# Taking the Strain

Mammalian cell culture systems are starting to dominate the production of vaccines for viral diseases such as rabies. Making use of existing biopharma infrastructure, these advances might even spell the end for the use of hens' eggs in future vaccine development

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Today, the rapidly expanding demand for vaccine products for viral diseases such as rabies has necessitated the development of more sophisticated production techniques based around cell culture systems. This article reviews vaccine production strategies, with a focus on rabies, looking specifically at the use of the Vero cell line – used worldwide and approved by the US Food and Drug Administration – as well as media technology and the bioreactor options available.

## Growing Demand

Viral diseases, including rabies, are worldwide challenges for the international biomedical community. The World Health Organization (WHO) notes that over 32,000 rabies-related deaths were reported in 1998, while annual deaths worldwide from the virus grew to 55,000 by 2006 (1,2).

Rabies is often transmitted to humans from infected domestic animals. Dogs infected with rabies can become extremely aggressive and attacks on humans are widespread, especially in certain Asian countries where using unleashed dogs for home security is common. It is spread through the saliva of infected animals and bites can be fatal.

Since the 18th century, vaccination has proven to be the most successful – and perhaps the only – route to the total elimination of viral diseases such as rabies. From the early work of pioneers such as Jenner and Pasteur, vaccination was put on a sound scientific footing. Demand for vaccine products has continued to increase ever since, prompting the advances in production technology that we see today.

## Viral Cultivation

The expanding vaccine requirements have led to techniques for growing large quantities of antigenic proteins. Traditionally, viruses have been grown in embryonated hens' eggs, but numerous shortcomings compromise their utility. These include a bottleneck in the availability of high-quality, pathogen-free eggs, as well as low titers of emerging viruses (3).

A major concern is that, when viruses are cultivated through extended passages in hens' eggs, there is an evolutionary process in the amnion or allantoic cavity of the egg, resulting in the selection of a virus subpopulation, antigenically and biochemically distinct from the original inoculum (4). Because of these and other factors, permanent cell lines are coming to dominate the field as an alternative method.

Mammalian cell culture systems provide much shorter lead times; a more controlled production process that takes advantage of closed-system bioreactors; a reduced risk of microbial contamination; and the opportunity to cultivate viral stocks without significant egg passage-dependent antigenic changes (5). These systems provide

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a flexible and scalable platform that can make use of existing biopharma infrastructure for vaccine production, and could replace egg-based vaccines in the foreseeable future (6).

## Cell Line Options

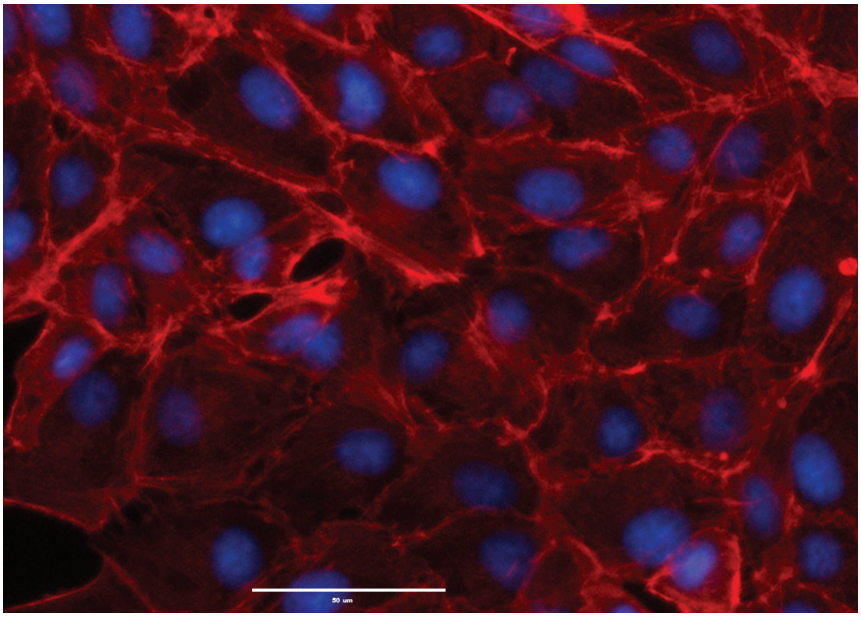
In the past few years, several continuous cell lines have been approved by regulatory authorities for virus production. These include the *Spodoptera frugiperda* insect cell line from Protein Sciences (7), Madin-Darby canine kidney (MDCK), the PER.C6 cell line, designed for growth to high densities (8), and the widely used Vero line.

It is important to note that certain cell lines may provide an environment favouring selection of viral subpopulations, and these types may be inappropriate for vaccine production. In light of this, various lines of investigation support the Vero cell line as the candidate of choice for viral vaccine production, including:

- Efficiency of primary virus isolation and replication to high-infectivity titers
- Genetic stability of the haemagglutinin molecule, while maintaining the antigenic properties of human-derived viruses
- Similarities in the pattern of protein synthesis and morphological changes between virus-infected Vero and MDCK cells (9)

## Media Alternatives

There is a variety of different Vero isolates available from commercial suppliers, but all are quite similar, and their nutritional needs are comparable (10). The search for the ideal mammalian cell culture medium began in the 1950s, with the holy grail being an economical, protein-free, serum-free medium that would provide strong growth support and have the property of scalability to large volumes, up to thousands of litres, while coming in at an affordable price.



Source: Eppendorf, Inc

**Figure 1:** Fluorescent image of confluent Vero cells; DAPI-stained nuclei appear blue, and actin filaments stained with rhodamine-conjugated phalloidin appear red

Serum provides a protective function to cultured cells, and binds toxins and other contaminating materials. As such, serum-free media must be extremely carefully formulated (11). Albumin can be substituted for serum, but it may impede the downstream steps of purification (12).

Chen *et al* tested five different serum-free media, combined with Cytodex 1 microcarriers (12). The following were evaluated: OptiPro SFM (Invitrogen), VP-SFM (Invitrogen), EX-CELL Vero SFM (SAFC Biosciences), Provero-1 (Lonza) and HyQ SFM4MegaVir (HyClone). EX-CELL Vero SFM gave one of the highest cell densities, demonstrating that the use of serum-free media has become routine for Vero cell cultivation. Comparable results were observed with a commercial serum-free medium MDSS2N (AXCEVIR-VeroTM by Axcell Biotechnologies).

## Rabies Strategies

The Brazilian group led by Frazatti-Gallina has been active in the field of rabies vaccine production (13). Using Vero cells adhered to microcarriers, and cultivated in a bioreactor with

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serum-free medium, they generated an effective rabies vaccine. With the aid of tangential filtration, they purified the rabies virus by chromatography and inactivated it using beta-propiolactone.

Their protocol states that 350cm$^2$ T-flasks were harvested and inoculated into a 3.7-litre CelliGen bioreactor, at a proportion of 16 cells per microcarrier (Cytodex 3-GE), yielding an initial seeding of 2.5×10$^5$ cell/ml. The cells were grown in serum-free MDSS2 medium.

The serum-free VP-SFM medium, according to the manufacturer, was developed for Vero, BHK-21 and Chinese hamster ovary cell growth. This medium drives the adherence of the Vero cells to the microcarriers. After four days of cultivation in VP-SFM medium, the cells were infected with Pasteur vaccins (PV) strain rabies virus (multiplicity of infection = 0.08). The harvests of the culture supernatant were carried out three days after the virus inoculation and four times thereafter at 24-hour intervals. During this period, culture conditions were maintained at 60rpm at a pH of 7.15 and 5 per cent dissolved oxygen. Only the temperature varied, from 36.5ºC in the cellular growth phase of the culture to 34ºC after virus inoculation. In the course of the programme, seven batches of virus suspensions were produced in the bioreactor (16 litre per cycle) at a mean viral titer of 104. FFD50/0.05ml.

The effectiveness of the preparation was demonstrated by immunising mice with three doses of the new vaccine, and comparing it with the commercial Verorab and human diploid cell rabies vaccine. Mean titers of neutralising antibodies of 10.3-34.6, 6.54 and 9.36 IU/ml were found, respectively.

The choice of the serum-free medium was fortunate. In this case, the amount of contaminating DNA was very low and tolerable – less than 22.8pg per dose of vaccine. The authors argue that this protocol is especially applicable in the developing world, where rabies is a constant hazard and a major public health problem.

## Increasing Yield

Yu *et al* sought methods to increase yield in Vero cell culture systems over that obtained using roller bottles (14). In a recent review, they summarised the production technology developed over the course of the last seven years. They have adopted the 30-litre BioFlo 4500 fermentor/bioreactor. The cells were cultivated in media containing 10 per cent serum, first grown as a monolayer, and when the cell density reached 1.0-1.2×10$^6$ cells/ml, they were transferred to the bioreactor containing 25g/litre of Cytodex-1 for perfusion culture. The virus preparations, also cultured in roller bottles, were infected with the PV2061 virus strain, harvested and transferred to the bioreactors.

## Purified Vaccine

Wang *et al* have described a purified Vero cell rabies vaccine that has been widely produced in China, referred to as ChengDa (Liaoning ChengDa Biological) (15,16). It is grown on a Vero cell line utilising the PV2061 strain, inactivated with beta-propiolactone, lyophilised, and reconstituted in 0.5ml of physiological saline. It fulfils WHO recommendations for potency.

The process used for ChengDa was developed by Aycardi (17). A single bioreactor was capable of producing one million doses of rabies vaccine per year. The method uses ultra-high-density microcarrier cell cultures adapted to a 30-litre CelliGen bioreactor equipped with a patented cell lift impeller, specifically configured for a perfusion system to feed the growth media into the bioreactor. A specially designed decanting column was used to prevent perfusion loss of microcarrier and keep the cells in high concentration. The system delivers high oxygen transfer, high nutrient level and low shear stress, allowing cell growth of up to 1.2x10$^7$ million cells/ml under continuous perfusion for up to 20 days.

## Important Step

The combination of advances reviewed here provides strong support for the use of cell culture systems for virus production of vaccines. The fact that Vero cells have been approved for clinical products represents an important step on the road to technologies that do not rely upon hens' eggs for generation of adequate quantities of viruses.

Advances in culture media enable the elimination of serum, thus driving the rapid and efficient purification of proteins. The use of carrier beads adds to the efficiency of culture technology, allowing greatly increased cell densities to be reached. Improvements in bioreactor design, combined with these various technological advances, results in a greatly improved and more functional production train.

References


1. World survey of rabies number 34 (for the year 1998), WHO, 2000. Visit: www.who.int/emc/diseases/zoo/wsr98/html version/wsr 98 index.html
2. Kaur M, Rai A and Bhatnagar R, Rabies DNA vaccine: no impact of MHC class I and class II targeting sequences on immune response and protection against lethal challenge, *Vaccine* 27(15): pp2,128-2,137, 2009
3. Genzel Y and Reichl U, Continuous cell lines as a production system for influenza vaccines, *Expert Rev Vaccines* 8(12): pp1,681-1,692, 2009
4. Oxford JS *et al*, Serological studies with influenza A(H1N1) viruses cultivated in eggs or in a canine kidney cell line, *Bull World Health Organ* 65(2): pp181-187, 1987
5. Doroshenko A and Halperin SA, Trivalent MDCK cell culture-derived influenza vaccine, Optaflu (Novartis Vaccines), *Expert Rev Vaccines* 8: pp679-688, 2009
6. Montomoli E *et al*, Cell culture-derived influenza vaccines from Vero cells: a new horizon for vaccine production, *Expert Rev Vaccines* 11(5): pp587-94, 2012
7. Visit: www.proteinsciences.com/BEVS.htm
8. Visit: www.crucell.com/Technology%20-%20Cell%20Technology

9. Govorkova EA *et al*, African green monkey kidney (Vero) cells provide an alternative host cell system for influenza A and B viruses, *J Virol* 70(8): pp5,519-5,524, 1996
10. Ammerman NC, Beier-Sexton M and Azad AF, Growth and maintenance of Vero cell lines, *Current Protocols in Microbiology*, Appendix 4E, November 2008
11. Chen A *et al*, Serum-free microcarrier based production of replication deficient influenza vaccine candidate virus lacking NS1 using Vero cells, *BMC Biotechnol*, 11th August 2011
12. Visit: www.invitria.com/cell-culture-products-services/serum-albumin.html
13. Frazatti-Gallina NM *et al*, Vero-cell rabies vaccine produced using serum-free medium, *Vaccine* 23(4): pp511-517, 2004
14. Yu P *et al*, Production and evaluation of a chromatographically purified Vero cell rabies vaccine in China using microcarrier technology, *Hum Vaccine Immunother* 8(9): pp1,230-1235, 2012
15. Wang C *et al*, Promising rabies vaccine for postexposure prophylaxis in developing countries, a purified Vero cell vaccine produced in China, *Clin and Vaccine Immuno* 17(4): p688, 2010
16. Visit: bariconsultants.com/projects.html
17. Aycardi E, Producing human rabies vaccines at low cost, *Genetic Engineering News* 22(8), 5th April 2002


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