Original Research Article **Microbiology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

PRELIMINARY ASSESSMENT OF ADAPTABILITY OF LENINGRAD-ZAGREB MUMPS VACCINE VIRUS TO HUMAN DIPLOID CELLS AND VERO CELLS.

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ABSTRACT

Mumps is a highly contagious childhood respiratory disease preventable by vaccination. Leningrad-Zagreb (L-Z) strain based mumps vaccine is one of the safe and effective vaccines, being used for successful immunization in many countries. It is being produced on the primary culture of chicken embryo fibroblast. This culture needs to be initiated using specific pathogen free (SPF) eggs, derived from SPF flocks of chickens. Primary cultures prepared from the avian source have inherent limitations such as variations in the supply of eggs and the potential risk of adventitious agents escaping possibility of detection. Above mentioned limitations could be overcome by using well characterized banks of cell substrates to grow the virus. MRC-5 cells and Vero cells formed excellent alternative cell substrates to assess the adaptability of the L-Z mumps virus. These banked cell substrates offer distinct advantages in vaccine production technology because of their ready availability, well characterized attributes such as homogeneity, stability, purity, identity, faster growth rates and consistent supply. Successful adaptation of the L-Z mumps vaccine virus to these cell substrates could therefore be translated into shortened cycle times and improved productivities, thus benefiting the vaccine industry and society at large.This report describes the preliminary assessment of adaptability of the L-Z mumps vaccine virus to the human diploid cell strain, MRC-5 and the Vero cell line in comparison with it's original cell substrate. The original CEC-grown L-Z mumps virus was consecutively passaged for few times through MRC-5 cells and Vero cells in two different sets of experiments. Adaptability of the passaged viruses was qualitatively checked by mumps-specific immune-fluorescence assay. It was also quantitatively assessed by performing the standard tissue culture infectivity dose titration. Adaptability of the original L-Z mumps virus to Vero cells was observed to be much better than that to MRC-5 cells.

KEYWORDS: ***Adaptation, L-Z mumps vaccine virus, MRC-5 cells, Vero cells, Immuno-fluorescence, Tissue Culture Infectivity Dose.***

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Received on: 28-09-2017
Revised and Accepted on: 31-10-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.4.b532-537

INTRODUCTION

Mumps is a viral disease mainly affecting children. It is highly contagious respiratory disease spread by aerosolized droplets containing the virus. Many countries routinely practice mumps immunization, which has resulted in controlling the global disease burden to quite an extent, however sporadic outbreaks of mumps are reported, highlighting the need for increasing the vaccination coverage and maintaining it consistently above threshold.[1,2] Live attenuated mumps vaccines are found to be superior than inactivated vaccines in terms of providing long lasting protection against the disease. Many live attenuated mumps vaccines such as *Jeryl Lynn, Leningrad-3, L-Zagreb, RIT-4385, RS-12* are being used in different countries. Mumps vaccine containing *Leningrad-Zagreb* strain and grown on chicken embryo primary culture was developed and characterized in Croatia as a further attenuated vaccine and is being used for active immunization in many countries all over the world.[3] Mumps vaccine containing *RS-12* strain was recently established on human diploid, MRC-5 cells and was shown to be safe, immunogenic and thermostable.[4] MMR vaccine containing Hoshino strain of mumps virus was recently shown to be safe and immunogenic.[5] Most of the above mentioned mumps vaccine strains are grown on the primary culture of chicken embryo fibroblast derived from SPF eggs. This process has inherent limitations such as long lead time, complex operations, biological variability and heterogeneity, susceptibility to contamination and dependency on the external source for the supply of SPF quality eggs. Fertile eggs from SPF chicken flocks should be freely available for the preparation of primary cultures of chicken embryo. Only a limited number of organizations have the capability to supply SPF quality eggs at industrial scale. It therefore requires a lot of advanced planning and robust arrangement for transportation of delicate eggs over long distances. Country specific restrictions, rules and regulations governing the import export policies of animal derived materials, make the process further complicated and expensive. Primary cultures also mandate addition of antibiotics to the culture media in order to mitigate the potential risk of presence of contaminating microorganisms originating from the starting tissues or organs. Considering the above mentioned limitations, it was very much prudent to explore an alternative cell substrate. Various cell substrates used for viral vaccine production are described in the literature.[6]Research efforts were therefore planned to assess the adaptability of the existing CEC-grown *L-Z* mumps vaccine virus to the new cell substrates. Adaptation of virus to new cell substrate is a complex task in itself that should be complemented with in-depth characterization of the adapted virus. There are many interacting variables which can influence the adaptation process. These include incubation temperature, growth medium composition, population doubling level of the cell substrate, multiplicity of infection (MOI) and passage number of the virus. Further, complete adaptation of a virus to a new cell substrate is difficult to define in general. Here, for the preliminary assessment purpose, we limit the definition of adaptation to the formation of sufficient quantities of infectious virus particles measurable in a bioassay and further confirmation of the identity of these infectious virus particles by immuno-fluorescence assay. In the recent past, vaccine manufacturing industry was found to be relying increasingly on the human diploid cells and continuous cell lines such as Vero cells as cell substrates of choice, because of their well characterized nature.[7,8] Human diploid cells such as *MRC-5* cells and the continuous cell line of African green monkey kidney cell such as *Vero* cell line are the most studied and hence the safest known cell substrate for the manufacture of human vaccines. These cells can be preserved in-house, in liquid nitrogen as a cell bank with ease of revival at any time with a short notice, thus eliminating the efforts on advanced planning, logistics and dependency on external source for the supply of the starting material. These anchorage dependant cells can be grown as static culture in tissue culture flasks (TCF) or cell factories (CF) as well as in roller bottles or bioreactors as dynamic culture, thus making them easily amenable to scale up. Their growth rate is higher than that of the primary culture and they are inherently more stable and more consistent in culture, thus making them a cell substrate of choice industrially. Therefore, it was decided to assess the adaptability of the mumps *L-Zagreb* vaccine virus to the two new cell substrates, viz. *MRC-5* cells and *Vero* cells, both of which were readily available in-house as well characterized cell banks.

MATERIALS AND METHODS

The in-house biological starting materials included *Leningrad-Zagreb* mumps virus, cell strains: *Vero-CCL-81,* human diploid -*MRC-5*cells and the chicken embryo culture derived from SPF eggs of Valo Germany. For cell and virus growth, Minimum Essential Medium with Hanks©salts from Gibco was used, supplemented with 10% and 2% Fetal Bovine Serum (Morgate Australia) respectively. Phosphate buffered saline (PBS) was from Sigma. For culturing the cells and virus, Tissue Culture Flasks (TCFs), 175 cm^2 from Corning Inc. were used in bench top CO_2 incubators (Shell Lab.)set at 36±1^oC and 33±1^oC respectively. Kalt is deep freezers (-60 to -80^oC) were used for storing viral harvests and samples. For microscopic examination of cultures, inverted photomicroscope and Fluorescence microscope (Axio) from Zeiss were used. For potency determination, 96 well plates from Corning Inc. (Catalogue No 3595) were used, whereas for qualitative assay of the virus, Immuno-Fluorescence Assay (IFA) kit from EMD Millipore (Catalogue No. 3140) was used.

Seed virus preservation and sampling

CEC-grown L-Z mumps vaccine virus at passage 26, virus progeny of subsequent passages generated during the adaptation experiments and the representative samples collected in 5 ml amber vials were stored in deep freezers at -60 to -80^oC till further use.

Adaptation of the L-Z mumps virus to Human Diploid Cells and Vero cells

CEC-grown L-Z mumps vaccine virus at passage 26 was used for infecting 72 hour old monolayers of MRC-5 cells and 24 hour old monolayers of Vero cells in 175 cm^2 TCF in separate experiments. Briefly, spent

medium from respective TCFs was decanted; monolayers were washed twice with 100 ml of PBS, before adding the virus on to the monolayer at an MOI of 0.01. The infected cultures were incubated at 36±1°C, for around 120 minutes in case of MRC-5 cells and 60 minutes for Vero cells for allowing adsorption of the virus, thereafter decanting the supernatant from the monolayers and adding around 100 ml of fresh virus medium in the TCF. Incubation was then continued at 33±1°C, with daily microscopic observation of the cultures for cytopathic effect (CPE). The infected TCFs were harvested on the 10th day post infection for MRC-5 cells and on the 6th day post infection for Vero cells. The harvest and samples were stored at -60 to -80°C till subsequent use. Additional two consecutive passages were performed on both the cell lines using around 10 ml of earlier passage virus for infection. MRC-5 cells of population doubling level 33 to 35 and Vero cells of population doubling level 152 to 154 were used for the study. Three biological replicates and four technical replicates were performed. Parallel passages on CEC were performed for relative comparison of adaptation.

Quantitative determination of infectious virus particles by cell culture method

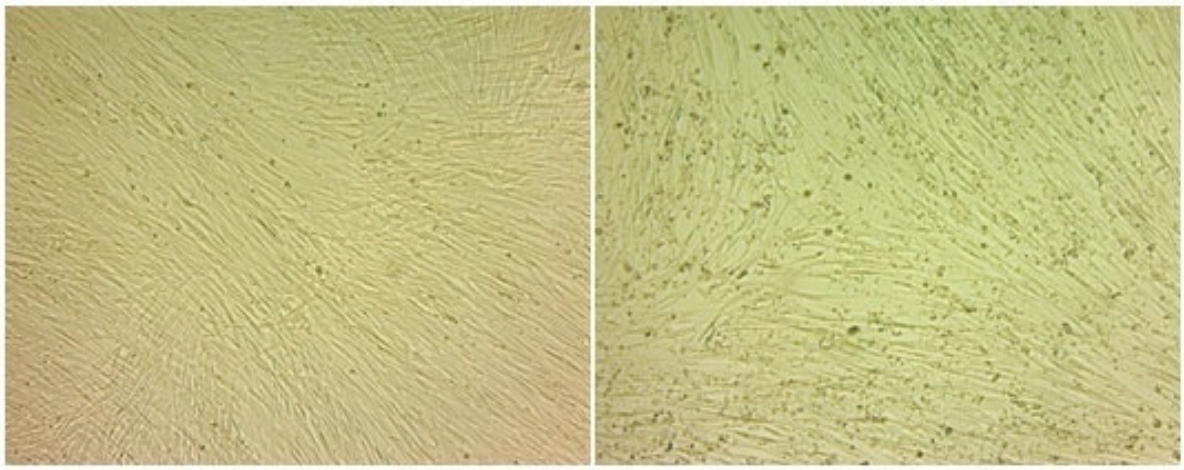
A ten days tissue culture infectivity dose ($TCID_{50}$) method was used for the purpose. Vero cells in the 96 well plates were infected with ten-fold dilutions of the test samples. The plates were incubated at 36±1°C for 10 days and then observed for the virus specific cytopathic effect. Confirmation by Haemagglutination (HA) was also done if the cell-virus system was not indicative of clear cut CPE. Statistical Spearman Karber method was used for calculation of the results as log $TCID_{50}$ per 0.5 ml. Mumps in-house reference standard was included in every $TCID_{50}$ assay.

Qualitative determination of the mumps virus by immuno-fluorescence method

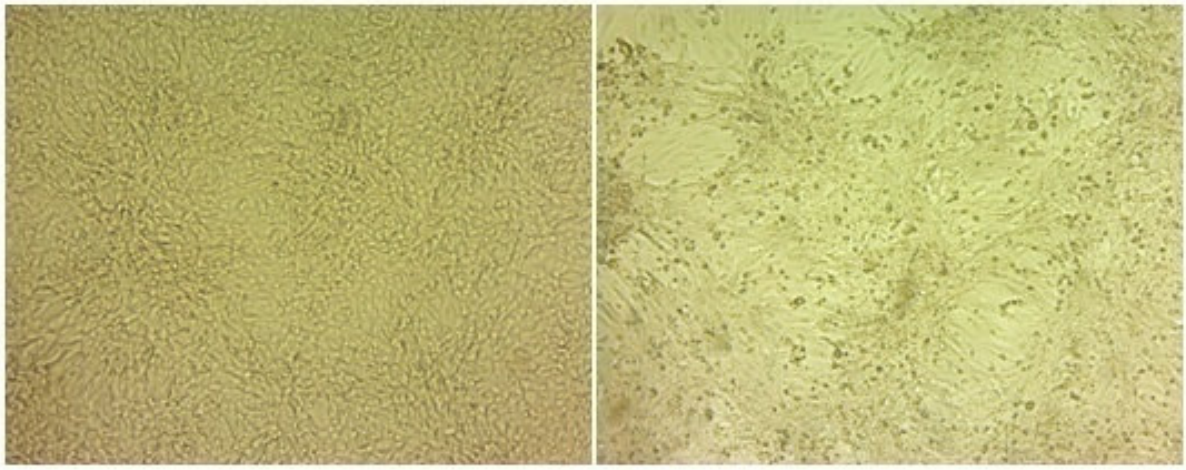
Mumps IFA kit from EMD Millipore Corporation was used for the purpose. The test was used as a confirmatory test for the presence of mumps virus in the infected cell culture. Briefly, confluent monolayers of Vero cells in 96 well plates were infected with harvested virus samples from all the passages from both the cell substrates. Plates were incubated at 36±1°C for 48-72 hours in case of Vero grown and CEC grown viruses and for 120-168 hours for MRC-5 cells grown viruses. Infected cells were then fixed by adding around 200 µl of 80% acetone per well and holding the plates at -20°C for 15 minutes, followed by rinsing the wells with the wash fluid from the kit. Subsequently 40 µl of primary antibody solution per well was added and the plates were incubated at 36±1°C for 30 minutes, followed by rinsing the wells with the wash fluid for 10-15 seconds. Then 40 µl of secondary antibody solution from the kit was added per well and the plates were incubated at 36±1°C for 30 minutes, followed by rinsing the wells with the wash fluid for 10-15 seconds. Finally, 100 µl of mounting fluid from the kit was added per well and the plates were observed under fluorescent microscope. Positive and negative controls were run simultaneously as per kit manufacturer's instructions.

RESULTS

Regular microscopic examination of infected and uninfected control cultures revealed slow and gradual but not readily discernible changes in the morphology of infected cells. Changes in cellular morphology attributable to the progressing infection was apparent much earlier in case of infected Vero cells and infected CEC, compared to the infected MRC-5 cells (Fig 1). Confluent mono layers of healthy MRC-5, Vero and CEC cells are seen in part (A) of the Fig 1, whereas disturbed morphological patterns of these cells are seen in part (B) of Fig 1. In case of MRC-5 cells, apparently, no distinguishable CPE was observed as late as 8 to 10 days post infection. Changes in cellular granularity, size and shape were only apparent after more incubation of these cultures. In case of L-Z infected Vero cells, CPE and progressing infection was more unambiguous as early as 5 days post infection and destruction of monolayer was evident by 10th day of incubation, similar to that of CEC. Immuno-fluorescence assay indicated an apple green fluorescence in the mumps infected cells, because of the fluorescein isothiocynate (FITC) labeled secondary antibodies. Fluorescence was absent and dull red color was evident in negative control cultures because of the Evans Blue counter stain. More number of fluorescent foci were visible per microscopic field in case of infected Vero cells and infected CEC than in infected MRC-5 cells. Further, the immuno-fluorescent foci started appearing much later in case of MRC-5 cells, with no immuno-fluorescence being observed till day 5 (Fig 2). Quantitative results obtained by $TCID_{50}$ method were supportive to the qualitative observations as above. Comparison of potency results in the last passage indicated around 500 fold more virus particles on Vero cells compared to MRC-5 cells but around 10 fold less virus particles when compared to those on CEC (Table1**)**. Further, with increasing passage number, the rate of increase in the yield of infectious virus was much less appreciable in case of MRC-5 cells than in Vero cells, which was nearer to that of CEC (Fig 3).



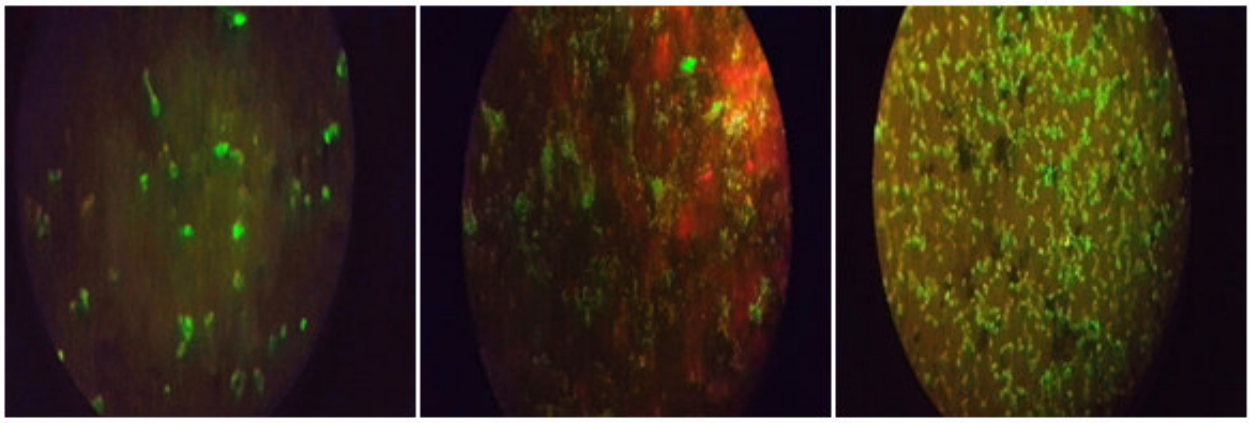
(A) *(B)*
Uninfected *L-Z infected*
MRC-5 cells: Virus Passage-27, 10 days post infection.



(A) *(B)*
Uninfected *L-Z infected*
Vero cells: Virus Passage-27, 5 days post infection.

(A) *(B)*
Uninfected *L-Z infected*
CEC: Virus Passage-27, 5 days post infection.

Figure1
Microscopic Examination of cultures for Cytopathic Effects (100X).



*Days Post Infection

(A)
*MRC-5 cells, 6 DPI**

(B)
Vero cells, 3 DPI

(C)
CEC, 3 DPI

Figure 2
Immuno-fluorescent foci of L-Z mumps virus (Passage-29) infected cells.

Table1
Potency of the L-Z Mumps virus adapted to MRC-5 cells and Vero cells in comparison to CEC.

Passage No.	Average Potency (± CI*), L-Z Mumps virus, $Log_{10}CCID_{50}$/0.5ml		
	MRC-5 cells	Vero cells	CEC
P-27	2.148(± 0.11)	4.398 (± 0.11)	5.710 (± 0.04)
P-28	2.408 (± 0.04)	4.94 (± 0.04)	6.075 (± 0.09)
P-29	2.721 (± 0.03)	5.471 (± 0.04)	6.575 (± 0.06)

*CI: Confidence Interval with 95 % confidence.

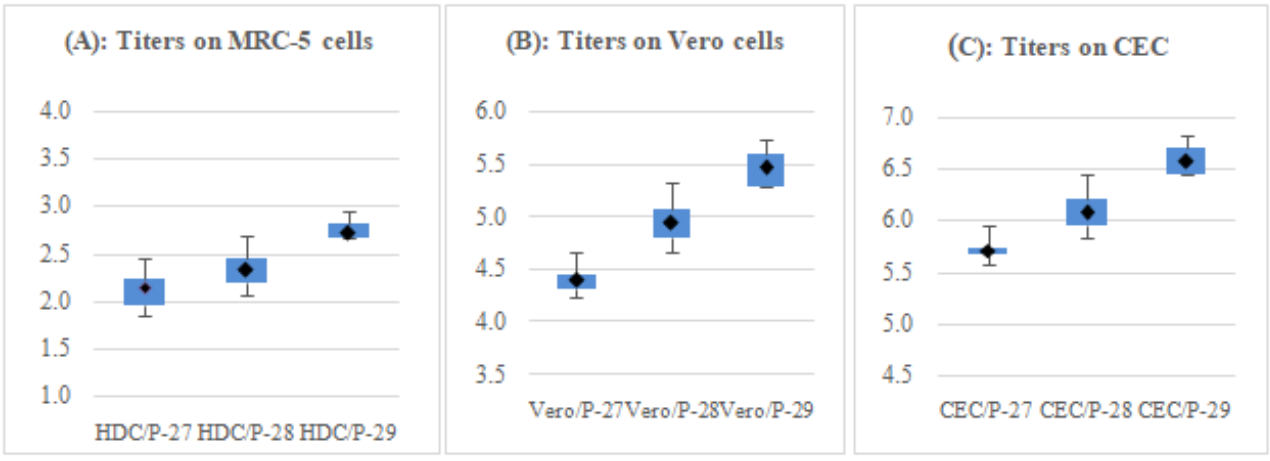


Figure3
Passage wise titers of L-Z Mumps Vaccine Virus on different cell substrates ($LogCCID_{50}$/0.5ml)

DISCUSSION

Preliminary assessment of adaptability of the L-Z mumps vaccine virus to MRC-5 cells and Vero cells showed that the virus could be adapted on these cell substrates. No previous report of adaptation of L-Z mumps virus on MRC-5 cells could be found. Rubini strain of mumps was adapted on MRC-5 cells but was observed to be less efficient as a vaccine[9]. Vero cells have been used for mumps titration. Better adaptability of L-Z mumps strain to Vero cells was therefore expected. This strain has been passaged on Vero cells to understand its attenuation[10]. It could be possible that the L-Z mumps virus tends to be latent in case of MRC-5 cells and hence showing less clear CPE and lower titers as compared to Vero cells. More research efforts will be needed in future to explain the latent behavior of the virus in MRC-5 cells. Cultivation conditions such as incubation temperature and growth medium composition were kept constant in this study. Only a limited number of passages of the L-Z mumps virus were assessed on both the cell substrates in this preliminary study. It could be possible to improve further the adaptability of the L-Z virus on MRC-5 cells and Vero cells by studying more number of passages but remains to be seen. The effect of changes in the cultivation of infected cultures such as change in incubation temperature or change in media

composition could also be undertaken as future studies to enhance the adaptability. Manipulation of culturing techniques such as high cell density perfusion or continuous cultures may be considered to improve yields and lower the cost. Further studies should be undertaken to evaluate the genotypic and the phenotypic characteristics of the adapted viruses in comparison to the original virus population.

CONCLUSION

The adaptability of the L-Z mumps vaccine virus on MRC-5 cells and Vero cells appeared to be reasonable, if not excellent as compared to the CEC grown virus. Vero cells were found to be more susceptible to this virus than MRC-5 cells. Future studies with respect to improving the yield on these cell substrates and characterizing the progeny viruses are recommended for thorough understanding of its adaptability.

ACKNOWLEDGEMENT

Authors wish to acknowledge the laboratory resources provided by Serum Institute of India Pvt. Ltd.

CONFLICT OF INTEREST

Conflict of interest declared none.

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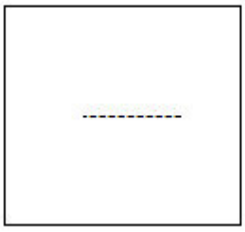

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We sincerely thank the above reviewers for peer reviewing the manuscript