ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
December 2018; 17(6):586-593.
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Induction of Immune Response and Protective Immunity by a Local Isolated Varicella Virus in Animal Model: A Future Candidates for Vaccine Production

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Received: 30 September 2018; Accepted: 14 November 2018

ABSTRACT

Preparation of the indigenous varicella zoster vaccine could significantly reduce the disease burden of varicella zoster virus especially in immunosuppressed children. To achieve this goal, the varicella zoster virus was isolated from an 8 years boy infected with chicken pox. The virus was cultivated in sensitive cell line and determined varicella zoster.

The adaptation and attenuation of virus was carried out after several passages in MRC-5 cell culture, Primary Guinea pig embryo fibroblast cell culture and again switching in MRC-5 cell culture. The challenged of vaccine dose was found 3LogCCID50. Following two doses of immunization in guinea pigs via inoculated cell culture-fluid attenuated- local isolated VZV at zero and 14 day, the humoral immune response, varicella-zoster virus (VZV) IgG and IgM were determined using enzyme-linked Immunosorbent and seroneutralization assays at 7, 14, 21, 30, 60, 90.120 days after receiving of the first and second dose of vaccine.

The results of immunization showed good 93% seroconversion in guinea pig which compared with vOKa vaccine was not significant ($p<0.05$).

The prepared attenuate varicella zoster virus promising a candidate Virus for our future plan to vaccine production.

Keywords: ELISA; Guinea pig; Immunogenicity; Local; Seroneutralization; Varicella

INTRODUCTION

Varicella Zoster Virus is an important pathogen causing severe disease in young children, and adult.[1] The worldwide impact of this virus on public health has led to efforts to develop vaccines to control varicella viral disease. Followed by the initial infection inducing a vesicular rash, pain and low grade fever, general weakness, prodromal malaise, pharyngitis, rhinitis, which is often the first sign of the disease. Varicella zoster virus (VZV), can remain latent in the dorsal root and cranial nerve ganglia following the initial infection.[1] Spreading along the nerves to the

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Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

skin, Reactivated and leads to the replication of the virus in the sensory ganglia, that causes herpes zoster (HZ).[2] Humoral and cellular immunity is therefore a critical factor in protection against varicella zoster virus after natural infection, suggesting that highly effective vaccines must induce Humoral immune responses. Oka strain vaccines were first licensed for use in high risk children, in 1984. Licensure for use in healthy children started in 1986 and after that distributed in many millions of doses to different part of world.[3]

Two live attenuated subcutaneous candidate VZV virus vaccines have been assessed for infants and adult, but with variable efficacy.[4] Varicella vaccine is ~80 to 85% effective in preventing any varicella disease and >95% effective in preventing severe disease. Therefore, about 15 to 20% of vaccinated children will develop breakthrough varicella. In this case the infection is occurs>42 days after vaccination. Most of breakthrough disease was described as being mild, with or without fever and fewer skin lesions.

This survey is on the humoral immune responses and its correlation to protective immunity by isolated local varicella virus infection in animal model. Guinea pigs is one of the best animal models have been used to study varicella virus pathogenesis and immunity.[5] This research may enable us to define similar correlates of protective immunity to VZV viral infections and to develop an effective vaccine strategies by new local isolate from our geographic distribution. All of the Methods design based on World Health Organization (WHO) requirement and our previous experiment.[6,7]

We selected a virulent (vesicular swab -passaged) and the attenuated (cell culture-passaged) derivative strain of $Pst1^{-}/Bgl1^{+}$ varicella virus for this study. Based on our study (under published data) and others seems to" $Pst1^{-}/Bgl1^{+}$ varicella virus "be dominant strain in Iran and our geographic position.[8,9] Our goal was to evaluate protection induced by manual exposure or vaccination, using the virulent vesicular swab for master as the challenge, and its attenuation by the cell culture-passaged as local isolate. In this process vOKa "(MERCK) commercial vaccine" as reference positive for VZV virus and phosphate buffer inoculated as negative control. The virulent master was used for simulation of natural exposure, and the derivative attenuated mimicked live injectable vaccine exposure. The immunoglobulin (Ig) isotypes of varicella virus-specific antibody in sera after inoculation and challenge via the experimental Guinea pigs were measured by using an Enzyme linked Immunosorbent (ELIZA) and Virus Neutralization (VN) antibody assay.

MATERIALS AND METHODS

Varicella Zoster Virus

The swab sample of VZV virus strain was prepared from an 8 years boy with chicken pox. Four serial passage was made after cultivation of virus in human diploid lung cell culture (MRC-5) used as a virulent varicella virus based on our previous experiment and world Health Organization (WHO) Requirement. The molecular diagnosis and genotyping determination of the isolated VZV was achieved using specific primers for Open Reading Frame (ORFs) 32, 58 as described previously (under publishing material). The following cultivation of virus was carried out to adapt and attenuate of VZV based on our previous experiment.[10]

The isolated virus was cultivated 27 passages in cultures included 10 passage in MRC-5, 10 passage in in primary Guinea Pig Embryo Fibroblasts kidney cells (GPEFC) (using 20 days pregnant fetus) and 7 passage in MRC-5.[11] Since the challenge of the varicella zoster vaccine is not well define, the following observations were done including the cellular morphological changes in the virulence phase and after 27 passages of virus in cell culture, the molecular pattern of RFLP of PCR products of ORF 32 digested by PstI restriction enzyme site and ORF 58 digested by BglI restriction enzyme site as described previously (under process of publishing).

To evaluate the immunogenicity and dose finding of treated virus used the procedure in our previous article,[12] the 50% cell culture infectivity dose (CCID50) assay was determined for each inoculum injection in guinea pigs.

Immunogenicity Assay on Ginea Pigs

To assess the immunogenicity of the treated varicella virus, three doses of 0.5 ml contain 3, 4, and 5, LogCCID 50/mL from passages 4^{th}, 17^{th}, and 27^{th}, were prepared and administrated one site of pelvic cavity via hypodermic injection subcutaneously (SC) in each groups of 3 male guinea pigs(eight-week-old male≤weight≤300 g) respectively. The commercial vOKa vaccine (Merck Germany), was used as a positive reference. Three Ginea pigs used as negative controls that were injected subcutaneously with single dose of 0.5 mL of Dulbecco's modification of Eagle

medium (DMEM). Their sera were tested for detection of varicella zoster IgG and IgM by ELISA and neutralizing antibody determination at 2, 4, 8, 12, 16.20, week's interval after post-inoculation.

Virus Transmissibility among Received to Non-Received Varicella Virus

To observe the evidence of transmissibility of varicella zoster from immunized animal by treated varicella zoster virus to non-inoculated/non immune animal, a group of animal (3 male guinea pigs) which received highest titers (5LogCCID50/mL of passages 27^{th}) of treated VZV virus were placed in one cage with non-inoculated/non immune group animal (3 male guinea pigs). The non-received varicella virus groups marked by colored ear tag. Daily the non-inoculated/non immune animal group were observed if any of them acquired varicella zoster infection via close contact with immunized group, for period of three months. The expected scored signs was evidence of, vesicle, fever, weight loss, and rash (scores: 6, normal [no sign infection among guinea pigs]; score 18, mild [signs of infection in 2-3/ 6 guinea pigs]; score 24 [moderate; sign of infection in 3-4 /6 guinea pigs; and score 36 [severe; all 6 guinea pigs showed sign of infection]). To confirm further clinical sign of infection, oral swabs and blood samples were collected from all guinea pigs 3 days' interval, and 2 week interval respectively.

Skin Test

To Measure Cell-Mediated Immunity (CMI) in immunized guinea pig by treated varicella zoster virus, the suspension of VZV-infected culture fluids passage 27^{th} was injected subcutaneously (SC) to three Guinea pigs. The second injection of virus (0.5 mL of p.27.3LogCCID50) was inoculated intradermal rout five days after first inoculum. Skin reaction including redness and swelling was measured in the site of injection.

Neutralizing Antibody Detection

Serial two-fold dilutions of sera including 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 titer were prepared from immunized Guinea pigs. Amount of 50 µL of each serum dilution was mixed with equal amount of VZV isolated passage 27^{th} (300-500 CCID50), the mixture (antigen-antibody) were shaken and incubated at $37^{\circ}C$ for 1hr. Then each separate mixture was inoculated onto four confluent well of Vero cells in 96-well plates. The duplicate test was carried out for each test. The cytopathic effect was observed daily. For each serum sample, the neutralizing antibody titer was determined by the highest dilution of serum capable to reduce the number of plaques virus by 50% or greater compared to control tests.[13]

Enzyme-Linked Immunosorbent Assay (ELISA)

An enzyme-linked Immunosorbent assay (ELISA) (Enzygnost anti-VZV/IgG, IgM Anti-*VZV* Virus ELISA Kit, Marburg, Germany) was used to determine VZV IgG and IgM in the immunized guinea pigs, according to manufacture instruction and manual for VZV detection.[13,14] Concurrently, the sera of immunized guinea pigs by vOKa as positive and immunized Guinea pigs with Phosphate Buffer saline (PBS) was used as a negative reference in test were also evaluated. A part of Blood samples were sent to Biobank Research Center (IRBSC/Iran) for cellular immunity evaluation and measuring the CD markers (CD4, CD8).

Statistical Analysis

The statistical comparison results between the antibody level after the inoculation and infection using positive result of SN, ELIZA, and assay in different particle Guinea groups was analyzed using Mann Whitney test the SPSS ver. 19 (SPSS Inc. Chicago, IL). Significance was assigned to calculate $p<0.05$.

Ethic consent

The research was conducted in accordance with the Declaration of Helsinki (as revised in 2008) and according to local guidelines and laws. The project was approved by ethic committee of Razi vaccine and Serum research institute Karadj Alborz I.R. IRAN and Faculty of Medical Sciences, Tarbiat Modares University Tehran, I.R. IRAN. Ethics committee approval code: IR TMU.REC 1394.18.

RESULTS

The Typical Cyto Pathic Effect (CPE) was appeared in MRC-5 cells after inoculation of the fourth passage of the isolated varicella zoster. The titer of the harvested virus at 4^{th} passage' was 2.3 LogCCID 50/mL and after passage 27 reached to 7.5 LogCCID 50/mL on human diploid fetal lung fibroblast (MRC-5),

primary guinea pig embryo fibroblast (GPEF) and human diploid fetal lung fibroblast (MRC-5). After each 5 passage interval, the virus was harvested and DNA virus was amplified using RFLP-PCR in two Open Reading Frame (ORFs) [32,58]. The results of immunization via subcutaneous and intradermal root in two group animal showed good humoral and neutralization antibody responses (p<0.05). With regard to efficacy of the treated virus, the level of antibodies responses after immunization of treated VZV at passage 4th, 17th and 27th revealed that the status of antibodies were increased slowly at passage 17 and increased one and half fold after virus passage at 27th in immunized animals. This phenomena exhibits by increasing the virus passage and its titer in tissue culture the immunogenicity of treated vzv provoked immune system to increase humoral response (Figure 1).

Following six months serological follow up the results of efficacy of three different doses of treated VZV at passage 17th, and 27th (3/ ID and 3/ SC) Guinea pigs showed good humoral responses compared with vOKa a commercial (Merck) controls vaccine. The level of neutralizing antibody response was varied from 1>16 to 1>32 titer of antibody correspond to virus particle 1000–100000/mL respectively after four weeks post vaccination. The persistence of antibody response was lasted up to 16 weeks post inoculation. Similar results of antibody IgG and IgM response was also obtained by ELISA test (Figure 2). The stimulation Cell Mediate Immunity by responses was observed in immunized Guinea pigs with treated VZV (Figure 3). Specific erythema started to occur at 8 h and remained until 48 h post injection. Erythema was observed also in the site injected with control antigen at 6 h but it diminished by 16 h post injection. As a control, five uninfected animals were challenged with the skin antigen, and no specific reaction was observed. On the other hand, antibody responses were not detected until day 7**,** but all animals showed antibody increases on day 10 (Figure 1). When the immunological examination was done 4 months after infection, both humoral and cellular immunities were found to persist.

The results of the skin test in immunized guinea pig revealed the reactivity of antigen to immune cell after 4 days observation and confirmed the immunization process (Figure 2). The level of VZV IgM was showed higher than VZV IgG 10 days after the single dose of VZV treated virus. (Figure 3, Table. 1). Two weeks after second dose of treated virus the titer of VZV IgG was twofold increased and the level of the VZV IgM was disappeared.

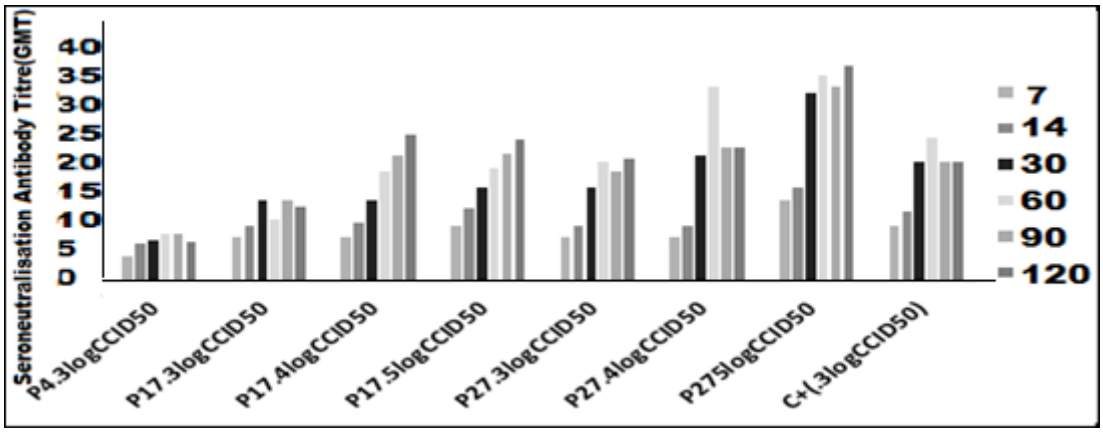

Figure 1. Antibody and neutralization assay of - 6 groups of guinea pig immunized against Local isolated VZV virus.
Development of VZV antibody in immunized guinea pigs. Geometric means of VZV antibody titers were measured by neutralization tests as described in the text, and the values of negative control group were all <2.The Higher and lower level of Neutralizing antibody was assessed from; p27.5LogCCID50 and P4.2LogCCID50. The level for P27.3LogCCID50 and Control positive (vOKa3LogCCID50) was same in different days post inoculation. The virus at P17 LogCCID50 was not significant in compare of P4.2LogCCID50. Increasing the particles number to 4LogCCID50 and 5LogCCID50there for P17 was linear to level of antibody but was not significant.

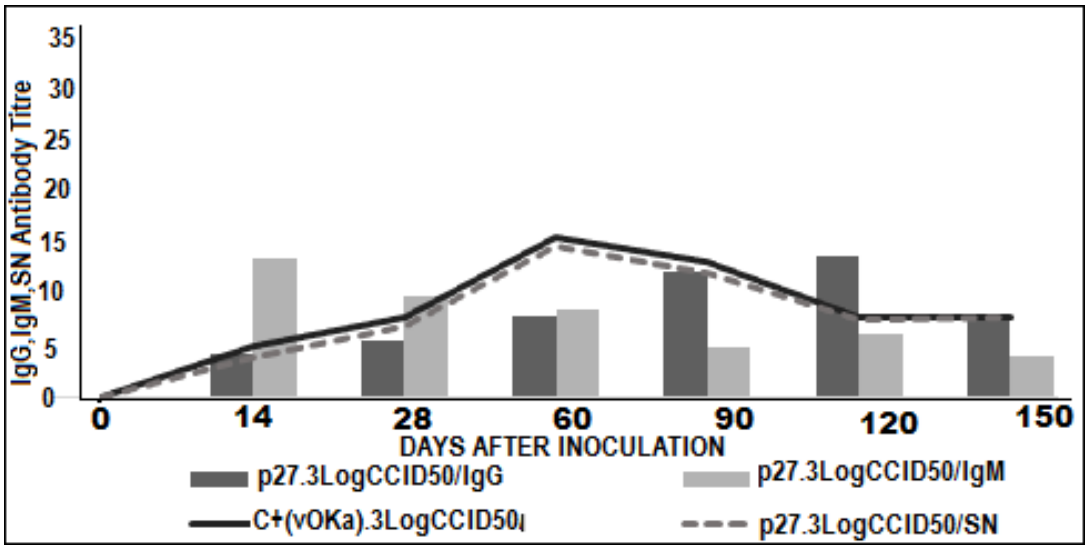

Figure 2. Comparative Qualitative test results obtained from two serological assays SN & ELISA.
Immune antibody titer of SN test for varicella is considered to be 1: 8. Cut off: the upper limit of the reference range of non-infected persons. In ELISA: Negative range=8 (IU/ml), Cut-off=11 (IU/mL), Positive range≥11 (IU/mL).

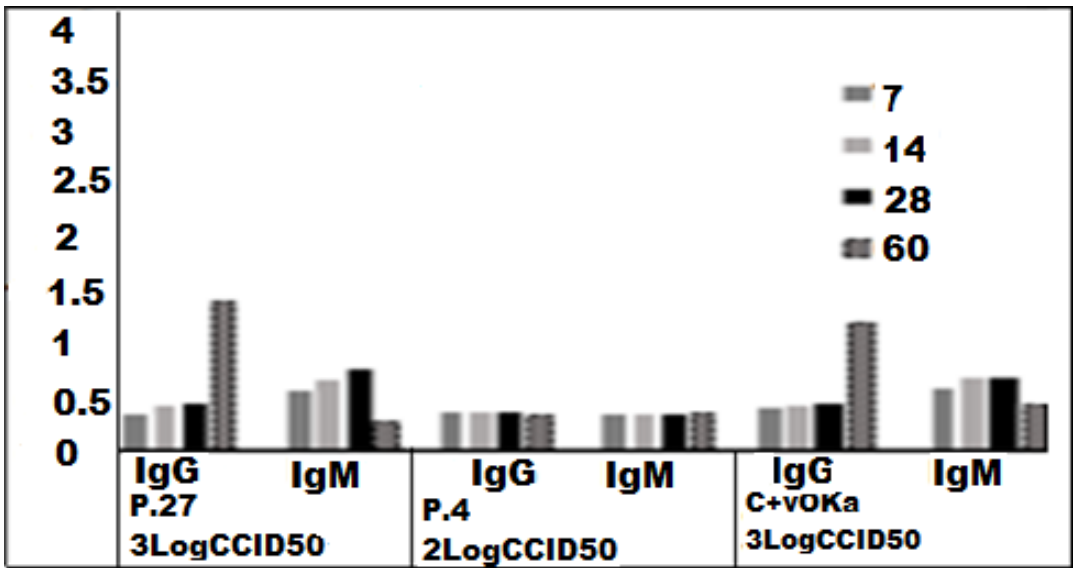

Figure 3: The levels, IgG and IgM after 2 inoculation of local isolated VZV in guinea pigs .The IgM was higher than IgG, 10 days after the single dose. Two weeks after second dose, the titer IgG was increased two fold and the level IgM was disappeared.

Table 1. Antibody levels and cell-mediated immunity after 1 and 2 inoculation of local isolated varicella-zoster virus (VZV) in guinea pigs

Days after Inoculation	Groups/specification, no.	1 st-inoculation		2 nd- inoculation	
		ELISA/titer/GMT	CMI/SI*	ELISA/titer/GMT	CMI/SI
7	3logCCID50.p27	12.5	28	12.5	32
14	4logCCID50.p27	23	23	33	24
30	5logCCID50.p27	25	21.7	28	22
60	3logCCID50.vOKa	35	25	34	32

* Antibody levels were determined by ELISA as the geometric mean titer (GMT); cell-mediated immunity was determined as a stimulation index (SI).

No clinical sign of VZV infection was observed among the non-immunized ginea pigs exposed to immunized group animals. These results showed, the confirmation of attenuation of treated VZV.[15,]

DISCUSSION

Vaccination is the most cost effective and the safest way to reduce disease complications for all infectious diseases. A licensed vaccine contains live, attenuated Oka strain varicella virus, registered more than 3 decades ago .The vaccine, can causes far milder disease than wild-type varicella and universally recommended for all susceptible children and adolescents.[5,11]

The results of cultivations of the local VZV in susceptible cell lines MRC-5 and GPEFC after more than 27 passages, showed a potent virus isolated .To confirm cross contamination including bacterial, fungus and viral infection which enter during VZV passages in tissue culture, the sample of the each five passage was tested in thioglycollate**,** soybean casein broth and PCR-Mycoplasmas assay. The results of cross contamination[10] and adventitious gent for each five passages showed no growth for bacteria, fungus and viral infection in the local isolated Varicella Virus (VZV).Our plan was to develop a varicella virus candidate for vaccine production.

Following serial passage of local VZV isolate the adaptation of virus in sensitive cell culture was exhibited. The appearance of severe cytopathic effect in the passage 4th with low titer (2.3 LogCCID50), changed with increasing passage, Such that the severity of cytopathic effect in the passage 17th decreased and the virus titer started to increase after 27 passages.

The attenuation in local varicella isolate also is the other result that confirmed via incidence rate of Transmissibility. Levin et al mentioned if the transmissibility at the time of living in a cage assessed to10%, it was indicated low virulence activity present in virus because the virus has capability of spreads from immunized to susceptible ones in similar space.[4]

The observation of transmissibility among the non-immunized group of guinea pigs closed with immunized group revealed that no VZV infection was observed among the non-immunized guinea pigs for three months period. In present study the infection was not transmitted clinically or serologically from local isolated varicella virus recipients to susceptible none recipient guinea pig at the time of living in one cage.

The results of the skin test confirmed the immunization process and the susceptibility of guinea pig to local isolated varicella virus antigens and correlated well with others experiment.[15] In natural varicella infection, skin test reactions is negative before the onset of the rash, while after the appearance of the rash obtained positive results usually.[16,17] As early as two days after SC immunization the skin reaction was positive in all of the 3 animals, which was a week prior to appearances of neutralizing antibody. It was also concluded that local isolated VZV virus has a good capability for stimulation immunoglobulin (IgG, IgM) in compare of Positive reference. A week after immunization, the stimulation of neutralizing antibody activated and elicited by one to three weeks after. This result expressed good Lymphocyte proliferative activity in new local isolated varicella virus.[18] The

cellular immune responses in guinea pig seems to be closely presented the protective efficacy of new local isolated varicella for immunization (Table. 1). Although after second dose of virus it seems to be an increase in immune response but it was not significant in compare of 1st injection. . However in order to present the final point , and assess the role of the boosting by second injection**,** it is better the immunization stability to be evaluated and repeated in under test guinea pig over a longer period of time. The results of subcutaneous injection for each dose of treated VZV, 3LogCCID50, 4LogCCID50 and 5LogCCID50 showed no occurrence of death found in injected animal, which indicated the safety of isolated varicella virus.[7] All three dose of different treated VZV particle showed the immunity in the immunized guinea pigs but, among them the dose of 3LogCCID50, of VZV particle showed good titer of antibody response as the 5LogCCID50 of VZV particle showed immunity in other group of guinea pigs, thus the optimum dose for immunization found to be 3LogCCID50 VZV particle. The dose of 1 $X10^3$ VZV particle of commercial vaccine (vOKa, Merck) was programmed in schedule of VZV vaccine immunization in children in different country.[3,7] The aforementioned results and evaluation of this study were showed in accordance with the results reported by.[11,19,20] The results of two doses of the treated VZV in immunized guinea pigs showed the raising specific IgM VZV followed by raising level of VZV IgG in the guinea pig. The similar results of raising VZV IgM and VZV IgG were found in immunized guinea pigs by two dose the commercial Merck VZV vaccine (vOKa).[3,20] Varicella is one of the viruses in which no method has been specifically developed to differentiate the wild-from attenuated type virus. The method for this type of distinguishing is the picture of cytopathic effect on cell culture and level of the immunogenicity in animal model which is included in this research and also the PCR-RFLP for different ORFs such as 32 and 58[21] publish in press. The results of PCR and RFLP assay showed changes of RFLP pattern as shown by the commercial control vaccine, and is different with prototype VZV isolated strain. The isolated VZV in this study showed genotyped E (publish in press). Unfortunately the published VZV genotyping in our country is limited and required more investigations from different region of Iran. The result of the variation between wild type VZV strains has been reported different RFLP profiles based on geographic region. This profiles revealed as PstI+, BglI+ or PstI−, BglI+ platform for Japanese isolates and PstI+, BglI+ or PstI-, BglI+, from the US, UK, Europe and PstI+, BglI- isolated from eastern Australia.[21,22] The isolated strain in tropical regions such as Africa, , Bangladesh, China, India, Central America and northern Australia reported as BglI+ strains.[23,24]

The results of the present study indicates promising first VZV attenuated virus strain development in Iran , although the stability, potency, clinical trial of the treated VZV as a vaccine strain, required to documented. Further criteria including dominant VZV genotypes in different region in Iran should be determined.

In summary the cytopathic effect of the isolated virus was showed at passage 4th and use as master virulent for immunization in guinea pig. The dose $1x10^3$ at passage 27th vzv showed good immune response in animal model compare of reference positive and virulent. the results of the present study indicates promising first vzv attenuated virus candidates for vaccine development in Iran , although further criteria including dominant vzv genotypes in different region of Iran should be determined . Interesting variation in viral titer during the attenuation process occurred but verification that any changes is in accordance of nucleotide mutations needs to complete genome sequencing that is the failure of this article Also the other study such as stability, potency, complete formulation of the treated vzv as a vaccinal strain, required to stabled.

ACKNOWLEDGEMENTS

This work was supported by Razi vaccine and serum research institute; and Tarbiat Modares University, as a part of thesis project for PhD Degree.

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