Development of an immunochromatography assay (QuickNavi™-Ebola) to detect multiple species of ebolaviruses

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Abstract

The latest outbreak of Ebola virus disease (EVD) in West Africa has highlighted the urgent need for the development of rapid and reliable diagnostic assays. We utilized monoclonal antibodies specific to the ebolavirus nucleoprotein to develop an immunochromatography (IC) assay (QuickNavi^{TM}-Ebola) for rapid diagnosis of EVD. The IC assay was first evaluated with tissue culture supernatants of infected Vero E6 cells and found to be capable of detecting 10^3-10^4 focus-forming units/ml of ebolaviruses. Using serum samples from experimentally infected nonhuman primates, we confirmed that the assay could detect the viral antigen shortly after disease onset. It was also noted that multiple species of ebolaviruses could be detected by the IC assay. Owing to the simplicity of the assay procedure and absence of requirements for special equipment and training, QuickNavi^{TM}-Ebola is expected to be a useful tool for rapid diagnosis of EVD.

Keywords: Ebola virus; ebolavirus; EVD; filovirus; nucleoprotein; monoclonal antibody; immunochromatography assay; diagnosis

INTRODUCTION

Ebolaviruses and marburgviruses are enveloped, nonsegmented, negative-stranded RNA viruses belonging to the family *Filoviridae*. These filoviruses are known to cause severe hemorrhagic fever in humans and nonhuman primates with human case fatality rates of up to 90% [1]. In contrast to the genus *Marburgvirus*, which contains only one known species, *Marburg marburgvirus* consisting of Marburg virus (MARV) and Ravn virus, five distinct species are known in the genus *Ebolavirus*, *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï Forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus*, represented by Ebola virus (EBOV), Sudan virus (SUDV), Taï Forest virus (TAFV), Bundibugyo virus (BDBV), and Reston virus (RESTV), respectively [2].

Ebola virus disease (EVD) poses a significant public health threat as implicated by the increase in the incidence of EVD outbreaks in Africa over the past two decades with some occurring in previously unaffected areas [3]. The most recent epidemic of EVD severely affected Sierra Leone, Guinea, and Liberia, and emphasizes the need for rapid, sensitive, reliable and virus-specific diagnostic tests to control the spread of the virus. Rapid and simple antigen-detection tests such as immunochromatography (IC) assays utilizing filovirus-specific monoclonal antibodies (mAbs) are likely one of the options for early diagnosis of filovirus infections in the field setting.

EBOV particles consist of seven structural proteins, the nucleoprotein (NP), viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, and polymerase [1, 4]. The NP appears to be an ideal target antigen for IC assays because of its abundance in filovirus particles, its strong antigenicity, and the presence of common epitopes among ebolavirus species [5]. The average EBOV particle contains about 3,200 NP molecules [6]. The NP plays an important role in the replication of the viral genome and is essential for formation

of the ribonucleocapsid [7]. Coexpression of NP VP40 and GP in cultured cells leads to efficient production of virus-like particles (VLPs) containing NP in the core [6, 8].

Previously, we generated a panel of mouse monoclonal antibodies (mAbs) recognizing the EBOV NP and identified their epitopes, some of which are shared among multiple ebolavirus species, whereas others are species-specific [5]. Using these mAbs, we generated an IC assay (QuickNavi^{TM}-Ebola) and evaluated its ability to detect the NP antigen in culture supernatants of infected Vero E6 cells and in sera collected from EBOV-infected nonhuman primates (NHPs).

MATERIALS AND METHODS

Viruses and Cells

EBOV (Mayinga, Kikwit, Makona C05, and C07), SUDV (Boniface), TAFV (Pauléoula), BDBV (Butalya), RESTV (Pennsylvania), and MARV (Angola) were propagated in African green monkey kidney Vero E6 cells and stored at -80°C. Virus titers were determined as focus-forming units (FFU) by immunoplaque assays. All experiments involving the use of infectious filoviruses were performed in the Biocontainment Level 4 (BSL4) laboratories of the Integrated Research Facility at the Rocky Mountain Laboratories (RML), Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA. Standard operating procedures for infectious work were approved by the RML Biosafety Committee.

Preparation of VLPs and purified recombinant NPs (rNPs)

Plasmids encoding NP, VP40, and GP of EBOV (Mayinga and Makona C07), SUDV, TAFV, BDBV, and RESTV were constructed as described previously [5, 9]. VLPs were produced by transfection of 293T cells with plasmids expressing ebolavirus NP, VP40, and GP as described previously [6, 8]. Forty-eight hours after transfection, supernatants containing VLPs were harvested and used for kit evaluation assays. For the preparation of purified NPs, 293T cells were transfected with the plasmids expressing the NPs of the respective ebolaviruses. Forty-eight hours later, the cells were lysed, and the NP fraction was collected by discontinuous CsCl gradient centrifugation as described previously [6, 8].

ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the reactivities of mAbs with rNPs as previously described [5]. Briefly, 96-well ELISA plates (Nunc, Maxisorp) were coated with purified ebolavirus rNPs (2 µg/ml in PBS, 50 µl/well) overnight at 4°C, followed by blocking with 1% bovine serum albumin, and purified mAbs (1 µg/ml or 4-fold serial dilutions from 10 µg/ml) were added. Bound antibodies were visualized with horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch) and 3,3',5,5'-tetramethylbenzidine (Sigma).

Evaluation of the IC assay

Tissue culture supernatants from Vero E6 cells infected with filoviruses (EBOV, SUDV, TAFV, BDBV, RESTV, or MARV), sera collected from NHPs (rhesus and cynomolgus macaques) experimentally infected with EBOV, and EBOV-infected patients were used to determine the sensitivity and specificity of the IC assay. For each assay, 30 µl (serum/plasma and culture supernatant sampels) or 10-20µl (whole blood samples) were used. Other human pathogens were used to test for cross-reactivity of the assay (Supplementary Table 1).

Ethical statement

NHP serum samples [10-13] were collected in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. EBOV-infected human blood samples were collected from patients during the 2014 EBOV outbreak in DR Congo.

Blood collection during outbreak investigations was approved under a special response protocol established between the World Health Organization and national authorities. The experiments involving the use of human samples were approved by the institutional ethics committee, (Denka Seiken Co., LTD, Niigata, Japan) in accordance with the Declaration of Helsinki.

RESULTS

Selection of mAbs for the IC assay

We first analyzed the binding affinities of 10 different clones of NP-specific mAbs in ELISA with purified rNPs of the representative isolates of all known ebolavirus species (EBOV, SUDV, TAFV, BDBV, and RESTV), including the 2014 EBOV Makona isolate, as antigens (Table 1 and Supplementary Figure 1). To select two mAbs employed in the IC assay (i.e., one labeled mAb and the other capturing mAb), we produced a test device based on a lateral-flow IC assay and assessed all possible combinations of mAbs suitable for labeled and capturing mAbs to detect EBOV VLPs. We found two combinations of mAbs giving the highest sensitivity (ZNP105-7/ZNP108-2-5 and ZNP105-7/ZNP62-7) (data not shown). Based on their cross-reactivity profiles, we selected the ZNP105-7 (labeled)/ZNP108-2-5 (capturing) mAbs since this combination was expected to have the potential to detect ebolaviruses from the different species (i.e., EBOV, TAFV, and BDBV), whereas the other mAb combination was EBOV-specific. The binding assay with the chimeric rNPs between EBOV and SUDV indicated that both mAbs ZNP105-7 and ZNP108-2-5 recognized amino acids at positions 632-739 of the NP molecule (Supplementary Figure 2). These amino acids are located in the C-terminal region of NP, which has been reported to have strong antigenicity [14].

Sensitivity and specificity of the IC assay to detect ebolaviruses in tissue culture supernatants

Using ZNP105-7 and ZNP108-2-5 as labeled and capturing mAbs, respectively, we produced an IC device (QuickNavi^{TM}-Ebola) (Fig. 1). For one test, 10-30 μl of samples can be applied onto the sample pad of this IC assay, followed by the addition of

2 drops (approximately 40 μl) of the sample buffer (saline-based reagent) supplied with the kit. The results can be interpreted 10 min later as positive by the appearance of both control and test lines and negative if only the control line appears. We first assessed the specificity and sensitivity of the IC assay by analyzing 10-fold serially diluted infectious filoviruses (10^1-10^6 FFU/ml) and found that the assay was reactive for EBOV Mayinga (10^3 FFU/ml), EBOV Makona (10^4 FFU/ml), TAFV (10^3 FFU/ml), and BDBV (10^3 FFU/ml), but not SUDV, RESTV, and MARV (Table 2). The spectrum of virus detection was consistent with the reactivity profile of the mAbs chosen for the IC assay. To further evaluate the sensitivity of the assay, we used purified rNPs and estimated that the limit of the detection (LOD) ranged from 0.03 to 0.3μg/ml depending on the isolates (Table 2). Other human pathogens, including viruses and bacteria, some of which potentially cause hemorrhagic fever, were also tested (Supplementary Table 1) but did not react in the assay. In addition, we confirmed that the presence of these pathogens in human plasma did not interfere with the reactivity of the IC assay to detect EBOV VLPs.

Application of the IC assay for EVD diagnosis

We next evaluated the utility of the IC assay using serum samples from experimentally infected NHPs (Table 3). Serum samples were collected on the indicated days after infection, and virus titers were determined. For the IC assay, undiluted serum samples (30 μl) were directly applied and the results were obtained 10 min later. We found that the IC assay was able to detect EBOV and BDBV antigens in most of the samples containing infectious viruses that were detectable in 50% tissue culture infectious dose ($TCID_{50}$) assays (Table 3). Consistent with the data of the tissue culture experiment, the LOD of the IC assay seemed to be 10^3-10^4 FFU/ml. It was noted that EBOV in the serum

189 became detectable simultaneously in $TCID_{50}$ and the IC assay on day 3 or 4 after infection,
190 at a time before significant hematologic changes (e.g., a rapid increase of ALT and
191 decrease of platelet counts) appeared (Figure 2), suggesting that our IC assay was capable
192 of detecting the viral antigen shortly after disease onset. We further confirmed that the IC
193 assay could specifically detect the EBOV NP antigen in plasma or blood samples
194 collected from EBOV-infected humans during the 2014 outbreak in DR Congo.
195 Unfortunately, we were only able to obtain a limited number of samples without
196 information about disease onset of the person; therefore a thorough evaluation of the IC
197 assay sensitivity could not be performed.
198
199 **Performance evaluation of the IC assay**
200 To evaluate the stability of the IC assay, we compared its performance at different
201 temperatures and humidity conditions mimicking mild, tropical, and severe conditions,
202 and noted that it was not significantly decreased even under tropical conditions
203 (Supplementary Tables 2-4). The influence of potentially interfering substances on the
204 sensitivity and specificity of the IC assay was also evaluated (Supplementary Table 5).
205 No interference was observed for hemoglobin, conjugated bilirubin, intrafat, triglyceride,
206 ibuprofen, ribavirin, promethazine, ampicillin, acetaminophen, or quinine. The following
207 substances also exhibited no significant effect on the test results up to the specified
208 concentrations: free bilirubin 25 mg/dl; doxycycline 195 μM; ciprofloxacin 10 mg/ml;
209 and aspirin 15 mg/ml. Rheumatoid factor negatively impacted the test results in a
210 concentration-dependent manner, causing false-positive and/or false-negative reactions at
211 a concentration higher than 200 IU/ml.
212

DISCUSSION

The 2014 EVD outbreak has raised many serious concerns regarding the development of effective strategies to control this deadly infectious disease. One of the key components to minimize the spread of EVD is early diagnosis. However, clinical signs and symptoms of EVD are mainly non-specific, often leading to misdiagnosis as other more frequent infectious diseases that are endemic to the area present with the same symptoms [15]. Consequently, there was usually a delay between the initial case identification and the laboratory-confirmed diagnosis of EVD in most of the past outbreaks. In addition, the initial cases and the subsequent spread of disease at the beginning of an outbreak often occur in remote, sometimes inaccessible areas in the affected countries, thereby making the collection of samples for laboratory diagnosis difficult and resulting in a long time-lapse prior to awareness of the EVD outbreak [16]. These facts further underline the need for diagnostic methods that can be used on-site, enabling immediate commencement of appropriate intervention measures upon recognition of suspected EVD cases.

For early diagnosis of EVD in suspected cases, detection of viral RNA by quantitative real-time RT-PCR (qRT-PCR) and viral antigens by ELISA are the recommended methods [17, 18]. Although these methods are highly sensitive, specific, and relatively rapid, they require a special laboratory setup, equipment and personnel training. Furthermore, the samples need to be transported to the main diagnostic laboratory, often leading to a delay in diagnosis and subsequent commencement of measures to control the spread of the disease. On the other hand, IC assays are successfully used for rapid diagnosis of various diseases such as influenza, human adenoviral infection, streptococcal infection, etc. [19]. The advantages of these assays are

their simplicity of use, lack of need for special training, and no electricity requirement, with significant reliability. In this study, we successfully developed an IC assay for easy and rapid diagnosis of EVD using mAbs specific to ebolavirus NPs.

Currently, there are three rapid diagnosis assays approved by the FDA and/or WHO, ReEBOV (Corgenix), OraQuick Ebola (OraSure Technologies, Inc.), and SD Q Line Ebola Zaire Ag (SD Biosensor Inc.) [20]. ReEBOV uses whole blood samples or plasma samples and has been most widely used in both point-of-care and reference laboratory facilities in West Africa. This assay uses caprine polyclonal antibodies against EBOV VP40. Two independent studies reported that the LOD of ReEBOV was 10^6 plaque-forming units/ml or 211 million RNA copies/ml (maximum CT value of 26.3). The OraQuick Ebola, which is also based on the detection of VP40, can detect EBOV, SUDV and BDBV. The LOD of this assay, according to the manufacturer, is 1.64×10^6 $TCID_{50}$/ml. The SD Q Line Ebola Zaire Ag has three capture lines coated with mouse mAbs specific for EBOV GP, NP, or VP40 and one control line. The LODs of GP, NP, and VP40 are 31.3 ng/ml, 3.9 ng/ml and 62.5 ng/ml, respectively. On the other hand, the LODs of QuickNavi^{TM}-Ebola were 10^3-10^4 FFU/ml for infectious ebolaviruses and 33 ng/ml (1 ng/30 μl/test) for purified EBOV rNP, suggesting that our IC assay has at least comparable or even better sensitivity than the above-mentioned rapid diagnostic assays.

It has been reported that viremia is often not detectable during the first few days after EBOV infection but increases steeply with severe disease onset in many EBOV-infected patients, and the viral load eventually reaches 10^6-10^9 plaque-forming units/ml in the blood [21, 22]. In a NHP model of EVD, a similar correlation between the disease onset and increased viremia was observed [10, 23, 24]. QuickNavi^{TM}-Ebola could detect 5.6 x 10^2 and 1.8 x 10^3 $TCID_{50}$/ml of EBOV (Kikwit) in the sera of the infected NHPs

before a significant increase of ALT and a decrease in platelet count were observed (Figure 2). While the virus was not detected in the blood or sera of these NHPs on day 2 after infection by our IC assay, qRT-PCR, or $TCID_{50}$ assay, samples collected on day 4 after EBOV infection became positive both in our IC assay and qRT-PCR. It should be noted that this correlation between increased viremia and onset of clinical symptoms was commonly seen in both Kikwit- and Makona- infected NHPs and there was not a remarkable difference in the timing of detectable viremia between these NHPs, although Makona-infected NHPs eventually showed a little longer survival [23]. Indeed, QuickNavi^{TM}-Ebola could detect EBOV (Makona) in the sera of infected NHPs concomitantly with a significant increase of viremia (Table 3). These results suggest that the sensitivity of QuickNavi^{TM}-Ebola is reliable and high enough for the practical use in EVD diagnosis, particularly for the detection of an initial EVD case in remote areas and also for the first screening of suspected patients in Ebola treatment units.

Moreover, the absence of cross-reactivity and interference with other tested pathogens such as hemorrhagic fever viruses (e.g., Lassa virus and Crimean-Congo hemorrhagic fever virus), bacteria causing acute diarrhea or dysentery (e.g., *Salmonella Typhimurium*, *Shigella sonnei*, and *Vibrio cholerae*), and *Plasmodium falciparum* suggests the high specificity and reliability of QuickNavi^{TM}-Ebola. Accumulatively, these findings support the suitability of our IC assay for clinical application for the diagnosis of EVD in endemic settings in Africa. Since the idea of developing IC assays comes from the on-site utility and performance under rough and tough field conditions, we confirmed its stability to be satisfactory in both high temperature and high humidity conditions. An ongoing study suggests a shelf-life time of at least 8 months at room temperature. These data indicate that QuickNavi^{TM}-Ebola does not require refrigeration for transportation and

storage, and can be used under severe conditions without special instruments in remote areas in Africa.

Although EBOV has caused the majority of human infections, other ebolaviruses (e.g., SUDV and BDBV) have also repeatedly caused outbreaks in Central Africa. Since it is not predictable which ebolavirus species will cause EVD in future, the first priority for the initial case identification should be to quickly clarify EVD or other viral diseases, enabling us to act immediately in response to suspected outbreaks, followed by ebolavirus species-specific countermeasures once it is confirmed. In summary, QuickNavi^{TM}-Ebola showed high sensitivity and high specificity for EBOV, TAFV, and BDBV. We have already produced anti-SUDV NP mAbs and determined the optimal mAb combination that could detect SUDV (10^3 FFU/ml) (data not shown). With the addition of these SUDV NP-specific mAbs, our IC assay will have the potential to readily detect all known African ebolaviruses. Although further analyses are needed to assess the clinical applicability of QuickNavi^{TM}-Ebola using human samples, our findings suggest that this IC assay based on the detection of NP is a valuable tool for the rapid diagnosis of EVD.

Notes

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392

393 **Figure legends**

394

395 **Figure 1.** Design of the IC assay. QuickNavi^{TM}-Ebola is a lateral flow-based IC kit
396 (85mm x 21mm x 6.9mm) for the direct detection of ebolavirus NP antigens. For each
397 assay, 30 μl of serum/plasma or 10-20μl of whole blood samples can be applied. When a
398 sample is added to the sample window of the kit, it migrates through the reagent pads via
399 capillary action. One of the reagent pads is a conjugate pad on which latex conjugated
400 with an anti-ebolavirus NP mAb has been dried. The ebolavirus NP antigens present in
401 the sample bind to the latex-conjugated mAbs on the pad. Another ebolavirus NP-specific
402 mAb is also immobilized on a nitrocellulose membrane at the Test line position to capture
403 the complexes of NP antigens and mAbs conjugated with latex. As the assay develops,
404 those complexes deposit a visible blue line.

405

406 **Figure 2.** Hematological changes and $TCID_{50}$ of NHPs infected with EBOV. Virus titers
407 in the blood samples were determined as $TCID_{50}$ in Vero E6 cells (Please also see #1 and
408 #2 in Table 3). Platelet counts were determined from whole blood samples. Alanine
409 aminotransferase levels (ALT) were determined from serum. The arrows indicate the time
410 at which the NHPs tested were positive in the IC assay.

Table 1

Table 1. Binding profiles of anti-NP mAbs.

mAb	Isotype	EBOV (Mayinga)	EBOV (Makona C07)	SUDV	TAFV	BDBV	RESTV
ZNP31-1-8	IgG1	++	++	++	++	++	++
ZNP41-2-4	IgG1	++	++	++	++	++	++
ZNP74-7	IgG1	++	++	++	++	++	++
ZNP24-4-2	IgG1	++	++	++	++	++	++
ZNP106-9	IgG1	++	++	+	++	++	-
ZNP108-2-5	IgG1	++	++	-	++	++	-
ZNP105-7	IgG1	++	++	-	+	++	++
ZNP98-7	IgG2a	++	++	-	-	++	-
ZNP35-16-3-5	IgG1	++	++	-	-	-	-
ZNP62-7	IgG2b	++	++	-	-	-	-

Antibody binding was evaluated based on ELISA OD_{450} values at a mAb concentration of 1 μg/ml. ++, $OD \geq 1.0$, +, $0.2 < OD < 1.0$; -, $OD \leq 0.2$.

Table 2. Detection of infectious ebolaviruses and rNP by QuickNaviTM-Ebola.

Virus	Limit of detection (LOD)	
	Virus titer (FFU/ml)	Purified rNP (μg/ml)
EBOV (Mayinga)	$1x10^3$	0.033
EBOV (Makona C07)	$1x10^4$	0.15
SUDV	$>1x10^5$	>30
TAFV	$1x10^4$	0.33
BDBV	$1x10^3$	0.15
RESTV	$>2x10^5$	>30
MARV	$>2x10^5$	>30

Serial dilutions of the samples (30 μl) were applied to the sample pad of the device, and LOD are expressed as the lowest titers (virus) and concentrations (rNP) that were positive in the IC assay.

Table 3. Detection of ebolaviruses in the sera of infected monkeys.

NHP	Virus	Days post infection	$TCID_{50}$/ml	CT value[b]	IC assay
#1 (rhesus)[a]	EBOV (Kikwit)	0	-[c]	-	-
		2	-	-	-
		4	$1.8x10^3$	34.94	+
		6	$5.6x10^6$	20.66	+
		8	$3.2x10^7$	17.6	+
#2 (rhesus)[a]	EBOV (Kikwit)	0	-	-	-
		2	-	-	-
		4	$5.6x10^2$	35.1	+
		6	$5.6x10^3$	25.32	+
		8	$3.2x10^6$	18.98	+
#3 (rhesus)[d]	EBOV (Makona C05)	0	-	ND[e]	-
		2	-	ND	-
		4	-	ND	-
		6	$1.8x10^6$	ND	+
		8	$5.6x10^4$	ND	+
#4 (rhesus)[d]	EBOV (Makona C05)	0	-	ND	-
		2	-	ND	-
		4	-	ND	-
		6	$3.2x10^6$	ND	+
		8	$5.6x10^5$	ND	+
#5 (rhesus)[d]	EBOV (Makona C05)	0	-	ND	-

		2	-	ND	-
		4	$1.8x10^4$	ND	+
		6	$5.6x10^6$	ND	+
		8	$1.8x10^4$	ND	+
#6 (cynomolgus)[d]	EBOV (Makona C07)	0	-	ND	-
		3	$3.2x10^2$	ND	-
		6	$3.2x10^6$	ND	+
#7 (cynomolgus)[d]	EBOV (Makona C07)	0	-	ND	-
		3	$3.2x10^3$	ND	-
		5	$1.8x10^7$	ND	+
#8 (cynomolgus)[a]	BDBV	3	ND	ND	-
		7	ND	ND	+
		10	ND	ND	+
#9 (cynomolgus)[d]	MARV	0	-	ND	-
		4	$1.8x10^5$	ND	-
		7	$3.2x10^7$	ND	-

[a] Gamma-irradiated serum samples were used.

[b] Real-time RT-PCR cycle threshold values.

[c] Not detected.

[d] Intact serum samples were used.

[e] Not determined.

Figure 1

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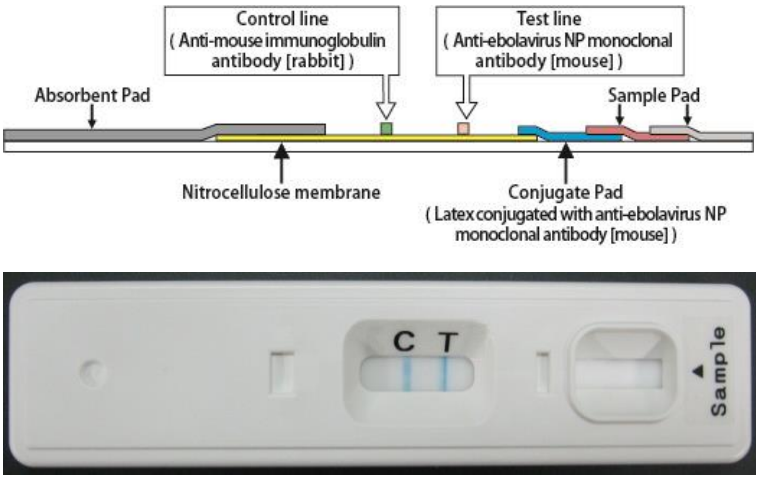

Fig. 1

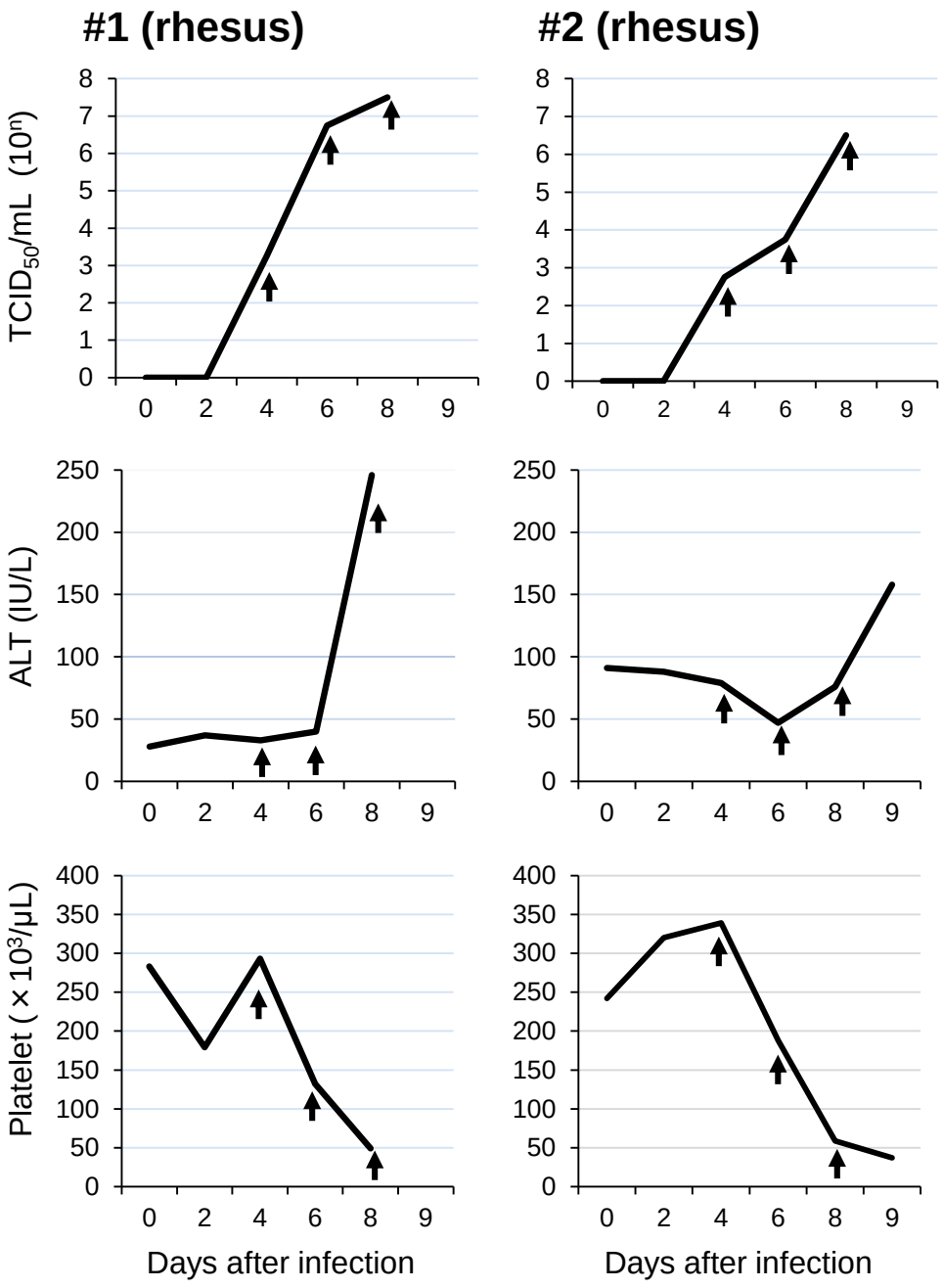

Fig. 2