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(71) Demandeur/Applicant:
JANSSEN VACCINES & PREVENTION B.V., NL

(72) Inventeurs/Inventors:
SANDERS, BARBARA PETRONELLA, NL;
CUSTERS, JEROME H.H.V., NL;
EDO-MATAS, DIANA, NL;
UIL, TACO GILLES, NL;
LEWIS, JOHN ALFRED, US

(74) Agent: NORTON ROSE FULBRIGHT CANADA

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(54) Title: COLD-ADAPTED-VIRAL-ATTENUATION (CAVA) AND NOVEL ATTENUATED POLIOVIRUS STRAINS

(57) Abrégé/Abstract:
A poliovirus (PV) strain was attenuated by a novel method of Cold-Adapted-Viral-Attenuation (CAVA). The resulting recombinant attenuated PV, CAVA-PV, shows wild- type replication at 30°C, but no substantial replication at 37°C. The inability to replicate at 37°C is defined by an inability to quantify virus during infection at this temperture by titration (infectious units), qPCR (viral RNA) or Electron Microscopy (visual signs of infection). CAVA-PV is genetically stable under production conditions and shows utility for use as the backbone to produce attenuated strains with the same antigenic profile as conventional vaccines by replacing the sequence coding for the capsid of CAVA-PV with sequences coding for capsids of different PV strains. Furthermore, mutations identified in CAVA-PV can be engineered into different, even wild-type and neurovirulent poliovirus background strains to obtain additional CAVA-PV strains.

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(71) **Applicant**: **CRUCELL HOLLAND B.V.** [NL/NL]; Archimedesweg 4, NL-2333 CN Leiden (NL).

(72) **Inventors**: **SANDERS, Barbara, Petronella**; Archimedesweg 4-6, NL-2333 CN Leiden (NL). **CUSTERS, Jerôme H.H.V.**; Archimedesweg 4-6, NL-2333 CN Leiden (NL). **EDO-MATAS, Diana**; Archimedesweg 4-6, NL-2333 CN Leiden (NL). **UIL, Taco, Gilles**; Archimedesweg 4-6, NL-2333 CN Leiden (NL). **LEWIS, John, Alfred**; 270 West Main Road, Little Compton, RI 2837 (US).

(74) **Agents**: **MANTEN, Annemieke** et al.; Archimedesweg 4-6, NL-2333 CN Leiden (NL).

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(57) **Abstract**: A poliovirus (PV) strain was attenuated by a novel method of Cold-Adapted-Viral-Attenuation (CAVA). The resulting recombinant attenuated PV, CAVA-PV, shows wild- type replication at 30°C, but no substantial replication at 37°C. The inability to replicate at 37°C is defined by an inability to quantify virus during infection at this temperture by titration (infectious units), qPCR (viral RNA) or Electron Microscopy (visual signs of infection). CAVA-PV is genetically stable under production conditions and shows utility for use as the backbone to produce attenuated strains with the same antigenic profile as conventional vaccines by replacing the sequence coding for the capsid of CAVA-PV with sequences coding for capsids of different PV strains. Furthermore, mutations identified in CAVA-PV can be engineered into different, even wild-type and neurovirulent poliovirus background strains to obtain additional CAVA-PV strains.

Cold-Adapted-Viral-Attenuation (CAVA) and Novel Attenuated Poliovirus Strains

TECHNICAL FIELD

[0001] The invention relates to the field of viral attenuation and the development of novel attenuated poliovirus strains for use as vaccines.

BACKGROUND OF THE INVENTION

[0002] The Poliovirus (PV) belongs to the enterovirus genus within the *Picornaviridae* family. These small RNA viruses have a single-stranded positive-sense RNA genome and are non-enveloped. They are subdivided into three serotypes; PV1, PV2 and PV3. Infection with PV is usually asymptomatic, however, 1-2 % of the cases result in paralytic poliomyelitis where viral-induced destruction of motor neurons causes paralysis, generally in limbs, termed Acute Flaccid Paralysis (AFP). Of these AFP cases, 5 – 10% are lethal as the virus spreads to regions of the brainstem resulting in respiratory arrest and, ultimately, death. (For reviews of PV and pathogenesis see, for example, (Minor 1999, Racaniello 2006, Pfeiffer 2010).

[0003] Today, poliomyelitis is on the verge of eradication with only 406 and 359 cases worldwide in 2013 and 2014, respectively. There are two vaccines available which have enabled this successful battle against PV; the Inactivated Poliovirus Vaccine (IPV) (Salk 1953) and the Oral Poliovirus Vaccine (OPV) (Sabin 1956). IPV consists of three formalin-inactivated (killed) wild-type (neurovirulent) PV strains from each serotype (typically Mahoney, MEF-1 and Saukett for PV1, PV2 and PV3 respectively). OPV is made up of three live attenuated strains called Sabin 1, Sabin 2 and Sabin 3. The Sabin strains are named after Albert Sabin, who generated the strains through serial passage of three parental wild-type viruses through cell culture and even whole organisms (Sabin 1973). Due to the ease of administration and low costs of OPV (John 2009), it was heralded by the World Health Organization (WHO) and Global Polio Eradication Initiative (GPEI) as the vaccine of choice for the eradication program in 1988 (WHO 1988). However, the use of OPV is at odds with eradication and the end game strategy because the OPV vaccine strains display reversion of the attenuated phenotype to neurovirulent and transmissible polioviruses (Henderson, Witte et al. 1964). These Vaccine Associated Paralytic Poliomyelitis (VAPP) cases occur with a frequency of 1

case per 500,000 vaccines (John 2004). Furthermore, circulating Vaccine Derived Polioviruses (cVDPV's) result when the reverted vaccine viruses become transmissible within a susceptible population. As OPV is now recognized as a potential source of poliomyelitis through the VAPP and cVDPV phenomena, there has been a call for the use of OPV to be eliminated if complete eradication of poliomyelitis is to be achieved. In fact, over the last decade, regulatory authorities have recognized that OPV use must be ceased for eradication of poliomyelitis (WHO 2005). With IPV, the vaccine immunogens are formalin-inactivated, and therefore VAPP and cVDPV are not observed with the use of this vaccine. However, the 20-fold higher cost of this vaccine renders it unfavourable for use in low- and middle-income countries (Heinsbroek and Ruitenberg 2010, Zehrung 2010). To that end, the WHO and its many collaborators have initiated multiple projects for the development of strategies to make IPV safer and more economical (Zehrung 2010, Hawken and Troy 2012, Resik, Tejeda et al. 2013).

[0004] IPV is indeed a safer alternative to OPV with respect to VAPP and cVDPV, however, the wild-type viruses that make up the vaccine could pose a threat to the global population during the post eradication era in the event of accidental escape of these viruses from a manufacturing facility. Furthermore, upon eradication, wild-type polioviruses will be subject to stringent bio-containment regulations which may further increase the costs of goods of this already expensive vaccine (WHO 2006, Verdijk, Rots et al. 2011). In fact, the WHO already stipulates the destruction of any wild-type PV strains from laboratories across the globe. These actions will presumably be enforced more vigorously as eradication draws nearer (WHO 2009). Development of IPV vaccines based on attenuated strains is a safer vaccine manufacturing procedure and mitigates risks of potential disease outbreaks in the case of industrial accidents. To that end, the production of an IPV based on attenuated strains has been proposed as a strategy for the safe and economical production of IPV for the post eradication era. Multiple attenuated strains have been proposed as a basis for a novel IPV vaccine, of which most research has been invested in making an IPV from the Sabin strains (Verdijk, Rots et al. 2011). Sabin-based IPV has recently undergone a successful Phase II clinical trial as a stand-alone vaccine (Liao, Li et al. 2012) and a Phase II and III trial in combination with diphtheria, tetanus and acellular pertussis (DTaP) (Okada, Miyazaki et al. 2013). Moreover, in Japan a Sabin based IPV has recently been licensed in combination with DTaP (Mahmood,

Pelkowski et al. 2013). However, the capsids of the Sabin viruses differ significantly from the conventional IPV wild-type viruses, and, upon formalin inactivation, Sabin viruses display different antigenic properties required to raise neutralizing antibodies (and therefore protection) against PV infection. Indeed it has been observed that the immune response to Sabin IPV is altered as compared to the one raised against conventional IPV, therefore, different dosing of the vaccine is necessary (Westdijk, Brugmans et al. 2011).

[0005] Several rationally engineered attenuated PV strains exist, however, their potential as IPV vaccine strains has, as of yet, not been fully explored. One set of such candidate attenuated strains is based on genetically stabilized Sabin strains as superior live vaccine strains as compared to the Sabin strains for a novel OPV development, as well as use for a basis of a novel IPV (e.g. Macadam *et al*, 2006; WO 2008/017870; WO 2012/090000). These strains are capable of growth at physiological temperatures, however they show significantly reduced infectivity at this temperature.

[0006] For the post-eradication era, there remains a need to develop an IPV based on safe, attenuated poliovirus strains which do not revert to neurovirulent forms and which induce a similar immune response as conventional IPV based on wild-type strains.

SUMMARY OF THE INVENTION

[0007] The present invention provides a novel method for developing recombinant attenuated poliovirus strains, as well as resulting novel recombinant attenuated poliovirus strains which can be used as a basis for an IPV. The attenuated backbone can be re-engineered to contain the wild-type capsids of strains used in current IPV vaccines, resulting in a wild-type antigenicity profile after inactivation whilst maintaining the attenuated phenotype during virus production and scale-up to produce a vaccine. The recombinant attenuated polioviruses are genetically stable under production conditions. Furthermore, due to their inability to replicate at 37°C it is highly unlikely that they can revert to a neurovirulent form upon accidental ingestion, in the event of (un)intentional escape from a manufacturing facility. The recombinant attenuated poliovirus strains of the present invention can be formalin-inactivated for use in an IPV vaccine.

[0008] The invention is directed to the general and preferred embodiments defined, respectively, by the independent and dependent claims appended hereto, which for the

sake of brevity are incorporated by reference herein. Other preferred embodiments, features, and advantages of the various aspects of the invention will become apparent from the detailed description below taken in conjunction with the appended drawing figures.

[0009] In one embodiment, the present invention provides a recombinant poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, comprising a capsid from a Mahoney, MEF-1, or Saukett strain.

[0010] In another embodiment, the present invention provides a recombinant attenuated temperature sensitive poliovirus type 1 strain that can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C.

[0011] In another embodiment, the present invention provides a recombinant attenuated temperature sensitive poliovirus type 2 strain that can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C.

[0012] In another embodiment, the present invention provides a recombinant attenuated temperature sensitive poliovirus type 3 strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C.

[0013] In another embodiment, the present invention provides a composition comprising a poliovirus strain and a pharmaceutically acceptable carrier or excipient, wherein the poliovirus strain is a recombinant attenuated temperature sensitive poliovirus strain that can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C, and wherein the poliovirus strain can optionally comprise a capsid from a Mahoney, MEF-1, or Saukett strain.

[0014] In another embodiment, the present invention provides a composition comprising first, second and third recombinant poliovirus strains, wherein each of the first, second and third recombinant poliovirus strains can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C, and wherein the first, second and third recombinant poliovirus strains respectively comprise a capsid from a Mahoney, a MEF-1, and a Saukett strain.

[0015] In another embodiment, the present invention provides a composition comprising first, second and third recombinant poliovirus strains, further comprising a pharmaceutically acceptable carrier or excipient, wherein the first, second and third recombinant poliovirus strains can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C, and wherein the first, second and third recombinant poliovirus strains respectively comprise a capsid from a Mahoney, a MEF-1, and a Saukett strain.

[0016] In another embodiment, the present invention provides an IPV composition, wherein the poliovirus in the composition is inactivated, and wherein prior to being inactivated the poliovirus is a recombinant poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, and wherein the first, second and third recombinant poliovirus strains respectively comprise a capsid from a Mahoney, a MEF-1, and a Saukett strain.

[0017] In another embodiment, the present invention provides an IPV composition, wherein the IPV composition comprises inactivated first, second and third recombinant poliovirus strains, wherein prior to being inactivated each of the first, second and third recombinant poliovirus strains can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C, and wherein the first, second and third recombinant poliovirus strains respectively comprise a capsid from a Mahoney, a MEF-1, and a Saukett strain.

[0018] In another embodiment, the present invention provides a combination vaccine composition comprising an IPV composition described *supra*, and one or more antigens from other pathogens, such as diphtheria, tetanus, pertussis, *Heamophilus influenzae* type b (Hib), Hepatitis B virus (HBV), etc.

[0019] In another embodiment, the present invention provides a method for vaccinating against poliovirus infection and/or poliomyelitis, the method comprising administering to a subject a composition comprising an IPV composition described *supra* or a combination vaccine composition comprising an IPV as described *supra*.

[0020] In another embodiment, the present invention provides a nucleic acid molecule comprising a sequence that codes for the genome or the complement of the genome of a poliovirus, wherein the poliovirus is a recombinant attenuated temperature sensitive

poliovirus that can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C, and wherein the recombinant attenuated temperature sensitive poliovirus comprises a capsid from a Mahoney, MEF-1, or Saukett strain.

[0021] In another embodiment, the present invention provides a method for preparing a preparation comprising a poliovirus, wherein the poliovirus is a recombinant attenuated temperature sensitive poliovirus that can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C, and wherein the poliovirus can optionally comprise a capsid from a Mahoney, MEF-1, or Saukett strain, the method comprising

a) infecting a cell in a cell culture with the poliovirus,

b) culturing the cells in the cell culture (at a temperature that is permissive for replication of the poliovirus, e.g. between about 20°C and about 33°C) to propagate the poliovirus; and,

c) isolating the poliovirus strain from the cells or from the cell culture to obtain the preparation comprising the poliovirus.

[0022] In another embodiment, the present invention provides a method for preparing a preparation comprising an inactivated poliovirus, wherein prior to being inactivated the poliovirus is a recombinant attenuated temperature sensitive poliovirus strain that can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C, and wherein the poliovirus can optionally comprise a capsid selected from a Mahoney, MEF-1, or Saukett strain, the method comprising:

a) infecting a cell in a cell culture with the poliovirus;

b) culturing the cells in the cell culture (at a temperature that is permissive for replication of the poliovirus, e.g. between about 20°C and about 33°C) to propagate the poliovirus;

c) isolating the poliovirus strain from the cells or from the cell culture to obtain the preparation comprising the poliovirus; and,

d) inactivating the poliovirus.

[0023] In another embodiment, the present invention provides a method for preparing an IPV comprising an inactivated poliovirus in a formulation suitable as a pharmaceutical composition, wherein prior to being inactivated the poliovirus is a recombinant attenuated

temperature sensitive poliovirus strain that can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C, and wherein the poliovirus can optionally comprise a capsid selected from a Mahoney, MEF-1, or Saukett strain, the method comprising:

5a) infecting a cell in a cell culture with the poliovirus;

b) culturing the cells in the cell culture (at a temperature that is permissive for replication of the poliovirus, e.g. between about 20°C and about 33°C) to propagate the poliovirus;

c) isolating the poliovirus strain from the cells or from the cell culture to obtain the preparation comprising the poliovirus;

10d) inactivating the poliovirus; and,

e) formulating the inactivated poliovirus into a pharmaceutical composition.

[0024] In another embodiment, the present invention provides a method for obtaining a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, the method comprising

15 the steps of:

a) passaging a parental (or starting) poliovirus strain at ≤30°C for sufficient passages to produce a virus with impaired growth at 37°C (e.g. for at least 5, 10, 15, 20, 25, 30 or 35 passages);

b) isolating two or more different temperature sensitive clones from the viral population

20 which display impaired growth at 37°C;

c) sequencing the genomes of the temperature sensitive clones;

d) identifying mutations in the sequences of the genomes of temperature sensitive clones by comparing the sequences of the temperature sensitive clones to the sequence of the parental poliovirus strain;

25e) synthesizing the recombinant attenuated poliovirus strain by combining mutations from two or more different temperature sensitive clones into the genome of a (e.g. the parental or another) poliovirus strain; and,

f) rescuing the recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C.

[0025] In another embodiment, the present invention provides a method for obtaining a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, wherein the recombinant attenuated poliovirus strain can optionally comprise a capsid selected from a different poliovirus strain, the method comprising the steps of:

a) passaging a parental poliovirus strain at ≤30°C for sufficient passages to produce a virus with impaired growth at 37°C;

b) isolating two or more temperature sensitive clones from the viral population which display impaired growth at 37°C;

c) sequencing the genomes of the temperature sensitive clones;

d) identifying mutations in the sequences of the genomes of temperature sensitive clones by comparing the sequences of the temperature sensitive clones to the sequence of the parental poliovirus strain;

e) synthesizing the recombinant attenuated poliovirus strain by combining mutations from the two or more different temperature sensitive clones into the genome of a (e.g. the parental or another) poliovirus strain;

f) rescuing the recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C; and

g) optionally replacing the sequence coding for the capsid from the rescued recombinant attenuated poliovirus strain with a sequence coding for a capsid from a different poliovirus strain.

In certain embodiments, the sequence coding for the capsid from the rescued attenuated poliovirus strain is replaced with a sequence coding for a capsid from a Mahoney, MEF-1, or Saukett strain.

In certain embodiments, the sequence coding for the capsid from the rescued attenuated poliovirus strain is replaced with a sequence coding for a capsid from a Sabin type 1, Sabin type 2 or Sabin type 3 strain.

[0026] In another embodiment, the present invention provides a method for obtaining a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C

and that cannot be substantially propagated in cell culture at 37°C, wherein the recombinant attenuated poliovirus strain can optionally comprise a capsid selected from a Mahoney, MEF-1, or Saukett strain, the method comprising the steps of:

h) passaging a parental poliovirus strain at ≤30°C for sufficient passages to produce a virus with impaired growth at 37°C;

i) isolating two or more temperature sensitive clones from the viral population which display impaired growth at 37°C;

j) sequencing the genomes of the temperature sensitive clones;

k) identifying mutations in the sequences of the genomes of temperature sensitive clones by comparing the sequences of the temperature sensitive clones to the sequence of the parental poliovirus strain;

l) synthesizing the recombinant attenuated poliovirus strain by combining mutations from the two or more different temperature sensitive clones into the genome of a (e.g. the parental or another) poliovirus strain;

15m) rescuing the recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C; and

n) replacing the sequence coding for the capsid from the rescued recombinant attenuated poliovirus strain with a sequence coding for a capsid from a Mahoney, MEF-1, or Saukett strain.

20 [0027] In another embodiment, the present invention provides a recombinant attenuated poliovirus strain obtainable by the methods described *supra*.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] **Fig. 1**: Schematic representation for a method of attenuating a poliovirus strain by Cold-Adapted-Viral-Attenuation (CAVA) to produce a recombinant attenuated poliovirus strain ($CAVA\text{-}PV_{Backbone}$). A representative line graph shows growth at 37°C for a parental or starting poliovirus strain and three different temperature sensitive clones. The parental virus is shown as filled circles and the 3 temperature sensitive clones are shown as empty diamonds, empty circles, and empty squares.

[0029] **Fig. 2**: Replication kinetics in suspension PER.C6 (sPER.C6) cells for a panel of 4 different Type 1 PV strains and a representative CAVA-$PV_{Backbone}$. 2A: growth at 30°C. 2B: growth at 37°C. Data for the CAVA-PV, Brunhilde, Mahoney, Brunenders, and Sabin 1 strains are shown as open circles, filled diamonds, empty squares, filled triangles, and filled squares, respectively.

[0030] **Fig. 3**: Average replication kinetics in sPER.C6 cells for Brunenders parental PV and a representative CAVA-$PV_{Backbone}$. The error bars represent standard deviation from the mean (N=3). Fig. 3A shows growth at 30°C and Fig. 3B shows growth at 37°C. Brunenders parental PV is shown as filled triangles and CAVA-$PV_{Backbone}$ is shown as open circles.

[0031] **Fig. 4**: Replication kinetics in HEK293 cells for Brunenders parental PV and a representative CAVA-$PV_{Backbone}$. 4A: growth at 30°C, 4B: growth at 37°C, and 4C: growth at 39.5°C. Brunenders parental PV is shown as filled triangles and CAVA-$PV_{Backbone}$ is shown as open circles.

[0032] **Fig. 5**: Replication kinetics in L20B cells for Brunenders parental PV and a representative CAVA-$PV_{Backbone}$. 5A: growth at 30°C, 5B: growth at 37°C, and 5C: growth at 39.5°C. Brunenders parental PV is shown as filled triangles and CAVA-$PV_{Backbone}$ is shown as open circles.

[0033] **Fig. 6**: Replication kinetics in HeLa cells for Brunenders parental PV and a representative CAVA-$PV_{Backbone}$. 6A: growth at 30°C, 6B: growth at 37°C, and 6C: growth at 39.5°C. Brunenders parental PV is shown as filled triangles and CAVA-$PV_{Backbone}$ is shown as open circles.

[0034] **Fig. 7**: Replication kinetics in Vero cells for Brunenders parental PV and a representative CAVA-$PV_{Backbone}$. 7A: growth at 30°C. 7B: growth at 37°C. Brunenders parental PV is shown as filled triangles and CAVA-$PV_{Backbone}$ is shown as open circles.

[0035] **Fig. 8**: Replication kinetics in SK-N-MC cells for Brunenders parental PV and a representative CAVA-$PV_{Backbone}$. 8A: growth at 30°C. 8B: growth at 37°C. Brunenders parental PV is shown as filled triangles and CAVA-$PV_{Backbone}$ is shown as open circles.

[0036] **Fig. 9**: Replication kinetics in adherent PER.C6 (adPER.C6) cells for Brunenders parental PV and a representative CAVA-$PV_{Backbone}$. 9A: growth at 30°C. 9B:

growth at 37°C. Brunenders parental PV is shown as filled triangles and CAVA-$PV_{Backbone}$ is shown as open circles.

[0037] **Fig. 10**: Replication kinetics for CAVA-$PV_{Backbone}$ (, closed circles) and after 8 (open circles) passages (A) under envisioned production conditions (A) at 30°C; and (B) at 37°C, at small scale.

[0038] **Fig 11:** Replication kinetics of (11A, B) CAVA-$PV_{Mahoney}$, (11C, D) CAVA-PV_{MEF-1} and (E, F) CAVA-$PV_{Saukett}$ and after 5 passages on sPER.C6 (N=3) under envisioned production conditions (11A, C, E) at 30°C; and (11B, D, E) at 37°C, at small scale. Filled diamonds represent the growth of the starting stock viruses whilst empty squares, circles and triangles represent each of the three passaged viruses (N=1, 2 and 3, respectively).

[0039] **Fig. 12**: Schematic overview of the different CAVA-PV vaccine strains after swapping the sequence coding for the capsid of the original CAVA-$PV_{Backbone}$ strain with the sequences coding for the capsids of the Mahoney, MEF-1, and Saukett strains. After the capsid swaps, the resulting vaccine strains are referred to as CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$, respectively.

[0040] **Fig. 13**: Replication kinetics of CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ and the parental Brunenders strain. 12A: growth at 30°C. 12B: growth at 37°C. The error bars represent standard deviation from the mean (N=2 or 3). Brunenders parental PV is shown as filled triangles, CAVA-$PV_{Mahoney}$ shown as open circles, CAVA-PV_{MEF-1} shown as open squares, and CAVA-$PV_{Saukett}$ shown as open diamonds.

[0041] **Fig. 14**: Virus Neutralization Titers of rats immunized with inactivated 14A: CAVA-$PV_{Mahoney}$, 14B: CAVA-PV_{MEF-1} or 14C: CAVA-$PV_{Saukett}$.

[0042] **Fig. 15**: Replication kinetics for parental Brunenders PV, CAVA-$PV_{Backbone}$ of Example 1 (having the 31 mutations of Table 1 compared to a Brunenders parental strain), and a further CAVA-PV of Example 10 (which corresponds to a parental Brunenders strain with the 14 mutations of Table 4). 15A: growth at 30°C. 15B: growth at 37°C. Data for the parental Brunenders PV are shown as filled triangles, data for CAVA-$PV_{Backbone}$ of Example 1 are shown as open circles, and data for CAVA-PV of Example 10 are shown as filled diamonds.

[0043] **Fig. 16**: Replication kinetics for parental wild-type MEF-1, CAVA-$PV_{Backbone}$ of Example 1 (having 31 mutations of Table 1 in a Brunenders background) and a further CAVA-PV strain of Example 11 (which corresponds to a parental MEF-1 PV with 13 mutations as indicated in Table 4). 16A: growth at 30°C. 16B: growth at 37°C. Data for MEF-1 are shown as filled squares, data for CAVA-$PV_{Backbone}$ of Example 1 are shown as open circles, and data for MEF-1 with 13 CAVA mutations (CAVA-PV of Example 11) are shown as open squares.

[0044] **Fig. 17**: Replication kinetics for parental Sabin 3, CAVA-$PV_{Backbone}$ of Example 1 (having 31 mutations of Table 1 in a Brunenders background) and a further CAVA-PV strain of Example 11 (which corresponds to a parental Sabin 3 PV with 12 mutations as indicated in Table 4). 17A: growth at 30°C. 17B: growth at 37°C. Data for Sabin 3 are shown as filled squares, data for CAVA-$PV_{Backbone}$ of Example 1 are shown as open circles, and data for Sabin 3 with 12 CAVA mutations (CAVA-PV of Example 11) are shown as open triangles.

[0045] **Figure 18:** EM micrographs of representative cells during infection with (A) PBS (Mock) at 37°C, (B) Mahoney at 37°C, (C) CAVA-$PV_{Mahoney}$ at 37°C, and (D) CAVA-$PV_{Mahoney}$ at 30°C.

[0046] **Figure 19:** CAVA-$PV_{Mahoney}$ infection over time in viral RNA levels as measured by qPCR expressed as genome copies (Log_{10} GC on Right Y-axis, circles) and infectious units as measured by titration (Log_{10} $TCID_{50}$/ml on Left-Y axis, squares) during infection at (open square and circle) 30°C and (filled square and circle) 37°C.

[0047] **Figure 20:** Replication kinetics of parental Brunenders, CAVA-$PV_{Backbone}$ of Example 1, CAVA-$PV_{Mahoney}$ of Example 6 and two intermediate viruses with either the 7 CAVA mutations in the 5'UTR or the 17 CAVA mutations in the Non-Structural proteins. 20A: growth at 30°C. 20B: growth at 37°C. Data for Brunenders parental strain are shown as filled circles, data for the intermediate virus with 7 CAVA mutations in the 5'UTR are shown as filled triangles, data for the intermediate virus with 17 CAVA mutations in the Non-Structural proteins are shown as filled squares, data for CAVA-$PV_{Backbone}$ of Example 1 are shown as open circles, data for CAVA-$PV_{Mahoney}$ are shown as open triangles.

DETAILED DESCRIPTION OF THE INVENTION

[0048] In the present invention a novel method of Cold-Adapted-Viral-Attenuation (CAVA) was used to produce recombinant attenuated poliovirus strains, Cold-Adapted-Viral-Attenuation Poliovirus (CAVA-PV). A CAVA-PV grows to high titers at low temperature (30°C), but does not grow substantially at physiological temperature (37°C). Due to the inability to replicate substantially at 37°C, CAVA-PV strains encounter a non-permissive temperature when inside a mammalian host, thus conferring an attenuated phenotype suitable for use as the basis for an IPV vaccine. The CAVA-PV genomic backbone is suitable for re-engineering to contain sequences coding for different virus capsids (e.g., the three wild-type capsids used in conventional IPV), thus conferring the antigenic, and therefore presumably the same immunogenic, profile of the conventional vaccine whilst maintaining the attenuated phenotype of CAVA-PV. Furthermore, formalin-inactivated recombinant attenuated poliovirus strains of the present invention can be used as an IPV providing a similar immune response compared to marketed conventional IPV vaccines, while the risks of VAPP and cVDPV are eliminated.

[0049] As defined herein, “does not grow substantially”, “inability to replicate substantially”, “no substantial replication”, and “cannot be substantially propagated” means that the increase in the number of infectious units of the virus compared to the theoretical input/inoculum (based on calculated MOI) is not more than 10%, or preferably not more than 5%, or more preferably not more than 1%, or even more preferably there is no measurable increase in the number of infectious units for the virus. The assay to measure/titrate viral infectious units ($TCID_{50}$ assay) has a limit of detection of 1.7 Log_{10} $TCID_{50}$/ml. In certain embodiments, “does not grow substantially”, “inability to replicate substantially”, “no substantial replication”, and “cannot be substantially propagated” is defined as no measurable increase in viral RNA (genome copies) as compared to the theoretical input/inoculum (calculated genome copies) as measured by quantitative Reverse Transcription PCR (RT-qPCR). In certain embodiments, “does not grow substantially”, “inability to replicate substantially”, “no substantial replication”, and “cannot be substantially propagated” is defined as the lack of visual signs of infection (cytopathic effect) as observed by light microscopy or the lack of visual signs of infection (dead or apoptotic cells with virus induced membrane vesicles or virus lattices) by Electron Microscopy (EM).

[0050] As defined herein, “recombinant” means that the nucleic acid molecule coding for the poliovirus has undergone a molecular biological manipulation combining genetic constituents from two or more different sources, e.g, different clones, different strains, or different organisms. The recombinant attenuated polioviruses of the present invention can be generated by molecular DNA cloning or can be chemically synthesized (*de novo* DNA synthesis), by techniques that are well known and common practice for those skilled in the art. This could even be done by providing the desired recombinant sequence information to a contract manufacturer(e.g. Genscript, GeneArt, BaseClear), which can then produce these molecules according to routine techniques. Any standard manual on DNA technology provides detailed protocols that can be used to produce the recombinant PV strains of the present invention. Brief, exemplary instructions for the construction of such recombinant viruses are provided below in the Examples.

[0051] As defined herein, “attenuated” means that the virulence of the poliovirus has been reduced such that the poliovirus is less pathogenic compared to a parental or starting virus but it is still viable. For instance an attenuated poliovirus of the invention is significantly less neurovirulent compared to wild-type strains and therefore has significantly decreased, if any, capability for causing paralysis. In particular, a preferred attenuated virus strain of the present invention would be safe for use as a vaccine strain, not only when administered in an inactivated form, as intended, but even in the case of accidental infection and/or escape from a manufacturing facility. In certain preferred embodiments, an attenuated virus of the present invention has a (P)LD_{50} of greater than $1x10^7$ $TCID_{50}$ when administered by intra cerebral (i.c.) administration in CD155 transgenic mice.

[0052] As used herein, the phrase “nucleotide”, “nucleic acid” or “nucleic acid molecule” refers to DNA, RNA, as well as any of the known base analogs of DNA and RNA or chimeras formed therefrom. Thus, a “nucleotide”, “nucleic acid” or a “nucleic acid molecule” refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; “RNA molecules”) or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; “DNA molecules”) in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and

secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, the term includes double-stranded DNA found in linear or circular DNA molecules (e.g., restriction fragments or plasmids), as well as single-stranded positive-sense RNA molecules of the poliovirus genome and fragments thereof. In discussing the structure of particular double-stranded DNA molecules or single-stranded RNA molecules, sequences may be described herein according to the normal convention of giving the sequence in the 5' to 3' direction along the single-stranded positive-sense RNA molecules of the poliovirus genome or the non-transcribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

[0053] The Brunenders poliovirus strain was used as a parental strain for the production of CAVA-$PV_{Backbone}$. The Brunenders strain is of serotype 1 and was originally derived from a clinical isolate called the Brunhilde strain. Passaging the Brunhilde strain through twelve serial passages in tissue culture of human origin by Dr. John Enders in 1956 resulted in the Brunenders strain. This strain has been shown to be partially attenuated (Enders 1952, Sanders, Liu et al. 2015) A representative sequence of the Brunenders strain is provided as SEQ ID NO:1. This Brunenders strain was used as the parental strain for the present invention, but some natural variation is common in a virus population.

[0054] It is also shown herein that a CAVA-PV strain can also be prepared using MEF-1 (a type 2 PV that is wild-type and neurovirulent) or Sabin 3 (an attenuated type 3 PV) as parental strains, showing the general applicability of the invention to create CAVA-PV strains that have the phenotype of significant growth at 30°C and no substantial growth at 37°C. A representative sequence of the MEF-1 strain is provided as SEQ ID NO: 5 and Sabin 3 as SEQ ID NO: 8. It will be clear to the skilled person that the mutations inducing the temperature sensitive phenotype are not serotype-specific and therefore other poliovirus strains, such as, for example, Mahoney or Saukett strains, can also be used as the parental strains for creating further CAVA-PV strains with the phenotype of the invention, according to the teachings provided herein.

[0055] As defined herein, a "parental strain" or "starting strain", can be a wild-type strain circulating in or isolated from nature, a known standard laboratory strain, or any other poliovirus strain that has not already been subjected to the CAVA method of the present invention. Non-limiting examples of parental strains are Brunenders, MEF-1,

Mahoney, Saukett, Sabin 1, Sabin 2 or Sabin 3 strains. Representative examples of sequences are provided for these strains herein as follows: Brunenders (SEQ ID NO: 1), MEF-1 (SEQ ID NO: 5), Mahoney (SEQ ID NO: 6), Saukett (SEQ ID NO: 7), Sabin 3 (SEQ ID: NO 8), Sabin 1 (SEQ ID: NO 9) and Sabin 2 (SEQ ID NO: 10).

[0056] The invention also provides a method for obtaining a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, comprising the steps of: a) passaging a (parental) poliovirus strain at a temperature of ≤32°C for sufficient passages to produce a virus with impaired growth at 37°C; b) isolating two or more (e.g. two, three, four, five, or more) different temperature sensitive clones that display impaired growth at 37°C; c) sequencing the genomes of the temperature sensitive clones, d) identifying mutations in the sequences of the genomes of temperature sensitive clones by comparing the sequences of the temperature sensitive clones to the sequence of the parental poliovirus strain; e) synthesizing the recombinant attenuated poliovirus strain by combining mutations from two or more different temperature sensitive clones into the genome of the parental poliovirus strain or into the genome of another poliovirus strain; and f) rescuing the recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C. The passaging in step a) is preferably performed by infecting at a low multiplicity of infection (MOI), e.g. at an MOI between 0.0001 and 1, e.g. between 0.001 and 0.1, e.g. at about 0.01. The temperature for passaging in step a) is 32°C or less, e.g. between about 20°C and 31°C, e.g. between about 24°C and 30°C, e.g. at about 30°C. Any cell line that is permissive to poliovirus growth at this temperature can be used, e.g. Vero, PER.C6, HEK293, etc. The skilled person will appreciate that the number of passages that is required is not critical and can be conveniently determined by screening for a phenotype of impaired growth at 37°C in clones of a dissected viral population, as mutations that would contribute to a cold-adapted phenotype can accumulate at any passage. After a limited number of passages such a phenotype may already be observed, and if not, further passaging may increase the chance of finding clones with this phenotype. Hence, in certain embodiments, the number of passages in step a) can be at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, or more. As used herein, “impaired growth” at 37°C, is defined as at least 10-fold, e.g. 100– to 1000-fold, reduction in maximum titer compared

to wild type virus. In addition, clones with impaired growth also may display slower growth, i.e. longer infection time required to reach the maximum titer, as compared to a wild type or parental virus. In certain embodiments, growth kinetics of the temperature sensitive clones at lower temperature (e.g. 30°C) may be faster as compared to the starting (parental) strain. In one embodiment, to develop a CAVA-PV, the Brunenders parental strain was serially passaged on PER.C6 cells more than 30 times at low temperature (≤30°C) and at low MOI (e.g., 0.01). The resulting virus population was dissected to identify temperature sensitive viral clones in the population with impaired growth at physiological temperatures (37°C) and wild-type growth at low temperature (30°C). Three temperature sensitive clones (which showed impaired growth at 37°C as well as faster growth kinetics at 30°C compared to the parental Brunenders strain) were found by screening of approximately 1000 clones in the viral population at 37°C.

[0057] The 3 temperature sensitive clones were sequenced and a total of 31 mutations were found across the three different clones. Each clone had 18 nucleotide mutations of which some were shared among the different clones and some were unique per clone. These mutations were identified in 3 of the 4 different regions of the PV genome, including the 5'UTR (untranslated region), the capsid, and the non-structural proteins. No mutations were identified in 3'UTR. The 5'UTR contains a cloverleaf structure which is necessary for linkage of the genome to the VpG protein (2B) to form an infectious virion and encapsidation of the RNA into the capsid. The remainder of the 5'UTR is the IRES (Internal Ribosomal Entry Site) which is essential for translation of the viral RNA. As the region does not encode any proteins (untranslated) the element performs its function by directly binding its interacting RNA/protein counterparts, therefore, the secondary structure of this domain is important for its function. The capsid region encodes the outer surface of the viral particle and can be subdivided into four proteins which make up this exterior of the virion, known as the capsid. These four proteins are termed VP1, VP2, VP3 and VP4. The non-structural proteins are subdivided into proteins 2A, 2B, 2C, 3A, 3B, 3C, 3D. The proteins are required for viral replication, polyprotein processing, translation and interactions with host cell components for successful infection. For example, the 2A protease shuts off host cell protein translation via cleavage of eIF4G (Skern and Liebig 1994). The 3'UTR is also an untranslated region required for initiation

of complementary strand synthesis. As with the 5'UTR, the structure of the element enables its function.

[0058] The 31 mutations identified across the three clones included 7 mutations in the IRES, 7 mutations in the capsid, and 17 mutations in the non-structural proteins. A first CAVA-$PV_{Backbone}$ was developed by combining all 31 mutations in the Brunenders wild-type background (and thus the first developed CAVA-PV is a Type 1 poliovirus). See Example 1, Table 1, for the 31 mutations that were combined to produce this CAVA-$PV_{Backbone}$. Also see Figure 1 for a schematic overview of the CAVA method and how the CAVA-$PV_{Backbone}$ was generated.

[0059] The combination of the 31 mutations in one virus genome had a synergistic effect. The CAVA-$PV_{Backbone}$ exhibited an accumulative temperature sensitivity compared to the 3 individual clones, by showing no substantial replication at 37°C in sPER.C6 cells. On the contrary, in the same cells, at 30°C, CAVA-$PV_{Backbone}$ showed similar growth kinetics, or even faster growth, compared to the Brunenders parental strain and other PV1 strains (Example 2, Figures 2 and 3). The growth characteristics for CAVA-$PV_{Backbone}$ were subsequently tested in various other mammalian cell lines and the inability of CAVA-$PV_{Backbone}$ to replicate at physiological temperature of 37°C was confirmed in all the mammalian cell types that were tested (Example 3, Figures 4-9).

[0060] The synthetic combination of mutations found in the clones is deemed essential for obtaining the CAVA-PV phenotype (ie similar growth at 30°C as compared to the parental Brunenders strain and inability to replicate at 37°C). Selection of a virus with a complete loss of replication at 37°C was not possible by serial passage of Brunenders and MEF-1 for more than 30 passages at low temperature. In fact, only 2 - 3 out of approximately 1000 screened clones obtained during passage of Brunenders and MEF-1 showed impairment of growth at 37°C (Fig. 1), whilst increased growth at 30°C as compared to the parental strain was observed in clones after passage of Brunenders and MEF-1. Therefore, the passaging conditions used here offered a selective advantage for improved growth at 30°C but did not favor selection of viruses with impaired replication at 37°C. Only upon synthetic combination of the mutations found in 3 individual clones into the Brunenders genome was the CAVA phenotype observed. Interestingly, the CAVA-PV strains did not display better growth as compared to the parental strain at

30°C (Fig. 3), which was observed with the clones. Therefore, natural combination of these CAVA mutations during passage at 30°C (which are necessary for the CAVA phenotype) would be disadvantageous compared to the starting clones and are therefore unlikely to occur spontaneously. This accentuates the prerequisite for synthetic combination of observed mutations to achieve the CAVA phenotype.

[0061] Thus, the present invention in certain particular embodiments provides a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, wherein the genome of the recombinant attenuated poliovirus strain comprises mutations at at least 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31 of the following positions as compared to the genome of a Brunenders strain (SEQ ID NO: 1): 133 (A), 142 (U), 146 (G), 163 (A), 579 (G), 597 (C), and 609 (G) in the 5'UTR; 805 (A), 1787 (C), 1905 (U), 2756 (U), 3236 (C), 3323 (C), and 3376 (A) in the capsid; and 3476 (C), 3486 (G), 3852 (A), 4120 (U), 4253 (C), 4301 (U), 4428 (A), 4563 (A), 4811 (A), 5436 (G), 5705 (A), 6059 (C), 6210 (A), 6488 (C), 6848 (G), 7079 (U), and 7102 (U) in the non-structural proteins (the nucleotide in the parental strain is indicated between brackets after its position, and thus this is mutated into a different nucleotide in this embodiment). In certain particular embodiments thereof, the genome of the recombinant attenuated poliovirus strain comprises at least 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31 of the following mutations compared to the genome of a Brunenders strain (SEQ ID NO:1): 133 (A to G), 142 (U to C), 146 (G to A), 163 (A to G), 579 (G to A), 597 (C to U), and 609 (G to A) in the 5'UTR; 805 (A to C), 1787 (C to U), 1905 (U to C), 2756 (U to C), 3236 (C to U), 3323 (C to U), and 3376 (A to G) in the capsid; and 3476 (C to U), 3486 (G to A), 3852 (A to U), 4120 (U to C), 4253 (C to U), 4301 (U to C), 4428 (A to G), 4563 (A to U), 4811 (A to G), 5436 (G to A), 5705 (A to G), 6059 (C to U), 6210 (A to G), 6488 (C to U), 6848 (G to A), 7079 (U to C), and 7102 (U to C) in the non-structural proteins.

[0062] In addition, in a transgenic CD155 mouse model (Koike, Taya et al. 1991) used to evaluate poliovirus neurovirulence *in vivo*, the CAVA-PV was shown to be highly attenuated (Example 4, Table 2). In fact, not one mouse infected with the highest possible dose of the CAVA-PV showed any paralysis or any other signs of disease. This is not the case for Sabin 1, a known attenuated PV strain that was also included in the test. In

decreasing order of virulence, the strains tested in the neurovirulence mouse model were Mahoney, Brunhilde, Saukett, Brunenders, Sabin 1-3 and CAVA-PV's. It was determined that the CAVA-PV's are at least 100 times more attenuated than Brunenders, which is a partially attenuated strain, and at least as attenuated as the Sabin strains, which is are well-known attenuated strain that is widely used in many countries for oral vaccination against poliomyelitis.

[0063] Furthermore, by re-engineering a CAVA-PV backbone to contain capsids from different poliovirus strains, CAVA-PV is suitable for use as an attenuated vaccine strain for development of IPV with an antigenic profile of the currently used wild-type IPV vaccines but with an attenuated CAVA-PV phenotype. For example, a CAVA-PV is suitable for use as an attenuated backbone for production of attenuated polioviruses containing the capsids of virulent wild-type PV strains (types 1, 2 and 3, e.g., the type 1 strain Mahoney, type 2 strain MEF-1, and type 3 strain Saukett). This can for instance be done by replacing the capsid sequence from a CAVA-PV strain with a desired capsid sequence using routine molecular biology technology (see e.g. WO 2012/090000 for examples of re-engineering the capsids of attenuated PV strain backbones to those of wild-type strains; however in the referenced case, the resulting strains are still capable of substantial replication at 37°C, in contrast to the CAVA-PV strains of the instant invention). Thus, a CAVA-PV can be used to produce recombinant attenuated polioviruses for IPV. Preferably, a CAVA-PV is engineered to contain the same capsid sequences as the wild-type IPV strains that have been used to successfully immunize the global population since 1952. This will circumvent an altered antigenic (and, therefore, presumed immunogenic) profile from other attenuated strains for IPV, as has been observed for the Sabin strains, upon formalin inactivation. In particularly preferred embodiments therefore, a CAVA-PV is used as the backbone, and the sequence coding for the capsid is exchanged for the sequence coding for the capsid from a Mahoney, MEF-1, or Saukett strain. This results in recombinant poliovirus strains that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, comprising a capsid from a Mahoney, a MEF-1 or a Saukett strain, respectively. In alternative preferred embodiments, mutations that cause the CAVA-PV phenotype (e.g. at least 10, 11, 12 , 13 or 14 of the mutations shown in Table 4 for the Brunenders background strain) are engineered into the corresponding positions of

parental Mahoney, MEF-1 or Saukett strains, which will result in respectively Mahoney, MEF-1 or Saukett strains (which thus have the original capsids of such strains) that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C. The present invention also provides such methods and poliovirus strains obtainable thereby. Also such strains can be used to swap capsids, e.g. a MEF-1 strain that has already been mutated to contain the mutations leading to the CAVA-PV phenotype (growth at 30°C, no substantial growth at 37°C, Fig. 11) can be used as a basis to swap the MEF-1 capsid for a Mahoney or Saukett capsid by further genetic engineering.

[0064] As described in Example 6 below, the sequences coding for the capsids from Mahoney, MEF-1 and Saukett were placed into the background of the CAVA-PV genome by replacing the sequence coding for the CAVA-PV capsid, which corresponds to the capsid of the parental Brunenders strain (nucleotides 747 to 3389 of SEQ ID NO:1). The resulting vaccine strains are CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$, which contain the Mahoney, MEF-1 and Saukett capsids, respectively. As described in Example 7, the growth kinetics of CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ in the suspension PER.C6 (sPER.C6) cells, a suitable production cell line, were all compared to the growth of the Brunenders parental strain. The CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ strains all showed growth kinetics that were similar to that of the parental Brunenders strain at 30°C. On the contrary, at 37°C, The CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ strains showed no substantial replication. These same growth kinetics were observed for the first CAVA-$PV_{Backbone}$ without capsid exchange, demonstrating that the temperature sensitivity of the viruses lies within the mutations outside of the capsid region.

[0065] Thus, the present invention in certain embodiments provides a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, wherein the genome of the recombinant attenuated poliovirus strain comprises mutations at at least 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 of the following positions as compared to the genome of a Brunenders strain (SEQ ID NO: 1): 133 (A), 142 (U), 146 (G), 163 (A), 579 (G), 597 (C), and 609 (G) in the 5'UTR; and 3476 (C), 3486 (G), 3852 (A), 4120 (U), 4253 (C), 4301 (U), 4428 (A), 4563 (A), 4811 (A), 5436 (G), 5705 (A), 6059 (C), 6210 (A), 6488 (C),

6848 (G), 7079 (U), and 7102 (U) in the non-structural proteins (the nucleotide in the parental strain is indicated between brackets after its position, and thus this is mutated into a different nucleotide in this embodiment). In certain particular embodiments thereof, the genome of the recombinant attenuated PV strain comprises at least 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 of the following mutations compared to the genome of a Brunenders strain (SEQ ID NO:1): 133 (A to G), 142 (U to C), 146 (G to A), 163 (A to G), 579 (G to A), 597 (C to U), and 609 (G to A) in the 5'UTR; and 3476 (C to U), 3486 (G to A), 3852 (A to U), 4120 (U to C), 4253 (C to U), 4301 (U to C), 4428 (A to G), 4563 (A to U), 4811 (A to G), 5436 (G to A), 5705 (A to G), 6059 (C to U), 6210 (A to G), 6488 (C to U), 6848 (G to A), 7079 (U to C), and 7102 (U to C) in the non-structural proteins. In certain embodiments, the capsid of such strains as compared to the Brunenders capsid has been replaced with a capsid from a Mahoney, MEF-1, or Saukett strain. Representative capsid amino acid sequences are provided here as Mahoney (SEQ ID NO:2), MEF-1 (SEQ ID NO:3), and Saukett (SEQ ID NO:4), and of course the skilled person is aware that some natural variation is common in a virus population.

[0066] It will also be apparent to those of skill in the art that the mutations identified here for CAVA-PV can be extrapolated to other poliovirus strains because there is a relatively high degree of homology between the genomes of many of the different types of PV strains. Thus, the positions corresponding to the mutations in CAVA-PV can be identified in different strains of poliovirus by aligning the genomic sequences of the different strains. In fact, an alignment has been made containing examples from all three poliovirus serotypes (Toyoda, Kohara et al. 1984). In this way, the attenuated phenotype of CAVA-PV can be transferred to other strains to produce an attenuated CAVA-PV phenotype in new and different backbones. For example, it was determined that of the 31 mutations in CAVA-PV, 11 are unique in CAVA-PV and the parental Brunenders nucleotide at those positions are conserved in all other PV strains used for the alignment (e.g. nucleotide 142 is a C (cytidine) in CAVA-PV, but the nucleotide at that position is a U (uridine) in the Brunenders parental strain and the Brunhilde, Mahoney, Sabin 1, Sabin 2, Sabin 3, MEF-1, and Saukett strains). Furthermore there are 6 other mutations in CAVA-PV for which the parental Brunenders nucleotide at those positions are conserved in all of the PV1 strains used for the alignment (Brunenders, Brunhilde, Mahoney and Sabin 1). There is also 1 mutation that is common in CAVA-PV and Sabin 1, but the

nucleotide at that position is conserved in all other strains. In addition, there are 6 mutations in CAVA-PV at positions that are not conserved in the other strains used for the alignment.

[0067] Based on the analysis of all of the CAVA-PV mutations, 14 mutations were identified that would likely provide a strong contribution to the temperature sensitive (and therefore attenuated) phenotype of CAVA-PV. The 14 mutations are shown in Table 4 (see also Example 10 below). The mutations were selected based on the following criteria: a) conservation among other PV strains; b) experimental evidence based on reversion in the clones after passage at 37°C; c) novel mutations in clones compared to preceding intermediate passage populations that were still capable of growing at 37°C; and, d) mutations that cause amino acid changes or in essential RNA structures (i.e. the IRES). The 14 mutations were engineered into different poliovirus background strains. Thus, a CAVA-PV of the present invention may comprise the 14 mutations in Table 4. A CAVA-PV of the present invention may also comprise the 14 mutations in Table 4 and optionally one more of the other 17 mutations in the first identified CAVA-$PV_{Backbone}$. A CAVA-PV of the invention may also comprise the 14 mutations in Table 4, and optionally further other mutations in the genome compared to a wild-type strain. A CAVA-PV of the present invention may also comprise the 14 mutations in Table 4 and optionally may also comprise a capsid from a Mahoney MEF-1, or Saukett strain. It will also be clear that it is possible for the skilled person, using the teachings herein, to make further CAVA-PV strains that have only a subset of the 14 mutations of Table 4 (e.g. 8, 9, 10, 11, 12 or 13 of these, either in the Brunenders background strain or on corresponding positions in other PV background strains) and test such strains for the CAVA-PV phenotype, and in such way potentially obtain additional CAVA-PV strains.

[0068] Thus, the present invention in certain embodiments provides a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, wherein the genome of the recombinant attenuated poliovirus strain comprises mutations at at least 10, 11, 12, 13 or 14 of the following positions as compared to the genome of a Brunenders strain (SEQ ID NO:1): 133 (A), 142 (U), 163 (A), 597 (C), and 609 (G) in the 5'UTR; and 3486 (G), 3852 (A), 4120 (U), 4428 (A), 4563 (A), 5436 (G), 6210 (A), 6848 (G), and 7102 (U) in the non-structural proteins (the nucleotide in the parental strain is indicated between

brackets after its position, and thus this is mutated into a different nucleotide in this embodiment). In certain particular embodiments thereof, the genome of the recombinant attenuated PV strain comprises at least 10, 11, 12, 13 or 14 of the following mutations as compared to the genome of a Brunenders strain (SEQ ID NO: 1): 133 (A to G), 142 (U to C), 163 (A to G), 597 (C to U), and 609 (G to A) in the 5'UTR; and 3486 (G to A), 3852 (A to U), 4120 (U to C), 4428 (A to G), 4563 (A to U), 5436 (G to A), 6210 (A to G), 6848 (G to A), and 7102 (U to C) in the non-structural proteins. In certain preferred embodiments, the capsid of such strains has been replaced with a capsid from a Mahoney, MEF-1, or Saukett strain.

[0069] The recombinant attenuated poliovirus strain of the present invention may also be derived from a poliovirus strain other than the Brunenders strain (e.g., Brunhilde, Mahoney, Sabin 1, Sabin 2, Sabin 3, MEF-1, or Saukett strain, or strains derived from any of these, or other strains) by transferring the mutations from CAVA-PV to the homologous nucleotides in the genome of the other poliovirus strain. Furthermore, the recombinant attenuated poliovirus strain of the present invention may be derived from a poliovirus strain other than the Brunenders strain and optionally comprise a capsid from a different poliovirus strain. As PV strains show some variation in their genome lengths the exact nucleotide position of a mutation can vary upon alignment of the various PV sequences. Therefore the CAVA mutations differ slightly in nucleotide number when extrapolated from the Brundeners background to the MEF-1 strain or other poliovirus strains, according to how the sequences align. The corresponding nucleotide positions of CAVA mutations for the Mahoney (PV1) , MEF-1 (PV2), Saukett (PV3), Sabin 1 (PV1), Sabin 2 (PV2) and Sabin 3 (PV3) strains, as compared to the numbering for the Brunenders strain, are provided herein (Table 4).

[0070] Thus, as a further non-limiting example, the present invention in certain embodiments provides a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, wherein the genome of the recombinant attenuated poliovirus strain comprises mutations at at least 10, 11, 12, 13 or 14 of the following positions as compared to the genome of a MEF-1 strain (SEQ ID NO:5): 134 (A), 143 (U), 164 (A), 598 (C), and 610 (G) in the 5'UTR; and 3481 (A), 3847 (A), 4115 (U), 4423 (A), 4558 (A), 5431 (G), 6205 (A), 6843 (G), and 7097 (U) in the non-structural proteins (the nucleotide in the parental

MEF-1 strain is indicated between brackets after its position, and thus this is mutated into a different nucleotide in this embodiment). In certain particular embodiments thereof, the genome of the recombinant attenuated poliovirus strain comprises at least 10, 11, 12, 13 or 14 of the following mutations as compared to the genome of a MEF-1 strain (SEQ ID NO: 5): 134 (A to G), 143 (U to C), 164 (A to G), 598 (C to U), and 610 (G to A) in the 5'UTR; and 3481 (A to A), 3847 (A to U), 4115 (U to C), 4423 (A to G), 4558 (A to U), 5431 (G to A), 6205 (A to G), 6843 (G to A), and 7097 (U to C) in the non-structural proteins. In certain embodiments, the capsid of such strains can be replaced with a capsid from a Mahoney, or from a Saukett strain.

[0071] In further non-limiting examples, the present invention in certain embodiments provides a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, wherein the genome of the recombinant attenuated poliovirus strain comprises mutations at at least 10, 11, 12, 13 or 14 of the following positions as compared to the genome of a Mahoney strain (SEQ ID NO: 6): 131 (A), 140 (U), 161 (A), 593 (C), and 605 (G) in the 5'UTR; and 3482 (G), 3848 (A), 4116 (U), 4424 (A), 4559 (A), 5432 (G), 6206 (A), 6844 (G), and 7098 (U) in the non-structural proteins (the nucleotide in the parental Mahoney strain is indicated between brackets after its position, and thus this is mutated into a different nucleotide in this embodiment). In certain particular embodiments thereof, the genome of the recombinant attenuated poliovirus strain comprises at least 10, 11, 12, 13 or 14 of the following mutations as compared to the genome of a Mahoney strain (SEQ ID NO: 6): 131 (A to G), 140 (U to C), 161 (A to G), 593 (C to U), and 605 (G to A) in the 5'UTR; and 3482 (G to A), 3848 (A to U), 4116 (U to C), 4424 (A to G), 4559 (A to U), 5432 (G to A), 6206 (A to G), 6844 (G to A), and 7098 (U to C) in the non-structural proteins. In certain embodiments, the capsid of such strains can be replaced with a capsid from a MEF-1, or from a Saukett strain.

[0072] In yet further non-limiting embodiments, the invention provides a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, wherein the genome of the recombinant attenuated poliovirus strain comprises mutations at at least 10, 11, 12, 13 or 14 of the following positions as compared to the genome of a Saukett strain (SEQ ID NO: 7): 133 (A), 142 (U), 163 (A), 596 (C), and 608 (G) in the 5'UTR; and 3472 (A), 3839

(A), 4107 (U), 4415 (A), 4550 (A), 5423 (G), 6197 (A), 6835 (G), and 7089 (U) in the non-structural proteins (the nucleotide in a parental Saukett strain is indicated between brackets after its position, and thus this is mutated into a different nucleotide in this embodiment). In certain particular embodiments thereof, the genome of the recombinant attenuated poliovirus strain comprises at least 10, 11, 12, 13 or 14 of the following mutations as compared to the genome of a Saukett strain (SEQ ID NO: 7): 133 (A to G), 142 (U to C), 163 (A to G), 596 (C to U), and 608 (G to A) in the 5'UTR; and 3472 (A to A), 3839 (A to U), 4107 (U to C), 4415 (A to G), 4550 (A to U), 5423 (G to A), 6197 (A to G), 6835 (G to A), and 7089 (U to C) in the non-structural proteins. In certain embodiments, the capsid of such strains can be replaced with a capsid from a MEF-1, or from a Mahoney strain.

[0073] In yet further non-limiting embodiments, the invention provides a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, wherein the genome of the recombinant attenuated poliovirus strain comprises mutations at at least 10, 11, 12, 13 or 14 of the following positions as compared to the genome of a Sabin 3 strain (SEQ ID NO: 8): 133 (A), 142 (U), 163 (G), 596 (C), and 608 (G) in the 5'UTR; and 3473 (A), 3839 (A), 4107 (U), 4415 (A), 4550 (A), 5423 (G), 6197 (A), 6835 (G), and 7089 (U) in the non-structural proteins (the nucleotide in a parental Sabin 3 strain is indicated between brackets after its position, and thus this is mutated into a different nucleotide in this embodiment). In certain particular embodiments thereof, the genome of the recombinant attenuated poliovirus strain comprises at least 10, 11, 12, 13 or 14 of the following mutations as compared to the genome of a Sabin 3 strain (SEQ ID NO: 8): 133 (A to G), 142 (U to C), 163 (G to G), 596 (C to U), and 608 (G to A) in the 5'UTR; and 3473 (A to A), 3839 (A to U), 4107 (U to C), 4415 (A to G), 4550 (A to U), 5423 (G to A), 6197 (A to G), 6835 (G to A), and 7089 (U to C) in the non-structural proteins. In certain embodiments, the capsid of such strains can be replaced with a capsid from a Mahoney , MEF-1, or from a Saukett strain.

[0074] In further non-limiting examples, the present invention in certain embodiments provides a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, wherein the genome of the recombinant attenuated poliovirus strain comprises mutations at at least

10, 11, 12, 13 or 14 of the following positions as compared to the genome of a Sabin 1 strain (SEQ ID NO: 9): 131 (A), 140 (U), 161 (A), 593 (C), and 605 (G) in the 5'UTR; and 3482 (G), 3848 (A), 4116 (C), 4424 (A), 4559 (A), 5432 (G), 6206 (A), 6844 (G), and 7098 (U) in the non-structural proteins (the nucleotide in the parental Sabin 1 strain is indicated between brackets after its position, and thus this is mutated into a different nucleotide in this embodiment). In certain particular embodiments thereof, the genome of the recombinant attenuated poliovirus strain comprises at least 10, 11, 12, 13 or 14 of the following mutations as compared to the genome of a Sabin 1 strain (SEQ ID NO: 9): 131 (A to G), 140 (U to C), 161 (A to G), 593 (C to U), and 605 (G to A) in the 5'UTR; and 3482 (G to A), 3848 (A to U), 4116 (C to C), 4424 (A to G), 4559 (A to U), 5432 (G to A), 6206 (A to G), 6844 (G to A), and 7098 (U to C) in the non-structural proteins. In certain embodiments, the capsid of such strains can be replaced with a capsid from a Mahoney, MEF-1, or from a Saukett strain.

[0075] In further non-limiting examples, the present invention in certain embodiments provides a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, wherein the genome of the recombinant attenuated poliovirus strain comprises mutations at at least 10, 11, 12, 13 or 14 of the following positions as compared to the genome of a Sabin 2 strain (SEQ ID NO: 10): 131 (A), 140 (U), 161 (A), 594 (C), and 606 (G) in the 5'UTR; and 3481 (G), 3847 (A), 4115 (U), 4423 (A), 4558 (A), 5431 (G), 6205 (A), 6844 (G), and 7098 (U) in the non-structural proteins (the nucleotide in the parental Sabin 2 strain is indicated between brackets after its position, and thus this is mutated into a different nucleotide in this embodiment). In certain particular embodiments thereof, the genome of the recombinant attenuated poliovirus strain comprises at least 10, 11, 12, 13 or 14 of the following mutations as compared to the genome of a Sabin 2 strain (SEQ ID NO: 10): 131 (A to G), 140 (U to C), 161 (A to G), 594 (C to U), and 606 (G to A) in the 5'UTR; and 3481 (G to A), 3847 (A to U), 4115 (U to C), 4424 (A to G), 4559 (A to U), 5432 (G to A), 6206 (A to G), 6844 (G to A), and 7098 (U to C) in the non-structural proteins. In certain embodiments, the capsid of such strains can be replaced with a capsid from a Mahoney, MEF-1, or from a Saukett strain.

[0076] The invention also provides a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell

culture at 37°C, wherein the genome of the recombinant attenuated poliovirus strain comprises the following nucleotides at at least 10, 11, 12, 13 or 14 of the following positions as compared to the genome of a parent poliovirus strain (wherein the parent poliovirus strain is for instance a Brunenders/MEF-1/Mahoney/Saukett/Sabin 3/Sabin 2/Sabin 1 strain, respectively, with respective genome sequences as in for instance SEQ ID Nos: 1/5/6/7/8/9/10): G at position 133/134/131/133/133/131/131, C at position 142/143/140/142/142/140/140, G at position 163/164/161/163/163/161/161, U at position 597/598/593/596/596/593/594, and A at position 609/610/605/608/608/605/606 in the 5'UTR; and A at position 3486/3481/3482/3473/3473/3482/3481, U at position 3852/3847/3848/3839/3839/3848/3847, C at position 4120/4115/4116/410741407/4116/4115, G at position 4428/4423/4424/4415/4415/4424/4423, U at position 4563/4558/4559/4550/4550/4559/4558, A at position 5436/5431/5432/5423/5423/5423/5431, G at position 6210/6205/6206/6197/5197/6206/6205, A at position 6848/6843/6844/6835/6835/6844/6843, and C at position 7102/7097/7098/7089/7089/7098/7097 in the non-structural proteins, or in corresponding positions in other PV parent strains based on alignment with the sequences for these four strains. In certain embodiments, the capsid of such strains comprises a capsid sequence from a Mahoney, from a MEF-1, or from a Saukett strain.

[0077] The invention thus also provides further methods for generating CAVA-PV strains, by introducing mutations (e.g. via routine genetic engineering, or via *de novo* synthesis of complete poliovirus genomes) in a wild-type poliovirus genome (e.g. from Brunenders, Mahoney, MEF-1, Saukett, or other PV strains), such that the genome comprises at least 10, 11, 12, 13 or 14 of the following nucleotides at the following positions: G at position 133, C at position 142, G at position 163, U at position 597, and A at position 609 in the 5'UTR; and A at position 3486, U at position 3852, C at position 4120, G at position 4428, U at position 4563, A at position 5436, G at position 6210, A at position 6848, and C at position 7102 in the non-structural proteins with reference to a Brunenders strain (SEQ ID NO: 1), or corresponding positions in other PV strains, for instance as provided in Table 4.

[0078] The recombinant attenuated CAVA-PV strains of the invention typically are also genetically stable under envisioned production conditions and due to their inability to replicate at physiological conditions of 37°C they are highly unlikely to revert to a neurovirulent form upon accidental infection and/or escape from a manufacturing facility. Serial passage of CAVA-PV strains at 37°C always leads to in ability to quantify virus after the first passage, indicating inability to revert at this temperature, even after more than 10 blind passages, to regain ability for replication at 37°C. This gives CAVA-PV an advantage over other attenuated strains that are capable of replication at physiological temperature. Thus, CAVA-PV provides for development of IPV vaccines with safer vaccine manufacturing procedures with potentially lower bio-containment thresholds because of the mitigated risk of potential disease outbreaks in the case of industrial accidents. In this way, the inherent safety of CAVA-PV as the basis of IPV may help to control costs of IPV manufacture, as well as may allow manufacturing in countries where manufacturing with wild-type PV is restricted or poses a high risk. In addition, the CAVA-PV strains can be grown in suspension cultures of PER.C6 cells at high densities, which provides high yields and therefore the use of this production cell line can also contribute to significantly lower the costs of IPV production compared to other cell lines. See, for example, U.S. Patent No. 8,546,123 and Sanders, Edo-Matas et al. (2012).

[0079] CAVA-PV strains can be propagated by methods that are well known by those skilled in the art. For example, CAVA-PV can be propagated by culturing in a permissive cell line (e.g. PER.C6, or Vero cells, HEK293 cells, HeLa, L20B, etc), and at permissive temperatures (e.g., 20-33°C, 26-33°C, 28-32°C, or preferably at about 30°C). Suitable culture media for such cell lines are widely known and available from various manufacturers. Preferably, serum-free culture media are used, and in certain embodiments cells are cultured in suspension. Harvesting of the virus is typically performed when the maximum titer is reached, this is dependent on the MOI used and the incubation temperature. In general an MOI of 1 will reach the maximum titer between 12-48 hours post infection (hpi), e,g, 18-30 hpi, e.g. around 24 hpi at 30°C.

[0080] Methods for harvesting and purifying poliovirus or viral components, and production of vaccines therefrom are used in the art for decades, and thus are well known and have been amply described, see, for example, WO 2007/007344; U.S. Pat. No.:

4,525,349; and (van Wezel, van Steenis et al. 1978, Montagnon, Fanget et al. 1984), all incorporated by reference herein.

[0081] In general, each of the poliovirus strains is cultured in a separate process, and if for instance a trivalent vaccine containing three types of poliovirus is prepared, the (inactivated, for IPV) viruses are mixed and formulated for preparation of individual dosages. In certain embodiments for example, a final vaccine per dose may for instance comprise different amounts of each CAVA-PV. In certain embodiments, this can be done with CAVA type 1 (CAVA-$PV_{Mahoney}$), type 2 (CAVA-PV_{MEF-1}) and type 3 (CAVA-$PV_{Saukett}$) strains. In certain embodiments, a final vaccine per dose (e.g. 0.5 ml) may for instance comprise 10-80, e.g. 40, D-antigen units (DU) of type 1, 2-20, e.g. 8 DU of type 2 and 8-64, e.g. 32 DU of type 3, as determined by comparison to reference preparations.

[0082] Inactivation of CAVA-PV can be done according to methods that are well known to those skilled in the art, for instance with formalin or with β-propiolactone (BPL), see, for example, (Jiang, Pye et al. 1986). In certain embodiments, inactivation is performed with formalin, for example by the following method: the purified viral suspension is filtered over a 0.22 μm membrane, heating to 37°C with steady magnetic stirring for 24 hours, after which a formalin solution is added to achieve a concentration of 1 per 4,000. While keeping the viral suspension at 37°C, the magnetic stirring is continued for the first four days. On the sixth day, the viral suspension is filtered over a 0.22 micron membrane, and inactivation is continued under suspension at 37°C until the twelfth day. The inactivated viral suspension is homogenized and may be stored, e.g., at 4° C. After this step, concentrated and/or final batches for administration may be prepared for instance by mixing the desired preparations.

[0083] In certain embodiments, the purified CAVA-PV or viral component is formulated into a pharmaceutical composition. This can be done according to a variety of methods and using a variety of buffers all according to routine methods well known to the person skilled in the art after reviewing the instant disclosure. In general, it entails bringing the poliovirus particles in a pharmaceutically acceptable composition, comprising the poliovirus and at least a pharmaceutically acceptable excipient. Such a composition may be prepared under conditions known to the skilled person, and in certain embodiments is suitable for administration to humans. In certain embodiments, the

composition may comprise buffered culture medium, which may optionally be Medium M-199, which is used as formulation buffer for certain registered conventional IPVs. Further, phosphate buffered saline may be used, and the final dosage formulations may comprise for instance 0.5% of 2-phenoxyethanol and a maximum of 0.02% of formaldehyde per dose as antimicrobial preservatives.

[0084] Pharmaceutically acceptable carriers or excipients and diluents are well known in the art and used extensively in a wide range of therapeutic products. Preferably, carriers are applied that work well in vaccines. In certain embodiments, the vaccines further comprise an adjuvant, e.g., alum. Adjuvants are known in the art to further increase the immune response to an applied antigenic determinant.

[0085] For administering to humans, the invention may employ pharmaceutical compositions comprising the CAVA-PV and a pharmaceutically acceptable carrier or excipient. In the present context, the term "Pharmaceutically acceptable" means that the carrier or excipient, at the dosages and concentrations employed, will not cause any unwanted or harmful effects in the subjects to which they are administered. Such pharmaceutically acceptable carriers and excipients are well known in the art.

[0086] The purified inactivated CAVA-PV or immunogenic parts thereof preferably are formulated and administered as a sterile solution. Sterile solutions may be prepared by, e.g., sterile filtration or by other methods known in the art. The solutions are then lyophilized or filled into pharmaceutical dosage containers. The pH of the solution generally is in the range of pH 3.0 to 9.5, e.g., pH 5.0 to 7.5. The poliovirus or immunogenic parts thereof typically are in a solution having a suitable pharmaceutically acceptable buffer, and the solution of poliovirus may also contain a salt. Optionally stabilizing agent may be present, such as albumin. In certain embodiments, detergent is added. In certain embodiments, the vaccine may be formulated into an injectable preparation. These formulations contain effective amounts of poliovirus or immunogenic parts thereof, are either sterile liquid solutions, liquid suspensions or lyophilized versions and optionally contain stabilizers or excipients.

[0087] The CAVA-PV vaccine obtainable according to the present invention can be monovalent, containing one type of poliovirus (type 1, 2 or 3), or bivalent (containing two

types of poliovirus, e.g., types 1 and 2, 1 and 3, or 2 and 3), or trivalent (containing three types of poliovirus, e.g., types 1, 2 and 3).

[0088] Furthermore, in addition to being used as a stand-alone IPV, the CAVA-PV based IPV obtainable according to methods of the present invention can be combined with other vaccines in the regular manner, e.g., in the form of a combined vaccine that can optionally include further vaccine components, e.g., against one or more of diphtheria, tetanus, pertussis, *Heamophilus influenzae* type b (Hib), Hepatitis B virus (HBV), etc, like is commonly done for conventional IPV (see e.g. "Vaccines." 5th edition. S. Plotkin, W. Orenstein, P. Offit, 2008, Section 2, for various components and combination vaccines; e.g., Chapter 25 describes IPV vaccines [Plotkin, pp 605-630]. Thus, the CAVA-PV vaccine obtainable according to the present invention is suitable for use in the expanded program on immunization (EPI), and can be combined with the vaccines in that program. Similarly to conventional IPV, the CAVA-PV vaccine according to the invention can be given as a single dose, or preferably in prime-boost regimens wherein multiple doses of vaccine are administered with appropriate time intervals. For example, as recommended by the WHO for countries with high immunization coverage (>90%), the schedule could include a primary series of three doses, beginning at 2 months of age (e.g. at 2, 3 and 4 months). Additionally, if the primary series begins earlier (e.g. with a 6-, 10- and 14-week schedule), a booster dose could be administered after an interval of at least 6 months, i.e., a four- dose schedule. Furthermore, a CAVA-PV vaccine according to the present invention could also be used in combination with OPV as has been suggested by WHO for use of conventional IPV. For example, WHO recommends that all countries currently using only OPV add at least 1 dose of IPV to the schedule. In polio-endemic countries and in countries at high risk for importation and subsequent spread, WHO also recommends an OPV dose at birth (also called 'zero dose'), followed by the primary series of 3 OPV doses and at least 1 IPV dose. Ultimately, the optimal dosage regime can be determined according to standard medical practice and will generally follow the same schemes as those for the available IPVs.

[0089] It will also be apparent to those of skill in the art that the therapeutically effective amount of the CAVA-PV vaccine obtainable according to the present invention will depend upon the administration schedule, the unit dose of recombinant polioviruses

administered, whether the recombinant attenuated polioviruses is administered in combination with other therapeutic agents, and the status and health of the patient.

[0090] The present invention also includes a kit for administering a composition comprising the CAVA-PV vaccine obtainable according to the present invention can and a pharmaceutically acceptable carrier. The kit comprises a recombinant attenuated poliovirus as disclosed herein. The kit can optionally further comprise a pharmaceutically acceptable carrier, an applicator, such as a syringe, and an instructional material for the use thereof. The instructions can provide any information that is useful for directing the administration of the recombinant attenuated poliovirus or for propagating the virus.

[0091] Various publications, which may include patents, published applications, technical articles and scholarly articles, are cited throughout the specification in parentheses, and full citations of each may be found at the end of the specification. Each of these cited publications is incorporated by reference herein, in its entirety.

[0092] Other embodiments, features, and advantages of the invention are further illustrated by reference to the following examples.

EXAMPLES

[0093] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. The following working examples therefore, specifically point out certain embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Example 1: Method for Cold-Adapted-Viral-Attenuation (CAVA) and production of a CAVA Poliovirus (CAVA-PV)

[0094] For this example, a Brunenders parental strain was serially passaged 34 times in PER.C6 cells at low temperature (≤30°C) and at low MOI (0.01) in Permexcis™ medium (chemically defined serum-free medium, e.g. available from Lonza, cat. # BE02-039Q) supplemented with 4 mM L-Glutamine. The resulting virus population was dissected to identify temperature sensitive viral clones in the population with impaired growth at physiological temperatures (37°C) and wild-type growth at low temperature

(30°C). In screening of approximately 1000 clones, 3 clones (named G12P5, F9P4, and G11P3) were found that showed temperature sensitivity with impaired growth at 37°C. Impaired growth was defined as a 100- to 1000-fold reduction in maximum titer compared to parental virus. The 3 clones were still capable of replicating at physiological temperature, but to lower titers. Growth kinetics of the 3 clones at lower temperature (30°C) were faster compared to the starting parental strain. The temperature sensitive clones were sequenced and a total of 31 mutations were found across the three different clones. Each clone had 18 nucleotide mutations of which some were shared among the different clones and some were unique per each clone.

[0095] To generate a novel recombinant poliovirus strain, referred to here as Cold-Adapted-Viral-Attenuation Poliovirus (CAVA-PV), all 31 mutations identified in the 3 clones were combined into one genome using the parental Brunenders sequence as the backbone. The parental Brunenders sequence is provided here as SEQ ID NO:1. A CAVA-PV sequence was synthesized and the CAVA-$PV_{Backbone}$ was rescued. In brief, the sequence for the recombinant attenuated poliovirus CAVA-$PV_{Backbone}$ strain was generated synthetically in the form of a cDNA plasmid, wherein the viral genome sequence is directly downstream of a phage T7 promoter which is necessary for production of viral RNA. For rescue, a cDNA plasmid containing the CAVA-$PV_{Backbone}$ genome sequence was used as a template for *in vitro* transcription mediated by the T7 polymerase to produce the viral RNA which was subsequently used for transfection of cells and virus rescue. This rescue procedure is used frequently in the art, see, for example, (van der Werf, Bradley et al. 1986). Table 1 shows the 31 mutations that were combined to produce a CAVA-$PV_{Backbone}$. Figure 1 is a schematic overview of the CAVA method and how a first CAVA-PV was generated.

Example 2: Growth kinetics of CAVA-$PV_{Backbone}$ in sPER.C6 cells compared to other Type 1 PV strains

[0096] Growth kinetics of CAVA-$PV_{Backbone}$ in the suspension PER.C6 (sPER.C6) cells, a production cell line, was compared to the growth of other Type 1 PV (PV1) strains at 30 and 37°C. The other PV1 strains were Brunhilde, Brunenders, Mahoney, and Sabin 1. Brunhilde is the parental strain of Brunenders which is in turn the parental strain of CAVA-$PV_{Backbone}$. Mahoney is a wild-type, neurovirulent PV strain which is typically

used as the vaccine strain for the type 1 component of Salk's IPV. Sabin 1 is an attenuated strain used as the vaccine strain for the live attenuated oral poliovirus vaccine (OPV). The suspension PER.C6 cells at time of infection had a cell density of $10x10^6$ cells/ml in Permexcis media supplemented with 4 mM L-Glutamine. Cells were infected with an MOI of 2, the infections were performed once (N=1). Viral harvests were titrated using a $TCID_{50}$ assay at 30°C to give the infectious dose where 50% of the samples showed infection (CPE) which is the Tissue Culture Infectious Dose 50% ($TCID_{50}$) per ml. Line graphs for the replication kinetics in the sPER.C6 cells are shown in Figure 2. CAVA-$PV_{Backbone}$ showed wild-type growth kinetics, or even faster growth, as compared to the other PV1 strains at 30°C. On the contrary, at 37°C, CAVA-$PV_{Backbone}$ surprisingly showed no significant replication indicating that there was a synergistic accumulative effect from combining the mutations from the 3 different temperature sensitive clones. The other PV1 strains all replicated normally and showed similar growth behaviour in sPER.C6 cells at 37°C.

[0097] The growth kinetics of CAVA-PV and Brunenders in sPER.C6 cells were further evaluated in three independent experiments and the average titer was plotted over time at 30°C and 37°C. The average titer is plotted in Figure 3, with error bars representing standard deviation from the mean. The growth curves show similar kinetics for both viruses at 30°C , but at 37°C CAVA-$PV_{Backbone}$ does not replicate; only the input virus is measured during this assay. The parental Brunenders strain showed no impairment in growth at 37°C. The average maximum titer of CAVA-$PV_{Backbone}$ at 30°C was 9.82 Log_{10} $TCID_{50}$/ml, which is similar to titers attained with wild-type strains on sPER.C6 cells (Sanders, Edo-Matas et al. 2012).

Example 3: Growth kinetics of CAVA-$PV_{Backbone}$ in various cell lines in comparison to Brunenders starting virus

[0098] A successful infection is a complex interplay between virus and host cell, hence, temperature sensitivity may be influenced by host cell factors. Therefore, a panel of various mammalian cell lines was examined for viral growth to confirm the inability of CAVA-$PV_{Backbone}$ to replicate at physiological conditions.

[0099] Figures 4, 5 and 6 show the replication kinetics of CAVA-$PV_{Backbone}$ and Brunenders in HEK293, L20B and HeLa cells, respectively. For each cell line $1x10^6$ cells

were infected at three different temperatures (30°C, 37°C and 39.5°C) with an MOI of 2 in DMEM medium+1% BCS. The viral titers were quantified by plaque assay on HelaR19 cells at 30°C which results in the number of Plaque Forming Units (PFU's) in the viral harvest. The results confirm that CAVA-$PV_{Backbone}$ does not replicate at 37°C or 39.5°C in all three cell lines. At 30°C, however, the growth kinetics of CAVA-$PV_{Backbone}$ are similar to parental Brunenders PV strain. The Brunenders virus shows no impairment in replication in any of the cell lines at any of the temperatures, but titers are slightly lower at 39.5°C compared to the lower temperatures.

[00100] Figures 7, 8 and 9 show the replication kinetics of CAVA-$PV_{Backbone}$ and Brunenders in Vero, SK-N-MC and adherent PER.C6 (adPER.C6) cells, respectively. These infections were performed at either 30°C or 37°C at an MOI of 2 in MEM medium +5% FBS (Vero and SK-N-MC cells) or DMEM+10%FBS+4.9 mM $MgCl_2$ (adPER.C6). Viral harvests were titrated using a $TCID_{50}$ assay at 30°C. The results for Vero and adPER.C6 concur with what has been observed previously: CAVA-$PV_{Backbone}$ only shows replication at 30°C, comparable to that of the Brunenders strain. Again, at 37°C a lack of replication is observed.

[00101] For the SK-N-MC cell line (Figure 8), CAVA-$PV_{Backbone}$ was unable to replicate at both temperatures. This cell line is a human neuronal cell line derived from a neuroepithelioma and has been used as an *in vitro* model for neurovirulence (Jahan, Wimmer et al. 2011). Although this is not a validated predictive *in vitro* assay for neuro-attenuation of a virus strain, the inhibited viral growth of CAVA-$PV_{Backbone}$ in this cell line at both 30°C and 37°C may indicate an inability to replicate in neuronal cell lines and predict neuro-attenuation.

Example 4: Neurovirulence testing in CD155 transgenic mice

[00102] To determine whether the *in vitro* temperature sensitive phenotype translates to neuro-attenuation, an *in vivo* transgenic CD155 mouse model was used (Koike, Taya et al. 1991). CD155 transgenic mice are genetically modified to express the poliovirus receptor (PVR or CD155) which results in susceptibility of the mice to PV infection. The CD155 mice were infected with CAVA-PV's and other selected PV strains. The mice were infected either intra cerebrally (i.c.), intra muscularly (i.m.) or intra peritoneally (i.p.) with varying doses to determine the (Paralytic or) Lethal Dose 50 ((P)LD_{50}), which

corresponds to the number of infectious units (expressed in$TCID_{50}$) needed to cause paralysis or death to 50% of the mice in a given test group. The lower the (P)LD_{50} the more neurovirulent the virus. Table 2 shows the results for the *in vivo* neurovirulence test. For the CAVA-PV's (active CAVA-$PV_{Backbone}$, CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$) the maximum amount of virus administered to mice did not result in any signs of paralysis in any of the mice injected, therefore, the (P)LD_{50} is given as more than (>) the maximum dose that could be administered. As an indication of neurovirulence the (P)LD_{50}'s of other PV viruses were also determined. Brunenders and Brunhilde are the parental strains of CAVA-PV while Mahoney, MEF-1, Saukett and the Sabin viruses are the vaccine strains of IPV and OPV, respectively. Mahoney is the most virulent virus, followed by Brunhilde, Saukett, Brunenders, MEF-1 and the Sabin strains. This concurs with the neurovirulence data in literature of these strains. The CAVA-PV's are less neurovirulent than any of the wild-type strains strains. CAVA-PV's are at least 1 million times more attenuated than Mahoney via the intra cerebral administration route (the most sensitive route used here, to measure the ability of a virus to destroy neuronal cells and cause paralysis). CAVA-PV's are at least 100 times more attenuated than Brunenders, which is a partially attenuated strain. To ascertain whether the CAVA strains are more attenuated than Sabin one would need to use a more sensitive model for determination of neurovirulence as this model is not sensitive enough to discriminate between Sabin and CAVA strains. However, the Sabin strains did induce paralysis in some of the mice administered with the highest dose, whereas the CAVA strains did not.

The (P)LD_{50}'s of two known attenuated viruses (RIPO and Sabin 1 have been reported to be $>10^8$ and $5x10^7$ PFU, respectively, in the same neurovirulence model (Bouchard, Lam et al. 1995, Jahan, Wimmer et al. 2011). However, these values were not determined in the same experiment, therefore caution should be exerted when directly comparing these (P)LD_{50}'s. The RIPO strain is an attenuated oncolytic poliovirus strain licensed for use in clinical trials to treat malignant glioma (Jahan, Wimmer et al. 2011). The CAVA-PV strains perform in a similar fashion as these attenuated strains. In fact, not one mouse infected with the highest possible doses of the CAVA-PV strains showed any signs of disease, while this is not necessarily the case for other attenuated viruses (e.g., Sabin 1, which is the type 1 component of OPV, a licensed live attenuated vaccine, administered

to millions of children per year). Thus, in this neurovirulence model, CAVA-PV is shown to be highly attenuated.

Example 5: Genetic stability of CAVA-PV's under production conditions

[00103] The CAVA-$PV_{Backbone}$ strain was passaged at small scale under envisioned production conditions (sPER.C6 cells with a cell density of $10x10^6$ vc/ml in Permexcis medium, at an MOI of 1, at 30°C) and harvested at 24 hpi. The passaging was done 8 times which represents 5 passages beyond a theoretical commercial manufacturing batch. After these passages the entire genome was sequenced. None of the 31 mutations that were introduced to the virus reverted. The entire genome after passaging was identical to the starting stock except for one nucleotide which showed a mixed population at nucleotide 5206 in the 3A gene, causing an amino acid substitution. Due to the large error rate of the RNA polymerase of polioviruses we assume this mutation to be random and not inducing any reversion of the temperature sensitive (and attenuated) phenotype. We subsequently confirmed the temperature sensitive phenotype by performing replication kinetics and observed that the 8x passaged CAVA-PV showed similar growth curves as compared to the CAVA-PV starting stock at both 30°C and 37°C (Fig. 10). Thus, the temperature sensitive phenotype of CAVA-$PV_{Backbone}$, an *in vitro* indication of neuroattenuation, is stable after multiple passages under production conditions. This would make CAVA-PV suitable for use in the manufacturing process for production of batches for commercial use.

[00104] The same genetic stability testing was performed using the envisioned vaccine strains namely; CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett.}$ Here, viruses were passages thrice (n=3) at small scale under envisioned production conditions (sPER.C6 cells with a cell density of $10x10^6$ vc/ml in Permexcis medium, at an MOI of 1, at 30°C and harvested at 24 hpi). The number of passages was 5, which represents two passages beyond a theoretical commercial lot. Fig. 11 depicts the *in vitro* phenotype of all passaged viruses as compared to the starting stock. All viruses retained their temperature sensitive phenotype, as well as their *in vivo* attenuation, where the PLD_{50}'s were still $>10^8$ for the three serotypes after extended passage. Sequencing of the passaged viruses of Fig 11 showed that only few mutations arose, and as described above, these mutations did not affect the attenuation *in vivo* and *in vitro*.

Example 6: Introduction of the conventional IPV antigenic profile into CAVA-PV

[00105] To generate a CAVA-PV based IPV that can display the same immune profile as the conventional IPV, the sequences coding for the capsids of some exemplary viruses were placed into the background of the CAVA-PV genome by replacing the sequence coding for the original CAVA-$PV_{Backbone}$ capsid. This capsid swap removes 7 of the 31 mutations deliberately engineered into CAVA-PV. The capsid swaps are first designed *in silico* and consequently DNA is generated by chemical synthesis of the novel genomes. Once the plasmid DNA was synthesized (outsourced to Genscript) this was used to generate viral RNA via *in vitro* transcription and consequently rescue the different novel recombinant CAVA-PV's via transfection. The resulting vaccine strains were named CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$, which contain the Mahoney, MEF-1 and Saukett capsids, respectively. Representative capsid amino acid sequences are provided here for PV type 1 strain Mahoney (SEQ ID NO:2), PV type 2 strain MEF-1 (SEQ ID NO:3), and PV type 3 strain Saukett (SEQ ID NO:4). Figure 12 shows a schematic overview of these different CAVA-PV vaccine strains.

Example 7: Growth kinetics of CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ in sPER.C6 cells compared to the parental Brunenders strain

[00106] Growth kinetics of CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ in the suspension PER.C6 (sPER.C6) cells, a production cell line, was compared to the growth of PV1 Brunenders at 30 and 37°C. The suspension PER.C6 cells at time of infection had a cell density of $10x10^6$ cells/ml in Permexcis media supplemented with 4 mM L-Glutamine. Cells were infected with an MOI of 1, the infections were performed twice for CAVA-$PV_{Saukett}$ and Brunenders (N=2) and three times for CAVA-$PV_{Mahoney}$ and CAVA-PV_{MEF-1} (N=3). Viral harvests were titrated using a $TCID_{50}$ assay at 30°C. Line graphs for the replication kinetics in the sPER.C6 cells are shown in Figure 13. The CAVA-PV's showed wild-type growth kinetics as compared to the parental Brunenders strains at 30°C, with error bars representing standard deviation from the mean. On the contrary, at 37°C, The CAVA-PV's showed no substantial replication, as was also observed for the CAVA-$PV_{Backbone}$ indicating that the temperature sensitivity of the viruses lies within the mutations outside of the capsid region. The Brunenders strain replicated normally and showed similar growth behaviour in sPER.C6 cells at 37°C.

[00107] The average maximum titer of CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ at 30°C was 9.94, 9.91 and 9.74 Log_{10} $TCID_{50}$/ml, respectively, which is similar to titers attained with wild-type strains on sPER.C6 cells (Sanders, Edo-Matas et al. 2012).

Example 8: *In vitro* antigenic content of CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ in sPER.C6 cells compared to the conventional IPV strains Mahoney, MEF-1 and Saukett

[00108] IPV dosing is based on D-antigen Units (DU) which is quantified by an *in vitro* D-Antigen ELISA (Beale 1961), performed as described in the European Pharmacopeia monograph 0214. For wild-type IPV, one dose of the vaccine is to contain 40, 8 and 32 DU's for the inactivated Mahoney, MEF-1 and Saukett viruses, respectively, corresponding to a dose which will induce a protective immune response in vaccine recipients (Grassly 2014). The D-antigen content, and therefore indirectly the immunogenic potency, of the active CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ viruses was quantified by D-antigen ELISA assay and compared to the conventional IPV strains, Mahoney, MEF-1 and Saukett. Infections were done in PER.C6 cells in suspension with a cell density of $10x10^6$ cells/ml at an MOI of 1. Infection temperature was 30°C for the CAVA-PV strains and 35°C for the wild-type strains, as this is the production temperature for conventional IPV.

[00109] Table 3 shows the D-antigen values obtained for the viruses, expressed per millilitre of infection harvest (DU/ml) and per infectious unit (DU/$TCID_{50}$). Since the D-antigenicity per millilitre (DU/ml) is dependent on the concentration of virus present in the sample (Titer or $TCID_{50}$/ml) the specific antigenic content per infectious unit of the CAVA-PV strains versus the wild-type strains were calculated. This corresponds to a fair comparison of the strains. The specific antigenic content was similar for the CAVA-PV strains versus their wild-type counter parts, indicating that a similar immunogenic profile between CAVA-PV and their wild-type counter parts may be expected. As the viruses contain the same capsid sequences the antigenicity is expected to be similar, this is corroborated by the D-antigen results obtained.

Example 9 *In vivo* immunogenicity of inactivated and purified CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ in rats.

[00110] The CAVA vaccine strains: CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} and CAVA-$PV_{Saukett}$ were purified and inactivated. Purification was performed by two subsequent chromatography steps using clarified crude harvest material from an infection under envisioned production conditions (2x250 ml in a roller bottle containing sPER.C6 cells with a cell density of $10x10^6$ vc/ml in Permexcis medium, at an MOI of 1, at 30°C). Cation Exchange Chromatography (CEX) was performed using Sartobind S cationic membranes after which the eluted material was consequently used for Size Exclusion Chromatography step for further purification (polish) and buffer exchange. The SEC eluate was conditioned using M199 and glycine prior to inactivation. Inactivation was performed by addition of 0.009% formalin (or 3.3mM formaldehyde) and incubated for 13 days at 37°C. Filtration was performed at days 6 and 13 of inactivation. The inactivated bulks were used for *in vivo* immunogenicity testing in rats. Four groups of Wistar female rats (n=10) were immunized with a dilution of 1:1 (full human dose), 1:2, 1:4 and 1:16 of each of the inactivated CAVA vaccine strains. The full human dose represents 40, 8 or 32 D-antigen units of Type 1, 2 and 3 respectively, which is the dosing of conventional IPV. Rats were left for 21 days after which they were bled and sera was used for Virus Neutralization Assay using Sabin viruses as challenge viruses and Hep2C cells. Figure 14 depicts the neutralizing antibody titers raised in rats after immunization with either inactivated CAVA-$PV_{Mahoney}$, CAVA-PV_{MEF-1} or CAVA-$PV_{Saukett}$. All three vaccine strains showed to be immunogenic as they induced production of high titers of neutralizing antibodies in a dose-dependent fashion upon dilution of the full vaccine dose.

Example 10: Generation of an additional CAVA-PV strain.

[00111] The 31 mutations that were derived from the three independent clones (see example 1, Table 1) were analyzed for their conservation amongst a panel of PV strains. We identified 14 mutations that would likely play a significant role in providing the temperature sensitive phenotype observed in CAVA-PV. These are depicted in Table 4. In this table, a single asterisk depicts mutations that are unique in CAVA-PV and where the nucleotides at those positions are conserved in all other PV strains used for the alignment, which were Brunenders, as well as Brunhilde, Mahoney, Sabin 1, Sabin 2,

Sabin 3, MEF-1, and Saukett. A double asterisk in this table depicts mutations that are unique in CAVA-PV and where the nucleotides at those positions are conserved in all of the other Type 1 PV strains used for the alignment: Brunenders, Brunhilde, Mahoney and Sabin 1. The nucleotide mutation at position 4120 in CAVA-PV is shared with Sabin 1, but the nucleotide is conserved at that position in all of the other PV strains used for the alignment.

[00112] We inserted these 14 mutations described in Table 4 into the parental sequence of Brunenders to determine the effects on temperature sensitivity. The virus was prepared as described in Example 1. Briefly; the recombinant cDNA plasmid containing the Brunenders genome with 14 mutations was synthesized. The resulting plasmid was subjected to *in vitro* transcription and subsequent RNA transfection. Replication kinetics was performed in PER.C6 cells by infecting cells at either 30°C or 37°C with an MOI of 1 and a cell density of $10x10^6$ cells per ml in Permexcis medium supplemented with 4mM L-Glutamine. Samples were taken at 0, 2, 8, 24 and 48 hours post infection and subjected to titration.

[00113] Fig. 15 shows the growth curves of the new CAVA-PV strain with 14 mutations as described in this Example, and the CAVA-$PV_{Backbone}$ strain of Example 1 (having 31 mutations), as compared to their wild-type counterpart (Brunenders parental strain). The Brunenders parental strain shows growth curves as is expected from wild-type strains at both 37°C and 30°C, reaching the plateau titer within 10 and 24 hours post infection, respectively. Upon introducing the 14 mutations, the new CAVA-PV strain displays a growth phenotype similar to the CAVA-$PV_{Backbone}$ strain of Example 1 that had more than twice the amount (i.e. 31) of mutations compared to the parental strain. No replication is observed at 37°C while at 30°C the replication was comparable to wild-type.

[00114] These results show that the loss of fitness at physiological temperature can already be induced by these 14 mutations. Moreover, further to the various embodiments described above, this example provides yet another embodiment of a CAVA-PV strain according to the invention, i.e. a recombinant poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated at 37°C.

Example 11: Generation of CAVA-PV's strain from a different parental strains from different serotypes

[00115] The previous examples all described the use of the Brunenders strain (a partially attenuated Type 1 PV strain) as the starting or parental strain for the generation of the various CAVA-PV strains. In this Example, two very dissimilar parental strains, namely the Type 2 neurovirulent, wild-type MEF-1 strain, and the Type 3 neuroattenuated Sabin 3 strains were used as a background (parental) strain. MEF-1 is routinely used as the Type 2 immunogen for conventional IPV preparations whilst Sabin 3 is a component of OPV preparations.

[00116] Thirteen of the 14 mutations described in Table 4 with respect to the Brunenders strain were inserted at the corresponding nucleotide positions into the MEF-1 strain (SEQ ID NO: 5; as the MEF-1 strain already contained an A at position 3481, this position was not mutated, hence the 13 instead of 14 mutations.) to determine the effects on temperature sensitivity. The same was performed using the Sabin 3 genomic sequence (SEQ ID NO: 8, as the Sabin 3 strain already contained a G and an A at positions 163 and 3473, respectively, these nucleotides were not mutated, hence the 12 instead of 14 mutation, see Table 4 for detailed description of CAVA mutations per strain).

[00117] The MEF-1 and Sabin 3 viruses were prepared as described in Example 1. Briefly; the recombinant cDNA plasmids containing the MEF-1 and Sabin 3 genomes with 13 and 12 mutations were first synthesized. The resulting plasmids were subjected to *in vitro* transcription and subsequent RNA transfection. Replication kinetics was performed in PER.C6 cells by infecting cells at either 30°C or 37 °C with an MOI of 1 and a cell density of $10x10^6$ cells per ml in Permexcis medium supplemented with 4mM L-Glutamine. Samples were taken at 0, 2, 8, 24 and 48 hours post infection and subjected to titration.

[00118] Fig. 16 shows the growth curves of the new strain based on MEF-1 with 13 mutations as described in this Example, and the CAVA-$PV_{Backbone}$ strain of Example 1 (i.e. a Brunenders strain having 31 mutations), as compared to the wild-type MEF-1 strain. The MEF-1 parental strain shows growth curves as is expected from wild-type strains at both 37°C and 30°C, reaching the plateau titer within 10 and 24 hours post infection, respectively. Upon introducing the 13 mutations, the new MEF-1 based strain

developed a growth phenotype similar to the CAVA-$PV_{Backbone}$ strain of Example 1 that was based on a Brunenders background strain and had more than twice the amount of mutations compared to its parental strain. No replication is observed at 37°C while at 30°C the replication was comparable to wild-type.

[00119] Fig. 17 shows the growth curves of the new strain based on Sabin 3 with 12 mutations described in this Example, as compared to the CAVA-$PV_{Backbone}$ of Example 1 (i.e. a Brunenders strain having 31 mutations), as compared to the attenuated Sabin 3 strain. The Sabin 3 OPV strain shows growth curves comparable to wild-type PV growth at both at both 37°C and 30°C, reaching the plateau titer within 10 and 24 hours post infection, respectively. Upon introduction of the 12 mutations, the new Sabin 3 based strain displayed a growth phenotype similar to the CAVA-$PV_{Backbone}$ strain of Example 1 that was based on a Brunenders background strain. No replication is observed at 37°C while at 30°C the replication was comparable to wild-type.

[00120] The data demonstrate that the new MEF-1 and Sabin 3 strains with 13 and 12 mutations, respectively, also have a CAVA-PV phenotype and is thus yet a further embodiment of a CAVA-PV strain according to the invention, i.e. a recombinant poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated at 37°C. Therefore, in total 14 mutations (of which 1 and 2 were already present in the new MEF-1 and Sabin 3 parental strains) are sufficient for conveying the CAVA-PV growth phenotype into a wild-type poliovirus strain. This example demonstrates yet an alternative way to create additional CAVA-PV strains, and it is shown that such strains can be prepared from different poliovirus parental strains. Notably, these data show that the parental strain neither needs to be a Type 1 PV strain, nor a wild-type or partially attenuated strain, since the parental strain for this example was a Type 2, neurovirulent and wild-type PV strain as well as a Type 3 attenuated strain. The new strain of this example has a MEF-1 wild-type capsid sequence, and can thus be used directly for generating a safe and effective IPV against Type 2 PVs. It can also be used as a starting point to swap this capsid for other wild-type capsids such as a Mahoney or a Saukett capsid. The skilled person will also appreciate that this example makes plausible that introduction of the 14 mutations (or a subset thereof) of Table 4 into the corresponding positions in the genome of a Mahoney, Saukett, Sabin 1 and Sabin 2

strains would result in further CAVA-PV strains that could be used as safe and effective vaccine strains for IPV production.

Example 12: Quantification of CAVA-PV infection by EM

[00121] Quantification of viral infection by titration ($TCID_{50}$) at 37°C of CAVA-PV strains does not result in the observation of cytopathic effects (CPE), hence titration must be performed at 30°C. This is corroborated by the replication kinetic curves of the CAVA-PV strains demonstrating no increase in infectious units during infection at 37°C (Figs. 2-11, 13, 15-17). This indicates that the viruses are unable to replicate at this temperature. However, to further understand the block in replication at 37°C, we performed Electron Microscopy (EM) as an alternative method to characterize PV infection. Figure 18 depicts results of the EM after infection of sPER.C6 cells with a cell density of $10x10^6$ cells/ml with an MOI 1 at 30 and 37°C using Mahoney, CAVA-$PV_{Mahoney}$ or PBS (mock infected).

[00122] EM samples were fixed in 1.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and stained with 1% osmium tetroxide. The samples were then washed in 0.14 M cacodylate buffer and pelleted in 3% agar, after which the pellets were gradually dehydrated with anethanol series. The samples were then infiltrated for one hour with a 1:1 mixture of propylene oxide and epoxy LX-112 resin (Ladd Research) and an additional hour in 100% epoxy LX-112, after which the samples were polymerized for 48 h at 60°C. Cell sections of 50 nm thickness were cut, placed onto carbon-coated formvar grids, and counterstained with 7% uranyl acetate and lead citrate. A Tecnai 12 BioTwin transmission electron microscope (FEI company) operated at 120 kV was used for imaging.

[00123] The EM data in Figure 18 depict representative cells from each infection. The cells infected with CAVA-$PV_{Mahoney}$ at 30°C resembled those infected with the wild-type Mahoney strains, most cells were already dead and lysed or apoptotic. The apoptotic cells had shrunk in size and were very dark in colour. These apoptotic cells contained rearrangements of ER membranes as well as virus induced vesicle formation. When poliovirus concentrations are high in a cell highly structured virus lattices can be formed which were visible in the Mahoney and CAVA-$PV_{Mahoney}$ (at 30°C) samples.

[00124] However, at 37°C the CAVA-$PV_{Mahoney}$ infected cells did not show any of these features associated to PV infection. In fact, these cells showed to be healthy with no signs of infection in any of the cells in the samples. The CAVA-$PV_{Mahoney}$ infected cell which were incubated at 37°C resembled the PBS mock infected samples. The CAVA-$PV_{Mahoney}$ titer of this 37°C incubated sample was measured to be 4.32 $TCID_{50}$/ml, which is less than the theoretical input (7.0 $TCID_{50}$/ml as calculated MOI of added volume of input virus of known titer), however it confirms that CAVA-$PV_{Mahoney}$ virus had indeed been added to the infection without inducing any signs of infection.

Example 13: Quantification of CAVA-PV infection by qPCR

[00125] The sample samples used for EM (Example 12) were subjected to quantitative PCR as to determine the levels of viral RNA during infection. Viral RNA was isolated from infection harvests using a QIAamp viral RNA isolation kit after which the PowerSYBR Green RNA-to-Ct 1 step Kit was uses for RT-qPCR, using primers binding to the 3D polymerase gene. Figure 19 depicts vRNA levels, as observed by titration of infection harvests, the viral RNA levels decrease when CAVA-1 Mahoney infection is maintained at 37°C, however, this is not the case when infection temperatures are at 30°C, where an increase of vRNA is observed. It can therefore be concluded that there are defects in RNA replication, and it is likely that it is blocked completely at 37°C. The block in CAVA-PV replication therefore takes place at RNA replication level, or earlier in the infection cycle.

Example 14: Generation of PV's which do not display the CAVA phenotype.

[00126] To further understand which mutations are involved in the CAVA-phenotype, new intermediate viruses were constructed either the 7 CAVA mutations in the 5'UTR or the 17 mutations in the Non-structural proteins in the background of the parental Brunenders strain (SED ID NO: 1, see Table 4 for the CAVA mutations in these two regions). The CAVA-PV strains of Example 6 has already demonstrated that the CAVA mutation in the capsid could be removed without effects on the CAVA phenotype, therefore only the mutations in the 5'UTR and Non-structural regions were examined here. The intermediate viruses were tested for replication kinetics at 30 and 37°C as compared to the parental Brunenders strain, the CAVA-$PV_{Backbone}$ of Example 1 and CAVA-$PV_{Mahoney}$ of Example 6.

[00127] The intermediate viruses were prepared as described in Example 1. Briefly; the recombinant cDNA plasmids containing the Brunenders genomes with either 7 CAVA mutations in the 5'UTR or 17 CAVA mutations in the Non-structural proteins were first synthesized. The resulting plasmids were subjected to *in vitro* transcription and subsequent RNA transfection. Replication kinetics was performed in PER.C6 cells by infecting cells at either 30°C or 37 °C with an MOI of 1 and a cell density of $10x10^6$ cells per ml in Permexcis medium supplemented with 4mM L-Glutamine. Samples were taken at 0, 2, 8, 24 and 48 hours post infection and subjected to titration.

[00128] Fig 20 shows the growth curves of the new strains based the Brunenders parental strains with mutations as described in this Example, as well as the CAVA-$PV_{Backbone}$ strain of Example 1 (i.e. a Brunenders strain having 31 mutations), as compared to the wild-type Brunenders strain. The intermediate strains show impaired growth curves at 37°C, where replication is hampered as compared to the Brunenders parental strains, but not abolished as compared to the CAVA-$PV_{Backbone}$. Here the intermediate viruses grow at a slower rate as compared to the Brunenders parental strain as well as to lower maximum titers. At 30°C, the intermediate viruses show identical growth curves as compared to the Brunenders parental strain as well as the CAVA-$PV_{Backbone}$ and CAVA-$PV_{Mahoney}$ strains of Example 1 and 6, respectively. The plateau titer was reached within 24 hours post infection. Upon introducing the 5'UTR or Non-structural mutations, the new strains did not have a phenotype identical to the CAVA-$PV_{Backbone}$ and CAVA-$PV_{Mahoney}$ strains, therefore it can be concluded that the CAVA phenotype can only be induced when mutations from both regions (5'UTR and non-structural proteins) are combined into one parental genome.

Table 1: CAVA-PV with a total of 31 mutations, including 7 mutations in the 5'UTR, 7 mutations in the capsid, and 17 mutations in the non-structural proteins. The nucleotide positions described in Table 1 are referenced to the genome of a Brunenders strain, for other PVs the exact nucleotide numbering may differ slightly.

7 mutations in 5'UTR

Where	nt#	nt change
5'UTR	133	A → G
5'UTR	142	U → C
5'UTR	146	G → A
5'UTR	163	A → G
5'UTR	579	G → A
5'UTR	597	C → U
5'UTR	609	G → A

7 mutations in the capsid

nt#	Where	nt change	aa Change
805	VP4	A → C	Y→ S
1787	VP3	C → U	silent
1905	VP3	U → C	silent
2756	VP1	U → C	silent
3236	VP1	C → U	silent
3323	VP1	C → U	silent
3376	VP1	A → G	E → G

17 mutations in the non-structural proteins

nt#	Where	nt change	aa Change
3476	2A	C → U	silent
3486	2A	G → A	V → I
3852	2B	A → U	I → F
4120	2B	U → C	I → T
4253	2C	C → U	silent
4301	2C	U → C	silent
4428	2C	A → G	I → V
4563	2C	A → U	I → L
4811	2C	A → G	silent
5436	3A	G → A	V → M
5705	3BC	A → G	silent
6059	3D	C → U	silent
6210	3D	A → G	M → V
6488	3D	C → U	silent
6848	3D	G → A	M → I
7079	3D	U → C	silent
7102	3D	U → C	V → A

Table 2: Mouse model for *in vivo* neurovirulence in CD155 transgenic mice

Virus	**Route**	**(P)LD$_{50}$ (TCID$_{50}$)**
CAVA-PV$_{Backbone}$	i.c. i.m. i.p.	$>9x10^7$ $>10^8$ $>10^8$
CAVA-PV$_{Mahoney}$	i.c.	$>2.4 \times 10^8$
CAVA-PV$_{MEF-1}$	i.c.	$>2.4 \times 10^8$
CAVA-PV$_{Saukett}$	i.c.	$>1.7 \times 10^8$
Brunenders	i.c. i.m. i.p.	$1.5x10^6$ $3.2x10^6$ $1.7x10^7$
Brunhilde	i.c. i.m. i.p.	$4.2x10^2$ $2.4x10^4$ $6.9x10^6$
Mahoney	i.c. i.m. i.p.	10^2 $1.6x10^4$ $5x10^5$
MEF-1	i.c. i.m i.p	$3.2x10^4$ $>10^8$ $>10^8$
Saukett	i.c. i.m i.p	$7.0x10^2$ $1.0x10^6$ $2.3x10^6$
Sabin 1	i.c.	$>2x10^7$
Sabin 2	i.c.	$>10^8$
Sabin 3	i.c.	$>10^8$

Table 3: Antigenic content of CAVA-PV$_{Mahoney}$, CAVA-PV$_{MEF-1}$ and CAVA-PV$_{Saukett}$

Poliovirus		**DU/ml**	**Titer (TCID$_{50}$/ml)**	**DU/TCID$_{50}$**
Type I	CAVA-1-Mahoney	2652	9.56	$7.3x10^{-07}$
	Mahoney	3288	10.09	$2.7x10^{-07}$
Type II	CAVA-2 MEF-1	705	9.91	$8.7x10^{-08}$
	MEF-1	501	10	$5.0x10^{-08}$
Type III	CAVA-3 Saukett	866	9.56	$2.4x10^{-07}$
	Saukett	1334	9.56	$3.7x10^{-07}$

Table 4: 14 mutations for temperature sensitivity of some CAVA-PV strains (e.g. Examples 10 and 11). *: mutations that are unique in CAVA-PV and the parental Brunenders nucleotides at those positions are conserved in all other PV strains used for the alignment. **: mutations that are unique in CAVA-PV and the parental Brunenders nucleotides at those positions are conserved in all of the other PV1 strains used for the alignment. Representative parent strain genome sequences are provided as SEQ ID NO: 1 (Brunenders), SEQ ID NO: 5 (MEF-1), SEQ ID NO: 6 (Mahoney), SEQ ID NO: 7 (Saukett), SEQ ID NO: 8 (Sabin 3), SEQ ID NO: 9 (Sabin 1) and SEQ ID NO: 10 (Sabin 2). All the nucleotide changes as described are identical for all strains and corresponding positions, with the exception of: mutation in the sequence encoding 2A protein which is already an adenine in MEF-1, Saukett and Sabin 3 strains at positions 3481 and 3473, respectively, annotated as "[a]" in the table. Mutation in IRES Domain II is already an guanine in Sabin 3 at position 163, annotated as "[b]" in the table. Mutation at position 4120 in the 2B protein in Sabin 1 was already a "C" in this position.

5 mutations in 5'UTR

Nucleotide position in respective PV genome								
Where	**Brunenders**	**MEF-1**	**Mahoney**	**Saukett**	**Sabin 1**	**Sabin 2**	**Sabin 3**	**nt change**
IRES Domain II	133	134	131	133	131	131	133	A → G*
IRES Domain II	142	143	140	142	140	140	142	U → C*
IRES Domain II	163	164	161	163	161	161	163[b]	A → G**
IRES Domain VI	587	589	593	596	593	594	596	C → U*
IRES Domain VI	609	610	605	608	605	606	608	G → A*

9 mutations in the non-structural proteins

Nucleotide position in respective PV genome								
Where	**Brunenders**	**MEF-1**	**Mahoney**	**Saukett**	**Sabin 1**	**Sabin 2**	**Sabin 3**	**nt change**
2A	3486	3481[a]	3482	3473[a]	3482	3481	3473 [a]	G → A**[a]
2B	3852	3847	3848	3839	3848	3847	3839	A → U*
2B	4120	4115	4116	4107	4116[c]	4115	4107	U → C
2C	4428	4423	4424	4415	4424	4423	4415	A → G*
2C	4563	4558	4559	4550	4559	4558	4550	A → U*
3A	5436	5431	5432	5423	5432	5431	5423	G → A*
3D	6210	6205	6206	6197	6206	6205	6197	A → G*
3D	6848	6843	6844	6835	6844	6843	6835	G → A*
3D	7102	7097	7098	7089	7098	7097	7089	U → C*

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CLAIMS

1. A recombinant poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, comprising a capsid from a Mahoney, MEF-1, or Saukett strain.

2. A recombinant attenuated temperature sensitive poliovirus type 1 strain that can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C.

3. A recombinant attenuated temperature sensitive poliovirus type 2 strain that can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C.

4. A recombinant attenuated temperature sensitive poliovirus type 3 strain that can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C.

5. A composition comprising a poliovirus strain according to any one of the preceding claims and a pharmaceutically acceptable carrier or excipient.

6. A composition comprising first, second and third recombinant poliovirus strains, wherein each of the first, second and third recombinant poliovirus strains can be propagated in cell culture at 30°C and cannot be substantially propagated in cell culture at 37°C, and wherein the first recombinant poliovirus strain comprises a capsid from a Mahoney strain, the second recombinant poliovirus strain comprises a capsid from a MEF-1 strain, and the third recombinant poliovirus strain comprises a capsid from a Saukett strain.

7. A composition according to claim 6, further comprising a pharmaceutically acceptable carrier or excipient.

8. An inactivated poliovirus vaccine (IPV) composition, comprising a composition according to any one of claims5-7, wherein the polioviruses in the composition are inactivated.

9. A combination vaccine composition comprising an IPV composition according to claim 8, and further comprising one or more antigens from diphtheria, tetanus, pertussis, *Heamophilus influenzae* type b (Hib), or Hepatitis B virus (HBV).

10. A method for vaccinating against poliomyelitis, the method comprising administering to a subject a composition according to claim 8 or 9.

11. A recombinant nucleic acid molecule comprising a sequence that codes for the genome or the complement of the genome of the poliovirus strain according to any one of claims 1-4.

12. A method for preparing a preparation comprising a poliovirus according to any one of claims 1-4, the method comprising:

a) infecting a cell in a cell culture with a poliovirus strain according to any one of claims 1-4,

b) culturing the cells in the cell culture to propagate the poliovirus, and

c) isolating the poliovirus from the cells or from the cell culture to obtain the preparation comprising the poliovirus.

13. A method according to claim 12, further comprising inactivating the poliovirus.

14. A method for preparing an IPV, the method comprising a method according to claim 13, and formulating the inactivated poliovirus into a pharmaceutical composition.

15. A method for obtaining a recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C, comprising the steps of:

a) passaging a parental poliovirus strain at 30°C or less for sufficient passages to produce a virus with impaired growth at 37°C;

b) isolating two or more different temperature sensitive clones that display impaired growth at 37°C;

c) sequencing the genomes of the temperature sensitive clones;

d) identifying mutations in the sequences of the genomes of temperature sensitive clones by comparing the sequences of the temperature sensitive clones to the sequence of the parental poliovirus strain;

e) synthesizing the recombinant attenuated poliovirus strain by combining mutations from two or more different temperature sensitive clones into the genome of a poliovirus strain; and

f) rescuing the recombinant attenuated poliovirus strain that can be propagated in cell culture at 30°C and that cannot be substantially propagated in cell culture at 37°C.

16. A method according to claim 15, further comprising:

g) replacing the sequence coding for the capsid from the rescued recombinant attenuated poliovirus strain with a sequence coding for a capsid from a different poliovirus strain.

17. A method according to claim 15 or 16, further comprising:

g) replacing the sequence coding for the capsid from the rescued recombinant attenuated poliovirus strain with a sequence coding for a capsid from a Mahoney, MEF-1, or Saukett strain.

18. A recombinant attenuated poliovirus strain obtainable by the method of any one of the claims 15, 16 or 17.

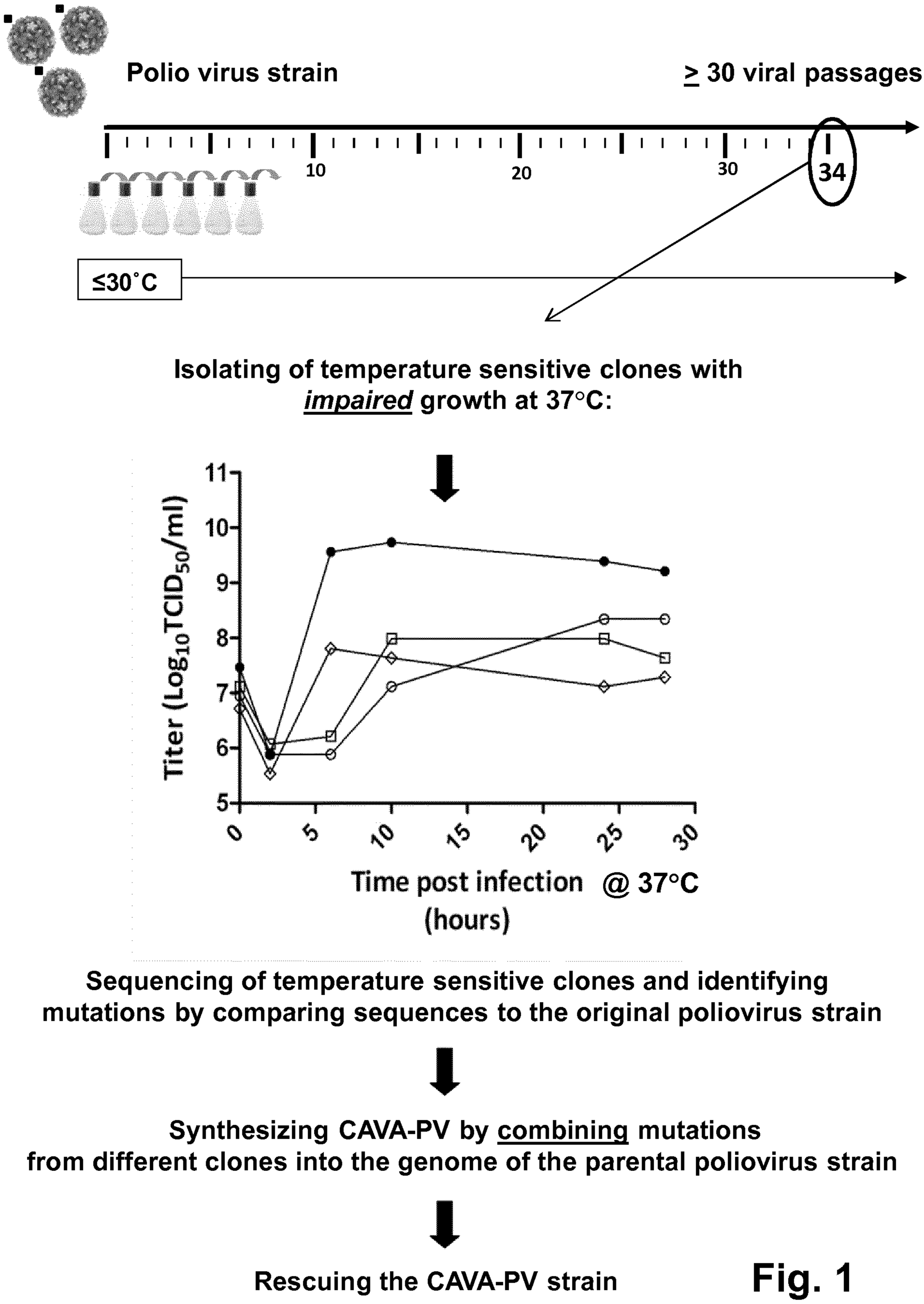

Polio virus strain
≥ 30 viral passages
10
20
30
34
≤30˚C
Isolating of temperature sensitive clones with
impaired growth at 37°C:
Titer (Log10TCID50/ml)
11
10
9
8
7
6
5
0
5
10
15
20
25
30
Time post infection @ 37°C
(hours)
Sequencing of temperature sensitive clones and identifying
mutations by comparing sequences to the original poliovirus strain
Synthesizing CAVA-PV by combining mutations
from different clones into the genome of the parental poliovirus strain
Rescuing the CAVA-PV strain

Fig. 1

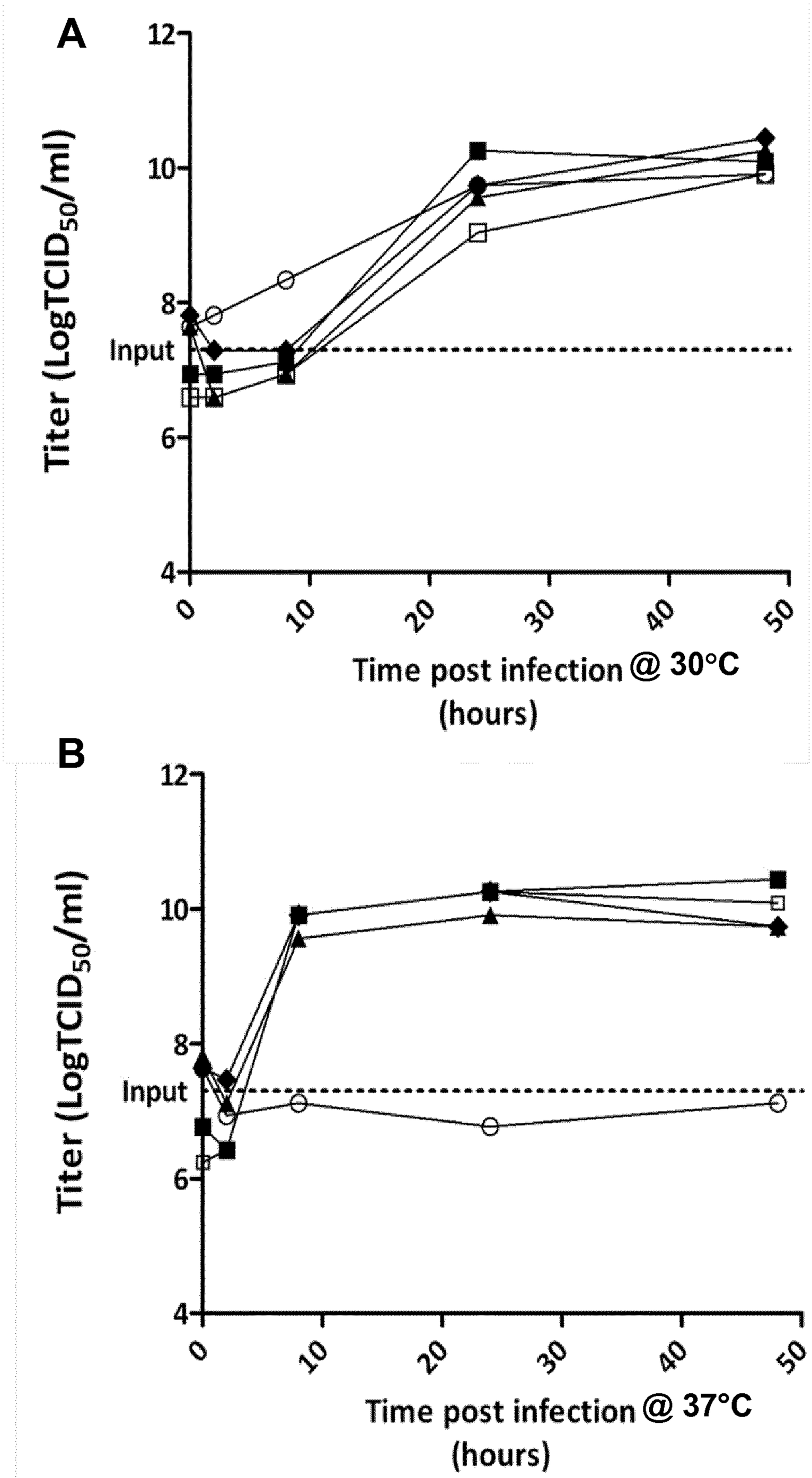

A
12
10
8
6
4
Input
Titer (LogTCID50/ml)
0
10
20
30
40
50
Time post infection @ 30°C
(hours)
B
12
10
8
6
4
Input
Titer (LogTCID50/ml)
0
10
20
30
40
50
Time post infection @ 37°C
(hours)

Fig. 2

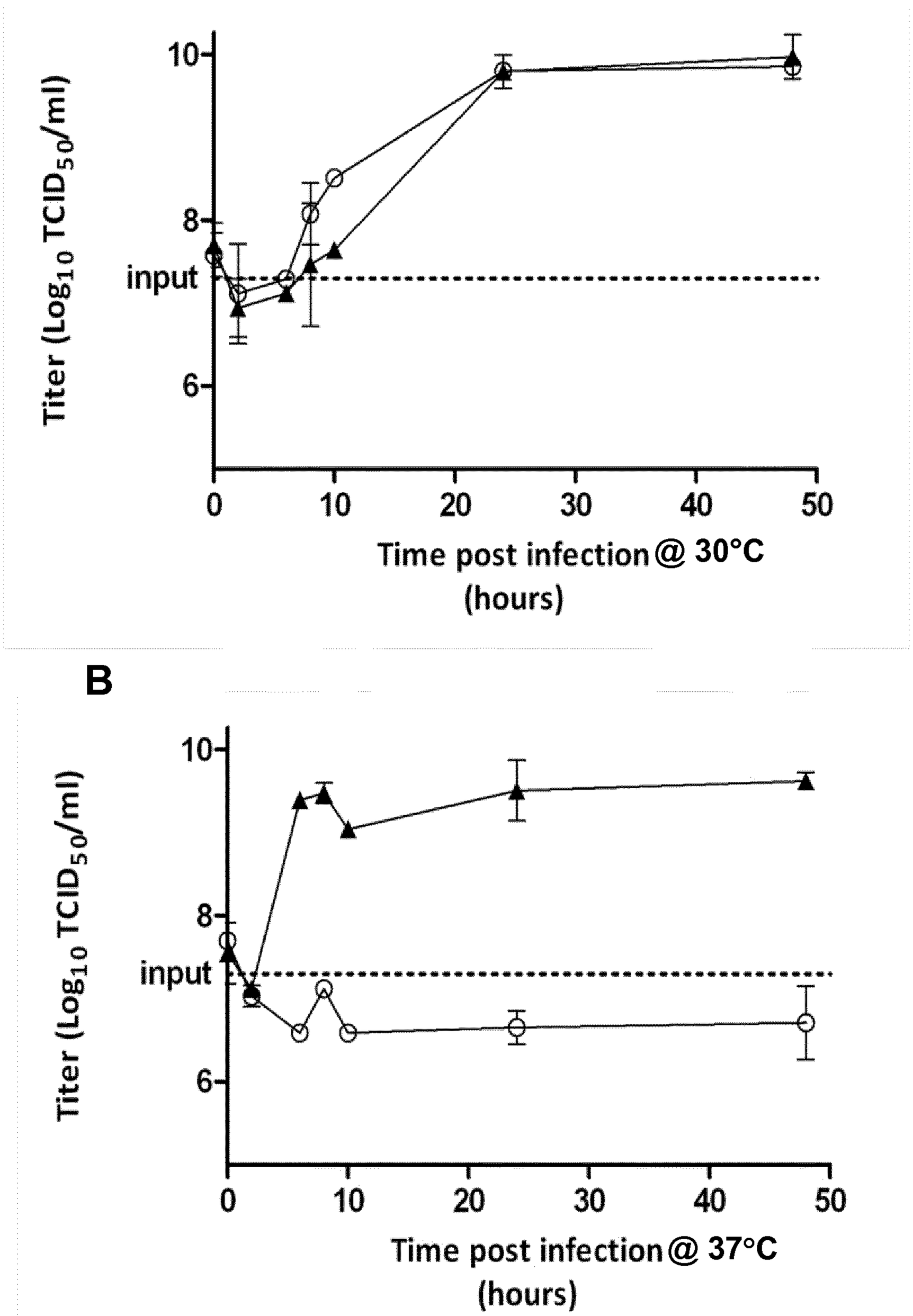

A
10
8
6
input
Titer (Log10 TCID50/ml)
0
10
20
30
40
50
Time post infection @ 30°C
(hours)
B
10
8
6
input
Titer (Log10 TCID50/ml)
0
10
20
30
40
50
Time post infection @ 37°C
(hours)

Fig. 3

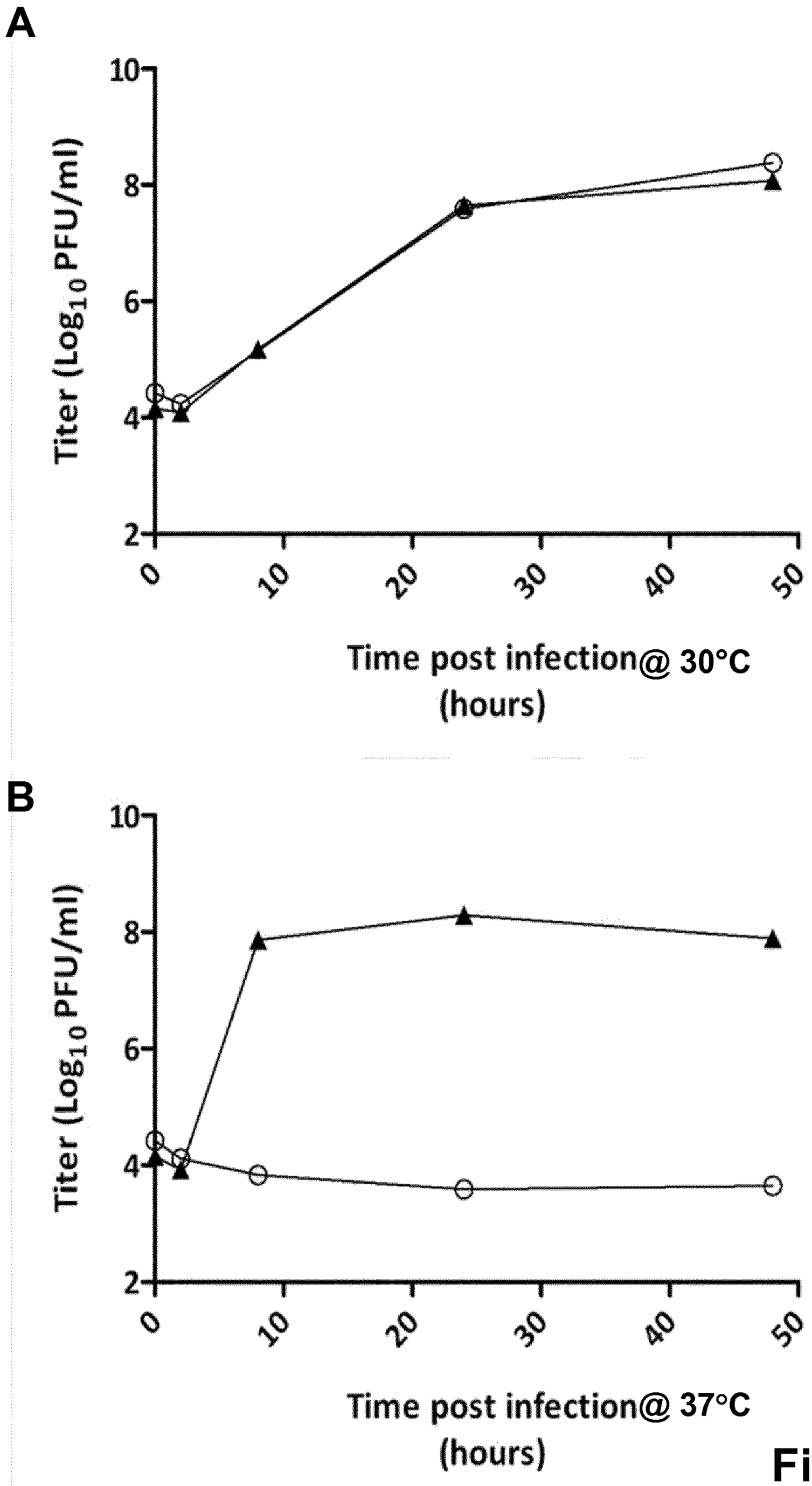

A
10
8
6
4
2
Titer (Log10 PFU/ml)
0
10
20
30
40
50
Time post infection@ 30°C
(hours)
B
10
8
6
4
2
Titer (Log10 PFU/ml)
0
10
20
30
40
50
Time post infection@ 37°C
(hours)

Fig. 4

C

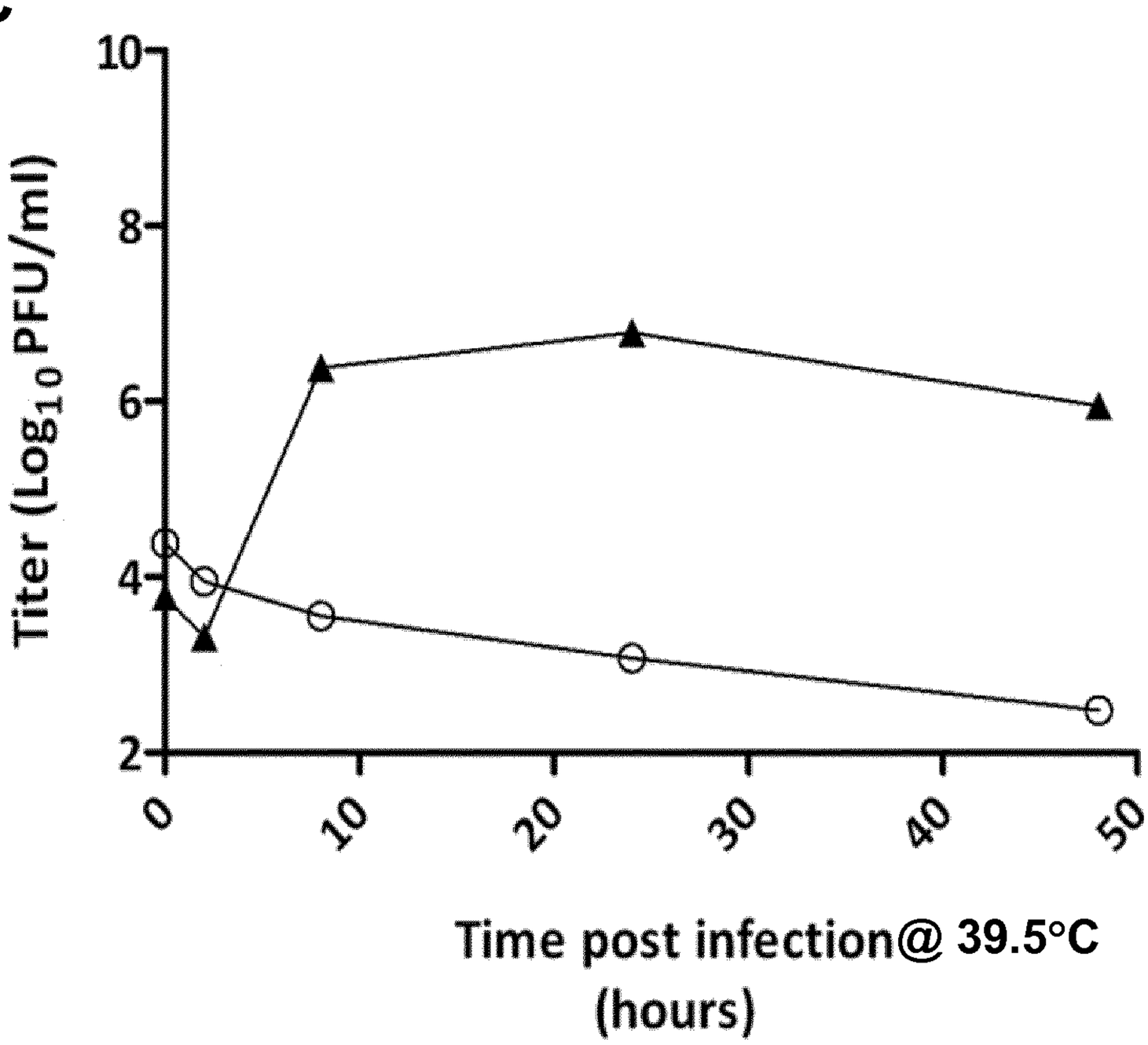


Fig. 4 - continued

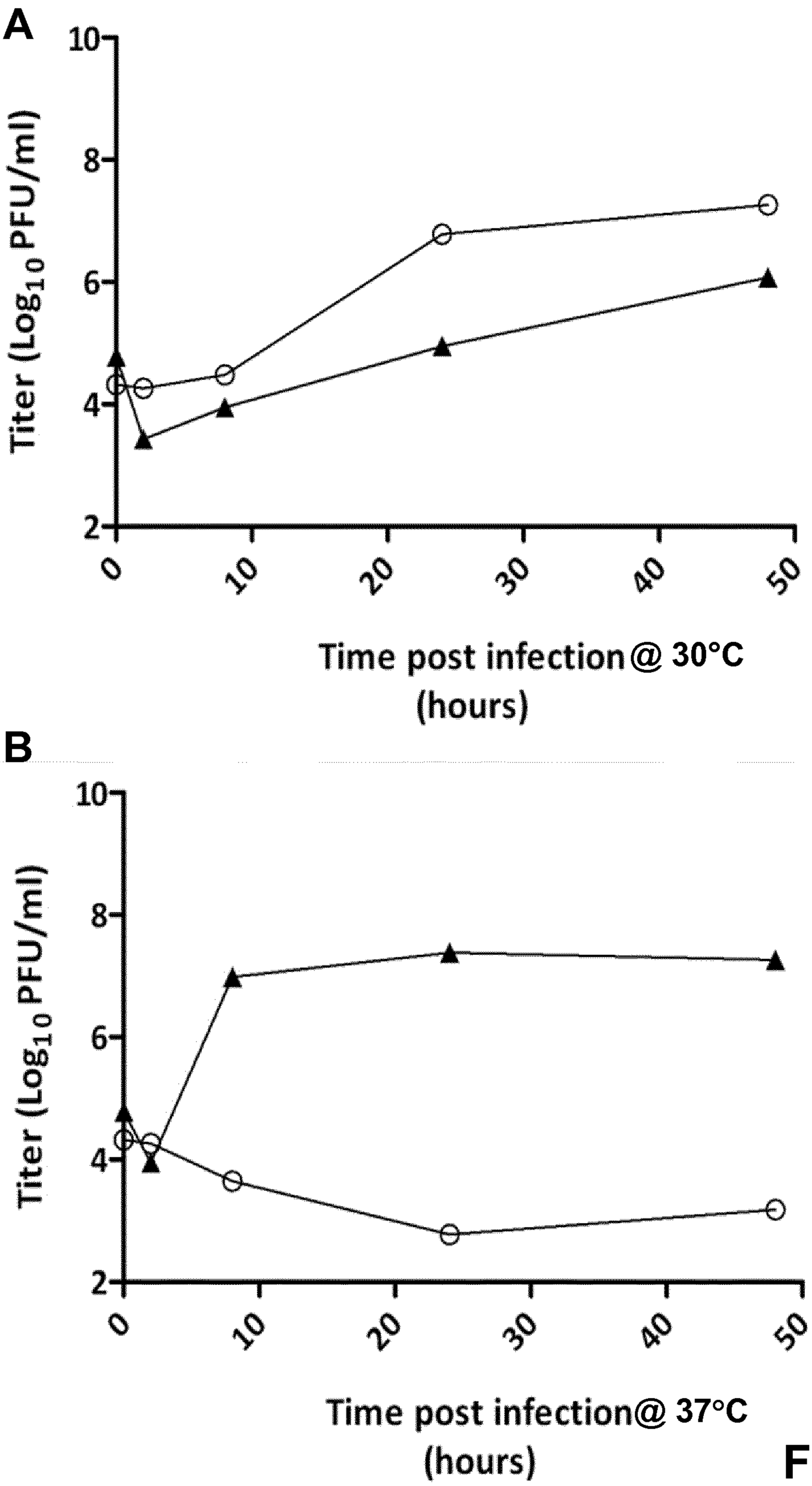

A
Titer (Log_{10} PFU/ml)
10
8
6
4
2
0
10
20
30
40
50
Time post infection@ 30°C
(hours)
B
Titer (Log_{10} PFU/ml)
10
8
6
4
2
0
10
20
30
40
50
Time post infection@ 37°C
(hours)

Fig. 5

C

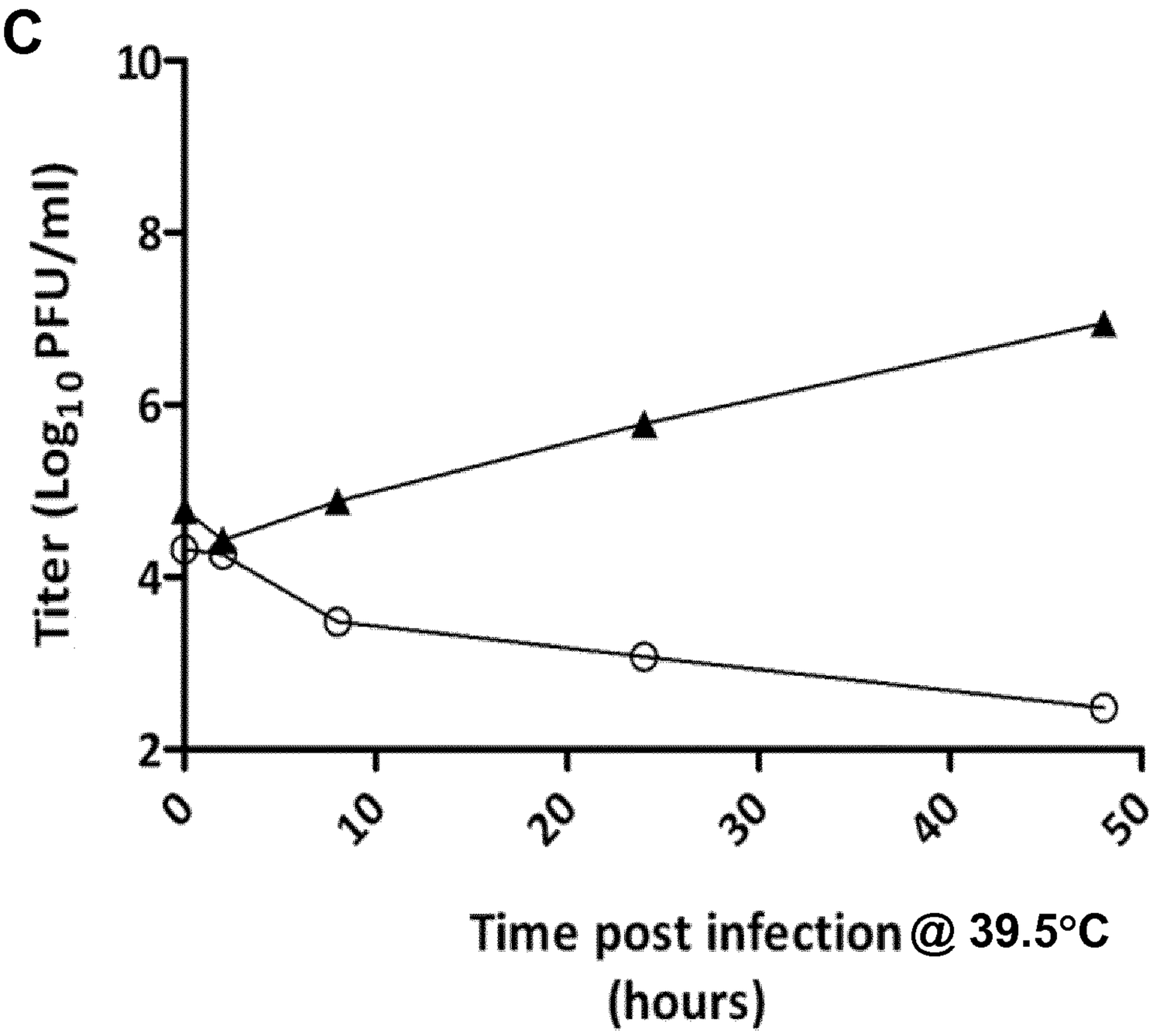


Fig. 5 - continued

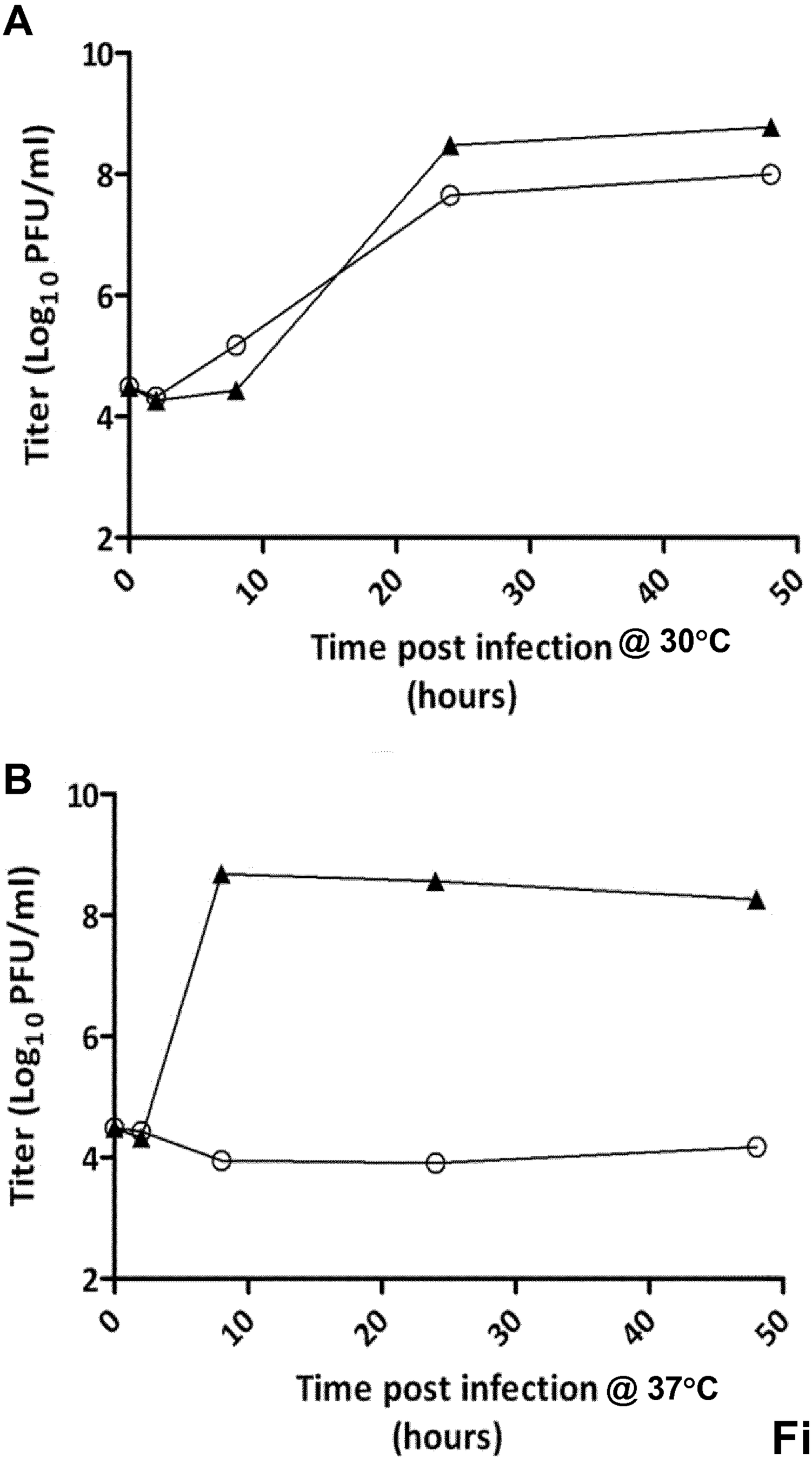

A
Titer (Log_{10} PFU/ml)
10
8
6
4
2
0
10
20
30
40
50
Time post infection @ 30°C
(hours)
B
Titer (Log_{10} PFU/ml)
10
8
6
4
2
0
10
20
30
40
50
Time post infection @ 37°C
(hours)

Fig. 6

C

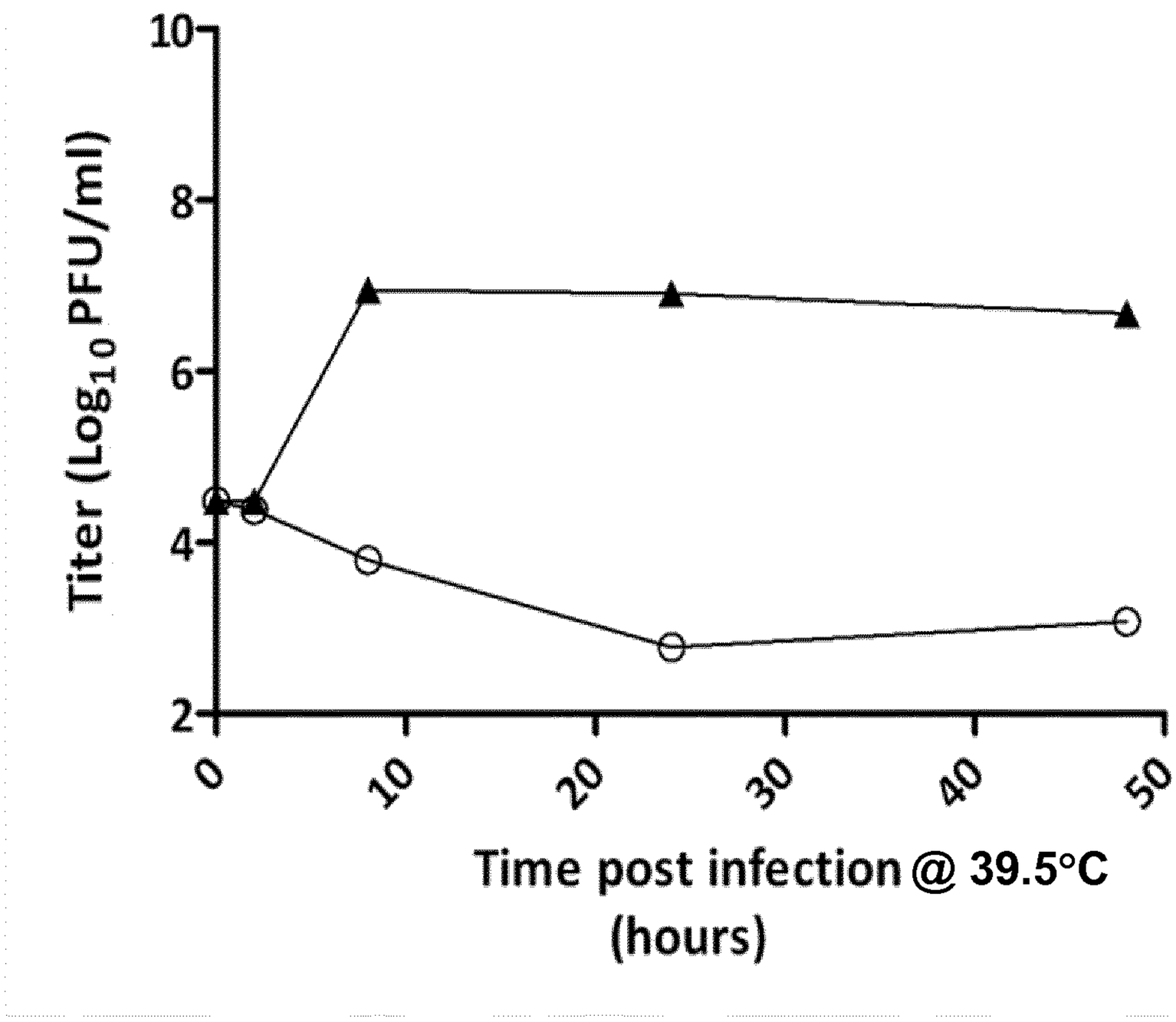


Fig. 6 - continued

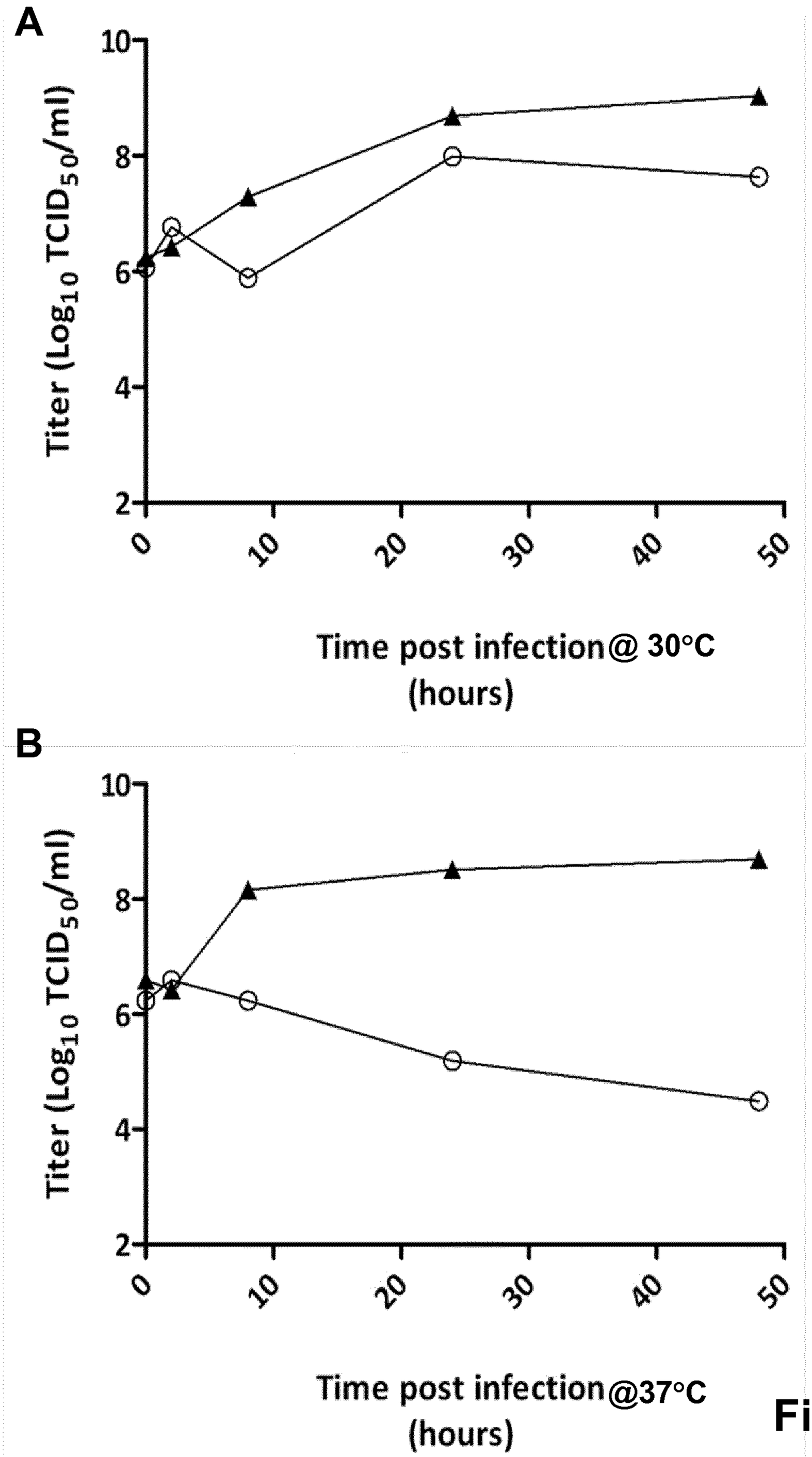

A
Titer (Log10 TCID50/ml)
10
8
6
4
2
0
10
20
30
40
50
Time post infection@ 30°C
(hours)
B
Titer (Log10 TCID50/ml)
10
8
6
4
2
0
10
20
30
40
50
Time post infection@37°C
(hours)

Fig. 7

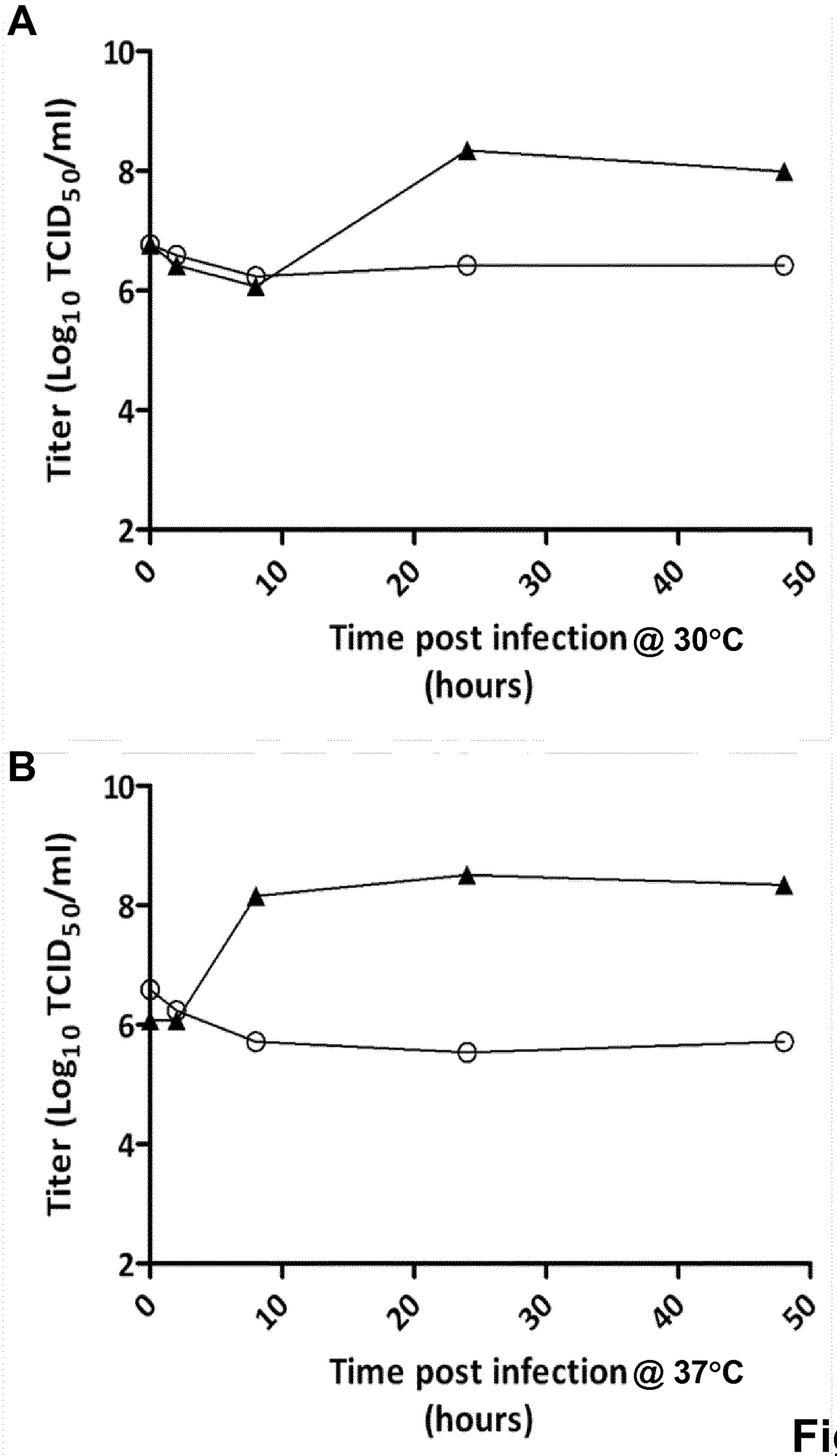

A
Titer (Log10 TCID50/ml)
10
8
6
4
2
0
10
20
30
40
50
Time post infection @ 30°C
(hours)
B
Titer (Log10 TCID50/ml)
10
8
6
4
2
0
10
20
30
40
50
Time post infection @ 37°C
(hours)

Fig. 8

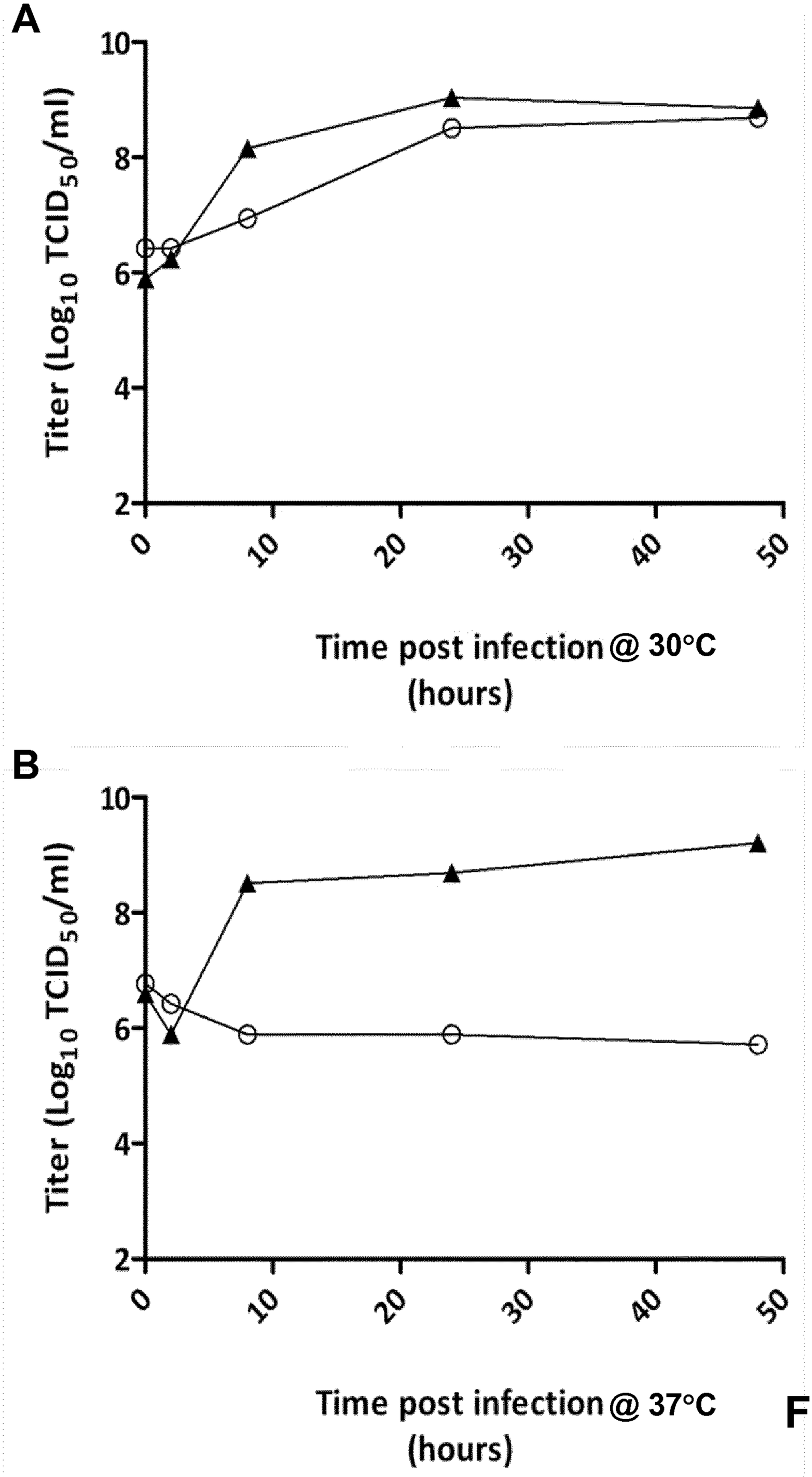

A
Titer (Log10 TCID50/ml)
10
8
6
4
2
0
10
20
30
40
50
Time post infection @ 30°C
(hours)
B
Titer (Log10 TCID50/ml)
10
8
6
4
2
0
10
20
30
40
50
Time post infection @ 37°C
(hours)

Fig. 9

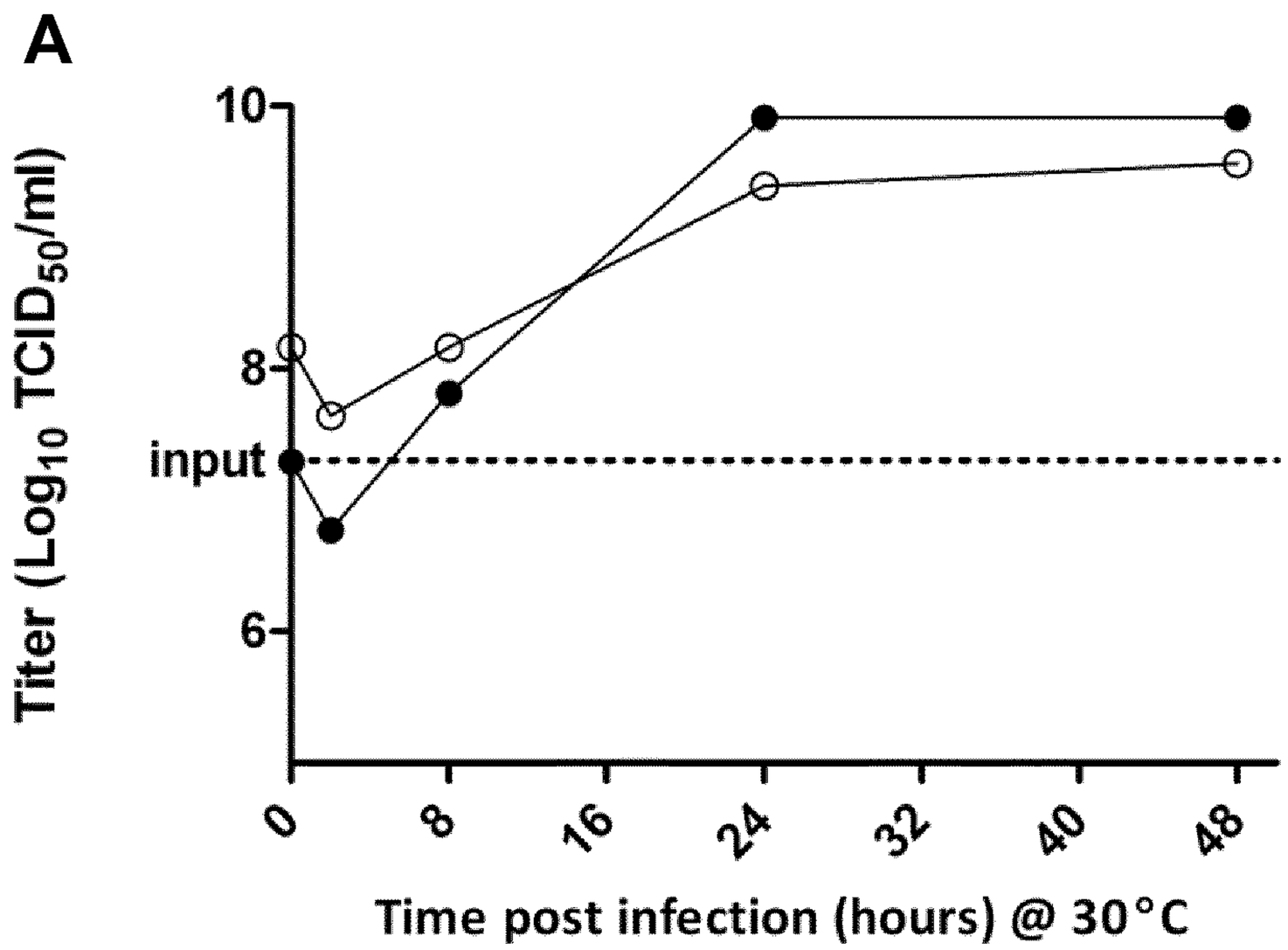

A
10
8
input
6
Titer (Log10 TCID50/ml)
0
8
16
24
32
40
48
Time post infection (hours) @ 30°C
B
10
8
input
6
Titer (Log10 TCID50/ml)
0
8
16
24
32
40
48
Time post infection (hours) @ 37°C

Fig. 10

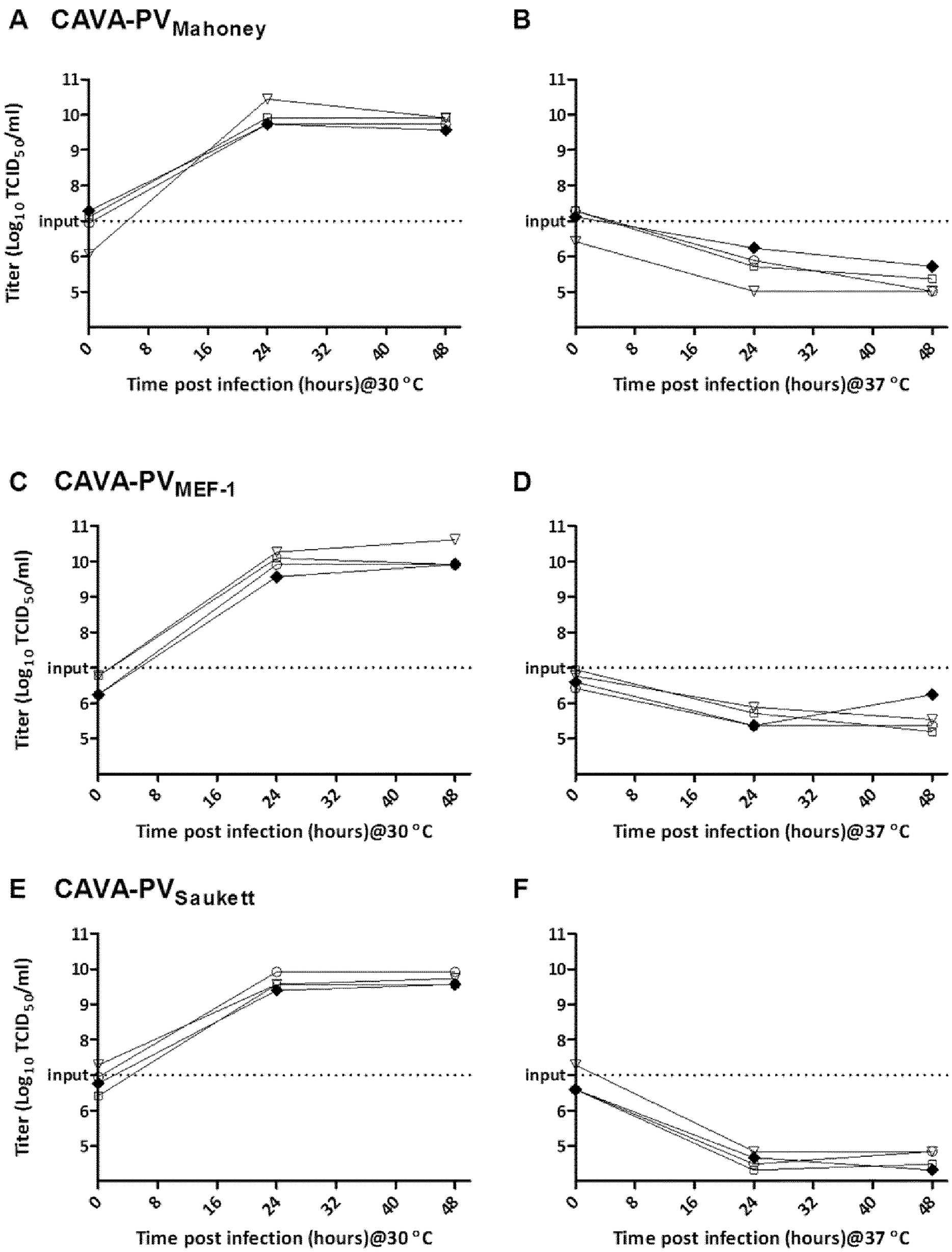

A CAVA-PV Mahoney
B
C CAVA-PV MEF-1
D
E CAVA-PV Saukett
F
Titer (Log10 TCID50/ml)
input
Time post infection (hours)@30 °C
Time post infection (hours)@37 °C

Fig. 11

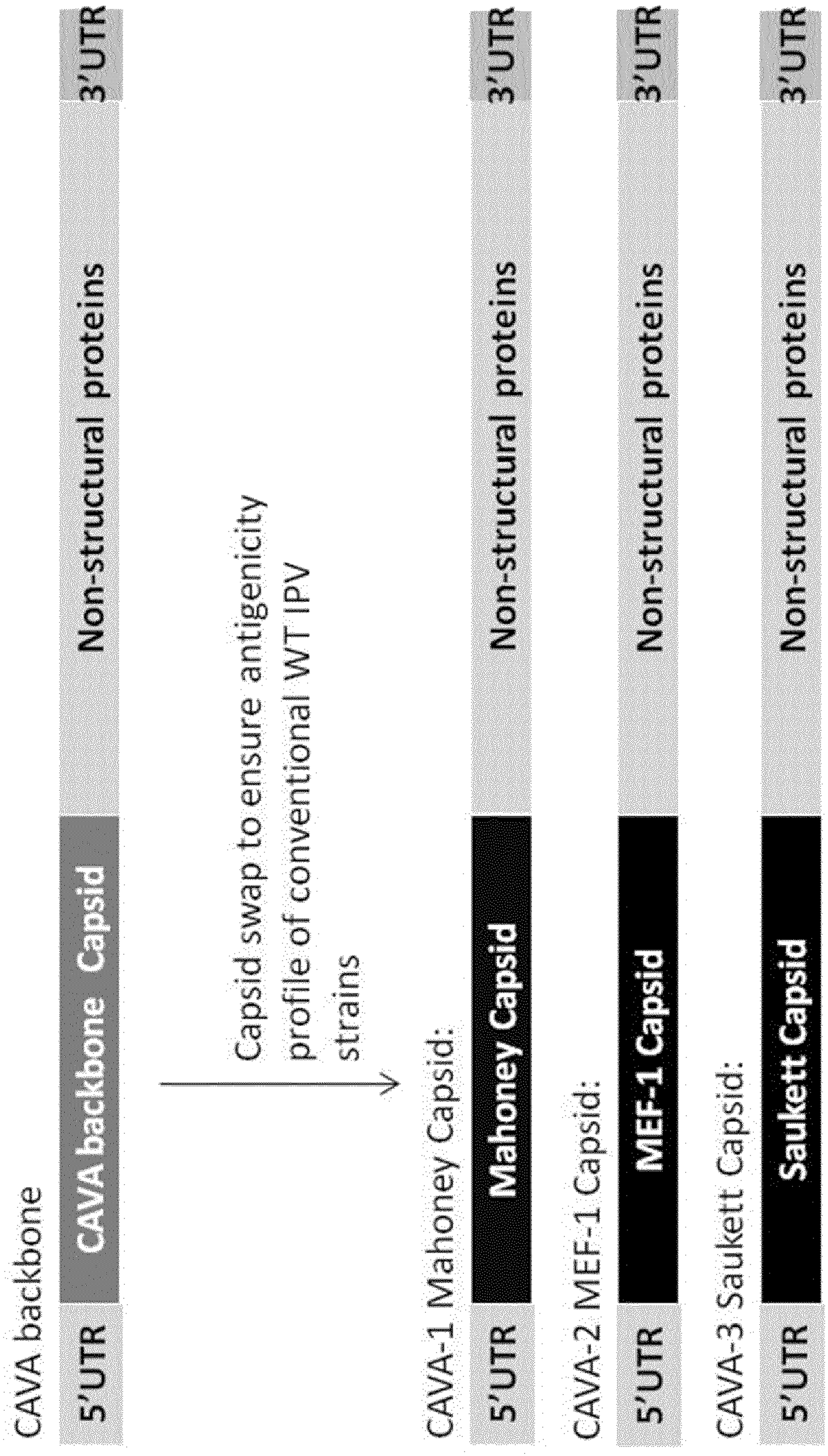

CAVA backbone
5'UTR
Non-structural proteins
3'UTR
CAVA backbone Capsid
5'UTR
Non-structural proteins
3'UTR
Capsid swap to ensure antigenicity profile of conventional WT IPV strains
Mahoney Capsid:
CAVA-1 Mahoney Capsid
5'UTR
Non-structural proteins
3'UTR
Mahoney Capsid
MEF-1 Capsid:
CAVA-2 MEF-1 Capsid
5'UTR
Non-structural proteins
3'UTR
MEF-1 Capsid
Saukett Capsid:
CAVA-3 Saukett Capsid
5'UTR
Non-structural proteins
3'UTR
Saukett Capsid

Fig. 12

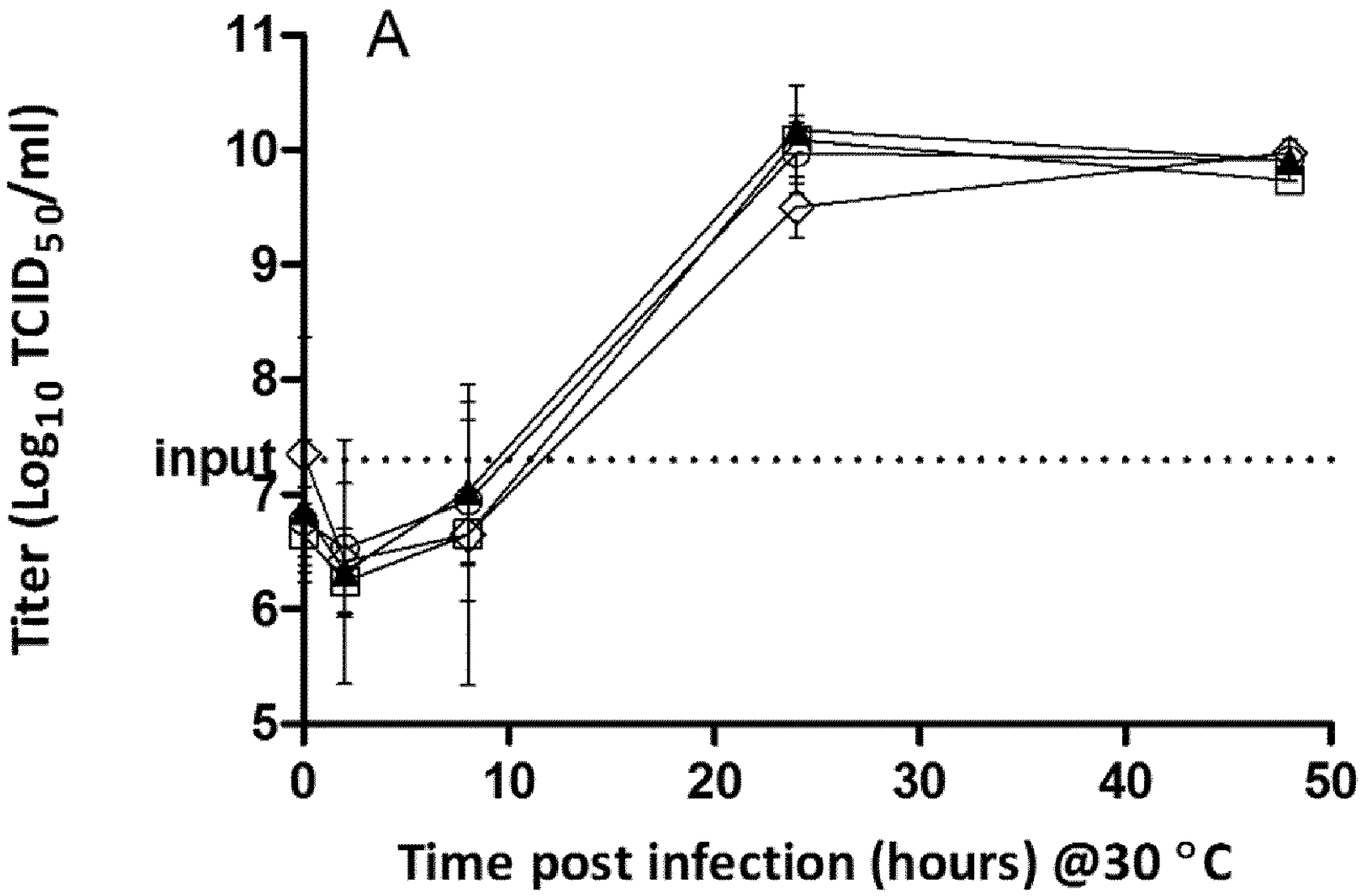

A
Titer (Log10 TCID50/ml)
11
10
9
8
input
7
6
5
0
10
20
30
40
50
Time post infection (hours) @30 °C
B
Titer (Log10 TCID50/ml)
11
10
9
8
input
7
6
5
4
0
10
20
30
40
50
Time post infection (hours) @37 °C

Fig. 13

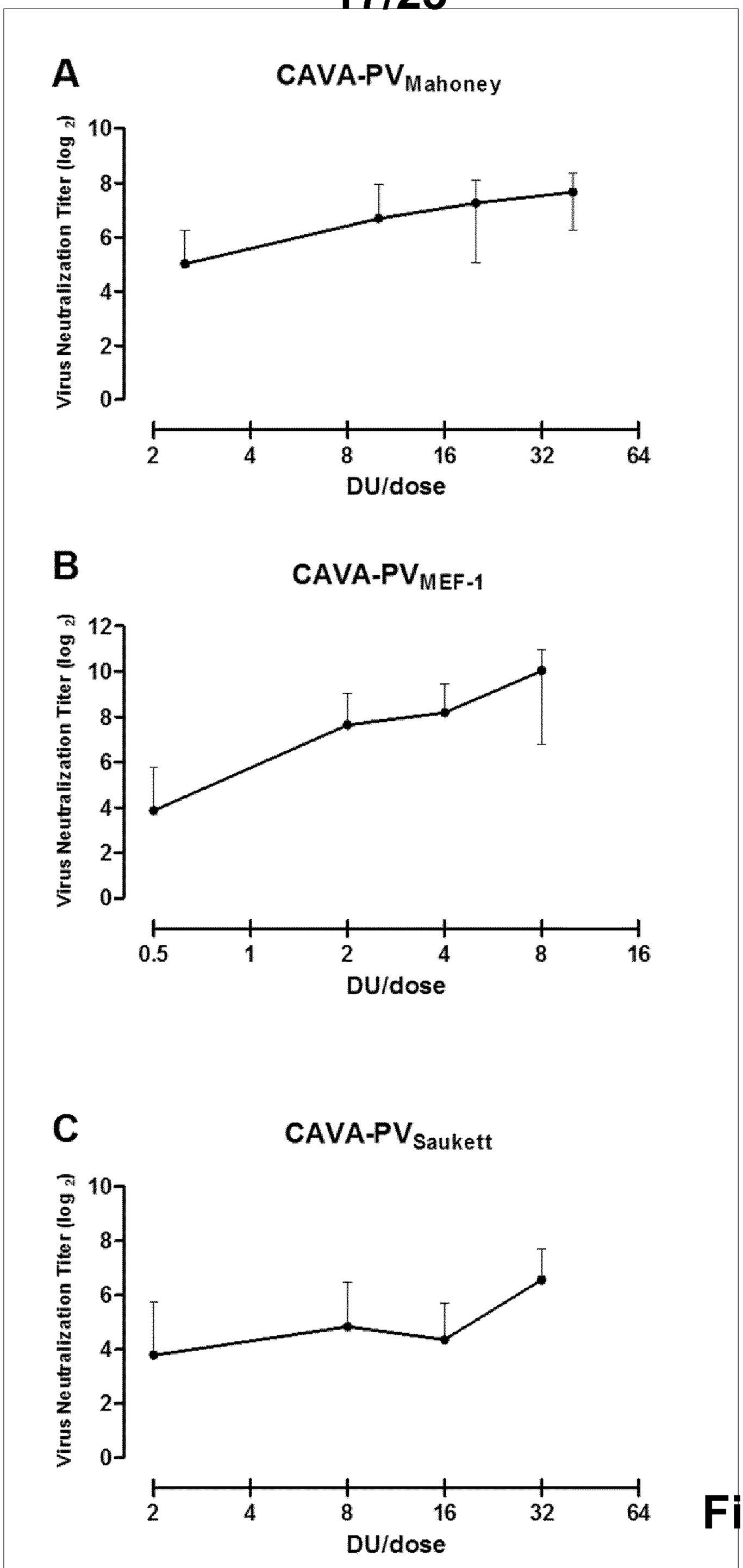
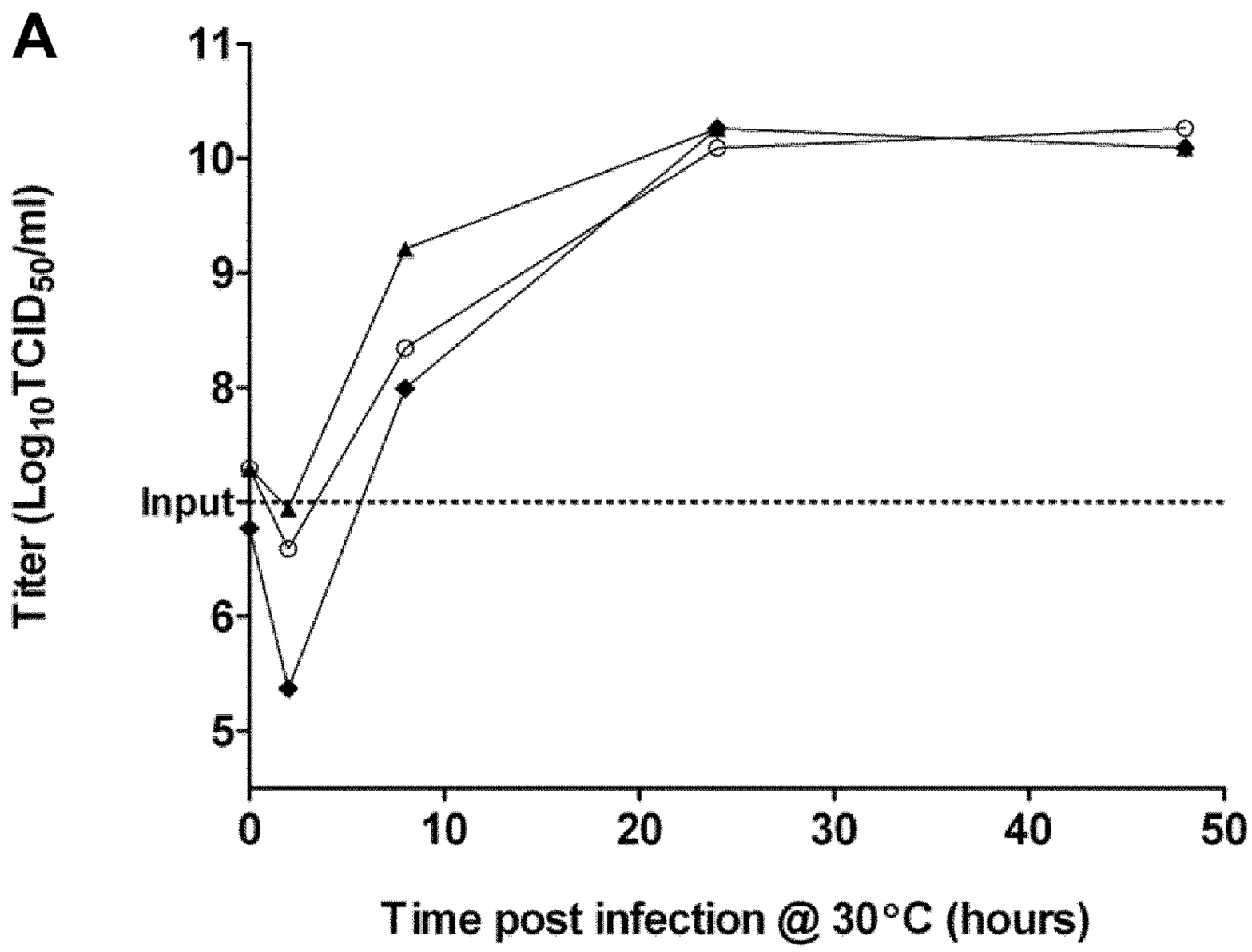

A
CAVA-PV Mahoney
Virus Neutralization Titer (log 2)
10
8
6
4
2
0
2
4
8
16
32
64
DU/dose
B
CAVA-PV MEF-1
Virus Neutralization Titer (log 2)
12
10
8
6
4
2
0
0.5
1
2
4
8
16
DU/dose
C
CAVA-PV Saukett
Virus Neutralization Titer (log 2)
10
8
6
4
2
0
2
4
8
16
32
64
DU/dose

Fig. 14


A
11
10
9
8
Input
6
5
Titer (Log10TCID50/ml)
0
10
20
30
40
50
Time post infection @ 30°C (hours)

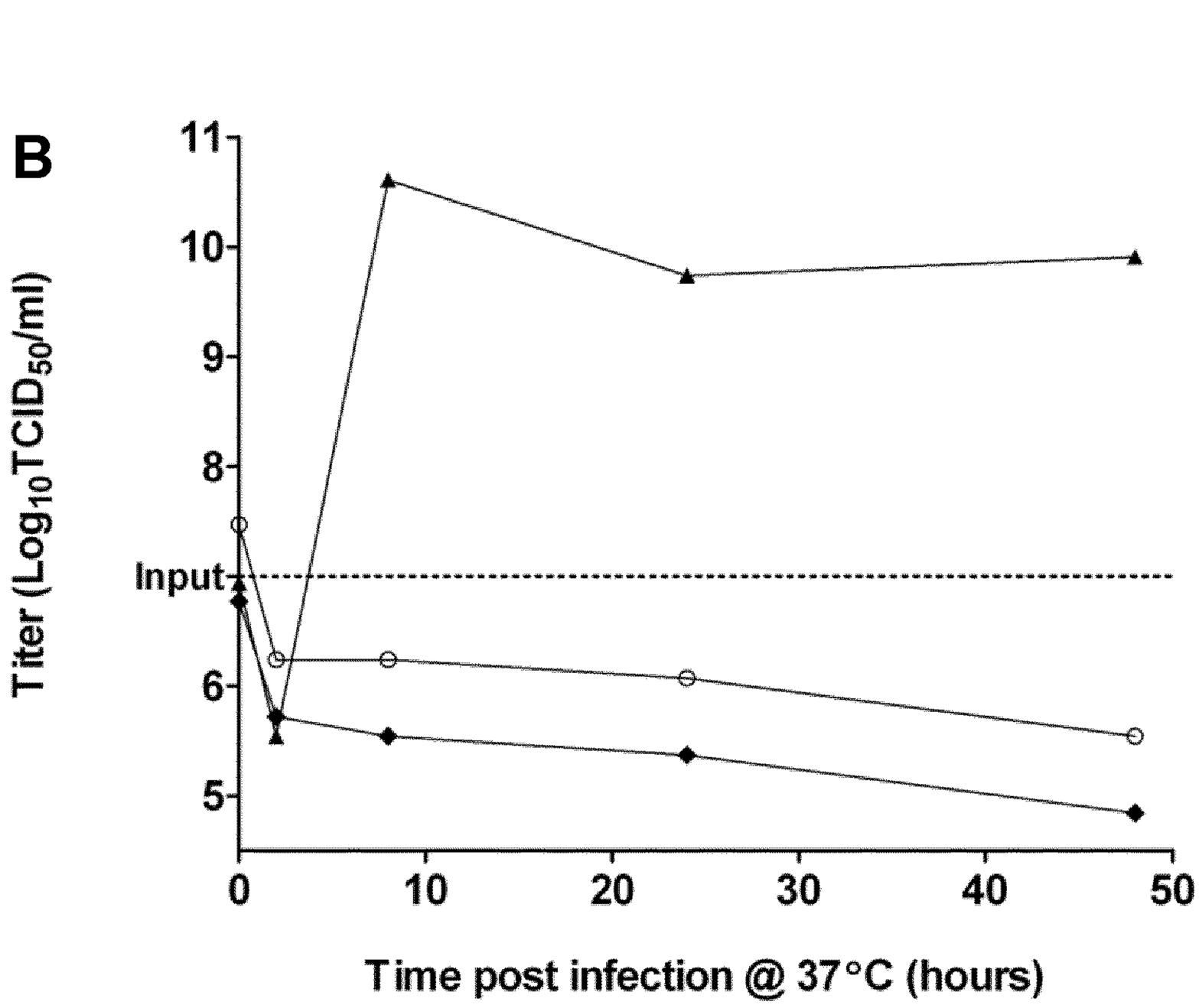
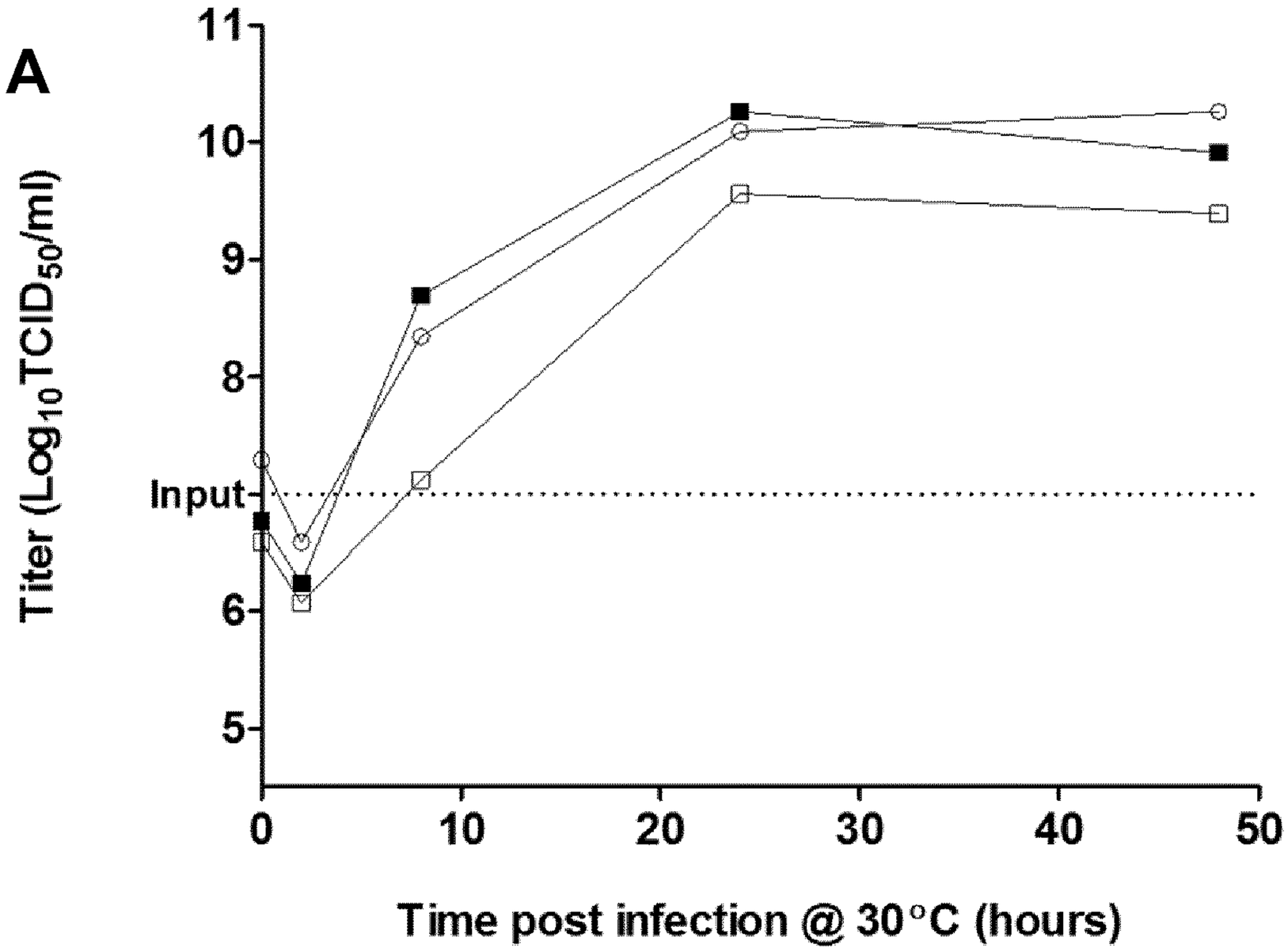

B
11
10
9
8
Input
6
5
Titer (Log10TCID50/ml)
0
10
20
30
40
50
Time post infection @ 37°C (hours)

Fig. 15


A
Titer (Log10TCID50/ml)
11
10
9
8
Input
6
5
0
10
20
30
40
50
Time post infection @ 30°C (hours)

B
Titer (Log10TCID50/ml)
11
10
9
8
Input
6
5
0
10
20
30
40
50
Time post infection @ 37°C (hours)

Fig. 16

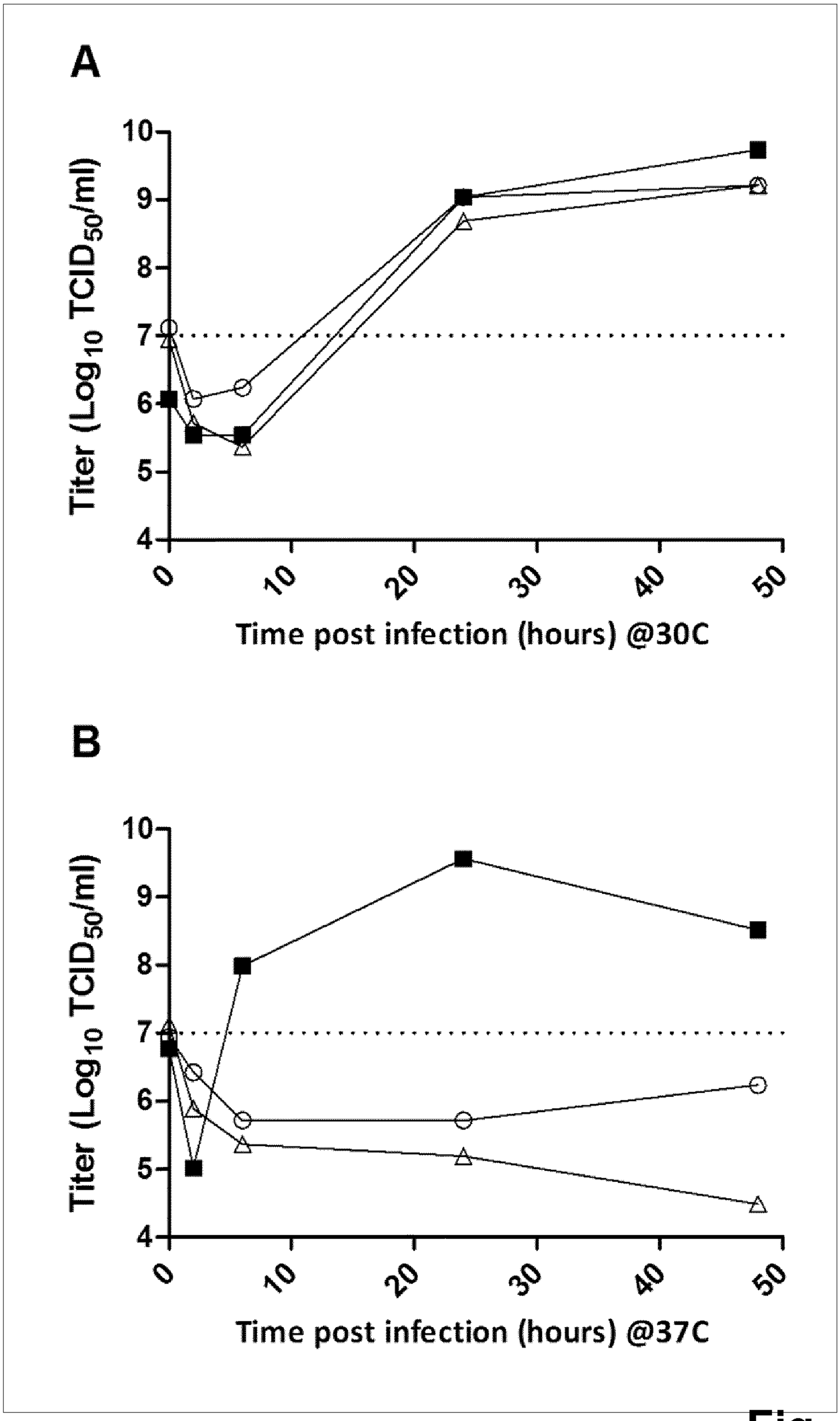
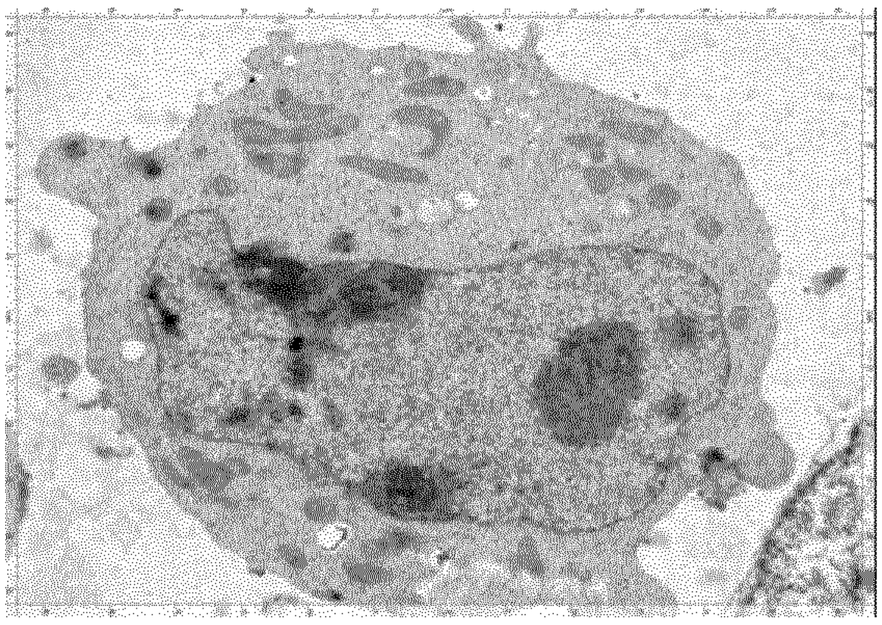
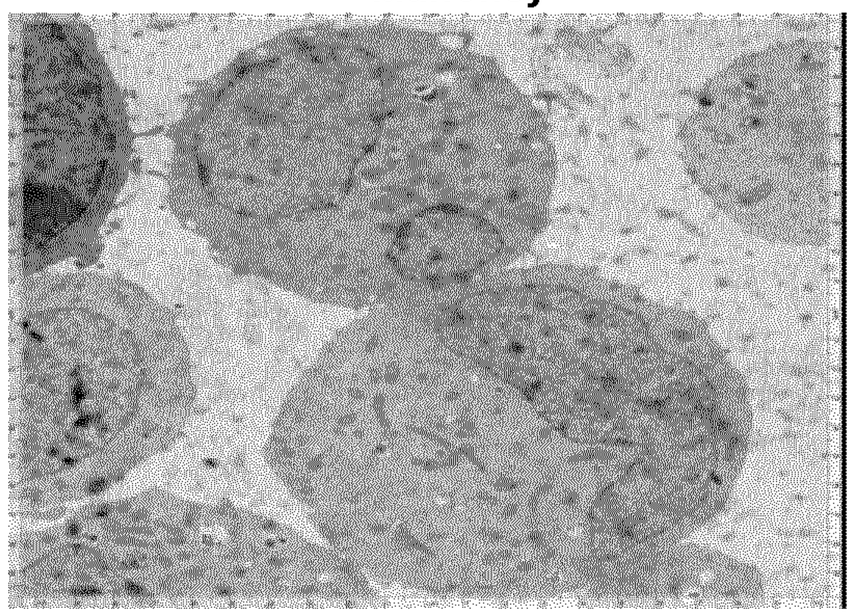

A
Titer (Log10 TCID50/ml)
10
9
8
7
6
5
4
0
10
20
30
40
50
Time post infection (hours) @30C
B
Titer (Log10 TCID50/ml)
10
9
8
7
6
5
4
0
10
20
30
40
50
Time post infection (hours) @37C

Fig. 17

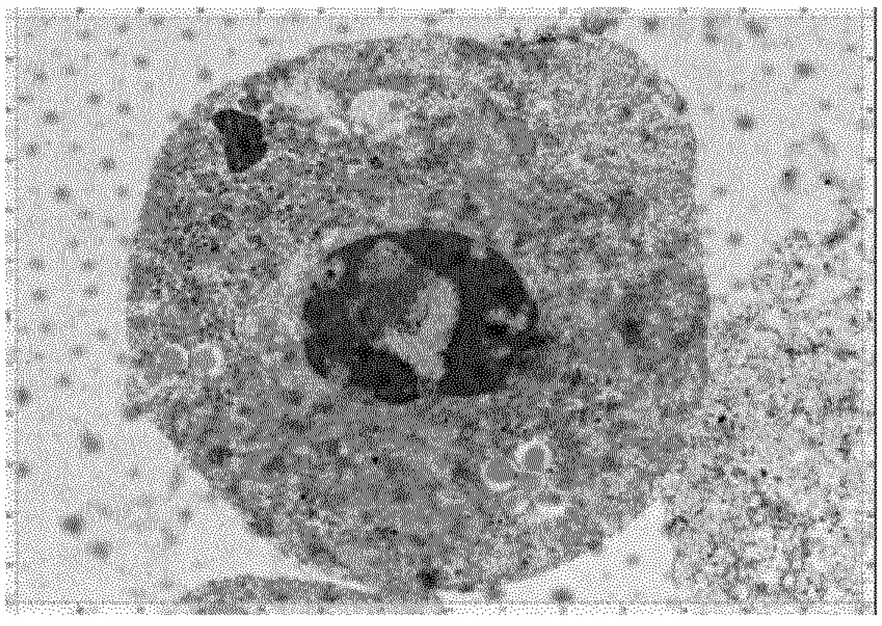
A) PBS/Mock@37°C



C) CAVA-PV$_{Mahoney}$@37°C



B) Mahoney@37°C



D) CAVA-PV$_{Mahoney}$@30°C

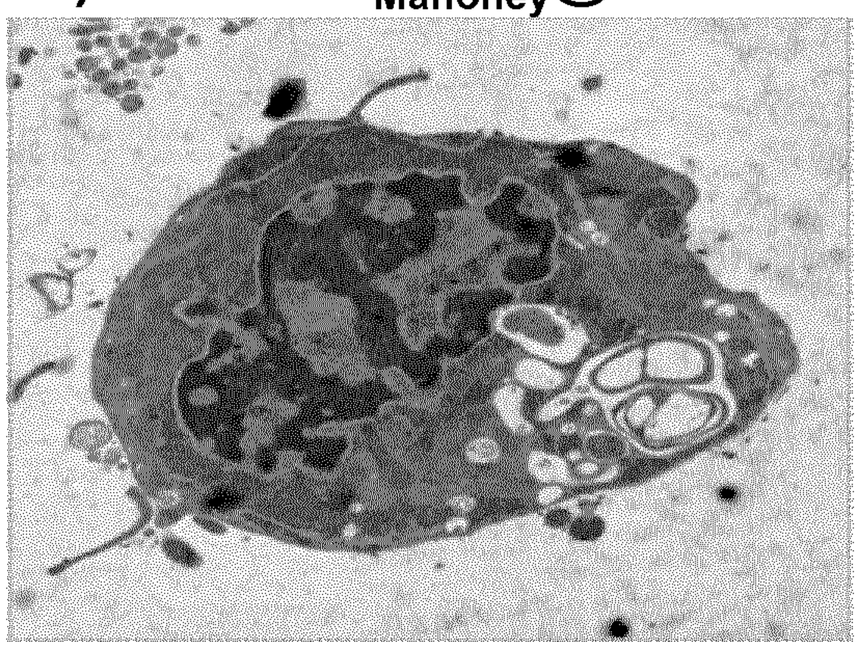


Fig. 18

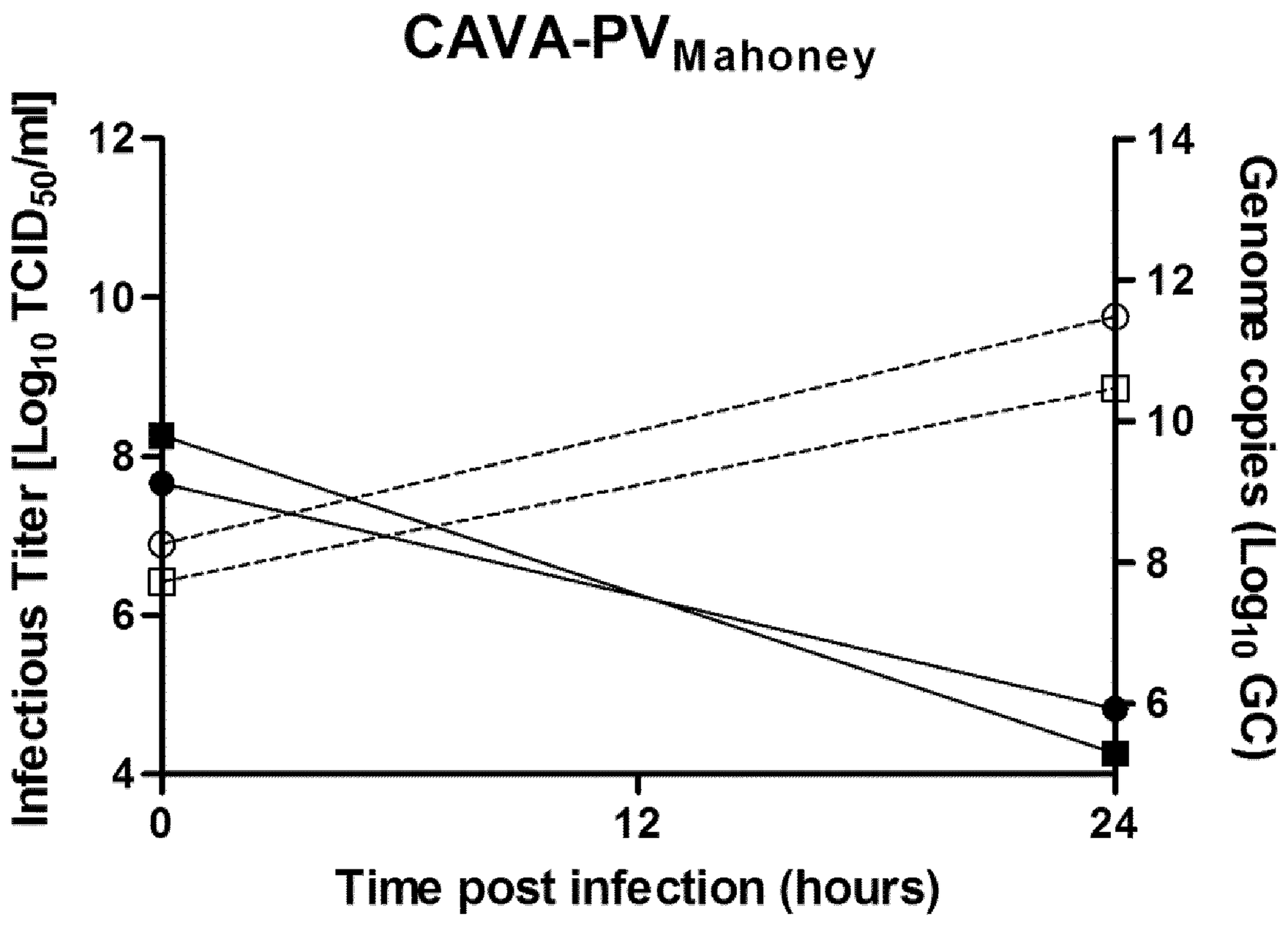
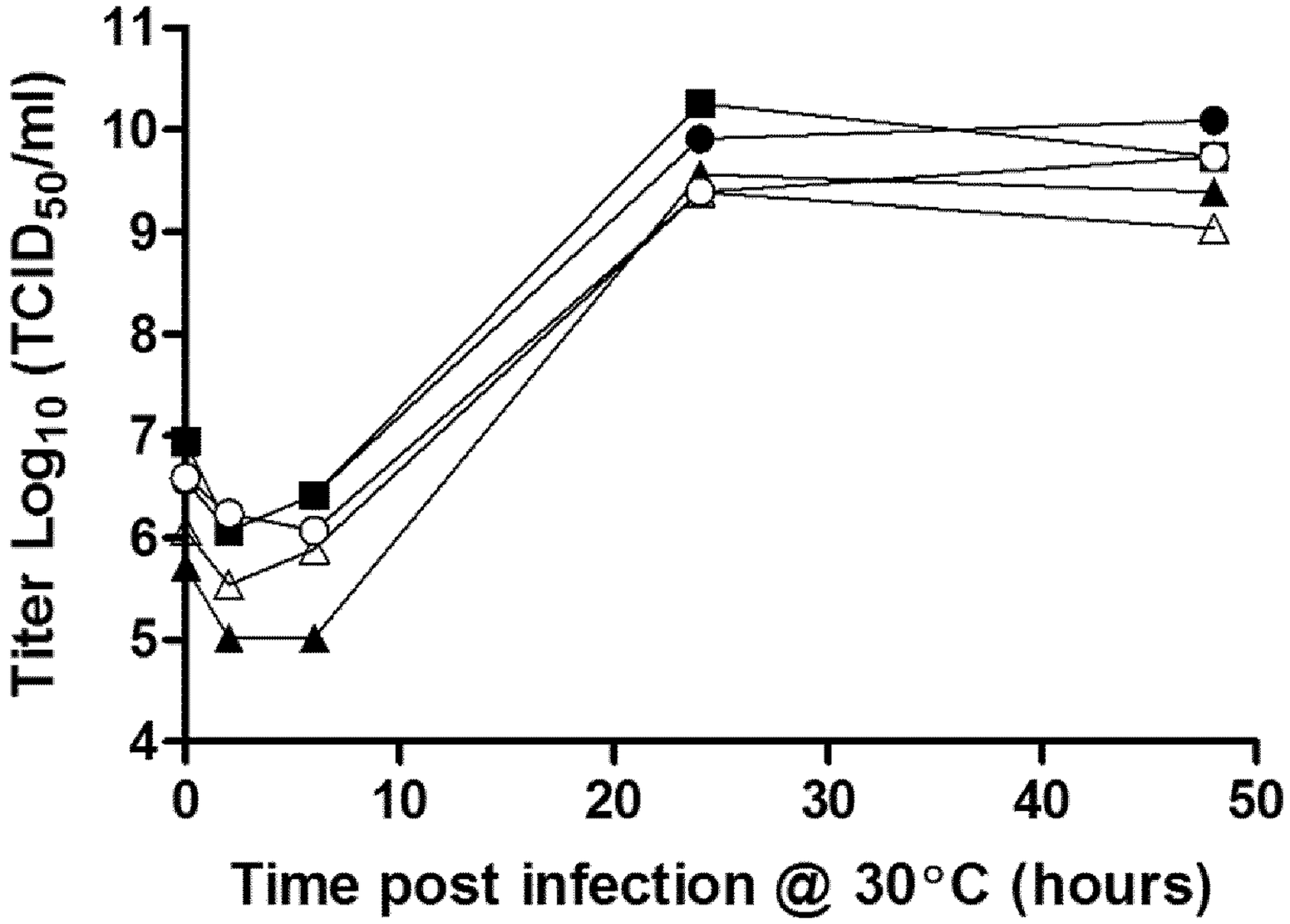

CAVA-PV Mahoney
Infectious Titer [Log10 TCID50/ml]
Genome copies (Log10 GC)
12
10
8
6
4
14
12
10
8
6
0
12
24
Time post infection (hours)

Fig. 19

A


Titer Log10 (TCID50/ml)
11
10
9
8
7
6
5
4
0
10
20
30
40
50
Time post infection @ 30°C (hours)

B

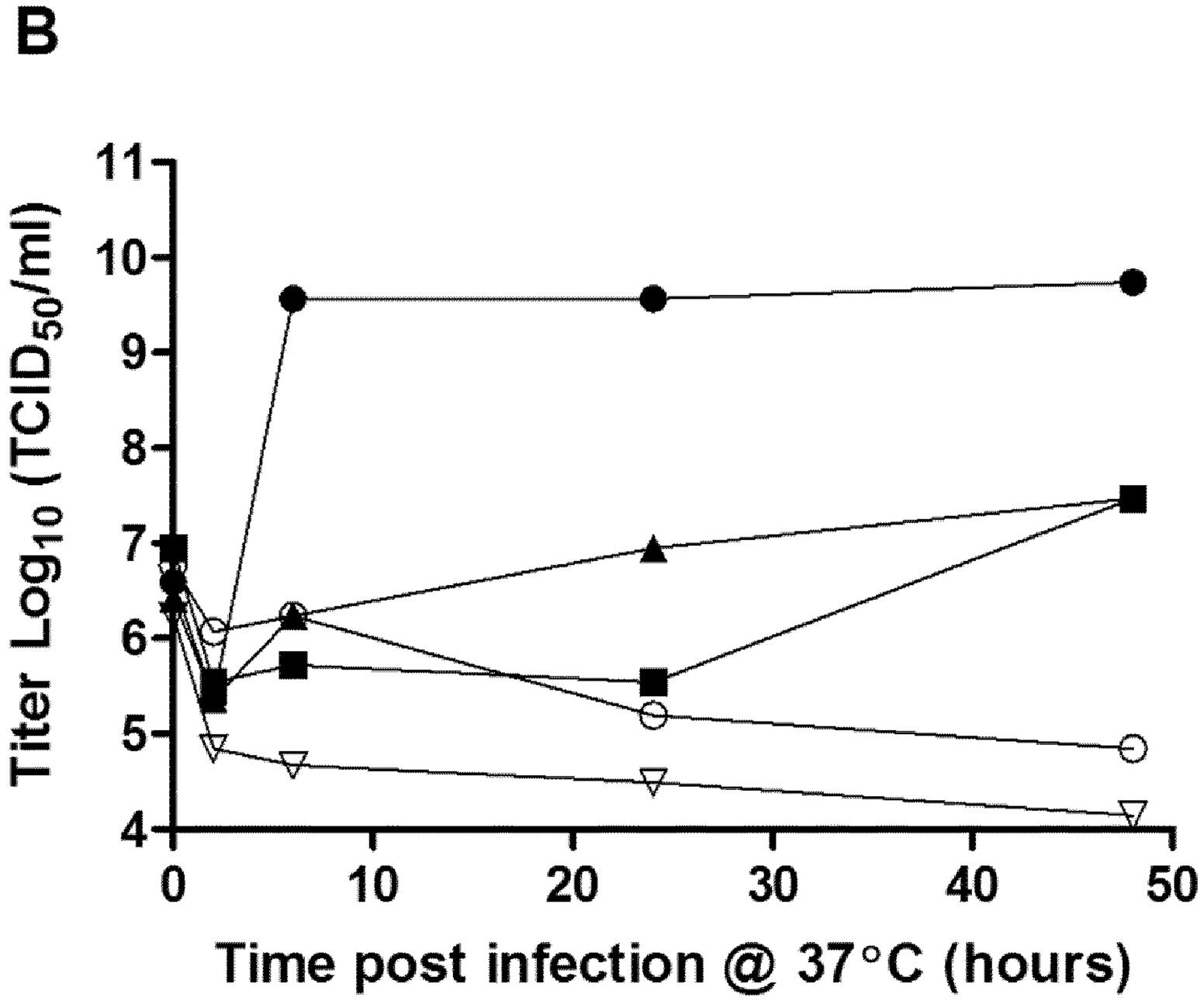

Titer Log10 (TCID50/ml)
11
10
9
8
7
6
5
4
0
10
20
30
40
50
Time post infection @ 37°C (hours)

Fig. 20