Vero Cell-based Vaccine Production: Rabies and Influenza Cell lines, Media and Bioreactor Options

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1. Abstract

We review strategies for optimizing vaccine production with examples given for rabies and influenza using cell culture systems. The Vero cell line is one of the most satisfactory based on its stability and well-documented performance in quality and quantity of viral yield. It has received FDA approval and is used throughout the world. Cell culture media technology has advanced dramatically in recent years, and a number of serum free and protein free options are available through commercial suppliers. Because serum tends to bind toxins and contaminants, its elimination calls for careful monitoring of culture conditions in order to achieve optimal performance.

Improvements in microcarriers have been important additions to the range of possible choices for optimizing in vitro production systems. With a series of bioreactor options available, we can foresee the elimination of hens eggs for virus production.

2. Introduction

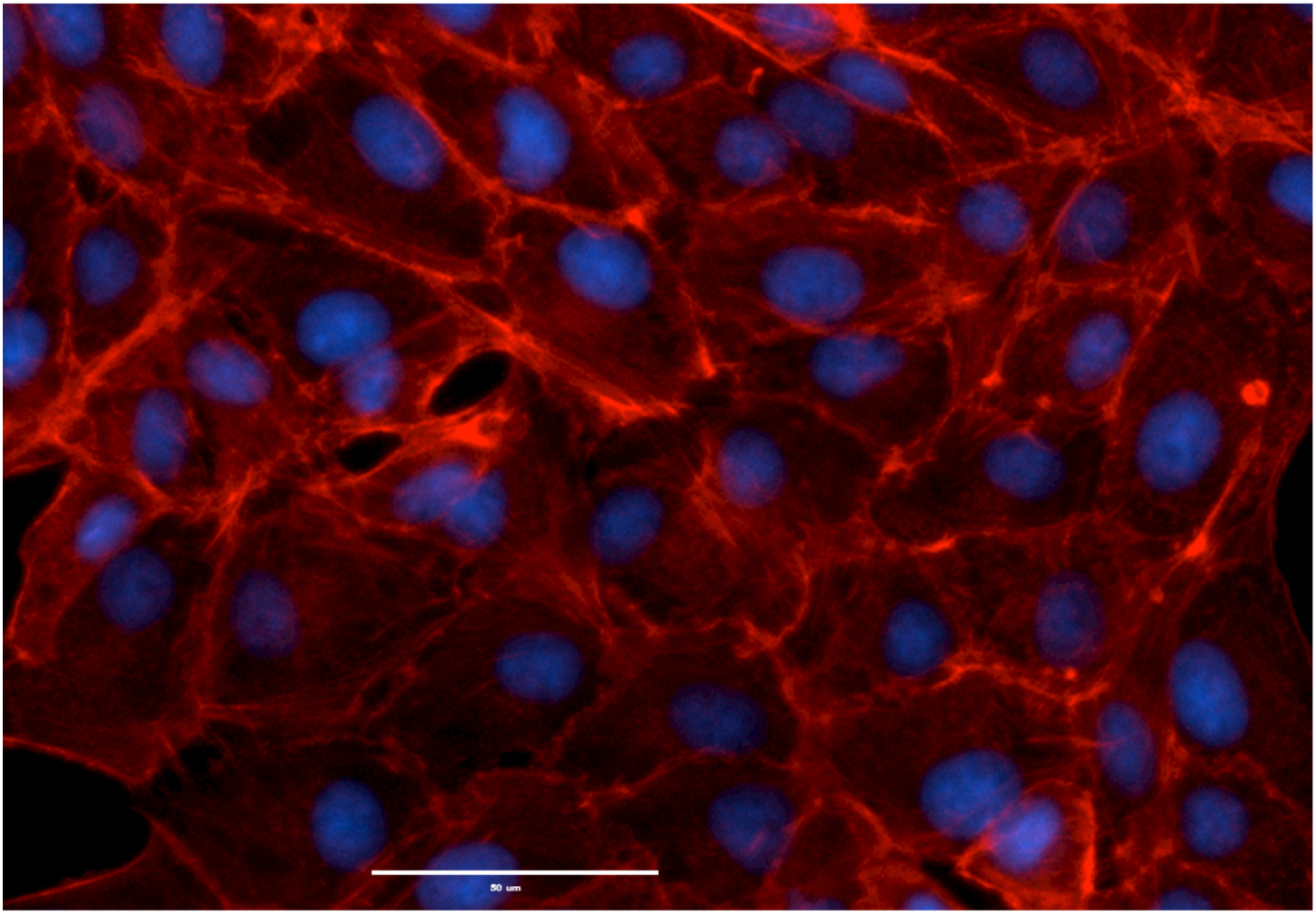

Figure 1. Fluorescent image of confluent Vero cells; DAPI-stained nuclei appear blue, and actin filaments stained with rhodamine-conjugated phalloidin appear red (Eppendorf Inc.).

Viral diseases, including rabies and influenza, are worldwide challenges to the international biomedical community. WHO notes that in 1998 over 32,000 deaths due to rabies were reported, while influenza has been responsible for millions of deaths worldwide over the course of the last century.[1]

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 Asia countries where using unleashed dogs for home security is common. The virus is spread through their saliva and bites by infected animals can be fatal. In China, the disease is referred to as “Kuang Quan Bing” (狂犬病 in Chinese), i.e. “Mad Dog Disease”. The annual number of deaths worldwide caused by rabies had grown to 55,000 by 2006.[2]

Influenza is a second worldwide scourge. The CDC Influenza Division reported an estimated range of deaths between 151,700 and 575,400 individuals resulting from the 2009 H1N1 virus infection during the first year the virus circulated.[3] These figures, however pale in comparison to reports of a half a million deaths *every year* throughout the world due to influenza.[3] Annual deaths in the United States top 36,000 with 114,000 hospitalizations accompanied by a staggering cost of $600 million in health care and an additional $1 billion in economic costs.[4] Anti-viral drugs are employed for acute treatment, but vaccination remains far and away the most effective approach for combating viral illnesses.

Moreover, there is a constant, underlying concern regarding the possibility of the emergence of a truly deadly influenza strain, on a level with the 1918 influenza outbreak, the “Spanish Flu" that caused ~50 million deaths worldwide. For this reason, existing technologies are being relentlessly evaluated and upgraded with the aim of avoiding a devastating pandemic.[5]

Since the 18th century, vaccination has proven to be the most successful (and perhaps the only) route to the total elimination of viral diseases. The history of smallpox is well known, as is the introduction of the use of cowpox virus from lesions in infected animals by Jenner in 1796.[6] Despite his work and that of others, smallpox epidemics continued throughout the 19th century, due to improperly applied or non-existent vaccination regimes. The work of Pasteur and others toward the end of the 19th century put vaccination on a sound scientific footing.[7]

3. Biological Systems for Viral Cultivation

Today throughout the world there is a rapidly expanding demand for vaccine products. These growing requirements have necessitated the development of a range of techniques for growing large quantities of antigenic proteins. Traditionally, viruses have been grown in embryonated hen’s 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.[8] A major concern is the fact 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 innoculum.[9] Because of these and other factors, permanent cell lines are coming to dominate the field.

As an alternative to egg-based vaccine production, the advantages of mammalian cell culture systems have been widely recognized. Cultured cells 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.[10]

A WHO conference some years ago enunciated concern regarding the rapid emergence of pandemic viral strains. It was concluded that insufficient time would be available to generate the large quantity of high quality, fertile hens’ eggs that would be required to the demands of a worldwide pandemic.[11] In the intervening years, the situation has only exacerbated. Thus the cell culture alternative provides a flexible and scalable platform that can make use of existing biopharmaceutical infrastructure for Influenza vaccine production. Indeed, Montomoli et al[12] argue that because of these inherent limitations, cell culture will replace egg-based vaccines within the foreseeable future.

4. Cell Line Options

In the past few years, several continuous cell lines have been approved by regulatory authorities for influenza virus production, such as the *Spodoptera frugiperda* insect cell line (Protein Sciences[13]), the Madin-Darby canine kidney (MDCK) and the Vero line, one of the most widely used. A fourth alternative is the PER.C6® cell line,[14] designed for growth to high densities. This property means that much more biological product can be harvested from much smaller bioreactors. The manufacturers claim that the PER.C6 cells, infected with virus for manufacturing purposes, produce at

least 10 times more virus per ml than other FDA approved cell lines. It should be noted that PER.C6 is a proprietary cell line, and licensing costs, obtained from Crucell, may be substantial.

It is important to be aware that certain cell lines may provide an environment favoring selection of viral subpopulations, and these types may be inappropriate for vaccine production. Anez et al attempted[15] production of Dengue virus vaccine candidates using FRhL-2 diploid fetal rhesus monkey lung cells. However, passage in this cell line resulted in the accumulation of a mutational variant which was responsible for reduced infectivity and immunity in Rhesus monkeys. This phenomenon was not observed in viruses passaged in the Vero cell line. Other 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 hemagglutinin molecule, while maintaining the antigenic properties of human-derived viruses; and similarities in the pattern of protein synthesis and morphological changes between virus-infected Vero and MDCK cells.[16] Vero is the only cell line that has received worldwide regulatory acceptance.[17]

There are claims that head to head comparison of growth performance in lab-scale bioreactors (stirred tank, wave bioreactor) resulted in lower yield for Vero cells as opposed to the MDCK line, although both displayed comparable productivity in small scale systems. However, this observation is applicable only under the specific conditions and specific cell lines employed in this study. Given the regulatory acceptance of the Vero cell line as well as the abundance of vaccines already successfully developed (Table 1), Vero remains one of the most attractive platforms for cell based viral vaccine production.

5. Media Alternatives

There are a variety of different Vero isolates available from commercial suppliers (Vero, Vero 76, Vero E6, Vero B4), but all are quite similar, and their nutritional needs are comparable.[18] 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 liters, while coming in at an affordable price.

Anti-viral Vero Cell-Based Vaccines

Study (year)	Disease	Vaccine Type	Genus
Wang et al (2008)	Chikungunya Fever	Live attentuated	Alphavirus
Howard et al (2008)	Chikungunya Fever	Inactivated	Alphavirus
Guirakhoo et al (2004) Blaney et al (2007) Blaney et al (2008)	Dengue Fever	Live attenuated or live chimeric	Flavivirus
Tauber et al (2007) Tauber et al (2008) Srivastava et al (2001)	Japanese encephalitis	Inactivated	Flavivirus
Kuzuhar et al (2003) Guirakhoo et al (1999) Monath et al (2003)	Japanese encephalitis	Live attenuated or live chimeric	Flavivirus
Vesikari et al (2006) Ruis-Palacios et al (2006)	Viral gastroenteritis	Live attenuated	Rotavirus
Montagnon (1989) Montagnon (1989)	Polio	Live attenuated Inactivated	Picornovirus
Montagnon (1989)	Rabies	Inactivated	Lyssavirus
Aycardi E (2002)	Rabies	Inactivated	Lyssavirus
Kistner et al (2007)[19]	Ross River fever	Inactivated	Alphavirus
Spruth et al (2006) Qu et al (2005) Qin et al (2006)	Severe acute respiratory syndrome	Inactivated	Cornovirus
Monath et al (2004)	Smallpox	Live attenuated	Orthopoxvirus

Lim et al (2008)	West Nile Encephalitis	Inactivated	Flavivirus
Monath et al (2006)	West Nile Encephalitis	Live attenuated	Flavivirus
Kistner et al (1998) Ehrlich et al (2008)	Influenza	Inactivated	Orthomyxovirus
Bonnie and William (2009)	Influenza	Inactivated	Orthomyxovirus
Chan and Tambyah (2012)	Influenza	Inactivated	Orthomyxovirus

Table 1. Anti-viral vaccines using Vero cell culture production technologies. Modified from Barrett et al[23].

Serum provides a protective function to cultured cells and binds toxins and other contaminating materials. Thus serum-free [20] media must be extremely carefully formulated. Albumin can be substituted for serum, but it may impede the downstream steps of purification.[21]

Chen et al[21] have tested five different serum free media, combined with Cytodex 1 microcarriers. The following were evaluated: OptiPro SFM (Invitrogen®), VPSFM (Invitrogen), EX-CELL™ Vero SFM (SAFC Biosciences®), Provero-1 (Lonza®) and HyQ SFM4MegaVir (HyClone®). The 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 (manufactured under the name AXCEVIR-VeroTM by Axcell Biotechnologies). In this case, Vero cells were compared with MDCK cells grown in T-flasks and microcarrier cultures.

6. Rabies Virus Cultivation Strategies

The Brazilian group led by Frazatti-Gallina[22] has been active in the field of Rabies vaccine production. Using Vero cells adhered to microcarriers, and cultivated in a bioreactor with 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 350 cm^2 T-flasks were harvested and inoculated into a 3.7 liter New Brunswick™ 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 (Axcell Biotechnologies).

The serum-free VP-SFM medium, according to the manufacturer, was developed for Vero, BHK-21 and CHO cell growth. This medium drives the adherence of the Vero cells to the microcarriers. After 4 days of cultivation in VP-SFM medium, the cells were infected with PV rabies virus (multiplicity of infection = 0.08). The harvests of the culture supernatant were carried out 3 days after the virus inoculation and four times thereafter at intervals of 24 h. During this period, culture conditions were maintained at 60 rpm at a pH of 7.15 and 5% dissolved oxygen. Only the temperature varied from 36.5 ^oC in the cellular growth phase of the culture to 34^oC after virus inoculation. In the course of the program, seven batches of virus suspensions were produced in the bioreactor (16L per cycle) at a mean viral titer of 104. FFD50/0.05 ml.

The effectiveness of the preparation was demonstrated by immunizing mice with three doses of the new vaccine (seven batches), comparing it with the commercial Verorab and HDCV (Rabies vaccine). Mean titers of neutralizing 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 22.8 pg 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.

Yu et al[23] sought methods to increase yield in Vero cell culture systems over that obtained using roller bottles. In a recent review, they summarized the production technology developed over the course of the last seven years. They have adopted the 30 L New Brunswick BioFlo® 4500 Fermentor/Bioreactor. The cells were cultivated in media containing 10% serum, first grown as a monolayer, and when the cell density reached $1.0–1.2 \times 10^6$ cells/ml, they were transferred to the bioreactor containing 25 g/L 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.

Wang[24] et al have described a purified Vero cell rabies vaccine that has been widely produced in China, which currently is responsible for almost two-thirds of the total rabies vaccines used in Asia. The most successful offering used in China is a purified Vero cell vaccine, referred to as ChengDa (Liaoning ChengDa Biological Co., Ltd., Shengyang, China[25]). It is grown on a Vero cell line utilizing the L. Pasteur 2061 strain of rabies virus, inactivated with β-propiolactone, lyophilized, and reconstituted in 0.5 ml of physiological saline. It fulfills the WHO recommendations for potency.

The process used at ChengDa was developed by Aycardi.[26] A single New Brunswick bioreactor was capable of producing one million dose of rabies vaccine per year. The method uses ultra-high density microcarrier cell cultures adapted to a 30 L New Brunswick CelliGen bioreactor equipped with a patented Cell Lift Impeller (Figure 2), specifically configured for a perfusion system to feed the growth media into the bioreactor. A specially designed decanting column (New Brunswick Scientific) 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, thus allowing cell growth up to 1.2 x 10^7 million cells/ml) under continuous perfusion for up to 20 days.

ChengDa was licensed by the Health Ministry of China and the State Food and Drug Administration of China (SFDA) in 2002 and has been marketed throughout the country since that time. Although not approved for sale in the United States, purified Vero cell rabies vaccine is permitted for use by US citizens if available in a destination country, according to the CDC[24].

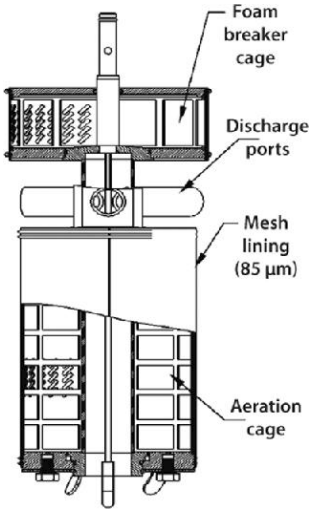

Figure 2. New Brunswick Cell Lift Impeller (Eppendorf Inc). Patented design consists of three discharge ports located on the impeller shaft to provide uniform circulation without traditional spinning blades for conducing microcarrier cultures under ultralow-shear conditions. The flow is driven by centrifugal force, the rotation of the three ports creates a low-differential pressure at the base of the impeller shaft, lifting microcarriers up through the hollow shaft and expelling them out through its ports (The discharge ports must be submerged during operation). Bubble-sheer is eliminated by the Cell Lift impeller, which utilizes a ring sparger generating bubbles only within the aeration cage, so that the oxygenation works without any bubbles coming into contact with the cells.

7. Influenza Virus Cultivation Strategies

The application of Vero cells for the propagation of influenza virus in animal-derived component free (ADCF) media was extensively described by Wallace et al in their US patent[27] (no. 7,534,596 B2). The patent application includes the steps of attaching ADCF-adapted cells to a microcarrier (SoloHill® Engineering Inc.) and infecting the cells with vaccine media, producing virus within the cells and harvesting of the virus. The influenza viruses produced by this method achieved higher titer than that of the egg produced vaccine (Table 2.).

Production System	Panama H1N1 Titers (log_{10} $TCID_{50}$/mL)
Egg	7.8
Vero: Serum-containing	7.9
Vero: Serum-free ADCF	8.0

Table 2. Comparing egg-based influenza production with Vero-cell–based production using Hillex II microcarriers (SoloHill Engineering).

A method for microcarrier-based expansion of cells from a 0.2 L spinner culture to a 2L and 10 L bioreactor culture was developed (Figure 3). A New Brunswick CelliGen 310 bioreactor with a 5 L vessel was used for the 2 L culture stage. The vessel was equipped with a ring sparger, spin filter, 3-segmented pitched blade impeller (up-pumping), and 4-gas control

at 100 mL/min (Air, N_2, CO_2, and O_2). This expansion strategy couple with the demonstration of viral productivity represents an ideal closed system platform for vaccine production.

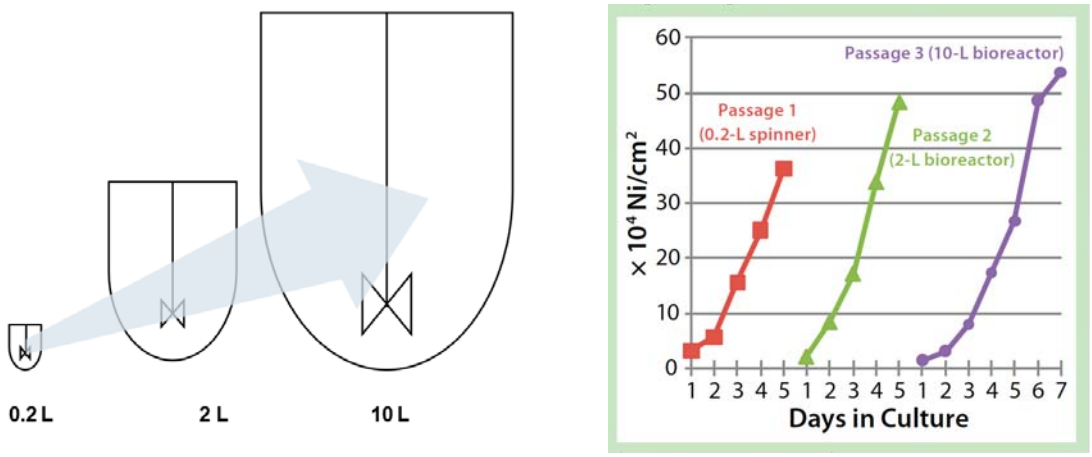

Figure 3. Vero-based expansion on microcarriers; seed train of Vero cells cultured on Hillex II micocarrier beads (SoloHill Engineering). Left: Diagram detailing bioreactor based expansion scheme; Right: Scale-up from Spinner flask to industrial bioreactors.

A similar method using Vero cell line for influenza vaccine production was demonstrated by Chen et al[4]. Using Cytodex 1 microcarrier beads, these investigators were able to achieve cell densities of 2.6 x 10^6/ml in serum free, protein free medium. These findings were obtained using a 250 ml Bellco microcarrier spinner flask equipped with a paddle impeller, inoculated with 2.5 10^5/ml Vero cells in 5% CO_2 atmosphere. In a subsequent expansion phase, starting from an initial number of 5 x 10^5 /ml, the cells were expanded in a 3L bioreactor. After 24 hours the cells had adhered to the microcarriers and the virus was added together with fresh medium. Using these procedures, the authors were able to obtain high virus titers up to 10 Log10 $TCID_{50}$/ml. They conclude that their approach could serve as a basis for large scale commercial production of influenza virus.

In 2011, Baxter International Inc. announced the approval for PREFLUCEL, the first Vero Cell based seasonal influenza vaccine, available for 13 participating European Union countries, including Germany, Spain, UK and the Scandinavian countries. Preflucel is comprised of purified, inactivated split influenza virions, manufactured using Baxter's adaptation of the Vero cell platform.

Although not approved for sale in the United States, data from a U.S. Phase III study with over 7,200 healthy individuals has shown that Preflucel provided 78.5% protective efficacy against subsequent culture-confirmed influenza infection, and robust immune responses against the three viral strains contained in the vaccine.

8. Conclusions

The combination of advances reviewed here provides strong support for the use of cell culture systems for virus production for 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 hen's 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. Whereas serum-containing media may continue to occupy a default position, it is now generally recognized that serum-free media are now the optimal choice. The use of carrier beads adds to the efficiency of culture technology, allowing greatly increased cell densities to be reached. Finally, improvements in bioreactor design combined with these various technological advances results in a greatly improved and more functional production train.

9. Acknowledgments

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