and animals even now. Other diseases related to the clostridia occur in humans. They include blood poisoning and gangrenous type diseases. Similar diseases are seen in animals and a good coverage of the diseases of animals caused by the clostridia can be found in Hungerford (1990). Farmers throughout the world use clostridial vaccines to protect their stock, particularly sheep and cattle, from disease.

Pioneers in the field showed that cultures of these bacteria that had been inactivated by the addition of formalin to culture and then mixed with an **adjuvant** (see Blending) would protect animals against infection by the native disease. An adjuvant is a substance that improves the immune response to a compound mixed with it. **Antigens** (molecules that stimulate an immune response) mixed with adjuvant give a higher antibody titre and more

prolonged response than antigens injected by themselves. It is not proposed to spend time on the mode of action of various adjuvant types but interested readers are referred to Bunn *et al* (1986) and Edelman *et al* (1980) for reviews.

VACCINE PRODUCTION

Overview

The production processes used by many companies have remained largely unchanged for a number of years. Until recently, the regulatory environment was relatively relaxed and manufacturing standards and practices were basic. The requirements have changed noticeably over the last ten years with standards as high as the pharmaceutical industry now commonplace. The industry is now highly regulated and any changes to products must be accompanied by substantial scientific proof showing that the vaccine efficacy is unchanged.

In simple terms, vaccine production requires growth, inactivation and processing of the organism, mixing the processed material with an adjuvant and then filling and packing of the blended vaccine. The discussion in this document is based around the methods used by Mallinckrodt Veterinary Ltd. We will look in more detail at each of the steps in their order of application. The overall process is summarised in **Figure 1**.

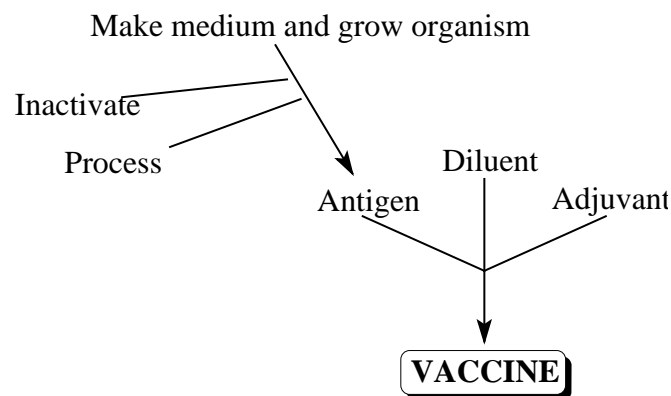


Figure 1 - Summary of the vaccine manufacturing process

Preparation of the Medium

Although the clostridia will grow in many different media, the amount of toxin they will produce in different media is highly variable. Most clostridial media are complex mixtures or peptones, salts, vitamins and buffers. The manufacturer tries to use a medium that is cheap and gives good toxin yield.

The core components of clostridial media are protein digests and extracts called **peptones**. A peptone is a length of amino acids joined by peptide bonds (**Figure 2**). The basic process of manufacture of peptones is to take a protein source (e.g. casein, vegetable protein or a meat source) and digest them with a proteolytic enzyme such as trypsin, pancreatin or papain. The degree of digestion and the resulting peptide lengths are a function of the digestion conditions (temperature, time, concentration of enzyme/protein) and the number of cleavage sites for the enzyme in the protein.

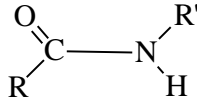


Figure 2 - The peptide bond
(R and R' are functional side chains)

Peptone manufacturers control the conditions carefully and can generate a remarkably uniform range of peptide lengths from batch to batch. This is important because the variation in peptone type, time of year of manufacture and even supplier of a peptone can have drastic consequences on yield of toxin by the end-user. Manufacturers of clostridial vaccines deal with the peptone variation in different ways. One of the most common is to test pre-purchase samples of a batch of a medium component. If yields are good from that batch of peptone, the manufacturer will buy a years supply from that batch. As it is often not clear exactly what component in the peptone (in terms of amino acids or peptide length) makes that batch “good” selection is often empirical.

A number of companies manufacture peptones for use in vaccine manufacturing processes (e.g. Unipath, Difco) and they are usually sold in a spray dried form. Other companies (such as Mallinckrodt Veterinary Limited) manufacture their own peptones using proprietary formulas.

The peptones are mixed together with salts, vitamins and water to specific concentrations. The clostridial media used by the various manufacturers of veterinary products are often quite different. The medium may have been refined over a number of years. The hallmark of a good medium is that it works in the manufacturer's hands reproducibly.

Sterilisation of the medium is normally by absolute membrane filtration to 0.2 µm or by heat sterilisation in the fermenter vessel. Both of these methods have disadvantages. Filters are very expensive and the batch heat process can cause significant damage to sensitive vitamins and cofactors in the media.

The sterilisation process needs to avoid causing damage to the proteins and important cofactors in the medium. Damage to proteins, vitamins and cofactors is a function of both heat and time. For a number of the clostridia, notably *C.septicum*, Mallinckrodt Veterinary Limited has shown that the degree of heat and the period of time for which the heat is applied can negatively affect yield. For this reason, many manufacturers still use sterile filtration, despite the expense.

The Bacterial Master Seed

One of the most critical components of the fermentation is the bacterial culture that produces the toxin in the controlled environment of the fermenter. Not all bacterial cultures are equal in their ability to produce toxin. Wellcome Biotechnology Limited tested a large number of bacterial strains in small trials to find strains that would reproducibly produce high levels of toxin. The selection continued over the years, with high-producing isolates from the original strain re-isolated and used in subsequent fermentations. The end result of this kind of program is a seed that is well adapted to a narrow range of conditions. This type of selection and re-selection is no longer acceptable under today’s strict regulatory climate.

Under the current regulatory standards, the clostridial seed strains are carefully and rigorously maintained by the manufacturer. Alteration of the strain of bacterium requires the consent of regulatory bodies (such as the Animal Remedies Board in NZ). The purpose of these regulatory agencies is simple; they act as guardians of the standard so that customers can be confident that the material they buy is of the highest quality.

In order to maintain the quality, a change to a seed culture requires stringent testing to demonstrate its equivalence to the old culture. The validation can often take the form of trial vaccine blends and efficacy trials in the target animal.

In the case of the clostridia, strain maintenance is especially important. Most of the genes present in the clostridia that are involved in toxin production are located on extrachromosomal elements such as **plasmids** (independently replicating DNA molecules found in bacteria) and **bacteriophage** (viruses that infect bacteria). Without these elements the toxins cannot be produced. These extrachromosomal elements can be lost if the selective pressures for their presence are not maintained.

Fermentation and Production of the Clostridial Toxins

The clostridia produce a huge range of toxins but only the major toxins are believed to be critical in the disease process. If the animal is protected from these critical toxins, the immune system will deal with the infection. A good review of the important clostridial toxins is found in Hatheway (1990)

The production of the toxin in the manufacturing process consists of two stages; scale up of the seed to give the final inoculum and the production-scale fermentation in which the toxin is actually produced in commercial quantities.

The scale up stages aim to produce a rapidly growing culture in successively larger volumes. It is possible to go from 100.0 millilitres to 200.0 litres in three steps. The number of steps should be kept to a minimum to avoid the loss of important genetic characteristics. Work with bacterial cultures has shown that the greater the number of generations that the organism goes through, the higher the probability of a mutation. Further, an increase in the number of steps also adds to the cost of the product. At each step, set parameters of bacterial density, culture condition (assessed microscopically) and pH are aimed for. By taking the very actively growing culture at late log phase, growth times in each stage of scale up is kept to a minimum (**Figure 3**). A rapidly dividing culture will continue to divide on sub-culture with minimal lag phase.

Once the culture is inoculated into the production fermenter, the fermentation will have to be controlled to produce product. As the bacteria grow, they use sugars, proteins and vitamins from the medium and convert them into cellular biomass. In the process, the bacterium will excrete extracellular enzymes (e.g. proteases for proteins, lipases for lipids) to digest nutrients and waste products from metabolism such as organic acids and alcohols. Once the bacterium enters stationary phase the by-products will inhibit toxin production and the extracellular enzymes may even degrade the toxin. The effect of the by-products on growth must be controlled until the fermentation has produced the required end-product (the toxin).

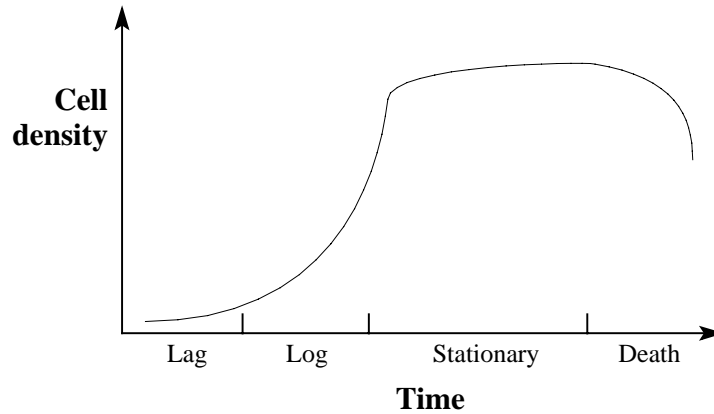


Figure 3 - Bacterial growth curve

Control of the anaerobic fermentation once in the production fermenter is relatively simple. The fermentation control is aimed to stabilise the growth long enough to produce the required end-product.

In order to best manage the fermentation, carbohydrate concentration and pH should be carefully controlled. If possible it is best to limit the addition of carbohydrate (usually glucose) to only enough to maintain logarithmic growth of the bacteria and no more. In practise this is difficult without expensive sensors. We have analysed several batches of each clostridial type to establish an optimum feed rate for that culture.

It is necessary for the clostridial culture to establish sufficient biomass to generate adequate amounts of toxin. With a rapidly dividing culture, pH control is usually required within an hour of inoculation of the production fermenter. The pH is controlled by the addition of sodium hydroxide in the production fermenter. **Figure 4** shows a typical pH control curve in the production fermenter, overlaid with the toxin production curve.

The curve of toxin production typically starts after the fermentation pH has dropped below pH 7.0. The pH of the culture is adjusted back to a pH of 7.8 and maintained at that position by a pH sensor in the fermenter. The sensor is attached to a microprocessor. The microprocessor opens a valve and causes sodium hydroxide to be pumped in until the pH is back in the set range at which point it shuts down. Using good control software and the correct settings of the microprocessor parameters it is possible to keep the pH variation within 0.1 unit. Maintaining the growth at the pH optimum extends the length of the fermentation.

The fermentation times vary for the clostridial species. Most of the fast growing *C. perfringens* group produce maximal toxin within 4 hours. The slow growing *C. tetani* takes 7 days to produce maximal toxin. Others take a matter of a few hours. *C. tetani* is a special case, as the bacterial cells must all lyse in order for the toxin to be released. *C. tetani* also produces a number of volatile inhibitors during growth which must be removed. These volatile inhibitors are removed by an air sweep across the medium surface. The air sweep serves to oxidise the inhibitors and more importantly flush them from the fermenter vessel. It seems strange to be adding air to an anaerobic fermentation but this step is critical for fermenter growth of *C. tetani*.

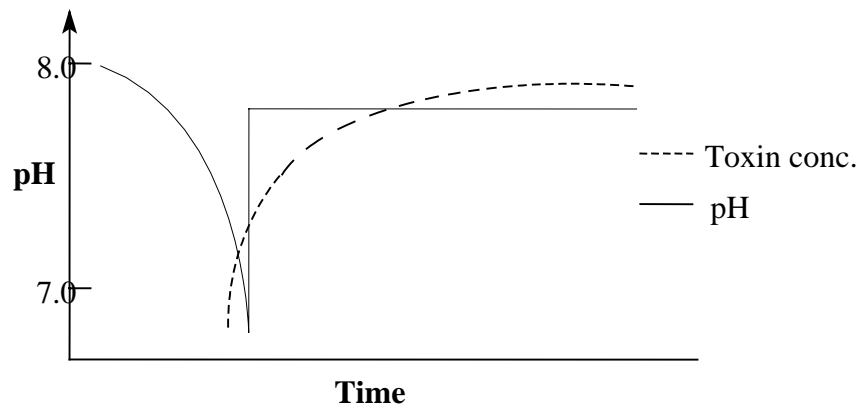


Figure 4 - The pH and toxin production curves for a *Clostridium profringens* culture

The fermentation processes were derived by trial-and-error and the critical environmental triggers that cause toxin production to be switched on or off are not known. Each clostridial species has a specific assay to quantify the required toxin that the fermentation produces. The tests are specific for one toxin and one toxin only.

Chemically speaking, many of the toxins produced by the clostridia are enzymes. The *C. profringens* toxin alpha is a phospholipase. Its basic effect is to “make a hole” in the membrane of a cell by cleaving a cell membrane component. The cell contents leak out and the cell dies. The toxin of *C.septicum* acts in a similar way, forming a pore in the cell, again allowing the cell contents to leak. This membrane-action seems to be a general trait of clostridial toxins.

Once the toxin level has been reached the fermentation is halted by the addition of formalin (36% v/v formaldehyde).

Inactivation and Processing

As we have already noted, it is the clostridial toxins that cause the damage in the disease process. These toxins are complex protein molecules, many with enzymatic activities. The native toxins are quite lethal, the tetanus and botulinum toxins are two of the most lethal molecules known. Therefore, a method is needed of inactivating the toxin that doesn't stop it providing protection for the vaccinated animal (it remains **immunogenic**). This non-toxic but immunologically active product is called a **toxoid**. It is this toxoid, once processed, that forms the heart of a vaccine. The toxoid is formed by the addition of formalin to toxin, combined with heating over set periods of time. A second purpose of the formalin is to kill the bacterial cells making the culture non-viable.

The process of forming the toxoid is poorly understood. Formaldehyde acts as a cross-linking agent and changes the conformation of the protein by permanently cross-linking amino acids that are not normally linked. The cross-linking process is not predictable. The critical part of the toxoiding process is that it must inactivate the toxin without destroying its protective ability. Trial and error is the simplest way to determine the conditions of formalin amount, time and temperature that will inactivate the cells and the toxin and this is the method most manufacturers have used.

Assays specific for each toxoid are used for confirming that the toxoid molecule is non-toxic and for determining what potency it has after the toxoiding process. Minimum potency levels

for each antigen process are set by the manufacturer and are used as the targets for the inactivated material. Like the toxin tests, the toxoid tests are highly specific.

The second effect of formalin is to inactivate the cells, that is to make them non-viable. The cells must either be dead or removed because live cells may later produce active toxins with consequent harm to the animals vaccinated.

The manufacturer must demonstrate that the inactivated material is potent, non-toxic and non-viable. The potency assays for the toxoid perform the first task, the non-toxicity test the second and the non-viability test the third.

In designing a non-viability test it is first necessary to show that the medium chosen to test the viability of the bacteria will support the growth of the bacteria in question. It is then necessary to demonstrate at what level (ie what number of cells per millilitre) the bacteria can be measured. This sensitivity analysis must be carried out with bacteria alone, in the presence of formalin and in the presence of inactivated culture. In this way the reliability of the test can be assessed. From there a safety margin (to ensure no live bacteria remain) is added to the test.

The test itself is simple; a sample of the inactivated culture is added to the growth medium and the medium is incubated for a period of time. If no growth occurs, the bacterium is inactivated and the product passes test. Growth of the organism indicates failure and will mean the product needs retreating.

The potency test for the antigen is, in essence, a back-titration. Toxoid is non-reactive (but immunogenic) and cannot be directly measured in the existing registered tests. The unknown toxoid is mixed with varying amounts of antibody which can recognise both toxin and toxoid. The antibody is bound and neutralised by the toxoid. Toxin (which can be detected) is added to the mixture. If there is excess antibody, the toxin will also be bound and will not be measured. If toxin is in excess it will have an effect and will be measured. The equivalence point is the value of the unknown toxoid.

Unfortunately, most of these toxins can only be measured by using an animal (a mouse) to determine their presence. The tests use mouse lethality to determine toxin levels. At this time, there is no *in vitro* measure that is reliable. Mallinckrodt Veterinary Limited has spent and continues to spend several hundred thousand dollars per annum developing tests that do not use animals.

The non-toxicity test is used to detect any residual toxic activity in the inactivated material, the toxoid. Obviously, a manufacturer cannot put toxic material in vaccines as it would kill the animals the vaccine is designed to protect. The presence of the toxin causes adverse effects on the indicator system (for some toxins, red blood cells can be used as the indicator). In order to “pass” the test, no toxic reactions must occur as a result of the toxoid.

Once the culture has been inactivated it is passed on for further processing. Mallinckrodt Veterinary Limited uses ultrafiltration as a means of purifying the toxoid molecules and has done so for more than ten years. Basically, membranes of molecular porosity smaller than the size of the native toxin are used. The stream of product is forced across the surface of the filter. Molecules smaller than the molecular weight cut-off of the filter are pushed through the membrane pores while larger molecules continue to circulate. As water is a small

molecule, this has the effect of reducing the volume of the product stream as the recirculation continues. Small media peptides, salts and superfluous molecules are removed from the product

This is the one stage of the process that cannot have the equipment sterilised by steam. Membranes of a commercial scale that can stand repeated steam sterilisation under pressure are not available. The membranes are cleaned by enzyme/detergent solutions after each use and sterilised by the recirculation of concentrated formalin solutions.

The product left after the ultrafiltration is commonly called an “Antigen” (a misnomer). The batch of antigen is stored at 4°C until used.

Blending

The final stage of the vaccine manufacture is blending. In this step the antigen is diluted to vaccine strength and combined with the adjuvant to form the vaccine. Two different aluminium based adjuvants are in common use by Mallinckrodt Veterinary Limited (and the industry generally), alum (potassium aluminium sulphate) and TASGEL® (aluminium oxy-hydroxide known mineralogically as boehmite). The central core to both vaccines is the aluminium and although these adjuvants have been in use for many years, their precise mode of action remains unknown.

How does an adjuvant work? This is still not completely clear. Adjuvants act as ‘irritant’ and attract cells of the immune system to the site of vaccination. They act as ‘depots’, doling out vaccine antigen to the immune cells and they also seem to have a role in presenting the vaccine antigens so that the immune cells can best recognise them. As well as the aluminium salts discussed below, various other adjuvants have been used. These include mineral oils (such as Drakeol®), plant extracts (such as saponin) and synthetic compounds such as poly-lactides.

Alum adjuvanted vaccines are often referred to as **alum-precipitated**. The antigens are diluted out to the vaccine input strength in physiological saline and the alum is added as a solution. The pH immediately drops. The solution forms a cloudy granular floc. At this point sodium hydroxide is added to raise the pH to the vaccine final pH of 6.0-6.5. As the NaOH is added the floc changes consistency and becomes much finer. The final result is a very fine suspension of metallic ions with proteins ionically bonded with them. If the vaccine is left to settle and the supernatant measured for antigenic protein very little is found. Greater than 90% of the immunogenic toxoid is associated with the suspended metal salts.

The alhydrogel adjuvanted vaccines are referred to as **aluminium-absorbed**. The aluminium oxy-hydroxide $(\text{AlOOH})_n$ compound is mixed with a buffer and NaOH is used to adjust the pH to 6.0-6.5. The adjuvant forms a crystalline-type lattice which is quite regular. The antigens are then added to this lattice and they absorb to the charged groups in the lattice. This lattice-protein compound is also insoluble. As with the alum adjuvant, the vast majority of the antigen is associated with the lattice.

What is the difference between these adjuvants? There is no simple answer to that question. Both seem to behave in similar ways in the host. A full discussion of the effect of these adjuvants is outside the scope of this article. In any case, alum adjuvants are the only ones licensed for human use and remain effective for most antigens made in traditional processes.

The antigens are mixed with the adjuvant and the vaccine formed, the vaccine is transferred and filled into sterile plastic packs of sizes from 20 mL to 1 L. These packs of vaccine are then thoroughly tested to ensure they meet exacting criteria. A schedule of testing is shown in Table 1. The final product specifications are usually set by the countries' Regulatory Authorities in conjunction with the manufacturer.

Table 1 - Schedule of final product testing for a veterinary vaccine

Test	Purpose of test
Sterility	Demonstrates that no live microorganisms are present in the product
Chemistry	Demonstrates that the product has the correct amount of adjuvant and preservative, and that the pH is correct
Safety	Demonstrates that an overdose of the product causes no harm
Residual toxicity	Demonstrates that the product contains no material that can cause harm
Efficacy	Demonstrates that each antigen in the product meets the recommended guideline level (or better) in internationally recognised tests

The Vaccine

The vaccine is now suitable for sale. In New Zealand some vaccines can be sold and administered either by veterinary practitioners or over the counter at wholesalers for farmers to use directly. All vaccines sold in New Zealand must be registered and this registration process involves not only support of the vaccine claims but rigorous inspection of the facility and its quality systems.

Mallinckrodt Veterinary Limited make a variety of vaccines and they have slightly different purposes. These purposes depend on the range of diseases present in the country or at the time of year and the need for other added compounds. Many New Zealand farmers still recognise the brand name COOPERS® and product names such as MULTINE . The differences in the products relate to their use and the components that make up the vaccine. A few examples are listed below (Table 2). The inclusion of minerals such as selenium and chemicals such as levamisole allows the farmer to combine treatments. The animal is not only vaccinated against the infectious disease but alleviation of other ailments is delivered at the same time.

Mallinckrodt Veterinary Limited manufactures more than 30 different clostridial and clostridial-combination products for countries all over the world. These products are formulated specifically for those countries and for the conditions that farmers there must face. Mallinckrodt Veterinary Limited is one of the very few suppliers to be able to make a claim of world-wide supply in the animal health business and have had to have convinced many different regulatory agencies of the quality of the products. The products themselves are well received by the customers and gives farmers the ability to limit loss to clostridial disease on their property.

Table 2 - Some veterinary vaccine products made by Mallinckrodt Veterinary Limited

Vaccine brand name	Active components	Diseases/agents protected against
LAMBVACC™	<i>C. perfringens</i> D tetanus antitoxin	Pulpy kidney Tetanus
MULTINE Selenised™	<i>C. perfringens</i> D <i>C. tetani</i> <i>C. septicum</i> <i>C. novyi</i> B <i>D. chauvoei</i> Selenium	Pulpy kidney Tetanus Malignant oedema Black disease Blackleg White muscle disease
NILVAX™	<i>C. perfringens</i> D <i>C. tetani</i> <i>C. septicum</i> <i>C. novyi</i> B <i>D. chauvoei</i> Levamisole phosphate	Pulpy kidney Tetanus Malignant oedema Black disease Blackleg Parasite infection (worms)

Conclusion

Much of the detail of testing and manufacture has been left out of this article, partly to maintain the overview nature of the article and partly because much of it is proprietary. The article has also tended to concentrate on the process used by Mallinckrodt Veterinary Limited.

The clostridial vaccine manufacturing process looks relatively simple and in principle it is. In practise, a vast number of things can go wrong and affect the efficacy of the vaccine. The challenge of a biological production process is that $A + B = C$ is true most (but not all) of the time. The goal of the manufacturer is to constrain the biological process so that $A + B = C$ often enough to be economically viable.

Article compiled by Dr. M. J. O'Hara and Ms. E. F. Bauer (Mallinckrodt Veterinary Ltd.), with reference to:

- Bunn, T.O., R. M. Nervig and J. R. Pemberton. 1986. *Metallic salts as adjuvants for veterinary biologics*. In: Advances in Carriers and Adjuvants for Veterinary Biologics. R. M. Nervig, P. M. Gough, M. L. Kaeberle and C.A. Whetstone. Iowa State University Press. 105-113.
- Edelmann, R. 1980. Vaccine adjuvants. *Reviews of Infectious Diseases*, 2, 370-383.
- Hatheway, C. L. 1990. Toxigenic clostridia. *Clin. Microbial. Reviews*, 3, 66-98.
- Hungerford, T. G. 1990. *Diseases of Livestock*. Ninth Edition. McGraw-Hill Book Company, Sydney, 25-38 and 305-316.