



# Epidemiological study of Kabuto Mountain virus, a novel uukuvirus, in Japan

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**ABSTRACT.** Kabuto Mountain virus (KAMV), the new member of the genus *Uukuvirus*, was isolated from the tick *Haemaphysalis flava* in 2018 in Japan. To date, there is no information on KAMV infection in human and animals. Therefore, serological surveillance of the infection among humans and wild mammals was conducted by virus-neutralization (VN) test and indirect immunofluorescence assay (IFA). Sera of 24 humans, 59 monkeys, 171 wild boars, 233 Sika deer, 7 bears, and 27 nutria in Yamaguchi Prefecture were analyzed by VN test. The positive ratio of humans, monkeys, wild boars, and Sika deer were 20.8%, 3.4%, 33.9% and 4.7%, respectively. No positive samples were detected in bears and nutria. The correlation coefficients between VN test and IFA in human, monkey, wild boar, and Sika deer sera were 0.5745, 0.7198, 0.9967 and 0.9525, respectively. In addition, KAMV was detected in one pool of *Haemaphysalis formosensis* ticks in Wakayama Prefecture. These results indicated that KAMV or KAMV-like virus is circulating among many wildlife and ticks, and that this virus incidentally infects humans.

**KEY WORDS:** Kabuto Mountain virus, tick, uukuvirus, zoonosis

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Ticks are the primary vectors for pathogens of domesticated and wild animals and the secondary vectors for pathogens of humans [30]. Tick-bites are considered an occupational health issue for forestry personnel and others working (e.g., agricultural industry, military) and/or participating in recreational activities (e.g., hunting, hiking) in forested areas [36]. Since the beginning of this century, tick-borne viral diseases increasingly have been reported in many parts of the world, with examples including Alkhurma haemorrhagic fever [2], African swine fever [35], Crimean-Congo haemorrhagic fever [23], severe fever with thrombocytopenia syndrome (SFTS) virus [37], Heartland virus infection [30], Powassan virus infection [13], Kyasasur forest disease [1], Yezo virus infection [21], Oz virus [7, 41] and so on.

Tick-borne viruses (TBVs) belong to two orders (*Bunyvirales* and *Mononegavirales*), as well as another nine families and at least 12 genera that remain unassigned to any order [18, 38]. The order *Bunyvirales* was established in 2017 and comprises more than 500 viruses classified into 12 families, including several viruses that are highly pathogenic to mammalian hosts [25]. The genus *Uukuvirus* belongs to the family *Phenuiviridae* of the order *Bunyvirales*. According to Kuhn *et al.* [22], the genus *Uukuvirus* includes Kabuto Mountain virus (KAMV) [6], Zaliv Terpeniya virus [27], Uukuvirus [8], American dog tick virus [40], Sunday Canyon virus [44], Grand Arbaud virus [14], Kaisodi virus [3, 42], Dabieshan virus [26], EgAn 1825-61 virus (also known as Nile warbler virus) [33], Precarious Point virus [39], Murre virus [33], Lihan virus [26], Pacific coast tick virus [4], Tacheng tick virus 2 [26], Rukutama virus [29], Silverwater virus [31], Yongjia virus 1 [26], and Toyo virus, a new member of this genus [20]. Some species of the genus can infect humans and animals, since anti-Uukuvirus antibodies have been detected in sera from humans [14, 28], cows, and reptiles [14]. The first case of human Tacheng tick virus 2 infection in China was reported to show symptoms of fever, headache, anorexia, nausea, vomiting, and erythema [5]. However, the epidemiology and pathology of many uukuviruses remain unclear.

KAMV, strain T32, was first isolated from a pooled sample of two larvae, 20 nymphs, and one adult male of *Haemaphysalis*

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*flava* ticks, collected by flagging from September to November 2009 in Hyogo, Japan [6]. Suckling mice inoculated intracerebrally with KAMV showed severe symptoms; the virulence of this isolate increased after passage, demonstrating 100% mortality rate in this mouse model [6]. However, there exists (to our knowledge) no further information on KAMV.

In the present study, serosurveillance was performed for KAMV infection among mammals, including humans, along with molecular screening for tick-borne KAMV.

## MATERIALS AND METHODS

### Cells

Vero cells (JCRB9013), which are derived from the kidney of an African green monkey, and BHK-21 cells (JCRB9020), which are derived from baby hamster kidneys, were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco™, Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum (FCS; Hyclone Laboratories, Logan, UT, USA), 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The cells were maintained at 37°C in 5% CO<sub>2</sub>.

### Virus

KAMV strain T32 was isolated from the *Haemaphysalis flava* hard ticks in Hyogo, Japan, as described previously [6]. The virus was propagated in BHK-21 cells in DMEM containing 2% FCS at 37°C in 5% CO<sub>2</sub> and stored at -80°C until use.

### Virus titration

The virus titer of KAMV was determined by 50% Tissue Culture Infectious Dose (TCID<sub>50</sub>) assay using 96-well flat-bottom microplates (Sumitomo Bakelite, Inc., Tokyo, Japan). Briefly, 100 µl of serial 10-fold dilutions of virus suspension (10<sup>-1</sup> to 10<sup>-10</sup>) and 100 µl of BHK-21 cells (1 × 10<sup>5</sup>/ml) were mixed in each well and incubated at 37°C with 5% CO<sub>2</sub>. After 7 days of incubation, cells were fixed with 10% phosphate-buffered formalin and stained with crystal violet (Wako Pure Chemical Industries, Osaka, Japan). After visual inspection of wells, viral titer was calculated by using the Reed-Muench calculation [24].

### Serum samples

Serum samples were collected from multiple species of wild mammals captured in Yamaguchi Prefecture from 2013 to 2019, including wild boars (*Sus scrofa*, n=171), Sika deer (*Cervus nippon*, n=233), monkeys (*Macaca fuscata*, n=59), Asian black bears (*Ursus thibetanus*, n=7) and nutria (*Myocastor coypus*, n=27). In total, 24 human sera in Yamaguchi Prefecture were used for detection of antibodies against KAMV. Monkeys, wild boars, Sika deer, bears, and nutria were hunter-harvested or culled for nuisance control under the Program of Prevention from the Bird and Animal Damages. No animals were killed specifically for this study. Blood samples were collected directly from the hearts of the animals using a sterile needle. Human sera were collected from 24 hunters who hunt wild animals in Yamaguchi Prefecture, Japan; human samples were collected under a protocol approved by the Center for Clinical Research, Yamaguchi University Hospital (Control Number: H26-116). Hunters were provided informed consent for participation in the study. The collected sera were stored at -20°C until use.

### Virus-neutralization (VN) test

Heat-inactivated sera (56°C, 30 min) were subjected to serial 2-fold dilutions from 1:5 to 1:160 in DMEM containing 10% FCS; the dilutions were distributed at 50 µl/well to duplicate wells in 96-well culture plates (Sumitomo Bakelite, Inc., Tokyo, Japan). An equal volume of 50 TCID<sub>50</sub> of KMAV diluted in DMEM containing 10% FCS was added to each well, and the plates were incubated for one hour at 37°C. A BHK-21 cell suspension (100 µl/well; 1 × 10<sup>5</sup> cells/ml) then was added to each well, and the plates were incubated at 37°C for 7 days. The cells then were fixed with 10% phosphate-buffered formalin at room temperature for 30 min and stained with crystal violet. Cytopathic effect (CPE) was confirmed visually and the CPE-positive wells were recorded. Titers lower than the 1:10 dilution were judged negative and below the detection limit of the assay. The neutralizing antibody titer was defined as the reciprocal of the highest serum dilution that inhibited the CPE in duplicate wells.

### Immunofluorescence assay (IFA)

Vero cells were infected with KAMV at 0.01 multiplicity of infection (or mock-infected) for 7 days. After washing twice with phosphate-buffered saline (PBS), the cells were resuspended in a small volume of PBS and 10 µl of cells were transferred to each well of a glass micro-slide (Matsunami Glass IND, Osaka, Japan). After fixation in chilled acetone (Fujifilm, Osaka, Japan) at -20°C for 30 min, the cells were incubated with serum samples diluted in PBS containing 0.05% sodium azide and 2% FCS in a moist chamber for 30 min at 37°C. Following three washes with PBS, the cells were incubated with Protein A/G-FITC (BioVision, Mountain View, CA, USA) diluted in PBS containing 0.05% sodium azide and 2% FCS in a moist chamber at 37°C for 30 min. After three washes with PBS, the slides were covered with buffered glycerin and a cover-glass and then observed using a fluorescence microscope (Olympus BX53 Digital Fluorescence Microscope). IFA titers were recorded as the highest serum dilution that showed specific fluorescence. Mock-infected cells were used as a negative control.

### Collection of ticks

The ticks were collected by flagging on vegetation from sites in four prefectures in Japan, including Yamaguchi (N34°10'59.99", E131°28'0.01"), Ehime (N33°50'20.98", E132°45'56.66"), Wakayama (N33°55'0.01", E135°25'0.01"), and Fukushima

(N37°45'0.00", E140°28'0.01). All tick specimens were morphologically identified and pooled by species, stage, sex, and sampling location such that each pool contained fewer than 5 adults, 20 nymphs, or 50 larval ticks. The pooled tick samples were homogenized in 600 µl of PBS using a Multi-beads shocker (Yasui Kikai Co., Osaka, Japan). Each homogenate was transferred to a 1.5-ml tube and centrifuged (1,500 × g, 5 min, 4°C); and the resulting supernatant was passed through a sterile 0.45-µm filter (Costar® Spin-X®, Millipore Sigma, New York, NY, USA). The filtrates were stored at -80°C until use.

#### *Viral RNA detection*

RNA was extracted from tick homogenates using a QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommended protocol. The extracted RNA was reverse transcribed using a One Step RT-PCR Kit (QIAGEN) with a specific primer pairs consisting of forward primer T32-SegL-6.5kF (5'- CTTGAGAGCTCGCTTAGGAGGAGG-3') and reverse primer T32-SegL-6.5kR (5'- AGAGGACTAAGTGCTCCTCATTCA-3'). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the following program: reverse transcription at 50°C for 30 min; denaturation at 95°C for 15 min; 40 cycles of denaturation at 94°C for 0.5 min, annealing at 53°C for 0.5 min, and extension at 72°C for 1 min; and final extension at 72°C for 10 min. The resulting PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet (UV) illumination. The estimation of infection rate from pooled samples was calculated by the maximum likelihood estimation (MLE) method of Chiang and Reeves [12], using the equation where Y is the number of positive pools, X is the number of pools, and m is the pool size.

$$MLE = 1 - \left(1 - \frac{Y}{X}\right)^{1/m}$$

#### *Sequence analysis*

The PCR products were purified by using a MinElute® PCR Purification Kit (QIAGEN) and directly sequenced using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Austin, TX, USA) according to the manufacturer's instructions.

#### *Statistical analysis*

For statistical analysis, McNemar's  $\chi^2$  tests were performed. *P* values of <0.05 were considered statistically significant.

## RESULTS

#### *Serosurveillance of KAMV infection in humans and wild animals in Yamaguchi Prefecture*

A total of 24 human sera and 497 sera of wild animals (including Japanese monkeys (n=59), wild boars (n=171), Sika deer (n=233), Asian black bears (n=7), and nutria (n=27) captured in Yamaguchi from 2013 to 2019 were analyzed by the VN test against KAMV (Table 1). Five human sera (20.8%) possessed VN antibodies, exhibiting VN titers of 1:10 (n=3) and 1:20 (n=2). Among the wild animals, 3.4% of monkeys (2 of 59 sera), 33.9% of wild boars (58 of 171) and 4.7% of Sika deer (11 of 233) in Yamaguchi were positive for VN antibody against KAMV. None of the bear and nutria samples were seropositive (Table 1). Although VN titers in humans and monkeys were 1:20 or less, the highest VN titers in wild boars and deer were 1:160 and 1:80, respectively.

#### *Comparison of seroprevalence in wild boar by sex and body weight*

Seropositivities of wild boars were compared by sex and body weight. The results showed that there was no significant difference in positivity between males (12.3%) and females (20.5%), but seroprevalence in wild boars of 30 kg or less (25%) was less than that in animals over 30 kg (41%) (Table 2).

#### *Comparison of the results between VN test and IFA*

To confirm the results of the VN test, sera of humans (n=24) and wild animals (n=497) in Yamaguchi Prefecture were examined by IFA. All VN-positive sera were positive for IFA using sera diluted to 1:10 or more, but one VN-negative human serum and six VN-negative monkey sera tested positive by IFA. Using IFA, the seropositivity of human sera was 25% (6 of 24 sera). Among

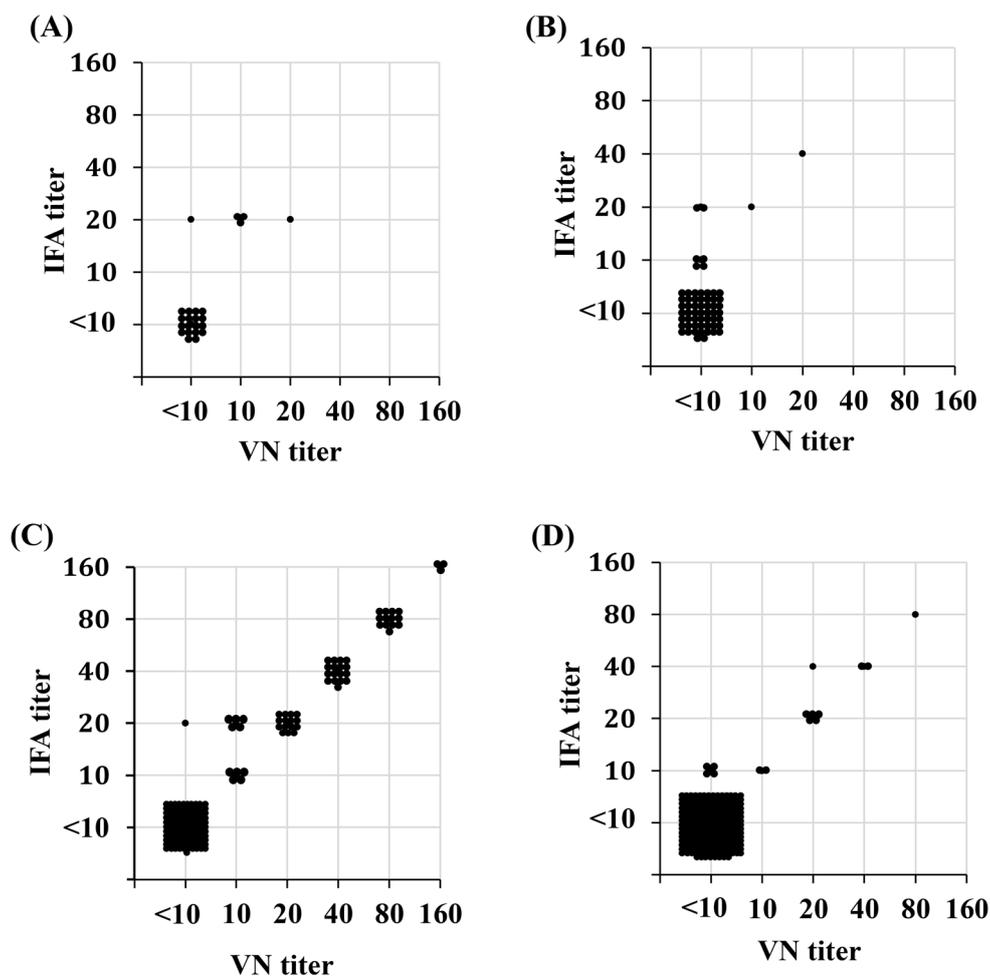
**Table 1.** Virus-neutralization test against Kabuto Mountain virus using sera from mammals in Yamaguchi Prefecture

Species	Scientific name	Year	Total	Virus-neutralization titer						% of positive rate
				<1:10	1:10	1:20	1:40	1:80	1:160	
Human	<i>Homo sapiens</i>	2015	24	19	3	2	0	0	0	20.8
Monkey	<i>Macaca fuscata</i>	2018–2019	59	57	1	1	0	0	0	3.4
Wild boar	<i>Sus scrofa leucomystax</i>	2013–2016	171	113	10	16	17	12	3	33.9
Sika deer	<i>Cervus nippon</i>	2013–2015	233	222	2	6	2	1	0	4.7
Bear	<i>Ursidae</i>	2013–2017	7	7	0	0	0	0	0	0
Nutria	<i>Myocastor coypus</i>	2015–2017	27	27	0	0	0	0	0	0

497 wild animals, 13.6% of monkeys (8 of 59 sera), 35.1% of wild boars (60 of 171) and 6.4% of Sika deer (15 of 233) possessed antibodies against KAMV. The correlation coefficients between the results of the VN test and IFA in human, monkey, wild boar, and Sika deer were 0.5745, 0.7198, 0.9967 and 0.9525, respectively (Fig. 1).

**Table 2.** Comparison of seroprevalence of Kabuto Mountain virus infections in wild boars by sexes and body weights

		No. of examined sera	No. of positive sera	% of positive sera
Sex	Male	67	21	31
	Female	93	35	38
	No information	11	2	18
Body weight (kg)	≤30	55	14	25
	>30	93	38	41
	No information	23	6	26
Total		171	58	33.9



**Fig. 1.** Comparison between results of the virus-neutralization (VN) test and the indirect immunofluorescence assay (IFA) against Kabuto Mountain virus. The correlation between VN test and IFA using human (n=24) (A), monkey (n=59) (B), wild boar (n=171) (C), and Sika deer (n=233) (D) sera collected in Yamaguchi Prefecture was analyzed. The x- and y-axes indicate VN titer and IFA titer, respectively. The correlation coefficient between the VN test and IFA data in humans, monkeys, wild boars, and sika deer were 0.5745, 0.7198, 0.9967, and 0.9525, respectively.

**Table 3.** Detection of Kabuto Mountain virus in ticks by RT-PCR

Prefecture	Species	No. of ticks*	No. of pools	No. of positive pools	Estimated infection rate (%)
Yamaguchi	<i>Haemaphysalis flava</i>	42 (40N, 2F)	4	0	0
	<i>Haemaphysalis formosensis</i>	3 (3M)	1	0	0
	<i>Haemaphysalis hystricis</i>	1 (1N)	1	0	0
	<i>Haemaphysalis longicornis</i>	212 (2L, 198N, 11F, 1M)	17	0	0
	<i>Ixodes turdus</i>	4 (2L, 2N)	2	0	0
	Total	262	25	0	0
Ehime	<i>Amblyomma testidunarium</i>	7 (7N)	2	0	0
	<i>Haemaphysalis flava</i>	5 (5N)	2	0	0
	<i>Haemaphysalis formosensis</i>	10 (7N, 1F, 2M)	4	0	0
	<i>Haemaphysalis hystricis</i>	10 (5N, 3F, 2M)	6	0	0
	<i>Haemaphysalis longicornis</i>	24 (24N)	5	0	0
	<i>Ixodes ovatus</i>	1 (1M)	1	0	0
Total	57	20	0	0	
Wakayama	<i>Amblyomma testidunarium</i>	53 (52N, 1M)	21	0	0
	<i>Dermacentor taiwanensis</i>	5 (2F, 3M)	5	0	0
	<i>Haemaphysalis cornigera</i>	36 (28N, 2F, 6M)	14	0	0
	<i>Haemaphysalis flava</i>	171 (113N, 19F, 39M)	27	0	0
	<i>Haemaphysalis formosensis</i>	76 (53N, 8F, 15M)	17	1 (Nymph)	1
	<i>Haemaphysalis hystricis</i>	15 (8F, 7M)	9	0	0
	<i>Haemaphysalis kitaokaii</i>	39 (21F, 18M)	17	0	0
	<i>Haemaphysalis longicornis</i>	169 (148N, 5F, 16M)	16	0	0
	<i>Haemaphysalis megaspinoza</i>	106 (87N, 10F, 9M)	13	0	0
	<i>Ixodes ovatus</i>	7 (3F, 4M)	7	0	0
	<i>Ixodes turdus</i>	2 (2N)	1	0	0
Total	679	147	1	0.1	
Fukushima	<i>Haemaphysalis flava</i>	21 (20N, 1M)	5	0	0
	<i>Haemaphysalis longicornis</i>	1 (1F)	1	0	0
	<i>Haemaphysalis megaspinoza</i>	6 (1L, 4N, 1F)	4	0	0
	<i>Ixodes ovatus</i>	6 (3F, 3M)	6	0	0
	<i>Ixodes persulcatus</i>	10 (2N, 5M, 3F)	10	0	0
Total	44	26	0	0	

\*The number and letter in the parenthesis indicate the number of ticks and their stages, respectively. L, N, F and M stand for larvae, nymph, female and male, respectively.

### Detection of KAMV from collected ticks

A total of 1042 larval, nymphal, and adult ticks collected by flagging on vegetation in Yamaguchi, Ehime, Wakayama, and Fukushima Prefectures were analyzed for KAMV. RNA was extracted from homogenates of animals pooled by species (and other parameters) and analyzed by RT-PCR. Only one pool of *H. formosensis* nymphs collected in Wakayama on May 13, 2015, was positive for KAMV RNA (Table 3). When compared with sequences in the National Center for Biotechnology Information (NCBI) database, the obtained sequence (398 bp) was 100% identical with that of KAMV strain T32 (LC153711.1).

## DISCUSSION

KAMV, a new member of the tick-borne phleboviruses, was isolated from *Haemaphysalis flava* in Hyogo Prefecture; this virus was shown to replicate in many mammalian cell lines and to cause lethal infection in suckling mice after intracerebral challenge [6]. Therefore, it was hypothesized that this virus may be a potential tick-borne zoonotic pathogen. Therefore, serological survey of the prevalence of KAMV infection in humans and wild animals in Yamaguchi Prefecture, in the western part of Japan, was conducted in the present study.

By the VN test, wild boars showed the highest positive rate (33.9%), but the positivities of monkeys and Sika deer were relatively low, 3.4% and 4.7%, respectively. Furthermore, all bears and nutria were seronegative (Table 1). All VN-positive sera were positive by IFA using infected cells, confirming that VN-positive animals indeed were infected with KAMV or a KAMV-like virus. In Japan, novel uukuviruses, Toyo virus and Okutama tick virus were recently discovered [19, 20]. In addition, fifteen wild boars possessed relatively high VN titers of 1:80 or more. In Sika deer, the frequency of seropositivity was low, but three deer possessed high titers of 1:40 or more. In monkeys, the seropositivity and VN titers were very low, but six VN-negative monkeys were positive by IFA, indicating that the VN test was less sensitive than IFA in monkey. The partial incompatibility between the results of VN test and IFA

may be explained by the general fact that the VN test only detects antibody that can block virus replication [34], while IFA detects all antibodies reactive with the fixed antigens in virus-infected cells [32]. Our serosurveillance showed that some (but not all) species of wild animals are naturally infected with KAMV (or a related virus), and that wild boars may be a natural host of KAMV. Interestingly, five and six hunters were seropositive as assessed by VN tests and IFA, respectively. All 24 hunters were negative (by VN test) for SFTS virus belonging to the Family *Phenuiviridae* (data not shown). Therefore, at least five hunters appeared to be infected with KAMV or a related viruses.

KAMV strain T32 was first isolated from a pooled sample of *H. flava* ticks comprising two larvae, 20 nymphs, and one adult male collected by flagging from vegetation in Hyogo [6]. Other strains of KAMV also were isolated from blood-feeding *H. flava* recovered from wild boars collected in Hyogo [6], and KAMV strain 17ISK-T11 was isolated from a pool of 26 nymphal *H. flava* ticks collected in Ishikawa Prefecture [19]. In the present study, KAMV was detected in a nymphal pool of *H. formosensis* ticks collected from vegetation in Wakayama by flagging (Table 3). Detection of KAMV previously was reported only from *H. flava* [6, 19]. *H. flava* must be a major vector tick for KAMV, but *H. formosensis* tick should be considered a candidate vector, given that RNA-positive ticks were collected from vegetation by flagging. Since KAMVs were detected from ticks in Ishikawa, Hyogo and Wakayama Prefectures and the positive sera were detected in human and wild animal in Yamaguchi Prefecture (Fig. 2), KAMV has been circulating in the western part of Japan.

The *H. flava* tick is distributed widely in Japan [9, 43], mainly in woodlands in urban/suburban areas [10]. In addition, *H. flava* ticks are active in all seasons [11], exhibiting a very broad host range that includes birds, small rodents, large animals, and humans [15, 17]. *H. formosensis* is distributed widely in tropical and subtropical areas of Japan [16] and seems to be the predominant species associated with wild boar [10]. This evidence suggests that KAMV transmitted by these two ticks might infect many mammalian species in Japan.

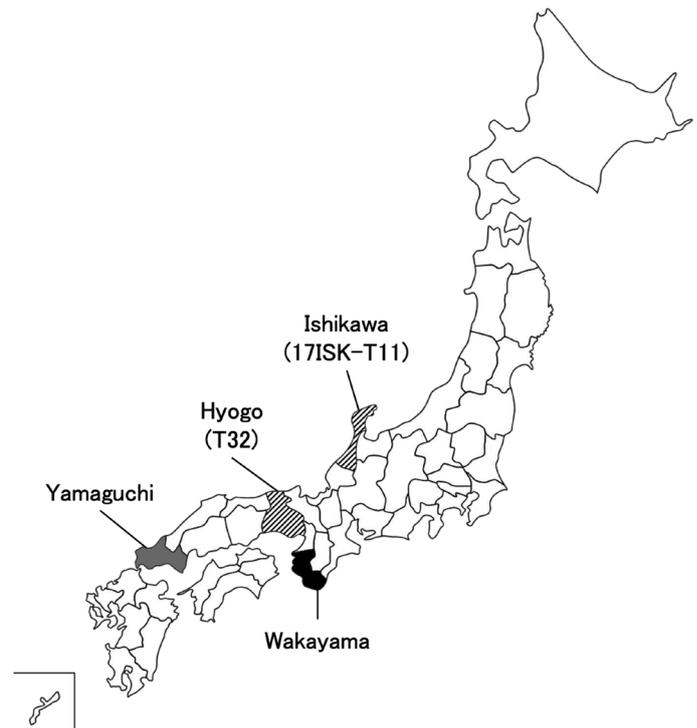
In conclusion, KAMV or a KAMV-like virus is circulating in Japan among mammals, including humans, and *Haemophysalis* ticks. Further investigation will be required to clarify whether KAMV is a human pathogen.

**CONFLICT OF INTEREST.** The authors declare no conflict of interest.

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**Fig. 2.** A map of Kabuto Mountain virus-detected areas. Black shows Wakayama Prefecture in which KAMV gene was detected from ticks. Grey shows Yamaguchi Prefecture in which serosurveillance was conducted in this study. Shaded areas indicate the prefectures in which KAMVs were isolated in the previous studies [6, 19]. Names of KAMV isolates are shown in the parenthesis.

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