separated proteins were electroblotted onto a PVDF membrane and blocked by incubating in blocking buffer (PBS containing 10% skim milk and 0.1% Tween 20) for 1 h. The membrane was incubated with the intestinal swab sample (1:10 dilution in blocking buffer) or serum sample (1:400 dilution in blocking buffer) for 1 h. After washing, the membrane was incubated with anti-dog IgA-alkaline phosphatase (AP) conjugate (1:3000 dilution in blocking buffer) for detection of mucosal IgA or incubated with anti-dog IgG-AP conjugate (1:3000 dilution in blocking buffer) for detection of serum IgG. The bound antibodies were detected with a BCIP/NBT immunodetection kit (PerkinElmer).

Preparation of Specific Antiserum Against the SRf1 Antigen

Preparation of mouse anti-SRf1 antiserum was performed as described previously [21]. SRf1 was heat-treated in the presence of 1% SDS to increase immunogenicity. Approximately 50 μg of SRf1 protein was administered to balb/c mice with Freund's complete adjuvant. Thereafter, 2 boosters with incomplete adjuvant were given to the animals at 2-week intervals.

Enzyme-linked Immunosorbent Assay

Specific serum antibodies to SRf1 were measured by an indirect enzyme-linked immunosorbent assay (ELISA) as described previously [22]. A 96-well ELISA plate was coated with the antigen preparation for 5 h at 37°C. The antigens were diluted in 0.05 M carbonate buffer (pH 9.6) to a concentration of 10 μg/mL. The protein concentration was calculated by measuring the optical density at 280 nm with BSA as a standard (0.7 OD at 280 nm = 1 mg/mL). Each well was washed with PBS containing 0.05% Tween 20 (PBS/Tween). The wells were reacted with sample sera diluted 1:100 with dilution buffer (1.0% [w/v] casein in PBS/Tween) overnight at 4°C. The wells were then washed with PBS/Tween and incubated with horseradish peroxidase-conjugated rabbit anti-dog IgG, IgA, or IgE at a dilution of 1:3000 in dilution buffer for 1 h at 37°C. Following the final wash with PBS/Tween, substrate solution containing 0.04% *o*-phenylenediamine and 0.006% H_2O_2 in 100 mM citrate phosphate buffer (pH 5.0) was applied to each well. The plate was incubated at room temperature, and the optical density was read at 492 nm.

Mucosal Immunization and Challenge Infection

A total of 17 dogs (female beagles, ages 3–4 months old) were used in this experiment. Six dogs were immunized nasally 4 times on days 0, 14, 28, and 42. Five hundred micrograms of SRf1 was mixed with 100 μg of cholera toxin (CT) subunit B (CTB, C9903, Sigma, St. Louis, MO, USA) in 400 μL PBS and incubated at 4°C overnight according to the method reported by Tuji et al. [23]. Before administration, carboxyvinyl polymer (CVP, SENKEN Co. LTD, Japan) was added to increase viscosity to a final concentration of 0.1%, and 0.1 μg of CT (Wako Pure Chemical Industries, Ltd.) was supplied to increase the immunogenicity of the antigen. The antigen (500 μg/animal) was administered to dogs nasally with a spray syringe (Nipro Corp., Japan). Thereafter, 3 boosters were given to each animal by oral administration of an enteric capsule (Sunsho Pharmaceutical Co. Ltd., Japan) containing 15 mg lyophilized SRf1 mixed with 100 μg CT on days 28, 42, and 56. Seven days after final administration of the capsule, all animals were orally administered 5×10^5 *E. multilocularis* protoscoleces. On day 35 postinfection, animals were euthanized, and necropsies were performed. The small intestine was divided into 6 sections and incubated in DMEM at 4°C for 7 days. Naturally released and scraped worms were counted after appropriate dilutions. Two control groups received the same schedule of administration with PBS alone (n = 5) or adjuvant alone (n = 6) instead of SRf1.

Evaluation of Protease Tolerance

Five hundred micrograms of SRf1 was digested in the presence of pepsin (1.0 mg/mL) or trypsin (0.4 mg/mL) and chymotrypsin (1.7 mg/mL) at 37°C for 1 and 4 h. The pH of the reaction mixture was adjusted to 2 for pepsin digestion or 7.4 for trypsin digestion. The reaction mixtures were analyzed by western blotting using sera from dogs infected 5 times and by gel filtration chromatography under identical conditions as mentioned above. The peak area was calculated with Unicorn ver. 3.10 equipped with an AKTA explorer system.

Determination of O-glycosylation

SRf1 was lyophilized in a reaction vial, and anhydrous hydrazine was added to the vial. After replacement with nitrogen under reduced pressure, the sample was heated at 60°C for 6 h under reduced pressure. Hydrazine was removed by 3 rounds of toluene azeotrope, and the sample was reacted with saturated $NaHCO_3$ and acetic anhydrate for 30 min. The reaction mixture was then applied to a Dowex AG50x2(H+) exchange resin and washed with distilled water 5 times. The eluate containing the released oligosaccharide was lyophilized.

Glycoblotting [24] and MALDI-TOF mass spectrometry (MS) analysis were performed according to the report described by Kato et al. [25], except for the use of 20 mM O-benzylhydroxylamine hydrochloric acid (BOA) for labeling. Purified BOA-labeled glycans were mixed with 2,5-dihydroxybenzoic acid solution and subsequently subjected to MALDI-TOF analysis by using an Autoflex III TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). The compositions of glycan structures were estimated with GlycoMod software (http://www.expasy.org/tools/glycomod/).

Localization of SRf1 by Immunostaining

Cyst tissues including protoscoleces were derived from infected cotton rats. Intestinal epithelial tissue harboring adult worms was derived from the small intestines of infected dogs on day 23 postinfection. The dogs were experimentally infected by oral administration of 5×10^5 *E. multilocularis* protoscoleces, as mentioned above. The small intestine was divided into 6 sections, and the central sections of the intestine were cut out. All tissues were fixed in 10% formalin-PBS and embedded in paraffin wax. Cryosections (thickness, 4 μm) were cut on a Retoratome REM-710 microtome (Yamato Kohki Co., Ltd., Japan) at 25°C and mounted on slides.

The slides were rehydrated in Tris-buffered saline (TBS), and endogenous peroxidase was inactivated by 10 min of incubation in 0.3% hydrogen peroxide (H_2O_2). Samples were washed with TBS for 5 min and incubated with mouse anti-SRf1 antibodies at a dilution of 1:3000 in 3% BSA/PBS for 1 h. After an additional washing step as above, the slices were incubated with EnVision (Dako Japan inc.) for 30 min. Slices were washed 3 more times, and the bound antibody was detected with diaminobenzidine (DAB) for 5 min. The stained samples were further stained with hematoxylin and washed with distilled water.

Statistics

A generalized linear model (GLM) was used to model the differences between treatments in the vaccine trial using the 'MASS' package [26] in R version 2.15.2 (R Foundation for

Statistical Computing, Vienna, Austria). Since the data were overdispersed (the mean parasite burden of each group was much less than the variance), a negative binomial distribution was applied. The optimal statistical model was chosen based on the lowest value for Akaike's information criterion (AIC).

Results

2D-PAGE and Western Blot Analysis

To search for vaccine candidates against *E. multilocularis* infection, reactivity of intestinal IgA from infected dogs to PCE or ACE was analyzed by 2D-PAGE and western blotting. Proteins from the PCE were separated on a 2D-gel with a pH gradient from 3 to 10. Approximately 250 protein spots were visualized by CBB staining (Fig. 1A–1). 2D-WB analysis demonstrated that all intestinal swab samples reacted with a smear band at the top of the membrane at a pI ranging from approximately 3.5 to 5 (Fig. 1A–2 to 1A–4), while no clear spots were detected with all intestinal swabs. These findings strongly suggest that intestinal IgA from infected dogs recognize glycoprotein(s) of the parasite. In addition, the intestinal swabs from dogs infected 3 or 5 times recognized a wider range of the smear band at the top of the membrane than the intestinal swab from dogs infected only once. Proteins from the ACE were separated on a 2D-gel as approximately 190 protein spots by CBB staining (Fig. 1B-5). All intestinal swabs from infected dogs reacted to a smear band from the ACE found at the same position as the reactive smear band from the PCE. The reactivity of all intestinal swabs to the ACE was significantly lower than their reactivity to the PCE, which suggested that the content of reactive antigens corresponding to the smear band in the PCE was higher than that of the ACE.

Purification of the SRf1 Antigen

To isolate the reactive antigens corresponding to the smear band in Fig. 1A, the PCE was applied to a Superose 6 gel filtration column (Fig. 1C). Two major peaks were eluted at 7.5–9.5 mL for the first peak and 15–19.5 mL for the second peak. The eluted position of the first peak corresponded to the void volume of the column; therefore, the molecules in this peak were predicted to be over 4000 kDa, which was judged by the molecular size standard in an identical gel filtration chromatography assay. These peak fractions were collected and designated as fractions 1 (SRf1) and 2 (SRf2), respectively. 2D-PAGE analysis revealed that almost all nontarget proteins of the PCE were present in SRf2, while 5 weak protein spots were detected in SRf1 (Fig. 1C–9 and 11). The concentrations of these impurities were calculated to be 16.9, 11.0, 3.6, 3.6, and 1.2 μg/mL in SRf1 (1 mg/mL) by densitometric analysis (data not shown). Thus, the glycoprotein component (SRf1) was purified over 95%. 2D-WB analysis using intestinal swab samples from dogs infected 5 times revealed that SRf1 showed a similar pattern as that observed in Fig. 1A–2, indicating that SRf1 contained abundant amounts of target antigens (Fig. 1C–10). In contrast, serum samples collected from dogs infected 5 times showed significant reactivities to many proteins found in SRf2 (Fig. 1C–12).

In this experiment, the SRf1 components were not stained with CBB. Similar results have been reported for the mucin glycoprotein, which is highly glycosylated [27,28]. In addition, the SRf1 components were always detectable by 2D-WB with infected dog sera [20] and glycoprotein staining with reagents from a Pro-Q Emerald 300 glycoprotein stain kit (Fig. 1D). Based on above-mentioned findings, we decided to use SRf1 for the vaccination experiments.

Protease Tolerance of SRf1

To determine whether SRf1 retained its immunogenicity after gastric protease digestion, the tolerance of SRf1 to gastric protease digestion was examined by analytical gel filtration and 1-dimensional (1D) western blot analysis (Fig. 2). SRf1 was incubated with pepsin at 37°C for 1 or 4 h. The peak areas on gel filtration were calculated to be 28.8 and 26.6 mAU, respectively. The peak area of the control with no proteases was calculated to be 31.1 mAU. Approximately 80% of the SRf1 protein was still intact after a 4-h digestion in the presence of 1 mg/mL pepsin. SRf1 was also digested at 37°C in the presence of trypsin and chymotrypsin for 1 or 4 h. The eluted peak areas were calculated to be 31.2 and 30.0 mAU, respectively. Western blot analysis using serum samples from dogs infected 5 times revealed only slight degradation of SRf1 depending on the digestion time with proteases.

Sequence Analysis of SRf1 *O*-glycans

Hülsmeier et al. reported that a major antigen of *E. multilocularis* was a mucin-type glycoprotein designated as Em2 [29]. However, information about carbohydrate moieties in the field of *E. multilocularis* research is still very limited. To address whether SRf1 was identical to the glycoprotein Em2, sequence analysis of SRf1 *O*-glycans was performed. MALDI-TOF MS spectra revealed that the carbohydrate moiety consisted predominantly of Hex and HexNAc, which were estimated with the GlycoMod Tool based on the mass spectra. Variations in the estimated carbohydrate moieties with S/N ratios over 4.0 are summarized in Table 1. Major peaks showing peak areas of over 1000 were Hex_2, $Hex_1HexNAc_1$, $Hex_2HexNAc_1$, and Hex_1HeNAc_2. These estimated glycan compositions were consistent with the reported carbohydrate moieties of Em2. Peaks showing peak areas of over 500 corresponded to Hex_1Pent_1, $HexNAc_2$, $Hex_1HexNAc_1$-$Sulph_1$, Hex_3, Hex_2NeuAc_1, Hex_4, $Hex_3HexNAc_1$, $Hex_2HexNAc_2$, and $Hex_4HexNAc_1$ and were not reported as Em2 carbohydrate moieties, with the exception of $Hex_2HexNAc_2$. A total of 28 peaks showing peak areas of over 100 in this assay were inconsistent with Em2 carbohydrate moieties. These results suggested that SRf1 shared some glycoprotein components with Em2, but was not identical in its glycoprotein composition.

Vaccine Trial of SRf1

The efficacy of SRf1 as a vaccine was evaluated by the reduction in the number of *E. multilocularis* adult worms in the small intestine of immunized dogs. Dogs were immunized nasally 4 times with CTB, and a CT booster was subsequently administered 3 times orally. After the final immunization, 5×10^5 protoscoleces were administered orally to each group of dogs. The numbers of adult worms in challenge infection were follows: 298,675, 349,875, 169,875, 289,000, and 291,000 for the group immunized with PBS alone; 201,450, 215,850, 175,800, 41,800, 73,800, and 145,125 for the group immunized with adjuvant alone; and 210, 7,700, 37,675, 20,670, 64,550, and 77,000 the group immunized with adjuvant plus SRf1. Figure 3 shows the mean number of adult worms in each group. The control group immunized with the adjuvant alone showed a 49.1% reduction in the mean number of adult worms compared with that of the group immunized with PBS alone. The group of dogs immunized with adjuvant plus SRf1 developed fewer adult worms (corresponding to a 87.6% reduction) than the control group. GLM analysis indicated that the model separating the SRf1 plus adjuvant group from the other 2 groups (PBS and adjuvant only) was the best among the tested models, with the lowest AIC value of 433.76 ($P<0.001$). Thus, we provided the first direct experimental evidence that

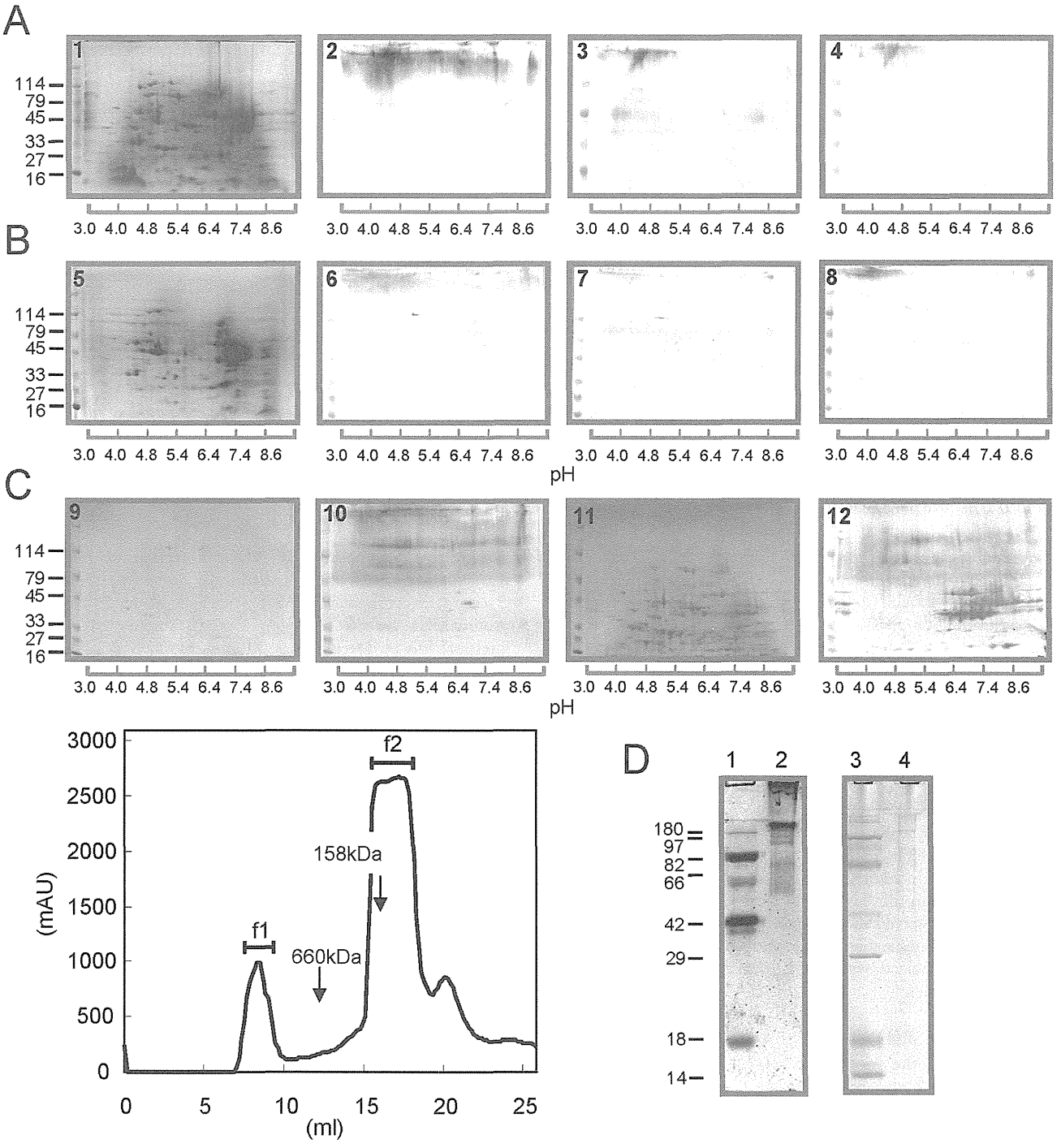

Figure 1. Identification and purification of vaccine antigen (SRf1) from *E. multilocularis*. Crude extracts were prepared from protoscoleces (PCE) and adult worms (ACE). A total of 120 μg protein was applied to 2D-PAGE. The proteins were blotted onto a PVDF membrane, and reactivity between proteins and intestinal IgA from dogs experimentally infected with *E. multilocularis* was examined. Panels A and B: PCE and ACE, respectively; panels 1 and 5: CBB-stained gels; panels 2 and 6: tested with intestinal IgA from dogs infected 5 times; panels 3 and 7: tested with intestinal IgA from dogs infected 3 times; and panels 4 and 8: tested with intestinal IgA from dogs infected once. Molecular size markers are indicated on the left (in kDa). Panel C: a gel filtration chromatogram of the vaccine antigen (SRf1) and 2D-western blot analysis; panels 9 and 11: CBB-stained gels of SRf1 and SRf2; panel 10: 2D-western blotting for SRf1 using intestinal swabs from dogs infected 5 times; panel 12: 2D-western blotting for SRf2 using sera from dogs infected 5 times. Panel D: SDS-PAGE analysis of SRf1; lanes 1 and 2: glycoprotein stained-gel of molecular size markers and SRf1; 3 and 4: CBB-stained gel with molecular size markers and SRf1. Glycoprotein detection was performed with a Pro-Q Emerald 300 gel stain kit and CandyCane glycoprotein molecular weight standards.
doi:10.1371/journal.pone.0069821.g001

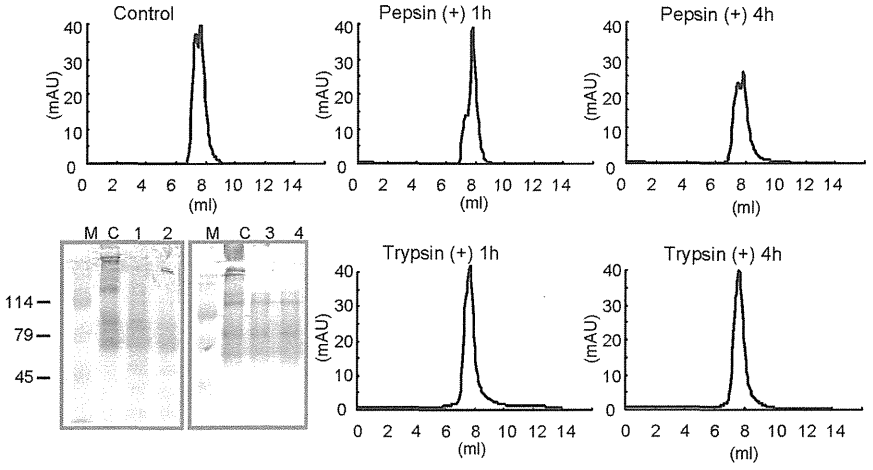

Figure 2. Tolerance of SRf1 against gastric protease digestion. SRf1 was digested in the presence of pepsin (1 mg/mL) at pH 2 or in the presence of trypsin (0.4 mg/mL) and chymotrypsin (1.7 mg/mL) at pH 7.4. After 1 or 4 h digestion, the reaction mixture was applied to a Superose 6 gel filtration column, and peak areas were compared to those of the control. Panel B: 1D-western blot analysis of digested SRf1s detected using sera from dogs infected 5 times. Lanes: M, molecular marker; C, controls (no proteases); 1 and 2, pepsin digestion for 1 and 4 h, respectively; 3 and 4, tryptic digestion for 1 and 4 h, respectively.
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SRf1 induced a host protective response in *E. multilocularis* infection. No significant suppression of growth was observed in dogs immunized with adjuvant alone or in dogs immunized with adjuvant plus SRf1.

Serum Antibody Response

Using serum samples collected during the course of immunization and infection, IgG, IgA, and IgE specific to SRf1 were examined by ELISA (Fig. 4). IgG levels in sera from dogs immunized with adjuvant plus SRf1 gradually increased from day 21 to day 63 after the first immunization, whereas no significant change was observed in sera from dogs immunized with adjuvant alone. On day 70, a sharp increase in IgG levels was observed in both groups, which corresponded to 7 days postinfection. Likewise, a sharp increase in IgA levels was observed in sera from dogs in both groups on day 70. No significant increase in IgA or IgE levels was observed in sera from dogs in both groups during the course of immunization. IgA could not be detected in saliva from dogs immunized with SRf1, but this was likely due to technical difficulties.

Localization of SRf1

To determine the localization of SRf1 in *E. multilocularis*, immunostaining of adult worms and protoscoleces was performed using mouse antiserum against SRf1. Western blot analysis using the PCE or ACE revealed that mouse anti-SRf1 antiserum recognized SRf1 alone (data not shown). As shown in Fig. 5A and B, anti-SRf1 antibodies enabled the visualization of SRf1 on the surface of adult worms collected from the epithelium of the small intestines of infected dogs. Anti-SRf1 antibodies also stained the surface and apical region of protoscoleces, including suckers, rostella, and hooks (Fig. 5C and D). Thus, in both the adult and larval stage, SRf1 was considered to be expressed in the tegument, including the suckers, rostella, and hooks.

Discussion

Despite the urgent need for new measures and tools to control *E. multilocularis* transmission, vaccine development for this parasite has been neglected [11]. The present study aimed to find a novel vaccine candidate that could induce protection against infection with adult *E. multilocularis* in definitive hosts. We found that a glycoprotein component, SRf1, purified from *E. multilocularis* protoscoleces by gel filtration, showed immunoreactivity with intestinal IgA in infected dogs. Moreover, mucosal immunization with this component induced significant reduction in worm burden in the immunized dogs. These findings suggested that further purification and immunological characterization of SRf1 could lead to the development of a novel vaccine candidate for the control of alveolar echinococcosis in humans.

In this study, we used combined nasal and oral mucosal immunization for delivery of the antigen. Some studies have investigated the immunoresponse in dogs immunized via mucosal administration of antigen candidates against *Echinococcus* infection. Carol et al. demonstrated that nasal immunization of immunostimulating complexes made from the *E. granulosus* tegumental antigen from protoscoleces showed significant induction of the secretory IgA antibody response detected in saliva and serum from dogs infected with *E. granulosus* [30]. Additionally, Gottstein et al. reported that subcutaneous and peroral vaccinated dogs showed strong humoral immune responses to antigens [31]. In both of these reports, no challenge infection data was available. However, Carol et al. suggested that more stringent and innovative search methods for appropriate immunogens or adequate immunization regimes were needed for successful development of protection against infection. Therefore, we decided to use a unique approach to identify vaccine candidates based on the reaction between parasite antigens and intestinal IgA from dogs repeatedly infected with *E. multilocularis*.

A series of egM recombinant antigens showed reactivity to the sera from dogs infected with *E. granulosus* [32]. EgA31 also possessed significant reactivity to the sera from infected dogs [33]. These facts indicated that these vaccine candidates, in their native forms in the infected parasite, were recognized by mucosal antigen-presenting cells in the small intestines of dogs infected with *E. granulosus* and induced a systemic antibody response. Such serum reactivity is important for vaccine candidates, and this was also observed in our study of SRf1. In our previous report, large glycoproteins with reactivity to sera from dogs infected with *E. multilocularis* were identified by the 2D-WB method [20]. Here,

Table 1. Estimated O-glycan compositions of SRf1 from mass spectrometric data.

Obsd. m/z	S/N	Area	δmass*	Estimated glycan composition
439.612	5.0	940	−0.54	$(Hex)_1(Pent)_1$
469.843	19.7	3095	−0.32	$(Hex)_2$
510.9	9.3	1417	−0.29	$(Hex)_1(HexNAc)_1$
511.886	16.5	2578	0.696	$(Hex)_1(HexNAc)_1$
552.941	7.9	812	0.724	$(HexNAc)_2$
572.96	4.3	383	0.765	$(Hex)1(Pent)_2$
591.963	9.5	837	0.816	$(Hex)_1(HexNAc)_1(Sulph)_1$
631.998	10.7	811	−0.22	$(Hex)_3$
655.025	4.8	352	0.772	$(HexNAc)_1(NeuAc)_1$
673.039	71.3	5243	−0.2	$(Hex)_2(HexNAc)_1$
715.068	68.6	4513	0.798	$(Hex)_1(HexNAc)_2$
776.119	10	549	0.84	$(Hex)_2(NeuAc)_1$
794.115	12.5	634	−0.15	$(Hex)_4$
818.143	6.2	301	1.837	$(Hex)_1(HexNAc)_1(NeuAc)_1$
835.149	10.3	476	−0.15	$(Hex)_3(HexNAc)_1$
836.137	19.1	893	0.841	$(Hex)_3(HexNAc)_1$
877.165	14.1	569	0.843	$(Hex)_2(HexNAc)_2$
956.201	12	367	−0.12	$(Hex)_5$
998.216	20.9	533	0.867	$(Hex)_4(HexNAc)_1$
1040.237	16.1	373	1.862	$(Hex)_3(HexNAc)_2$
1080.226	6.3	132	0.864	$(Hex)_2(HexNAc)_3$
1122.275	5.3	115	0.854	$(Hex)_1(HexNAc)_1(NeuAc)_2$
1159.285	8.7	192	−0.07	$(Hex)_2(HexNAc)_3(Sulph)_1$
1160.279	20.5	370	0.878	$(Hex)_5(HexNAc)_1$
1242.316	8.7	141	0.861	$(Hex)_3(HexNAc)_3$
1284.327	5.7	113	1.846	$(Hex)_2(HexNAc)_4$
1322.33	15.4	237	0.919	$(Hex)_3(HexNAc)_3(Sulph)_1$
1404.365	9.8	166	0.858	$(Hex)_4(HexNAc)_3$
1446.377	7.1	129	1.843	$(Hex)_3(HexNAc)_4$
1556.409	9.7	163	1.817	$(Hex)_2(HexNAc)_2(NeuAc)_2(Sulph)_1$
1608.415	10.2	176	1.828	$(Hex)_4(HexNAc)_4$
1650.413	4.8	109	1.807	$(Hex)_3(HexNAc)_2(NeuAc)_2$
1770.420	5	107	1.78	$(Hex)_5(HexNAc)_4$
1932.456	4.3	126	1.764	$(Hex)_6(HexNAc)_4$

*δmass = [observed m/z] − [theoretical m/z]. Abbreviations: Hex, hexose (e.g., mannose, galactose); HexNAc, N-acetylhexosamine (e.g., GlcNAc, GalNAc); NeuAc, N-acetylneuraminic acid; Sulph, sulfated glycan; Pent, pentose.
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SRf1 was detected on the top of the membrane as a smear, at the same position as the large glycoproteins on 2D-WB analysis. Therefore, this suggested that SRf1 was recognized by both serum IgG and intestinal IgA from dogs infected with *E. multilocularis*. Similarly, Zhang and McManus [11] found local mucosal and systemic immune responses generated against *E. granulosus* in infected dogs. In addition, SRf1 showed significant tolerance against gastric proteases. Taken together, these data suggest that effective vaccine antigens, capable of oral administration, can be generated against *Echinococcus* infection.

In a preliminary experiment using 6 dogs, subcutaneous administration of SRf1 with Freund's adjuvant did not show a significant level of protection, although the immunized dogs

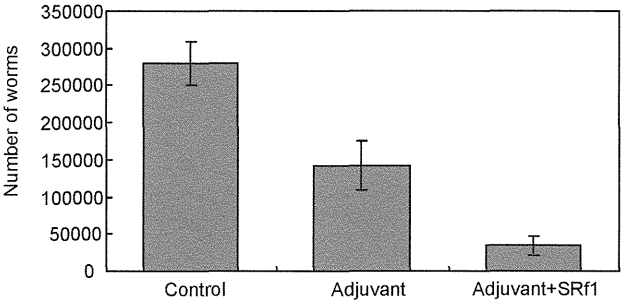

Figure 3. Challenge infection in dogs mucosally immunized with SRf1. Each dog was nasally immunized with SRf1 with CTB adjuvant 4 times. A booster was given orally 3 times with CT. Protoscoleces (5×10^5) were administrated orally after the final immunization. As controls, groups were immunized with PBS or PBS plus adjuvant. The values are the mean number of adult worms ± the S.D. The model separating the group immunized with SRf1 plus adjuvant from the control group best fits the data according to generalized linear modeling ($P<0.001$).
doi:10.1371/journal.pone.0069821.g003

exhibited a significant IgG response in sera as well as obvious physiological responses, such as diarrhea and inflammation of the skin (data not shown). In this study, to identify an adequate immunization regime, we used mucosal immunization combined with 4 nasal and 3 oral immunization doses. As a result, administration of SRf1 with mucosal adjuvant induced an 87.6% reduction in worm numbers in the immunized group compared to the control group, indicating that SRf1 was a promising antigen for use as a mucosal vaccine against *E. multilocularis* in dogs. In addition, to prevent transmission of *E. multilocularis*, the development of a deliverable oral vaccine, such as in fox baits, like the rabies vaccine, is required. Therefore, studies are needed to further support the immunogenicity of SRf1 and its application as a bait oral vaccine.

In this study, we also examined the effectiveness of SRf1 by using CTB and CT, some of the strongest mucosal adjuvants available. Many researchers have attempted to develop mucosal vaccines using nasal or oral administration of antigen with CTB or CT as a mucosal adjuvant [34,35]. Pierce et al. evaluated CT as an oral immunogen against experimental canine cholera. Dogs were immunized orally with a 0.1 mg dose of purified CT and demonstrated marked protection. However, they reported that most dogs experienced moderate diarrhea following administration of CT [35]. Unexpectedly, no symptoms were observed in all experimental dogs in our study. This may be due to differences in the route of administration for CT. Unfortunately, the use of CT is limited by its promiscuous binding to GM1 ganglioside receptors present on all nucleated cells, including epithelial cells and nerve cells [36]. Indeed, a commercial intranasal flu vaccine with a CT as an adjuvant revealed an increased incidence of Bell's palsy in vaccinated subjects [36]. An alternative strategy has proven that mutant CT, which has no or very little enzyme activity, can act as a mucosal adjuvant. CpG oligonucleotides [37] would be also a better strategy for mucosal vaccine development.

In this study, a weak but clear IgG response specific to SRf1 was observed in the immunized group (adjuvant plus SRf1), whereas no specific antibody response was observed in the control group. Mucosal immunization with antigen, co-administered with a mucosally active adjuvant, such as CT, induces both systemic and mucosal immunity [36]. In this study, the mucosal IgA response evoked by immunization of SRf1 could not be detected, most likely due to technical difficulties. However, consistent with a

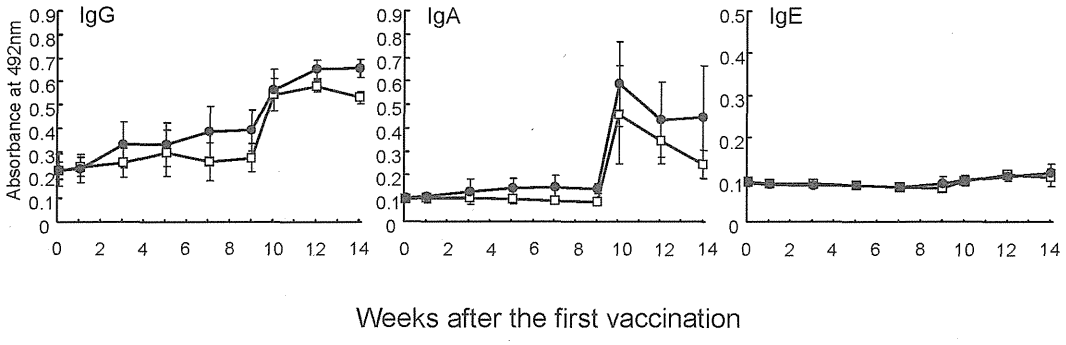

Figure 4. Serum antibody response in dogs immunized via the nasal and oral route with SRf1. Serum antibody responses against SRf1 evoked by nasal and oral immunization were detected by ELISA. The closed circle and open square indicate groups immunized with SRf1 plus adjuvant and adjuvant alone, respectively. Compared with the adjuvant control, an increase in the IgG response was detected in the group immunized with SRf1. No significant IgA or IgE responses were detected during immunization. A sharp increase in IgG and IgA responses were detected in each group after challenge infection.
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previous study [38], we observed a sharp increase in serum IgG and IgA responses just after challenge infection. Moreover, Tanaka et al. observed a similar antibody (IgG and IgA) response in sera from dogs experimentally infected with *E. multilocularis*. However, the detailed mechanisms through which this process leads to protection are still unknown.

Immunostaining revealed that the SRf1 antigen localized at the surface of both larval and adult forms of *E. multilocularis* (Fig. 5).

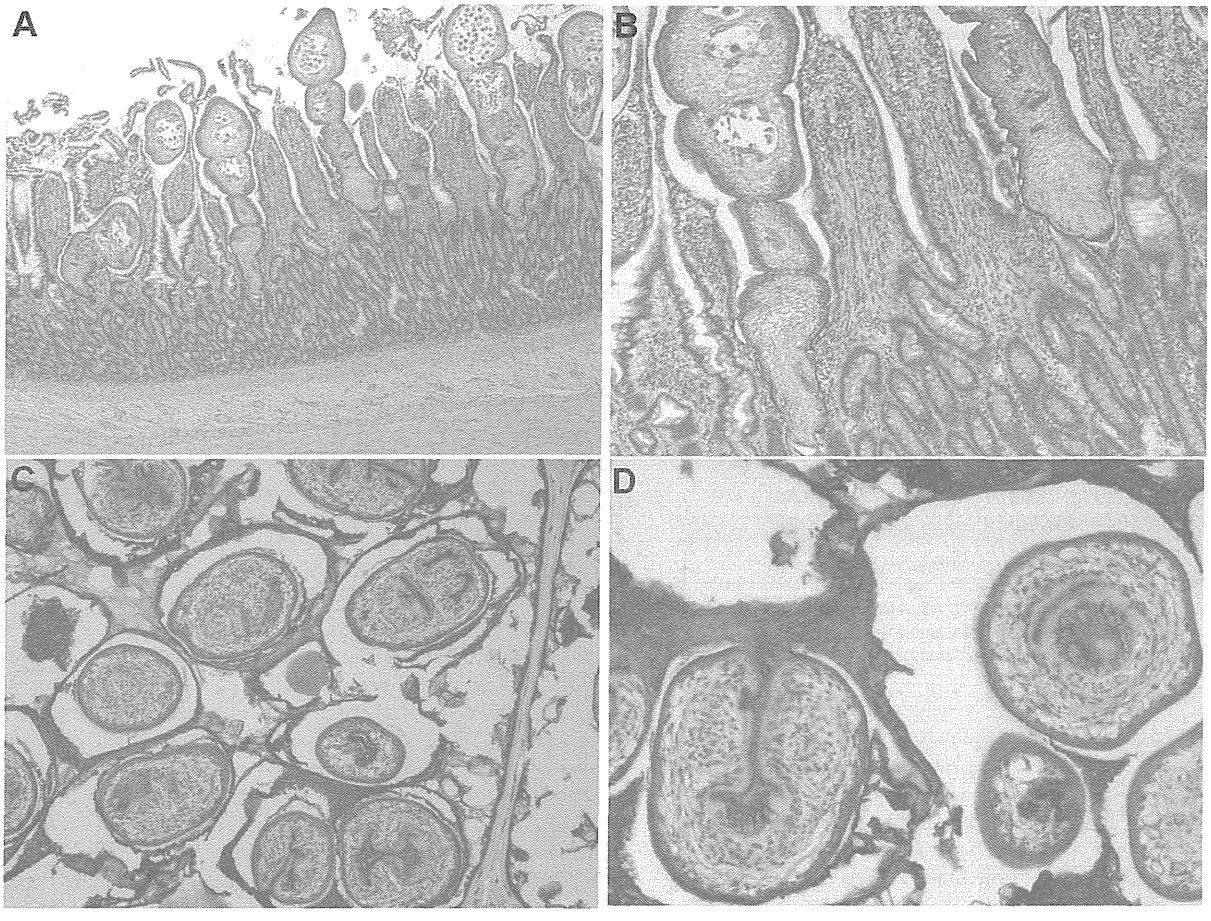

Figure 5. Localization of SRf1 in the larval and adult stages of *E. multilocularis*. Immunostaining was performed using polyclonal mouse anti-SRf1 antiserum. Panels A (40×) and B (100×) show adult worms harbored in the epithelium of the small intestine of infected dogs at 23 days postinfection. Panels C (100×) and D (200×) show protoscoleces in cysts derived from infected cotton rats. The brown color indicates specific antibody reactivity; the blue color indicates hematoxylin staining of nuclei. Anti-SRf1 antibodies were detected on the surface, including suckers, rostella, and hooks in both stages of worm development.
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Notably, SRf1 was also detected on the suckers and rostella, suggesting that SRf1 was recognized by the intestinal immune system during the course of infection. In a previous study, the EgA31 clone was shown to encode a paramyosin protein that also showed very similar localization in adult *E. granulosus* organisms [33]. Thus, surface antigens could represent a class of strong immunogens that are able to induce protection via the mucosal immune response. In addition, a significant reduction in worm numbers was observed in the adjuvant-immunized group. In line with this, infection with the gastrointestinal nematode *Strongyloides* is known to induce host protective immunity by accumulation of mucosal mast cells and activation of mucin release from goblet cells [39,40]. Studies have also suggested that mucin release is induced by CT via interactions with intestinal goblet cells [41,42]. This nonspecific protection induced by mucosal immunization with CTB and CT may provide insights to promote our understanding of the mechanisms of protection induced by mucosal immunization.

Our glycosylation analysis and immunostaining results revealed that the SRf1 antigen could be distinguished from Em2, a known mucin-type glycoprotein that localizes to the laminated layer of metacestode-stage *E. multilocularis* worms. The SRf1 antigen comprised 1 or more highly glycosylated tegument proteins.

Here, we provide evidence that SRf1, a large glycoprotein component from *E. multilocularis* protoscoleces, has vaccine potential to induce significant reduction in the worm burden in experimentally immunized dogs. However, this component, obtained by a relatively simple procedure, does not consist of a single molecular structure, as shown in our results. Thus, further purification and immunological characterization should be performed to identify the precise molecular component that is responsible for inducing the protection. Additionally, this vaccine candidate should be examined for its dose dependency and longevity of efficacy after immunization by performing experimental challenge infections. Successful characterization of the molecular structure of the vaccine candidate would open the way to large-scale preparation of the material by *in vitro* expression or synthesis, which is essential not only for further experimental studies but also for practical application in controlling the parasite.

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Author Contributions

Conceived and designed the experiments: HK K. Yagi YO. Performed the experiments: HK K. Yagi RN. Analyzed the data: HK RN K. Yamano. Contributed reagents/materials/analysis tools: HK K. Yagi. Wrote the paper: HK JM K. Yagi K. Yamano RN YO.

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FULL PAPER *Parasitology*

Epidemiological Study and Control Trial of Taeniid Cestode Infection in Farm Dogs in Qinghai Province, China

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ABSTRACT. An epidemiological study and control trial were conducted to assess taeniid infection in farm dogs in Qinghai Province, China. To improve egg detection by fecal examination, a deworming step with praziquantel was incorporated into the sampling methodology. As a result, a marked increase in the number of egg-positive samples was observed in samples collected at 24 hr after deworming. Then, the fecal examination and barcoding of egg DNA were performed to assess the prevalence of taeniid species in dogs from Xinghai, Haiyan, Gangcha and Chengduo counties. Analysis of 277 dog feces revealed that taeniid cestodes, including *Taenia* spp. and *Echinococcus granulosus*, were highly prevalent in Xinghai (34.4%), but eggs were not found in Haiyan where a control trial on canine echinococcosis had been conducted 20 years previously. A control trial involving the administration of 5–10 mg/kg praziquantel to 90 farm dogs at 45-day intervals was conducted in Xinghai. The prevalence of taeniid cestodes in the dogs was reduced to 9.6% and 4.9% after one and two years, respectively, indicating that some dogs were not administered praziquantel properly. A questionnaire survey of farmers in Xinghai and Haiyan revealed that most farmers in Xinghai were not familiar with echinococcosis or the transmission route of the disease, while most farmers in Haiyan had a more thorough understanding of the disease. The findings implied that a program for educating local farmers would be important for efficiently controlling canine taeniid infection in the region.

KEY WORDS: canine, China, control, *Echinococcus*, taeniid cestodes.

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The Chinese province of Qinghai on the Qinghai-Tibetan Plateau is one of the most highly endemic areas for *Echinococcus* in the world [5] with *E. granulosus*, *E. multilocularis* and *E. shiquicus* having a sympatric distribution in the region [13, 21, 27]. Nomadism of high-altitude adaptive animals, such as yak and sheep, is very popular, and most farmers have dogs for protection and for herding. Together, these conditions favor both endemism and the lifecycle of *E. granulosus*. Dogs are also infected with *E. multilocularis* by eating rodents in the pasture.

The nomadic lifestyle of Tibetan inhabitants is considered to be one of major risk factors associated with *Echinococcus* infection [26], and the reported prevalence in Tibetan nomads reached to 6.1% with *E. granulosus* and 5.1% with *E. multilocularis* in endemic areas in Qinghai Province [9]. The prevalence in domestic animals was also high, and the reported value reached to over 50% with *E. granulosus* and 5% with *E. multilocularis* in dogs [5, 25] and over 50% with *E. granulosus* both in yaks and sheep [24, 30, 31]. Dogs also serve as the definitive host of *Taenia* spp. that can infect to domestic animals, such as yaks and sheep, and thus would reduce animal health and meat quality. *Taenia* spp., such as *T. hydatigena* and *T. multiceps,* were commonly found in sheep and yaks at abattoirs in Qinghai Province [11, 12, 20].

Various control trials on taeniid cestodes, especially *E. granulosus*, incorporating periodic anthelmintic dosing have been conducted on dogs. In some countries, such as Iceland, New Zealand and Tasmania in Australia, government campaigns achieved complete or provisional eradication of *E. granulosus* [17]. However, in most endemic areas, efficient control has not been achieved or even attempted. In Qinghai Province, a similar trial combined with an education program was conducted in Haiyan County between 1991 and 1994. In that study, the prevalence of *Echinococcus* spp. in dogs and sheep was reduced from 63.6% to 0% and 87% to 10%, respectively [14]. The local government supported this

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Fig. 1. Study sites. A: Map of China showing Qinghai Province shaded in grey. B: Map of Qinghai Province. Dark area in Map B indicates Qinghai Lake. Bold lines represent district borders, and thin lines represent county borders. 1: Gangcha County, 2: Haiyan County, 3: Xinghai County (Heka Town), 4: Chengduo County. *: Xining (provincial capital).

campaign, and local veterinarians and epidemic prevention officers properly administered individual dogs with praziquantel at monthly intervals. However, after the termination of the trial, the prevalence in sheep increased to 46.1% by 2009 [14]. No follow-up study on changes in *Echinococcus* prevalence was conducted in dogs.

A variety of methods have been used to diagnose taeniid infection in dogs. Fecal egg examination is a convenient method for diagnoses in live dogs. The reliability of the method is relatively low, particularly for the diagnosis of tapeworm infection because of its discrete excretion of gravid segments. However, the reliability of this method can be increased, if the feces is collected shortly after deworming [19]. Alternatively, coproantigen and coproDNA detection techniques have been developed and employed in various field studies [6, 7, 10, 19, 29], but reports of cross-reactivity between taeniid species and the presence of inhibitory factors in the feces have meant that the results are not always reliable [6, 15, 16, 23].

In this study, we collected dog feces before and after deworming at a pilot site in Qinghai Province and then determined an optimal sampling schedule for egg examination. Using this method, we evaluated the prevalence of taeniid cestodes in dogs at 4 different sites in Qinghai Province. We then selected the most endemic site and conducted a control trial of taeniid infection in dogs with periodic anthelmintic dosing. Finally, in order to identify fundamental problems with the control trial, we conducted a questionnaire survey to assess farmers' knowledge of echinococcosis and the management of their dogs.

MATERIALS AND METHODS

Study sites: This study was conducted in four counties (Gangcha, Haiyan, Xinghai and Chengduo counties) in northeastern and southern Qinghai Province, China (Fig. 1). All of the study sites were inhabited by nomadic Tibetans and other minority ethnic groups grazing yak and sheep on the vast grasslands of the Qinghai-Tibetan Plateau. The altitudes of the study sites ranged from 3,000 to 4,000 m.

Collection of dog feces: To collect dog feces, we first asked local farmers to chain their dogs to prevent them from running away to ensure that they would defecate nearby. At Heka Town in Xinghai County, dog feces were collected before administration of 5 to 10 mg/kg praziquantel and then at 24 and 48 hr thereafter to determine the optimal time for detecting eggs from feces by fecal egg examination. Upon finding tapeworms in feces after deworming, they were collected and identified to species by DNA barcoding. Except for Chengduo County where deworming was not performed, dog feces were collected at 24 hr after praziquantel administration in all study sites. All feces samples were frozen at −80°C for at least 10 days to render eggs uninfective.

Fecal egg examination: Fecal egg examination was performed by the centrifugal sucrose flotation technique using 1.0 g of feces. Upon observing taeniid eggs, 2 to 40 eggs were collected from each feces sample under a stereomicroscope, and the eggs were identified to species by DNA barcoding.

Molecular identification of parasite species (DNA barcoding): DNA was extracted from adult cestodes or eggs using a QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) following the manufacturer's instructions. As reported previously [8], partial segments of the parasite mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and NADH dehydrogenase (*nad1*) gene were amplified by polymerase chain reaction (PCR) using primers 2575 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and 3021 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′) [3] for *cox1* and nad1T-Fw (5′-GGK TAT TCT CAR TTT CGT AAG GG-3′) and nad1T-Rv (5′-ATC AAA TGG AGT ACG ATT AGT YTC AC-3′) [1] for *nad1*. The amplified products (443 and 507 base pairs for *cox1* and *nad1*, respectively) were examined by agarose gel electrophoresis, and their nucleotide sequences were determined as described previously [8]. The obtained sequences were aligned using CLC Sequence Viewer 6.1 (CLC Bio Japan, Tokyo, Japan), and a Basic Local Alignment Search Tool (BLAST) similarity search was conducted for species and genotype identification.

Deworming of dogs: A control trial to assess taeniid infection in farm dogs was conducted in the town of Heka in Xinghai County where the prevalence of taeniid cestodes in dogs was the highest. A total of 90 dogs were selected for the trial. With support of a local veterinarian, dog owners were requested to administer the required dose of praziquantel (5–10 mg/kg) to each dog at 45-day intervals from July 2010 for two years. Change in the prevalence of infection with taeniid cestodes was then assessed by comparing the prevalence before the beginning of the trial and those at 1 and 2 years after starting the trial. In addition, in order to confirm the efficacy of praziquantel treatment, feces samples were collected and examined again from all dogs 1 month after

Table 1. Comparison of taeniid egg detection rate by fecal examination in feces samples collected before and after deworming dogs and the number of dogs that excreted cestodes after deworming

Taeniid species	No. dogs	No. positive in fecal examination			No. dogs excreting cestodes
		Timing of sampling			
		Before	After 24 hr	After 48 hr	
Taenia hydatigena	18	8	17	6	11
Taenia multiceps	1	1	1	1	1
None	24	0	0	0	0
Total	43	9 (21%)	18 (43%)	7 (17%)	12 (29%)

Table 2. Prevalence of taeniid cestodes determined by faecal egg examination in dogs from four counties in Qinghai Province, China

County	No. dogs examined	No. positive	Taeniid species		
			Echinococcus granulosus	*Taenia hydatigena*	*Taenia multiceps*
Haiyan*a)	38	0	NA	NA	NA
Gangcha*a)	77	14	1	12	1
Xinghai*a)	90	31	12	18	1
Chengduo*b)	72	9	6	3	0
Total	277	54	19	33	2

NA: Not applicable. *a) Fecal egg examination with deworming. *b) Fecal egg examination without deworming.

the 2nd year examination when all dogs were dewormed properly for sampling feces.

Questionnaire survey: Since the disparity in taeniid cestode prevalence in dogs was greatest between the counties of Xinghai and Haiyan, a questionnaire survey was conducted among 30 farmers in these 2 counties to assess their knowledge of echinococcosis and their feeding and contact with their dogs.

Statistical analysis: Difference in the questionnaire responses between the 2 counties was evaluated by Fisher's exact test implemented in the program R [22]. $P<0.05$ was considered to be significant.

RESULTS

Fecal egg examination: The results of fecal egg examination performed on feces collected from 43 dogs before praziquantel administration and then at 24 and 48 hr thereafter are shown in Table 1. Taeniid eggs were found in 9 (21%), 18 (43%) and 7 (17%) feces samples, respectively, indicating detection rate was highest in feces collected at 24 hr after deworming. After deworming, 12 dogs excreted tapeworms. Except for one dog, all of these dogs excreted taeniid eggs. DNA barcoding of the excreted eggs and adult cestodes revealed that 18 dogs were infected with *T. hydatigena* and one dog with *T. multiceps*. Eggs of other parasite species were not found.

Prevalence of taeniid cestodes in dogs at four different study sites: The prevalence of taeniid eggs in the feces of dogs from 4 counties examined in 2010 is shown in Table 2. The highest prevalence was found in Xinghai County, and no positive samples were found in Haiyan County. DNA barcoding of egg DNA showed that *E. granulosus* and *T. hydatigena* were detected in 3 of the counties and that Xinghai County had the highest prevalence of both parasite species. All *nad1* sequences of *E. granulosus* were identical to that registered in GenBank as G1 genotype (accession No. JF946624). In Gangcha and Xinghai counties, *T. multiceps* was detected in 1 dog feces sample in each county. In addition to taeniid eggs, *Trichuris vulpis* eggs were found in 1 dog from Gangcha County and in 2 dogs from Xinghai County.

Control trial of taeniid infection in dogs: Although the trial was started with 90 dogs, the number of animals decreased to 41 over the course of the trial due to death of dogs, transfer of dogs, not having of dogs chained at the time of sampling, disappearance of dogs and owners and owners rejecting further deworming treatments because of changes in dog health after dosing (Table 3). The overall prevalence of taeniid cestodes was reduced from 34.4% at the beginning of the trial to 9.6% and to 4.9% 1 and 2 years after starting the trial, respectively. Changes were also observed in the prevalence of individual parasites. For example, *E. granulosus* prevalence changed from 13.3% to 3.6% and to 2.4%, *T. hydatigena* prevalence changed from 20.0% to 2.4% and to 2.4%, and *T. multiceps* prevalence changed from 1.1% to 3.6% and to 0% 1 and 2 years after starting the trial, respectively. Only 2 dogs were positive for taeniid eggs in consecutive years of the study, but both dogs were infected by different taeniid species in different years; 1 dog was infected with *T. hydatigena* before anthelmintic treatment, but with *E. granulosus* at the end of the first year, and the other dog was infected with *E. granulosus* at the end of the 1st year, but with *T. hydatigena* at the end of the 2nd year. When dogs were examined for the last time, i.e. 1 month after the 2nd year examination, no eggs were detected in any

Table 3. Change in taeniid cestode prevalence in dogs over the course of a 2-year anthelmintic control trial in Xinghai County, China

Taeniid species	Pre-trial		One year post-trial		Two years post-trial	
	No. positive / No. examined	Prevalence (%)	No. positive / No. examined	Prevalence (%)	No. positive / No. examined	Prevalence (%)
E. granulosus	12 / 90	13.3	3 / 83	3.6	1 / 41	2.4
T. hydatigena	18 / 90	20.0	2 / 83	2.4	1 / 41	2.4
T. multiceps	1 / 90	1.1	3 / 83	3.6	0	0
Total	31 / 90	34.4	8 / 83	9.6	2 / 41	4.9

Table 4. Comparison of questionnaire responses from farmers in Haiyan and Xinghai counties

1. Feeding viscera of sheep and yaks to dogs

	Frequency (P=0.195)		Treatment of viscera (P=0.424)	
County	Sometimes	Not at all	Raw	heated
Haiyan	25	5	25	5
Xinghai	29	1	28	2

2. Frequency of contact with (patting) dogs (P=0.561)

County	Seldom	Sometimes	Frequent
Haiyan	20	4	6
Xinghai	19	7	4

3. Awareness of echinococcosis as a disease (P<0.001)

County	No	Only name	Yes
Haiyan	3	10	17
Xinghai	21	7	2

4. Awareness of infection risk of echinococcosis to oneself and his/her family (P<0.001)

County	No	Yes
Haiyan	7	23
Xinghai	28	2

5. Awareness of *Echinococcus* hosts (P<0.001)

Host	County	No	Yes
Definitive host (P<0.001)	Haiyan	11	19
	Xinghai	29	1
Intermediate host (P<0.001)	Haiyan	14	16
	Xinghai	30	0

of the dog feces.

Farmers' awareness of echinococcosis and animal management: The responses to the questionnaire are shown in Table 4. Although obvious differences were not observed in the feeding behavior of the respective dogs or contact frequency with their dogs, significant difference was observed in the awareness of echinococcosis and the knowledge of transmission risk and lifecycle.

DISCUSSION

Infection by *E. granulosus* and *T. hydatigena* in dogs was detected in 3 of the 4 counties, indicating the species are distributed widely in Qinghai Province. These findings are consistent with reports of cystic echinococcosis in humans and domestic animals elsewhere on the Qinghai-Tibetan Plateau [5, 9, 13, 14, 26, 29–31]. Finding *T. hydatigena* metacestodes at abattoirs seems to be very common, and the prevalence was reported to be 8.2–30.8% in sheep [11, 20]. While, *T. multiceps* was only found in 2 dogs, and the prevalence of this parasite in Qinghai Province is moderate and has been reported in 1.44 and 3.79% of the sheep and yaks examined, respectively [12]. However, the pathogenicity associated with *T. multiceps* infection in sheep is very high with 97% of the Tibetan sheep showing clinical signs dying after developing neurologic disorders due to cerebral coenurosis [12]. Measures should therefore be implemented to prevent infection of dogs both by *Echinococcus* spp. and

T. multiceps.

Other species of *Echinococcus* were not detected in this study. However, previous reports showed that the prevalence of *E. multilocularis* in humans and dogs was 1.3–3.3 and 2.5–3.1%, respectively, in southern Qinghai Province where Chengduo County is located [25, 29]. Infection with *E. shiquicus* was not detected either. This parasite is exclusively reported in Qinghai-Tibetan Plateau and maintained by Tibetan sand fox and plateau pika [27]. Dogs are not considered to be a suitable host for this parasite [28]. Recently, however, *E. shiquicus* DNA was found in the feces of dogs [2]. This suggests the possibility of the parasite infecting to dogs, although the coproDNA found in dog feces may have been derived from a diet.

Mixed infection, i.e. infection with multiple species of taeniid cestodes, was not detected in this study. However, it should be noted that the method used to identify taeniid species in this study could not always detect cases of mixed infection because the DNA examined was obtained from up to 40 eggs.

In Haiyan County, no eggs were found in the 38 dog feces samples examined. Although an anthelmintic dosing campaign in this county was terminated in 1994, some of the local residents were aware of the risk of echinococcosis and continued to dose their dogs. Following a report stating that the prevalence of *E. granulosus* in yaks and sheep increased after the termination of the campaign [14], the current prevalence in dogs is unlikely to be 0%; nonetheless, it is expected to be low. Our observation was contradicted with the previous observation in Peru where the termination of a control program may have contributed to a marked increase in infection prevalence in intermediate and definitive hosts as well as in humans [17, 18].

The sensitivity with which parasite eggs, especially cestode eggs, can be detected by fecal examination increases shortly after anthelmintic dosing of animals [19]. In this study, taeniid eggs were most frequently detected in dog feces collected 24 hr after dosing. In the 1 dog from which no taeniid eggs were obtained, a tapeworm (*T. hydatigena*) was excreted after praziquantel dosing. The failure to detect eggs in this animal may have been due to infection with an immature tapeworm, but we did not check to see if the excreted tapeworm contained any mature eggs (gravid segments).

The control trial conducted as part of this study reduced the prevalence of taeniid cestodes in the dogs examined. However, it was appeared that some of the dog owners did not dose their dogs properly because taeniid cestodes including *E. granulosus* and *T. hydatigena* were still detected in 9.6% and 4.9% of the dogs at the end of the 1st and 2nd years of the trial. Since the prepatent periods of *E. granulosus*, *T. hydatigena* and *T. multiceps* are about 45–60, 51 and 30 days, respectively [4], praziquantel doses with 45-day intervals can allow only *T. multiceps* to become patent infection during the trial. In addition, the observation that no taeniid eggs were detected in any of the dog feces samples one month after the final examination at the end of 2nd year when all of the dogs were properly dewormed using praziquantel, indicated that praziquantel was highly effective for treating taeniid cestodes.

The number of dogs used for the control trial was reduced from 90 to 41 at the end of the trial. Although a variety of reasons were responsible for this decrease in the number of animals, we did not anticipate such a marked reduction at the beginning of the trial. Many of the farmers in the study obtained new dogs to replace those that went missing, and it appears that replacing or trading dogs is common among farmers; this practice should be therefore considered, if controls are designed in the future.

The questionnaire survey revealed that an awareness of the risk of echinococcosis infection in humans, as well as knowledge of the transmission route of the parasite, would likely affect the efficacy of any control measures. For example, in Heka Town, where the control trial of this study was conducted and where local farmers did not know much about echinococcosis, deworming of all of the target dogs was not achieved. Conversely, in Haiyan County where a control trial combined with an education program had been conducted previously and where the level of disease awareness among local farmers was high, taeniid eggs were not detected in any of the dog feces samples examined. These findings suggested that education programs should be an integral component of any measures designed to control canine echinococcosis or initiatives directed at reducing the prevalence of *Taenia* infection in dogs.

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Short Communication

Serological evidence of infection of dogs with human influenza viruses in Japan

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HISTORICALLY, influenza virus has not been regarded as a major pathogen of dogs. However, recent infections of racing and pet dogs with H3N8 virus of equine origin in the USA after 2004 (Crawford and others 2005) and retrospectively in the UK in 2002 (Daly and others 2008), as well as with highly pathogenic H5N1 avian virus in Thailand in 2004 (Songserm and others 2006), revealed that dogs are susceptible to influenza A viruses. These infections caused respiratory disease in the dogs and several proved fatal. Moreover, H3N2 virus of avian origin infected pet dogs in Korea in 2007 (Song and others 2008) and China in 2010 (Li and others 2010), supporting the belief that dogs should be included among the animals that are responsible for interspecies transmission of influenza A virus (Kim and others 2013). Furthermore, there were reports in various parts of the world (Dundon and others 2010, Lin and others 2012) of dogs infected with A(H1N1)pdm09 virus of swine origin, possibly due to contact with people infected with this H1N1 virus, suggesting human-to-dog transmission. Together, these reports suggest that dogs may play a role as an intermediate host in which a mutant virus with pandemic potential could emerge. To partially address this possibility, we conducted a serological survey of human influenza virus infection in domestic dogs in Japan.

A total of 366 serum samples were collected from mostly indoor domestic dogs between January 2009 and February 2010 at animal hospitals in the prefectures of Yamaguchi (162 samples) and Kanagawa (204 samples), which are located in western and eastern Japan, respectively. The dogs came to the hospitals with various symptoms, and their serum samples were randomly collected for the study with the pet owners' permission. To detect antibodies specific to human influenza viruses in the sera, we performed a virus-neutralisation (VN) test (Itoh and others 2009) with a former seasonal H1N1 virus (A/Kawasaki/UTK4/09), seasonal H3N2 virus (A/Kawasaki/UTK20/08), A(H1N1)pdm09 virus, and influenza B virus (B/Tokyo/UT-E2/08), after receptor-destroying enzyme treatment and heat-inactivation of the sera to remove non-specific inhibitors. The VN antibody-positive sera (titres ≥10) were then further tested in an immunoblot assay using the same virus antigens to confirm reaction specificity.

In these assays, 14 sera were positive for VN antibody to A(H1N1)pdm09 virus, representing 3.8 per cent positivity (Table 1). One serum sample was also positive for VN antibody to the former seasonal H1N1 virus, but the reaction was weak, suggesting it may have been due to cross-reactivity. Eight other samples were seropositive to seasonal H3N2 virus, representing 2.2 per cent positivity; however, these sera did not react with dog H3N8 virus (A/canine/NE/52-14/06) or avian H3N2 virus (A/duck/Mongolia/301/01), suggesting human-to-dog transmission of the human virus. Notably, six sera were positive for VN antibody to type B virus, representing 1.6 per cent positivity. None of the sera was antibody-positive for both type A and B viruses. Moreover, all samples were negative for VN antibody to highly pathogenic H5N1 avian virus (A/whooper swan/Mongolia/4/05; clade 2.2.). None of the seropositive dogs showed any typical signs of acute respiratory illness according to the clinical records, suggesting that they were asymptomatic or had very minor infections, as supported by the low VN titres for the positive cases.

We conclude that dogs can be infected with human influenza viruses, including type B virus. To our knowledge, there is only one previous report of a possible natural influenza B virus infection of a dog (Chang and others 1976), and even in this case, the infection was not proven with established criteria. There has also been a report of influenza C virus infection of dogs (Ohwada and others 1987). Thus, domestic dogs may act as a vector for human influenza virus transmission within households, posing a potential public health concern. Previous reports that dogs are susceptible to avian viruses, such as H3N2 and H5N1, raise the possibility that dogs, like pigs, may act as an intermediate host for the emergence of new, potentially pandemic viruses. Continued surveillance of influenza viruses in dog populations will be important to achieve the "one health" concept for this zoonotic disease.

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TABLE 1: Dogs in the study that were seropositive to human influenza viruses

Prefecture	Number of samples	Virus	Number of positive samples (%)	VN titres (number)	Species (number)
Yamaguchi	162	A(H1N1)pdm09	4 (2.5)	10 (3), 20 (1*)	Miniature dachshund (1), Chihuahua (1), toy poodle (1*), shih tzu (1)
		H1N1	1 (0.6)	10 (1*)	Toy poodle (1*)
		H3N2	1 (0.6)	40 (1)	Miniature dachshund (1)
		B	4 (2.5)	10 (2), 20 (2)	Mongrel (2), toy poodle (1), miniature dachshund (1)
Kanagawa	204	A(H1N1)pdm09	10 (4.9)	10 (4), 20 (5), 80 (1)	Miniature dachshund (3), papillon (2), toy poodle (1), mongrel (1), shiba (1), springer spaniel (1), long coat Chihuahua (1)
		H1N1	0 (0)	–	–
		H3N2	7 (3.4)	10 (4), 40 (3)	Miniature dachshund (3), toy poodle (2), Welsh corgi (1), Yorkshire terrier (1)
		B	2 (1.0)	10 (1), 20 (1)	Miniature dachshund (1), Maltese (1)

*The same dog
VN, virus-neutralisation

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MAJOR ARTICLE

The First Identification and Retrospective Study of Severe Fever With Thrombocytopenia Syndrome in Japan

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Background. Severe fever with thrombocytopenia syndrome (SFTS) is caused by SFTS virus (SFTSV), a novel bunyavirus reported to be endemic in central and northeastern China. This article describes the first identified patient with SFTS and a retrospective study on SFTS in Japan.

Methods. Virologic and pathologic examinations were performed on the patient's samples. Laboratory diagnosis of SFTS was made by isolation/genome amplification and/or the detection of anti-SFTSV immunoglobulin G antibody in sera. Physicians were alerted to the initial diagnosis and asked whether they had previously treated patients with symptoms similar to those of SFTS.

Results. A female patient who died in 2012 received a diagnosis of SFTS. Ten additional patients with SFTS were then retrospectively identified. All patients were aged ≥50 years and lived in western Japan. Six cases were fatal. The ratio of males to females was 8:3. SFTSV was isolated from 8 patients. Phylogenetic analyses indicated that all of the Japanese SFTSV isolates formed a genotype independent to those from China. Most patients showed symptoms due to hemorrhage, possibly because of disseminated intravascular coagulation and/or hemophagocytosis.

Conclusions. SFTS has been endemic to Japan, and SFTSV has been circulating naturally within the country.

Keywords. Severe fever with thrombocytopenia syndrome; SFTS; SFTS virus: Japan; tick borne virus infection; bunyavirus; Hemophagocytosis.

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Severe fever with thrombocytopenia syndrome (SFTS), an infectious disease with a high case-fatality rate, is caused by SFTS virus (SFTSV), a novel bunyavirus reported to be endemic to central and northeastern parts of China [1, 2]. SFTSV, which is classified into the genus *Phlebovirus* and the family *Bunyaviridae*, is suspected to be a tick-borne virus owing to evidence of its presence in 2 species of ticks: *Haemaphysalis longicornis* and *Rhipicephalus microplus* [2, 3]. Approximately 2% of *H. longicornis* organisms collected from sheep, cattle, and dogs in Shandong Province tested positive for SFTSV in virus genome amplification assays [4]. A similar disease, which was named fever, thrombocytopenia and leukopenia syndrome (FTLS), was independently reported to be caused by a novel virus, Henan fever virus (HNFV) [1]. Despite the different names, "SFTS" and "FTLS" represent the same condition and "SFTSV" and "HNFV" represent the same virus. The case-fatality rate of SFTS is reported to be approximately 12% [1, 2]. Human-to-human transmission of SFTSV was reported to occur through close contact with the blood and/or body secretions of infected patients [5–9]. To our knowledge, there were no published reports of SFTS outside of China before we performed the study described here. Another tick-borne phlebovirus, the Heartland virus, which was detected in Missouri, is phylogenetically associated with SFTSV, caused severe febrile illness with thrombocytopenia, leukopenia in the total blood cell count, and elevated levels of liver enzymes [10].

We report the first identification of SFTS in Japan, which was detected in a previously healthy woman aged 50–59 years who died of multiple-organ failure in the autumn of 2012, and findings from a subsequent retrospective study of SFTS in Japan.

MATERIALS AND METHODS

Next-Generation Sequencing

Culture supernatants were subjected to viral RNA extraction using High Pure Viral Nucleic Acid Kit (Roche Diagnostics). Complementary DNA (cDNA) was synthesized using SuperScript III (Invitrogen) with random primers and then randomly amplified using the illustra GenomiPhi V2 Kit (GE Healthcare Life Sciences). A cDNA library was prepared using the Nextera DNA Sample Prep Kit (Illumina). A sequencing run for 50 nucleotides was performed with MiSeq (Illumina), using the MiSeq Reagent sequencing kit (Illumina). The assembled nucleotide sequences were used to determine homologous sequences by tBlast at the National Center for Biotechnology Information Web site (available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Viral Genome Detection, Virus Isolation, and Antibody Detection in Sera

The SFTSV genome was detected by reverse-transcription polymerase chain reaction (RT-PCR) in total RNA extracted from patient sera, using a High Pure Viral RNA kit (Roche Applied Science). Reverse transcription was performed with Ready-to-Go RT-PCR beads (GE Healthcare), using random nucleotide hexamers. PCR was performed to amplify SFTSV-specific cDNA fragments. PCR primer sets were designed to amplify the nucleoprotein (NP) region of the SFTSV genome (Supplementary Table 1). The PCR conditions were as follows: 1 cycle at 55°C for 30 minutes followed by 95°C for 2 minutes; 45 cycles at 94°C for 30 seconds, 52°C for 30 seconds, and 68°C for 30 seconds; followed by 1 cycle at 68°C for 5 minutes.

Vero cells were inoculated with RT-PCR–positive patient sera for virus isolation, cultured for 4–7 days, and examined for SFTSV antigen detection by indirect immunofluorescence assay (IFA) with a polyclonal antibody raised against SFTSV recombinant NP (rNP; rabbit anti-SFTSV rNP serum), which was produced as follows. SFTSV rNP tagged with histidine-tag on the C-terminus was expressed in a baculovirus expression system, as previously described [11, 12]. The NP gene of SFTSV strain HB29 (GenBank accession no. NC_018137) was artificially synthesized. Anti-SFTSV rNP serum was raised in rabbits by immunization with the purified SFTSV rNP, as previously described [13–15].

The neutralizing antibody to SFTSV Chinese isolate HB29 strain [2, 16] and Japanese isolate YG1 (this study) was detected as reported previously, except for the target virus [15]. With the exception of the antigen preparation, immunoglobulin G (IgG) antibody titers to SFTSV were determined by indirect IFA, using SFTSV HB29–infecting Vero cells as previously described [14].

Pathologic Studies With Histopathologic, Immunohistochemical, and In Situ Hybridization AT-Tailing (ISH-AT) Methods

Histopathologic studies of formalin-fixed and paraffin-embedded specimens were performed using hematoxylin-eosin stain.

Immunohistochemical analysis was performed as previously described, with some modifications [17]. The rabbit anti-SFTSV rNP serum and the peroxidase-labeled, polymer-conjugated anti-rabbit immunoglobulin (EnVision/HRP, Dako) were used in the immunohistochemical analysis as the primary and the secondary antibodies, respectively. Normal rabbit serum and lymph nodes of necrotizing lymphadenitis without SFTSV infection were used as negative controls for the antibody and tissue specimens, respectively.

ISH-AT was used for detection of SFTSV genomic RNA (negative-strand RNA) as reported previously, with the exception of the strand-specific oligonucleotide probes [18–20], which were designed for the S and L segments of the SFTSV genome (Supplementary Table 1).

Quantitative Amplification of the SFTSV Genome in the Autopsied Tissues and Organs

The SFTSV copy number was determined by performing quantitative real-time RT-PCR analysis of RNA samples extracted from paraffin-embedded sections as previously described, with

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some modifications [17]. The amount of human β-actin messenger RNA (mRNA) in the DNase-treated RNA extracted from each section was also determined and used as an internal reference for normalization [21]. The primers and labeled probes are shown (Supplementary Table 1).

Electron Microscopic Analysis

The culture supernatant from Vero cells inoculated with SFTSV isolated from the first patient was used for electron microscopic analysis. The samples were fixed with 4% glutaraldehyde. The fixed samples were negatively stained with 2% phosphotungstic acid and then observed using a JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan).

Recruitment of Patients With Suspected SFTS

The first diagnosis of SFTS in Japan was made public through an announcement from the Ministry of Health, Labor, and Welfare of Japan on 30 January 2013. Physicians were asked to volunteer information if they had treated patients who satisfied the following case definition: (1) fever of >38°C; (2) gastrointestinal tract symptoms, such as nausea, vomiting, abdominal pain, diarrhea, and melena; (3) thrombocytopenia, with $<100 \times 10^9$ platelets/L; (4) leukopenia, with $<4 \times 10^9$ white blood cells/L; (5) elevated levels of aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase; (6) absence of other causes; and (7) death or admission to an intensive care unit because of the severity symptoms. Information about patients was collected from their physicians. The retrospective recruitment of patients with suspected SFTS was conducted from 30 January to 31 March 2013. Written informed consent was obtained from patients or responsible relatives.

Serum samples, which had been collected from the patients for future analyses to clarify the etiology and had been stored in each hospital by the respective physicians, used for the present study were sent to the Department of Virology 1 at the National Institute of Infectious Diseases (NIID; Tokyo, Japan) for virologic analyses. Clinical and laboratory data of the patients with SFTS were also sent to the corresponding author (M. Saijo) without information that made it possible to identify individuals.

Phylogenetic Analysis

cDNAs prepared from the patients' sera samples were used to determine SFTSV genome sequences. The terminal sequences of SFTSV genomes were determined by rapid amplification of cDNA ends (RACE) at the 3′ and 5′ ends, performed by RT-PCR after 3′ and 5′ linker ligation using a DynaExpress miRNA Cloning Kit, High Efficient (BioDynamics Laboratory, Tokyo, Japan). Purified RNA from the culture supernatant of SFTSV (YG1)–infected Vero cells was subjected to the 3′ linker ligation according to the manufacturer's protocol. Next, the 5′ linker was ligated to the 5′-phosphate end of the purified 3′ linker ligated RNA, according to the manufacturer's protocol, following RNA purification by NucleoSpin RNA Clean-up XS (Takara Bio, Shiga, Japan). The 3′ and 5′ linker ligated RNA was then reverse transcribed according to the manufacturer's protocol by SuperScript III (Invitrogen, Carlsbad, CA), using either the 3′ forward RT primer, which is the antiparallel sequence of the 3′ linker sequence, or the pd(N)6 primer (random hexamer). RT-PCR was performed using Q5 Hot Start High-Fidelity DNA Polymerase (NEB, Ipswich, MA) and SFTSV gene-specific primers (Supplementary Table 1) with the 3′ RT primer or the 5′ primer, which is the antiparallel sequence of the 5′ linker sequence.

Nucleotide sequences of each full segment of SFTSV in patients' sera were aligned using MUSCLE, an en suite program in the Molecular Evolutionary Genetics Analysis 5.1 software (MEGA Team, Japan). Evolutionary distances were estimated using Kimura's 2-parameter method, and phylogenetic trees were constructed using the neighbor-joining method. The robustness of the trees was tested using 1000 bootstrap replications. Accession numbers of the nucleotide sequences of SFTSV L-, M-, and S-segments are described in Supplementary Table 2.

Ethics Statement

Serum samples were used for virologic analysis after obtaining written informed consent from the patients themselves (for those who survived) or their responsible family members (for those who died). The clinical and laboratory data of the patients with SFTS were sent to the corresponding author without personally identifying information. All of the protocols and procedures were approved by the research and ethics committees of the NIID.

The polyclonal antibody to SFTSV rNP was produced by immunizing rabbits with purified SFTSV rNP, with approval from the Institutional Animal Care and Use Committee of the NIID (no. 111 124).

RESULTS

The First Patient in Japan Who Received a Diagnosis of SFTS

A previously healthy woman aged 50–59 years who lived in the Yamaguchi prefecture of Japan was hospitalized with high fever, fatigue, vomiting, and melena in the autumn of 2012. Her body temperature was 39.2°C. Tick bite wounds were not observed anywhere on her skin. Laboratory tests revealed a low platelet count of 89×10^9 platelets/L (normal range, $150–250 \times 10^9$ platelets/L) and a low white blood cell count of 0.4×10^9 cells/L (normal range, $4.0–8.0 \times 10^9$ cells/L). Serum levels of alanine aminotransferase, aspartate aminotransferase, and creatine kinase were abnormally high, while the C-reactive protein level was normal. A coagulation study revealed a prolonged activated partial thromboplastin time and a high

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