1 # Full Title:

2   **A new isolated local varicella virus: isolation, identification, comparative**
3 **growth characteristics and immunological evaluation on an animal model**

4 # Short title:

5 **A new isolated local VZV, characterization and immunological evaluation**

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Abstract

A panel of 4 different cell lines was optimized for isolation, identification, and authentication of a VZV virus from a swab sample of an 8-year-old boy suspected to varicella zoster infection. The system enabled highly efficient and rapid isolation of viruses in 33^0C by serial sub culturing to more than 25 passages. The technique relies on isolation of viral genes by increasing the number of particles that are statistically represented in cell culture and verified by $CCID_{50}$, FAM-RT-PCR, and IE62 antibody in IF test. The viral genes (ORF38, ORF54) confirmed the new isolate as VZV and revealed the amino acid sequence of viral-encoded proteins after 27 passages, identical with positive control virus, in RFLP-PCR test. Utilization of successive serial passages at temperatures lower than the normal body temperature would reduce the virus virulence and directly cause virus attenuation. As a result, the attenuated virus is adapted to growth in vitro and presented higher replication at 33^0C. Our goal was to determine if the targeted virus with a large double-stranded DNA genome, varicella virus, is isolated and can be attenuated by cold adapting in vitro, Using vOka as attenuated VZV golden standard, in two quantitative tests, including CCID50 and FAM-RT-PCR. Finally, when compared with the local isolated virus, these results were strongly confirmed. We recorded plaque forming assay to show phenotypic changing which encodes the attenuation regarding size of plaque. Although in plaque forming assay, the size of plaque seemed smaller at first glance, the statistical distribution of the plaque size did not show any change between the virus in the first and last passages. In cell culture, the local VZV isolated viruses formed clear plaques and grew to higher titers compared with lower passages as parental virus. Due to lack of access to human fetal lung cells (MRC-5) and an alternative to vaccine production in the future, a new authenticated local foreskin cell substrate (RFSC) was used for virus cultivation. In comparison to, MRC-5-optimized and cloned- viruses replicated in vitro with kinetics that were similar to those of the RFSC. Laboratory animals that were infected with the optimized virus fluid as vaccine showed a good neutralization antibody against local VZV

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isolated as compared to vOka as control positive virus. These results demonstrate that the virus isolated from swab sample was authenticated as VZV virus, and this cold adapted attenuated virus may be an applicable candidate for future plan.

Keywords: local, VZV, isolation ,identification, immunological, new ,cell substrate

Author summary

We used different cell substrates for isolation, identification, and attenuation of a new local VZV virus from vesicle swab of suspected patient to varicella zoster diseases. The technique involves serial sub culturing of virus in cell culture. Our goal was to determine if the targeted virus with a large double-stranded DNA genome, varicella virus, is isolated and can be attenuated by cold adapting in vitro, using vOka as attenuated VZV golden standard, The virus was cloned and purified by serial dilution cloning and plaque assay. Different techniques were used regarding verification as differentiation from other members in family, potency, and sterility for isolated virus. The virus can grow well in new local foreskin cell substrate and produce good titre as compared with MRC-5. The isolated VZV potentially induced neutralization antibody in animal model. The results of our study imply that local VZV might be an applicable isolate for future plan in research and developing a varicella vaccine.

Introduction

Infectious pathogens are a serious problem in human life and varicella Zoster Virus (VZV) is a particularly challenging germ behind the disease. Varicella "Human Herpes Virus type 3 (HHV3)" is a cell-associated DNA virus belong to Alpha-herpes viridae. The virus causes varicella, a lymph proliferative disease with severe clinical symptoms in human. This is an acute infection which is characterized by fever, malaise, headache, and abdominal pain 1-2 days before appearance of Macula popular rash. During several days' rash involves 3 or more successive crops; each crop usually progresses within less than 24 h from macules

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to papules, vesicles, pustules, and crusts. In this case, there are lesions in different stages of development on any part of the body [1]. These clinical signs usually start on face and trunk, then spread to extremities. These features involve 250-500 lesions that are pruritic. The lesions are typically crusted 4-7 days after rash onset, and affects both children and young adults. Varicella disease is classified based on the virulence exhibited in infection through unvaccinated or vaccinated susceptible adults or teenage people. The infection caused by this virus is generally considered severe and self-resolving even in the absence of treatment but in some cases, it needs special treatment and Medicare related to pain and skin itching.

 This viral disease as primary infection causes chickenpox (varicella), is characterized by a generalized vesicular rash in children [2]. Virus antibodies in serum and skin lesions of affected children, react with some of the structural proteins of varicella virus where viral immune complex has been demonstrated in sera of patients .The VZV virus can establish latency in the host sensory nerve ganglia, and can reactivate at a later time to cause shingles (zoster) in adults with some problems in immunity, after years or decades [3]. This rate manifested in 30% of usual onset in varicella infections and has been named to varicella zoster (VZV) [3]. Mild chickenpox has been shown to be a rare vaccine associated adverse event, and the vaccine virus strain has also capacity for reactivating and causing mild shingles [4, 5]. In addition, break-through wild-type VZV infections have been shown to occur occasionally in individuals vaccinated previously [6, 7]. The latent infection after reactivation comes with unilateral vesicular rash, ophthalmic, neurologic, viremia, and dermatologic complications. The risk of transmission from zoster to varicella is far lower than from varicella. Varicella develops in people with no history of varicella disease or vaccines.

Serious and painful complications are known to occur regarding chickenpox and shingles. Although both infections are rarely fatal, during the first trimester in pregnancy, varicella can result in congenital malformations in the fetus or disseminated varicella in the neonate depending on the time of infection. There

135 is an effective vaccine for VZV employed a live attenuated by vOka strain, which

136 was developed in Japan in the mid-1970s [3, 8]. This vaccine was licensed for

137 use from 1995 [3].

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139 In the present study, Vero, MRC-5, GPEFC (Primary Guinea Pig Embryo

140 Fibroblast Culture) were used for isolation and characterization of a local

141 varicella virus from suspected varicella patients. A locally authenticated foreskin

142 derived cell line (RFSC) [9] was also introduced as a novel cell substrate for VZV

143 virus production and used for the first time as compared against MRC-5. The

144 virus was cloned after serial dilution by plaque assay. The size of the plaque did

145 not change between 4th and 27th passages. In the upper passages, the yield of

146 virus increased and revealed a higher titer. The presence of varicella virus

147 replication was proved in immunoflurocent using IE62 antibody, and verified

148 through SDS –PAGE, western blotting. The isolated VZV virus was adapted to

149 in-vitro culture in two different cell substrates, during successive passages.

150 Characterization of the virus genome was evaluated in RFLP-PCR using 2

151 different ORFs (38 and 54). We investigated on, comparative Potency by CCID50

152 and Real Time after serial passages in Vero cell line. The harvested virus fluid

153 was used for immunological evaluation in guinea pigs. Several VZV challenge

154 trials were also carried out in guinea pigs. The effect of vaccination on mortality

155 and viral load was evaluated. The efficacy of virus in vaccinating guinea pig after

156 subcutaneous (SC) and intra muscular (ID) as compared with intraperitoneal (IP)

157 administration, as well as the humoral and cellular immunogenicity were tested.

158 These immunogenicity results have already shown good results. In this article,

159 we will mention the processes applied and the results regarding isolation and

160 verification, plus identification of a local candidate for the varicella vaccine.

161 **Ethical consent**

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163 The research was conducted in accordance with the Declaration of Helsinki (as

164 revised in 2008) and according to local guidelines and laws. The project was

165 approved by the ethics committee of Faculty of Medical Sciences, Tarbiat

166 Modares University Tehran, and I.R. IRAN. The animal used for the experiment
167 was based on The US Public Health Service's "Policy on Human Care and Use
168 of Laboratory Animals," and "Guide for the Care and Use of Laboratory
169 Animals.

Results

Sample collection

172 Varicella virus was isolated from skin lesion in 4 samples. There were different
173 samples showing a positive result for varicella antibodies. Meanwhile, four
174 isolated viruses from all of the samples revealed better result in ELISA test than
175 others, which were selected for primary inoculation in cell culture. The results of
176 IgM and IgG antibodies to varicella virus in serum of these 4 patients are shown
177 in Table 1 and documented as a part of local isolated history. This article is about
178 isolation and characterization of sample number four belong an 8-year-old boy
179 which was negative in mycoplasma testing and after inoculation, it showed better
180 viral lesions in the cell culture.

Table 1. Summary of serological results of 4 serum samples which the related vesicular lesions used to isolate the virus in this study.

No	Sampling date (mo/day/yr.)	Sex	Age	IgG		IgM	
				LL^a	UL^b	LL	UL
1	9/19/2015	F	8	12	20.7	0.6	1.0
2	8/28/2016	F	7	12	17.6	0.3	1.9
3	4/10/2017	F	9	9.2	26.1	1.9	2.7
4*	9/6/2018	M	8	11	22.6	2.4	4.2

A: Lower Limit
B: Upper Limit
* Isolation of virus was successfully performed from sample number 4, belong an 8 years' boys.

Specific PCR to differentiate local VZV from other Herpesviridae members

195 After inoculation of the vesicular swab sample/VTM on cell culture by designing
196 the specific pair primer and cloning a conserve sequence in pBHA vector as
197 control positive in PCR test for differentiation the isolate from other member of
198 the Herpesviridae family and the identification of isolated virus as Varicella

199   zoster was performed [1]. In Table 2, the primer set sequence and thermal cycle,

200   as well as the conserve sequence are shown as positive controls.

a. Primer Set and Thermal Cycle in Specific PCR to Differentiate VZV from Other Herpes Viruses			
Specific Conserve sequence	Specific Primer set	Product size (bp)	Thermal cycle
GeneBank:X04370.1 DUMAS STRAIN: 9796-10071	5'-CACACGATAATGCCTGATCGG-3' 3'-TGCTGATATTTCCACGGTACAGC-5'	276-bp	1-95°C, 5min
			2-95°C, 1min
			3-46^0·1min
			4-72°C, 1min
			5-repeat 2 & 3,4 in 35 cycles
			6- 72°C, 10min
2b: Primer Set and Thermal Cycle in PCR-RFLP			
RFLP-PCR	Primer set	Product size (bp)	Thermal cycle
ORF32	FVZV32/ (GGAACCCCTGCACCATTAAA RVZV32/ TCCCTTCATGCCCGTTACAT		1-94^{0}c, 3 min 2: 56.7°C, 1min
ORF58	FVZV58/ (TTGAACAATCACGAACCGTT RVZV58/(CGGGTGAACCGTATTCTGAG		3-72°C, 1min 4-repeat 2 & 3 in 30 cycles 5- 72°C, 10min
2c: Primer Set and Thermal Cycle in Quantitative Real Time			
RT-PCR	Primer set	Product size (bp)	Thermal cycle
ORF 62 (101)	FVZV62/ATGTGGTTTCCAAGGCCAAGAG		1-95^0, 2min
			2-50^{0}C, 10min
	RVZV62/ TCCGTCAAGTGGCATCGTTATT		3- 60^{0}C ,1 min
	probe (FAM) CCCGTGGAGCGCGTCGAAA		4-repeat 2 & 3 in 60 cycles
			5-95^{0}C, 15 s,

Table   Primer **2.**   set and

211   thermal cycle in specific PCR to differentiate VZV from other herpes viruses.

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226 The result of a specific PCR test for differentiation of the isolated sample from
227 other Herpesviridae member has been shown 276 bp after electrophoresis on 1.5
228 % agarose gel, and staining by safe stain. This result was confirmed in
229 comparison with the size of construct as control positive in test.

230 In Fig 1 the pBHA vector, its primer set and conserve sequence of DUMAS strain
231 cloned as positive control and gel electrophoresis of sample are given the same
232 size.

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234

235 **Fig 1. Differentiation of local VZV DNA by specific-PCR.** A, pBHA vector; B, Analysis PCR
236 amplification: M lane, Ladder; lane 2, PCR products from control positive (DUMAS Strain: 9796-

237 10071) VZV DNA in dilution 1/10; lane 4, PCR products from local VZV DNA in dilution 1/10; lane
238 6, PCR products from local VZV DNA in dilution 1/100.

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240 After observing a dense layer of cell line, the transfer medium contain swab in

241 used as sample and inoculated to cell culture monolayer. The culture left in room

242 temperature for 2 hours regarding adsorption of virus. Then, DMEM was added

243 to culture and incubated at 37^0C. After 6-7 days, when the cytopathic effects

244 (CPE) appeared on culture, the flask was frozen and thawed. The cells and the

245 flask supernatant were centrifuged at 3000 RPM for 20 minutes. The supernatant

246 and pellet were extracted by Qiagen DNA extraction kit and underwent the

247 extraction by Qiagen DNA extraction kit according to the manufacturer's user

248 manual. The DNA was amplified by specific PCR. At each step, using specific

249 primer pair of varicella zoster with appropriate concentration. DNA detected in

250 agarose gel using ethidium bromide and comparison with markers to verify the

251 presence or absence of the virus. Positive control was tested along with positive

252 infectious sample. Comparison of the results showed that the components were

253 similar to the positive control.

254 **Virus isolation and attenuation**

255 CPE in cell culture systems is not always clear enough on primary inoculation,

256 thus tissue culture fluids may have to be passaged at least 2-3 times for full

257 adaptation and more than 20 times for isolation and attenuation as we did such

258 process for VZV virus from swab sample in this research. To follow the microbial

259 and viral adventitious agent, for every 5 passages, a sample from isolated virus

260 was inoculated in three different cell lines. The mycoplasma and sterility testing

261 were done based on requirement [5]. Two days post inoculation the cultures were

262 examined microscopically to monitor the virus growth. The culture media

263 changed with fresh ones every 2 days. Once the virus cytopathic effect spreads

264 on the culture, the media were harvested and stored at -60^{0}C for next step. Serial

265 sub cultivation was carried out and continued more than 25 times to reduce virus

266 virulence and adapted in growth at 33 ^{0} C. The first eleven passages were on the

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267 MRC-5 cells, then the cell substrate was changed by primary guinea pig embryo

268 fibroblasts cells (GPEFC) (using 20-day pregnant fetus) and viruses were

269 passaged from 12 to 24 on this cell substrate. In the third stage, MRC-5 cells were

270 used again and virus culture continued until passages number 30. In parallel, all

271 the processes were performed with the local foreskin cell line, as an alternative

272 to MRC-5 for future production [10]. For this purpose, the growth kinetics of two

273 cells in terms of higher titer production of isolated virus were also compared (Fig

274 2).

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277 **Fig 2. VZV on human diploid lung and human foreskin cells.** (A) Comparative cell division
278 number between two human cell substrate during seven days after seeding. MRC-5 as standard
279 human diploid cell strain and RFSC as a continuous local human foreskin cell substrate: The
280 local RFSC at passage 65 and MRC-5 at passages 26. (B) Replication characteristics of local
281 VZV isolate in human diploid lung (MRC-5) and human foreskin (RFSC) cells: Two human
282 cell lines were inoculated with the local VZV and its CPE assessed by $CCID_{50}$ method.

283 Replication kinetics of isolated VZV were compared with RFSC as an alternative cell substrate
284 for future research and production.

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287 Comparative evaluation of local virus replication on two different cell
288 substrates MRC-5 and RFSC

289 In the present study, concerning the limitation to access to MRC-5 causes, RFSC,
290 a local cell substrate that developed based on requirement by Razi Vaccine and
291 Serum Research Institute from the foreskin, was used to produce VZV vaccine
292 using the local virus.

293 In the present experiment, the replication kinetics of the two different passages
294 viruses were compared on MRC-5 and RFSC cells infected at two MOI: 0.01 and
295 MOI :0.1. Local isolated VZV showed peak virus titers by day post-infection, for
296 high and low passages post infection (Table 3).

297

298   **Table 3**. Viral ORF-62 genes expression levels on MRC-5 and RFSC cell substrate.

VZV/ORF62/ Hours post infection(hpi)	VZV/MRC-5/P5		VZV/RFSC/P5		VZV/MRC-5/P27		VZV/ RFSC -/P27	
	MOI 0.1	MOI 0.01	MOI 0.1	MOI 0.01	MOI 0.1	MOI 0.01	MOI 0.1	MOI 0.01
24	31.85	31.90	31.75	32.15	19.24	19.26	19.24	19.24
48	31.72	31.75	31.62	31.65	18.34	18.24	18.12	18.14
72	31.54	32.53	31.44	31.44	15.04	15.17	15.14	15.37
96	31.18	32.21	31.38	31.38	14.03	14.03	14.33	14.08
120	30.29	31.28	31.75	31.75	13.32	13.34	13.22	13.44
144	29.88	31.41	30.58	31.58	12.75	12.21	12.65	12.44

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300   The growth of local VZV was evaluated by quantitative RT-PCR at the indicated time point for different MOI:
301   0.1 and 0.01. Ct values were determined for viral ORF-62 genes. Experiments were performed in triplicate and
302   data represent mean ± standard error of the mean (SE).The result represent no statistically different replication
303   kinetics of isolated VZV were compared with RFSC (a local foreskin cell line) .

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306   Infection with each of the three (high and low passages local isolated beside
307   vOKa) viruses proved to be typical syncytium-cytopathic in these cells, which
308   remained persistently infected for the 10 days follow-up period, though virus
309   titers started to increase from 3 DPI onwards. In addition, peak virus titers were
310   measured by 9-10 DPI in MRC-5 and RFSC cells for high and low passages
311   viruses and by 7 DPI for vOKa as control positive. The result showed similar
312   titers were obtained from MRC-5 and RFSC cells inoculated by low passages
313   (5th) compared to strong higher titers obtained from P: 17, P: 21, P: 27, infected
314   MRC-5 cells. To study the short-term dynamics of virus replication, a one-step
315   growth curve was performed on MRC-5 (human lung diploid cell strain) at an
316   MOI of 0.1. The eclipse period for all stage high passages viruses was between
317   16 and 24 hours on MRC-5 cells. Slightly different eclipse periods were found on
318   human foreskin cells (between 18 and 30 hours), but it was not significant
319   compared to MRC-5. At 7 DPI, 60% of the cells were inoculated with MOI of
320   0.1 showing the cytopathological effect and high titers detected in the culture
321   supernatant of both MRC-5 and RFSC cells.

322   In a comparative evaluation of MRC-5 and RFSC cell substrate, although the
323   growth of the virus has increased, it was not statistically significant. Viability and
324   recovery rates of stored cells were assessed, in both RFSC and MRC-5 cell
325   categories, where survival rates were 94% and 92%, respectively, which is same
326   as the levels we expected since both cell lines had the same characteristics
327   regarding the growth kinetics(Fig 3).

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Fig 3. Replication and potency assay of Local isolated VZV virus during different passages on MRC-5. (a) Development of VZV virus inoculated on MRC-5 cell substrate. $CCID_{50}$/mL of VZV virus titers were measured by potency tests as described in the text. The values of, higher and lower level of virus titer was assessed from; p27. (8.75-9.40 $LogCCID_{50}$) and P4. (2.75-3.40 $LogCCID_{50}$). This level for Control positive was (vOKa3.7-4.3$LogCCID_{50}$) after 9 days' post inoculation. The virus at P.27 and P17 (6.5-7.4 $LogCCID_{50}$) was significant in compare of P9. (3.40-4.5 $LogCCID_{50}$) and P4. (2.75-3.40 $LogCCID_{50}$). Increasing the particles number to 6 and 5$LogCCID_{50}$ for p 27 and P17 was linear to level of virus in RT-PCR but was significant. (b) Replication and potency assay of local isolated VZV during different passages of VZV on local cell substrate (RFSC). After successive serial passages, the virus has become adapted to growing in new cell substrate. This procedure increases the proliferation and number of virus particle. The virus titer has increased by about 6 log $CCID_{50}$/ml in high passages (p: 27), compare to low passages (p: 4) and produces more particle in potency testing at hours post inoculations daily and also after 9 days in high passages The titer of the virus was measured using Karber method .

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354 To evaluate the effect of the adaptation on virus production, we performed
355 potency assay in the cell culture. We harvested fluid containing isolated virus
356 from MRC-5 and RFSC and inoculated on Vero cells through logarithmic serial
357 dilution. We obtained the result of $CCID_{50}$ by the Karber method in Vero cell
358 lines (Fig 4). We used Vero cells as it is highly sensitive to quality control of the
359 harvested virus. Successive passages showed the complete adaptation of RFSC
360 to virus as well as to MRC-5. It was observed that after 3 consecutive passages,
361 the complete adaptation of the virus to RFSC cell was established. Regarding
362 MRC-5 on first passages, the cytopathic effect of isolated local VZV and control
363 positive was visible after 8-9 days post-infection. Although viral cytopathies were
364 seen in the first passage, the rate of virus adaptation to the cell substrate was not
365 high enough to produce the high yield varicella zoster for future vaccine
366 production, and several passages were given to the isolated virus to accustom to
367 the in vitro culture.
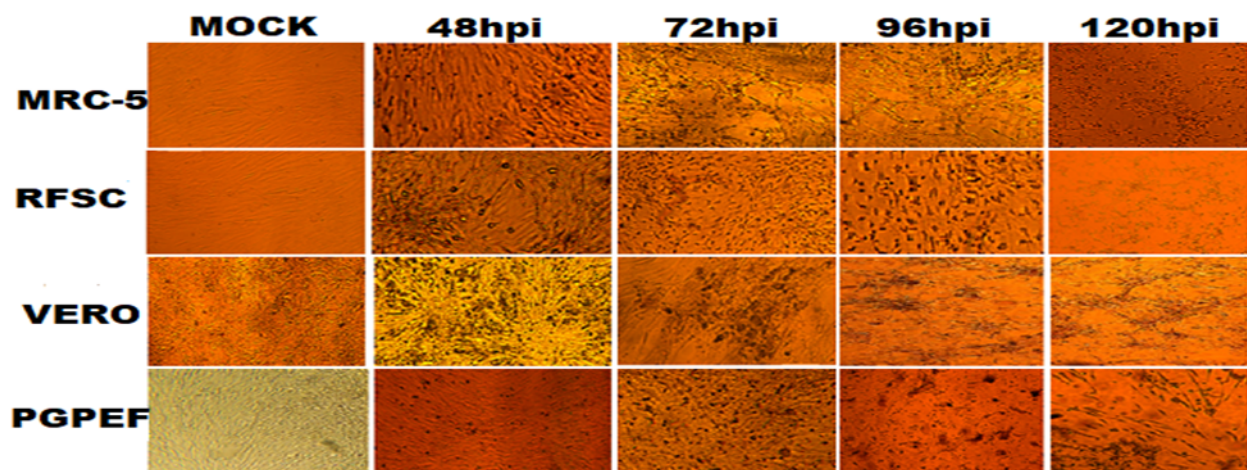
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370 **Fig 4. The local VZV cytopathogenicity.** Human lung diploid cell (MRC-5) and human
371 foreskin fibroblast (RFSC), Vero, and primary guinea pig fibroblast cells **(PGPFC)** to local
372 VZV virus infection at 48 and 72and 96 and 120 hours after infection (100x
373 magnification).The virus inoculate as MOI: 0.1 and was in 17th passage.

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377 In this procedure, vOKa was used as control positive. As the passage of the virus

378 increases on the target cells, so does the number of viral particles. The number of

379 particles in passage 27 as the high shows a huge difference with the low passage

380 4 , The virus does not need adaptation to the MRC-5 and its growth was achieved

381 after first passages by sub-culturing of the virus during 8-9 days. Local VZV virus

382 presented typical CPE and caused RFSC-cell lysis in a monolayer, which were

383 easily observed through microscope visualization. The pH of the media for the

384 virus culture was optimized on 6.6±0.2. The total cell per $25cm^2$ flask, were

385 $4.2 \times 10^6 \pm 1.5 \times 10^5$ as same as for MRC-5. These optimized growth conditions

386 for local VZV on RFSC cell substrate caused achieving a higher yield of virus in

387 potency. Maximum virus productivity of the local VZV virus was high in RFSC

388 $(10^{9.42} CCID_{50}/mL)$.

Plaque Assay

390 The most appropriate method for isolating a single particle from a virus in a

391 sample from different genetic populations is the plaque method. Using this

392 method, through measuring the plaque size in some viruses, an attenuated from

393 virulent parent of a virus can be indicated and differentiated. This phenomenon

394 has been reported for many viruses after induction of serial passaging in cultures

395 [6, 11].

396 In the plaque test, the fresh monolayer of Vero cell was infected with a serial

397 dilution (10^6-10^{12}) at MOI equal to 0.1 from local VZV virus. The culture

398 incubated at room temperature allowed the virus to be absorbed into the cells and

399 then covered with a nutrient medium containing agar. In this procedure, virus-

400 infected cells released viral particles, which limit the spread of new viruses only

401 to adjacent cells. As a result, infection with any viral particle creates only one

402 area of infected cells that can be seen with the naked eye called plaques. The

403 plaque test using neutral red as vital dyes help see the boundary between the

404 plaque and the surrounding single-layer cells, thereby absorbing the dye in living

405   cells. Thus, the bright plaques resulting from the virus replicating in a field of red,

406   and the living or non-living infected cells are distinguished. Since a virus particle

407   is sufficient to form a plaque, the virus titer can be presented as a plaque-forming

408   unit (PFU) per milliliter. In addition to titration, we used the plaque method to

409   purify and clone the virus. In the present study, the above method was used to

410   evaluate the titer and clone the isolated virus (after 25th passage) and also to

411   evaluate the size of the plaque in relation to its attenuation. We determined plaque

412   sizes of the passaged viruses. We did not detect any changes of the virus

413   phenotype. Plaque areas were measured using electronic size counter (Fig 5).

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Fig 5. Plaques formed by the viruses that were passaged in cell culture (Scale bar, 200 μ). (a) Representative Images of plaques formed by viruses that were passaged 5 times serially at low multiplicity of infection in cell culture. (b) Representative Images of plaques formed by viruses that were passaged 17 times serially at low multiplicity of infection in cell culture. (c) Representative Images of plaques formed by viruses that were passaged 27 times serially at low multiplicity of infection in cell culture. Serial passaging of viruses has not altered phenotype of plaque of viruses. Although the plaque produced in the 27th passages of virus were larger, and plaques formed by the 4th passages of virus were smaller. But the size distribution of relative plaque diameter normalized against the average plaque diameter of the parental virus. P-values were calculated using multiple comparison test, indicates $P<0.05$.

Characterization of isolated VZV viruses by RFLP-PCR after different passages in tissue culture

429   The isolated viruses were examined by PCR and restriction fragment length

430   polymorphism (RFLP) analysis, using the ORF38 and ORF54 region. Initially,

431   we performed PCR by designing two pairs of specific primers for these two ORFs

432   (Table 3). Then, via digestion through two restriction enzymes, BglI and PstI with

433   a fully -optimized thermal cycle, digestion was performed on the PCR product.

434   In electrophoresis of PCR and RFLP products on 1.5% agarose, the results were

435   confirmed after safe staining (Fig 6).

436

437   **Fig 6. Results of electrophoresis following RFLP test on PCR products of high passage**
438   **local VZV DNA by RFLP-PCR.** (A) PCR amplification of ORF 34 and ORF58, contain 222-
439   bp and 350-bp fragmentlane1, ladder; lane 2and 3, PCR products from vOKa as reference
440   positive ,350-bp and 222-bp; lane 4and5, PCR products from isolated varicella, 350-bp and
441   222-bp. (B) RFLP on PCR product to analysis the fragment size differentiates wild from
442   attenuated isolated; lane1 and 2 ladder; lane 3, vOKa profile after digestion by PstI (350-bp
443   and 222-bp); lane 4, vOKa profile after digestion by BglI as reference positive (85,100,137bp
444   fragment size); lane 5, digested products from local isolated VZV by BglI (85,100,137bp
445   fragment size); lane 6, digested products from isolated varicella (222 and 350-bp fragment).
446   (C) Schematic RFLP fragment size BglI and PstI.

447

448

449   **SDS-PAGE Western blotting analysis**

450   The activation of ORF62 was evaluated by Western blotting following SDS-

451   PAGE. In this study, a band was identified related to IE62 protein in varicella

452   virus. The protein is the most important viral activator of VZV gene transcription
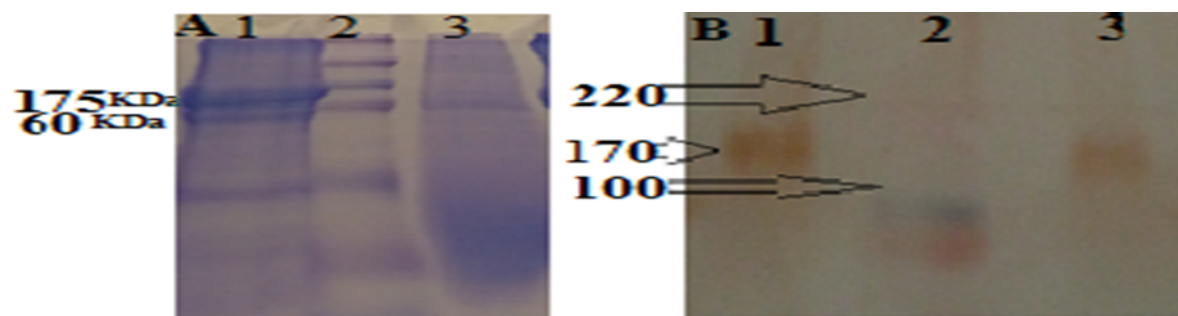
453   (Fig 7).

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Fig 7. Comparative SDS-PAGE and Western blotting results between isolated VZV and vOKa as control positive. (A) SDS-PAGE of isolated VZV and control positive; Lane 1, 175 kDa band of IE62 protein in the local VZV; Lane 2, Protein Prestain marker; Lane 3, 175 kDa band of IE62 protein in positive control. (B) Western blotting of isolated VZV and control positive; Lane 1, 175 kDa band of IE62 protein confirmed with specific antibody in the local VZV; Lane 2, Protein Prestain marker; Lane 3, 175 kDa band IE62 protein confirmed with specific antibody in positive control. This result confirmed identification of IE62-specific protein as the most important viral transcription activator of VZV gene between isolated VZV and vOKa as control positive. Its presence was examined using labeled specific antibodies, in the Western blot experiment with color band observation. Binding of the labeled secondary antibody was associated with the anti-C-terminal monoclonal antibody near the C-terminal IE62 protein, along with the identification of positive control.

470

Study on expression of the local isolated VZV gene by specific antibody (anti-IE62) in IF test

Our goal was to determine whether cold adaptation is an applicable strategy for attenuation of local isolated VZV herpes viruses. To study the effect of growth condition (viral replication) and its optimization at a lower temperature (viral attenuation), we recorded the IE62, as DNA Pol is essential for virus replication in vitro and in vivo, and we expected that reduction or increase of the effective protein on its function and production might change the biological properties of the isolated viruses, which could be easily observable both in vitro and in vivo. As observed previously the replication of high passages increases, if the conditions for the growth of the virus in 300C are optimized. This feature markedly presented in potency testing following that, but the number of fluorescent particles in IE62 antibody testing showed a sharp decrease in P:30 compared with parental P:5 because of occurring probably attenuation. The reduction of expression of IE62, following sub culturing of the virus, from low to

18

486 high passage for cell-substrate MRC-5 in IE62 antibody testing by IF has been
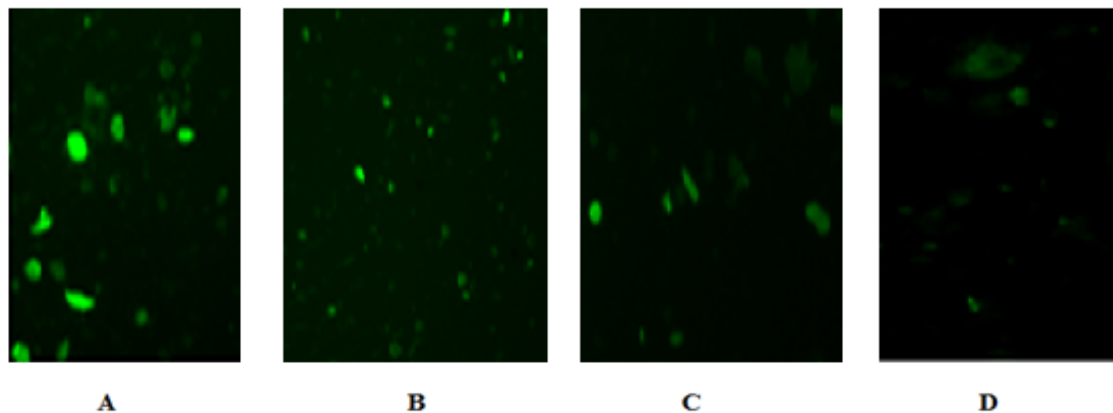
487 shown in Fig 8.

488

Fig 8. **The local VZV in IF test after serial passages.** A, the 5th passage of local VZV; B, the 30th passage of local VZV; C, control positive virus; D, the uninfected cell.

492

The comparative replication rate of local isolate VZV virus by $CCID_{50}$ and RT-PCR (Real Time PCR).

495 The replication rate of local isolate VZV virus in human cells was evaluated by

496 quantitative RT-PCR for 0.1 and 0.01 MOI.

497 The kinetic replication of ORF62 gene in infected cells was similar to the virus

498 titration results. VZV infection induced a rapid increase in the levels of ORF62

499 gene in MRC-5 cells. There were not any significant differences in the

500 transcription levels of the gene in VZV-infected cells. In this test, the levels of

501 viral ORF62 genes from infected cells were analyzed by one-step quantitative

502 RT-PCR. The reactions were conducted in duplicate and carried out for 30 cycles.

503 The relative expression threshold cycle (Ct) value was normalized using internal

504 control. Reactions were repeated three times for each sample. Representative

505 images in Fig 3 show MRC-5-transfected with a culture fluid at MOI: 0.1. To

506 quantify virus production was driven by different passages, the harvested fluid

507 was inactivated and we analyzed DNA production from the parental and high

508 passages 72 h after cell culture transfection. qPCR analysis showed that 27th

509 passages well adapted and produced significantly more as compared with the 4th

510 passage(Fig 9). This difference was completely significant. The purpose of the

511 above test was to evaluate the rate of ORF62 gene replication, which reported

512 that the increase in gene proliferation could be attributed to limit reduction.
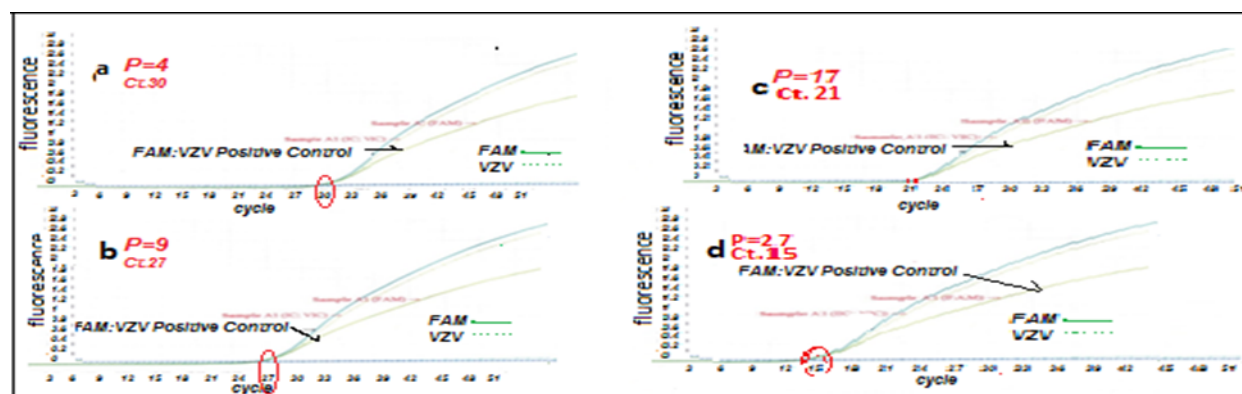
513

515 **Fig 9.Viral particles in current VZV isolate from Iran by RT-PCR on MRC-5 (72hpi).** (a)
516 Isolated VZV-infected MRC-5 cells P.4. (b) Isolated VZV-infected MRC-5 cells P.9. (c)
517 Isolated-infected MRC-5 cells in P.17. (d) Isolated-infected MRC-5 cells P.27.

518

519 **Immunological studies and serum antibody development**

520 This procedure was done to evaluate the immunogenicity of local isolated virus

521 and its efficiency for producing the antibody as dose finding.

522 Several studies have demonstrated the serological and pathogenesis of VZV virus.

523 We sought to determine the kinetics and optimization of minimum dose of local

524 isolated VZV that can induce antibody production. We found that isolated VZV

525 was serologically positive in the animal model inoculated with $10^{5.0}$ CCID$_{50}$/mL

526 (group A), $10^{4.3}$ CCID$_{50}$/mL (group B), $10^{3.3}$ CTCID$_{50}$/mL (group D), and $10^{3.0}$

527 CCID$_{50}$/mL (group E), either ID or SC. In the animals as early as 6 days post

528 inoculation, no clinical signs, fever, redness, and weight loss were apparent from

529 1st days post-inoculation onward. There is a significant increase in antibody

530 titters between the first stages of blood sampling belonging to pre-inoculation of

531 the virus and the second stage which is 6th days following the first inoculation.

532 Similarly, blood samples were taken on 21th and 44th and 162th days after the virus

533 was injected an ascending trend is also evident in almost all groups in their

534 antibody titters. More animals that received $10^{4.7}$ CCID$_{50}$ /dose, 10^4 CCID$_{50}$ /dose

535 10^3 CCID$_{50}$ /dose or $10^{2.7}$ CCID$_{50}$ /dose from high passages (30th) developed IgG

536 antibody compared to animals receiving $10^{2.7}$ CCID$_{50}$/dose low passages (5th :

537 group F). As shown in Fig 10, an increase in Ig M was observed for up to 21 days.

538 Then, a gradual decline from 44**th** days to 166**th** days after injection was recorded.

539 An increase in Ig G was clearly seen after 44 days and progressed to 162 days

540 post-injection (Fig 11). Note that group H includes animals exposed to vaccinated

541 group A without any injection, to assess the transmission of isolated virus. A case

542 of death or disease symptoms, and an increase in VZV not observed in the last

543 group. The result of serum antibody development revealed the differences

544 between high and low passaged viruses.

545

546

Fig 10. Development of local VZV-specific IgM over time in (350-405 gr) Guinea Pig.
Guinea Pig were inoculated ID or SC with (A) $10^{5.0}$CCID$_{50}$, (B) 104.3 CCID$_{50}$, (D)
$10^{3.3}$ CCID$_{50}$ and (E) $10^{3.0}$ CCID$_{50}$ of high (30th) passages isolated VZV virus , (F)10^3
CCID$_{50}$ of low passages (5th) and (C+) $10^{3.3}$ CCID50 of vOka vaccine as control. The lines
indicate threshold level of the assay (P>0.05).

552
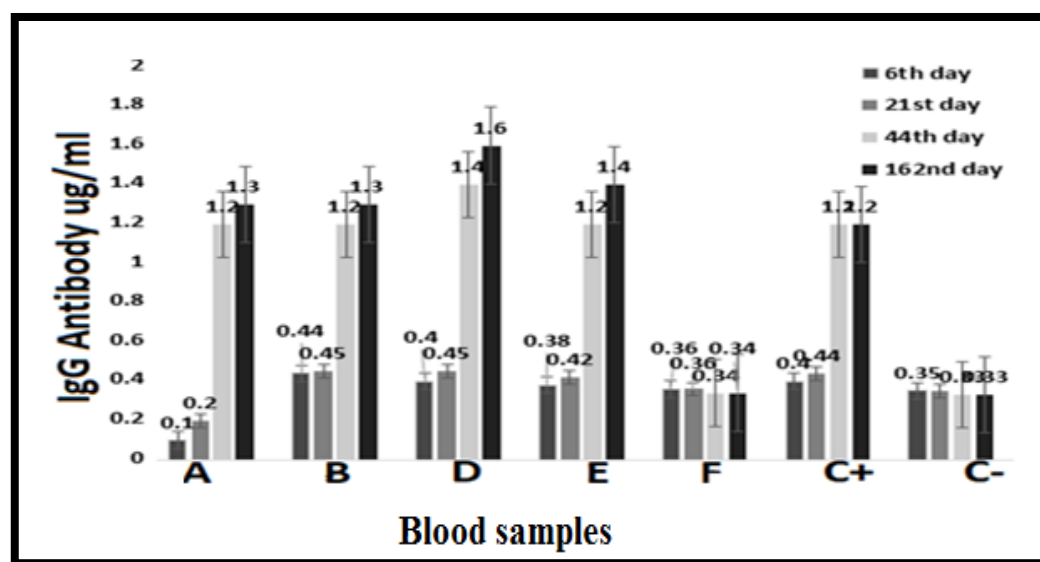
Fig 11. Development of local VZV-specific IgG over time in Guinea Pig (350-405 gr). Guinea Pig were inoculated ID or SC with (A) $10^{5.0}$CCID$_{50}$, (B) 104.3 CCID$_{50}$, (D) $10^{3.3}$CCID$_{50}$ and (E) $10^{3.0}$CCID$_{50}$ of 30th) passages isolated VZV virus , (F)10^3 CCID$_{50}$ of 5th passage and (C+) $10^{3.3}$ CCID50 of vOKa vaccine as control. The lines indicate threshold level of the assay (P>0.05).

The virus did not antibody in the serum samples of animals inoculated with 10^3 CCID$_{50}$/mL $_{of}$ low passages (5th). We compared antibody production in animals inoculated SC with high as attenuated and low as wild isolated virus and vOka commercial vaccine (group C$^+$) as positive controls. All animals had to be alive until 9 months after inoculation and monitored weekly. The development of antibodies was not detected in negative group animals (group C$^-$) which had received water for injection (WFI). The route of inoculation did not influence significantly the antibody production of animals receiving any dose of virus at 6, 21, 44, or 162 days post-inoculation (P>0.05). The use of isolated virus as antigen in the viral neutralization (VN) could explain the neutralizing antibody titres that high passages isolated virus (P: 30) inoculated guinea pig developed after inoculation with $10^{3.3}$CCID$_{50}$ or $10^{3.0}$ CCID$_{50}$ (Fig 12) [12].
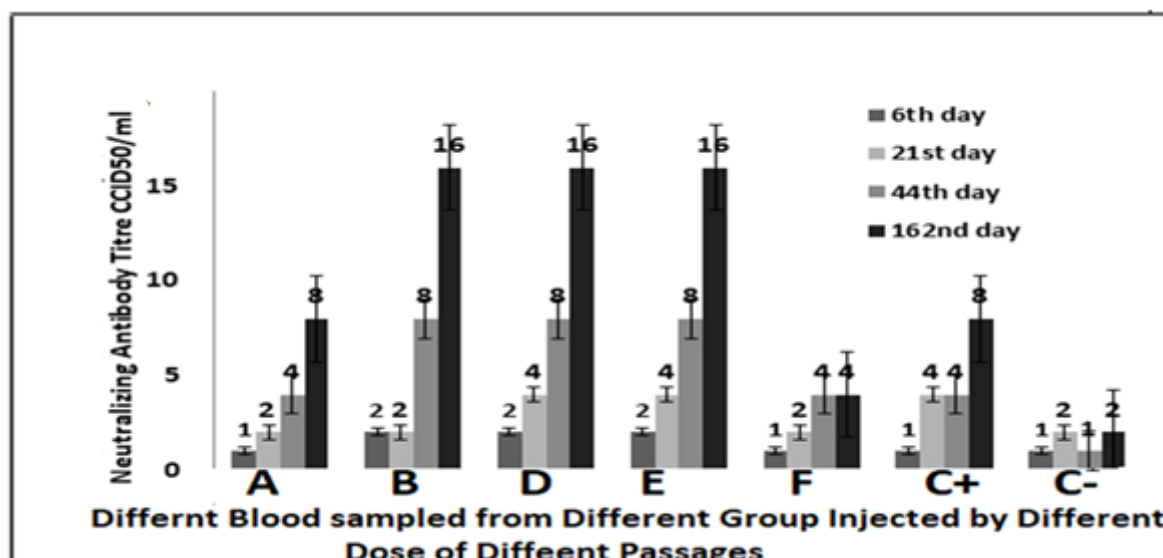
Fig 12. Development of Local VZV-specific neutralizing antibodies over time in (350-405 gr) Guinea Pig. Guinea Pig were inoculated ID or with (A) $10^{5.0}$CCID$_{50}$, (B) $10^{4.3}$ CCID$_{50}$, (D) $10^{3.3}$ CCID$_{50}$ and (E) $10^{3.0}$ CCID$_{50}$ of high (30th) passages isolated VZV virus, (F)10^3 CCID$_{50}$ of low passages (5th) and (C$^+$) $10^{3.3}$ CCID$_{50}$ of vOKa vaccine as control. The lines indicate threshold level of the assay.

Skin test

On the other hand, stimulation of cellular immunity following receiving isolated VZV virus was shown in test animals, on the 21st day using ID injection of positive control virus suspension, after 24-72 hours with a skin reaction in the form of redness and stiffness more than 5 mm in diameter, in the injection site.

Discussion

The live attenuated varicella vaccine using the vOka strain is the only vaccine licensed to prevent human herpes virus infection. Immunity and Safety to this vaccine has been demonstrated in early clinical trials in Japan [13]. Clinical evidence for attenuation of vOka has shown that vaccine-related rashes occur in only about 5% of healthy children and adults. Proliferation of the vOka VZV virus during immunization of immunocompromised individuals or those using immunosuppressive agents is relatively limited [14, 15].

23

595 Lower yields of infectious virus and slowness of skin lesions associated with
596 decreased viral protein synthesis of skin xenografts in SCIDhu mice compared to
597 pOka proposed that the attenuation of vOka vaccines results from a reduced
598 capacity to replicate in skin, which allows a longer interval for the adaptive
599 immune response after viral inoculation. According to this hypothesis, infectious
600 vOka may be cleared before skin lesions formed and before cell-associated
601 viremia develops [16, 17].

602 There are different experiences in which surveyed succeeded to isolate the viral
603 agent from the swab lesion, sera, CSF of affected patients by cultivation of sample
604 in different cell line. In some of them the isolated virus remained highly potent
605 and went under following passage for future vaccine [6, 18].

606 In this article, via a similar technique, we describe the in vitro isolation of local
607 varicella zoster from an 8-years boy skin vesicle swab. This sample selected from
608 four other because not only blood serum of patient presented acceptable antibody
609 level but also presented good condition regarding sterility, mycoplasma, cell
610 culture, as well as its subsequent molecular and biological characterization. The
611 differentiation from other Herpesviridae family member was 100% identical to
612 the primary isolate as varicella zoster by specific PCR assay. Chickenpox
613 infection is highly contagious from days 7-21 of the commune (average 14 days),
614 and studies suggest that the VZV particle is transmitted through the air. Airborne
615 particles from skin lesions are the main source of infection and are more involved
616 in the spread of the disease than the patient's respiratory secretions [19, 20]. The
617 risk of transmitting chickenpox is about five times higher than in those with
618 shingles [21, 22].

619 The disease begins with symptoms such as lethargy and fever from one to two
620 days before and after the rash. The fever usually lasts for 5-7 days in parallel with
621 the rash. Each rash appears as an itchy lesion within 24 hours of the stage from
622 macular to papule and then to the vesicle and then the pustule and eventually to
623 the wound. Thus, all of these lesions are seen in a person with chickenpox, where

624 mainly the trunk, scalp, and even epithelium of the internal organs (endothelium)

625 also   become   infected   [23,   24].

626 In the case of VZV, the phenomenon of escape from the immune system as well

627 as escape from binding to antibodies has been observed, which leads to

628 asymptomatic recurrences [12]. The virus invasion might not be dependent on the

629 amount of virus replication. Given the nature of transmission via usually close

630 contacts superficial scratches or respiratory secretion, in the human infection, it

631 is generally believed that the VZV virus is invasive at low doses [25, 26]. The

632 amount of virus that has been mentioned for human infection in various articles

633 is closely related to the immune status of the elderly or other terrestrial diseases.

634 Indeed, varicella zoster virus causes infection in a substantial number of adults or

635 infants. Further comparisons have revealed that either route of infection or the

636 age of the case led to marked differences in the clinical outcome, antibody

637 response, or amount of viral RNA recovered from the patients' infectious

638 material. Although the incidence of varicella disease is declining as a result of

639 vaccination but there is not including as expanded program for immunization in

640 Iran yet. In order to study the replication potential of the isolated virus, different

641 experiments were performed in this research. The shortage and limitation to

642 access to MRC-5cell strain caused to candidate an authenticated local cell line

643 from foreskin, as alternative for future research and development [10]. The

644 growth curve of VZV could reveal no significant differences in replication

645 kinetics between the titre in different passages of viruses, either on skin epithelial

646 or human fibroblast cells. The mechanisms underlying these differences are not

647 completely clear. It is possible that the two different human cell lines differ in

648 viral receptor density or qualitative or quantitative differences in anti-viral

649 response to infection. Alternatively, differences in the rate of cell division or split

650 level in culture could also explain the differences observed in the yield virus

651 potential of the two human cell substrates. On the other hand, RFSC is a

652 continuous cell line and MRC-5 is a diploid cell strain capable of limiting the

653 number of passages for production. In this regard RFSC, can be cultivated

654   continuously and will have a higher value in terms of production. The results

655   revealed that the increase in virus yield in these two human cell substrates: MRC-

656   5 and RFSC was due to the isolated virus adapted to cultivation at 30^0C. Our data

657   confirm the high sensitivity of the two different human cell lines after inoculation

658   by different passages of local isolated VZV virus.

659   One of the factors that can determine the rate of adaptation to the cell is the rate

660   at which the cells become infected with the virus. Initially, the virus is attached

661   to the cell surface, and then the number (percentage) of cells that the virus has

662   been able to spread is determined as virus replication. A higher adaptation rate is

663   equal to the higher virus growth and ultimately the virus yield. The specific rate

664   of virus production in cell culture is actually the rate at which the virus

665   proliferates in the cell over a specific period of time before the cell was lysis or

666   disrupted. The higher the rate of virus replication, the greater the rate of virus

667   production in the culture and the shorter the time required in terms of high yield

668   virus production. To examine the process of replication and viral yield in a cell

669   line, determining the rate of potency efficiency will be one of the important

670   factors for vaccine production. The higher this factor, the higher the viral particles

671   in a given volume of cell substrate, which is very important from the point of

672   view of vaccine production.

673   Herpes viruses, as many other large DNA viruses, are equipped with their own

674   DNA replicase, which renders them independent of the host DNA replication

675   machinery. The catalytic subunit of the herpesvirus DNA polymerase is a pivotal

676   enzyme responsible for genome replication, and thus, for successful transmission

677   of genetic information from one generation to the next [4, 7, 27].

678   IE62 protein is the most important Trans activator for VZV DNA polymerase.

679   Some studies have suggested that the level of expression of IE62 protein inversely

680   correlates with virulence. However, more recent data suggest that IE62 protein

681   expression levels are not critical for pathogenicity [28]. It remains to be seen if

682   the IE62 protein of VZV would have the capacity to induce such signals and

confer virulent or attenuated phenotype. IF test showed serial passages at low temperature associated with decreasing IE62 expression in high passage of the local isolated VZV, in contrast low as wild isolated virus. Thus, as other investigations on vOka have suggested one of the characteristic signs of VZV attenuation can be reduction of IE62 function, it was investigated by other researchers [29].

The virus has had more than 30 passages on both cells in parallel. Virus stocks were prepared by 30 additional passages of the primary isolate on human cells (MRC-5, RFSC) for each 5 passages interval. The purpose of this phase was to induce in vitro adaptation of viral isolate at lower temperatures than what would occur in the body during natural infection. Due to successive passages, not only has the virus become accustomed to growing at lower temperatures, but the result has seen an increase in the titer of the virus in higher passages than in lower ones. Remember that different amounts of defective interfering particles in the harvested viral fluid may have affected the replication kinetics of viruses. The result of such an adaptation in many articles as a method that reduces virulence and produced attenuated virus vaccine strains has been reported [4, 30].

To study the cytopathogenicity of human cells to local VZV virus, MRC-5, RFSC, GPFE and Vero cells were infected at MOI: 0.1. Nevertheless, the marked CPEs were evident 48 hpi for MRC-5 cells infected at targeted MOI, which increased with time. At 72 hpi, approximately half of the infected cells appeared smaller and irregularly shaped compared to mock cells (Fig 4). Infection of MRC-5, RFSC, and also GPFE cells by local VZV virus caused a fast CPE and a rapid fall in the pH of cell in culture. The granular and fragmented cells became obvious within 48 to 72 hpi. In contrast, infected Vero cells showed the rate increased slightly from 24 to 48 hpi. Note that higher viral titers were observed in infected MRC-5 cells, and the peak of titer reached 124 hpi. Thus MRC-5 indicates more sensitivity for VZV proliferation. This is important in terms of optimizing the best time for cell passages and virus inoculation. The isolated virus passaged

712   more than 25 times in MRC-5 cell strain, regarding reduction in virulence. Also,

713   subsequent experiments were carried out with (P: 5) as the parent isolate (P: 5)

714   that had been passaged fifth on MRC-5 and (P: 30) that we hope was attenuated.

715   The results mainly showed a significant difference between primary characterized

716   isolate (P: 5) and the current passaged virus (P: 30) virus in term of potency and

717   RT-PCR.

718   One feature of the serial sub culturing in human diploid fibroblast that differed

719   from the others was the adaptation of the strain to growth at 300C which we

720   included in our procedure. The idea behind this was to rapidly induce attenuation,

721   on the basis of previous experience with attenuated measles, mumps and rubella

722   strains [11, 31-33]. By following the work of others showing that replication at

723   low temperatures selected against virulence. By the 27th passage in a human

724   diploid cell strain, we expected local VZV had clearly become attenuated for

725   humans.

726   In the follow up by potency testing in CCID50, it was found that the infectivity

727   titer of the isolated VZV became higher as the passaging in cell substrate

728   progressed. This feature was clearly confirmed in the RT-PCR test results (Fig

729   9). In other words, we found a correlation between virus levels determined by

730   CCID50 /mL and mRNA levels determined by qPCR. The differences between

731   primary characterized isolate (P: 4) virus mainly showed significant in results of

732   potency and RT-PCR of the high passaged virus (P: 27). Consistently, viruses or

733   RNA could be detected in inoculated cell culture in-vitro and laboratory animals

734   in vivo. Similarly, in the present article the virus was isolated from infectious

735   material such as vesicular swab and throat, especially in 3-4 first days of infection

736   from VZV patient case. We could determine the virus titer in the infectious

737   material (blood serum) of the patient by endpoint dilution. The quantitative PCR

738   data suggest that the sample used for detection of patient was enough containing

739   approximately 10 TCID50/ml of infectious virus. These results are in agreement

740   with results from plaque assay, which showed that the number of plaques

741   increased. Since decreasing the virus growth temperature is one of the factors

742   influencing the reduction of the virulence, we have accustomed the isolated VZV,

743   to growth at 300C. We expected that, increasing the virus titre at a temperature

744   lower than normal in human body (300C) should result in phenotypic changes,

745   which would be easily observable in cell culture. Fortunately, these results were

746   confirmed by three different quantitative tests. Increasing the virus titre

747   determined by CCID50 /between higher (27th) and lower passage (4th) and

748   increasing the Ct in FAM-q PCR related to ORF 62. Although the above results

749   show that the increase in passage has led to an increase in virus titre, the size of

750   the plaque has not been affected. The plaque size between different passages

751   resulted serial passaging of viruses has not altered phenotype of plaque in local

752   isolated VZV viruses. Although at a glance the plaque produced in the 27th

753   passages of virus were larger, and plaques formed by the 4th passages of virus

754   were smaller in size, the size distribution of relative plaque diameter normalized

755   at 27th against the average plaque diameter of the parental (4th) virus. In

756   calculating the test the P-values resulted $P<0.05$. This marker characteristic was

757   applied by many of other researchers regarding the attenuation of different

758   isolated virus [11, 30].

759   As prior in vivo study of the virus proved to be necessary to further study the

760   characteristics of the virus, we studied on the local isolate VZV by PCR-RFLP,

761   after more than 25 passages. The restriction fragment length polymorphism

762   analysis (RFLP) on DNA is recommended as one of the most valuable molecular

763   techniques that can distinguish the complete analysis between different VZV

764   strains [8, 34, and 35]. The isolated strain in tropical regions such as Africa,

765   Bangladesh, China, India, Central America and northern Australia also reported

766   as BglI+ strains. The result of many studies also reported RFLP profiles of wild

767   type VZV strains in Japanese isolates as, PstI+, BglI+ or PstI-, BglI+ as in US,

768   UK, Europe PstI+, BglI+ or PstI-, BglI+, meanwhile in eastern Australia result of

769   varicella RFLP-PCR showed PstI+, BglI- .Using this method, RFLP profile of

770   isolated varicella in this article ,after more than 20passages showed PstI⁻, BglI⁺

771   (PstI site and BglI site in many Asian strains) [36-39].

772 Our findings are in agreement with previous studies. RFLP-PCR of the local
773 isolate proved to be highly identical at nucleotide level to the other isolates from
774 Asia for which the complete genome sequences are available and published. The
775 identity between this isolate and others belonging to Asia, Europe, African and
776 other region isolates is necessary to repeat the experiment and for accurate
777 analysis [39, 40].The main limitation of this article was the lack of complete
778 sequence result of from local isolate presented in this article and other isolates
779 from different provinces in the country. If we could examine other isolations from
780 other provinces in terms of complete sequence, we could be more certain about
781 the isolation of the country's circulation. These limitations were due to financial
782 and logistical reasons. These processes by excess financial support is under
783 second repeat for isolated virus in this article. Phylogenetic analysis of viral
784 particle has been largely based on the complete genome sequence [41]. At the
785 time of analysis there were some financial limitations to repeat the process for
786 accurate analysis but the problem solved right now the result is under work.
787 Further studies are required to clarify the biological relationship between the
788 differences observed between local isolated in the present paper and its evaluation
789 with various other VZV isolates.

790 In our studies we did not see differences in antibody development between the
791 different routes of inoculation. In skin test we could not detect **"redness and
792 inflammations"** side effect of replicating virus at the site of inoculation early after
793 infection via ID. There was no association between development of seropositivity
794 and route of inoculation. In animals inoculated with $10^{2.7}$ $CCID_{50}$/dose of isolated
795 virus at 5th passages no specific antibody response was detected. In order to assess
796 whether the inoculated virus was sufficient to induce antibody response, in the
797 skin test with positive control virus injection, the safety status of animal models
798 receiving the high dose virus was tested with the result obtained indicating that it
799 was stimulated [42, 43,44]. Comparison of in vitro and in vivo characteristics and
800 virulence after adaptation to growth in $30^{0}C$ and more than 30 sub passaging with
801 those of vOKa vaccine (commercial vaccine) showed clear similarities with

802   vOKa –VZV strain [13]. In contrast, low passages (p5), act as non-attenuated and

803   exhibited a different tropism for cultured cells, different antibody development,

804   and nearly 100% different from its daughter at 30th passages. Overall, all animals

805   exhibited signs of progression developing neutralizing antibodies by the three

806   different doses of high passages isolated viruses. Extensive development of the

807   antibody in guinea pigs by VZV correlated with the characteristic signs of

808   attenuation. Given the differential antibody development patterns of the three

809   high passages isolated virus with these viruses in low passage, it would be

810   interesting to compare the receptor usage of the parent low passages as wild with

811   high passages daughter as attenuated. These findings as compared with positive

812   control suggest that in immunogenicity, the three high passages viruses are most

813   likely similar to vOka as attenuated commercial vaccine [13]. Our findings are in

814   agreement with previous studies suggesting the used method serial passages and

815   following results.

816   Both the in vitro and in vivo systems for isolated propagation described in the

817   present study will be valuable tools to further elucidate the isolation and

818   adaptation, as well as the process for developing a candidate for research.

819

Materials and Methods

820

821

Sample collection

822

823   The samples contained skin lesions belonging to an 8-year-old boy. The swab

824   sample was received in viral transmission medium (VTM) which contained

825   sufficient amounts of antibiotics.

826

Sample screening by PCR

827

31

828 To confirm the existence of the varicella and its differentiation from other herpes

829 virus, a pair of primers was designed along with positive control. It was used to

830 test the samples and differentiate it from other Herpes viruses in the sample [, 45,

831 46, 47].

832

Virus isolation and attenuation by monitoring cell infection

834 Four different cell lines were used for isolation of varicella virus in tissue culture:

835 a) African green monkey cell line (Vero) prepared in tissue culture tube,

836 measuring the potency of isolated virus b) human diploid cells (MRC-5) prepared

837 in tissue culture flasks (25cm2), c) local human foreskin cell line (RFSC), d)

838 Primary Guinea Pig Embryo fibroblast cell culture (GPEFC) grown in tissue

839 culture flasks (25cm2). The growth medium for all cultures was DMEM

840 containing 5-8% of in house irradiated bovine calf serum. All of cultures tested

841 for sterility and mycoplasma and were negative. The cell sheets were washed

842 once with PBS before inoculation. The specimen was inoculated as 0.5 ml into

843 each cell line. The maintenance media was composed of DMEM with 1-2% of in

844 bovine albumin serum added after 2h adsorption at room temperature. The

845 cultures were incubated at $300^{o}C$ in stationary phase. The isolation methods were

846 designed based on our previous experiment and World Health Organization

847 WHO requirement (Two days' post inoculation the cultures were examined

848 microscopically to monitor the virus growth. The culture media changed with

849 fresh ones every 2 days. Once the virus cytopathic effect spread on the culture,

850 the media were harvested and stored at -600C for the next step. Serial sub-

851 cultivation was carried out and continued more than 25 times to reduce virus

852 virulence and adapted in growth at 30 0C.

853
854

855 **Viral growth kinetics, specific virus production rate, and overall virus**

856 **productivity at $30^{0}C$ by CCID $_{50}$ method**

857 Specific growth characteristics of MRC-5 cell and the yield of the isolated VZV

858 virus in this cell strain were captured as follows. Tripsinated 4×175 Cm2 flask

859 of MRC-5 and counting the cells. Inoculated with MOI: 0.1 from local isolated

860 VZV virus. The culture flask was stirred at room temperature for 15 minutes at

861 20 rpm, after which a sufficient amount of DMEM containing 5% FCS + 50μg

862 Gentamycin was added as culture medium. This mixture was divided as 2×10^6

863 cell / 25cm^2 flasks (80000 cell / cm2) and incubated at 36 ^{0} C. After 48 h, the

864 culture medium was replaced with a serum-free medium. The flasks were

865 incubated at 32-33 ^{0}C. At 24-hour intervals, three flasks were removed from the

866 incubator and sampled from the supernatant to determine the titer of the virus.

867 Then, the flask was Tripsinated and the cells counted the rate of virus production

868 was calculated. The specific rate of virus production in the cell culture was

869 determined by the same method.

870 The higher the growth rate, the more cells it can produce over a period of time;

871 thus, the faster the product can be produced and the more efficient it can be.

872 The culture fluid was harvested from cell line daily for 240 hours. To assess the

873 comparative adaptation and replication number of viral particles in the cell line,

874 potency assay was performed The 0.1 ml of culture fluid, inoculated in 10-fold

875 dilutions (10^{-1} - 10^{-9}) on Vero cell substrate cultured in tube. All dilutions were

876 done in 4 repeats. The viral growth and its cytopathic effect were investigated for

877 12–14 days using an inverted microscope. The potency of virus was calculated as

878 $CCID_{50}$/mL. This method was done simultaneously for three times in parallel for

879 both passages (4th and 27th) in 300c (Fig 2). The commercial (**v**OKa) vaccine

880 strain was used as a positive control.

881

882 **Plaque assay for purification of isolated virus**

883 Briefly, MRC-5 (1×10^5) seeded in a well of a 24-well plate was infected with 100

884 PFU of local isolated virus. The entire inoculation was performed in duplicate.

885 Cells were frozen and thawed 2, 3, 4, 5, 6 and 7 days post infection with 10-fold

886 serial dilutions inoculated onto fresh MRC-5 and Vero. Plaques were stained and

887   counted six days post infection. The plaque on MRC-5 from the highest dilution

888   of the isolated virus was used for cloning. To analyze cell-to-cell spread of

889   viruses' plaque areas were determined. Briefly, the fluid harvested from MRC-5

890   containing 50 PFU was mixed with Vero ($1\times10^{5/ml}$) and seeded into a well of a

891   24-well plate. After 6 days, plaques were visualized via indirect

892   immunofluorescence (see below). For each virus, images of 50 randomly selected

893   plaques were taken at 100-fold magnification. Plaque areas were measured using

894   image software from which, assuming that ideal plaques would have a circular

895   shape. Plaque diameters were calculated, plotted, and analyzed using an

896   electronic counter. Plaque sizes were determined in three independent biological

897   repeats.

898

899   **Virus characterization by RFLP-PCR**

900   To confirm the presence of targeted virus and evaluate the changes in the virus

901   genome due to intermittent passage, the harvested fluid was assayed by RFLP-

902   PCR using a specific primer designed for ORF 38 and ORF 54 of varicella-zoster

903   virus (Table 3). The infected cell cultures were removed using a rubber policeman

904   72 h post-inoculation and pooled in 1.5 mL micro tube. The suspension culture

905   fluid was centrifuged at 8000 rpm for 1 min. Total viral DNA was extracted using

906   Qiagen viral DNA extraction kit (QIAamp DNA Mini Kit, Hilden, Germany) and

907   DNA polymerase Tag (Takara) according to the manufacturer's instructions. The

908   thermal cycle and appropriate primer sequence are mentioned in Table 3. The

909   amplified fragments were electrophoresed on 2.0% Gel agarose and visualized

910   after staining with safe stain. A panel of two restriction enzymes, PstI and BglI,

911   conducted RFLP. The RFLP reaction mixture was performed in a 32 μL

912   containing 15 μL PCR product, 6 μL buffer, 6 μL deionized water, and 2.5 μl of

913   each BglI (Thermo scientific, Massachusetts, U.S.A.) as well as PstI (Thermo

914   scientific). The mixture was incubated for 16 h in a 37°C water bath. The RFLP

915   product was electrophoresed on a 4% agarose gel. The commercial vaccine

916 (vOKa) was used as a positive control. The size of digestion fragment for various

917 types of varicella is presented in Fig 6.

918

SDS-PAGE and Western blotting analysis

920 Once SDS-PAGE was performed western blotting to detect IE62 proteins of

921 VZV, the proteins were transferred onto a PVDF membrane. The membrane was

922 blocked at room temperature with a blocking reagent (Tris-buffered saline, TBS,

923 containing 5% nonfat milk) for 2 h. It was then incubated for 1 h at 4°C with IE62

924 antibody (Santa Cruz Biotechnology.Inc; sc-17525) and washed three times with

925 TBS containing 0.05% (v/v) Tween-20. The membrane was exposed to HRP-

926 conjugated anti-goat IgG (Santa Cruz Biotechnology. Inc; sc-2020) second

927 antibody for 1 h, washed four times with gentle shaking, and visualized by DAB

928 (3,3'Diaminobenzidine Tetrahydrochloride: Santa Cruz Biotechnology. Inc; sc-

929 209686) staining [46].

930

931

Indirect immunofluorescence assays for cell infection monitoring

933 Monolayers of Vero cells (10^5 cells per well) in Lab-Tec tissue culture chamber

934 slides (Miles Scientific, Naperville, IL.) were infected with three different

935 passages of VZV local isolate and vOKa separately at a concentration of 30 to 50

936 particles per well of virus. After 72 h, when CPE became visible, cells were

937 washed twice with PBS. The cells were fixed with methanol 100% for 30 min at

938 room temperature, 100 ul of diluted antiserum (1:100 in PBS) per well was added,

939 with the chamber slides incubated for 4 h at 37°C in a humid atmosphere. Cells

940 were rinsed extensively with PBS and incubated for 1 h at 37°C with 100μl of

941 diluted FITC-conjugated monoclonal antibody (1:700 in PBS). After being

942 washed with PBS, the slides were examined with a fluorescence microscope

943 equipped with a 25X/0.75 PL-Fluotar objective and a filter for light with a

944 wavelength of 450 to 490 nm. Photographs were taken by Motic 2828.

Quantitative PCR for viral load between different passages

The test was performed by designing a primer set for VZV ORF62 and a probe (FAM) using the ABI Prism 7700. Total cellular DNA was extracted from cell culture harvested fluids using a specific Kit (Genoproof; Cat No ISIN/025 Kit, Czech Republic) containing a protected specific sequence for each Herpes virus such as ORF62 for VZV. The kit generated by an internal control for each virus. The viral gene from infected cells at different passages (4th, 9th, 17th, and 27th) was analyzed via quantitative RT-PCR. The quantity of dye FAM released during amplification was calculated as the values of real time fluorescence. In each reaction, the threshold cycle (Ct) is the value showing the cycle number in which the fluorescence exceeds the threshold [9, 45]. Reactions were repeated three times for each sample, and standard deviations were calculated.

In vivo testing of local VZV in animal model

The antibody development upon subcutaneous (SC) and intradermal inoculation (ID) was tested regarding high and low passages of local isolated VZV, in 350-450-gram guinea pigs as laboratory animal. There were three guinea pigs in each group. The group were named as: A, B, and D and E injected by four doses, from 30th passages of local isolated viruses. The F group received$10^{3.0}$ $CCID_{50}$ /dose from 5th passages as parent virus. The C^+ group injected by$10^{3.0}$ $CCID_{50}$ /dose of vOka vaccine as a standard positive control. The C^- as negative control group received 0.5 mL (1 dose) of the water for injection. The blood samples were taken from animal hearts at four stages. Zero-day was pre-inoculation of the virus, involving the first blood sampling. The other four stages of blood sampling were taken at certain time intervals.

Sample screening by ELISA

Antibody measurement in the blood serum samples was assayed using standard ELISA test [45, 46]. EUROIMMUN anti-VZV virus ELISA Kit was used to

974 measure antibody titer against varicella virus. The procedure was performed

975 based on manufacturer's instructions (Euro immune Elisa Kit) for IgG and IgM.

976

Skin test

978 To evaluate the stimulation of cellular immunity due to injection of isolated VZV

979 virus, positive control virus suspension was used for injection of ID with a

980 volume of 0.1 mL, in A, B, D, and E groups. This test was performed through

981 injection in the gluteal region. The injection site was then inspected for 24 hours

982 to a week. The area was marked.

983

Acknowledgments

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1126   **S5: Fig 5. Plaques formed by the viruses that were passaged in cell culture (Scale bar,**
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Supporting information

Title manuscript: A new isolated local varicella Virus: Isolation, Identification, Comparative growth characteristics and immunological evaluation on animal model

List of Figures captions
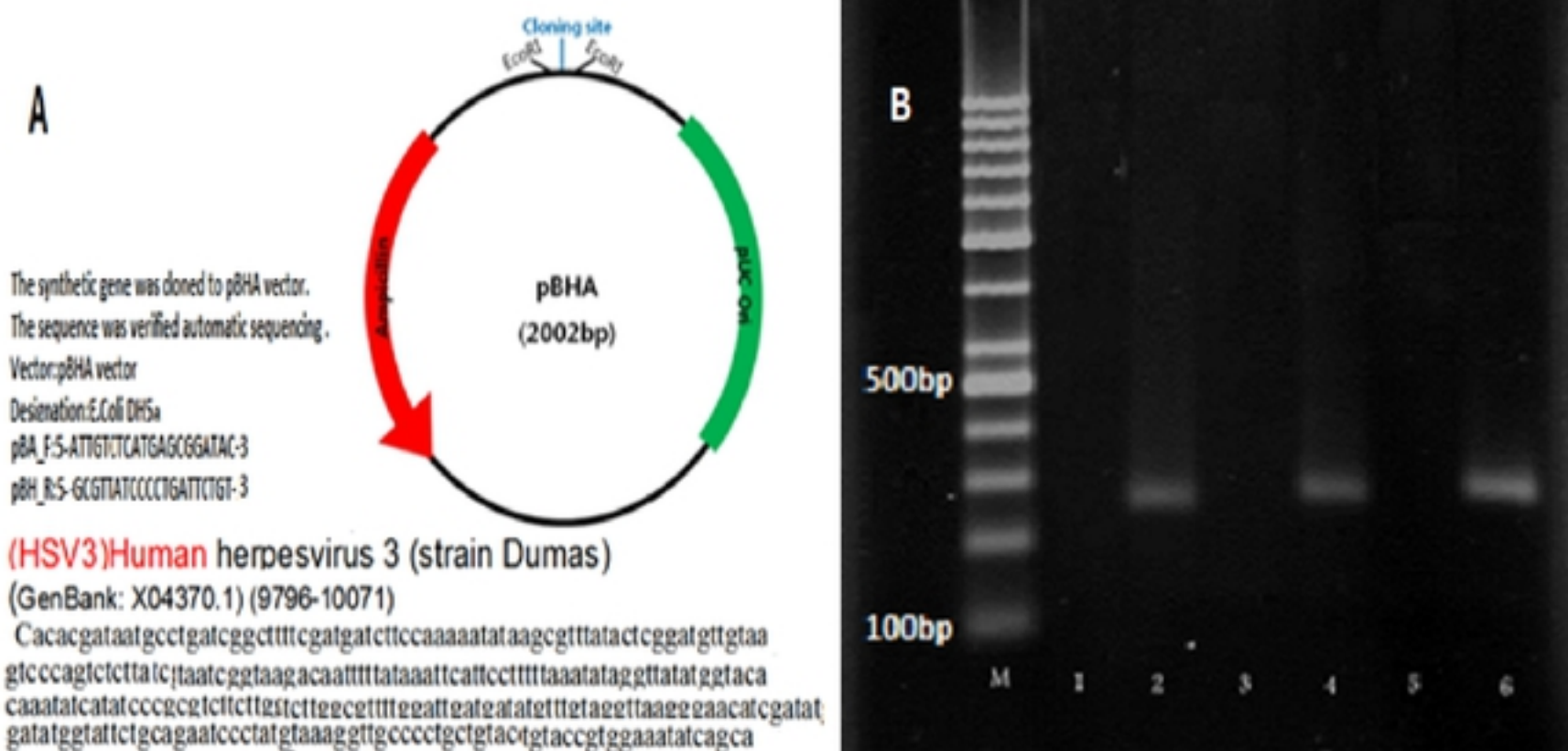
S1: Fig 1. Differentiation of local VZV DNA by specific-PCR. A, pBHA vector; B, Analysis PCR amplification: M lane, Ladder; lane 2, PCR products from control positive (DUMAS STRAIN: 9796-10071) VZV DNA in dilution 1/10; lane 4, PCR products from local VZV DNA in dilution 1/10; lane 6, PCR products from local VZV DNA in dilution 1/100.
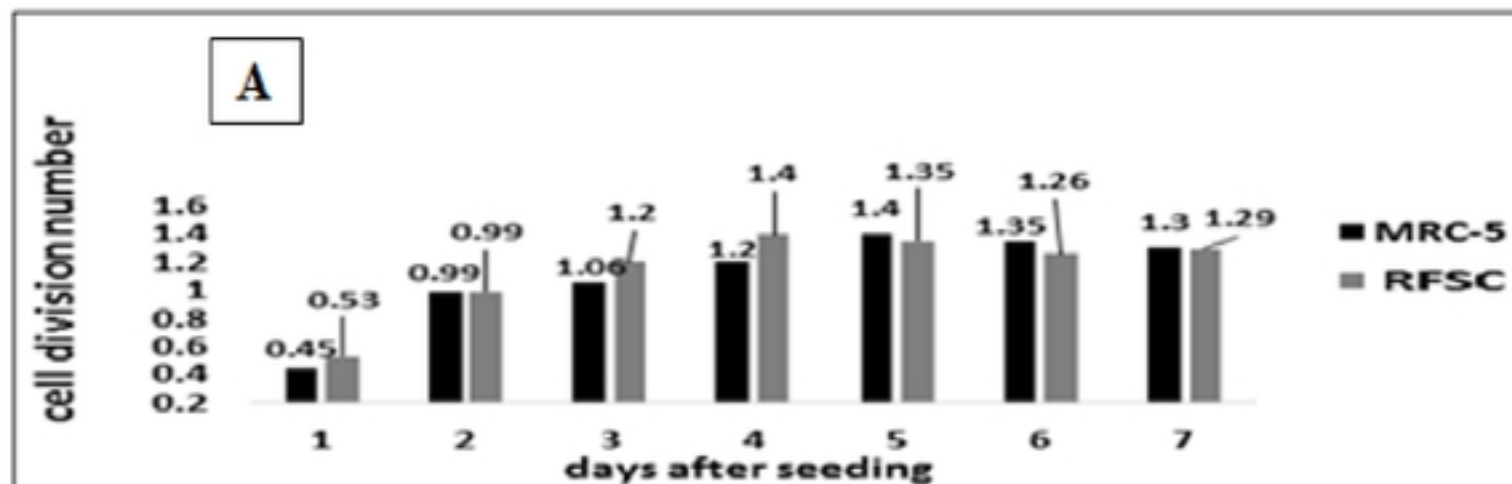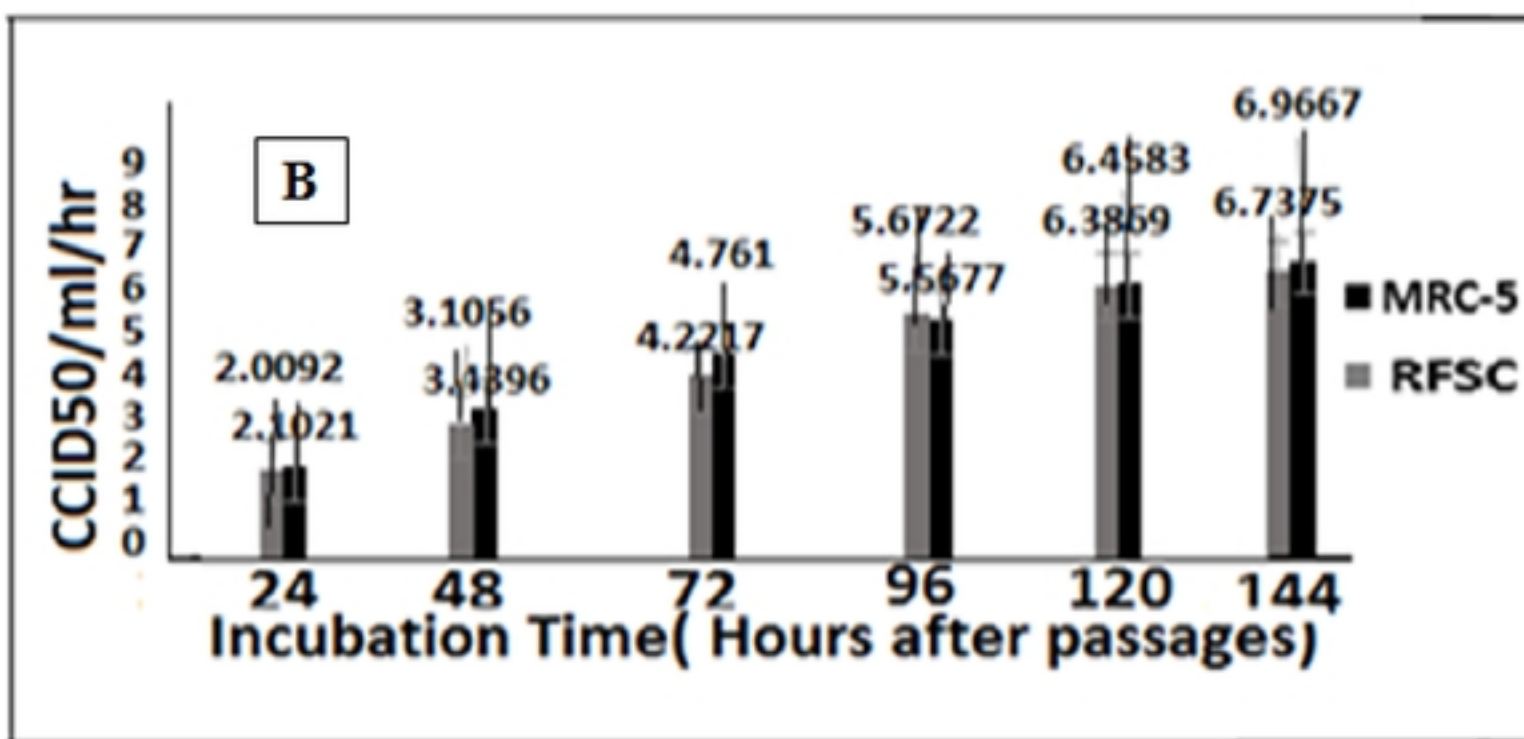
S2: Fig 2. VZV on human diploid lung and human foreskin cells. (A) Comparative cell division number between two human cell substrate during seven days after seeding. MRC-5 as standard human diploid cell strain and RFSC as a continuous local human foreskin cell substrate: The local RFSC at passage 65 and MRC-5 at passages 26. (B) Replication characteristics of local VZV isolate in human diploid lung (MRC-5) and human foreskin (RFSC) cells: Two human cell lines were inoculated with the local VZV and its CPE assessed by $CCID_{50}$ method. Replication kinetics of isolated VZV were compared with RFSC as an alternative cell substrate for future research and production.

S3: Fig 3. Replication and potency assay of Local isolated VZV virus during different passages on MRC-5. (a) Development of VZV virus inoculated on MRC-5 cell substrate. $CCID_{50}$/mL of VZV virus titers were measured by potency tests as described in the text. The values of, higher and lower level of virus titer was assessed from; p27. (8.75-9.40 $LogCCID_{50}$) and P4. (2.75-3.40 $LogCCID_{50}$). This level for Control positive was (vOKa3.7-4.3$LogCCID_{50}$) after 9 days' post inoculation. The virus at P.27 and P17 (6.5-7.4 $LogCCID_{50}$) was significant in compare of P9. (3.40-4.5 $LogCCID_{50}$) and P4. (2.75-3.40 $LogCCID_{50}$). Increasing the particles number to 6 and 5$LogCCID_{50}$ for p 27 and P17 was linear to level of virus in RT-PCR but was significant. (b) Replication and potency assay of local isolated VZV during different passages of VZV on local cell substrate (RFSC). After successive serial passages, the virus has become adapted to growing in new cell substrate. This procedure increases the proliferation and number of virus particle. The virus titer has increased by about 6 log $CCID_{50}$/ml in high passages (p: 27), compare to low passages (p: 4) and produces more particle in potency testing at hours post inoculations daily and passages The titer of the virus was measured using Karber method .
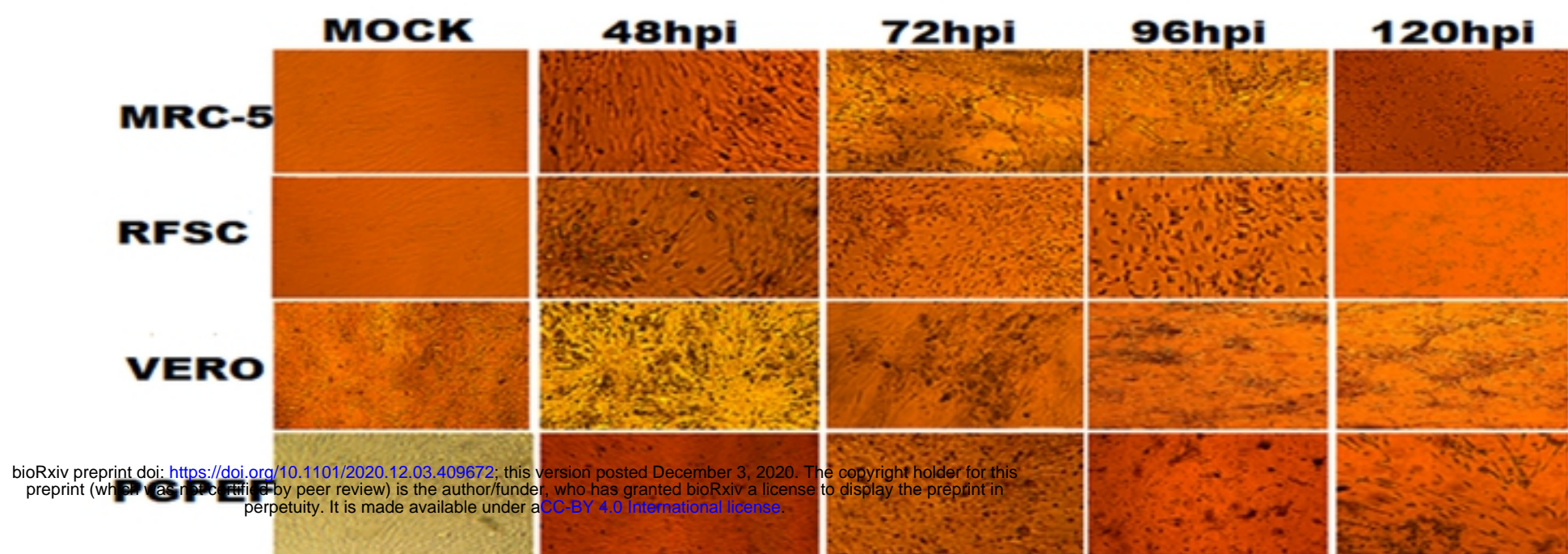
S4: Fig 4. The local VZV cytopathogenicity. Human lung diploid cell (MRC-5) and human foreskin fibroblast (RFSC), Vero, and primary guinea pig fibroblast cells (PGPFC) to local VZV virus infection at 48 and 72and 96 and 120 hours after infection (100x magnification). The virus inoculate as MOI: 0.1 and was in 17th passage .

S5: Fig 5. Plaques formed by the viruses that were passaged in cell culture (Scale bar, 200 μ). (a) Representative Images of plaques formed by viruses that were passaged 5 times serially at low multiplicity of infection in cell culture. (b) Representative Images of plaques formed by viruses that were passaged 17 times serially at low multiplicity of infection in cell culture. (c) Representative Images of plaques formed by viruses that were passaged 27 times serially at low multiplicity of infection in cell culture. Serial passaging of viruses has not altered phenotype of plaque of viruses. Although the plaque produced in the 27th passages of virus were larger, and plaques formed by the 4th passages of virus were smaller. But the size distribution of relative plaque diameter normalized against the average plaque diameter of the parental virus. P-values were calculated using multiple comparison test, indicates $P<0.05$.

S6: Fig 6. Results of electrophoresis following RFLP test on PCR products of high passage local VZV DNA by RFLP-PCR. (A) PCR amplification of ORF 34 and ORF58, contain 222-bp and 350-bp fragmentlane1, ladder; lane 2and 3, PCR products from vOKa as reference positive ,350-bp and 222-bp; lane 4and5, PCR products from isolated varicella, 350-bp and 222-bp. (B) RFLP on PCR product to analysis the fragment size differentiates wild from attenuated isolated; lane1 and 2 ladder; lane 3, vOKa profile after digestion by PstI (350-bp and 222-bp); lane 4, vOKa profile after digestion by BglI as reference positive (85,100,137bp fragment size); lane 5, digested products from local isolated VZV by BglI (85,100,137bp fragment size); lane 6, digested products from isolated varicella (222 and 350-bp fragment). (C) Schematic RFLP fragment size BglI and PstI.

S7:

Fig 7. Comparative SDS-PAGE and Western blotting results between isolated VZV and

and control positive; Lane 1, 175 KDa band of IE62 protein in the local VZV; Lane 2, Protein Prestain marker; Lane 3, 175 KDa band of IE62 protein in positive control. (B) Western blotting of isolated VZV and control positive; Lane 1, 175 KDa band of IE62 protein confirmed with specific antibody in the local VZV; Lane 2, Protein Prestain marker; Lane 3, 175 KDa band IE62 protein confirmed with specific antibody in positive control. This result confirmed identification of IE62-specific protein as the most important viral transcription activator of VZV gene between isolated VZV and vOKa as control positive. Its presence was examined using labeled specific antibodies, in the Western blot experiment with color band observation. Binding of the labeled secondary antibody was associated with the anti-C-terminal monoclonal antibody near the C-terminal IE62 protein, along with the identification of positive control.

S8: Fig 8. The local VZV in IF test after serial passages. A, the 5th passage of local VZV; B, the 30th passage of local VZV; C, control positive virus; D, the uninfected cell.
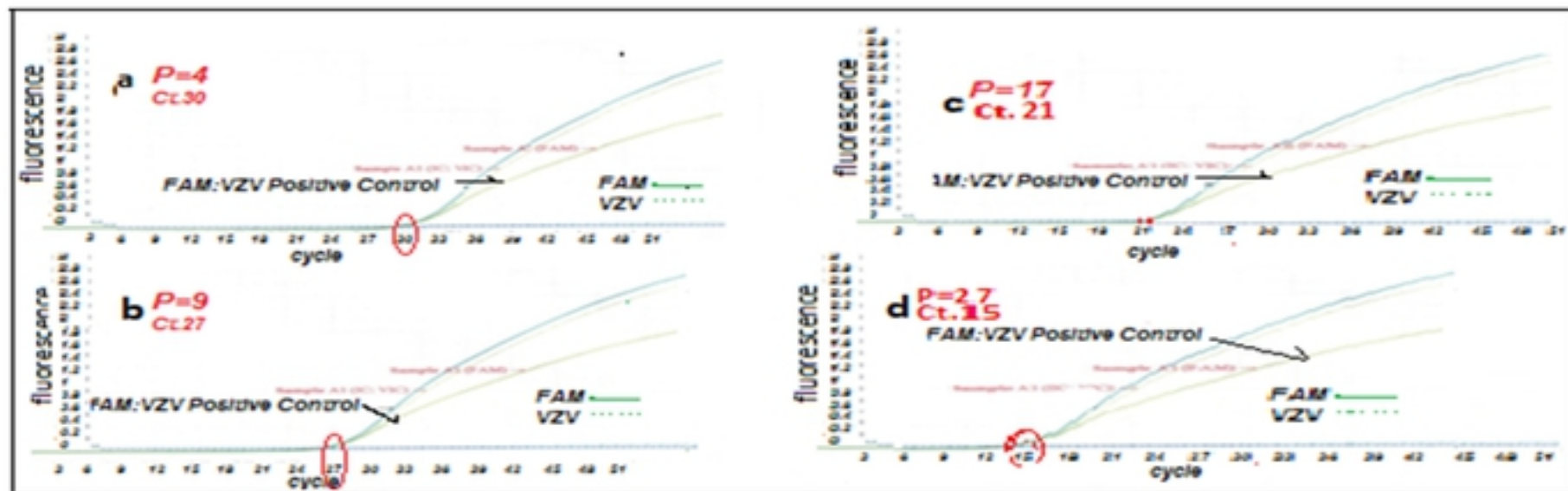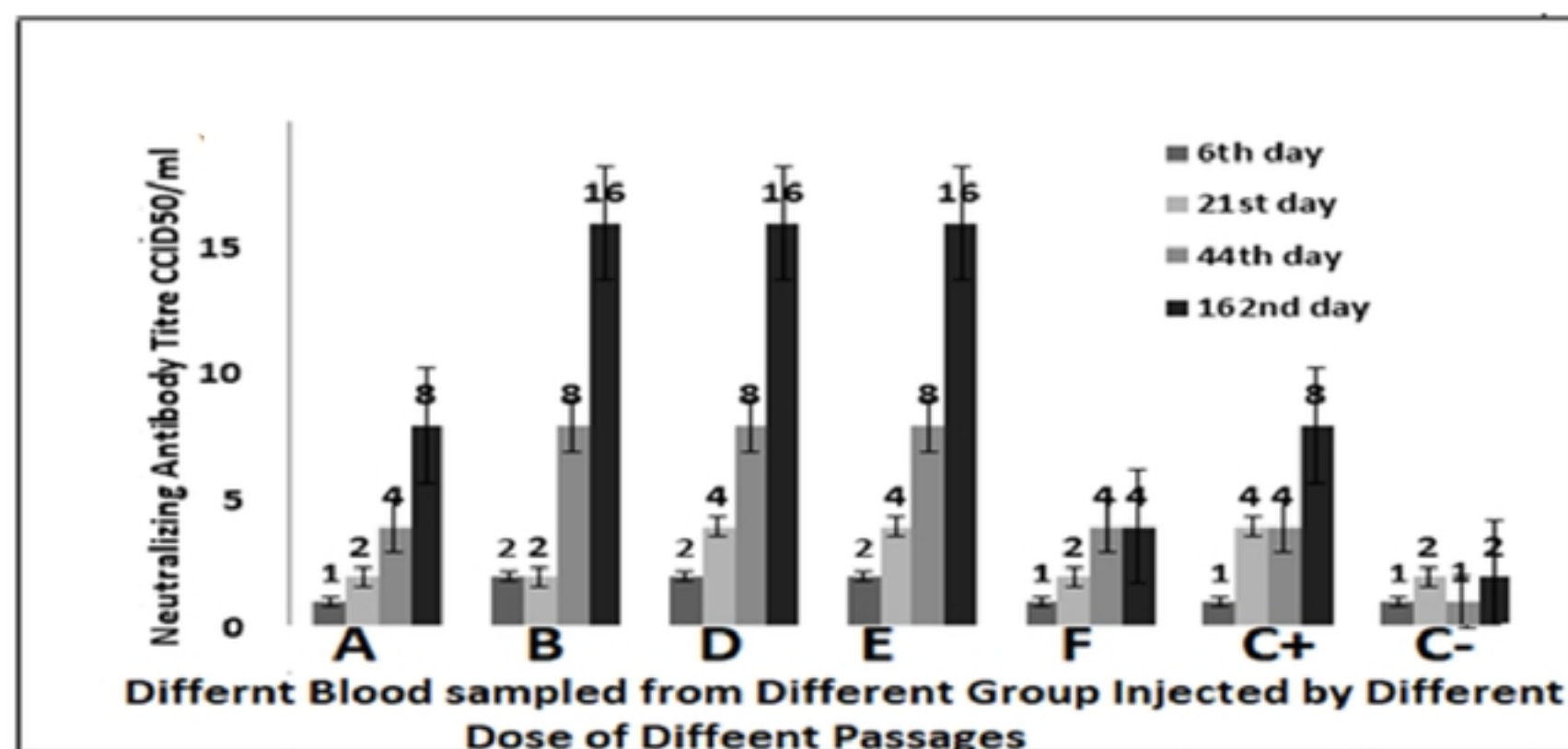
S9: Fig 9. Viral particles in current VZV isolate from Iran by RT-PCR on MRC-5 (72hpi). (a) Isolated VZV-infected MRC-5 cells P.4. (b) Isolated VZV-infected MRC-5 cells P.9. (c) Isolated-infected MRC-5 cells in P.17. (d) Isolated-infected MRC-5 cells P.27.
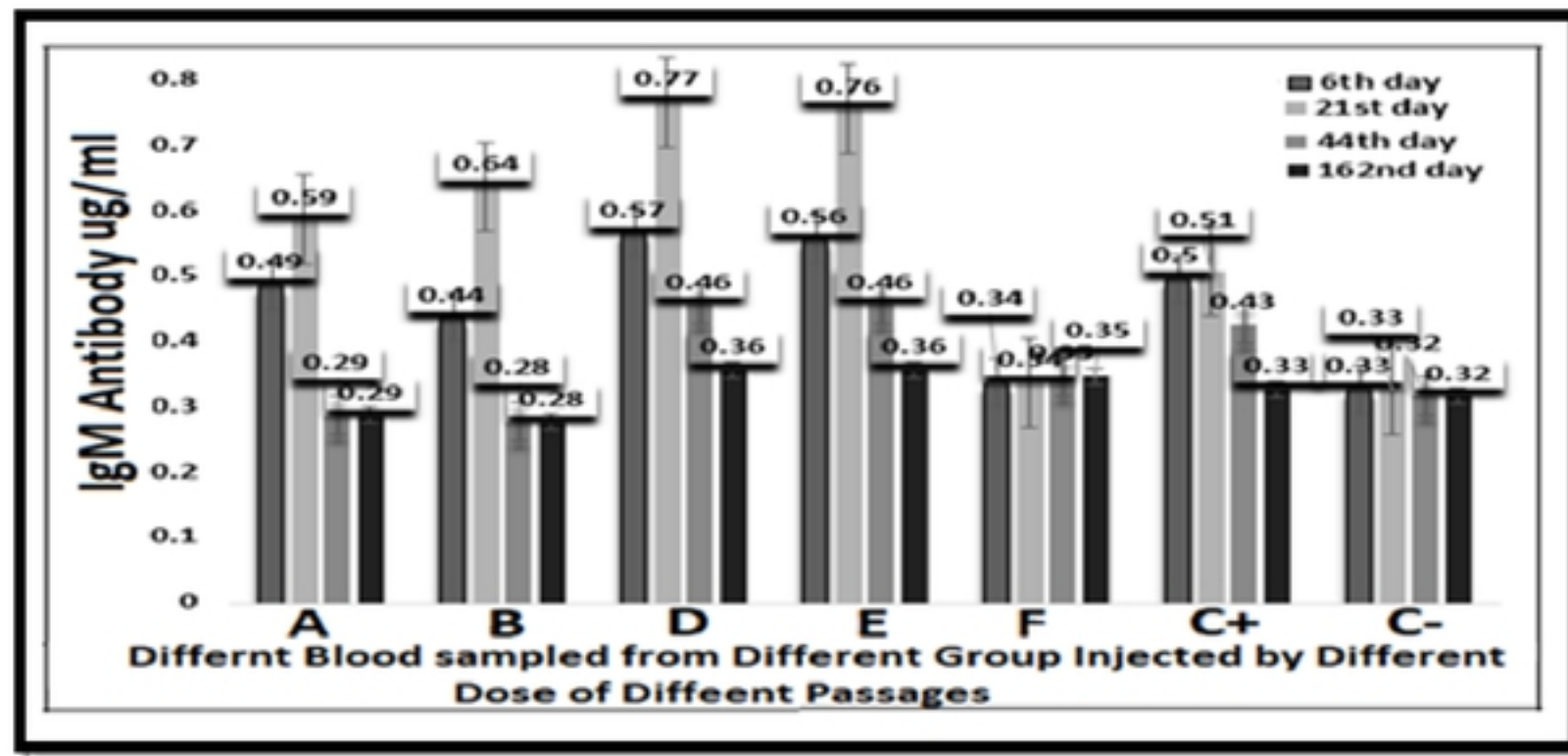
S10: Fig 10. Development of local VZV-specific IgM over time in (350-405 gr) Guinea Pig. Guinea Pig were inoculated ID or SC with (A) $10^{5.0}$CCID$_{50}$, (B) 104.3 CCID$_{50}$, (D) $10^{3.3}$ CCID$_{50}$ and (E) $10^{3.0}$ CCID$_{50}$ of high (30th) passages isolated VZV virus , (F)10^3 CCID$_{50}$ of low passages (5th) and (C+) $10^{3.3}$ CCID50 of vOka vaccine as control. The lines indicate threshold level of the assay (P>0.05).
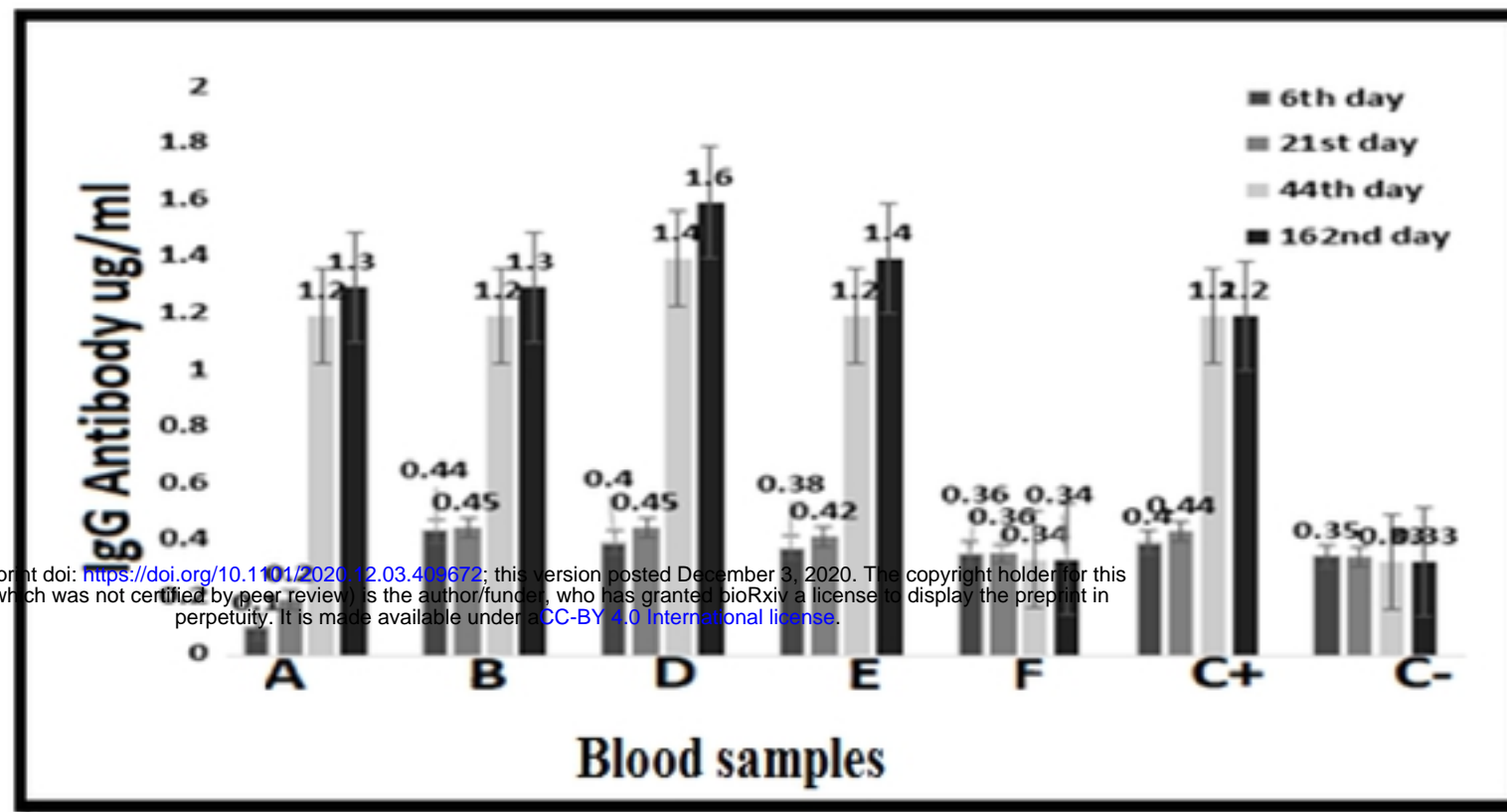
S11: Fig 11. Development of local VZV-specific IgG over time in Guinea Pig(350-405 gr). Guinea Pig were inoculated ID or SC with (A) $10^{5.0}CCID_{50}$, (B) $104.3\ CCID_{50}$, (D) $10^{3.3}\ CCID_{50}$ and (E) $10^{3.0}\ CCID_{50}$ of 30th) passages isolated VZV virus, (F)$10^3\ CCID_{50}$ of 5th passage and (C+) $10^{3.3}\ CCID50$ of vOka vaccine as control. The lines indicate threshold level of the assay (P>0.05).

S12: Fig 12. Development of Local VZV-specific neutralizing antibodies over time in (350-405 gr) Guinea Pig. Guinea Pig were inoculated ID or with (A) $10^{5.0}CCID_{50}$, (B) $10^{4.3}\ CCID_{50}$, (D) $10^{3.3}\ CCID_{50}$ and (E) $10^{3.0}\ CCID_{50}$ of high (30th) passages isolated VZV virus, (F)$10^3\ CCID_{50}$ of low passages (5th) and (C$^+$) $10^{3.3}\ CCID_{50}$ of vOka vaccine as control. The lines indicate threshold level of the assay.

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