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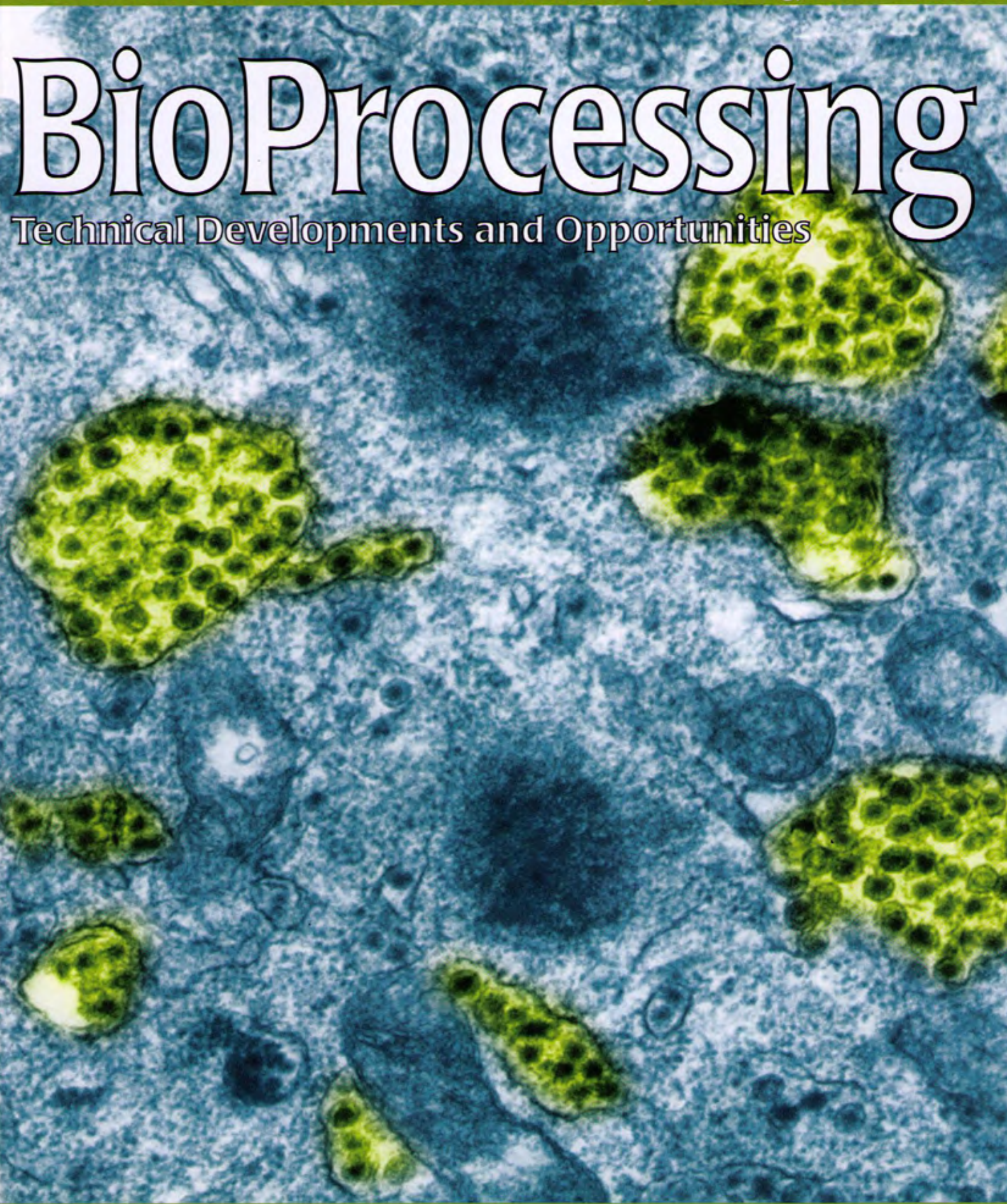

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Animal Viral Vaccine Manufacturing

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This paper reviews the manufacturing of veterinary viral vaccines and discusses the industry regulatory frameworks in both the European Union and the United States, the world's two largest regulatory markets. We also address specific technical and regulatory issues associated with viral vaccine inactivation. Finally, we present two case histories for conventional viral vaccines: Foot and Mouth Disease Virus (FMDV) and Marek's Disease, which are both long-established conventional vaccines, but nevertheless of great interest.

Introduction

The animal health care industry is a very diverse and competitive sector. In 2001, the world market, which is made up of at least ten major players and many smaller niche companies, was worth in excess of US$12 billion. Frequently, the industry is broken down to pharmaceuticals (chemical entities) and biologics, such as vaccines. For animal health products, these business areas are again split into sectors for production animals and pets. Notable features that distinguish the animal and human healthcare markets include market values and the multiplicity of products. Most often, human medicinal products are only developed if their annual market value is predicted to exceed US$100 million (orphan drugs excepted). On the other hand, veterinary vaccines often have market values in the range US$5 million to US$25 million per year, with occasional "blockbusters" exceeding US$50 million. The diversity of animal pathogens results in vaccines that are bacterial, viral, and parasite-based, plus they may use the original organism (conventional), or be based on recombinant DNA technologies. But all animal vaccines have one common theme – they must be developed quickly and have processes that are as simple as possible.

In this article we look briefly at some regulatory issues associated with vaccine manufacturing, and then proceed to some case histories where process issues are considered. An overview of two viral products, Foot and Mouth Disease Virus (FMDV) and Marek's Disease, is presented. These are long-established and conventional vaccines, which illustrate how this type of product is manufactured.

Some Regulatory Issues Associated with Veterinary Vaccine Production

Regulatory Authorities and Standards

The main issues consist of determining the scope (national or global) of product development, and the geographical location of the manufacturing facility. For instance, the European Commission, plus the European Agency for the Evaluation of Medicinal Products (EMEA) and its Committee for Veterinary Medical Products (CVMP) regulate vaccines developed in the European Union (EU). The main regulatory texts are The Rules Governing Medicinal Products in the EU, volume four (GMPs) and volumes 5-8 (controls and clinical testing). Essentially, these documents dictate that products marketed in the EU will be developed according to Good Laboratory Practice (GLP) and manufactured according to Good Manufacturing Practice (GMP) standards. Average development times, based on past experiences with these standards, range between five to seven years.

The United States Department of Agriculture (USDA) and its Animal and Plant Health Inspection Service (APHIS) regulate the development of animal vaccines in the United States. The main regulatory text is the Code of Federal Regulations Title 9 (9 CFR). GLPs and GMPs are not necessary for products developed, manufactured, and marketed in the US, although GLPs are normally followed. Average development times are generally much quicker than in the EU, and typically run between two and five years. Despite these regulatory differences, today's markets are increasingly global, and the tendency, where feasible, is to develop one product that complies with all market regulations, irrespective of where it's developed or produced.

Inactivation of Vaccines : General Strategies

Although the subject of this discussion is limited to viral vaccines, the principles apply to most veterinary vaccines, with the exception of recombinant DNA technologies such as DNA vaccines. Conventional veterinary vac-

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cines can be manufactured with live attenuated viral strains (e.g. Marek's Disease Vaccine), or they can be formulated as a killed or inactivated vaccine (e.g. Foot and Mouth Disease Vaccine).

Chemical agents are typically used to inactivate such vaccines, and care must be taken during this procedure to preserve the structural integrity of the viral particle and its immunogenicity. Inactivating agents can also have an action on the viral nucleic acid (RNA or DNA). As for any chemical reaction, the kinetics of the inactivating agent on viral nucleic acid is driven by the concentration of the substrate (DNA or RNA) and the inactivating agent. Depending on the virus and the chemical agent, different kinetic curves can be observed (Fig. 1). Most viral inactivation reactions are explained by second order kinetics, giving a Y=1/X type curve.

Second order inactivation kinetics can prove difficult to manage, as the decreasing slope of the viral titer diminishes dramatically during the process. This decrease means that the curve presents a significant "tailing effect," as can be seen in Figure 1. When designing an inactivation process, researchers must pay close attention to this "tailing effect" and find the best compromise between the initial inactivating agent concentration and virus particle stability. The more inactivating agent you add at time zero, the faster the viral titer will decrease, however, a high concentration tends to denature viral proteins. If possible, you should design the inactivation process in two phases (double inactivation), with two successive chemical additions that are separated by several hours to avoid high initial concentrations. In order to provide a margin of safety, define the final inactivation process so that the culture is 100% inactivated at 67% of the specified time.

Regulatory Analytical Aspects of Inactivation

Depending on the viral strain and the final product (target species, virus infectivity…), regulatory requirements for inactivation processes may differ. For example, for FMDV, the Ph. Eur. monograph indicates that there must be less than one infectious particle per 10,000 liters of culture at the end of the inactivation time. This represents a concentration of less than 10^{-8} particles/mL. With this value in mind, it is worth modeling the inactivation curve in order to predict the final viral suspension titer at the end of inactivation. The detection thresholds of many analytical titration techniques are frequently too high to measure very low virus concentrations. Inactivation control techniques are thus used to show that the product is actually inactivated. This technique is based on virus amplification in an appropriate cell line. If there are still sufficient infectious particles in the suspension, the virus will infect the cells, multiply, and show cytopathic effect (CPE). The amplification effect carried out over several passages produces detectible virus concentrations.

Manufacturing Case Histories

Foot and Mouth Disease

Foot and Mouth disease (FMD) has been with us for many centuries (Fracastorius, 1546).[1] It is a highly infectious viral infection that affects cattle, pigs, sheep, goats, buffalo, and other wildlife species. FMD induces fever plus vesicles in the mouth, muzzle, teats, and feet. It also results in death in young animals. The disease runs rampant in many parts of the Middle East, Africa, Asia, and South America. Europe has been free of FMD for many years, except for occasional outbreaks such as the one that occurred in the United Kingdom in 2001. An aphthovirus of the family *Picornaviridae* (single stranded RNA virus) causes FMD, and seven immunologically distinct serotypes exist today: A, O, C, Asia 1, and SAT (Southern African Territories) 1, 2, and 3. Within each serotype, a large number of strains exhibits a spectrum of antigenic characteristics. Therefore, covering the antigenic diversity requires a number of vaccine strains for each serotype, particularly O and A.

Contact between susceptible and infected animals facilitates transmission of FMD, although other routes have been noted. FMD incubates for 2-14 days. Infected animals produce a large

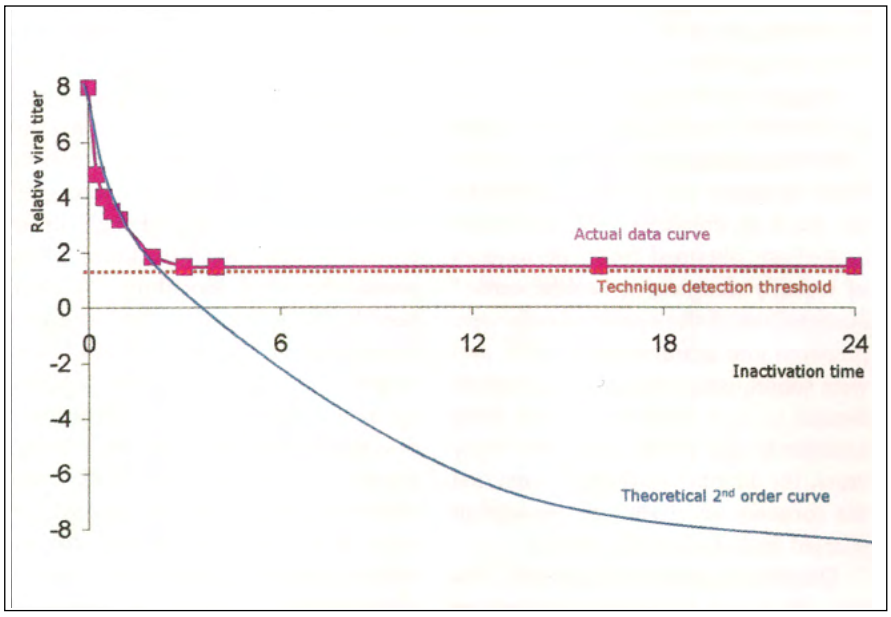

Figure 1: Viral Vaccine Inactivation Examples of inactivation curves. A theoretical second order curve is shown in blue (lower curve), and actual data obtained for an inactivation process is shown in purple, and in this case showing that an assay detection threshold is reached (upper curve). The detection threshold is an artifact of the methodology employed, and the vaccine is shown to be completely inactivated by inoculating inactivated product in control cells and looking for cytopathic effect (see text).

amount of aerosolized virus in their exhaled air, which can infect other animals via respiratory or oral routes. All excretions and secretions from infected animals contain virus, and virus shedding may occur as many as four days before clinical signs appear. Aerosol FMDV can spread a considerable distance as a plume, depending on weather conditions, relative humidity, and surface topography. The occurrence of FMD in countries previously free of the disease can have a devastating effect on local and international trading arrangements. Many countries free of FMD have a policy of slaughtering all affected animals, plus those susceptible animals that have been in contact with sick animals. Typically there are also strict restrictions on animal and vehicle movement around infected premises. After slaughter, the carcasses are burned, and the buildings are thoroughly washed and disinfected with mild acid, or alkali, and then fumigated.

Production Process Description: Historical Issues

Attempts to develop FMD vaccines started in the early 1900s. The first practical inactivated vaccine, developed by Waldmann et al (1937), used virus from the epithelium and vesicular fluid of tongues of deliberately infected cattle.[2] But the need to deliberately infect cattle was undesirable, so production of FMD vaccines was greatly assisted by the work of Frenkel (1947) who used epithelium obtained from the tongues of recently slaughtered healthy cattle.[3] Suspensions of the epithelial cells were prepared and maintained *in vitro*, and were subsequently infected in a manner similar to that used today with baby hamster kidney (BHK) cells. For many years, the Frenkel procedure remained the cornerstone of vaccine production (Girard and Mackowiak, 1953).[4]

Disadvantages associated with the use of bovine tongue epithelium included the logistics of collecting sufficient material, as well as maintaining sterility throughout the process. This prompted researchers to find a more appropriate cell line for production needs. Mowat and Chapman (1962) adopted the baby hamster kidney monolayer cell line, BHK-21, which had previously been developed by MacPherson and Stoker (1962), and had demonstrated that it could be used for the growth and titration of FMDV.[5,6]

Current Manufacturing Process

Given the relatively high risk of dissemination, and the highly infectious nature of FMDV, particular precautions must be observed when handling this virus. Production of FMD antigens and vaccines must be carried out within the framework of a Quality System, and under conditions that fully comply with internationally recognized standards of GMP and biocontainment. Researchers must put efficient decontamination procedures in place to prevent any possibility of virus escape from the manufacturing facility. Personnel working in Merial's FMDV manufacturing facilities follow a very strict code of conduct to avoid cross-contamination. These codes forbid contact with ruminants, and ban visits to farms or zoos for three days following work in the virus production suite of the manufacturing facility.

The process takes place in a completely closed system. The host cell for virus multiplication, BHK21, is grown in suspension culture. Using a seed train, cells are amplified to a final volume of several cubic meters. When the cell population is adequate, the spent medium is discarded and the cells are re-suspended in fresh medium. At this point, the viral inoculum (Working Seed Virus) is added to the bioreactor. Depending on the virus strain, infection time typically runs about 24 hours, but may need adjustment. Virus infection and multiplication kill the cells and result in a characteristic CPE when observed under the microscope. As soon as the majority of cells die, the viral suspension is clarified by centrifugation, and/or filtration, to remove cell debris and aggregates prior to the inactivation process. According to GMP requirements, the inactivation process is carried out in a two-tank system to ensure complete contact between virus particles and inactivating agent. The inactivating agent is added to the first stirred tank, which contains the bulk virus, and the suspension is then transferred to a second tank. While formaldehyde inactivation proved an acceptable process for many years, it became increasingly apparent that its use carried a slight risk of allowing the inactivated vaccines to be contaminated with live virus (Lombard, 2001).[7] The use of an aziridine acetylethyleneimine as a first order inactivant of the virus was first described by Brown and Crick (1959), and the basic aziridine methodology grew in popularity because of the effectiveness and reliability of the process at the industrial scale.[8] Acetylethyleneimine is no longer used, and the most widely employed inactivation process is based on binary ethyleneimine (BEI), which is generated by the action of alkali on bromoethylamine hydrobromide shortly before it is required (Bahnemann, 1973).[9] From a regulatory standpoint, BEI inactivation is invariably demanded by national and international authorities, and formaldehyde inactivation is no longer acceptable. Using BEI in a two-tank system and following GMP, it is possible to achieve the Ph. Eur. requirement of less than one infectious particle per 10,000 liters of FMD antigen preparation.

After inactivation, ultrafiltration is used to concentrate the product to where it is amenable to chromatographic purification. Chromatography is then used to purify the product. This purification process presents a major difference, and advantage, over other manufacturers' vaccines, because it ensures excellent safety, improved efficacy, and long-term stability. The active ingredient is then stored in the gaseous phase of liquid nitrogen until formulation.

The formulation of the final product depends on the strains required in the vaccine and the species to be vaccinated. FMD vaccines commonly contain multiple strains of the virus to reflect the epidemiological situation in the customer's country (Doel, 2002).[10] South American vaccines, for example, are commonly trivalent, containing single isolates from the region of serotypes O, A, and C. Other regions of the world may require more or less complex products. Selection of the most appropriate strain(s) necessitates a sound knowl-

edge of the FMD situation worldwide, plus the resources to determine and match the vaccine strain to the strains prevalent in a given region. Once the selection of strains is made, the antigen concentrates are removed from the liquid nitrogen freezer, diluted with buffers, and then blended with either an oily adjuvant, or with aluminium hydroxide and saponin. Up to eight vaccine strains can be associated in a combination FMD vaccine, provided the inactivated antigens are purified by chromatography.

Marek's Disease

Marek's Disease is a transmissible lymphoproliferative disease in poultry that is caused by a *herpesvirus*, a double stranded DNA virus (Witter, 1997).[11] The disease is seen worldwide and its effects are related to the pathotypes present in a specific country or area. Clinical signs of Marek's Disease include depression, weight loss, starvation, and paralysis. Marek's Disease may occur in chickens after three weeks of age, but occurs most often in birds between 12 and 24 weeks of age. Malignant tumors appear in internal organs and peripheral nerves. Feather follicles in the skin are the only sites of virus replication. Feathers, dander, dust, and litter contain the virus, and airborne dispersion of this refuse spreads it. The disease causes significant economic losses due to mortality, loss of egg production, and condemnation at slaughter. Good hygiene practices play an important role in controlling the disease. In addition, vaccination can effectively control Marek's Disease. There are three main serotypes having different pathogenicity, including CV1988 – Rispens (serotype 1), SB-1 (serotype 2), and HVT (serotype 3). Two forms of vaccine are available – a live, attenuated, cell-associated "wet" vaccine, and a cell-free, lyophilised version for HVT only. Most people use the "wet" vaccine where production logistics permit because it is much more effective.

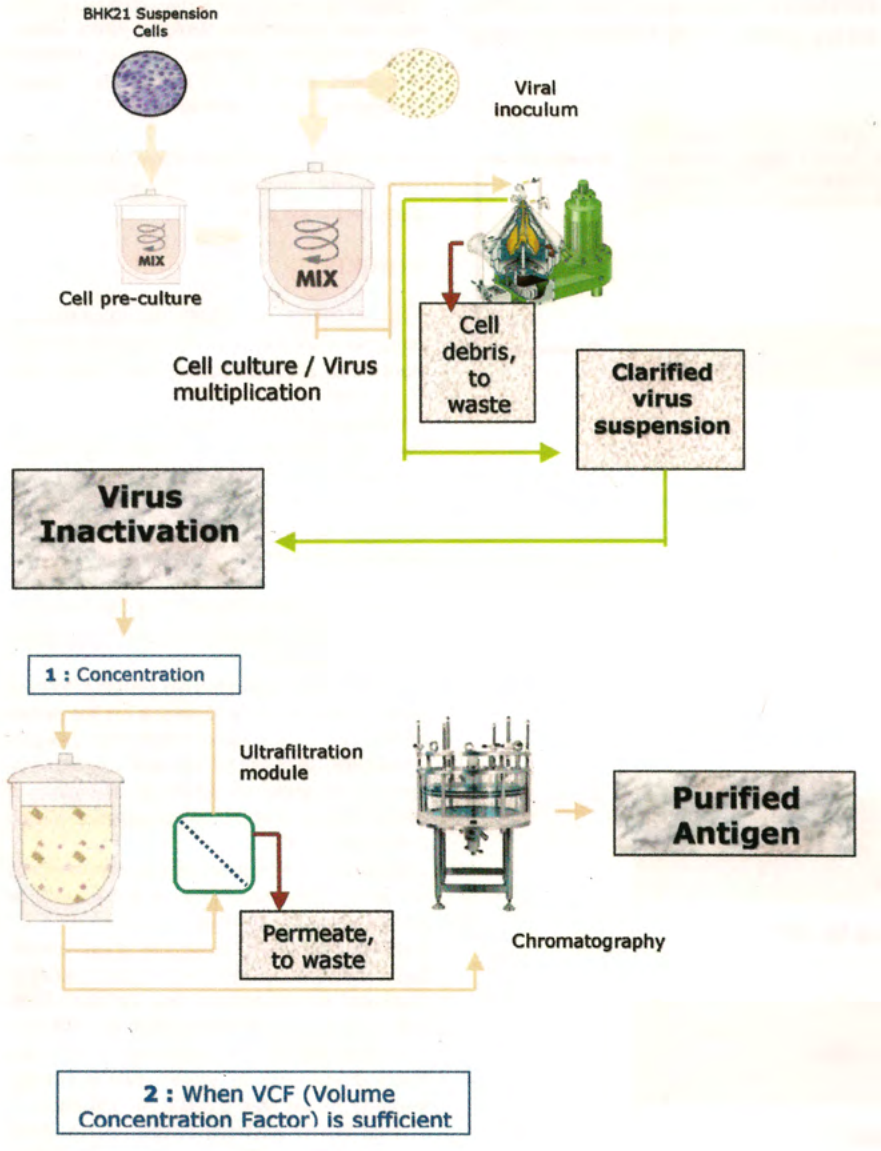

Figure 2: Foot and Mouth Disease Vaccine Production
Cells are grown in a bioreactor. When the desired concentration is reached they are transferred to a second production train and inoculated with Working Seed Virus. When the cytopathic effect is complete, the culture is harvested and inactivated. Following concentration, the elutate is purified by chromatography and stored, where it is ready for formulation and filling.

Production Process Description

As mentioned above, Marek's Disease vaccine is an attenuated live vaccine. A Master Virus Seed is prepared using virus attenuated by successive passages. No return to virulence should be observed during six serial passages on subsequent control, as tested on SPF chicks. Birds inoculated with a 10x field dose should have no significant lesions after 120 days (as per 9CFR). Subsequent vaccine manufacturing must take place within X + 5 passages of Master Seed Virus. This vaccine is manufactured in roller bottle culture using Chicken Embryo Fibroblasts (CEF) as shown in Figure 3 (page 52). CEFs are prepared in bulk prior to seeding for different productions. A typical medium employed in production might be EMEM or F199 with 5% serum. The subsequent virus production process varies slightly depending on the strain. A deceptively simple process hides small process "tricks" which can make a significant difference in product quality and viral yield. Finally, dilution for the

freeze-dried and cell-associated vaccines is based on previous experience, because the titer can only be assessed after freezing the final product. Final product ships in bottles of approximately 1,000 dose units.

Conclusions

This paper provides a brief overview of the regulatory environment for the manufacture of veterinary viral vaccines, and focuses on some practical aspects associated with vaccine inactivation. The two case histories of conventional vaccines elucidate some of the issues involved with the manufacture of these types of vaccines. Each product varies, requiring careful attention to detail during both process design and subsequent scale-up to avoid manufacturing failures at full production scale. Overall, we hope that these case histories help to clarify the steps animal vaccine manufacturers should follow to stay competitive in today's global markets.

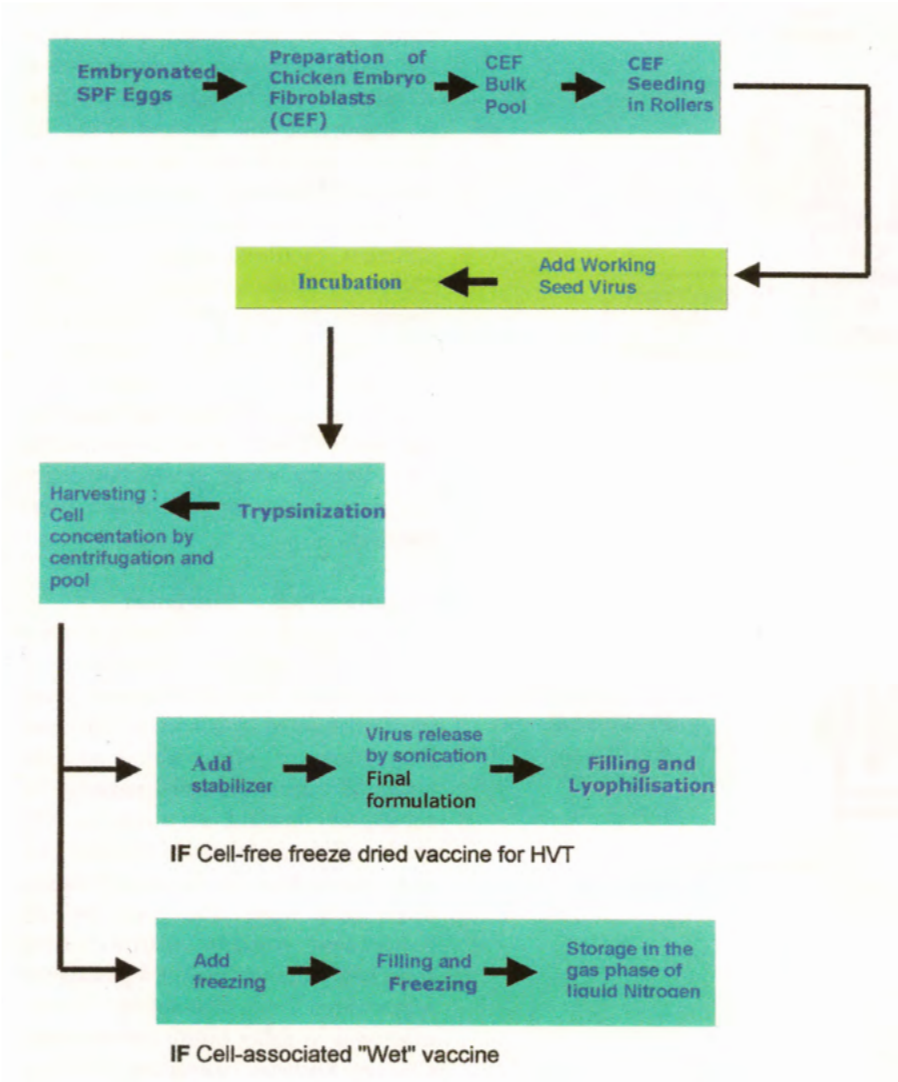

Figure 3: Marek's Disease Vaccine Process
Marek's Disease vaccine is manufactured according to EU regulatory requirements at Merial's Gainesville site in Georgia, USA. A bulk pool of Chicken Embryo Fibroblasts are prepared from SPF embryonated eggs, and are seeded into roller bottles. Working Seed Virus is added. The actual process is slightly different depending on the virus serotype manufactured (see text). After harvesting, cells are either sonicated and freeze-dried in the case of HVT, or filled as a "wet," cell-associated vaccine in the case of any of the other serotypes.

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REFERENCES

1.FRACASTORIUS H (1546). De sympathia et antipathia rerum liber unus. De contagione et contagiosis morbis et eorum curatione liber I. Venice, Heirs of L.A. Junta.
2.WALDMANN O, KÖBE K and PYL G
Die aktive Immunisierung des Rindes gegen Maul - und Klauenseuche mittels Formolimpfstoff. Zent. Bakt. Parasit. Infekt (1937) 138 : 401-412.
3.FRENKEL HS. La culture du virus de la fièvre aphteuse sur l'épithélium de la langue des bovidés. Bull. Off. int. Epiz (1947) 28 : 155-162.
4.GIRARD H and MACKOWIAK C. La Culture du virus Aphteux au stade industriel. La Revue d'Immunologie (1953) 17 : 224-238.
5.MOWAT GN and CHAPMAN WG. Growth of foot and mouth disease virus in a fibroblastic cell line derived from hamster kidneys. Nature (1962) 194 : 253-255.
6.MACPHERSON IA and STOKER MGP. Polyoma transformation of hamster cell clones - an investigation of genetic factors affecting cell competence. Virolog (1962) 16 : 147-151.
7.Lombard M. Une revue des développements récents des vaccines de la fièvre aphteuse. Bull Acad Vét de France (2001) 154 : 381-394.
8.BROWN F and CRICK J. Application of agar - gel diffusion analysis to a study of the antigenic structure of inactivated vaccines prepared from the virus of foot and mouth disease. J. Immunol (1959) 82 : 444-447.
9.BAHNEMANN HG. The inactivation of foot and mouth disease virus by ethyleneimine and propyleneimine. Zbl. Vet. Med. B (1973) 20 : 356-360.
10.DOEL TR. Foot and Mouth Disease : vaccine strains and field isolates. FMD control strategies (2002). Mérieux Foundation IABS and OIE Internal Symposium, Lyon, June 2002. Editors, Elsevier (in press).
11.Witter RL. Marek's Disease : an evolving pathogen, current and future strategies for its control. Presentation at the AAAP Symposium : Infections Poutry diseases, Athens, Ga (1997).