The widely distributed hard tick, *Haemaphysalis longicornis*, can retain canine parvovirus, but not be infected in laboratory condition

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ABSTRACT. Ticks are known to transmit various pathogens, radically threatening humans and animals. Despite the close contact between ticks and viruses, our understanding on their interaction and biology is still lacking. The aim of this study was to experimentally assess the interaction between canine parvovirus (CPV) and a widely distributed hard tick, *Haemaphysalis longicornis*, in laboratory condition. After inoculation of CPV into the hemocoel of the ticks, polymerase chain reaction assay revealed that CPV persisted in inoculated unfed adult female ticks for 28 days. Canine parvovirus was recovered from the inoculated ticks using a cell culture, indicating that the virus retained intact in the ticks after inoculation, but significant positive reaction indicating virus infection was not detected in the tick organs by immunofluorescence antibody test using a monoclonal antibody. In the case of ticks inoculated with feline leukemia virus, the virus had shorter persistence in the ticks compared to CPV. These findings provide significant important information on the characteristic interaction of tick with non-tick-borne virus.

KEY WORDS: canine parvovirus, experimental inoculation, feline leukemia virus, ixodid tick, vector

Ticks are widely notorious ectoparasites, considered second to mosquitoes on transmitting pathogens, including bacteria, protozoa, helminths and viruses, to humans and animals. The unique property of ticks that differentiates them from other arthropod vectors is their capability of long-term co-evolution with pathogens, which contributes to the influence of ticks on tick-borne pathogens' infection, replication, persistence and transmission [12, 35].

Tick-borne viruses can be divided into two groups: arboviruses and non-arboviruses [12], which are either replicable or non-replicable in ticks. Tick-borne arboviruses are currently found in six or seven different taxonomic virus families [23] and are known to be in close contact with ticks in terms of long-term co-evolution. Meanwhile, non-arboviruses are also known to interact with ticks profoundly, because the viruses could persist transstadially, intrastadially and transovarially in ticks [25]. This variability of association between ticks and viruses makes our understanding on interaction between pathogens and their vectors more controversial, and thus, further studies on biology of viruses in ticks as well as on potential roles of ticks under infection are needed.

Canine parvovirus (CPV) belongs to the genus *Parvovirus* within the family *Parvoviridae* and has spread worldwide, causing severe enteritis and myocarditis in dogs, since its first emergence in the mid-1970s [13, 37]. Canine parvovirus binds to the canine transferrin receptor for cell entry [14, 15, 32] and replicates in the cell nuclei of rapidly dividing cells. The general route for infection and the distribution of CPV in its hosts in nature are described as follows; after infection through the oronasal route, CPV first localizes in the tonsil, retropharyngeal and mesenteric lymph nodes. Subsequently, viremia occurs, which is associated with the isolation from a variety of organs and tissues, and then finally, CPV is excreted through the feces to the environment [27]. Recently, it has been also suggested that insect vectors, such as flies, play a role in the virus transmission in terms of indirect exposure to susceptible animals, which reflects the ability of CPV to persist in the environment with stable [4].

Although there has been no empirical study focusing on the interaction between ticks and CPV, these described characteristics of ticks and CPV can raise the possibility of their interaction both under natural and experimental conditions, with some other properties of them as follows. With respect to CPV, CPV causes viremia, which may enable blood-feeding arthropods to take up CPV in their bodies. In addition, canine parvoviral disease can be produced experimentally by injection of viruses intravenously [27, 34], theoretically supporting further possibility of CPV transmission by hematophagous arthropods. As for ticks, it was demonstrated that vertebrate transferrin is taken into the midgut and the ovary [30], which leads to a hypothesis that ticks may have the vertebrate transferrin receptor needed when CPV enters and infects host cells. In this study, we performed detailed systematic experiments on the features of CPV in the hard tick *Haemaphysalis longicornis* (*H. longicornis*) after inoculation into the hemocoel of the ticks to further clarify the interaction between tick and non-tick-borne virus. This hard tick species is distributed mainly in East Asia and Australia

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and is the most widespread tick species on wild and domestic animals in Japan [9, 19]. In addition, we performed a parallel experiment with feline leukemia virus (FeLV), a well-known viremia-causing pathogen in cats [39], using the same inoculation method to highlight the distinctive features of CPV in the ticks. This is the first report demonstrating the interaction of CPV and ixodid tick.

MATERIALS AND METHODS

Ticks and animals: The pathogen-free parthenogenetic *H. longicornis* female ticks (Okayama strain), which had been maintained by feeding on the ears of Japanese white rabbits (Kyudo, Tosu, Japan) [9] at our laboratory, were used throughout this study. All ticks had been stored at 15°C and 85% relative humidity before they were used for experiments or attached to rabbits for blood feeding and subsequent molting, oviposition and hatching. Rabbit care was approved by the Animal Care and Use Committee of Kagoshima University (Approval number: VM13007).

Viruses: The CPV strain Cp49 (CPV type 2) [3] grown in the Crandell–Rees feline kidney cell culture was stored at −80°C until used. The infective titer of the virus stock solution determined by forming of intranuclear inclusion bodies in *felis catus* whole fetus (fcwf-4) cells was $10^{5.5}$ TCID$_{50}$/m*l*.

The FeLV strain F422 (subgroup A) [33] was supplied by Kyoritsu Seiyaku Corporation (Tokyo, Japan) and stored at −80°C until used. The viral titer of the virus stock solution determined by forcus forming assay using C81 cells and feline embryonic fibroblast cells [18], and by real-time polymerase chain reaction was $1.6 \times 10^{5.0}$ forcus-forming units/m*l* and $10^{11.4}$ copies/m*l*, respectively.

Capillary tube inoculation: Unfed adult ticks were used after at least one month had passed since their last engorgement on rabbits. Also, engorged nymphs and adults were used after 1 day had passed since their last engorgement. All ticks were placed at room temperature (RT) for appropriate time before use. Subsequently, the ticks were immobilized with their ventral side up, using a double-sided adhesive tape on glass slides (Matsunami Glass, Osaka, Japan).

Capillary tubes (Drummond Scientific Co., Broomall, PA, U.S.A.) were prepared by cutting to a length of 60–70 mm by a capillary pipette pullar (NARISHIGE, Tokyo, Japan) according to the manufacturer′s protocol, and then, the end of each tube was further cut so that the compatible internal diameter was prepared with individual ticks. After the preparation of tubes and ticks, the thawed out virus stock solution was immediately introduced into the tubes and inoculated through the fourth coxae into the hemocoel of the ticks using a microinjector (NARISHIGE) under a dissecting microscope (Olympus, Tokyo, Japan), as previously described [2]. Sterilized high-purity water (Merck Millipore, Billerica, MA, U.S.A.) was also inoculated into negative control ticks for verification of test accuracy. The ticks were then examined for distended abdomens as an indicator for whether the ticks successfully received the inoculum.

Experimental design and sample collection: The numbers of ticks used in each experiment are indicated in Table 1.

Table 1. Numbers of ticks used for the inoculation tests

Experimet no.	Inoculum	Unfed adults	Engorged adults	Engorged nymphs
1[a]	CPV	33		
	Sterilized water	2		
2[b]	CPV	3		
	Sterilized water	3		
3[c]	CPV	15 (15)[f]		
	Sterilized water	15 (20)[g]		
4[d]	CPV		12	23
	Sterilized water		8	14
5[e]	FeLV	48		
	Sterilized water	3		

a) Assessing the persistence and distribution of CPV in the ticks. All ticks were inoculated with 0.5 μ*l*, respectively. b) CPV recovery. All ticks were inoculated with 0.5 μ*l*, respectively. c) Assessing the infection and replication of CPV in the tick organs. All ticks were inoculated with 0.5 μ*l*, respectively. d) Assessing transstadial and transovarial transmission of CPV in the ticks. Engorged adults and nymphs were inoculated with 2.0 μ*l* and 0.5 μ*l*, respectively. e) Assessing the persistence of FeLV in the ticks. All ticks were inoculated with 0.5 μ*l*, respectively. f, g) In parenthesises, the numbers of ticks used for assessment in unfed ticks are indicated.

The viral inocula were not diluted, and all experiments were conducted with the same titers of the stock solutions. The ticks after inoculation were kept at 25°C and 85% relative humidity to keep their physiological processes until used, to allow engorged adults to lay eggs, or to allow eggs to hatch. Ticks died or exhibiting abnormal behavior after inoculation were removed from the study after counting of the numbers, as this might suggest that the injection had caused excessive trauma, and thus, only active ticks were applied for the subsequent experiments. All experiments, except for detection of CPV from the tick samples by polymerase chain reaction (PCR), were performed at least twice, and the shown data are representatives of each two independent experiment.

Whole bodies and eggs, collected on each appropriate day post-inoculation (dpi) in each experiment, the organs obtained by dissecting each tick under the microscope and the hemocytes collected from the hemolymph samples as previously described [2] were immediately washed with sterilized phosphate buffered saline (PBS) thoroughly after each collection and stored at −30°C until used.

DNA extraction and PCR for CPV: Homogenized samples were suspended in an extraction buffer [100 mM Tris–HCl (pH 8.0), 0.5% SDS, 100 mM NaCl and 10 mM EDTA], and after adding proteinase K (10 mg/m*l*) (KANTO CHEMICAL, Tokyo, Japan), samples were incubated overnight at 55°C. After removal of proteins using Phenol:Chloroform:Isoamyl Alcohol (Sigma-Aldrich, St. Louis, MO, U.S.A.), ethanol precipitation was performed to collect DNA. All DNA samples were purified with an RNaseA solution (4 mg/m*l*) (Promega, Madison, WI, U.S.A.).

PCR was carried out using AmpliTaq Gold® 360 Master Mix (Life Technologies, Carlsbad, CA, U.S.A.) and the primer set of a forward primer 1 (5′-GTACATTTAAATAT-GCCAGA-3′) and a reverse primer 52 (5′-ATTAATGTTC-

TATCCCATTG-3′), which is able to amplify a 452-bp fragment of the gene encoding for the capsid VP2 protein [29]. To confirm the presence of DNA in the samples, control amplification of the *H. longicornis* ribosomal protein *L23* gene [10] was also performed using the primer set as follows; a forward primer (5′-AGATCCGCACGTCGGT-TAAG-3′) and a reverse primer (5′-TTGTTAGCCACATC-CAACGC-3′). The preparation of a reaction mixture and the DNA amplification followed the manufacturer′s recommendation. PCR products were detected by electrophoresis through a 1.5% agarose gel and visualization under UV light after ethidium bromide staining.

RNA extraction and reverse-transcriptase PCR (RT-PCR) for FeLV: Homogenized samples were added with TRI® reagent (Sigma-Aldrich). Complementary DNA synthesis was performed with ReverTra Ace-α-® (TOYOBO, Osaka, Japan) following the manufacturer′s protocol using 2.0 μg of total RNA. Subsequently, 1.0 μl of an RT reaction mixture was added to 9.0 μl of the PCR reaction mixture. The preparation of a PCR mixture and the DNA amplification were performed with the primer set of a forward primer, FeLV_standard_f (5′-CTACCCCAAAATTTAGC-CAGCTACT-3′) and a reverse primer, FeLV_standard_r (5′-AAGACCCCCGAACTAGGTCTTC-3′) [36], which were designed from the unique region of the long terminal repeat (U3-LTR), using Hot Start DNA Pol (Jena Bioscience, Jena, Germany), following the manufacturer′s recommendation. The efficiency of RNA extraction, subsequent reverse transcription and DNA amplification from the samples was confirmed using the specific primer set of a forward primer (5′-CCAACAGGGAGAAGATGACG-3′) and a reverse primer (5′-ACAGGTCCTTACGGATGTCC-3′) for *H. longicornis actin* gene (*Hlactin*) [5].

Virus recovery: The centrifugally separated supernatant of a pooled sample consisting of 3 homogenized tick samples in 0.25 ml of sterilized PBS was filtered through 0.45 μm pore size filter (Merck Millipore) and inoculated into freshly trypsinized fcwf-4 cell suspension in RPMI-1640 medium (Sigma-Aldrich) with 10% fetal bovine serum, 2.0 μM L-Glutamin (Life Technologies) and antibiotics. The cell culture was incubated, and the supernatant was examined for CPV by PCR. In addition, the fcwf-4 cells were stained with May-Grüenwald Giemsa for detection of intranuclear inclusion bodies by CPV.

Indirect immunofluorescence antibody test (IFAT): The salivary glands, midgut, fat body and the ovary dissected from the ticks were fixed overnight in 4% paraformaldehyde in PBS containing 0.1% glutaraldehyde. After a series of washings with different concentrations of sucrose in PBS solution, the organs were embedded in Tissue-Tek® O.C.T compound (Sakura Finetek Japan, Tokyo, Japan) and frozen at −80°C. The frozen sections were cut 4.0–5.0 μm thick using a cryostat (Leica CM 3050; Leica Microsystems, Wetzlar, Germany) and mounted on MAS-coated glass slides (Matsunami Glass). After air-drying at RT and subsequent treatment in acetone for 20 min, the sections were washed with PBS followed by blocking with 5% skim milk in PBS at RT for 1 hr. The preparation of the hemocytes from unfed

adults was followed by the method previously described [2]. The CPV specific monoclonal antibody (MAb) 2D9, which is considered to be directed at epitopes on the virus capsid protein [28], diluted with PBS in 1:200, and goat anti-mouse IgG conjugated with Alexa Fluor 488 (Life Technologies; 1:400 dilution with PBS) were applied as the first and second antibodies, respectively. Both antibodies were incubated at RT for 1 hr. The sections were mounted in Vectashield® with 4′,6-diamino-2-phenylindole mounting medium (DAPI) (Vector Laboratories, Burlingame, CA, U.S.A.), observed under a fluorescence microscope (IX71; Olympus) mounted with a DP71 camera and then processed using DP Controller software (Olympus).

RESULTS

Detection of CPV gene in the whole bodies, organs and the hemocytes of inoculated ticks: During the observation period, 2 and 1 of 33 CPV-inoculated ticks died on 2 and 5 dpi, respectively. On 1 through 28 dpi, CPV gene was detected from all the CPV-inoculated unfed adults (Fig. 1A). With respect to individual organs, the salivary glands, midgut, fat body, ovary, synganglion and the Malpighian tubules were the primary locations of CPV detection (Fig. 1B). In the hemocytes, PCR showed the persistence of CPV through 28 days after inoculation (Fig. 1C). It should be also noted that the hemolymph, in which CPV was inoculated directly, stably retained CPV for as 28 days. Detection of *L23* gene from all samples confirmed that DNA was extracted and amplified precisely (data not shown). However, it has to be also considered that some of the bands of organ samples, especially of the third sample on 21 dpi, were faint, compared to those of whole tick samples. Those stronger band intensity of whole samples than organ samples seemed to be also derived from other tissues or organs, especially the cuticles as well as the hemolymph, where the duration and the intensity of positive CPV bands matched the ones of shown organs from our preliminary PCR experiment (data not shown), indicating that other favorable spots for CPV existed. Taken together, after inoculation into the hemocoel of unfed adult ticks, CPV gene could persist at least for 28 days.

Recovery of CPV from inoculated ticks: To confirm whether CPV can maintain viability during its existence in the ticks, virus recovery using a cell culture was performed from the homogenized pooled sample of three individual adults on 28 dpi. At the second passage of the cell culture, CPV specific intranuclear inclusion bodies were detected in the cells inoculated with the homogenized solution of CPV-inoculated ticks (Fig. 2A). Positive isolation of CPV from the supernatant of the cell culture was further confirmed by PCR (Fig. 2B).

Detection of CPV antigens from the tick organs by IFAT: To show the infection and subsequent replication of CPV in the ticks, some organs of partially-fed adults inoculated with CPV were sectioned and stained with the CPV specific MAb. However, no significant positive reaction was observed in all tested organs (Fig. 3). In addition to the above experiment using partially-fed adults, we also attempted to
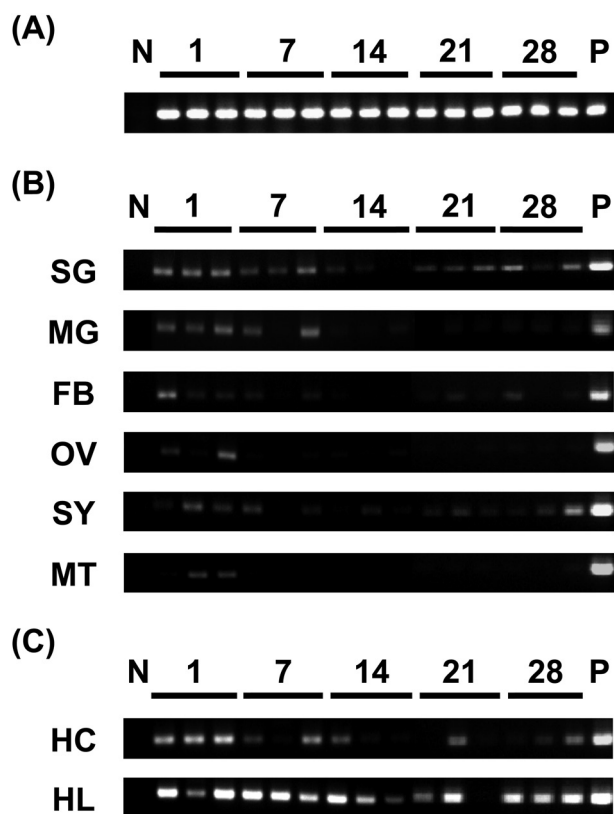
(A)

(B)

(C)

Fig. 1.   Detection of CPV gene from tick samples of CPV-inoculated unfed adult ticks. (A) Detection of amplified PCR products of CPV from CPV-inoculated unfed adult ticks, (B) from different organs, and (C) from the hemocytes and the hemolymph of CPV-inoculated unfed adult ticks. The numbers indicate each dpi, and each dpi consists of 3 individual tick samples. N, negative control ticks; P, undiluted CPV stock solution; SG, salivary glands; MG, midgut; FB, fat body; OV, ovary; SY, synganglion; MT, Malpighian tubules; HC, hemocytes; HL, hemolymph.

(A)

(B)

Fig. 2.   Recovery of CPV from CPV-inoculated unfed adult ticks on 28 dpi was confirmed by fcwf-4 cells (A) and by PCR (B). (A) Intranuclear inclusion bodies (arrowheads) were detected at the second passage of the cell culture. Scale bars indicate 50 μm. (B) Detection of CPV gene from supernatants of the cell culture by PCR. The numbers indicate each passage level of cell culturing. N, cells inoculated with the solution of a pooled sample of 3 homogenized negative control ticks; P, cells inoculated with undiluted CPV stock solution.

Fig. 3.   Detection of CPV antigens from tick organs of 4-day fed adult ticks through IFAT. The sections were incubated with MAb 2D9 followed by goat anti-mouse IgG conjugated with Alexa Fluor 488 (Alexa 488 sections), and nuclei were visualized using DAPI (DAPI sections). Each picture of the organs shows the representative section of the observed. Scale bars indicate 100 μm.

explore the same organs, including the hemocytes, of unfed adults inoculated with CPV, on 14 dpi. As shown in partially-fed adults, no positive signal was again detected from all the samples by the IFAT (data not shown).

Demonstration of transstadial and transovarial transmission by PCR: During the experimental period, 3 and 2 nymphs, inoculated with CPV and with high-purity water as controls, respectively, died on day 30 post-engorgement. Among the dead nymphs, only 1 nymph of each group underwent molting. Further, 3 of 12 CPV-inoculated engorged adults and 2 of 8 control adults did not lay enough eggs for analysis. PCR revealed that CPV gene was detected from all tick samples after the inoculation into the engorged nymphs (Fig. 4A). Tick samples that were collected on days 20 and 30 post-engorgement molted and reached adulthood between days 10 and 20 post-engorgement.

Meanwhile, all the three pooled samples each consisting of 150 eggs, derived from the individual CPV-inoculated engorged adults, and all the three pooled samples of larvae
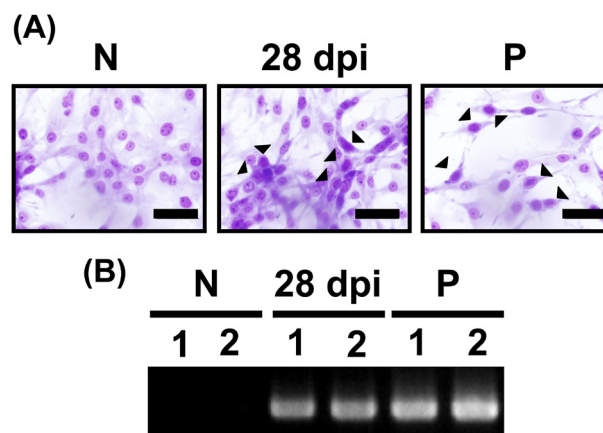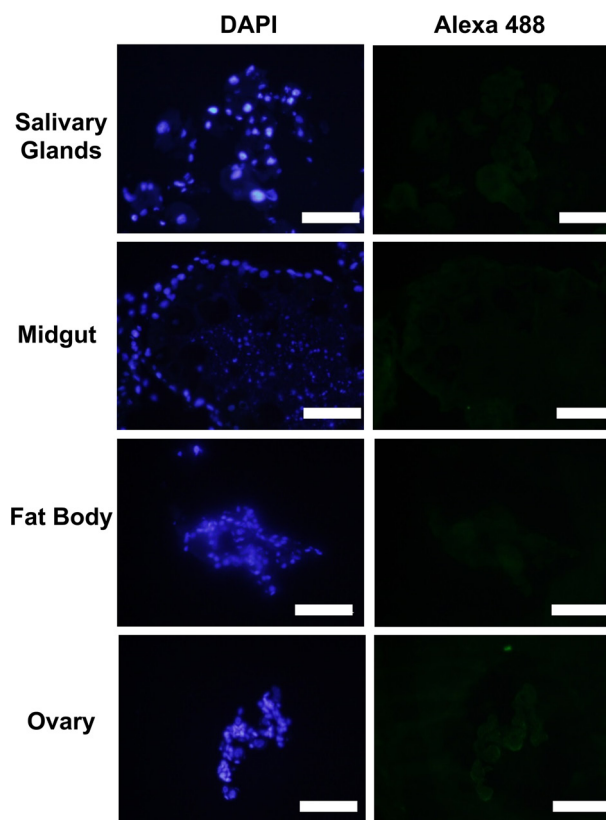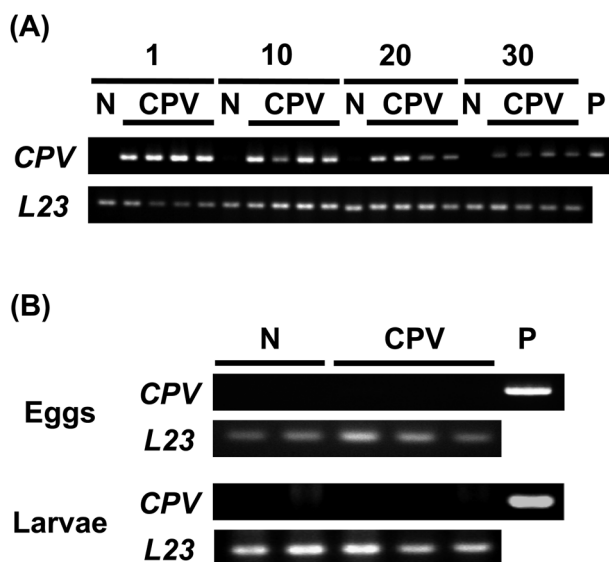
(A)

(B)

Fig. 4. Assessment on transstadial and transovarial transmission of CPV in the ticks by PCR. (A) Detection of amplified PCR products from engorged nymphs and newly emerged adult ticks after inoculation with CPV on day 1 post-their last engorgement. The numbers indicate each day post-engorgement, and each day consists of 4 CPV-inoculated and 1 control ticks. Molting was completed between days 10 and 20 post-engorgement. (B) Detection of amplified PCR products from the eggs and larvae originated from CPV-inoculated engorged adult ticks. Three tested and 2 control samples are shown as representatives. The tick *L23* gene was amplified as a loading control. N, samples obtained from negative control ticks; CPV, samples obtained from CPV-inoculated ticks; P, undiluted CPV stock solution.
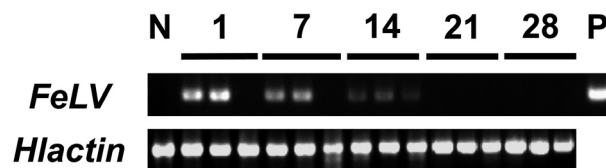

Fig. 5. Detection of FeLV gene from the FeLV-inoculated unfed adult ticks by RT-PCR. The tick *actin* mRNA was detected and amplified as a loading control. The numbers indicate each dpi, and each dpi consists of 3 individual tick samples. N, negative control ticks; P, undiluted FeLV stock solution.

each originated from the adults, did not show any specific amplification of CPV gene (Fig. 4B). Detection of CPV from other three engorged adults collected immediately after inoculation confirmed that the inoculation had been performed properly (data not shown).

Detection of FeLV from inoculated ticks: During the course of sample collection, 3 ticks inoculated with FeLV died on 29 dpi. RT-PCR targeting to the region of U3-LTR of FeLV amplified the indicated region from whole bodies of FeLV-inoculated unfed adults on 1, 7 and 14 dpi, but not on 21 and 28 dpi (Fig. 5).

DISCUSSION

Tick-borne diseases cause significant damage to vertebrates, being considered of great importance in economies and in public health in many countries [19]. Thus, besides controlling ticks and tick-borne diseases by using chemical acaricides and vaccines, now presented with several challenges, studying the biology of tick vector-pathogen interactions, including the determination of vectors of *de novo* pathogens, is useful for prophylaxis [26]. Experimental inoculation of pathogens into ticks by needles or capillary tubes has been rather widely performed [8, 17, 22], and some studies succeeded in indicating pathogenesis and biology of

hemocoel-inoculated ticks resemble those of ticks introduced via normal feeding [7, 20, 24]. Thus, intra-hemocoel inoculation method as we performed in the present study is a powerful tool for validation of interaction between pathogens and ticks, especially for a part of which is currently difficult through feeding of infected animals or artificial feeding techniques [11]. Indeed, in the present study using capillary tubes for inoculation into laboratory-reared pathogen-free ticks, available results were acquired with only minimal mortality of ticks for assessing the features of a non-tick-borne pathogen in the ticks.

CPV is a pathogen that causes life-threatening diseases in dogs throughout the world. Although it is known that viruses belonging to *Densovirinae*, another subfamily of *Parvoviridae*, can infect arthropods, such as insects, still, there has been few reports that CPV, which belongs to the other subfamily of *Parvoviridae*, infects or interacts with arthropods. Here, PCR assay provided clear evidence of the persistent existence of CPV in *H. longicornis* ticks and of transstadial transmission from nymph to adult stages. In addition, attempts to recover CPV in a cell culture from the inoculated ticks showed that CPV did maintain its viability in the ticks. However, it was also revealed that transovarial transmission of this virus might not occur in the ticks. Meanwhile, the evidence of infection and replication of CPV in the ticks was not obtained at the viral protein level by IFAT. To our knowledge, this is the first report demonstrating the features of CPV in an arthropod vector in detail.

Although the present study was performed under artificial condition, the persistent existence and infectivity of CPV for at least 28 days in the ticks suggest the possibility that there might be some kind of interaction between CPV and tick tissues. This possibility is reinforced by our comparable RT-PCR result of detecting another non-tick-borne virus, FeLV, from the inoculated ticks by the same method for CPV inoculation, which showed that FeLV disappeared from 21 dpi. The feature of FeLV in the ticks may agree with the previous reports on human immunodeficiency virus and bovine leukosis virus of *Retroviridae* within hematophagous arthropods, showing short existence and infectivity, thus further suggesting that biological and mechanical transmission, as well as transstadial transmission, of these viruses is unlikely [16, 31]. This difference between the two viruses on stable existence in the ticks seems to be explained by the difference of stability of the two viruses in nature, in other

words, that of a non-enveloped DNA virus and an enveloped RNA virus. However, some other enveloped RNA viruses, which have been also recognized to belong to non-tick-borne virus families, were isolated from wild ticks [23]. Additionally, bluetongue virus, which is an RNA arbovirus but not a tick-borne virus, was detected in ixodid ticks for 21 days in experimental condition, leading to an appropriate suggestion that the virus can interact with ticks [6]. These reports are very suggestive, because even vulnerable non-tick-borne RNA viruses can interact with both wild and laboratory-reared ticks, thus leading to highlight the difference between CPV and FeLV. Hence, our results provide further recognition on the characteristics of the two different viruses in tick.

Interestingly, our study also revealed that CPV could spread into various organs soon after the intra-hemocoel inoculation, despite the variability of its favorable existence spots according to the individual inoculated ticks. The general favorable spot of CPV appears to be the hemolymph, since the PCR bands for CPV gene were clearly detected compared to other organs. Nevertheless, persistent detection of CPV gene in the salivary gland samples suggests the possibility of transmission of CPV to new hosts, raising the need for additional investigations on the tick ability of transmission.

Our previous study showed the incorporation of vertebrate transferrin into the oocytes of the ovary in *H. longicornis* ticks [30]. Therefore, it could be postulated before the present experiment that the oocytes of the ticks might express rather a primitive, no species-specific transferrin receptor to utilize vertebrates' ferroprotein. Consequently, we assumed that CPV inoculated into the ticks could infect the oocytes, and thus resulting in transovarial transmission, though the optimized functional receptors for CPV infection have not been identified. As opposed to our expectation, we could not gain the positive reaction against the viral capsid in the tick cells, even in partially fed ticks, which contain active dividing cells compared to unfed ticks [1]. Further, we could not detect any CPV band from the eggs and larvae derived from CPV-inoculated adults. These negative results may suggest the lack of any receptor for efficient infection of CPV [14, 15, 32], and subsequent replication is not likely to occur in the ticks.

In conclusion, the present study showed a long persistence of CPV in the ticks by inoculation into the hemocoel. However, the infection routes of African swine fever virus and Japanese encephalitis virus were shown to have crucial effect on virus persistence and/or infection in ticks [21, 38], and thus, further studies are necessary to conclude, if CPV infects ticks in the other infection route. A potential of ticks as vectors should be considered for a virus species that is generally regarded as a non-vector-transmitted pathogen. Our study may lead to a major trend for future research on interaction between ticks and viral pathogens.

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