Received: 8 May 2021 | Revised: 1 July 2021 | Accepted: 5 July 2021

DOI: 10.1111/1348-0421.12929

ORIGINAL ARTICLE

Microbiology and Immunology

# Validation of serum apolipoprotein A1 in rabies virus-infected mice as a biomarker for the preclinical diagnosis of rabies

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**Funding information**
Science and Technology Research Partnership for Sustainable Development (SATREPS), Grant/Award Number: 17823721; Japan Agency for Medical Research and Development (AMED)/JICA

## Abstract

Rabies is a type of acute fetal encephalitis caused by rabies virus (RABV). While it becomes incurable after symptom onset, it can be prevented by post-exposure prophylaxis (PEP) during the long incubation period. While preclinical diagnosis aids the appropriate PEP administration, it is mostly nonfeasible owing to the absence of viremia or a specific antibody response during the incubation period. Here, an attempt was made to identify a serum biomarker for the preclinical diagnosis of rabies. Using the serum from a mouse inoculated intramuscularly (i.m.) with $5 \times 10^5$ focus-forming units (FFU) of recombinant RABV expressing red firefly luciferase (1088/RFLuc) immediately before symptom onset, two-dimensional differential gel electrophoresis was conducted, followed by mass spectrometry, and it was confirmed that apolipoprotein A1 (ApoA1) was up-regulated. ELISA showed that the serum ApoA1 and specific antibody levels increased during the incubation period and on the day of symptom onset. Since a lower infectious dose can be used to induce the unstable and long incubation period generally observed in natural infection, the ApoA1 level in mice inoculated i.m. with $10^3$ FFU of 1088/RFLuc was examined by monitoring viral dynamics using in vivo imaging. The serum ApoA1 and specific antibody levels were

**Abbreviations:** Actb, β-actin; ApoA1, apolipoprotein A1; CSF, cerebrospinal fluid; dpi, days post-inoculation; FFU, focus-forming unit; HDL, high-density lipoprotein; IHC, immunohistochemical; i.m., intramuscularly; MS, mass spectrometry; N protein, nucleoprotein; PEP, post-exposure prophylaxis; RABV, rabies virus; RFLuc, red firefly luciferase; Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; 2D-DIGE, two-dimensional differential gel electrophoresis.

up-regulated in 50% and 58.3% of mice exhibiting robust RABV replication, respectively, but not in mice exhibiting weak RABV replication. In addition, it was reported that ApoA1 was found to be a biomarker for neuronal damage. Additional biomarker candidates will be needed for the effective preclinical diagnosis of rabies.

KEYWORDS
ApoA1, biomarker, in vivo imaging, proteomics, rabies

## INTRODUCTION

Rabies is a zoonotic viral disease and a global epidemic. It is caused by the rabies virus (RABV), which is a highly neurotropic virus from the family *Rhabdoviridae* and genus *Lyssavirus*. The RABV genome encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase, also referred to as the large protein (L). In general, RABV is transmitted via the bite of infected animals and spreads from the peripheral nerves to the brain.[1] The incubation period for rabies ranges from a few days to several years, but typically from 3 weeks to 3 months.[2] The case-fatality rate of rabies is approximately 100%, and there is no cure after the onset of clinical symptoms. However, disease development can be prevented using the appropriate repeated vaccination routine (post-exposure prophylaxis [PEP]) that must be completed during the incubation period, even after exposure to RABV.[3] Nevertheless, rabies is estimated to cause approximately 59,000 human deaths worldwide per year.[4]

While the antemortem diagnosis of rabies is available, its preclinical diagnosis is uncommon. Specific laboratory methods, such as the detection of viral antigen/RNA from specimens (e.g., skin and saliva) and specific antibodies in the serum or cerebrospinal fluid (CSF), are used for antemortem diagnosis.[3] However, because neither viremia nor specific antibody responses occur during the incubation period,[5,6] conventional virological methods cannot be used for the preclinical diagnosis of rabies. In this regard, preclinical diagnosis will be helpful for patients with a history of potential exposure to RABV, such as via animal bite or contact with bats,[7] who did not receive PEP. Consequently, it is expected that the rate of rabies-associated mortality will improve.[2]

Blood sampling is less invasive than skin biopsy and CSF sampling, and the use of serum biomarkers has been attempted in the diagnosis of various viral diseases.[8–12] The proteomics analysis, two-dimensional differential gel electrophoresis (2D-DIGE) followed by mass spectrometry (MS) for protein identification, is used widely to identify disease biomarkers.[13,14] In 2D-DIGE, a pair of protein samples to be compared are labeled separately using distinguishable fluorescent dyes, and the labeled samples are mixed and separated using 2D polyacrylamide gel electrophoresis (PAGE). Protein spots with different intensities can be used to identify potential biomarkers.[14]

In a previous study involving in vivo imaging analysis in RABV infection, we observed the robust replication of RABV in the spinal cord during the incubation period in infected mice.[15] Based on this, we predicted the occurrence of specific host responses (not limited to immune responses) that may alter the serum proteome. Li et al.[16] reported an alteration of the serum peptide profile during the early phase of RABV or Irkut lyssavirus infection in mice; however, proteins have not been identified. In this study, we screened serum factors using 2D-DIGE/MS in a mouse model of RABV infection. We successfully identified and evaluated the candidate biomarker apolipoprotein A1 (ApoA1).

## MATERIALS AND METHODS

### Virus, cell, and mouse

1088/RFLuc, the recombinant RABV strain 1088 expressing red firefly luciferase, was used, and the viral titer was determined previously and expressed in terms of focus-forming units (FFUs).[15] Strain 1088 is a street virus strain that was isolated from a rabid woodchuck in North America.[17] High Five cells were maintained in Gibco Grace's Insect Medium (Thermo Fisher Scientific) supplemented with 10% fetal calf serum. Hairless mice (Hos: HR-1, female, 6-week-old) were purchased from Hoshino Laboratory Animals, Ibaraki, Japan.

### Animal experiments

In vivo bioluminescent imaging analysis of RABV-infected mice was conducted as described previously.[15] Briefly, the mice were inoculated in the right hindlimb with $5 \times 10^5$ or $10^3$ FFUs of 1088/RFLuc. At the time points of interest, the mice were intraperitoneally administered D-luciferin potassium salt (Wako Pure Chemical Industries) and imaged under inhalation anesthesia (2% isoflurane) using the Lumazone imaging system (Nippon Roper). The obtained 16-bit TIFF images were processed and analyzed using ImageJ software.[18] Whole blood was sampled using cardiac puncture under inhalation anesthesia with 5% isoflurane. If the mice did not die during the blood sampling process, they were immediately euthanized by cervical dislocation. Serum samples were collected after blood clotting. Tissue samples were obtained from the dead mice and processed for further analysis.

All animal experiments were approved by the Animal Experiment Committee of Oita University (approval No. 171001). All mice were killed before they exhibited severe neurological signs.

## 2D-DIGE

The serum samples were purified using the BioMag ProMax albumin removal kit (Polysciences) to eliminate albumin and eluted with a lysis buffer (8 M urea, 40 mM Tris, and 4% CHAPS [pH 8.0]) in the final step. 2D-DIGE was conducted as follows. Briefly, the purified samples were labeled with the Cy2 or Cy3 dye using the CyDye DIGE Fluor Cy2 or Cy3 minimal dye (GE Healthcare), respectively. The labeled samples were mixed and subjected to isoelectric focusing for first-dimension separation using the ReadyStrip IPG (pH 3–10, 7 cm) and PROTEAN IEF Cell (Bio-Rad Laboratories). Subsequently, SDS-PAGE was conducted for second-dimension separation using Mini-PROTEAN TGX precast gels and Mini-PROTEAN tetra cell (Bio-Rad Laboratories). The fluorescent gel was imaged using LuminoGraph I with a VariRays fluorescence imaging unit (ATTO). The image data were processed and analyzed using ImageJ.

## Protein identification using MS

The protein spots of interest in the 2D-DIGE analysis were selected and digested in-gel using the InGel tryptic digestion kit (Thermo Fisher Scientific). The digested protein samples were subjected to peptide mass fingerprinting analysis using MALDI-TOF MS AXIMA (Shimadzu). The obtained mass spectral data were used to query the Swiss-Prot database using the MASCOT program for protein identification.

## Preparation of recombinant N protein

The N gene of the RABV strain CVS from its expression plasmid was amplified using a high-fidelity DNA polymerase, KOD -Plus- Ver.2 (Toyobo), and the following primers: forward, 5′-CGCGCGGATCCATGTCGTACTAC CATCACCATCACCATCACGATTACGATATCCCAACG ACCATGGATGCCGACAAG-3′; reverse, 5′-CTGCAGGC GGCCGCTTATGAGTCATTCGAATACG-3′. The forward primer contained a BamHI site and 6× His tag and linker sequences (DYDIPTT), and the reverse primer contained the NotI site. The amplicon was cloned into the pFastBac dual vector (Thermo Fisher Scientific) under a polyhedron promoter. The plasmid was then introduced into DH10Bac competent cells (Thermo Fisher Scientific) containing a baculovirus shuttle vector (bacmid). The recombinant bacmid DNA containing the N gene was purified using a NucleoBond Xtra Midi kit (Macherey-Nagel GmbH & Co. KG) and used to transfect High Five cells using the TransIT-Insect transfection reagent (Mirus Bio). After several days of incubation, the culture supernatant was collected when a cytopathic effect was observed, and the remaining cells were subjected to fluorescence assay using FITC Anti-Rabies Monoclonal Globulin (Fujirebio Diagnostics) to detect N protein expression. The High Five cells were inoculated with the recombinant baculovirus expressing the N protein at a multiplicity of infection of 3, and the infected cells were collected several days post-inoculation (dpi). The recombinant N protein was purified from the collected cells using a HisTALON gravity column purification kit (Takara Bio Inc.). The purity of the recombinant protein was confirmed using SDS-PAGE followed by Coomassie Brilliant Blue staining, and the quantity of protein was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

## ELISA

The serum ApoA1 levels in mice were measured using the mouse apolipoprotein A1 ELISA$^{pro}$ kit (Mabtech AB) according to the manufacturer's instructions. The absorbance was measured at 450 nm (and at 600 nm for reference) using a multimode microplate reader (Varioskan Lux; Thermo Fisher Scientific).

The levels of serum IgM and IgG against RABV N protein were measured using in-house ELISA as follows. One hundred microliters of the recombinant N protein solution (8 μg/mL) in carbonate buffer (0.05 M, pH 9.6) was added to each well of a Nunc-Immuno 8-well strip Polysorp (Thermo Fisher Scientific), following which the strip was incubated at 4°C for overnight. The coated wells were blocked with 5% skim milk solution containing 0.1% Tween 20. Next, 100 μL of the 50-fold-diluted serum sample was added to each well. mAbs against the N protein (10-41-F2 and 87-3E2)[19] and the G protein (4-12)[20] were used in the positive and negative controls, respectively, and 100 μL of each mAb solution (1 μg/mL) was applied. The strips were incubated at 28°C for 1 h and washed with PBS containing 0.1% Tween-20. Subsequently, horseradish peroxidase-conjugated anti-mouse IgM or IgG was added and incubated at 28°C for 1 h. The secondary antibody was detected using KPL SureBlue TMB microwell peroxidase substrate (SeraCare Life Sciences) and the reaction was terminated by adding 1 N HCl. The absorbance was measured as described above.

## RNA extraction and RT-qPCR

Each tissue sample was immersed in the lysis buffer provided with the High Pure RNA tissue kit (Roche Molecular Systems Inc.) and disrupted by bead beating. The total RNA was isolated and purified using the kit according to the manufacturer's instructions. Purified RNA was stored at −80°C until use.

RT-qPCR was performed as follows. The total RNA was reverse transcribed to cDNA using the oligo(dT) primer with a SuperScript III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific). The cDNA samples were subjected to qPCR using the THUNDERBIRD SYBR qPCR Mix (Toyobo) and PikoReal 96 Real-time PCR System (Thermo

Fisher Scientific). Relative ApoA1 mRNA expression was measured using the comparative Ct method and mRNA levels of the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (Ywhaz) and β-actin (Actb) genes as references. These housekeeping genes have been shown to be suitable for the gene expression analysis in a liver disease model.[21,22] We used previously reported primer sets for mouse ApoA1 and Ywhaz genes[21,23] and primer sequences for the mouse Actb gene used were as follows: forward, 5′-CCATGAAGATCAAGATCATTGC-3′; reverse, 5′-AAGCACTTGCGGTGCAC-3′.

RT-qPCR was also conducted to detect the viral RNA in the serum of infected mice without RNA extraction using the inhibitor-tolerant RT-qPCR Mix (Veritas Technologies) and PikoReal 96 real-time PCR system. A previously reported primer/probe set was used against the viral N gene (RABVD2For, RABVD2Rev, and RABVD-RCprobe).[24] The standard curve for absolute quantification was generated using the purified PCR product, which was a part of the N gene amplicon obtained using Ex Taq (Takara Bio Inc.) and the following primers: forward, 5′-GAACACCATACT CTAATGACAACTC-3′; reverse, 5′-AGATTGTCCACTT CTATGGGTTC-3′.

## Immunohistochemical (IHC) analysis

The harvested tissues were fixed in 10% neutral-buffered formalin, and IHC analysis was performed as reported previously[25] with minor modifications. Anti-mouse ApoA1 goat polyclonal antibody (ab7614; Abcam) and anti-RABV-P rabbit polyclonal antibody[26] were used. The Histofine Simple Stain Mouse MAX PO (G) (Nichirei Biosciences Inc.) and Histofine Simple Stain Mouse MAX PO (R) (Nichirei Biosciences Inc.) were used to detect the primary antibodies. The antigen–antibody reaction in the tissue sections was visualized using the Simplestain DAB solution (Nichirei Biosciences Inc.), and the sections were counterstained using hematoxylin. Image data were acquired using the EVOS FL Auto 2 imaging system (Thermo Fisher Scientific).

## Statistical analysis

Dunnett's multiple comparisons test was performed using GraphPad Prism (version 8.4.3; GraphPad Software).

# RESULTS

## Identification of serum proteins with altered expression during the incubation period of rabies using 2D-DIGE/MS

Figure 1a shows the in vivo bioluminescence images of mice inoculated with 1088/RFLuc in the right hindlimb at a high dose ($5 \times 10^5$ FFU). The RABV replication dynamics were monitored from 1 dpi. The infected mice developed clinical signs (weight loss without neurological symptoms) at 7 dpi, when high viral replication was detected in the head region. Viral replication was detected in the spinal cord region from 4 to 6 dpi, but no signs were observed until 6 dpi; however, a weak signal was detected in the head region at 6 dpi. We anticipated the occurrence of host responses that altered the serum proteome; hence, serum samples from the infected mice were collected at 6 dpi for proteomic analysis, and we planned to examine whether identified molecules with altered expression can be detected in the early phase of infection using a highly sensitive method. Figure 1b shows the results of the serum 2D-DIGE analysis. We selected four spots for MALDI-TOF/MS identification: spot 1 was up-regulated in the infected mice, spots 2 and 3 were down-regulated in the infected mice, and spot 4 appeared to exhibit altered migration. We successfully identified spot 1 as mouse ApoA1 (Figure 1c); however, the proteins corresponding to the other spots could not be identified owing to the low concentration.

## Validation of ApoA1 as a serum biomarker in high-dose infection

We confirmed serum ApoA1 as a biomarker in mice inoculated with a high dose of 1088/RFLuc. Serum and tissue samples were collected at 4, 6, and 7 dpi for analysis. In this experiment, the viral replication dynamics exhibited a pattern similar to that shown in Figure 1a (Figure 2a). The serum ApoA1 levels were observed to increase from the incubation period until the day of symptom onset; the levels at 6 and 7 dpi were significantly higher than that in mock-infected mice (Figure 2b). Since ApoA1 is primarily produced in the liver and intestine,[27] we measured the ApoA1 mRNA levels in tissues from these organs. As shown in Figure 2c, the ApoA1 mRNA level did not change in the small intestine of mice from the different groups, whereas it decreased in the liver as the viral infection progressed. A similar trend was obtained when the Actb gene was used as the reference gene (Figure S1). IHC analysis revealed strong ApoA1 expression in the livers of infected mice at 6 dpi (Figure 3). Reportedly, ApoA1 expression increases after neuronal injury.[28] However, ApoA1 expression was not altered in the spinal cord at any of the time points (Figure 3), even though the viral antigen (P protein) was detected in the spinal cord at 6 and 7 dpi (data not shown).

We also measured the serum antibody levels against RABV using an in-house ELISA with the recombinant N protein (Figure 2d). The anti-N IgG levels were elevated in one of four infected mice at 4 dpi and in all infected mice at 6 and 7 dpi. This ELISA system could detect the anti-N mAbs 10-41-F2 and recognize the antigenic sites III and II, respectively,[19] but not the anti-G mAb. Meanwhile, IgM seroconversion was detected at 4, 6, and 7 dpi in one, two, and four mice, respectively. In addition, the viral RNA was

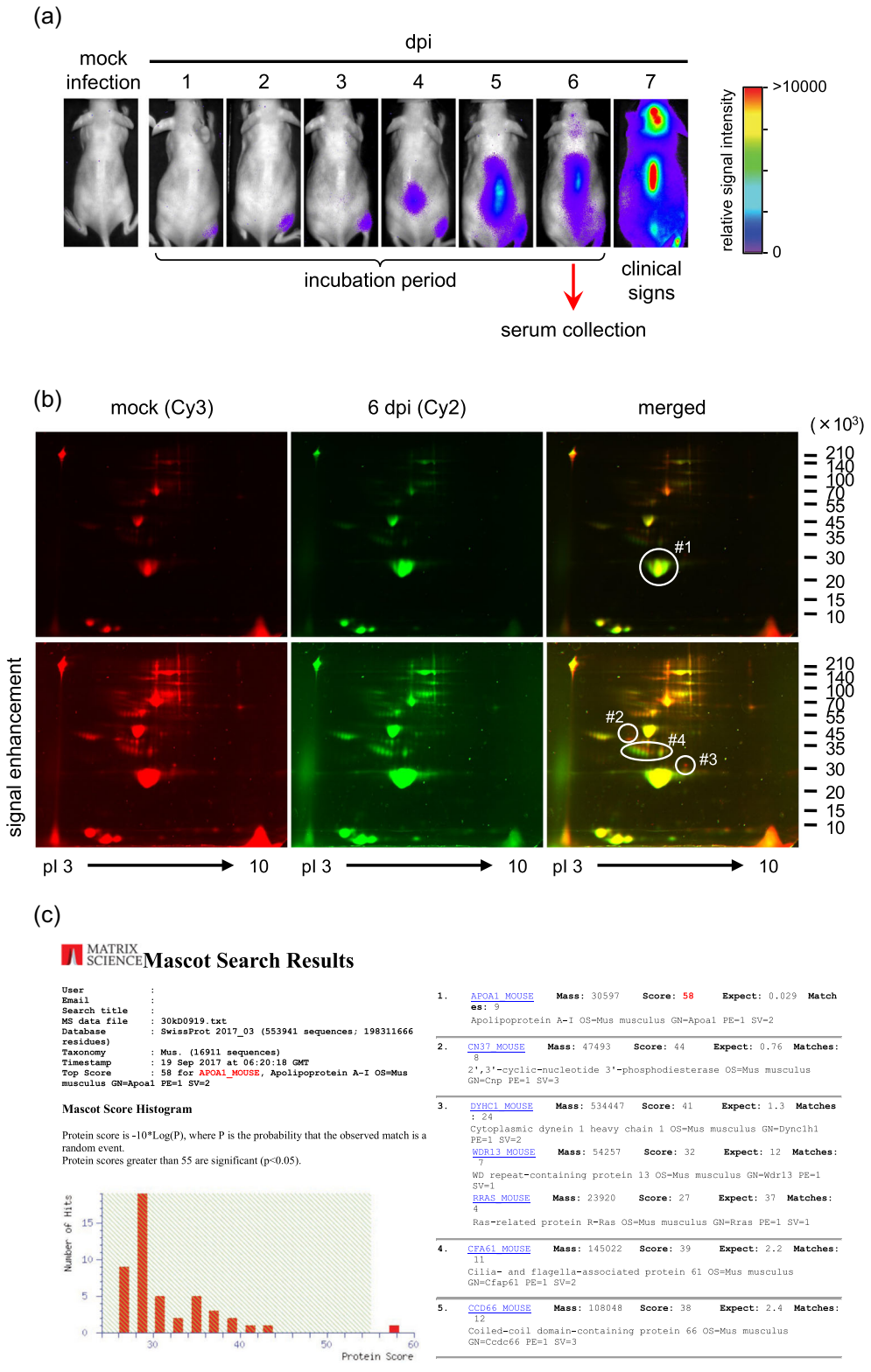


**FIGURE 1** (See caption on next page)

not detected in the serum of the infected mice at any time point using the qRT-PCR assay, even though the assay could detect four copies in the reaction (data not shown).

## Validation of ApoA1 as a marker in low-dose infection

We also validated the serum ApoA1 as a biomarker in the low-dose infection model, in which an unstable and longer incubation period can be reproduced, similar to that in natural infection. Figure 4 shows the in vivo bioluminescence images of mice injected with $10^3$ FFU of 1088/RFLuc in the right hindlimb. We killed the mice and collected serum samples at 6, 9, 12, 15, and 28 dpi. The serum ApoA1 and specific antibody levels were measured using ELISA, and the results are summarized in Table 1. The elevation of serum ApoA1 and specific antibody levels was not observed in infected mice that were sacrificed when a faint signal was observed (ID nos. 14, 17, 18, and 19). In contrast, the levels of both IgG and IgM against the N protein were elevated in 7 of 12 (58.3%) infected mice that were killed upon observing an apparent signal. The serum ApoA1 levels increased in 6 of 12 (50%) infected mice that were killed when an obvious signal was observed. Antibody responses were also detected in four mice (ID nos. 3, 12, 15, and 16), but not in the other two mice (ID nos. 4 and 7). No responses were detected in two mice (ID nos. 10 and 13), even though they were killed when an obvious signal was observed. All infected mice survived until 28 dpi (ID nos. 1, 2, and 8) exhibited the seroconversion of anti-N IgG, but not of IgM. No signal was observed at 18, 21, and 24 dpi in these mice (data not shown).

# DISCUSSION

ApoA1 is the major component of high-density lipoprotein (HDL) particles and participates in lipid metabolism and immune response regulation.[27,29] ApoA1 is considered to play a role in the healing of injured neurons by promoting neurite outgrowth and suppressing inflammation that leads to deleterious effects.[28,30] Reflecting this aspect, ApoA1 is commonly identified as a biomarker of neural injuries and diseases.[31–34] While we considered that identification of ApoA1 as a biomarker candidate was rational in the neurotropic viral infection, such as that caused by RABV, it cannot be used specifically for rabies. In fact, in the high-dose RABV infection, the serum ApoA1 level increased during the incubation period as the infection progressed. However, in the low-dose infection, the elevation of the serum ApoA1 level during the incubation period was observed in only 50% of the mice killed when robust viral replication was observed, whereas it was not observed in any of the mice sacrificed when faint viral replication was detected. In the course of mild RABV infection, some host responses may not be adequately strong to induce an elevation of serum ApoA1 levels.

In the present study, we analyzed the expression profile of ApoA1 in RABV-infected mice, but the results were difficult to interpret. ApoA1 is primarily expressed in the liver and small intestine.[27,29] Therefore, we measured the ApoA1 mRNA levels in both tissues; however, despite the increase in the serum ApoA1 level, the relative level was unexpectedly low in the liver, even as the infection progressed. A high-fat diet induces ApoA1 production via Toll-like receptor 5 activation through an increase in flagellin-positive bacteria in the gut microbiota;[35] hence, the reduction in the hepatic ApoA1 mRNA level might be attributable to reduced feed intake with infection progression. Meanwhile, IHC analysis revealed that ApoA1 expression increased in the liver at 6 dpi (Figure 3). In the acute phase, inflammatory cytokines induce the expression of serum amyloid A, which displaces ApoA1 from HDL, following which lipid-poor ApoA1 is catabolized in the liver and kidneys.[29,30] Therefore, lipid-free ApoA1 may have accumulated in the liver at 6 dpi. In addition, although ApoA1 expression was previously shown to be enhanced in injured neural cells,[28] ApoA1 expression in the spinal cord was unaltered during RABV infection. Hence, we could not identify the source of the increase in serum ApoA1 levels during infection progression.

We believe that the detection of specific antibodies as indicators in the preclinical diagnosis of rabies is challenging. According to a manual for rabies diagnostics, serological assays are not suitable because of the absence of a detectable immune response during the incubation period.[6] However, seroconversion against the viral protein was observed during the incubation period in this study. This is not surprising because we have previously detected

**FIGURE 1** Identification of differentially expressed serum proteins in rabies virus (RABV)-infected mice during the incubation period. (a) In vivo bioluminescence imaging of mice inoculated with 1088/RFLuc (red firefly luciferase) ($5 \times 10^5$ focus-forming units [FFU]) in the right hindlimb. Bright-field images were merged with pseudocolored bioluminescence images; the scale bar indicates the relative signal intensity. Since the onset of clinical signs was observed in the infected mice at 7 days post-inoculation (dpi), 0–6 dpi was considered the incubation period. The serum sample was collected from the infected mouse for the identification of candidate biomarkers at 6 dpi, when the bioluminescent signal was detected in the head region. (b) Two-dimensional differential gel electrophoresis (2D-DIGE) analysis of differentially expressed proteins in the serum of the infected mice. After removal of albumin, serum samples were labeled with Cy3 (for mock infection) or Cy2 (for infection, 6 dpi). The labeled samples were mixed and then subjected to isoelectric focusing (pH 3–10), followed by SDS-PAGE. The gel was imaged using the fluorescence gel imager and processed. The spots (1–4) were subjected to protein identification. The relative molecular mass is indicated on the right. (c) Spot 1 was identified as mouse apolipoprotein A1 (ApoA1) using MALDI-TOF/MS. The mass spectral data were subjected to MASCOT database search, and the results are shown

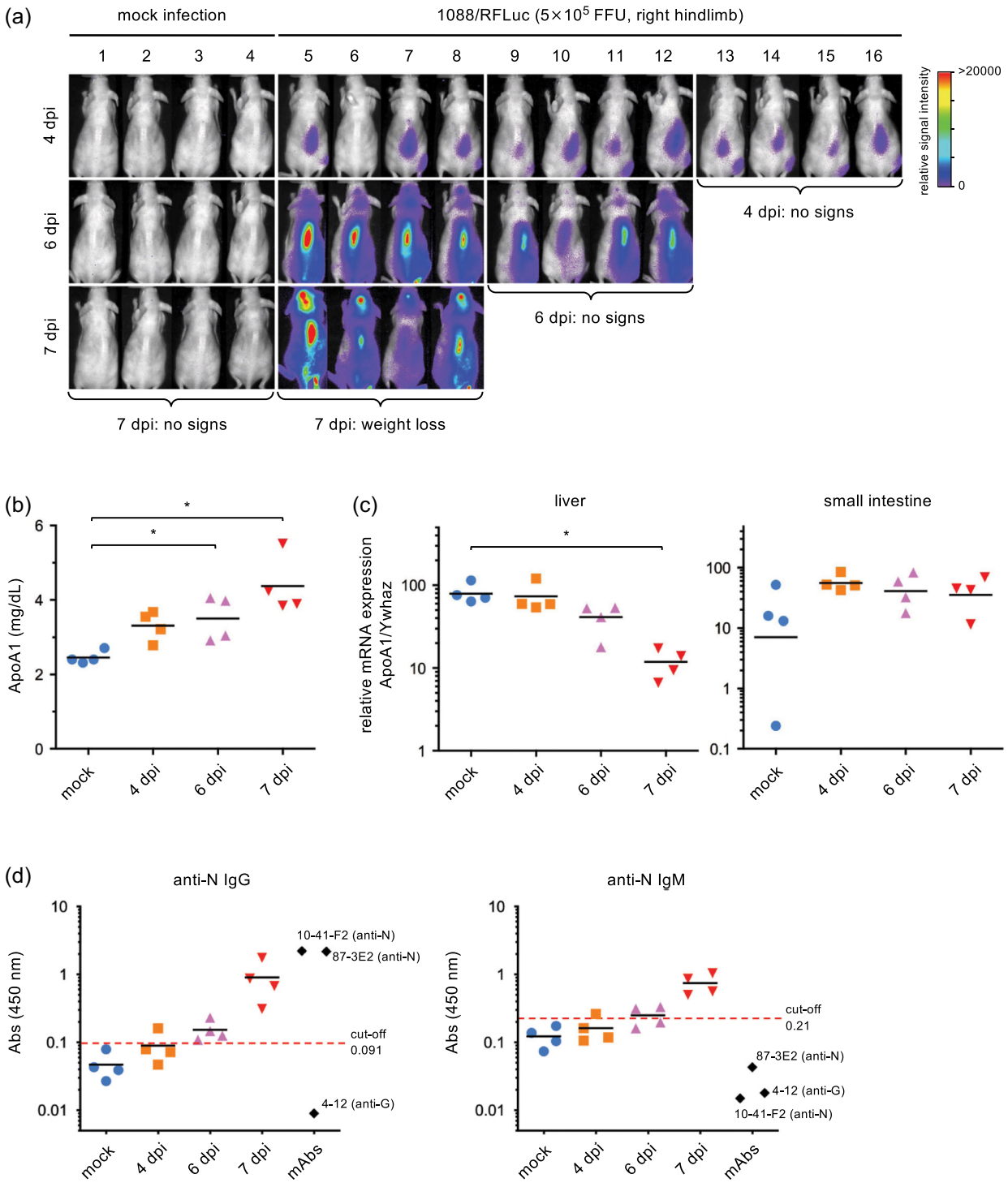


**FIGURE 2** Evaluation of candidate biomarkers in mice with high-dose rabies virus (RABV) infection. (a) The mice were inoculated with 1088/RFLuc ($5 \times 10^5$ focus-forming units [FFU]) in the right hindlimb, and in vivo bioluminescence imaging was conducted, as shown in Figure 1. Mice from different groups ($n = 4$) were killed at 4 (ID nos. 13–16), 6 (ID nos. 9–12), and 7 (ID nos. 1–4 and 5–8) days post-inoculation (dpi), and the serum and tissue samples were harvested. (b) The apolipoprotein A1 (ApoA1) levels in the serum were measured using ELISA at the indicated time points. (c) The ApoA1 mRNA levels in the liver and intestine were measured using RT-qPCR and normalized to the Ywhaz mRNA level. (d) The anti-RABV nucleoprotein (N) IgG and IgM levels in the serum were measured using ELISA. The y axis indicates absorbance (Abs), and values greater than the cut-off value are considered as positive. The cut-off was the mean + 2× SD of the mock infection group. The mAbs against N (10-41-F2 and 87-3E2) or glycoprotein (G) (4-12) proteins were used in the positive or negative controls, respectively. Horizontal bars indicate the mean of values. $^*P < 0.05$ (Dunnett's multiple comparisons test)

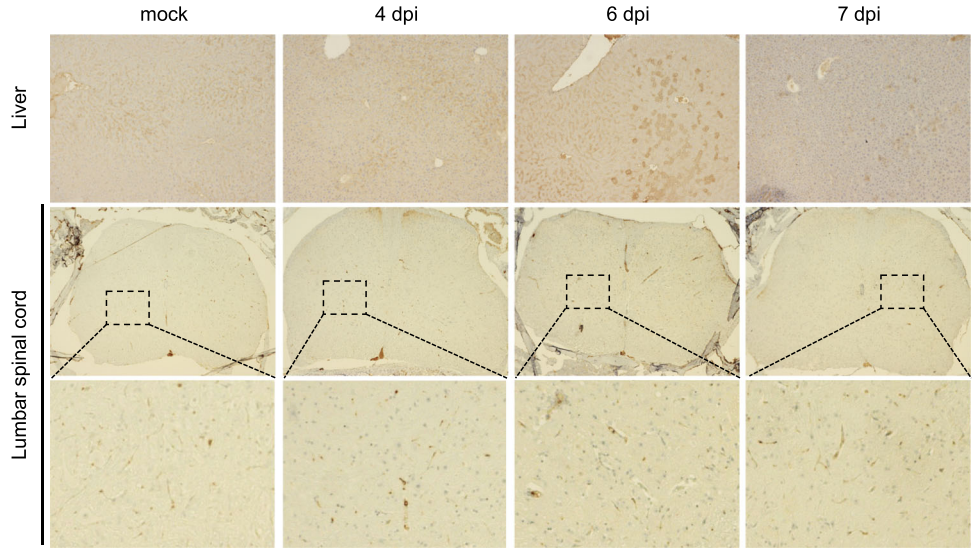


**FIGURE 3** The apolipoprotein A1 (ApoA1) expression in the tissues of mice with high-dose rabies virus (RABV) infection. The liver and lumbar spinal cord samples were collected from mice inoculated with 1088/RFLuc (red firefly luciferase) ($5 \times 10^5$ focus-forming units [FFU]) in the right hindlimb at the indicated time points and then were subjected to IHC analysis with the anti-ApoA1 polyclonal antibody. The lower panels show higher magnifications of the squares in the middle panels for the lumbar spinal cord

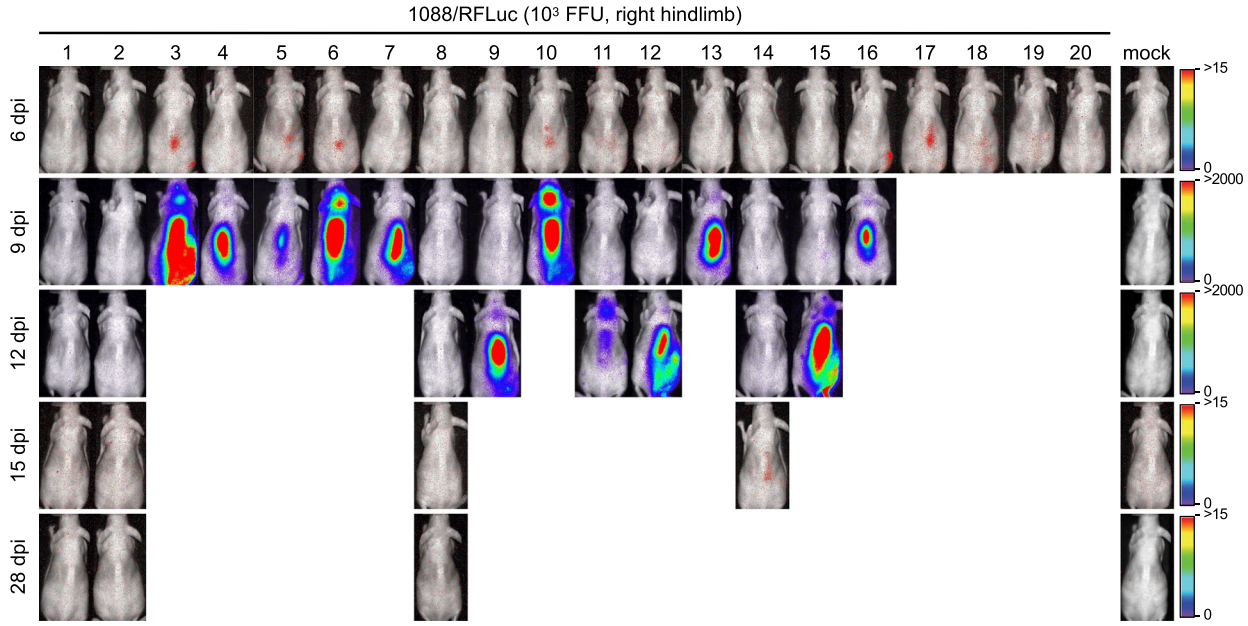


**FIGURE 4** In vivo bioluminescence imaging of mice inoculated with 1088/RFLuc (red firefly luciferase) at a low dose ($10^3$ focus-forming units [FFU]) in the right hindlimb. The mice were killed to collect the whole blood samples at 6, 9, 12, 15, and 28 days post-inoculation (dpi). The scale bars indicate the relative signal intensity; enhanced signals were shown for 6, 15, and 28 dpi. The serum apolipoprotein A1 (ApoA1) and anti-nucleoprotein (anti-N) antibody levels of mice indicated in this figure are shown in Table 1.

neutralizing antibodies against RABV during the incubation period in the serum of mice infected with strain 1088.[36] In the present study, the elevation in the serum anti-N IgG level was observed in 100% of the mice infected at a high dose 1 day before disease onset (6 dpi; Figure 2d) and in 58.3% of the mice infected at a low dose, who were killed when an obvious signal was observed (Table 1). On a side note, it was likely that the elevation in anti-N IgM levels was not observed in 50% of the mice from the former group owing to the low sensitivity of our ELISA system. Meanwhile, specific antibodies were not detected in the sera of patients with rabies collected within several days of illness onset, but were detected in sera collected during a later phase.[37] The diagnostics manual mentions that specific antibody detection has low sensitivity even in antemortem diagnosis owing to the delayed appearance of the antibodies

**TABLE 1** The anti-N antibodies and ApoA1 levels in sera from mice infected with 1088/RFLuc at a low dose

| Group | ID | Anti-N IgM (Abs)[b] | Anti-N IgG (Abs)[b] | ApoA1 (mg/dL)[b] | Replication[c] | Sampling date dpi) |
|---|---|---|---|---|---|---|
| 1088/RFLuc $10^3$ FFU i.m. | 1 | 0.111 | **0.189** | 3.154 | – | 28 |
| | 2 | 0.069 | **0.281** | 3.140 | – | 28 |
| | 3 | **0.420** | **0.135** | **6.249** | ++ | 9 |
| | 4 | 0.152 | 0.044 | **5.292** | ++ | 9 |
| | 5 | 0.145 | 0.057 | 2.791 | ++ | 9 |
| | 6 | **0.624** | **0.323** | 4.581 | ++ | 9 |
| | 7 | 0.095 | 0.032 | **5.431** | ++ | 9 |
| | 8 | 0.117 | **2.052** | 4.697 | – | 28 |
| | 9 | **0.369** | **0.732** | 3.523 | ++ | 12 |
| | 10 | 0.127 | 0.131 | 3.859 | ++ | 9 |
| | 11 | **0.507** | **0.776** | 4.617 | ++ | 12 |
| | 12 | **0.251** | **0.671** | **5.286** | ++ | 12 |
| | 13 | 0.151 | 0.078 | 2.664 | ++ | 9 |
| | 14 | 0.157 | 0.083 | 3.668 | + | 15 |
| | 15 | **0.322** | **0.197** | **5.119** | ++ | 12 |
| | 16 | **0.338** | **0.593** | **5.648** | ++ | 9 |
| | 17 | 0.112 | 0.058 | 2.775 | + | 6 |
| | 18 | 0.112 | 0.072 | 3.437 | + | 6 |
| | 19 | 0.122 | 0.063 | 3.885 | + | 6 |
| | 20 | 0.068 | 0.054 | 4.898 | – | 6 |
| Mock[a] ($n = 4$) | Mean | 0.123 | 0.075 | 3.742 | – | 28 |
| | SD | 0.041 | 0.028 | 0.616 | | |
| | Mean + 2 SD | 0.205 | 0.132 | 4.973 | | |

ApoA1, apolipoprotein A1; dpi, days post-inoculation; FFU, focus-forming unit; i.m., intramuscularly; N, nucleoprotein; RFLuc, red firefly luciferase.

[a]For the mock infection group, the mean and SD are indicated, and the mean + 2 SD was used as the cut-off.

[b]Each ELISA was performed as described in Figure 2. Values higher than the cut-off value are indicated in bold letters.

[c]Replication levels were referred to the in vivo imaging analysis described in Figure 4; –, no signal when sampling; +, faint signal when sampling; ++, obvious signal when sampling.

(>8 days),[6] indicating that the detection of specific antibodies for the preclinical diagnosis of rabies is not practical, unlike that in the mouse model. In addition, antibody detection must be distinguished from ongoing infection and cases, such as that observed in virus clearance without disease onset and in previously vaccinated patients. In fact, anti-N IgG was detected in serum samples from persons who received a rabies vaccine using our ELISA system (our unpublished data). Moreover, the fact that infected mice that survived until 28 dpi (ID nos. 1, 2, and 8) exhibited seroconversion of anti-N IgG (Table 1) indicates the virus clearance without onset, and the IgM level may have reduced to levels lower than the cut-off value in these mice during the long time period post-seroconversion. Several reports have shown that RABV-neutralizing antibodies were detected in apparently healthy unvaccinated humans, indicating potential cases of nonlethal RABV infection.[38]

In general, RABV is not considered to cause viremia, indicating that viral RNA detection in the serum during the incubation period is not feasible. In fact, in this study, we could not detect RABV RNA in the serum during incubation period and on the day of disease onset at the high-dose infection using the sensitive real-time RT-PCR method. Meanwhile, Lodmell et al. mentioned the possibility of RABV viremia after disease onset based on previous reports and their experimental results.[5] Conversely, they could not detect viral RNA in the serum during the incubation period, and stated that the risk of RABV transmission from blood might be minimal if animals do not exhibit clinical signs. Therefore, the

detection of serum RABV RNA is not a practical method for preclinical diagnosis.

In conclusion, while we successfully identified ApoA1 as a serum factor up-regulated during the incubation period of RABV infection, it was not highly sensitive and specific to RABV infection. To establish a preclinical diagnosis for rabies, additional biomarker candidates need to be identified using high-sensitivity methods, such as isobaric tag for relative and absolute quantitation (iTRAQ).[39] In fact, we could not identify the spots with low protein concentrations. Since the serum peptide profile was reportedly altered in the early phase of infection in RABV-infected mice,[16] we believe that a biomarker set for the preclinical diagnosis of rabies can be developed if the proteins with altered expression are identified. Lastly, we recommend the use of in vivo imaging as a powerful tool for monitoring viral dynamics during the incubation period of an infection and for evaluating biomarkers for preclinical diagnosis.

## ACKNOWLEDGMENTS

We thank Ms Mami Koya (Oita University) for helping us to conduct 2D-DIGE and protein identification experiments. We also thank Dr Kazuaki Mannen (Oita University) for providing the High Five cells. This work was supported by Science and Technology Research Partnership for Sustainable Development (SATREPS), Japan Agency for Medical Research and Development (AMED)/JICA to Akira Nishizono (No. 17823721).

## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Yamada K, Kuribayashi K, Inomata N, et al. Validation of serum apolipoprotein A1 in rabies virus-infected mice as a biomarker for the preclinical diagnosis of rabies. Microbiol and Immunol. 2021;65:438–448.
https://doi.org/10.1111/1348-0421.12929