Vaccine 27 (2009) 5332–5337

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Effects of hydrostatic pressure on the stability and thermostability of poliovirus: A new method for vaccine preservation

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ARTICLE INFO

Article history:
Received 19 October 2008
Received in revised form 8 June 2009
Accepted 29 June 2009
Available online 17 July 2009

Keywords:
Poliovirus
Hydrostatic pressure
Vaccine stability

ABSTRACT

Viruses are a structurally diverse group of infectious agents that differ widely in their sensitivities to high hydrostatic pressure (HHP). Studies on picornaviruses have demonstrated that these viruses are extremely resistant to HHP treatments, with poliovirus appearing to be the most resistant. Here, the three attenuated poliovirus serotypes were compared with regard to pressure and thermal resistance. We found that HHP does not inactivate any of the three serotypes studied (1–3). Rather, HHP treatment was found to stabilize poliovirus by increasing viral thermal resistance at 37 °C. Identification of new methods that stabilize poliovirus against heat inactivation would aid in the design of a more heat-stable vaccine, circumventing the problems associated with refrigeration during storage and transport of the vaccine prior to use.

1. Introduction

Poliovirus belongs to the *Picornavirus* genus of the *Picornaviridae* family and is known to cause poliomyelitis (polio), a highly infectious disease. The trivalent oral polio vaccine (OPV) is a live attenuated virus vaccine currently used in several countries for the prevention of poliomyelitis. The OPV is heat-labile, and thus, must be stored frozen and used immediately after thawing to ensure effective immunization against poliomyelitis.

Most live vaccines are temperature-sensitive, and the live attenuated polio vaccine is among the least stable of all common childhood vaccines. OPV is a liquid formulation composed of a single attenuated strain from each of three serotypes, which rapidly lose infectivity at room temperature [1–3]. Attempts to stabilize OPV have been strictly empirical, and only in the last decade have more rational approaches been introduced to tackle this problem. Previously tested stabilizers for attenuated polioviruses include sucrose, milk, gelatin, amino acids and magnesium chloride ($MgCl_2$) [4]. $MgCl_2$ is the major stabilizer used in commercial OPV vaccine [1,5–8]. Although other alternative methods have been tested, including stabilization of poliovirus with fatty acids [4], pirodavir [9] or heavy water (D_2O) [10], none have shown favorable results for commercial vaccine production.

Limited information is currently available concerning the potential of HHP methods to stabilize poliovirus. Most studies continue to focus on the use of HHP as a method for viral inactivation and vaccine production [11–19]. Previous studies on picornaviruses indicated that the inactivation profiles of these viruses change after hydrostatic pressure treatment. Eight picornaviruses have been evaluated for high pressure effects including foot-and-mouth disease virus (FMDV), hepatitis A virus (HAV), rhinovirus serotype 14, poliovirus type 1, coxsackievirus B5, coxsackievirus A9 (CAV9), human parechovirus type 1 (HPeV-1) and Aichi virus (AiV) [20–23]. The currently known sensitivity hierarchy is as follows: FMDV (6.0 log reductions at 240 MPa for 120 min), HAV (5.2 log reductions at 400 MPa for 5 min, inactivation at 460 MPa for 5 min), CAV9 (3.4 log reductions at 400 MPa for 5 min), HPeV-1 (1.3 log reductions at 400 MPa for 5 min), CBV5, AiV and PV-1 (essentially resistant to pressure treatments of ≥600 MPa) [23]. Poliovirus serotype 1 is one of the most HHP-resistant viruses [21,24–26]. However, the effect of HHP treatment on poliovirus serotypes 2 and 3 was not investigated prior to this study.

The aim of the present study was to compare pressure sensitivity and thermal resistance in three attenuated poliovirus serotypes. Our results demonstrated that live attenuated poliovirus (serotypes 1–3) preparations did not inactivate and became even more heat-resistant after HHP treatment without loosing titer under any condition tested. For the first time, we show that HHP stabilizes poliovirus against heat inactivation, strongly suggesting this treatment method as a potential vaccine stabilizer. The implications of

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doi:10.1016/j.vaccine.2009.06.099

these findings for vaccine storage and preservation are also discussed.

2. Materials and methods

2.1. Cells and virus

Hep2C and Vero (African green monkey kidney, CCL81) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Both cell types were grown and maintained in Medium 199 with Earle's salts (M199; Gibco, Invitrogen Co., CA, USA), buffered with sodium bicarbonate and supplemented with 5% fetal bovine serum (Gibco, Invitrogen Co., CA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma–Aldrich Co., MO, USA).

The three attenuated poliovirus serotypes initially used in this study [poliovirus type 1 (LSC, 2ab), poliovirus type 2 (P712 CH 2ab) and poliovirus type 3 (Leon 12 a 1b)] were obtained from GlaxoSmithKline Biologicals (GSK, Rixensart, Belgium) (vaccine batches SB 1003A, SB 238B and SB 359A, respectively). The commercial vaccine is grown in MRC-5 (human fetal lung fibroblast cells) and contains 90–100 mg/mL of $MgCl_2$ and approximately 10 mg/mL of L-arginine. Viruses in the absence of any thermostabilizers (L-arginine and $MgCl_2$) were obtained by culturing in Vero cells. Supernatants from infected cultures were cleared of cell debris by centrifugation at 1000 × *g* for 10 min.

2.2. Virus propagation

Working stocks were generated by passaging the three poliovirus serotypes five times in Vero cells. Briefly, confluent cell monolayers were inoculated at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell for each of three poliovirus serotypes in growth medium. Viruses were allowed to adsorb for 1 h at 37 °C, and monolayers were fed with 100 mL of maintenance medium in T-175 cell culture flasks. Each assay was performed a minimum of three times for reproducibility. Standard deviations were calculated for each condition analyzed.

2.3. Virus titration

An "in house," standardized multiwell plate assay was used to obtain virus infectivity titers by resuspending freshly trypsinized Hep2C cells (1.5×10^5 cells/mL) into 96-well plates. Subsequently, 100 μL of 10-fold serial viral dilutions were added to appropriate wells. Plates were incubated at 34 °C for 7 days at 5% CO_2. Virus titers were obtained by 50% Tissue Culture Infectious Dose ($TCID_{50}$/mL) and calculated as previously described by Reed and Muench [27].

2.4. High-pressure studies

The high-pressure reactor was purchased from ISS (Champaign, IL) and has been previously described [28]. The system was fixed at various temperatures with the aid of a circulator bath. Samples were subjected to 310 MPa for different intervals of time at various temperatures (−10, 25 or 37 °C). Virus titers were also measured in samples not subjected to pressure treatments, but still incubated for 8 h at the same temperatures as the pressure-treated samples.

2.5. Thermostability studies

The temperature sensitivity of polioviruses (vaccine batches and viruses cultivated in Vero cells) was assayed using an electrical thermostatic water bath (Eyella Thermopet NTT-211). Samples subjected to temperatures between 4 and 52 °C were monitored for 65 h, and virus titers were calculated using the method of Reed and Muench [27]. Standard deviations were calculated for each condition analyzed.

2.6. Statistics

Statistical analyses were performed using Statistica software, version 6.0 (Stata Corporation, College Station, TX, 1999). Student's *t*-tests were used for comparing means, and differences were considered statistically significant at *P*-values ≤ 0.05.

3. Results

3.1. Viral growth in culture

Several studies have shown that viral replication differs when particles are grown in different cell lines. Currently, little is known about viral replication of the three attenuated poliovirus serotypes in Vero cells, a cell line that can be used for production of the poliovirus vaccine. In order to characterize viral growth kinetics in this cell line and to best define the optimal conditions for virus infection, different MOIs of the three attenuated viruses were used. Individual poliovirus types differed in their growth characteristics. We found that the maximum titer for poliovirus type 1 replication occurred at 78 h post-infection, independent of MOI (Fig. 1A), while poliovirus types 2 and 3 peaked at 126 h post-infection (Fig. 1B and C). Based on these results, the optimum conditions for viral growth in Vero cells were determined to be at an MOI = 0.01 $TCID_{50}$/cell for the three poliovirus strains.

3.2. HHP studies

In order to analyze differential sensitivities and thermostabilization after HHP treatment of the three poliovirus serotypes grown in Vero cells, virus samples were subjected to 310 MPa of pressure at various temperatures and durations. Previous studies have shown that poliovirus serotype 1 is one of the most HHP-resistant viruses [21,24–26]. Here, we also investigated the effect of HHP treatment on poliovirus serotypes 2 and 3. Hydrostatic pressure did not induce a detectable decrease in virus titer for the three serotypes even after 65 h of treatment. We also investigated effects of high pressure at various temperatures (−10, 25 or 37 °C) to assess more drastic conditions (Table 1A and B, Fig. 2). No statistical difference among virus titer values was observed when samples were pressurized at −10, 25 or 37 °C (Table 1A and B). Interestingly, these results show that increased pressure actually stabilizes the three poliovirus serotypes, independent of the temperature during pressurization (Fig. 2). This behavior differs from that of other picornaviruses, which generally show decreases in virus titer after incubation at subzero temperatures under pressure [20]. On the other hand, titers of poliovirus types 2 and 3 were not detected when viruses were incubated at 37 °C (Fig. 3B and C). The loss of poliovirus infectivity in the absence of $MgCl_2$ was consistent with previously described results [1,5,6,8]. Taken together, these data demonstrate that polioviruses are very resistant to high-pressure treatments [20,21,24,25], suggesting that pressure resistance may be related to the shape or size of virus particles [21] or their thermodynamic stability [20]. Most importantly, the data demonstrate that HHP induces thermostabilization of the three poliovirus serotypes (Table 1B).

3.3. Thermostability assays

In order to study viral thermostability, the three poliovirus strains were incubated between 4 and 52 °C for 65 h, and the viral titers were compared in the absence (grown in Vero cells) or presence of thermostabilizers (commercial vaccines) (Fig. 3). In the

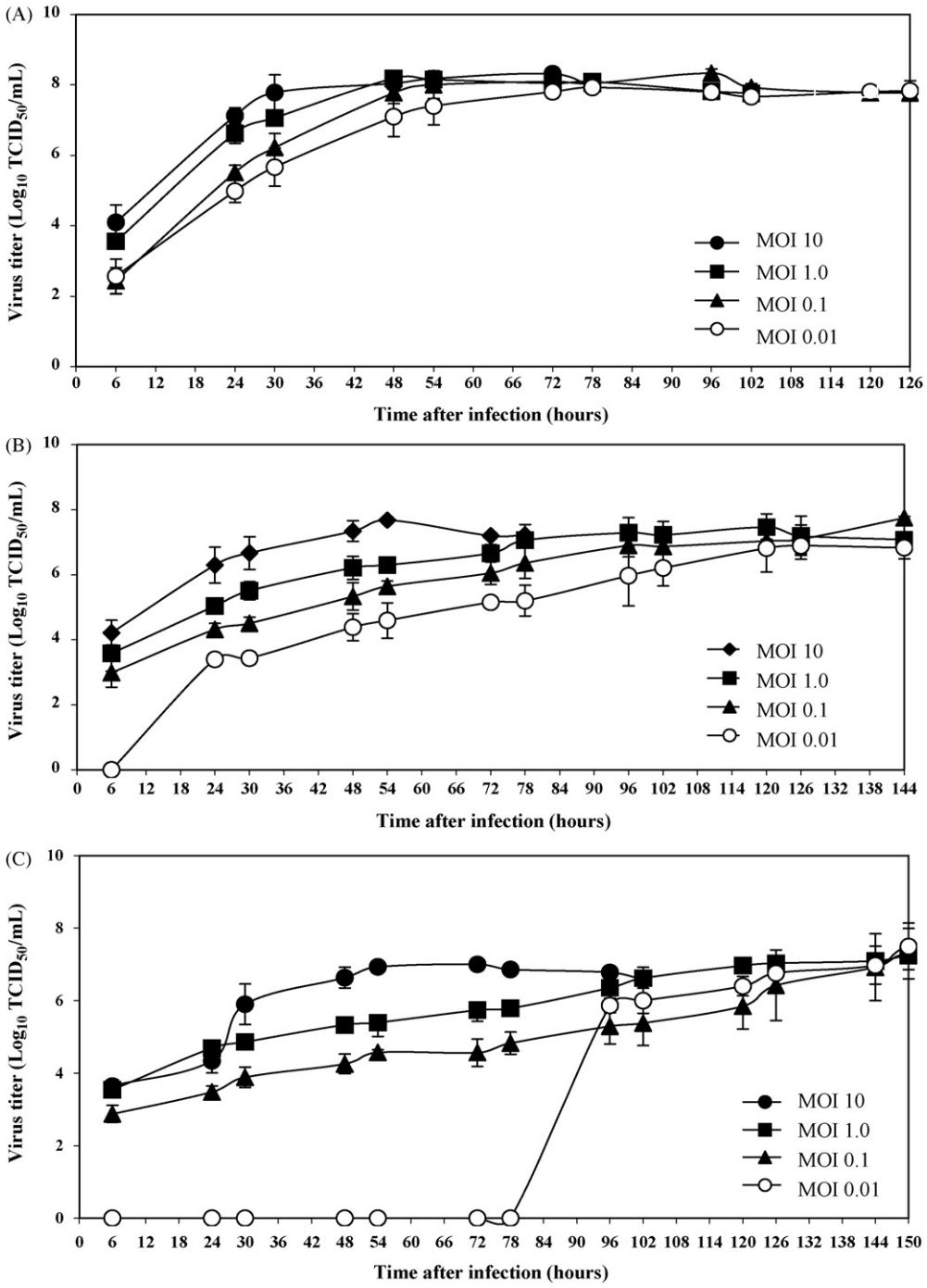

Fig. 1. Comparison of three attenuated poliovirus serotypes grown in Vero cells. (A) Poliovirus type 1, (B) Poliovirus type 2, (C) Poliovirus type 3. Different MOI values were used for each assay (10, 1.0, 0.1, 0.01). Symbols represent mean values from three independent experiments, and infectivity titers were determined by $TCID_{50}$ in Vero cells as described in Section 2.

absence of $MgCl_2$ and L-arginine, poliovirus types 2 and 3 lost infectivity after incubation at 37 °C for 65 h. Poliovirus type 1 had the same reduction in titer after incubation at 42 °C (Fig. 3). On the other hand, a slight reduction in virus titer was observed after incubation at 37 °C in the presence of stabilizers, and infectivity was abolished with incubation at 52 °C. These results demonstrate that all three virus types lose titer after incubation at 52 °C for 65 h, even in the presence of commercial stabilizers.

The infectivity profile for these viruses was expected based on previous results pertaining to viral thermostability [3,29–32], as polioviruses were often found to be stabilized by the presence of cations. In particular, addition of 1 M $MgCl_2$ to attenuated poliovirus strains enables vaccines to be stored at 4 °C for three months without significant loss of titer or human antibody response after vaccination [4].

Pressure-induced stabilization was also observed when the three polioviruses were incubated at 310 MPa for 65 h at 37 °C in the presence of commercial stabilizers. No statistically significant difference was observed among virus titer values before or after pressurization (Table 2). Furthermore, these results show that HHP treatment in the presence of stabilizers also promotes viral stabilization.

Table 1
Analysis of pressure effects on infectivity of the three attenuated poliovirus serotypes at different temperatures in the absence of thermostabilizers. (A) Virus pressurized at 310 MPa for 8 h. (B) Virus pressurized at 310 MPa for 65 h.

Virus grown in Vero cells	Virus titer before pressurization ($TCID_{50}$/mL) ($x \pm sd$)/T (°C)[a]		Virus titer after pressurization at 310 MPa for 8 h ($TCID_{50}$/mL) ($x \pm sd$)	Student's t-test of virus titers before and after pressurization
A				
Poliovirus type 1	8.10 ± 0.03	−10	8.25 ± 0.07	0.20
	7.51 ± 0.07	37	7.45 ± 0.12	0.84
Poliovirus type 2	8.17 ± 0.35	−10	8.14 ± 0.23	0.94
	7.64 ± 0.03	37	7.64 ± 0.04	1.00
Poliovirus type 3	8.72 ± 0.12	−10	8.51 ± 0.12	0.43
	7.91 ± 0.17	37	7.85 ± 0.04	0.84
Virus grown in Vero cells	Virus titer before pressurization ($TCID_{50}$/mL) ($x \pm sd$)/T (°C)[a]		Virus titer after pressurization at 310 MPa for 65 h ($TCID_{50}$/mL) ($x \pm sd$)	Student's t-test of virus titers before and after pressurization
B				
Poliovirus type 1	8.02 ± 0.11	−10	8.30 ± 0.28	0.26
	8.30 ± 0.28	25	7.99 ± 0.09	0.13
	8.20 ± 0.25	37	8.11 ± 0.32	0.94
			5.69 ± 0.03[b]	
Poliovirus type 2	8.20 ± 0.14	−10	8.37 ± 0.37	0.50
	7.89 ± 0.12	25	7.89 ± 0.18	0.80
	8.13 ± 0.21	37	7.89 ± 0.03ND[c]	0.49
Poliovirus type 3	8.25 ± 0.35	−10	8.08 ± 0.03	0.42
	7.14 ± 0.05	25	7.24 ± 0.26	0.70
	8.26 ± 0.23	37	8.06 ± 0.31ND[c]	0.28

[a] Temperature of experimental conditions.
[b] Virus titer after treatment for 65 h at atmospheric pressure ($TCID_{50}$/mL) ($x \pm sd$).
[c] Virus titer after treatment for 65 h at atmospheric pressure was not detected (ND).

4. Discussion

Although the epidemiology and molecular structure of picornaviruses have been characterized, little is known about the stability of the three polioviruses serotypes after HHP treatment. HHP is used to promote viral assembly, disassembly [33] and inactivation in most viruses studied [11–19]. Here, we show that pressure treatment not only prevents inactivation of polioviruses, but also promotes viral thermostabilization.

Viruses are known to differ widely in their intrinsic susceptibility to HHP; however, the mechanism of viral pressure resistance is not fully understood. Poliovirus, in particular, demonstrates a wide range of resistance to HHP treatments, with no significant reductions in infectivity reported after relatively severe treatments (e.g., 600 MPa at 20 °C for 60 min) [20,21,25]. Here, we show that resistance of the three poliovirus strains subjected to various pressure and temperature treatments in the absence of thermostabilizers was similar in all conditions tested, including pressurization at 310 MPa for up to 65 h and incubation at 37 or −10 °C. Many studies have demonstrated that loss of picornavirus infectivity, which occurs at 37 °C, is caused by fragmentation of viral RNA *in situ* [30,31,34] suggesting that inhibition of RNA polymerase activity would stabilize the infectivity of poliovirus as well as other picornaviruses. One possible mechanism for poliovirus stabilization could be mediated through pressure-induced RNA polymerase inhibition at 37 °C. Additionally, increased pressure can induce conformational changes of structural viral proteins, which may be protecting these proteins from heat effects.

The thermal liability of live vaccines has been a serious concern since 1960. Since OPV is a very heat-labile vaccine, the use of an efficient cold chain system is often required. The thermostabilization effect of $MgCl_2$ on infectivity of oral poliovirus vaccine strains was discovered about 43 years ago [1,6]. $MgCl_2$ has been used for years to stabilize Sabin oral poliovirus vaccine strains against thermal inactivation [1,5,6,8]. Here, we investigated viral stability at a pressure of 310 MPa for 65 h at 37 °C in the presence or absence of commercial stabilizers. Our results showed that pressurized particles were able to resist exposure to 37 °C for 65 h in the absence of $MgCl_2$ and L-arginine. Taken together, our results demonstrate that HHP treatment can increase poliovirus stability in the absence of $MgCl_2$. This effect was observed for all three poliovirus strains examined, as demonstrated by viral titrations in cell culture. The accelerated degradation test satisfactorily confirmed OPV stability, including incubation at 37 °C for 48 h. The vaccine passes the test if the loss of infectivity upon exposure is not greater than a factor of $10^{0.5}$ infectious units per human dose [4].

The mechanism of poliovirus thermostabilization by $MgCl_2$ has been elucidated [35]. $MgCl_2$ increases rigidity of poliovirus capsid conformation and diminishes the extent of water penetration into the capsid. Moreover, thermostabilization of poliovirus by L-arginine appears to be dependent on a single intermolecular contact that mediates the intramolecular stabilization of both RNAs and proteins. Electrostatic interactions between positively charged tandem amino groups with negatively charged residues on the viral capsid result in noncovalent crosslinks, which increase the stability of poliovirus conformations [36].

Table 2
Pressure effects on poliovirus at 37 °C in the presence of commercial stabilizers.

Virus in the presence of commercial stabilizers ($MgCl_2$ and L-arginine)	Virus titer before pressurization ($TCID_{50}$/mL) ($x \pm sd$)	Virus titer after pressurization at 310 MPa for 65 h ($TCID_{50}$/mL) ($x \pm sd$)	Student's t-test of virus titers before and after pressurization
Poliovirus type 1	8.10 ± 0.12	8.27 ± 0.05	0.35
Poliovirus type 2	8.00 ± 0.11	7.87 ± 0.11	0.46
Poliovirus type 3	8.30 ± 0.22	7.80 ± 0.05	0.50

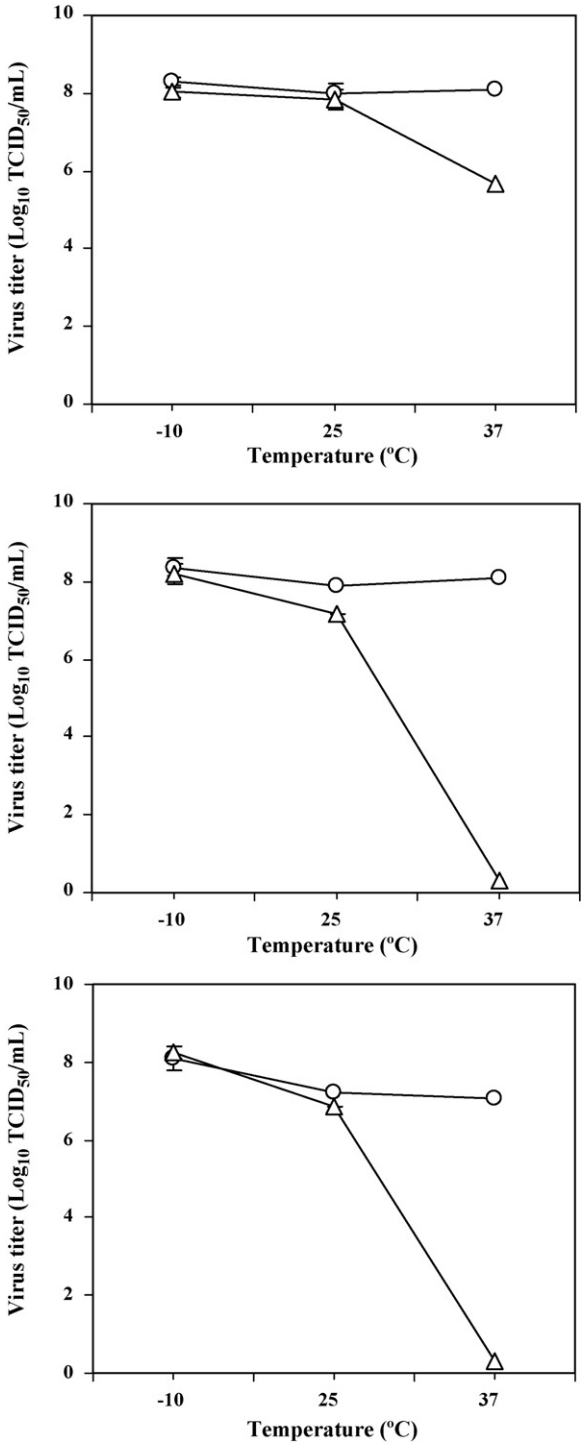

Fig. 2. Pressure effects on the three serotypes of attenuated poliovirus infectivity at different temperatures. Circles represent viruses pressurized at 310 MPa for 65 h. Triangles represent viruses at atmospheric pressure. All symbols are mean values from three independent experiments. Infectivity titers were determined by $TCID_{50}$ in Vero cells.

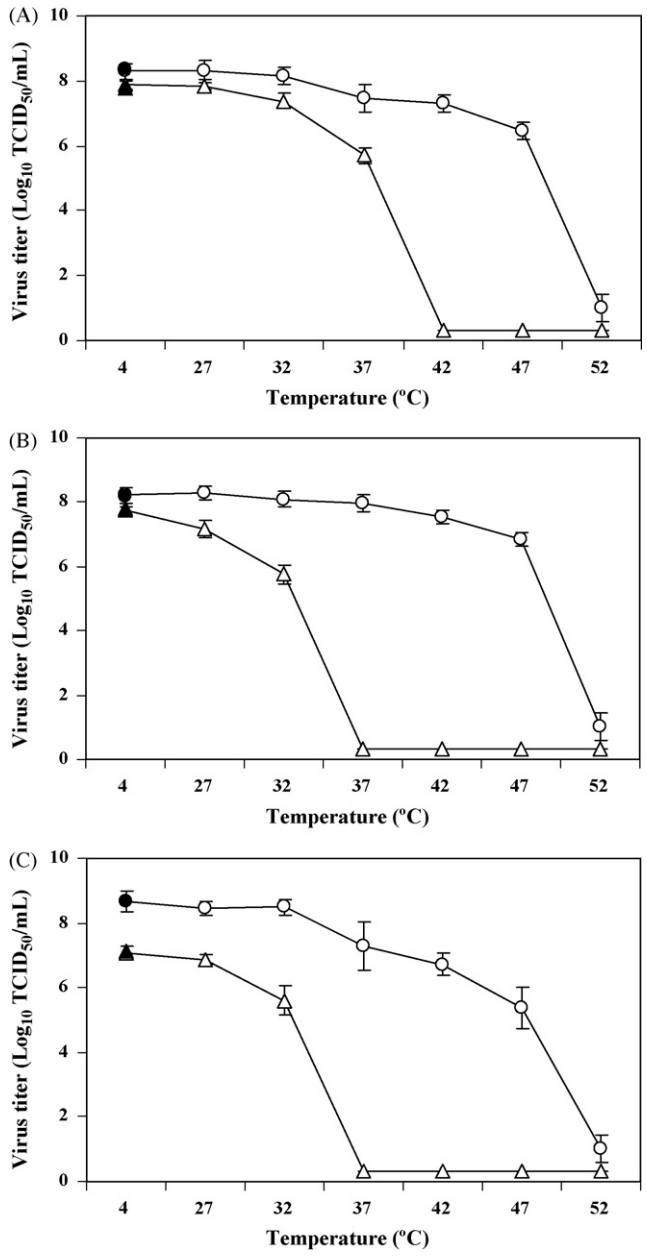

Fig. 3. Effects of high temperature on infectivity of the three attenuated poliovirus serotypes after treatment for 65 h. (A) Poliovirus type 1, (B) Poliovirus type 2, (C) Poliovirus type 3. Circles represent viruses stabilized with $MgCl_2$ and L-arginine; triangles represent viruses without stabilizers. All symbols are mean values from three independent experiments. Infectivity titers were determined by $TCID_{50}$ in Vero cells.

The potency of OPV vaccines is greatly affected during the manufacturing, transportation and storage processes. During the summer months in most parts of the world, electric power is only supplied for a few hours a day, which can adversely affect OPV potency [37]. Here, we propose high hydrostatic pressure treatment for 65 h as an alternative for increasing thermostability of three attenuated poliovirus serotypes. In addition to being both extremely secure and cheap, this technology can be used alone or with other stabilizers to guarantee effective stabilization of commercial poliovirus vaccines. Furthermore, pressure treatment is known to preserve important structural epitopes on the virus, enabling treated particles to elicit the appropriate neutralizing antibody response [13,18,19]. This is particularly important for OPV and inactivated poliomyelitis vaccine (IPV), as the ability of these viruses to elicit neutralizing antibodies is destroyed through heat treatment and freeze/drying. D-antigen content for type 1 in IPV dropped significantly after incubation at 24 °C for 20 days and was undetectable with storage at 32 °C for the same duration [4]. The thermostabilization effect induced by HHP on the three poliovirus serotypes was not analyzed with regard to durability after HHP treatment. Our next step will be to correlate pressure effects with the absence of significant virus titer loss after long periods of sample storage.

Reports on the poor performance of biological substances in addition to progressive climate changes in the Americas, South Asia and Africa thus justify vigorous and continued efforts toward improving the stability of conventional and genetically produced vaccines [37,38].

Acknowledgements

We thank Rafael B. Gonçalves for technical assistance and the Instituto de Tecnologia em Imunobiológicos for continued interest and support. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Rio de Janeiro (FAPERJ) and Instituto do Milênio de Biologia Estrutural em Biomedicina e Biotecnologia (IMBEBB) of Brazil. The author(s) have declared that the methodology presented here is the subject of a current patent application (authors LPG, ACO, JLV and MSF and the Fundação Oswaldo Cruz sponsoring institution).

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