



Production of Bovine Herpesvirus-1 Vaccine Strains in MDBK Cells Using BelloCell Bioreactor

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Authors' contributions

This work was carried out in collaboration with all authors. Author PKG conceived and designed the experiments. Authors LSR, AK, BRP, TP, LRA and MS performed the experiments. Authors LSR and PKG analyzed the data. Author LSR wrote the manuscript. Authors PKG and MS revised the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To produce bovine herpesvirus-1 (BoHV-1) vaccine strains in MDBK cells using an oscillating bioreactor, BelloCell.

Place and Duration of Study: Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar-243122, India, between November 2013 and June 2015.

Methodology: MDBK cells were cultured in BelloCell bioreactor seeded with MDBK cells. After 6 days incubation at 37°C under 5% CO₂ tension, the MDBK cells were infected with 0.001 multiplicity of infection (MOI) of either wild-type (wt)-BoHV-1 or glycoprotein E deleted (Δ gE)-BoHV-1 vaccine strains and incubated further for 96 h. To analyse the vaccine potential of the harvests, inactivated wt-BoHV-1 and Δ gE-BoHV-1 were inoculated in guinea pigs. Sera from immunized guinea pigs were collected at 90 days after primary immunization and analysed for virus neutralizing (VN) antibody response specific to wt-BoHV-1.

Results: Using BelloCell bioreactor, the MDBK cell density reached to a maximum number of

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1.5×10^9 to 2.0×10^9 cells after 6 days cultivation. After infection with wt-BoHV-1 and Δ gE-BoHV-1 strains, the total virus yield at 96 h post-infection was $0.5 \times 10^{11.25}$ and $0.5 \times 10^{11.83}$, respectively. There was no significant difference in VN antibody response in sera collected from immunized guinea pigs.

Conclusion: This study indicated that BelloCell bioreactor can be used as a simple system for high population density cell culture and large scale production of vaccines.

Keywords: Bioreactor; BelloCell; MDBK cells; bovine herpesvirus-1; immune response; guinea pig.

1. INTRODUCTION

Infectious bovine rhinotracheitis (IBR) is an economically important disease of domestic and wild cattle. It is caused by bovine herpesvirus-1 (BoHV-1), a DNA virus of the genus *Varicellovirus*, subfamily *Alphaherpesvirinae* and family *Herpesviridae*. The disease causes high morbidity and low mortality and has been associated with other clinical manifestations like, encephalitis, conjunctivitis, enteritis, abortions and reduction in milk yield [1,2]. IBR control programmes are running in many parts of the world for the eradication and many countries like, Austria, Denmark, Finland, Sweden and the province of Bolzano in Italy have been considered as free from this disease [3]. Prevention and control of IBR through vaccination is recommended in countries where prevalence of IBR infection is high. For IBR control programme currently available vaccines are live attenuated, inactivated and subunit (DNA or vector based) which are used parenteral or intranasal [4-6]. For initiating the efficient IBR control programme, requirement of large quantity of vaccine with deletion in marker gene for differentiation of the infected from vaccinated animals (DIVA) and competent enough to produce the immune response similar to the wild-type virus is required. Glycoprotein E (gE) deletion mutant of BoHV-1 has been used as marker vaccine in many countries in European union for the IBR control and eradication programme [7].

Cell culture based production techniques have been widely used to manufacture viral vaccine biologicals. MDBK cell line represents suitable choice for propagation and vaccine production of BoHV-1 [8]. Almost all the cell lines used for virus propagation, including MDBK are anchorage dependent, therefore the virus infection depends on the attached cells, and the scaling up of these cells relies on the available surface area for cell attachment [9,10]. Microcarriers and culture in bioreactors can be used for large scale production of vaccines but is limited by the

maximum possible cell density [11] because of limited oxygen supply, physical damage to the cells due to agitation and accumulation of the toxic metabolites. The BelloCell bioreactor by Cesco Bioengineering Co. (Hsinchu, Taiwan) is a disposable bioreactor with unlimited oxygen supply and maximum surface area for high density cell production. The oscillating movement helps in proper nourishment of the cells and gas exchange. One BelloCell bioreactor can replace dozens of roller bottles and requires 20 times less space and labour. Different cells, including BHK-21, Vero, MDCK, HEK-293, insect cells [12-17] have been used successfully in the production of different vaccines, like hepatitis delta virus (HDV)-like particles [12], inactivated Japanese encephalitis virus (JEV) vaccine [14] and inactivated Influenza H5N1 vaccine [15].

In this study, we optimized the culture conditions for MDBK cells in BelloCell bioreactor and achieved a high population cell density of MDBK cells that resulted in high titre for both wild-type (wt)-BoHV-1 and glycoprotein E deleted (Δ gE)-BoHV-1 vaccine strains. We analysed the immunogenicity of these vaccine virus strains as inactivated vaccine in guinea pigs.

2. MATERIALS AND METHODS

2.1 Cells and Viruses

MDBK (Madin Darby bovine kidney) cell line was obtained from National Center for Cell Science (NCCS), Pune, India and propagated in minimal essential medium Eagles modification (EMEM) supplemented with 5% foetal bovine serum (Invitrogen). Wild type (wt)-BoHV-1 [18] and a mutant virus (Δ gE-BoHV-1) [19], adapted to grow in MDBK cells were used throughout this study for demonstrating the scale up using BelloCell bioreactor system.

2.2 Bioreactor

The BelloCell bioreactor (Cesco Bioengineering Co., Hsinchu, Taiwan) is pre-packed with

gamma-irradiated 865 BioNOCII microcarrier. The BioNOCII microcarrier chips are non-woven fabric strips made of 100% PET with a specific surface area of 2400 cm²/g and was specifically surface treated to make it hydrophilic for the efficient attachment of the adherent cells.

2.3 Optimization of Growth Requirements

MDBK cells were cultured in two batches for optimizing the culture conditions and requirements. The BelloCell bioreactors with microcarrier were seeded with $1.5-2.0 \times 10^8$ MDBK cells in 100 ml growth medium and placed in BelloStage. The BelloStage was maintained at an up/down speed of 1.0 mm/sec with a top and bottom holding time of 10 sec and 30 sec, respectively for cell attachment. When more than 90% cells were attached, additional 400 ml pre-warmed growth medium was added to the BelloCell and incubated further for 6 days at 37 °C under 5% CO₂ tension. On day 3, the growth medium was changed. Total number of live MDBK cells was monitored daily using Cellometer Auto T4 cell counter (Nexcelom).

2.4 Virus Production Using BelloCell Bioreactor

The MDBK cells ($1.5-2.0 \times 10^9$) grown on BioNOCII microcarrier in BelloCell bioreactor were infected with 0.001 multiplicity of infection (MOI) of either wt-BoHV-1 or Δ gE-BoHV-1 vaccine strains. For infection, the medium in the BelloCell bioreactor was removed and replaced with either wt-BoHV-1 or Δ gE-BoHV-1 strains as inoculum in 100 ml growth medium. After one hour adsorption, additional 400 ml growth medium was supplemented to BelloCell bioreactor and incubated for 96 h at 37°C under 5% CO₂ tension. The MDBK cell count in bioreactor was monitored daily. Aliquots of infected cell culture supernatants were collected at different time intervals and virus titer was estimated using Reed and Muench method described earlier [20]. At the end of incubation, the microcarriers in BelloCell Bioreactor were freeze thawed three times and cell debris from infected cell culture supernatant was removed by centrifugation at 5,000 rpm for 20 min at 4°C and used for vaccine preparation.

2.5 Immunogenicity Test

To analyse the vaccine potential of the harvests, inactivated wt-BoHV-1 and Δ gE-BoHV-1 vaccine

strains were inoculated in 3-4 months guinea pigs along with Freund's incomplete adjuvant (FIA). Briefly, $2.0 \times 10^{7.0}$ 50% tissue culture infective dose (TCID₅₀) of wt-BoHV-1 or Δ gE-BoHV-1 strains were mixed with 1% of 0.1 M binary ethyleneamine (BEI) and incubated at 37°C for 12 h for inactivation. To neutralize the residual BEI, freshly prepared sodium thiosulphate (1M) solution was added. Complete inactivation was confirmed by inoculating aliquots of inactivated virus in MDBK cell monolayer. The inactivated wt-BoHV-1 or Δ gE-BoHV-1 vaccine strains ($2.0 \times 10^{7.0}$ TCID₅₀ in 0.5 ml before inactivation) were mixed with equal volume of FIA and inoculated to guinea pigs intramuscularly in quadriceps muscle. Two groups (n=4, each) were inoculated with one ml of either wt-BoHV-1 or Δ gE-BoHV-1 vaccine preparation as primary immunization and booster dose was given on 21 days post-immunization. The control group (n=4) was immunized with healthy MDBK cell supernatant mixed with FIA. Sera from immunized guinea pigs were collected at 90 days after primary immunization and analysed for VN antibody response specific to wt-BoHV-1.

2.6 Virus Neutralization (VN) Test

To analyse the virus neutralization (VN) antibody titer, the 2-fold diluted sera from immunized guinea pigs were mixed with 100 TCID₅₀ of wt-BoHV-1 and incubated overnight at 37°C for neutralization. The VN titer was estimated by incubating un-neutralized wt-BoHV-1 with 0.5×10^5 MDBK cells in duplicate wells of 96-well plate at 37°C for 6 days. The VN titer was defined as reciprocal of the highest 2-fold dilution of sera that completely neutralized 100 TCID₅₀ of wt-BoHV-1.

3. RESULTS

3.1 Growth of MDBK Cells in BelloCell Bioreactor

In this study, in two independent experiments, $1.5-2.0 \times 10^8$ MDBK cells in 100 ml were used for inoculation in BelloCell bioreactor. It took 4 h incubation at 37°C under 5% CO₂ tension to achieve >90% MDBK cells attachment to microcarriers. The culture volume in BelloCell bioreactor was increased to 500 ml and incubated for 6 days at 37°C under 5% CO₂ tension. On day 3, a growth medium change was done when pH of the medium was below 6.8 (Fig. 1). At the end of 6 days, the MDBK cell

number in two different batches increased to 1.5×10^9 and 2.0×10^9 , respectively.

3.2 Virus Production

For production of BoHV-1 vaccines, the BelloCell bioreactor with 1.5×10^9 and 2.0×10^9 MDBK cell density was inoculated with wt-BoHV-1 and Δ gE-BoHV-1 strains, respectively. The virus titre markedly increased from 12 h post-infection and reached maximum at 96 h post-infection. The kinetics of virus production with both the BoHV-1 strains used was same as there was no significant difference in virus titer at different time interval (Fig. 2). The total yield of wt-BoHV-1 and Δ gE-BoHV-1 in the final harvest was $0.5 \times 10^{11.25}$ and $0.5 \times 10^{11.83}$, respectively.

3.3 Immunogenicity of BoHV-1 Vaccines

When inactivated wt-BoHV-1 and Δ gE-BoHV-1 strains were inoculated as vaccines into guinea pigs, there was sero-conversion in all the animals. The VN titer reached to $9 \log_2$ to $12 \log_2$ in both the immunized groups. There was no significant difference in VN titer in both the immunized groups showing no difference in immunogenicity of both BoHV-1 vaccines. There was no sero-conversion in control guinea pigs (Fig. 3).

4. DISCUSSION

For large-scale production of anchorage-dependent cells, roller bottles, CellCube and Cell Factory systems are routinely used with limited cell proliferation. Various bioreactors such as the hollow-fibre bioreactor, Celligen Plus Bioreactor (New Brunswick Scientific, Edison, NJ) and Wave Bioreactor (Wave Biotech, Somerset, NJ) are also available, but all of them involve complicated operation and provide low population densities of cells. To achieve the maximum yield of MDBK cells and production of two different BoHV-1 vaccine virus, in this study, the BelloCell bioreactor was evaluated. The BelloCell bioreactor by Cesco Bioengineering Co. (Hsinchu, Taiwan) is a user-friendly system for obtaining both the maximum cell cultivation and large scale virus production. In BelloCell bioreactor system, the oscillating compression and relaxation of the bellows enables the immobilized cells on BioNOCII microcarriers intermittently submerged and emerged between the media and air, similar to a roller bottle [17]. When the microcarrier are in air, oxygen present in fresh air exposes the large surface area on microcarrier. When the microcarrier is submerged in media, the nutrients are provided to cells attached to microcarriers. There is very low shear force to cells on microcarriers and no foaming [15,21].

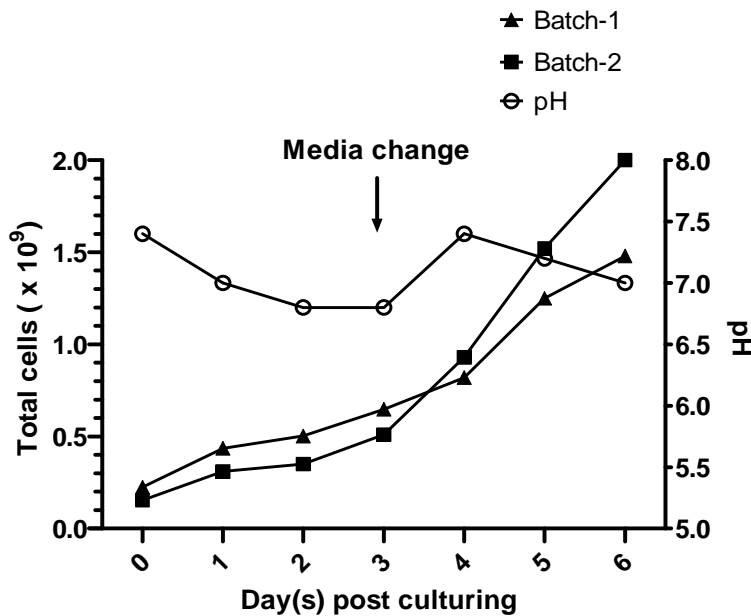


Fig. 1. Pattern of cell growth and pH change during MDBK cell cultivation using BelloCell bioreactor

Arrow indicated media change

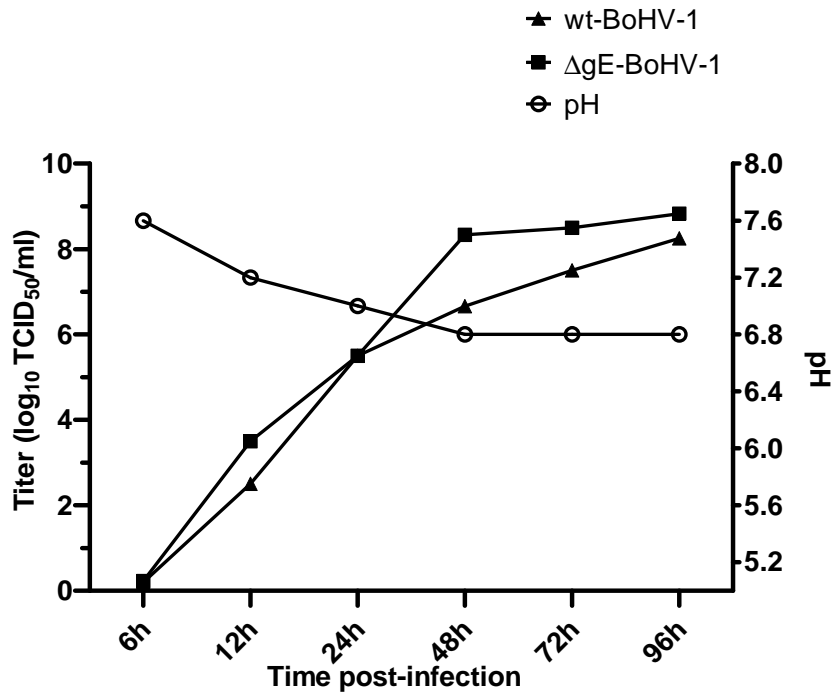


Fig. 2. Multiplication kinetics and pH change during propagation of wt-BoHV-1 and ΔgE-BoHV-1 vaccine strains in MDBK cells using BelloCell bioreactor

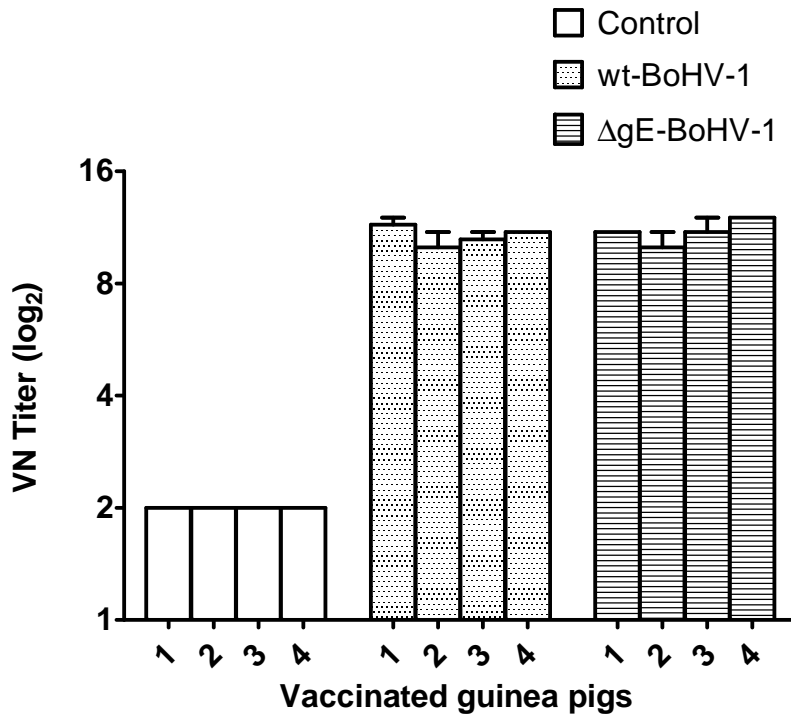


Fig. 3. Induction of virus neutralizing (VN) antibody response in guinea pigs immunized with inactivated wt-BoHV-1 and ΔgE-BoHV-1 vaccine strains

For determining the standard culture conditions for the growth of MDBK cells in BelloCell bioreactor, we performed two independent experiments. In one experiment, 1.5×10^8 and in another 2.0×10^8 MDBK cells were seeded in BelloCell bioreactor. We monitored the change in pH and glucose concentration in the medium. The pH was reduced to 6.8 on day 3 and we found that a change of medium was necessary. The glucose concentration was maintained above 1.0 g/L during incubation. Under these conditions, the total number of cells reached to 1.5×10^9 and 2.0×10^9 at the end of day 6. Using BelloCell bioreactor, Vero cells [14] and insect cells [16] were produced up to 2.8×10^9 after 7 days of culture with an initial inoculation of 1.5×10^8 cells.

For production of different BoHV-1 vaccine virus strains, the wt-BoHV-1 and Δ gE-BoHV-1 strains were produced as batch culture without change of medium after virus inoculation. The amount of virus was $0.5 \times 10^{11.25}$ and $0.5 \times 10^{11.83}$ for wt-BoHV-1 and Δ gE-BoHV-1, respectively, indicating no significant difference in the total yield. Further, BoHV-1 strains produced in the BelloCell were inactivated and injected into guinea pigs to test the ability to induce virus neutralizing antibodies as vaccine. When guinea pigs were immunized with the same amount of both the inactivated BoHV-1 vaccines, there was no significant difference in VN titer against wt-BoHV-1. Thus, BelloCell culture could provide high population density of MDBK cells and yielded high virus production.

5. CONCLUSION

The BelloCell bioreactor can be adopted as a simple system for high population density cell culture and for large scale production of viral vaccines.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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