Studies on the survival dynamics of tick-borne viruses in the hard tick *Haemaphysalis longicornis* and the effects of tick antimicrobial peptides on viral replication

フタトゲチマダニにおけるダニ媒介性ウイルスの媒介能とマダニ由来抗微生物ペプチドのウイルス増殖に与える影響

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ABBREVIATIONS

AMPs: antimicrobial peptides
BHK: baby hamster kidney
BSL2, 3: biosafety level 2 or 3
cDNA: complementary DNA
CCHFV: Crimean-Congo hemorrhagic fever virus
DAI: days after infestation
DAC: days after challenge
DPI: days post-injection
DMEM: Dulbecco's modified Eagle's medium
EMEM: Eagle's minimum essential medium
FFA: focus formation assay
FFU: foci-forming-unit
FL: full length
dsRNA: double-stranded RNA
EST: expressed sequenced tag(s)
FBS: fetal bovine serum
HPLC: High Performance Liquid Chromatograph
IFA: immunofluorescence assay
IFAT: indirect immunofluorescent antibody test
IgG: immunoglobulin G
IMD: immune deficiency
JAK/STAT: Janus kinase-signal transducers and activators of transcription
Luc: firefly luciferase gene
LGTV: Langat virus
MOI: multiplicity of infection
MEM: minimum essential medium
NC: negative control
PAMPs: pathogen-associated molecular patterns
PBS: phosphate-buffered solution
PC: positive control
qPCR: quantitative polymerase chain reaction
RF%: percentage of foci reduction
RNAi: RNA interference
RT-PCR: reverse transcription polymerase chain reaction

SFTSV: severe fever with thrombocytopenia syndrome virus
TCID50: 50% tissue culture infectious dose
TAE: Tris-Acetate EDTA buffer
TBEV: tick-borne encephalitis virus
TBFVs: tick-borne flaviviruses
TBVs: tick-borne viruses
THOV: Thogoto virus

GENERAL INTRODUCTION

Ticks are important vectors of disease-causing microorganisms, rickettsiae, spirochaetes, protozoa and viruses affecting humans, livestock, wild and companion animals [1]. Among the important pathogens that ticks transmit, viruses form a major constituency of the transmitted pathogens and remain to be a big threat to both human and animal populations as they can produce diseases with high morbidity and mortality [2, 3].

Tick-borne viruses (TBVs) comprise a wide range of viruses classified into six virus families and these include *Asfarviridae*, *Bunyaviridae*, *Flaviviridae*, *Orthomyxoviridae*, *Rhabdoviridae*, and *Reoviridae* [4]. Among these viral families, *Bunyaviridae* and *Flaviviridae* are considered to have the most important TBVs of public health importance, including tick-borne encephalitis virus (TBEV) and Crimean-Congo hemorrhagic fever virus (CCHFV) which are known to cause severe clinical symptoms in humans [4 – 6].

A tabular summary of select virus families transmitted by ticks of medical and veterinary importance is shown in the next page.

Table 1. Select tick-borne viruses listed by virus family of medical or veterinary importance

Family	Representative virus	Vectors[a]	Distribution	Disease[b]
Asfarviridae	African Swine Fever	*Or. moubata*, *Or. erraticus*	Africa	Fever (pigs)
Bunyaviridae	CCHFV	*Hy. marginatum* *De. marginatus*	Africa, Asia, Southern Europe	Hemor. fever (humans)
Flaviviridae	TBEV	*Ix. ricinus,* *Ix. persulcatus*	Europe, Northern Asia	Encepha. (humans)
Orthomyxo-viridae	Bourbon	Unknown	United States	Hemor. fever (humans)
Rhabdoviridae	Sawgrass	*De. variabilis*	United States	Unknown
Reoviridae	Colorado Tick Fever	*De. andersoni*	United States	Fever (humans)

[a]De.: *Dermacentor*; Ix.: *Ixodes*; Hy.: *Hyalomma*; Or.: Ornithodoros, [b]Encepha.: Encephalitis; Hemor.; Hemorrhagic. (Table adapted from Brackney and Armstrong (2016) and dela Fuente et al. (2017).

Of the 900 currently known tick species, only less than 10% are implicated as virus vectors and these included the *Ornithodoros* and *Argas* Genera for the argasid ticks and *Ixodes, Haemaphysalis, Hyalomma, Amblyomma, Dermacentor,* and *Rhipicephalus* Genera in ixodid ticks [6, 7].

Although the role of ticks in the transmission of viruses has been known for over a century [3] and ticks being second to mosquitoes in transmitting human diseases [8], the diversity of tick-borne viruses has been less thoroughly studied than that of mosquito-borne viruses [9]. Even until now, detection of new pathogenic viruses are

still being reported and known viruses continuously spread to new geographical locations [3].

The flaviviruses account for the majority of arthropod-borne viruses worldwide, which includes the TBEV serocomplex [10, 11]. TBEV serocomplex is consists of closely related flaviviruses that cause important diseases in animals and humans which include Kyasanur Forest virus, Langat virus (LGTV), Louping-ill virus, Omsk hemorrhagic fever virus, Powassan virus, and TBEV [12]. The transmission of this group of viruses is commonly associated with tick species which include hard ticks *Ixodes ricinus, I. persulcatus, Dermacentor* spp., and *Hyalomma* spp., [10].

Haemaphysalis longicornis is also a hard tick mainly distributed in East Asia and Australia and a known vector of theileriosis, non-zoonotic babesiosis [13, 14] and was also recently established as the potential reservoir and vector of a bunyavirus, the severe fever with thrombocytopenia syndrome virus (SFTSV) [15].

Detection and isolation of flaviviruses have been previously reported in *H. longicornis*. Powassan encephalitis virus was isolated in *H. longicornis* in Canada and USA [16], while TBEV has been molecularly detected in the tick in South Korea [17]. Though these reports suggest that *H. longicornis* can successfully harbor and transmit flaviviruses, studies showing survival dynamics of any tick-borne flavivirus in *H.*

longicornis are lacking, most especially experiments demonstrating the capacity of the tick to maintain and successfully transmit flaviviruses to animals.

Since tick vectors of TBEV and other pathogenic members of TBEV serocomplex circulate in East Asia [10] where *H. longicornis* is also mainly distributed, it is interesting to know whether *H. longicornis* is a competent vector of these viruses in East Asia. Vector competence is defined as the ability of an arthropod to become infected with the virus and transmit the virus following blood feeding [18]

Since most TBFVs require at least a biosafety level 3 (BSL3) containment facility, the use of the naturally attenuated LGTV provides a convenient BSL2 model of TBEV and other highly pathogenic TBFVs [19].

LGTV was isolated from pools of *I. granulatus* from Malaysia [20]. LGTV shares more than 74% nucleotide identity with TBEV which prompted the use of the virus as a vaccine candidate against TBE for several years [21], but the vaccination was later on abandoned due to development of unexpectedly high incidence of neurologic disease in vaccinated patients (around 1:10,000) [21 - 23]. In natural environment, LGTV does not cause disease in rodents, but in young laboratory mice inoculated intracerebrally with the virus, encephalitis develops [24].

On the other hand, Thogoto virus (THOV), a tick-borne virus not previously reported in East Asia, was recently isolated from a *H. longicornis* in Kyoto, Japan [9]. THOV is a type of species of the genus Thogotovirus in the family Orthomyxoviridae [25]. The virus was first isolated in 1960 from *Rhipicephalus (Boophilus) decoloratus* and *Rhipicephalus* spp. ticks collected on cattle in Thogoto Forest, Nairobi, Kenya [26], and is reported to affect vertebrate hosts such as cattle, camels, and, sporadically, humans. Other ticks that are known to be vectors of this virus include *R. annulatus, R. sanguineus, R. appendiculatus, R. bursa, R. evertsi, Amblyomma variegatum, H. truncatum*, and *H. a. anatolicum* [27]. The virus can cause afebrile leucopenia in cattle and abortion in sheep [28]. THOV can also affect humans, with one fatality already recorded from two reported human cases [29].

Another aspect of TBVs that is still at an early stage of understanding is the tick-virus interaction. As carriers of several pathogenic microorganisms, protozoa, rickettsiae, spirochaetes, and viruses [1, 30], ticks need to employ broad spectrum innate immunity mechanisms that will allow them to maintain the pathogens and commensal microbes without impairing their viability and further development [31, 32]. With the exceptional longevity of ticks, they can harbor TBVs over prolonged periods of time, making them not only vectors but also excellent reservoir hosts for the viruses

[6]. Although tick immune responses against bacteria have been widely studied, the knowledge about antiviral immunity pathways is generally limited in ticks [33, 34]. RNA interference (RNAi) is considered to be the most important antiviral response in mosquitoes and also the most likely important antiviral response in all arthropods [35]. It is a conserved biological response to double-stranded RNA (dsRNA) which results in the production of small RNAs that mediate regulation of expression of genes by degradation or by translation inhibition of homologous messenger RNA [36]. Aside from RNAi, the other signaling pathways in arthropods which respond to virus infection include Immune deficiency (IMD), Janus kinase-signal transducers and activators of transcription (JAK/STAT), and Toll [35, 37 – 39]. In ticks however, the only antiviral innate immune response described to date is RNAi [40, 41]. In the case of melanisation, it remains unclear whether phenoloxidase (PO) cascade is present in ticks [35], although, the Toll signaling pathway can be activated in ticks after a flavivirus infection [42].

In this study, I explored the role of humoral defenses in ticks particularly the antimicrobial proteins and peptides which play a major role in protecting ticks against microorganisms [43, 44]. Antimicrobial peptides (AMPs) are ancient immune molecules that are important in invertebrate and vertebrate host defenses [45, 46]. These peptides display broad-spectrum biological activity against bacteria, yeast, fungi, protozoan

parasites, and enveloped viruses [47 - 49] and have been demonstrated to possess immunomodulatory properties [50]. Numerous small molecules such as defensins, lysozymes or by tick-specific antimicrobial compounds such as microplusin provide the direct antimicrobial defense in ticks [31].

This dissertation describes studies on survival dynamics of TBVs in *H. longicornis* and the effects of tick antimicrobial peptides in viral replication with the following specific objectives:

1. To demonstrate the survival dynamics of Langat virus in *H. longicornis*;
2. To evaluate the vector competence of *H. longicornis* for Thogoto virus isolated in Japan;
3. To evaluate the antiviral activity of *H. longicornis* antimicrobial peptide, longicin;
4. To identify and characterize a new antimicrobial peptide, HEdefensin, with focus on antiviral activity.

CHAPTER 1

Demonstration of the survival dynamics of Langat virus in *Haemaphysalis longicornis* using anal pore microinjection

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1.1 Introduction

Majority of arthropod-borne viruses worldwide belongs to Flaviviruses which also include the TBEV serocomplex that can cause important diseases in animals and humans [10, 11]. Tick species of the hard ticks *Ixodes ricinus*, *I. persulcatus*, *Dermacentor* spp., and *Hyalomma* spp., commonly transmit this group of viruses [10].

Haemaphysalis longicornis is also a hard tick mainly distributed in East Asia and Australia and a known vector of theileriosis and non-zoonotic babesiosis [13, 14]. The hard ticks was also the established reservoir and vector of the severe fever with thrombocytopenia syndrome virus (SFTSV) [15]. The detection and isolation of flaviviruses have been reported previously in *H. longicornis* [17, 51]; however, studies showing the survival dynamics of any tick-borne flavivirus in *H. longicornis* are still lacking. While ticks can be naturally infected with tick-borne viruses by feeding them on viraemic animals, this method requires a sufficient level of viraemia for transmission to a naïve tick [52]. Moreover, synchronization of tick infection with a defined viral inoculum is a notable limitation in this method [2]. Another method of tick infection is through percoxal microinjection, however, it bypasses the midgut barrier which makes it non-representative of natural route of infection and may not ensure consistent infection rates among fed ticks [53]. Immersion method, on the other hand, can also successfully

infect ticks. This infection method is simpler and relatively inexpensive; however, generating cohorts of infected ticks with equal pathogen burden is its major limitation [54].

In this study, I have demonstrated a consistent infection and maintenance of Langat virus (LGTV), a naturally attenuated member of the TBEV serocomplex of flaviviruses, in adult *H. longicornis* using anal pore microinjection originally used to infect ticks with *Borrelia burgdorferi* [54]. Although no transovarial transmission was observed in this study, the ticks infected by this method successfully established horizontal transmission of LGTV to mice making this method an additional tool in studying tick–virus–host interactions.

1.2 Materials and Methods

1.2.1 Ticks and animals

Parthenogenetic *H. longicornis* (Okayama strain) ticks were maintained for several generations by feeding on the ears of Japanese white rabbits (KBT Oriental Co., Saga, Japan) at the Experimental Animal Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan [55]. Alternatively, ticks were capsule/tube

fed using six-week-old, female, ICR mice (Kyudo, Fukuoka, Japan) [56]. The animals were kept in a temperature- and humidity-controlled room, with a constant supply of water and commercial feeds. The use and care of animals in this study was in accordance with approved guidelines (approval numbers VM 15055 and VM 15058) from the Animal Care and Use Committee of Kagoshima University.

1.2.2 Cells and virus

Baby hamster kidney (BHK-21) cells (ATCC CCL-10) (ATCC, Manassas, VA, USA) were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 5% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA) and 1% antibiotic/antimycotic (Nacalai Tesque, Kyoto, Japan). Cell cultures were maintained at 37 °C under 5% CO_2 until use. To amplify the LGTV TP21 strain, BHK-21 cells were utilized in this study. The LGTV stock titer was determined through focus formation assay (FFA), as described previously [57], and later aliquoted and stored at −80 °C.

1.2.3 Tick infection

Adult ticks were infected with LGTV by anal pore microinjection [54], basically as described. Briefly, several 10 μL calibrated capillary tubes (Drummond

Scientific Co., Broomall, PA, USA) were fabricated into microinjection needles by heating and pulling in a capillary pipette puller (model PN-30) (Narishige, Tokyo, Japan), which were eventually stored on adhesive tape in a petri dish. The ticks were then immobilized using a double-sided adhesive tape on glass slides, with the tick's ventral side up. Under a dissecting microscope (Olympus, Tokyo, Japan), the tick's anal aperture area was focused. Then, after connecting the microinjection needle in the IM 300 microinjector (Narishige, Tokyo, Japan) equipped with automated foot control, the tip of the tube was snapped where the diameter is slightly smaller than that of the anal aperture of the tick by gently touching the tip of the needle. Then microinjection needle was loaded with 0.3 μL of virus stock containing approximately 15,000 focus forming units (ffu) of LGTV. With the immobilized ticks under the dissecting microscope and focused on the anal aperture, a very mild pressure was gently applied to any area near the anal aperture using fine forceps, allowing the separation of the anal plates and opening the anal pore. The tip of the needle was then carefully inserted slightly into the anal aperture through the forced opening of the anal plates, while keeping the needle insertion to a minimum to prevent any damage to the hindgut. Then using the microinjector, the virus inoculum was injected to each tick, wherein, each tick received a single injection. For the control group, EMEM was injected. After the injection, the

ticks were held for 24 h in a 25 °C incubator to check for any mortality arising from possible injury due to the injection.

1.2.4. Detection of Langat virus RNA

To determine viral infection, ticks and mouse samples were collected at indicated time points and subsequently homogenized to isolate the total RNA for cDNA synthesis. Real-time PCR using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) with a 7300 real-time PCR system (Applied Biosystems, CA, USA) was used to detect the viral RNA. LGTV membrane associated glycoprotein precursor (Pre-M) gene-specific primers were used to detect LGTV RNA, while mouse β-actin-specific primers were used for normalization (Table 1.1). Likewise, to quantify the change of viral RNA in ticks post-infection, real-time PCR was also used. LGTV negative-sense RNA-specific primers described elsewhere [2] were used to detect LGTV RNA, while *H. longicornis* ribosomal protein *L23* gene-specific primers were used for normalization (Table 1-1). Alternatively, viral infection in different stages of ticks (egg, larva, and adult) was also determined using reverse transcription PCR (RT-PCR). Homogenization of tick samples, cDNA synthesis, and PCR product visualization were already described elsewhere [58]. Detection of viral RNA was carried out using universal flavivirus

forward (5′-AATGTACGCTGATGACACAGCTGG CTGGGACAC-3′) and reverse (5′-TCCAGACCTTCAGC ATGTCTTCTGTTGTCATCCA-3′) primers [59], while *H. longicornis actin* gene-specific forward (5′-ATCCTGCGTCTCGACTTGG-3′) and reverse (5′-GCCGTGGTGGTGAAAGAGTAG-3′) primers [60] were used as internal control.

1.2.5. Langat virus titration among LGTV-infected adult ticks

Ticks inoculated with LGTV were collected and individually homogenized at 0, 1, 3, 7, 14, 21, 28, 60, and 120 days post-injection (dpi). The collected individual homogenate was eventually titrated as previously described [57].

1.2.6. Detection of Langat virus antigens in tick organs using indirect immunofluorescent antibody test

The indirect immunofluorescent antibody test (IFAT) was performed to demonstrate the localization of LGTV in salivary glands, midguts, and hemocytes of *H. longicornis* (28 dpi via anal pore microinjection), following the method described previously [61, 62].

1.2.7. Langat virus transmission from ticks to mice

To determine whether LGTV could be transmitted to mice by tick bite, LGTV-infected adult ticks (28 dpi) were allowed to feed on 20 mice (one tick per mouse) until fully engorged by feeding capsule method, as previously described [56]. Blood samples collected from each mouse at 28 days after infestation (dai) were used for LGTV detection using specific real-time PCR primer pairs (Table 1). Then, using the previously described immunofluorescence assay (IFA) [15], I also detected LGTV antibodies in serum samples from mice fed upon by infected adult ticks. Likewise, mice were observed for up to 28 dai for any clinical signs, including hunchback posture, ruffled fur, and hind-limb paralysis [63, 64]. Brain samples were collected from mice that exhibited paralysis (considered terminal) and from survivors (live mice 28 dai) to detect viral RNA, as described above. Lastly, five mice infested with EMEM-injected ticks and another five mice injected with 10,000 ffu of LGTV intraperitoneally served as negative and positive controls, respectively.

1.2.8. Transovarial transmission of Langat virus in ticks

All the fully engorged ticks collected from the tick infestation experiment using feeding capsule method were collected and allowed to lay eggs. Fifty percent of the

individual egg clutch produced from both LGTV- and EMEM-injected groups was homogenized separately, while the remaining 50% of each egg clutch was allowed to hatch into larvae. Both egg and larval homogenates were used to isolate total RNA for cDNA synthesis. LGTV RNA was detected in each sample using RT-PCR.

1.2.9. Statistical Analysis

All samples were tested at least in triplicate and statistically analyzed using Welch's *t*-test in GraphPad Prism version 3.0 software (GraphPad Software, San Diego, CA, USA), wherein p-values of less than 0.05 and 0.01 were regarded as significant and highly significant, respectively.

1.3 Results

1.3.1 Langat virus infection in ticks

I initially observed that LGTV injected ticks via anal pore microinjection remained positive to LGTV RNA even after 28 dpi (Table 1.2). Additionally, infectious virions can be detected in the midgut (20/20) and carcass (without the midgut and salivary gland; 3/20), but not in the salivary gland (0/20) (Table 1.3).

These observations may suggest that the virus can be maintained in the ticks, specifically in the midgut, even after 28 dpi without any difference in mortality compared to the control. However, since the mere presence of infectious virions may only suggest retention and not necessarily replication, I later on confirmed the LGTV replication through time point determination of LGTV RNA and titer among the anal pore microinjected ticks. The LGTV negative-sense RNA strand, an obligatory marker for virus replication [2], increased over the time course of the infection (Figure 1.1A), and increasing viral titers were also observed beginning 3 dpi, with the maximum titer recorded at 28 dpi, while the virus remained detectable for at least 120 dpi (Figure 1.1B). On the other hand, viral antigens at 28 dpi were consistently detected in the cytoplasm of midgut cells of LGTV-injected ticks (Figure 1.2A). I also detected LGTV antigens in the hemocytes, but not in the salivary glands.

1.3.2 Langat virus transmission to mice

I also tested whether anal pore microinjected, unfed, adult ticks can successfully transmit the virus to a susceptible host. Infected adult ticks (28 dpi) were allowed to feed on mice, and then the presence of both viral RNA and LGTV antibodies was checked 28 dai. Mice were also observed for clinical signs for the entire duration of

the study, wherein paralyzed mice were sacrificed to collect the brains for LGTV RNA detection through real-time PCR. Mice inoculated with LGTV or EMEM through intraperitoneal inoculation served as positive and negative controls, respectively. One mouse from the positive control group and two mice infested with the LGTV-infected ticks that showed hind-limb paralysis were positive for viral RNA in the brain (Table 1.4). In the blood and brain samples collected from the surviving mice at 28 dai, no LGTV RNA was detected; however, 16 of 18 mice (88.8%) infested with LGTV-infected ticks and 4/4 (100%) of the positive control mice showed LGTV-specific antibodies. As expected, no LGTV-specific antibodies were detected from the EMEM-inoculated ticks (Table 1.4). Representative IFA detection images of LGTV antibodies from all experimental groups are shown in Figure 1.2B.

1.3.3 Transovarial transmission of Langat virus in ticks

To observe the vertical transmission of LGTV in ticks, eggs, and larvae from the infected adults were examined for viral RNA using RT-PCR. In summary, 85% (17/20) and 100% (5/5) of the engorged ticks from the LGTV- and EMEM-injected groups successfully laid eggs, respectively. In addition, a 100% hatching rate was observed in both groups. However, as shown in Table 1.5, no LGTV RNA was detected

in both egg (0/17) and larval (0/17) samples from LGTV-injected ticks. As expected, no viral RNA was detected in the control group (0/5).

1.4 Discussion

One of the most important determinants of vector competence is the susceptibility of midgut cells to virus infection [6], and anal pore microinjection method may prove to be an important technique in evaluating vector competency, since the virus will first come in contact with the tick gut. As shown in Figure 1.1, LGTV successfully replicated in the *H. longicornis* as shown by increasing viral RNA and titer. I also managed to clearly demonstrate that the virus successfully infected the midgut cells and hemocytes (Figure 1.2) and infectious virions can be consistently isolated from the midgut (Table 1.3). The isolation of LGTV in some tick carcasses may suggest infection of some tick organs or may be due to incomplete washing of infected hemocytes.

On the other hand, LGTV localization and isolation in the salivary glands (Figure 1.2 and Table 1.3) was not observed in the current study. I can only speculate that the virus replicated in a sub-detectable level or may have not yet entered the organ, since I have not yet checked for the presence the virus in the salivary glands during or after blood feeding. The latter observation was previously reported in Thogoto virus

(THOV), wherein the virus was not detected in the salivary glands of *Rhipicephalus appendiculatus* (transstadially infected) until the ticks had fed on a host for about seven days [65]. It was previously reported for TBEV that feeding enhances salivary gland infection; thus, partially fed ticks have significantly higher infection prevalences than unfed ticks from the same collection site [66]. It is also during a subsequent meal, that the virus enters the saliva through the salivary gland epithelium for eventual transmission [67]. Thus, despite the absence of detection of LGTV in the salivary glands of the LGTV-injected ticks, the horizontal transmission of the virus was still observed as shown in Table 1.4.

On the other hand, despite the failure to detect viraemia in all the experimental groups of mice, almost 90% of the naïve mice infested with infected ticks seroconverted, suggesting that subclinical infection may have occurred. Such detection of antibodies in mice already suggests virus transmission, since host infection has usually been detected by virus isolation and/or seroconversion [68]. Likewise, the two paralyzed mice infested with infected ticks showed the presence of LGTV RNA in their brains, indicating further the successful transmission of virus from ticks to mice; however, I failed to determine whether meningitis or encephalitis was present in their respective brains. What is also notable in the present study was the absence of transovarial transmission as

determined from the eggs and hatched larvae collected from the engorged infected adult ticks (Table 1.5). Although not all infected ticks successfully transfer the virus to their eggs [53], the absence of detection from eggs and larvae could be explained by previous reports that a large proportion of larvae may become infected by non-viraemic transmission when they co-feed with infected nymph or larvae [69]. Thus, evaluation of the co-feeding transmission between adult infected ticks via anal pore microinjection and naïve nymphs, can further establish if the current method of infection can mimic the natural spread virus of among tick populations.

In summary of this Chapter, *H. longicornis* can be efficiently infected with LGTV through anal pore microinjection. Moreover, infected *H. longicornis* can effectively transmit LGTV to a susceptible host, as shown by the presence of viral RNA in the brain of infected mice and the presence of LGTV antibodies in almost 90% of infested mice. However, demonstration of LGTV transmission through co-feeding between an infected adult and immature naïve ticks are needed to demonstrate the possible mechanism on how LGTV can circulate in the tick population, especially that no transovarial transmission was observed in the study using anal pore microinjection. Taken together, the results presented highly suggest that the anal pore microinjection method can be a useful technique in studying tick, virus, and host interaction

Tables and Figures in CHAPTER 1

Table 1.1 List of real-time PCR primers used to detect Langat Virus RNA

Primers	Sequence (5'→3')
LGTV *Pre-M* Forward	GGATGGATTGTTGCCCAGGA
LGTV *Pre-M* Reverse	CCCAGCTCGAGAACCAATGT
LGTV Neg. Forward	GTCTCCGGTTGCAGGACTGT
LGTV Neg. Reverse	CTCGGTCAGTAGGATGGTGTTG
H. longicornis L23 Forward	CACACTCGTGTTCATCGTCC
H. longicornis L23 Reverse	ATGAGTGTGTTCACGTTGGC
Mouse *β-actin* Forward	TTCTTTGCAGCTCCTTCGTT
Mouse *β-actin* Reverse	ATGGAGGGGAATACAGCCC

Table 1.2 Detection of Langat virus RNA from ticks injected with LGTV and Eagle's Minimum Essential Medium via anal pore microinjection using reverse transcription PCR

Inoculum	LGTV detection	
	Absolute value	Percentage
EMEM	0/20	0
LGTV	20/20	100

Table 1.3 Comparative Langat virus titers from selected organs of unfed adult *Haemaphysalis longicornis* at 28 days post-injection (dpi)

Tissue	No. positive/total (%)	Mean titer ± SD (log_{10} ffu/tick)
Midgut	20/20 (100)	3.37±0.16
Salivary gland	0/20 (0)	-
Carcass	3/20 (15)	2.53 ±0.85

Table 1.4 Langat virus transmission from *Haemaphysalis longicornis* to mice

Treatment	Moribund mice[a]		Survivors		
	Mortality (%) (death/total)	Viral RNA in brain[b]	Seroconversion (%) (positive/total)	Viral RNA in brain[b]	Viral RNA in blood[b]
Medium-injected ticks	0 (0/5)	N.A.	0 (0/5)	-	-
LGTV-injected ticks	10 (2/20)	+ (2/2)	88.8 (16/18)	-	-
LGTV Inoculated mice	20 (1/5)	+ (1/1)	100(4/4)	-	-

[a]Mice were considered terminal and later on sacrificed at the first signs of disease.

[b]Real-time-PCR was used for detection of LGTV in mouse tissues as represented by presence (+), absence (-) or not applicable (N.A.).

Table 1.5 Detection of Langat virus RNA in eggs and larvae using reverse transcription PCR

Sample	Group	LGTV detection	
		Absolute value	Percentage
Eggs	EMEM	0/5	0
	LGTV	0/17	0
Larvae	EMEM	0/5	0
	LGTV	0/17	0

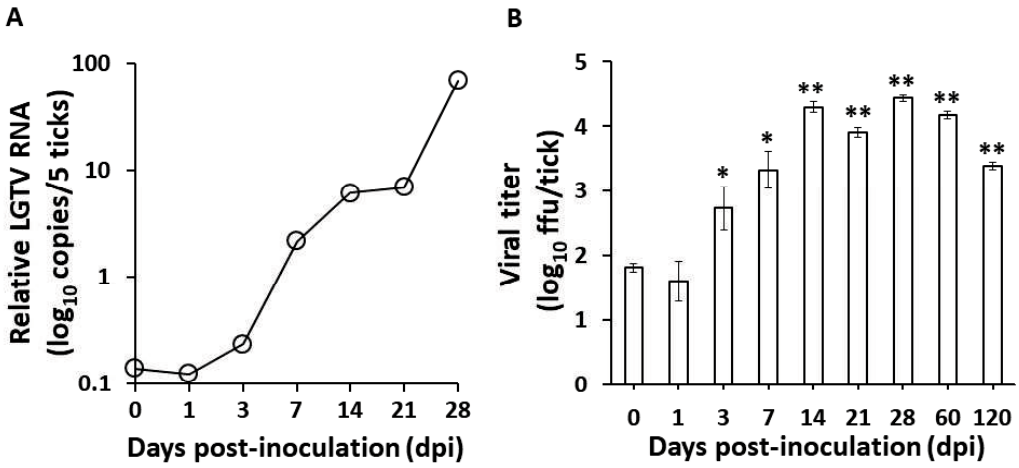

Fig. 1.1 Replication of Langat virus in *Haemaphysalis longicornis* after infection via anal pore microinjection. (A) Real-time PCR was used to quantify the changes in the negative-sense strand of LGTV RNA collected from groups of five ticks at each time point. The *H. longicornis L23* gene was used to normalize the data at each time point. (B) Virus titration after LGTV infection via anal pore microinjection. Error bars in virus titers indicate the SD in mean values of three ticks at each time point. **P* < 0.05, ** *P* < 0.01, as compared to day 0.

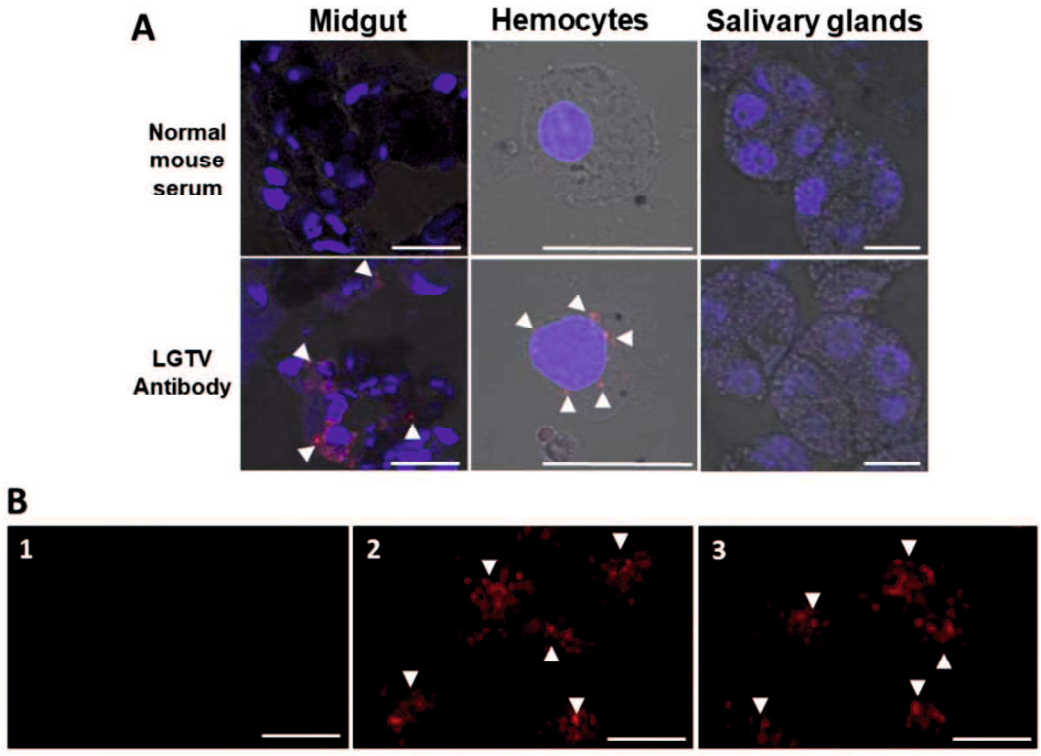

Fig. 1.2 Localization of Langat virus in selected organs from unfed adult ticks after infection via anal pore microinjection and immunofluorescence assay detection of LGTV antibodies in serum samples from mice. (A) Viral antigens were detected using a specific LGTV polyclonal antibody, while normal mouse serum served as a control. Nuclei counterstaining (blue) was done using DAPI, and arrowheads denote LGTV antigens (red) (bar = 20 μm). (B) Sera collected from mouse infested with EMEM-injected tick (1) (1:200, No.1); mouse inoculated with 10,000 ffu of LGTV (2) (1:12,800, No.1); mouse infested with LGTV-injected tick (3) (1:6,400, No.18) reacting with LGTV-infected baby hamster kidney cells. Arrowheads denote LGTV ffu detected by LGTV antibodies (red) (bar = 10 μm).

CHAPTER 2

Vector competence of *Haemaphysalis longicornis* ticks for a Japanese isolate of the Thogoto virus

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2.1 Introduction

Ticks are important vectors of viruses of public health importance, including TBEV, CCHFV, and African swine fever virus, which are known to cause severe clinical symptoms in humans and domestic animals [4, 5, 6]. In Japan, the only known endemic tick-borne viruses include TBEV and SFTSV [70, 71]. However, THOV, a tick-borne virus not previously reported in East Asia, was recently isolated from a *H. longicornis* in Kyoto, Japan [9]. THOV is a type of species of the genus Thogotovirus in the family Orthomyxoviridae [25]. THOV has a genome consisting of 6 negative-sense, single-stranded RNA segments, and it is structurally and genetically similar to influenza viruses [72, 73]. The virus was first isolated in 1960 from *Rhipicephalus decoloratus* and *Rhipicephalus* spp. ticks collected on cattle in Thogoto Forest, Nairobi, Kenya [26], and is reported to affect vertebrate hosts such as cattle, camels, and, sporadically, humans. Other ticks that are known to be vectors of this virus include *R. annulatus, R. sanguineus, R. appendiculatus, R. bursa, R. evertsi, Amblyomma variegatum, Hyalomma truncatum,* and *H. a. anatolicum* [27]. The virus can cause afebrile leucopenia in cattle and abortion in sheep [28], and can also affect humans, with one fatality already recorded from two reported human cases [29].

Since this is the first reported isolation of THOV in Japan, I am greatly

interested in knowing the survival dynamics of THOV in *H. longicornis*. Moreover, it is also important to determine whether *H. longicornis* is the principal vector of THOV in Japan, since the Japanese THOV isolate was originally obtained from *H. longicornis*, the major tick species at the collection site [9]. Ultimately, I aimed to demonstrate the vector competency of *H. longicornis* for harboring and transmitting THOV to further enhance our understanding of the public health importance of this newly emerging virus in Japan.

2.2 Materials and Methods

2.2.1 Ticks and animals

Parthenogenetic *H. longicornis* (Okayama strain) ticks were maintained for several generations by feeding on the ears of Japanese white rabbits (KBT Oriental Co.) at the Experimental Animal Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan [55]. Conversely, ticks were infested on 6-week-old female BALB/c mice (Kyudo) using the feeding capsule method as previously described [56]. Animal experiments were conducted in accordance with approved guidelines (approval numbers VM 15055 and VM 16016) of the Animal Care and Use Committee of Kagoshima University.

2.2.2 Cell culture and virus

BHK-21 cells were grown in EMEM containing 5% FBS and 1% antibiotic/antimycotic and maintained at 37 °C under 5% CO_2 until use. The THOV Kamigamo strain was amplified in BHK-21 cells, and the virus stock titer was determined by FFA as previously described [57] with some modifications.

2.2.3 Infection of ticks with THOV

Adult ticks were infected experimentally with THOV by anal pore microinjection as previously described [54, 74]. Infection by microinjection was accomplished by injecting 0.3 µl of virus stock containing approximately 100 ffu of THOV into the tick's anal aperture using a microinjector (Narishige Group). In contrast, EMEM was injected in the control group. After the injection, ticks were kept for 24 h in a 25 °C incubator to observe for any mortality arising from possible injury due to the injection.

Alternatively, naïve nymphs were also evaluated to determine whether they can acquire THOV through feeding on THOV-injected mice or via co-feeding with an adult infected via anal pore microinjection. For feeding in THOV-injected mice, 3 mice were injected subcutaneously with 10,000 ffu of THOV, and then immediately infested with

30 naïve nymphs each. Three EMEM-injected mice, which served as the negative controls, were infested with the same number of nymphal ticks per mice. For the co-feeding experiment, 5 THOV-infected adult ticks (28 dpi) were infested individually onto 5 mice together with 20 naïve nymphs/mouse. Four EMEM-injected adult ticks served as negative controls with the same number of nymphs/mouse. After feeding, all engorged nymphs were collected and allowed to molt. Twenty-one days post-molting, adult ticks were examined for either the presence of THOV RNA or infectious virions via real-time PCR and FFA, respectively.

2.2.4 Detection of THOV RNA in mouse and tick tissues

Total mRNA extraction and analysis of THOV RNA in mice and ticks through real-time PCR using THUNDERBIRD™ SYBR® qPCR Mix (TOYOBO) with a 7300 real-time PCR system (Applied Biosystems) were performed as previously described [60]. Briefly, gene-specific primers were designed to target THOV segment 6 (matrix protein) and tick ribosomal protein L23 or mouse β-actin (internal control) genes. To generate standard curves, eightfold serial dilutions of the cDNA from THOV, adult ticks, or mouse tissue were used. Each sample was run in triplicate, and the data were analyzed using 7300 System SDS software (Applied Biosystems). Lastly, normalized

gene expressions were computed by dividing the amount of THOV gene expression by the amount of L23 or mouse β-actin expressions for each sample.

2.2.5 Isolation and titration of THOV from tick tissues and whole adult ticks

Ticks inoculated with THOV were collected and individually homogenized at 0, 1, 3, 7, 14, 21, 28, 60, and 120 dpi). The collected individual homogenate was eventually titrated as previously described [57].

On the other hand, collected tick organs such as salivary glands, midguts, and carcasses (without midguts and salivary glands) were washed 3 times with PBS, homogenized individually, and then diluted with 300 µl of E-MEM with antibiotics, centrifuged, and subsequently filtered as previously described [74]. The collected supernatants were directly used for titration

2.2.6 Detection of THOV antigens in tick organs using IFAT

IFAT was performed to demonstrate the localization of THOV in salivary glands and midguts of unfed, THOV-injected ticks, while, for partially fed ticks, synganglia were also used, following the method described previously [61, 62].

2.2.7 Tick transmission of THOV to mice

To determine whether THOV introduced into ticks via anal pore microinjection can be transmitted to mice by tick bite, I allowed THOV-infected adults to feed on mice via the feeding capsule/tube method [56]. Twenty-two infected adult ticks (28 dpi) were allowed to feed individually on 22 naive mice until engorgement. EMEM-injected ticks were also allowed to feed on 8 mice for a negative control (NC), while another 8 mice were injected with 10,000 ffu of THOV to serve as a positive control (PC). Seven days after infestation (dai), spleen and liver tissues were obtained from 10 mice infested with THOV-injected ticks and examined by real-time PCR to detect THOV RNA as described above. Three NC and PC mice were also sacrificed for spleen and liver collection. Then, the remaining mice from each group were observed for up to 28 dai. At the end of the observation period (28 dai), blood samples were collected for the detection of THOV-specific antibodies from the different experimental groups using IFA [15]. Briefly, serum samples were assayed by using THOV-infected BHK cells as antigens. The cells were cultivated in 48-well plates, fixed with 4% paraformaldehyde, and used as antigens for IFAs to detect THOV antibodies in the collected serum samples. Each serum was initially assayed at a 1:200 dilution (5% skimmed milk in PBS), and then a two-fold diluted, thereafter.

Alternatively, I also allowed the molted adult ticks from nymphs co-infested with THOV-infected adults via anal pore microinjection to feed on mice. Twenty (20) molted adult ticks (28 days post molting) were allowed to feed individually on 20 naive mice until engorgement via the feeding capsule/tube method. EMEM-injected ticks were also allowed to feed on 5 mice for a negative control (NC), while another 5 mice were injected with 10,000 ffu of THOV to serve as a positive control (PC). At 28 dai, blood samples were collected for the detection of THOV-specific antibodies from the different experimental groups using IFA as described above.

2.2.8 Determination of transovarial transmission of THOV in ticks

All fully engorged ticks collected from the tick infestation experiments (experimental anal pore microinjection and experimental virus acquisition through co-feeding) using the feeding capsule method were allowed to lay eggs. Fifty percent of the individual clutch of eggs produced from both LGTV- and EMEM-injected groups was homogenized separately, while the remaining 50% of each clutch of eggs was allowed to hatch into larvae and, later, homogenized 14 days after hatching. Both egg and larval homogenates were used to isolate total RNA for cDNA synthesis. LGTV RNA was detected in each sample using real-time PCR as described above.

2.2.9 Statistical analysis

All samples were tested at least in triplicate unless otherwise stated and statistically analyzed using Welch's *t*-test in GraphPad Prism software, wherein *P* values of less than 0.05 and 0.01 were regarded as significant and highly significant, respectively.

2.3 Results

2.3.1 THOV replication in ticks

Figure 2.1 shows that THOV can successfully replicate in *H. longicornis*. Increasing viral RNA levels can be observed beginning at 3 dpi, and eventually peaking at 21 dpi (Figure 2.1A). On the other hand, Figure 2.1B, also shows an increasing virus titer pattern, peaking at 28 dpi. Even 4 months post-THOV inoculation, infectious virions can still be detected. I also conducted organ titration to determine which organ is the target site for virus replication in unfed and feeding states. In unfed adult ticks, THOV was consistently isolated from the midguts of infected ticks (20/20) (Table 2.2). On the other hand, I can also isolate infectious virions from 10% of the collected salivary glands (2/20), while no virus was isolated from the carcass (0/20). However,

the percentage of infected salivary glands and carcasses increased notably during blood feeding, at 93.3% (14/15) and 100% (15/15), respectively. The midgut remains 100% (15/15) infected during blood feeding.

For IFAT in the unfed ticks, THOV was consistently detected in the midgut (Figure 2.2B) and salivary glands (Figure 2.2D). During blood feeding, the virus can still be detected both in the midgut (Figure 2.3B) and salivary glands (Figure 2.3D), although the synganglia were also notably infected (Figure 2.3 F).

Meanwhile, acquisition experiments showed that H. *longicornis* can experimentally acquire THOV in an experimentally infected mouse, wherein 3.3% (1/30) of molted adult ticks from engorged nymphs fed on THOV-injected mice turned positive with THOV RNA, while 2.7% (1/36) turned positive for infectious virions (Table 2.3). In contrast, co-feeding between an infected adult and naïve nymphs showed much higher detection rates in the molted adults for viral RNA at 22.5% (9/40) and infectious virions at 7.9% (3/38) (Table 2.4).

2.3.2 Tick transmission of THOV to mice

To establish that *H. longicornis* infected with THOV through anal pore microinjection can transmit the virus to a susceptible host, I tested the vector capacity of *H. longicornis* in transmitting THOV to mice. At the end of the virus transmission experiment (28 days), all mice were apparently healthy, and no mortality was recorded in any experimental group. As shown in Table 2.5, THOV-injected ticks managed to infect the infested mice, as manifested by the presence of THOV RNA in both the spleen and liver, as determined by real-time PCR. Moreover, all mice (12/12) infested with THOV-injected adult ticks also showed the presence of THOV-specific antibodies after 28 dai. As expected, all mice (5/5) inoculated with THOV seroconverted, while those of the NC group did not. Among the positive serum samples from mice infested with THOV-infected ticks and the positive control group, the maximum detectable IFA titer was determined to be 1:25600. Representative IFA detection images of THOV antibodies from sera collected from all experimental groups are shown in Figure 2.4.

Since the ticks injected with THOV using anal pore microinjection were not infected via the normal tick infection route, I then alternatively utilized adult ticks that had molted from nymphs co-fed with THOV-injected ticks to also demonstrate the transmission of the virus to mice. As observed previously in our initial virus

transmission experiment, no sign of disease and mortality was recorded in any experimental animal group during the entire duration of the study. However, only one infested mouse (1/18) showed THOV-specific antibodies (titer at 1:12800) at 28 dai from the THOV co-feeding group. As expected, all mice inoculated with THOV seroconverted (5/5) (maximum at titer 1:25600), while those in the EMEM co-feeding group did not (0/5).

2.3.3 Detection of THOV RNA in eggs and larvae

Lastly, eggs and larvae collected from THOV- and EMEM-injected engorged ticks were also checked for THOV RNA to establish transovarial transmission; however, no viral RNA detection was observed in either group (0/22) and (0/5), respectively. The same results were also observed in the infected ticks via experimental acquisition, wherein no THOV RNA was detected (0/18) in eggs and larvae. As expected, molted adult ticks from nymphs co-infested with EMEM-injected ticks did not produce infected eggs and larvae.

2.4 Discussion

In this study, anal pore microinjection proved to be an effective method for infecting ticks with THOV with no apparent mortality. As shown in Figure 2.1, THOV can successfully infect and replicate in *H. longicornis* as observed in increasing levels of THOV RNA and virus titers.

On the other hand, IFAT results show that THOV mainly localizes in the midgut and salivary glands and, additionally, in the synganglia during feeding. However, organ titration revealed that digestive cells could be the primary replication site of the virus, since THOV was only consistently isolated from the midgut, especially in the unfed state. Such an observation is crucial, since one of the most important determinants of vector competence is the susceptibility of midgut cells to virus infection [6].

However, blood feeding greatly influenced the distribution of the virus in tick organs, most especially in the salivary gland. In the unfed state, only 10% of salivary glands were positive for infectious virions, as compared to 93.3% in partially fed ticks. These observations show that the virus readily transfers from the midgut to the salivary gland during feeding to facilitate the transmission of the virus to the host. For most tick-borne pathogens present in the tick gut, dissemination into the hemolymph and migration to the salivary glands can happen immediately after acquisition or after the

stimulus of a new blood meal [76]. Such a phenomenon was previously reported for TBEV, wherein feeding enhances salivary gland infection; thus, partially fed ticks have significantly higher infection prevalences than unfed ticks from the same collection site [66]. Infected synganglia, which could have also been infected from the virus present in the hemolymph during blood feeding, are consistent with the tropism of the virus in the organ. Synganglion infection by tick-borne viruses has already been reported previously, especially for THOV in *R. appendiculatus* [65] and for Langat virus and Powassan virus in *I. scapularis* [76].

Virus transmission experiments also showed that *H. longicornis* is a competent vector of THOV, as virus-injected ticks managed to infect the infested mice, as manifested by THOV RNA expression in both the spleen and liver and THOV-specific antibodies, but with no sign of disease and mortality for the whole duration of the study. Likewise, the absence of clinical signs and deaths in the present results still remains consistent with the previous results indicating that THOV-Kamigamo strain has a low-pathogenic characteristic [9], as previously shown by the absence of mortality in the challenged groups.

The current study also managed to demonstrate that naïve nymphs can be infected by THOV through natural routes of infections. The infection of nymphs

through feeding on THOV-injected mice proved to be possible in the present study; however, co-feeding the naïve nymphs with an infected adult showed a higher infection rate, at 22.5%. This occurrence was previously reported for THOV that was transmitted more efficiently by *R. appendiculatus* via non-viraemic guinea pigs (co-feeding between infected and uninfected ticks) than via highly viraemic hamsters [77]. The transmission between co-feeding infected and uninfected ticks without systemic infection was also demonstrated in other tick-borne viruses, such as the Crimean-Congo hemorrhagic fever virus, Kyasanur Forest Disease virus, Louping ill virus, and TBEV [78]. Transstadial maintenance of THOV in *H. longicornis* was also established in the present study, since the engorged nymphs produced infected adults, suggesting that the virus can survive the harsh molting process. Moreover, the non-detection of THOV in eggs and larvae in the present study suggests that no vertical transmission occurred; however, the infection of nymphal ticks through co-feeding with infected adults was clearly established. It was previously reported that in the presence of weak vertical transmission, the infection of nymphal ticks plays an important role in the virus transmission cycle that is critical in pathogen maintenance [79]. On the other hand, as compared to the experimentally infected ticks, the group of transstadially infected adults showed a considerably lower transmission rate as compared to that of experimentally

infected adults.

In summary, this chapter demonstrated that *H. longicornis*, a widely distributed tick in Japan, is a competent vector or even possibly the main tick carrier of the Japanese THOV isolate. However, additional studies are needed to identify which animals potentially serve as the reservoir host of the virus and to elucidate animal susceptibility and the geographic distribution of THOV infections in Japan. Our findings would also be of great help since emerging tick-borne viruses warrant better understanding for potential disease and vector control.

Tables and Figures in CHAPTER 2

Table 2.1 List of real-time PCR primers used for the detection of THOV in ticks and mice

Primer name	Primer sequence (5'-3')
THOV Forward	CGGATGGCAACAAGAAGCTG
THOV Reverse	AATCAGCACAACATCCCGGT
Mouse *β-Actin* Forward	TTCTTTGCAGCTCCTTCGTT
Mouse *β-Actin* Reverse	ATGGAGGGGAATACAGCCC
H. longicornis L23 Forward	CACACTCGTGTTCATCGTCC
H. longicornis L23 Reverse	ATGAGTGTGTTCACGTTGGC

Table 2.2 Titration of THOV isolated from tick organs of unfed and partially fed infected ticks via focus formation assay

Organs	Positive/Total (%)		Mean titer ± SD (log_{10} ffu/tick)	
	Unfed	Partially fed	Unfed	Partially fed
Midgut	20/20 (100)	15/15 (100)	2.85 ± 0.30	1.66 ± 0.57
Salivary gland	2/20 (10)	14/15 (93.3)	2.83 ± 0.29	1.38 ± 0.34
Carcass	0/20 (0)	15/15 (100)	-	1.42 ± 0.45

Table 2.3 Detection and isolation of THOV from whole adult ticks that molted from nymphs fed on either EMEM- or THOV-injected mice

Group	Viral RNA detection (%)	Virus isolation (%)
EMEM-injected mice	0/30 (0)	0/32 (0)
THOV-injected mice	1/30 (3.3)	1/36 (2.7)

Table 2.4 Detection and isolation of THOV from whole adult ticks that molted from nymphs co-fed with either EMEM- or THOV-injected adult ticks

Co-feeding group of nymphs	Viral RNA detection (%)	Virus isolation (%)
EMEM-injected adult ticks	0/20 (0)	0/45 (0)
THOV-injected adult ticks	9/40 (22.5)	3/38 (7.9)

Table 2.5 THOV transmission from ticks injected with THOV through anal pore microinjection to mice

Treatment	Viral RNA in liver and spleen*	Seroconversion (%) (positive/total)
Medium-injected ticks	- (0/3)	0 (0/5)
THOV-infected ticks	+ (10/10)	100 (12/12)
THOV-inoculated mice	+ (3/3)	100 (5/5)

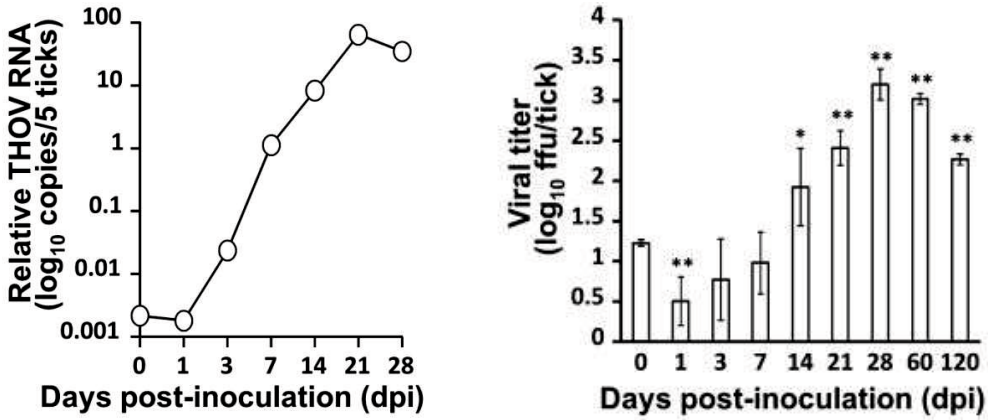

Fig. 2.1 Replication of the Thogoto virus (THOV) in *Haemaphysalis longicornis* after infection using anal pore microinjection. To quantify the changes in THOV RNA collected from groups of five ticks at each time point, real-time PCR was used (A). Virus titration after THOV infection, wherein error bars indicate the SD in mean values of five ticks at each time point (B). * $P < 0.05$, ** $P < 0.01$, as compared to day 0.

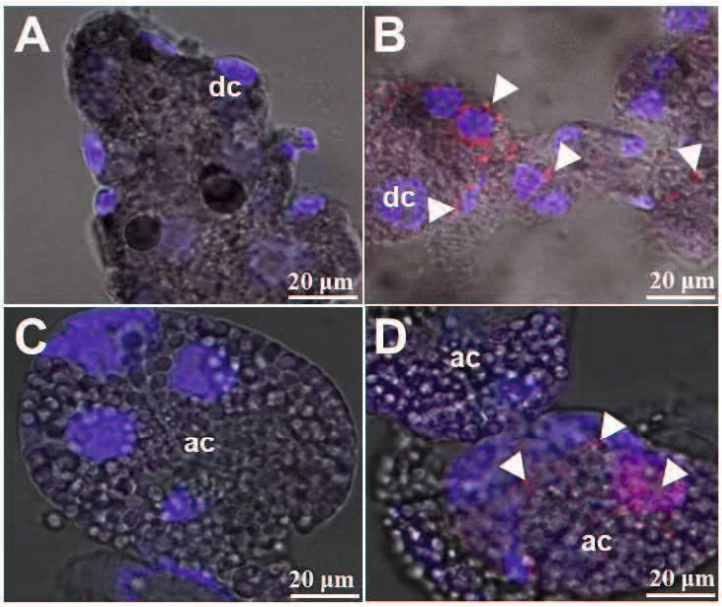

Fig. 2.2 Localization of the Thogoto virus (THOV) in the midgut (A,B) and salivary glands (C,D) of unfed adult ticks after infection via anal pore microinjection. Viral antigens were detected using a specific THOV polyclonal antibody (B,D), while normal mouse serum served as a control (A,C). Nuclear counterstaining (blue) was done using DAPI, and arrowheads denote THOV antigens (red) (bar = 20 µm). dc: digestive cells; ac: acinus.

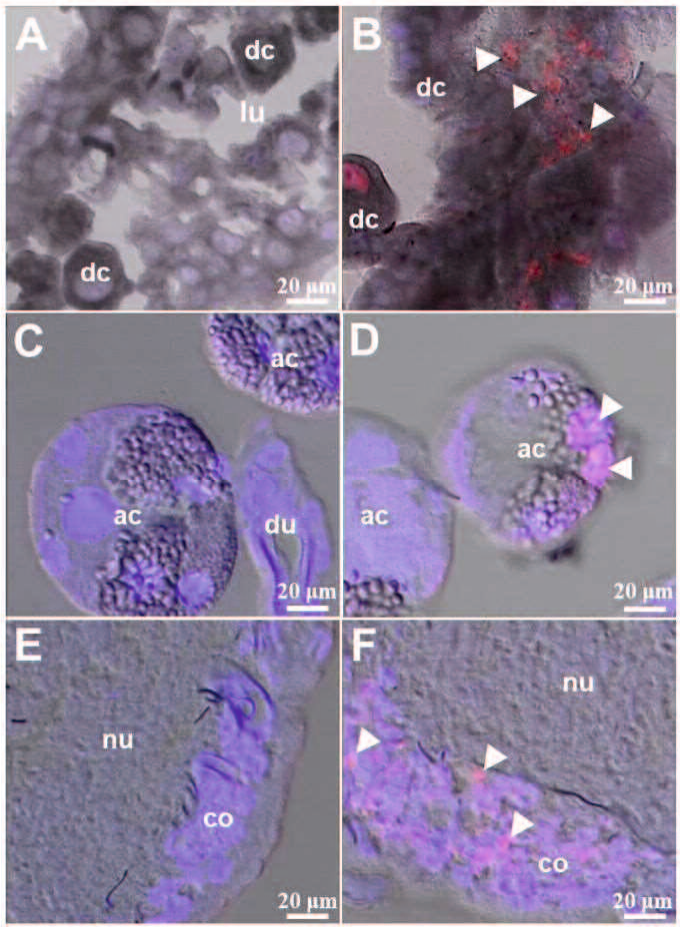

Fig. 2.3 Localization of the Thogoto virus (THOV) in the midgut (A,B), salivary glands (C,D), and synganglia (E,F) of partially fed, infected adult ticks via anal pore microinjection. Viral antigens were detected using a specific THOV polyclonal antibody (B,D,F), while normal mouse serum served as a control (A,C,E). Nuclei counterstaining (blue) was done using DAPI, and arrowheads denote THOV antigens (red) (bar = 20 µm). dc: digestive cells; ac: acinus; du: duct; lu: lumen; nu: neuropile; co: cortex.

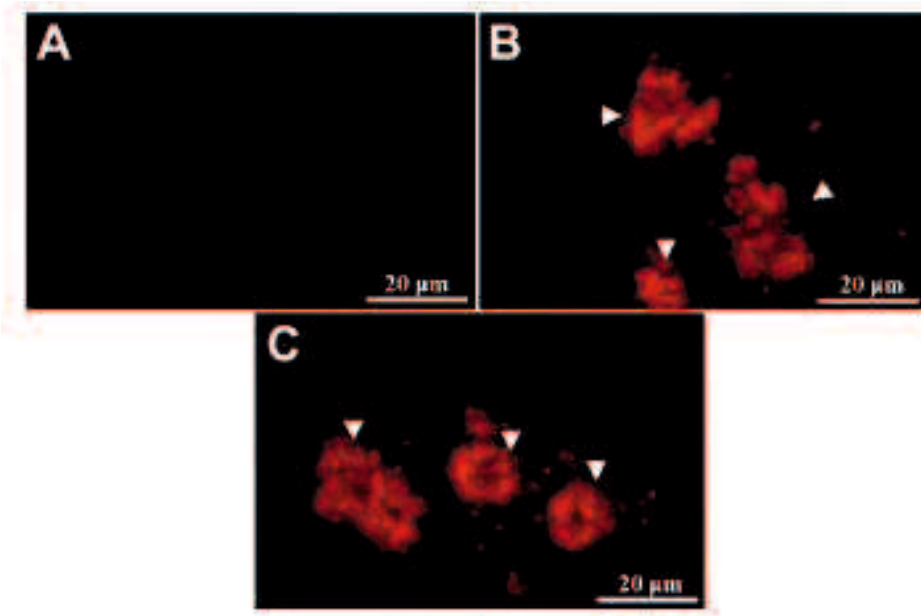

Fig. 2.4 Detection of Thogoto virus (THOV) antibodies in serum samples from mice using immunofluorescence assay. Sera collected from a mouse infested with EMEM-injected ticks (A) (1:200, No.1); mouse inoculated with 10,000 ffu of THOV (B) (1:3200, No.1); mouse infested with THOV-injected ticks (C) (1:3200, No.6) reacting with THOV-infected baby hamster kidney BHK-21 cells. Arrowheads denote THOV ffu detected by THOV antibodies (red) (bar = 20 μm).

CHAPTER 3

Evaluation of the role of antimicrobial peptide, longicin, from *Haemaphysalis longicornis* against Langat virus

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3.1 Introduction

Ticks are hematophagous arachnids capable of transmitting several disease-causing pathogens in domestic and wild animals, including humans [8, 31]. As carriers of several pathogenic microorganisms, protozoa, rickettsiae, spirochaetes, and viruses [1, 30], ticks need to employ broad spectrum innate immunity mechanisms that will allow them to maintain the pathogens and commensal microbes without impairing their viability and further development [32, 31]. As previously demonstrated, antimicrobial proteins and peptides play a major role in protecting ticks against microorganisms [43, 44].

Antimicrobial peptides are ancient immune molecules that are important in invertebrate and vertebrate host defenses [45, 46]. These peptides display broad-spectrum biological activity against bacteria, yeast, fungi, protozoan parasites and enveloped viruses [47 - 49] and have been demonstrated to possess immunomodulatory properties [50]. Numerous small molecules such as defensins, lysozymes or by tick-specific antimicrobial compounds such as microplusin provide the direct antimicrobial defense in ticks [31].

Longicin, a defensin-like peptide identified from the midgut epithelium in the hard tick Haemaphysalis longicornis, is a promising cationic antimicrobial peptide.

Many studies have shown that longicin and one of its synthetic partial analog (longicin P4) have antimicrobial, fungicidal, and parasiticidal properties [32, 80, 81]. Thus, making them attractive molecules to be used as therapeutic agents, not only against tick-borne pathogens, but also to important human and animal disease-causing agents. On the other hand, interest in the therapeutic applications of antimicrobial peptides or their synthetic analogues is increasing due to the rise in resistance to commonly used antibiotics [30, 82].

Tick-borne flaviviruses (TBFVs) cause considerable disease and death worldwide, wherein infections are characterized by mild to severe neurological symptoms, such as meningitis and encephalitis [19, 83]. For Europe, Russia and up to the eastern coast of Japan, TBEV is considered as one of the most medically important arboviruses with 10,000 to 15,000 cases recorded each year [83, 84]. Since most TBFVs require at least a biosafety level 3 (BSL3) containment facility, the use of the naturally attenuated LGTV provides a convenient BSL2 model of TBEV and other highly pathogenic TBFVs [19]. In this study, I investigated the virucidal activity of longicin P4 against LGTV, a member of TBEV serocomplex of the Flaviviridae family.

3.2 Materials and methods

3.2.1 Cell culture and virus

BHK-21 cells (ATCC CCL-10) were maintained in EMEM (Wako) containing 10% FBS (Equitech) and 1% antibiotic/anti-mycotic (Nacalai Tesque), while HeLa cells (ATCC CCL-2) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co., Japan) supplemented with 10% FBS, 1% antibiotic/anti-mycotic and 1% L-glutamine (Wako). Both cells were maintained at 37 °C under 5% CO_2 until use.

The LGTV TP21 used in this study was amplified in BHK cells and the virus stock titer was determined by FFA as previously described [57] with some modifications. Briefly, serial 10-fold dilutions of the virus stock were plated on 1×10^5 cells/well of BHK-21 cells in 24-well plates. The infected cells were overlaid with 1.5% methylcellulose containing MEM (Gibco) with 1% FBS and 1% antibiotic/anti-mycotic. Viral foci were detected by a primary antibody against Langat virus surface proteins (hyper immune mouse polyclonal IgG) followed by Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen), 3-4 days after infection. The number of foci was counted using a fluorescence microscope and the titer of virus stock was expressed as FFU. The virus

stock was then aliquoted and stored at -80˚C.

Human adenovirus 25 (ATCC VR-1103) was propagated in HeLa cells and the virus stock titer was quantitated by a 50% tissue culture infectious dose ($TCID_{50}$) assay as previously described [85] with some modifications. Briefly, serial 10-fold dilutions of the virus stock were plated (8 wells per dilution) on 1×10^4 cells/well of HeLa cells in 96-well plates. Cytopathic effect was scored 6-7 days after infection. The TCID50 was calculated as the inverse of the dilution at which 50% of the wells showed cytopathic effect, calculated by the method of Reed & Muench [86].

3.2.2 Ticks and animals

Adult parthenogenetic (Okayama strain) H. longicornis ticks were used in this study. These ticks were maintained for several generations by feeding on the ears of Japanese white rabbits (Kyudo, Japan) at the Experimental Animal Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan [57]. The rabbits were solely used for tick feeding and were not infected with any virus at any point during the conduct of this study. The use of the animals in my experiments was in accordance with the approved guidelines from Animal Care and Use Committee of Kagoshima University (approval number VM 15055).

3.2.3 Peptide synthesis

Peptides were synthesized using a Perkin-Elmer Applied Biosystems 431 A Synthesizer with prederivatized polyethylene glycol polystyrene arginine resin (Sigma Genosys, Ishikari, Japan) and double coupling for residues. The reduced peptides were purified using reverse HPLC. The partial peptides are as follows: longicin P1 (QDDESDVPHVRVRRG 15 mer, Mw: 1764.8, pI: 5.43) and longicin P4 (SIGRRGGYCAGIIKQTCTCYR 21 mer, Mw: 2306.7, pI: 9.50). Peptide purity and integrity were assessed by MALDI-TOF Mass. The peptides were dissolved in normal saline (0.85% w/v of NaCl) with a final concentration of 1 mmol/ml. The solutions were stored at -30˚C until use [81].

3.2.4 Cell proliferation assay

The CellTiter 96® Non-Radioactive Cell Proliferation Assay System (Promega, USA) was used to examine the toxicity of longicin P1 and P4 peptides on BHK-21 cells as per manufacturer's instructions. In brief, BHK-21 cells grown in EMEM were harvested and resuspended in a fresh medium at a final concentration of 1×10^5 cells/ml. Fifty microliters of the cell suspension was dispensed into each well of a 96-well microtiter plate containing an equal volume of either the two-fold serially diluted

longicin P1 and P4 peptides or only the growth medium. After 72 h of incubation, dye solution was added to each well followed by 4 h of additional incubation. The reaction was then stopped by adding a solubilization solution, and absorbance was recorded at 570 nm in a microplate reader. Cytotoxicity was expressed as the percentage inhibition of cell growth (%) and was calculated as follows: Percentage cell growth inhibition (%) = (1–A/B) × 100, where A and B represent the absorbance value in the presence or absence of the peptide [80].

3.2.5 Focus formation unit reduction assay

Antiviral activity of longicin peptides was determined by measuring the reduction in the number of viral foci. Briefly, BHK-21 cells were prepared in 24-well plates (1×10^5 cells/ml). The infected cells were overlaid with 1.5% methylcellulose containing MEM with 1% FBS. Viral foci were detected by a primary antibody against Langat virus surface proteins (mouse polyclonal IgG) followed by a secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG), three to four days after infection. The number of foci was counted using a fluorescence microscope and the titer of virus was expressed as FFU. The baseline for medium-treated infected cells was the mean of the viral foci number ± SD. Then the percentage of foci reduction (RF%) compared to

medium-treated cells was calculated as follows: RF(%) = (C-T) × 100/C. Where, C is the mean of the number of foci in medium-treated wells (without peptide) and T is the mean of the number of foci in peptide-treated wells [87].

3.2.6 Virucidal assay

Extracellular anti-LGTV activity of longicin P1 and P4 peptides was investigated by incubating 0.01 multiplicity of infection (MOI) of LGTV suspension with 1.25 μM longicin P1 or P4 for 2 h at 37°C. For each assay, an EMEM-treated LGVT and an EMEM only control culture were included. Then, BHK-21 cells in 24-well plates were infected with the treated viral suspension for 1 h at 37°C. Cells were washed with PBS to remove the unadsorbed viruses. Then, the plates were incubated at 37°C for 3-4 days. Antiviral activity was determined by the reduction in number of viral foci [87]. For the dose-dependent virucidal assay, LGTV was treated with two-fold dilutions of longicin P4 starting from 0.16 to 2.5 μM for 2 h at 37°C. On the other hand, to determine the time-dependent effect of longicin P4, LGTV was treated with 1.25 μM at different exposure times (0, 15, 30, 60, 120, 240 mins) at 37°C. Foci forming unit reduction assay was used to determine the dose- and time-dependent antiviral effects of longicin P4 peptides.

The virucidal activity of longicin P4 was also tested against a non-enveloped virus by incubating 10 $TCID_{50}$ of adenovirus suspension with 1.25 μM of longicin P1 or P4 for 2 h at 37°C. For this assay, a DMEM-treated adenovirus and a DMEM only control culture were included. Then, HeLa cells in 24-well plates were infected with the treated viral suspension for 1 h at 37°C. Cells were washed with PBS to remove the unadsorbed viruses. The microplate was then incubated at 37°C and observed daily under inverted microscope until CPE was more than 50% in the virus group. Images were taken 6-7 days after infection and at same time, respective supernatants were collected for titration.

3.2.7 Prophylactic antiviral assay

The prophylactic antiviral assay was performed by treating BHK-21 cells in 24-well plates with EMEM or 1.25 μM of longicin P1 or P4 for two hours at 37°C prior to virus infection. After washing with PBS, cells were infected with 0.01 MOI of LGTV for one hour for virus adsorption. Then cells were washed by PBS and overlaid with 1.5% methylcellulose containing MEM with 1% FBS. After 3-4 days after infection, the antiviral activity was determined by foci reduction as described above [87].

3.2.8 Post-adsorption antiviral assay

The antiviral activity of longicin peptides against intracellular replication of LGTV was performed by treating BHK-21 cells in 24-well plates with EMEM or 1.25 μM of longicin P1 or P4 after virus adsorption of 0.01 MOI of LGTV for 1 h at 37°C. Unadsorbed viruses were washed with PBS and then the cells were treated with medium or 1.25 μM of peptides for 3-4 days. Antiviral activity was determined by viral foci reduction assay as described above [87].

3.2.9 Virus yield reduction assay

Briefly, BHK-21 cells in 24-well plates were infected at a MOI of 0.01 with the LGTV pre-treated with longicin P1, P4 or medium for two hours at 37°C. After 1 h of viral adsorption at 37°C, cells were washed with PBS to remove the unabsorbed viruses and consequently replaced with EMEM with 1% FBS. Three days post-infection, supernatant from the infected cells were collected for titration [88].

For the adenovirus virucidal assay, virus yield was quantitated by a 50% tissue culture infectious dose ($TCID_{50}$) assay. Briefly, HeLa cells infected with adenovirus, which were previously treated with longicin P1, P4 and medium were sampled at 7 days after infection. Supernatants were harvested and centrifuged to remove debris. Serial

10-fold dilutions of the supernatants were plated (5 wells per dilution) on 1×10^4 cells/well of HeLa cells in 96-well plates. Cytopathic effect was scored 6-7 days post-infection. The $TCID_{50}$ was calculated as the inverse of the dilution at which 50% of the wells showed cytopathic effect, calculated by the method of Reed and Muench [86].

3.2.10 RNA interference (RNAi) and subsequent virus challenge

The PCR primers used for the synthesis of double-stranded RNA (dsRNA) are listed in Table 3.1. The longicin fragments were amplified by PCR from cDNA clones using oligonucleotides including T7-forward and T7-reverse primers to attach the T7 promoter recognition sites on both the forward and reverse ends. The firefly luciferase (Luc) was amplified from a vector DNA of pGEM-luc (Promega, Madison, WI, USA) through PCR using oligonucleotides containing T7-forward and T7-reverse primers. PCR products were purified using the GENECLEAN II kit (MP Biomedicals, Ohio, USA). The T7 RiboMAX Express RNA System (Promega) was used to synthesize dsRNA by *in vitro* transcription following the manufacturer's protocol. Successful construction of dsRNA was confirmed by running 0.5 μl of the dsRNA products in a 1.5% agarose gel in a TAE buffer. Microinjection of dsRNA was performed as previously described [89]. Briefly, 1 μg of *longicin* dsRNA in 0.5 μl of distilled water

was injected into the hemocoel of unfed adult female ticks through the fourth coxae, while *Luc* dsRNA was injected in the control group. A total of 92 ticks per group were injected. After injection, the ticks were held for 18 h in a 25°C incubator to check for mortality resulting from possible injury during injection. To initially confirm gene-specific silencing, 3 ticks from each group were collected at 0 and 4 dpi, and then total RNA was prepared for RT-PCR. RNA extraction was performed as previously described [89] and PCR was carried out using *longicin* and *actin* gene-specific primers (Table 2). PCR products were subjected to electrophoresis in 1.5% agarose gel in a TAE buffer, and bands were visualized after staining the gel with ethidium bromide using Quantity One 1-D Analysis Software (Quantity One Version 4.5, Bio-Rad Laboratories, Milan, Italy). Positive confirmation of longicin silencing was monitored until 60 dpi of *longicin* dsRNA.

To further check the *longicin* gene silencing efficiency, expression analysis of the *longicin* mRNA was also performed through real-time PCR using THUNDERBIRD™ SYBR® qPCR Mix (TOYOBO) with a 7300 real-time PCR system (Applied Biosystems), as previously described [60]. Briefly, gene-specific primers were designed to target *the H. longicornis longicin* and ribosomal protein *L23* (internal control) genes, as shown in Table 3. Four-fold serial dilutions of cDNA of adult ticks

were used to generate standard curves. The real-time PCR conditions were as follows: 95 °C for 10 min, 40 cycles of a denaturation step at 95 °C for 15 s, and an annealing/extension step at 60 °C for 60 s. The amount of *longicin* expressions was divided by the amount of *L23* expressions for both respective groups to obtain the normalized *longicin* expressions. Each sample was run in triplicates and the data were analyzed using the 7300 system SDS software (Applied Biosystems).

The percentage of gene silencing efficiency (%) of ticks injected with *longicin* dsRNA compared to *Luc* dsRNA-injected ticks was calculated as follows: Gene silencing efficiency (%) = (1–LLong/LLuc) × 100. Where, LLong and LLuc represent the normalized *longicin* expressions of *longicin* dsRNA-injected and *Luc* dsRNA-injected ticks, respectively [90].

Lastly, at 4 dpi of longicin dsRNA, both *longicin* gene-silenced and *Luc* gene-injected ticks were challenged with LGTV (approximately 2.9×10^4 ffu/0.5 μl) via percoxal microinjection. Right after the challenge, the ticks were held for 18 h in a 25°C incubator to check for mortality resulting from possible injury during injection. Thirty ticks per group were monitored for tick mortality for up to 35 days after challenge (dac), while the remaining ticks per group were used for virus titration done at 0, 1, 3, 7, 14, 21, 28 dac.

3.2.11 Statistical analysis

Data were statistically analyzed using Student's *t*–test wherein *P* values less than 0.05 were regarded as significant. All samples were tested at least in triplicate.

The Mantel-Cox log-rank test was also performed using GraphPad Prism software to determine significant difference in mortality ($P < 0.05$) between control and longicin gene-silenced ticks challenged with LGTV.

3.3 Results

3.3.1 Cytotoxicity activity of the longicin P4 peptide

To eliminate the possibility that foci reduction was due to reduction in the number of viable host cells, I examined the cell growth inhibitory effect of partial peptides longicin P1 and P4 on BHK-21 cells (Fig. 3.1). Longicin P1 did not show any significant cytotoxicity on BHK-21 cells. In contrast, longicin P4 only demonstrated a non-significant cytotoxic effect at 1.25 μM concentration. Based on these results, the 1.25 μM concentration was used for both peptides in the succeeding tests.

3.3.2 Antiviral effect of longicin P4 peptide against LGTV

Since defensin molecules accumulate in microbial membranes resulting in formation of pores in the targeted membrane, I checked first the extracellular virucidal activity of longicin peptides against LGTV. As shown in Fig. 3.2A, co-incubation of 1.25 μM longicin P4 with the virus at 37°C for two hours reduced the number of fluorescence-positive viral foci which is equivalent to a 70% foci reduction (Fig. 3.2B). However, the same effect was not observed from longicin P1 treatment.

Likewise, to further support that longicin P4 has a virucidal activity against LGTV, I further checked if the longicin P4 can lower down the virus yield post-infection as compared to medium- and longicin P1-treated LGTV. As shown in Fig. 3.2C, cells infected with virus treated with longicin P4 produced almost two-fold lower titer as compared to cells infected with either medium- or longicin P1-treated virus. Although the difference may seem low, the virus titer of longicin P4 group corresponds to more than 90% foci reduction in contrast to medium or longicin P1 treatment.

In addition, to further support that the possible mechanism of action of longicin P4 against LGTV is through extracellular virucidal activity and exclude alternative possibilities, I also conducted prophylactic and post-adsorption antiviral assays. In the

prophylactic antiviral assay, cells exposed to longicin P1 and P4 for two hours at 37°C prior to virus infection showed statistically non-significant foci reduction at 0.76% and -1.820%, respectively (Fig. 3.3A). Similarly, no significant antiviral activity was also recorded for both longicin peptides against LGTV in the post-adsorption antiviral assay, wherein, longicin P1 and P4 showed 0.71% and 4.4% foci reduction, respectively (Fig. 3.3B). Moreover, after establishing that co-incubation of longicin P4 with LGTV can successfully reduce foci formation and virus yield, I then checked the dose-dependent and time-dependent virucidal capacity of longicin P4. As shown in Fig. 3.4A, 1.25 μM of longicin P4 can significantly produce more than 50% foci reduction against LGTV, while the lowest concentration to show a significant foci reduction was 0.65 μM. Likewise in Fig. 4.4B, I can also observe that before adding to the cells, at least 30 minutes of close contact between the virus and longicin P4 at 37°C is required to achieve significant foci reduction and the optimum virucidal activity can be achieved at 2 h treatment.

3.3.3 Antiviral effect of longicin P4 peptide against human adenovirus

My results showed that longicin P4 has a virucidal activity against LGTV and it is supported by previous findings that suggest that cationic antimicrobial peptides

such as longicin P4 can only target pathogens possessing a membrane [47, 48]. Thus, to check if longicin P4 can only exert antiviral activity against membrane-bound or enveloped viruses, I determined its virucidal activity against a non-enveloped virus. As shown in Fig. 3.5A, co-incubation of 1.25 μM of longicin P4 and P1 failed to reduce adenovirus infectivity leading to a successful viral infection and marked cell death. In addition, no significant difference can be observed in the virus yield from medium-, P1- and P4-treated adenovirus (Fig. 5.5B).

3.3.4 Effect of longicin gene silencing in LGTV replication in adult H. longicornis

To evaluate the importance of longicin in the innate immunity of *H. longicornis* adult ticks against LGTV, gene silencing through RNAi was performed. Ticks were individually injected with either *longicin* dsRNA or with *Luc* dsRNA for the control group. Silencing of *longicin* gene was confirmed visually by regular RT-PCR and gel electrophoresis (Fig. 3.6A). In addition, gene silencing efficiency was also determined using real-time PCR (Fig. 3.6B), wherein more than 90% of *longicin* mRNA reduction can be observed for at least 60 days after microinjection of *longicin* dsRNA.

Although mean virus titers from whole ticks (Fig. 3.6C) demonstrate significant differences at 7 and 21 dac between *longicin* dsRNA and *Luc* dsRNA

injected ticks, the titers for both groups eventually equalized at the end of the observation period. In addition, no significant difference was observed in tick mortality on both groups after 35 dac (Fig. 3.6D).

3.4 Discussion

Several studies have already shown the antimicrobial, fungicidal, and parasiticidal activities of longicin and one of its synthetic partial analogs (longicin P4). A previous study has shown that some known defensins have a common motif (G3RRGG5) which could be related to their antiparasitic activity [91]. Such motif was also found in longicin P4 suggesting that this motif is the one responsible for its antiparasitic activity [81, 92]. Since longicin P4 has been shown to possess antimicrobial, fungicidal, and parasiticidal properties, I decided to test the peptide for its antiviral activity against a tick-borne flavivirus.

In this study, my data show that co-incubation treatment of LGTV with longicin P4 at 1.25 μM concentration prior to infection resulted in significant foci reduction. And to clarify if this antiviral activity is also present in the full-length (FL) longicin peptide, I also checked the antiviral capacity of the FL longicin against LGTV. Initially, I determined first the non-cytotoxic concentration of FL longicin on BHK cells

and used this concentration as the treatment concentration. As expected, FL longicin also exhibited an almost 40% viral foci reduction (Fig. 3.7).

On the other hand, the virus yield from cells infected with virus co-incubated with longicin P4 produced lower titer as compared to cells infected with either medium or longicin P1 treated virus, resulting in more than 90% foci reduction as compared to medium or longicin P1 treatment. The lower virus yield from cells infected with virus co-incubated with longicin P4 further confirm that the virucidal activity of longicin P4 is extracellular, through close contact, as previously shown in another study that longicin P4 impairs parasite membranes (*Toxoplasma gondii*) resulting in the reduction of infection in cells [81]. In addition, the failure of longicin P4 to significantly reduce foci formation in the prophylactic and post-adsorption antiviral assays clearly supports that the antiviral activity of the peptide is exerted through extracellular inactivation of the virus particle. LGTV, being an enveloped virus, has an outer coating that is composed of a lipid bilayer. Viral envelope can be a target for longicin as defensin molecules accumulate in microbial membranes resulting in the formation of pores in the targeted membrane [93]. However, additional experiments to show the binding between extracellular virus and longicin P4 are needed to fully establish the exact mechanism of membrane targeting of longicin in enveloped viruses. Nevertheless, to confirm that the

effect of longicin P4 may only be limited to membrane-bound targets, a common trait for cationic antimicrobial peptides, I tested its virucidal activity against human adenovirus 25, a non-enveloped virus. As shown in Figure 3.5, longicin P4 failed to inhibit virus replication, thus supporting the claims that although cationic antimicrobial peptides have diverse targets, their activity is generally limited to targets with membranes [48, 92].

However, previous findings by Smith and Nemerow [94], showed that human α-defensin can inhibit adenovirus infection by directly binding to non-enveloped adenoviral capsid, inhibiting virus disassembly. Such binding ultimately leads to inhibition of endosomal membrane penetration during cell entry. This finding further explains that the virucidal activity of longicin P4 may only be limited to enveloped viruses and the synthetic peptide does not target the capsid of adenovirus. Likewise, it is also possible to suppose that longicin P4 can directly bind on the membrane of LGTV without disrupting the viral envelope, and in effect inhibits the binding of the virus to cellular receptors for viral entry. However, this mechanism remains to be elucidated.

Lastly, to fully elucidate the importance of longicin in the innate immunity of *H. longicornis* ticks against LGTV, gene silencing through RNAi was performed. After successfully silencing the *longicin* gene, ticks were challenged with LGTV via

microinjection. Preliminary results on the effect of longicin gene silencing on virus titer show that significant difference can be observed at 7 and 21 dac. At 7 dac, *longicin* gene-silenced ticks produced higher viral titer as compared to *Luc* dsRNA injected ticks, which may be attributed to longicin gene silencing. However, at 21 dac, *longicin* gene-silenced ticks showed lower viral titer as compared to the control. Although a relatively high gene silencing efficiency (more than 90%) can be observed for at least 60 dpi, the lower viral titer in the longicin gene-silenced group at 21 dac remains to be answered, thus suggesting that the effect of *longicin* gene silencing on the survival dynamics of LGTV in vivo still remains unclear. In addition, a complete knockdown of the longicin gene may also be needed to fully assess the function of longicin against LGTV in vivo.

On the other hand, for the whole duration of the study, no significant difference in mortality was observed on both *longicin* gene-silenced and *Luc* dsRNA-injected ticks. Failure to observe any significant effect on tick mortality on the *longicin*-silenced ticks, may suggest that the activity of longicin in the tick is not related to the control LGTV infection or the virus model used for this study may not be suitable to test my hypothesis. Likewise, the sturdiness of ticks against any harmful effect due to LGVT infection may be expected since ticks act as highly efficient reservoirs of flaviviruses

[95]. Moreover, the failure of *longicin* silencing to affect the tick's resistance to LGTV suggests that the peptide's activity may only be effective *in vitro*. As previously observed, activities of antimicrobial peptides can be undesirably affected by various relevant factors present in vivo and these may include proteases, polyanions and high mono- and divalent cation concentrations [49].

In summary, this chapter established the extracellular virucidal activity of longicin P4 against LGTV in vitro, and to our knowledge, this is the first report of an antiviral activity of a native or synthetic antimicrobial peptide derived from *H. longicornis*. However, the role of the endogenous tick longicin in the antiviral defense of *H. longicornis* still remains to be demonstrated.

Tables and Figures in CHAPTER 3

Table 3.1 List of PCR primers used for the synthesis of double-stranded RNA.

PrimerName	Primer sequence
Longicin RNAi forward	CCTCATCTTCGTCCTTGTAG
Longicin RNAi reverse	ATTATGACGACACACATAAT
Longicin T7 forward	GGATCCTAATACGACTCACTATAGGCCTCATCTTCGTCCTTGTAG
Longicin T7 reverse	GGATCCTAATACGACTCACTATAGGATTATGACGACACACATAAT
Luc T7 forward	GTAATACGACTCACTATAGGGCTTCCATCTTCCAGGGATACG
Luc T7 reverse	GTAATACGACTCACTATAGGCGTCCACAAACACAACTCCTCC

Table 3.2 List of PCR primers used for the detection of *longicin* gene

Primer Name	Primer sequence
Longicin forward	ATGAAGGTCCTGGCTGTTGC
Longicin reverse	CTACTTGCGGTAGCACGTGC
Hlβ-actin forward	ATCCTGCGTCTCGACTTGG
Hlβ-actin reverse	GCCGTGGTGGTGAAAGAGTAG

Table 3.3 List of real-time PCR primers used for the determination of *longicin* gene

Primer Name	Primer sequence
Longicin real-time forward	ACATGAAGGTCCTGGCTGTTG
Longicin real-time reverse	TCTCGTCATCTTGAGCTGCTG
L23 real-time forward	CACACTCGTGTTCATCGTCC
L23 real-time reverse	ATGAGTGTGTTCACGTTGGC

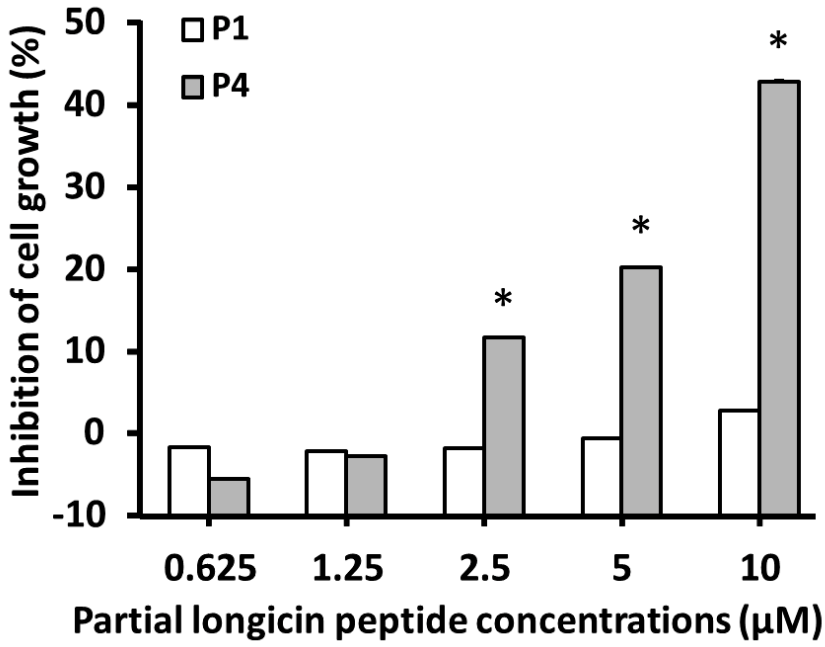

Fig. 3.1 Cytotoxicity of longicin P1 and P4 against BHK-21 cells. MTT assay was used to evaluate the cytotoxicity of the peptides. Values are representative of triplicate samples and error bars indicate the range of values obtained. **P* < 0.05, longicin P1 vs longicin P4.

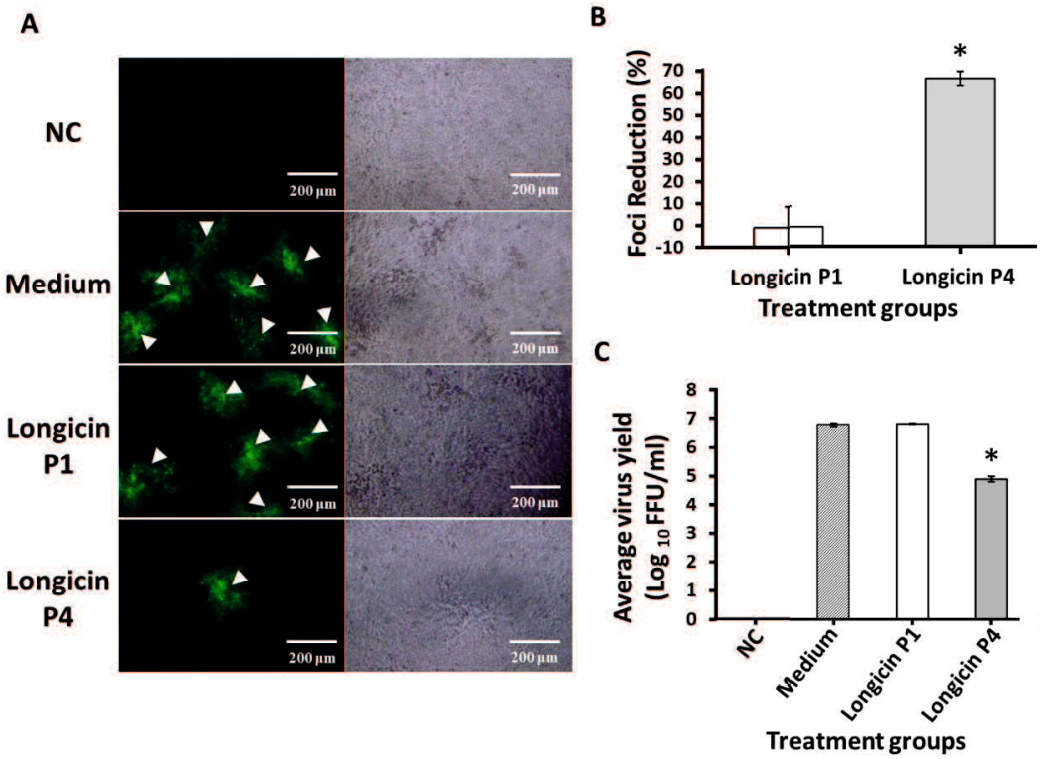

Fig. 3.2 Virucidal effect of longicin P1 and P4 against Langat virus. (a) Fluorescence images of BHK-21 cells infected with Langat virus (TP-21) treated with medium only, 1.25 µM of P1 and P4 for 2 h at 37 ˚C. Arrowheads point to positive fluorescence FFU. (b) Foci reduction and (c) yield reduction assays were used to determine extracellular virucidal effect of longicin peptides. The percentage of foci reduction (%) was obtained by comparing against medium-treated cells maintained in parallel. All experiments were conducted in triplicates and error bars indicate the range of values. NC refers to cells with no treatment and no infection. *$P < 0.05$, longicin P4 vs longicin P1 or medium.

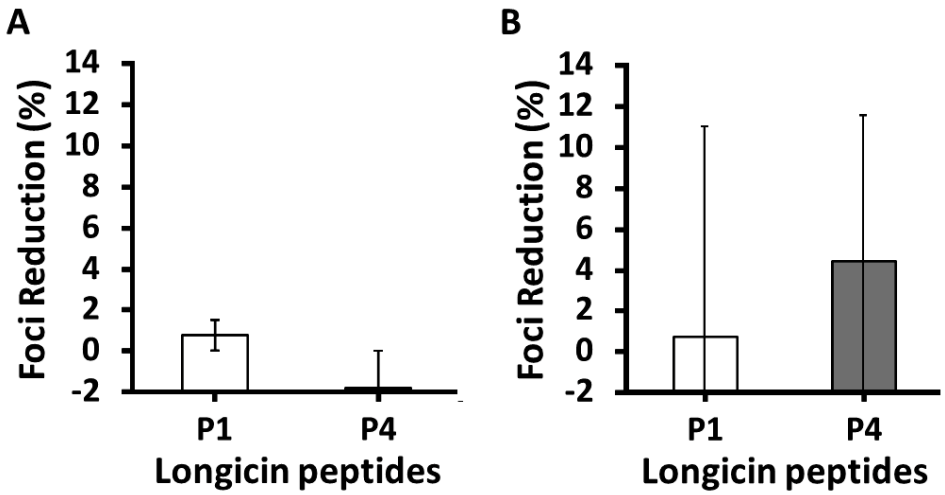

Fig. 3.3 Prophylactic (a) and post-adsorption (b) antiviral effects of longicin P1 and P4 against Langat virus. Experiments were conducted in triplicates and error bars indicate the range of values. The percentage of foci reduction (%) was obtained by comparing against medium-treated cells maintained in parallel. *$P < 0.05$, longicin P4 vs longicin P1 or medium.

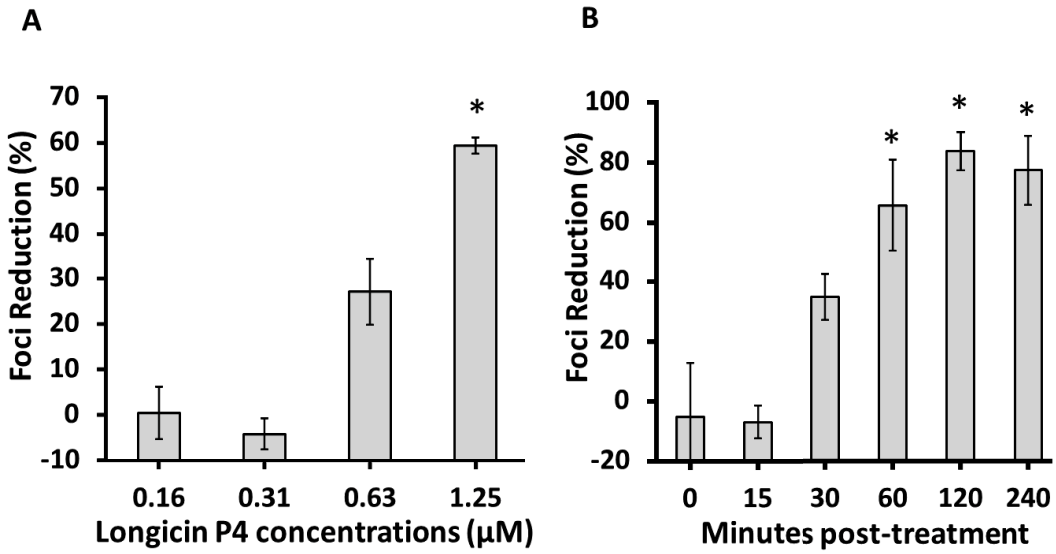

Fig. 3.4 Dose-dependent (a) and time-dependent (b) virucidal effects of longicin P4 against Langat virus. Experiments were conducted in triplicates and error bars indicate the range of values. The percentage of foci reduction (%) was obtained by comparing against medium-treated cells maintained in parallel. *$P < 0.05$, as compared to the lowest concentration or to 0 min.

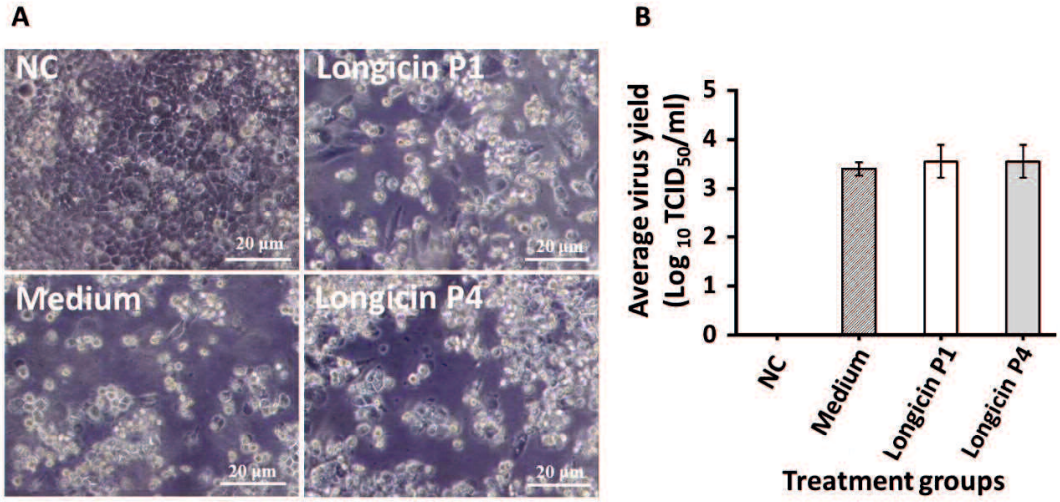

Fig. 3.5 Virucidal activity of longicin P1 and P4 against adenovirus. (a) Images of HeLa cells infected with human adenovirus 25 treated with medium only, 1.25 µM of longicin P1 and P4 for 2 h at 37 ˚C. Images were taken 7 d after infection (200 x magnification). (b) $TCID_{50}$ was used to determine the virus yield titers of the collected supernatants of the treatment groups. Data expressed as means ± SD.

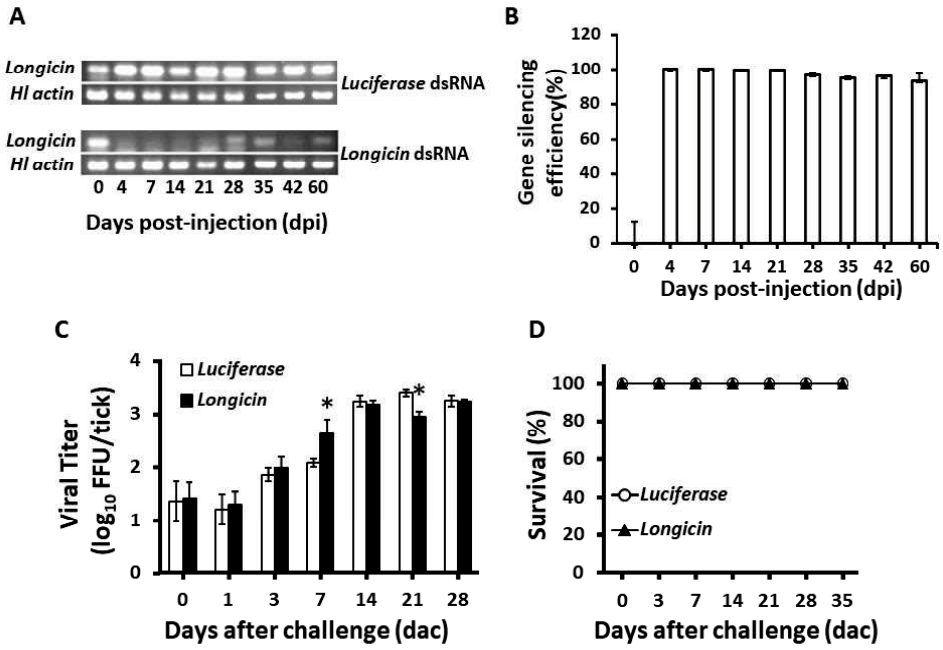

Fig. 3.6 Effect of *longicin* silencing in tick mortality and virus titer. (a) To confirm gene-specific silencing, 3 ticks from each group were collected at 0, 4, 7, 14, 21, 28, 35, 42 and 60 dpi of dsRNA. Initial confirmation of *longicin* silencing was carried out through RT-PCR and gel electrophoresis (a), while gene silencing efficiency was determined by real-time PCR (b). Virus titers (c) and tick survival (d) were monitored after injecting LGTV on 4-day *luciferase* dsRNA- or *longicin* dsRNA-inoculated ticks. Values for mortality (n=30 ticks per group) were expressed as the percentage of live ticks remaining to the number of ticks used at the beginning of the experiment in different time courses. Significant difference (*$P <$ 0.05) was determined using the Mantel-Cox log-rank test, while error bars in virus titers indicate SD in mean values of 5 ticks. *$P < 0.05$, *luciferase* vs *longicin*.

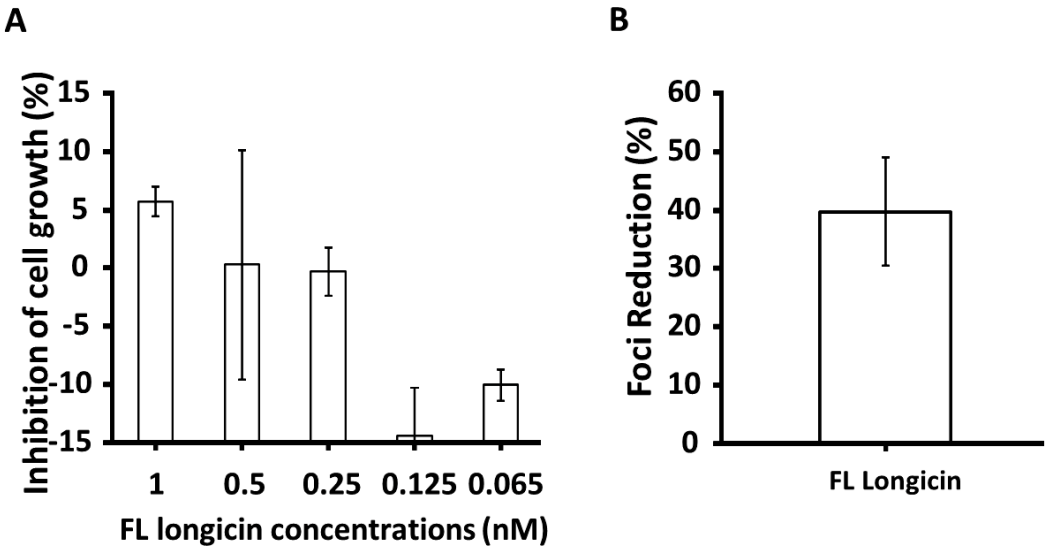

Fig. 3.7 Virucidal effect of full-length (FL) longicin against LGTV. Foci reduction assay was used to determine the extracellular virucidal effect of baculovirus-expressed FL longicin peptide using 0.5 nm concentration. Based from the cell proliferation assay (A), 0.5 nM of FL longicin showed no significant cytotoxicity on BHK cells that may affect the result of the foci reduction assay. (B) The percentage of foci reduction (%) was obtained by comparing against medium-treated cells maintained in parallel. All experiments were conducted in triplicates and error bars indicate the range of values.

CHAPTER 4

Evaluation of the role of antimicrobial peptide, HEdefensin, from *Haemaphysalis longicornis* against Langat virus

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4.1 Introduction

Ticks, being obligate hematophagous ectoparasites, are usually exposed to several pathogenic microorganisms and parasites [1, 8, 30, 31]; thus, they must possess broad-spectrum innate immunity mechanisms that can sustain pathogens and commensal microbes without any adverse effect on their viability and biological development [30, 32].

Antimicrobial peptides (AMPs) are important innate immunity molecules widely found in invertebrates and vertebrates [45, 46]. Genes of AMPs are normally expressed mainly in mucosal surfaces and skin, while AMPs are found at least as often in bodily fluids and primarily lyse bacteria, yeast, fungi, protozoan parasites and enveloped viruses by disrupting their biological membranes [46 - 49]. However, they also demonstrate immunomodulatory properties [50, 96]. AMPs play a major role in protecting ticks against microorganisms [43, 44]. Several AMPs have already been identified in ticks, and they have been shown to have broad biological activities against a wide range of pathogens [31]. Among the identified and widely characterized tick AMPs, defensins and their isoforms have been considerably well studied [32, 43, 44, 80, 81, 97 - 103]. Generally, arthropod defensins are cationic AMPs that contain six disulfide-paired cysteines [104 – 107]. In the hard tick *Haemaphysalis longicornis*,

longicin, a defensin-like, cationic AMP, has been shown to have biological activities against Gram-positive and -negative bacteria, fungi, protozoa and enveloped viruses through membrane disruption [32, 81, 108]. In this study, I focused on characterizing a new defensin-like peptide deduced from a *defensin*-like gene identified from the expressed sequence tags (EST) database of hemolymph from *H. longicornis*. Furthermore, multiple sequence alignments between the newly identified defensin-like peptide and known defensin-like peptide sequences from ticks were also generated.

I evaluated through real-time PCR the gene transcription of the defensin-like gene in the adult tick and in different organs during blood feeding and after Langat virus (LGTV) challenge. LGTV is a naturally attenuated member of the tick-borne encephalitis virus (TBEV) complex [109]. For most tick-borne flaviviruses (TBFVs), a biosafety level 3 (BSL3) containment facility is commonly required; however, the use of LGTV offers a convenient BSL2 model of highly pathogenic TBFVs [19]. I previously reported that the partial peptide of longicin exhibited anti-LGTV activities [108]; thus, I also tested the antiviral activity of the synthetic peptide against LGTV. Finally, in this Chapter, I demonstrated the antiviral activity of the newly identified defensin-like peptide against LGTV *in vitro*, thus making the peptide a possible candidate for a therapeutic agent against tick-borne flaviviruses.

4.2 Materials and Methods

4.2.1 Ticks and animals

Parthenogenetic *H. longicornis* (Okayama strain) ticks were maintained for several generations by feeding on the ears of Japanese white rabbits (KBT Oriental Co., Japan) at the Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan [55]. The use of animals in our experiments was in accordance with the approved guidelines (approval number VM 15055) from the Animal Care and Use Committee of Kagoshima University.

4.2.2 Sequence of the defensin-like peptide and bioinformatic analysis

The construction of a full-length cDNA library of *H. longicornis* was accomplished using the vector-capping method [110], while the expressed sequence tags (EST) were prepared in our laboratory as described previously [111]. cDNA clones encoding for a defensin-like gene were identified and selected from the EST database of the hemolymph for further investigation.

Using a Qiagen Plasmid Mini Kit (Qiagen, Germany), a pGCAP1 plasmid containing a *defensin*-like gene-encoding insert was extracted and subsequently analyzed using plasmid-specific primers through an automated ABI PRISM 3500XL Genetic Analyzer (Applied Biosystems, USA) for sequence determination. A

GENETYX-WIN DNA analysis software system (Genetyx, Japan) was used to determine the deduced amino acid sequence of the defensin-like gene. The predicted molecular mass, isoelectric point (pI) and net charge for the peptide were determined using the ProtParam server (http://web.expasy.org/cgi-bin/protparam/protparam), while the signal peptide was predicted and possible glycosylation sites were located using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) and the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively.

Finally, the protein homology search for the translated amino acids was performed using the Protein BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and multiple sequence alignments between the newly identified defensin-like peptide and known defensin-like peptide sequences from ticks were generated using the ClustalW 2.1 server (http://clustalw.ddbj.nig.ac.jp/) alignment program.

4.2.3 Expression profile analysis

The expression of the *defensin-like* gene in whole ticks and selected tick tissues such as salivary glands, midgut, ovary, fat body, synganglion and hemocytes was analyzed by real-time PCR using *defensin-like* gene-specific primers (Table 4.1). Total RNA was extracted from the above-mentioned tissues of adult female ticks before,

during and after blood feeding. Hemocytes were collected as previously described [55,61]. Dissected tissues and collected hemocytes were washed in PBS, placed directly in tubes containing TRI Reagent (Sigma-Aldrich, USA) and then disrupted using a pellet pestle motor. For the whole ticks, samples were collected before, during and after blood feeding. The collected samples were later homogenized using an Automill homogenizer (Tokken, Japan), and then TRI Reagent was added. mRNA expression was also determined after LGTV challenge by real-time PCR. After injecting LGTV (approximately 2.9×10^4 ffu/0.5 µl) directly into the hemocoel of each adult tick through the fourth coxae, three ticks from the LGTV-challenged group were collected 2, 4, 6, 12, 24, 48 and 72 h post-infection. Non-infected ticks served as a control group, and tick homogenization was done as described previously.

RNA extraction and the expression analysis of the *defensin-like* mRNA via real-time PCR using THUNDERBIRD™ SYBR® qPCR Mix (TOYOBO) with a 7300 real-time PCR system (Applied Biosystems) were performed as previously described [60]. Briefly, gene-specific primers were designed to target *H. longicornis HEdefensin* and ribosomal protein *L23* (internal control) genes, as shown in Table 2. To generate standard curves, four-fold serial dilutions of the cDNA of adult ticks were used. Real-time PCR conditions were as follows: 95°C for 10 min, 40 cycles of a denaturation

step at 95°C for 15 s and an annealing/extension step at 60°C for 60 s. Each sample was run in triplicate, and the data were analyzed using the 7300 System SDS software (Applied Biosystems, Japan). Lastly, normalized gene expressions were computed by dividing the amount of *defensin-like* gene expressions by the amount of *L23* expressions for each sample.

4.2.4 Peptide synthesis

The defensin-like peptide was synthesized using a Perkin-Elmer Applied Biosystems 431 A Synthesizer with prederivatized polyethylene glycol polystyrene arginine resin, FastMoc chemistry and double coupling for residues as discussed in Chapter 3. The reduced peptides were purified using reverse-phase high-performance liquid chromatography (RP-HPLC), while peptide purity and integrity were assessed using MALDI-TOF mass spectrometry (MALDI-TOF MS) [81]. The peptide was dissolved in normal saline (0.85% w/v of NaCl) with a final concentration of 1 mmol/ml and stored at -80°C until use.

4.2.5 Cells and virus

HeLa cells were maintained in DMEM (Nissui Pharmaceutical Co.) supplemented with 10% FBS (Equitech-Bio), 1% antibiotic/antimycotic (Nacalai Tesque) and 1% L-glutamine (Wako), while baby BHK-21 cells were maintained in EMEM containing 10% FBS and 1% antibiotic/antimycotic. Cell cultures were maintained at 37°C under 5% CO_2 until use.

BHK-21 cells were utilized to amplify and titrate the LGTV TP21 used in this study as previously discussed in Chapter 3. Likewise, the human adenovirus 25 was propagated in HeLa cells, and a 50% tissue culture infective dose ($TCID_{50}$) assay was used to quantify the virus stock titer as previously described in Chapter 3.

4.2.6 Cell proliferation assay

Following the manufacturer's protocol, the CellTiter 96® Non-Radioactive Cell Proliferation Assay System was used to examine the cytotoxic effect of the HEdefensin peptide on BHK-21 cells as previously enumerated in Chapter 3.

4.2.7 Focus formation unit reduction assay

The antiviral activity of the defensin-like peptide was determined by measuring the reduction in the number of viral foci as previously described in Chapter 3.

4.2.8 Direct virucidal assay

To demonstrate the extracellular anti-LGTV activity of the defensin-like peptide, a 0.01 multiplicity of infection (MOI) of LGTV suspension was incubated with 5 μM of the peptide for 2 h at 37°C. BHK-21 cells in 24-well plates were infected with the treated viral suspension for 1 h at 37°C, and the cells were then washed with PBS to remove the unadsorbed viruses. The plates were then incubated at 37°C for 3–4 days. For each assay, an EMEM-treated LGVT and an EMEM-only control culture were included, and the antiviral activity was determined by the reduction in the number of viral foci [87]. The LGTV was treated with two-fold dilutions of the defensin-like peptide in EMEM, from 0.0425 to 5 μM, for 2 h at 37°C to evaluate the dose-dependent antiviral activity of the peptide. Meanwhile, the LGTV was treated with 5 μM of the peptide at 37°C for different exposure times (0, 15, 30, 60, 120 and 240 min) to determine the time-dependent antiviral effect of the defensin-like peptide.

The virucidal activity of the peptide was also tested against adenovirus, a non-enveloped virus, as previously described in Chapter 3.

4.2.9 Prophylactic and Post-adsorption antiviral assays

Evaluation of the potential prophylactic and anti-intracellular replication of LGTV by the peptide was also conducted as previously described in Chapter 3.

4.2.10 Virus yield reduction assay

The virus yield reduction for both LGTV and adenovirus virucidal assay was quantitated by a $TCID_{50}$ assay as previously described in Chapter 3.

4.2.11 RNAi and virus challenge

Table 4.2 lists the PCR primers used for the synthesis of double-stranded RNA (dsRNA) with the procedure previously described in Chapter 3.

As described previously [89], 1 μg of *HEdefensin* dsRNA in 0.5 μl of distilled water was microinjected into the hemocoel of unfed adult female ticks through the fourth coxae. For the control group, *Luc* dsRNA was injected. A total of 160 ticks (80 ticks per group) were used in the dsRNA silencing confirmation and LGTV challenge.

After dsRNA injection, the ticks were held for 18 h in a 25°C incubator to check for mortality arising from possible injury after injection. Fifteen ticks from each group were exclusively allocated (non-infected) for gene-specific silencing at 4, 10, 14, 28 and 30 dpi. Three ticks per indicated time period were used to isolate total RNA for reverse-transcription PCR (RT-PCR), to confirm gene-specific silencing. RT-PCR was carried out using a *defensin-like* gene [89] and *actin* gene-specific primers (Table 4.1).

After the positive confirmation of *defensin-like* gene silencing, both groups were challenged with LGTV (approximately 2.9×10^4 ffu/0.5 µl) via percoxal microinjection 4 dpi of dsRNA. Tick mortality in 30 ticks per group was monitored for up to 35 dac, while for the virus titration done at 0, 1, 3, 7, 14, 21 and 28 dac, the 35 ticks remaining in each group were used.

4.2.12 Statistical analysis

All samples were tested at least in triplicate and statistically analyzed using Student's *t*-test, wherein *P* values of less than 0.05 were regarded as significant. Using GraphPad Prism software, the Mantel–Cox log-rank test was also performed to determine the significant difference in mortality ($P < 0.05$) between *defensin-like* and *Luc* gene-silenced ticks challenged with LGTV.

4.3 Result

4.3.1 Identification of defensin-like gene

cDNA encoding a defensin-like protein, designated as HEdefensin (*HEdefensin*; GenBank accession number LC169559), was isolated from an EST clone in the cDNA libraries of *H. longicornis*. The prefix HE denotes hemolymph, the tissue of origin of the EST database. The full-length gene consists of 566 bp that encode 74 amino acid residues (Fig. 4.1). The predicted amino acid sequence also showed a putative signal peptide composed of 23 amino acid residues. The conserved cysteine residues normally found in arthropod defensins were identified at positions 40, 47, 51, 61, 69 and 71. No glycosylation site was predicted from the predicted sequence.

Through BLAST analysis, HEdefensin was found to show high amino acid sequence identity with some known defensins from *Dermacentor variabilis* (Q86QI5, 78% identity), *D. marginatus* (ACJ00433, 77% identity), *Rhipicephalus microplus* (Q86LE4, 73% identity), *H. longicornis* (BAD93183, 68% identity), *Ixodes persulcatus* (BAH09304, 67% identity) and *I. scapularis* (XP_002436103, 65% identity) (Fig. 4.2). On the other hand, the synthesized 51-mer (signal peptide excluded) HEdefensin peptide (EEESEVAHLRVRRGFGCPLNQGACHRHCRSIRRRGGYCSGIIKQTCT

CYRN) has a predicted molecular mass of 5865.69 Da, with a theoretical isoelectric point of 9.43 and a net charge of positive 6.0 at pH 7.0.

4.3.2 Whole tick and tissue-specific expression profiles of HEdefensin in H. longicornis

HEdefensin mRNA levels in whole ticks and in various organs before, during and after blood feeding were investigated using real-time PCR. In whole ticks, unfed ticks showed the highest expression level; however, upon feeding, the initially low expression level of *HEdefensin* mRNA eventually increased to full engorgement after 4 days of feeding (Fig. 4.3A). In the midgut, fat body, ovary, synganglion, salivary gland and, most notably, hemocytes, the *HEdefensin* gene was upregulated during blood feeding, especially in the fully engorged state (Fig. 4.3B). *HEdefensin* mRNA expression was also determined post-LGTV infection using real-time PCR (Fig. 4.3C). mRNA expression increased slightly at 6, 12 and 72 h post-LGTV infection; however, no significant difference was observed between the challenged and control groups ($P < 0.05$).

4.3.3 Cell growth inhibition effect of the synthetic HEdefensin peptide

I examined the cell growth inhibitory effect of the HEdefensin peptide on BHK-21 cells (Fig. 4.4). The peptide did not significantly inhibit the growth of BHK-21 cells beginning at a 5 μM concentration. Based on the MTT assay result, the 5 μM concentration of the peptide was used in the proceeding tests.

4.3.4 Antiviral activity of the HEdefensin peptide against LGTV

I initially tested the biological activity of the peptide by evaluating its potential extracellular virucidal activity against LGTV, an enveloped virus. As shown in Fig. 4.5A, co-incubation of the virus with 5 μM of HEdefensin at 37°C for 2 h reduced the number of fluorescence-positive viral foci as compared to the medium-treated virus. Such a reduction is equivalent to a 99.2% foci reduction (Fig. 4.5B). Similarly, cells infected with LGTV treated with HEdefensin produced almost a five-fold lower virus yield as compared to cells infected with a medium-treated virus (Fig. 4.5C).

I also checked the dose- and time-dependent virucidal capability of HEdefensin. As shown in Fig. 4.6A, 0.081 μM was the lowest concentration of HEdefensin that could significantly reduce foci formation, while at least 30 min of close contact between the virus and HEdefensin at 37°C before infection of the cells is needed to produce

significant foci reduction (Fig. 4.6B). In addition, the virucidal activity of HEdefensin was maintained at the different temperatures tested, wherein the peptide showed at least a 98% foci reduction at any temperature tested from 4°C to 37°C (Fig. 4.7).

Lastly, to exclude any alternative mechanism of action of HEdefensin peptide against LGTV, I also conducted prophylactic and post-adsorption antiviral assays. As shown in Fig. 4.8, cells exposed to HEdefensin for 2 h at 37°C before virus infection showed no foci reduction as compared to the medium-treated LGTV at -0.026%. Similarly, no significant antiviral activity ($P < 0.05$) was recorded against LGTV in the post-adsorption antiviral assay, wherein the peptide showed a 0.41% foci reduction (Fig. 4.8).

4.3.5 Antiviral activity of the HEdefensin peptide against human adenovirus

To test whether the HEdefensin peptide has antiviral activity against a non-enveloped virus, I determined its virucidal activity against the human adenovirus. In Fig. 4.9A, co-incubation of the HEdefensin peptide and adenovirus prior to cell infection showed no reduction in adenovirus infectivity, similar to medium treatment. Furthermore, no significant difference ($P < 0.05$) was observed in the virus yield from the medium- and HEdefensin-treated adenovirus (Fig. 4.9B).

4.3.6 Effect of HEdefensin gene silencing on LGTV replication in adult H. longicornis

Gene silencing through RNAi was performed to evaluate the importance of HEdefensin in the innate immunity of adult female ticks against LGTV. Ticks were individually injected with either *HEdefensin* or *Luc* dsRNA for the control group. Silencing of the *HEdefensin* gene was initially confirmed 4 dpi. However, the effective gene-specific silencing was only maintained for up to 28 dpi, as shown in Fig. 4.10A. No significant differences in blood feeding, engorged body weights, egg production or egg hatching were observed between the HEdefensin- and Luc dsRNA-injected ticks (data not shown). The mean virus titers from whole ticks (Fig. 4.10B) demonstrate significant differences ($p < 0.05$) at 7 and 14 dac between *HEdefensin* dsRNA- and *Luc* dsRNA-injected ticks; however, the titers for both groups eventually equalized at 21 and 28 dac. In addition, no mortality was observed in either group 35 days post-LGTV challenge (Fig. 4.10C).

4.4 Discussion

As carriers of disease-causing agents and obligatory blood-feeders, ticks are easily exposed to several pathogenic microorganisms. With such constant threats, ticks need effective innate immunity mechanisms to coexist with their commensal microbes and pathogens [31, 32]. As previously reported, antimicrobial proteins and peptides are considered to be among the major innate immunity molecules utilized by ticks to provide protection against microorganisms [43, 44].

In this study, I have identified a defensin-like gene, HEdefensin, from the EST database of hemocytes of *H. longicornis*. The predicted signal peptide (17 amino acid residues) from the deduced amino acid sequence strongly suggests that the translated HEdefensin is a secretory peptide. The translated HEdefensin peptide also presented six conserved cysteine residues normally found in tick defensins, as shown in the amino acid sequence alignment of selected tick defensins. In addition, the peptide is considered cationic, like most defensins, since it has a predicted positive isoelectric point of 9.43 and a predicted positive net charge of 6.0. The predicted charge or isoelectric point is considered one important element in the defensin function [98, 112]. With these characteristics, I hypothesized that HEdefensin might also have biological activities comparable to other tick defensins.

In addition, the mRNA expression of *HEdefensin* was noticeably increased after blood feeding in whole adult ticks. The same pattern was observed in all collected organs, with the highest expression in hemocytes after engorgement. These observations might indicate that HEdefensin plays an important role in blood feeding. In the present study, the newly molted ticks showed the highest mRNA expression level. This indicates that right after molting, *HEdefensin* mRNA expression continues to increase. As previously reported, tick defensin expression increases after blood feeding and during molting [43, 100]. It should also be noted that during blood feeding, many possible pathogens and non-self objects can be encountered by the tick [100, 113].

Thus, to test my hypothesis, I initially checked whether the HEdefensin peptide can permeabilize a membrane-bound target. With their cationic nature, defensins are generally known to attach to the cell membranes of microorganisms and, in return, generate multimeric pores, leading to cell content leakage [48, 93]. Thus, after determining the non-growth inhibitory concentration of the HEdefensin peptide on BHK cells, I checked whether HEdefensin has antiviral activity against LGTV, an enveloped virus. The co-incubation treatment of LGTV with 5 μM of the peptide prior to infection resulted in a foci reduction of almost 100%. Moreover, the virus yield from cells infected with the virus co-incubated with HEdefensin showed significantly lower

titers as compared to cells infected with a medium-treated virus. These results indicate that the HEdefensin peptide has virucidal activity against LGTV that has the viral envelope as the potential target.

However, AMPs may also present immunomodulatory properties [50, 96]; thus, extracellular virucidal activity may not be the only biological activity of HEdefensin against LGTV. However, in our prophylactic antiviral assay, no significant viral foci reduction was observed after HEdefensin peptide treatment. A similar result was observed in the post-infection antiviral assay. Taken together, these results further support the idea that the antiviral effect of HEdefensin is most likely extracellular in nature. However, the precise mechanism of the membrane targeting of HEdefensin in enveloped viruses needs further evaluation. I also tested the virucidal effect of HEdefensin against a non-enveloped virus. As shown in Fig. 4.9, HEdefensin failed to inhibit the virus replication of human adenovirus 25, suggesting that the peptide's virucidal activity may be limited to enveloped viruses. Such an observation may not be unusual, since cationic antimicrobial peptides generally require membranes as targets to produce antimicrobial effects [48, 92].

Finally, to clearly evaluate the role of the HEdefensin gene in the antiviral immunity of *H. longicornis* ticks against LGTV, gene silencing through RNAi was

conducted. Although significant differences in virus titers were observed between the *HEdefensin* gene-silenced ticks and the control group at 7 and 14 dac, the differences were not sustained in succeeding weeks. I may be able to attribute the higher viral titer at 7 dac in the *HEdefensin*-silenced ticks to effective gene silencing; however, the lower viral titer in the *HEdefensin* gene-silenced group at 14 dac still requires explanation. Likewise, no mortality was observed in either the *HEdefensin* gene-silenced or the control group for the duration of study. Interestingly, I also observed no significant upregulation in *HEdefensin* mRNA expression post-LGTV infection, which suggests that HEdefensin may not be involved in the tick's antiviral protection against LGTV.

Conversely, mortality due to LGVT infection may be less likely, considering that ticks are tolerant of the adverse effects of LGTV, since they are efficient reservoirs of flaviviruses [95]. It was also reported that proteases, polyanions, high mono- and divalent cations and other important diverse factors present in vivo may undesirably affect the antimicrobial peptides' activities [49]. In addition, a recent study on longicin, a defensin-like gene from the midgut of *H. longicornis*, failed to demonstrate the biological activity of the longicin gene in vivo against LGTV, despite its translated peptide exhibiting significant antiviral activity against LGTV in vitro [108].

In summary, this chapter demonstrated that the recently identified *HEdefensin* gene from the hemolymph EST database of *H. longicornis* can be detected in different tick organs, chemically synthesized and biologically characterized. Although HEdefensin's role in the antiviral immunity against LGTV in ticks was not fully elucidated, the significant extracellular antiviral activity of the peptide against LGTV in vitro offers a new potential therapeutic agent for tick-borne pathogens, particularly flaviviruses.

Tables and Figures in CHAPTER 4

Table 4.1 List of PCR primers used for detection of the *HEdefensin* gene

Primer Name	Primer Sequence
HEdefensin Forward	ATGCGGGTGCTTGTGCTTT
HEdefensin Reverse	TGCCACTTCGCTTTCCTCCT
Hl-L23 Forward	CACACTCGTGTTCATCGTCC
Hl-L23 Reverse	ATGAGTGTGTTCACGTTGGC
HEdefensin Forward RT	ATGCGGGTGCTTGTGCTTT
HEdefensin Reverse RT	CGATACATGGGCGAAATTGT
Hlβ-actin Forward RT	ATCCTGCGTCTCGACTTGG
Hlβ-actin Reverse RT	GCCGTGGTGGTGAAAGAGTAG

Table 4.2 List of PCR primers used for the synthesis of double-stranded RNA

Primer Name[a]	Primer Sequence
HEdefensin RNAi For	TCGCTGTCATTCTTCTTTGC
HEdefensin RNAi Rev	CGATACATGGGCGAAATTGT
HEdefensin T7 For	TAATACGACTCACTATAGGTCGCTGTCATTCTTCTTTGC
HEdefensin T7 Rev	TAATACGACTCACTATAGGCGATACATGGGCGAAATTGT
Luc T7 For	GTAATACGACTCACTATAGGGCTTCCATCTTCCAGGGATACG
Luc T7 Rev	GTAATACGACTCACTATAGGCGTCCACAAACACAACTCCTCC

[a]For.: Forward; Rev.: Reverse.

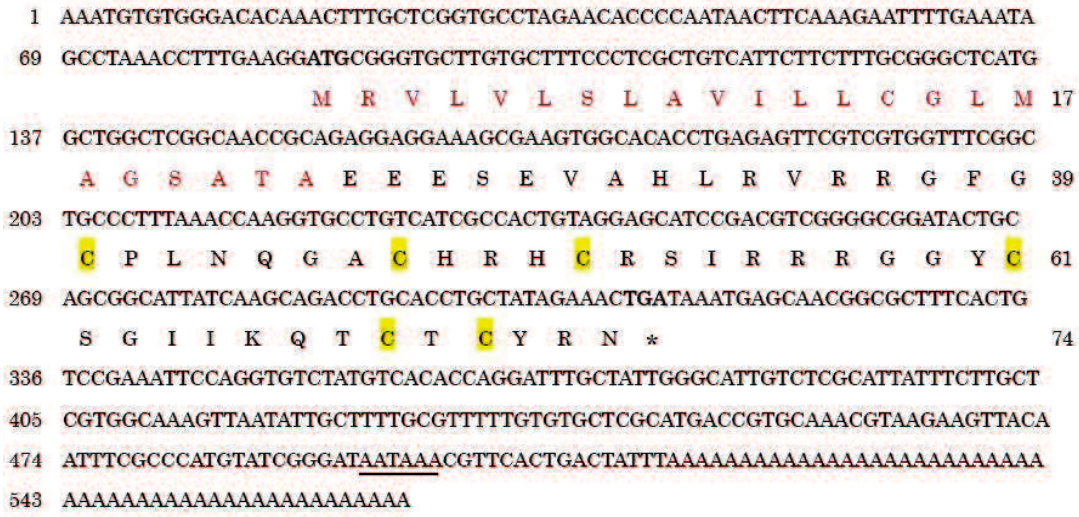

Fig. 4.1 Characterization of *HEdefensin* cDNA. Nucleotide and predicted amino acid sequences of *HEdefensin* cDNA. *HEdefensin* nucleotide sequences are numbered on the left, while the deduced amino acid sequences are numbered on the right. The red lettering indicates the signal peptide, and the yellow highlighting indicates the location of the conserved cysteine residues found in arthropod defensins. The stop codon is indicated by an asterisk. The putative polyadenylation after the stop codon has been underlined.

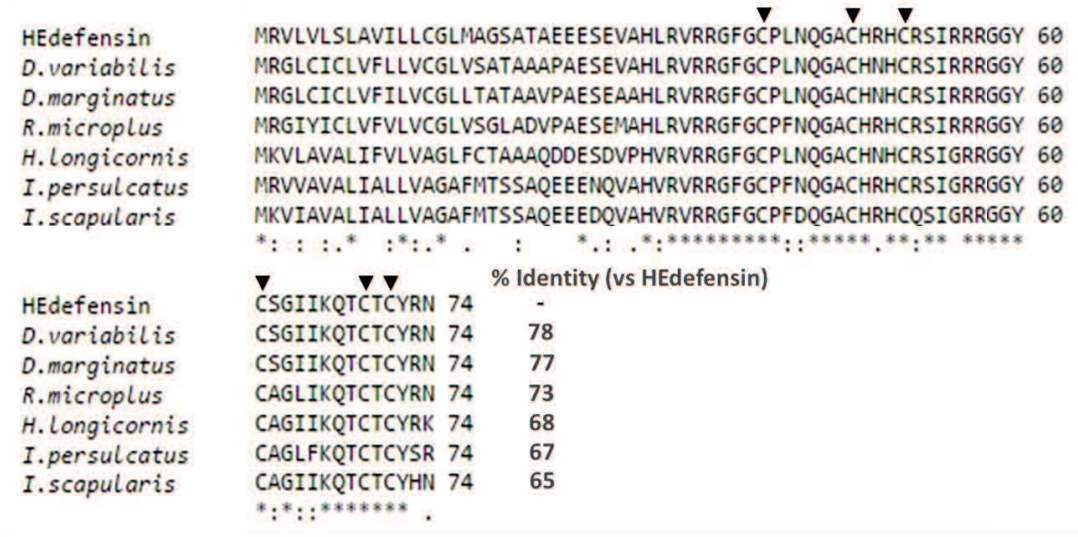

Fig. 4.2 Alignment of the amino acid sequences of HEdefensin and defensins from *Dermacentor variabilis* (Q86QI5), *D. marginatus* (ACJ00433), *Rhipicephalus microplus* (Q86LE4), *H. longicornis* (BAD93183*), Ixodes persulcatus* (BAH09304) and *I. scapularis* (XP_002436103). Identical residues are marked with an asterisk, while colons and periods indicate conservation between groups of strongly similar and weakly similar properties, respectively. To the bottom right is the summary of the percentage of identity of each aligned tick defensin with HEdefensin. The conserved cysteine residues found in tick defensins are marked with arrowheads.

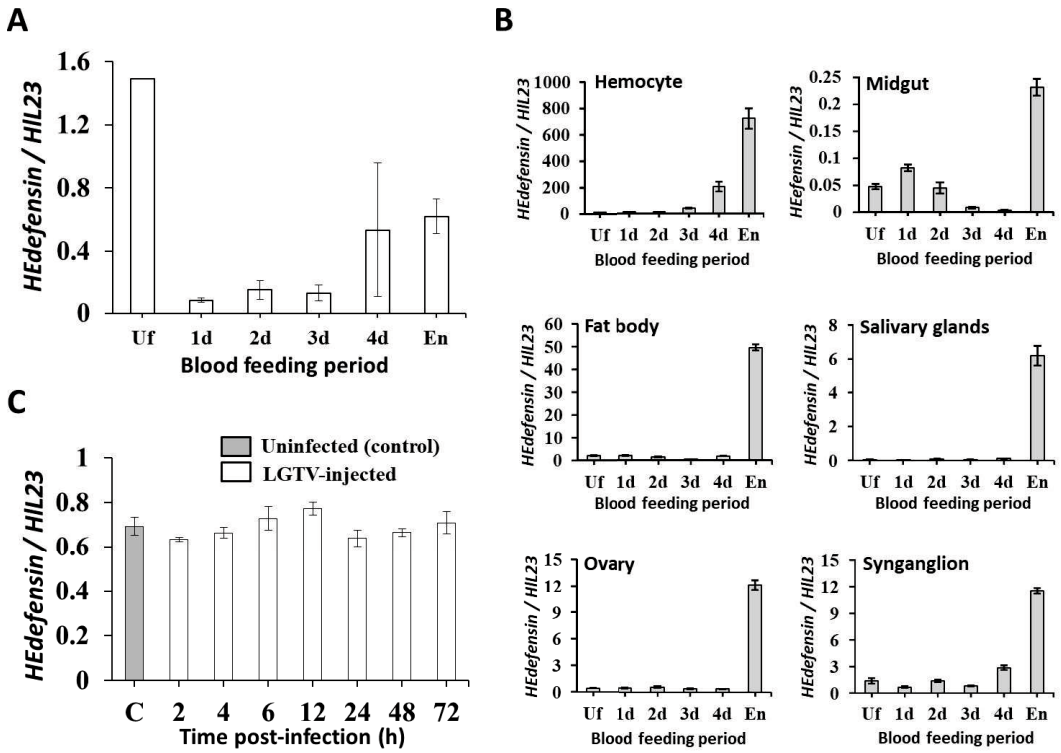

Fig. 4.3 Transcription profiles of *HEdefensin* analyzed by real-time PCR. (A) *HEdefensin* gene expression in whole ticks before, during and after feeding. (B) Expression profile of the *HEdefensin* gene in various organs of ticks at different stages of blood feeding. (C) *HEdefensi*n gene expression in whole ticks after the LGTV challenge. Uf, unfed; Pf, partially fed; En, engorged ticks; 1d, 1-day-fed; 2d, 2-day-fed; 3d, 3-day-fed; 4d, 4-day-fed. The transcription profiles of Hl-L23 were used as internal control.

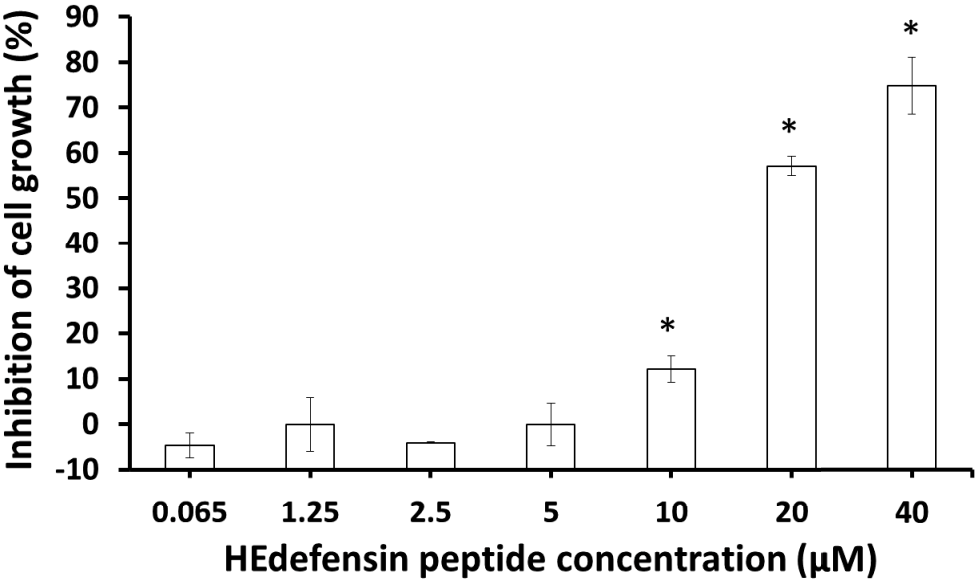

Fig. 4.4 Cell growth inhibition effect of the HEdefensin peptide on BHK-21 cells. Values are representative of triplicate samples, and error bars indicate the range of values obtained. *$P < 0.05$, as compared to the lowest concentration.

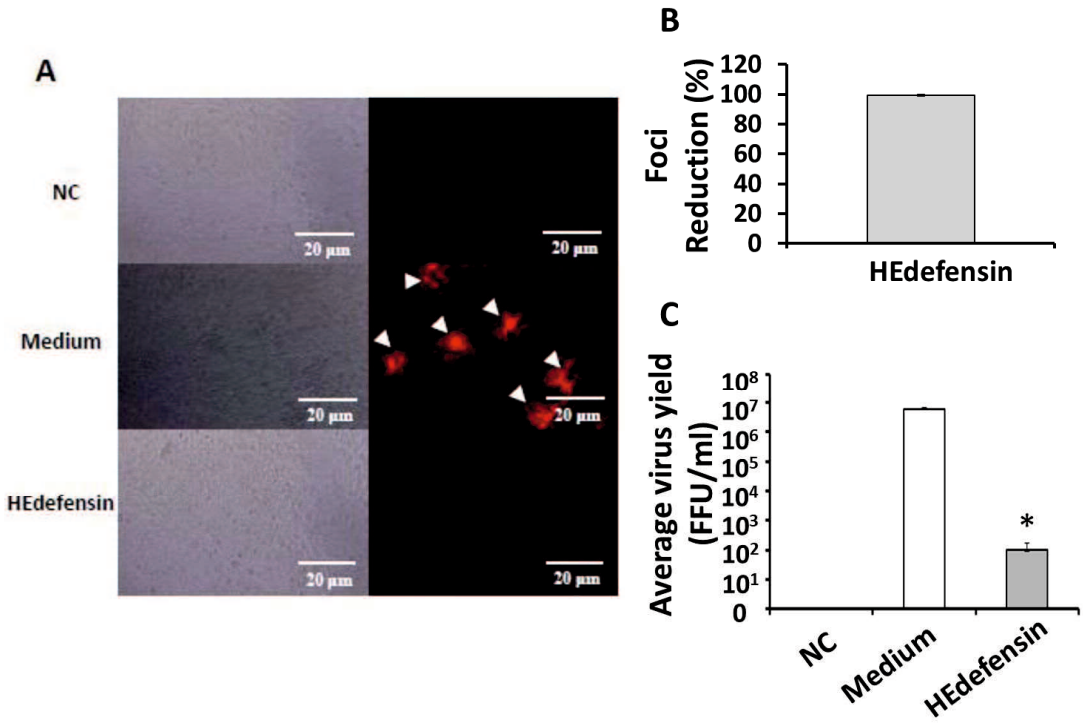

Fig. 4.5. Virucidal activity of HEdefensin against Langat virus. (A) Fluorescence images of BHK-21 cells infected with LGTV treated with medium only or HEdefensin for 2 h at 37°C. Positive fluorescence FFUs are indicated with arrowheads. To further establish the extracellular virucidal effect of HEdefensin against LGTV, (B) foci reduction and (C) virus yield reduction assays were conducted. The percentage of viral foci reduction (%) of the peptide was obtained by comparison with medium-treated cells maintained in parallel. All of the experiments were conducted at least in triplicate, and error bars indicate the range of values. Negative control (NC) refers to non-treated and non-infected cells. *$P < 0.05$, HEdefensin vs. medium.

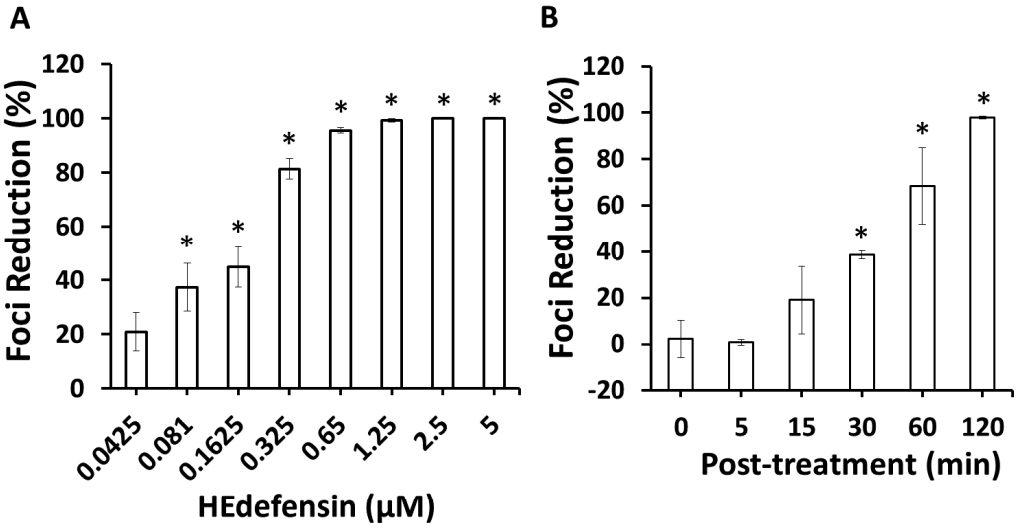

Fig. 4.6 (A) Dose-dependent and (B) time-dependent virucidal effects of HEdefensin against Langat virus. The percentage of viral foci reduction (%) of the peptide was obtained by comparison with medium-treated cells maintained in parallel. Experiments were conducted in triplicate, and error bars indicate the range of values. *$P < 0.05$, as compared to the lowest concentration or to 0 min.

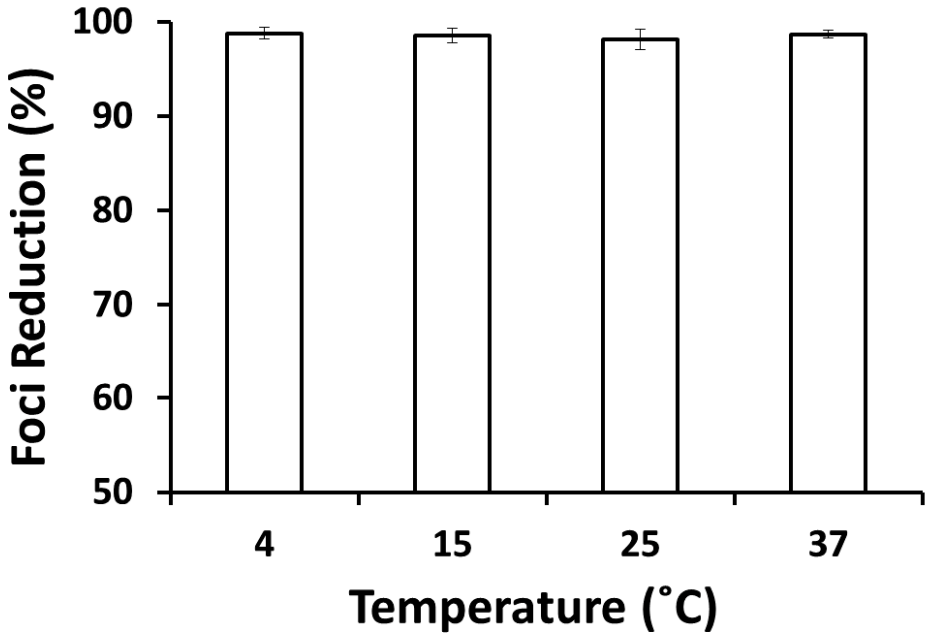

Fig. 4.7 Temperature-dependent virucidal effect of HEdefensin against Langat virus. To determine the temperature-dependent antiviral effect of HEdefensin, LGTV was treated with 5 μM of the peptide at 4, 15, 25 and 37°C for 2 h. A focus formation unit reduction assay was also used.

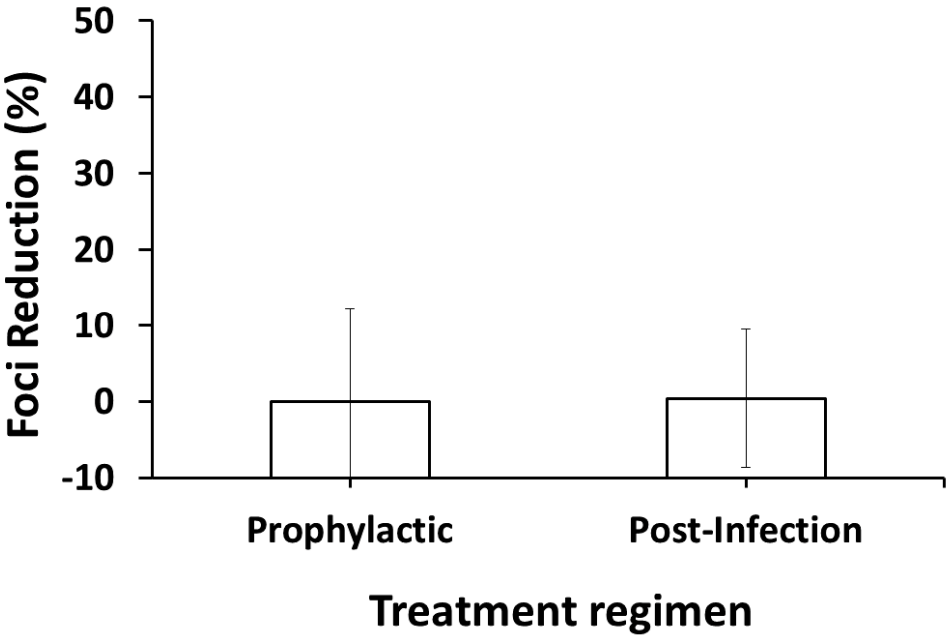

Fig. 4.8 (A) Prophylactic and (B) post-adsorption antiviral activity of HEdefensin against Langat virus. The percentage of viral foci reduction (%) of the peptide was obtained by comparison with medium-treated cells maintained in parallel. Experiments were conducted in triplicate, and error bars indicate the range of values. *$P < 0.05$, HEdefensin vs. medium.

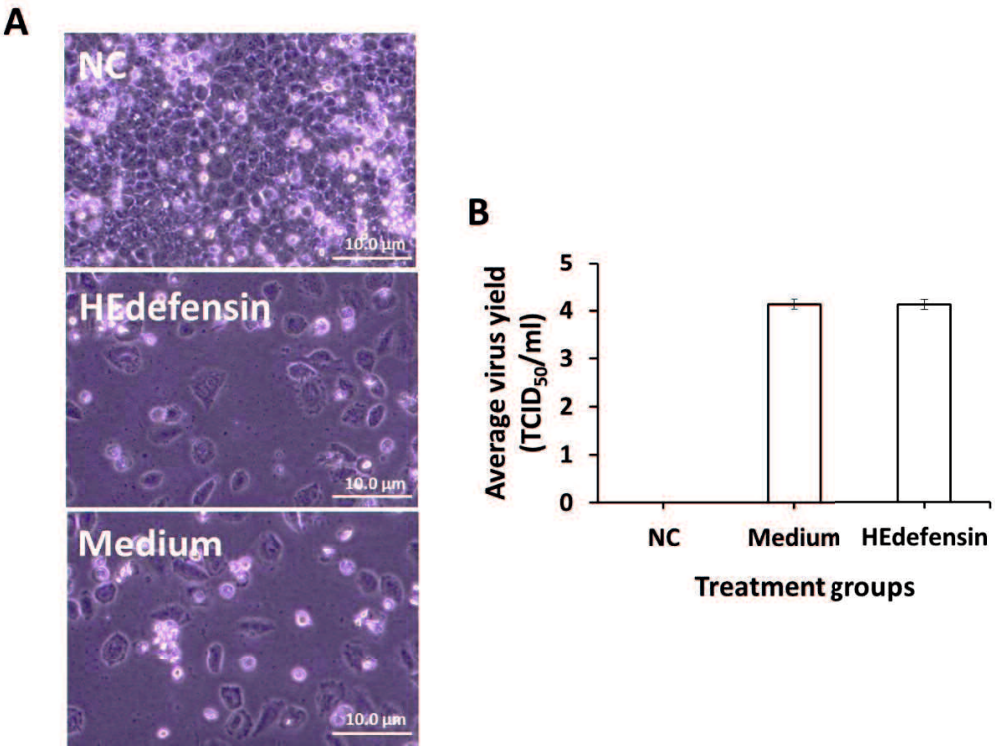

Fig. 4.9 Virucidal activity of the HEdefensin peptide against an adenovirus. (A) HeLa cells infected with human adenovirus 25 treated with medium only or HEdefensin for 2 h at 37°C. (B) To determine the virus yield titers (expressed as means ± SD) of the collected supernatants of the treatment groups, $TCID_{50}$ was used. NC refers to non-treated and non-infected cells.

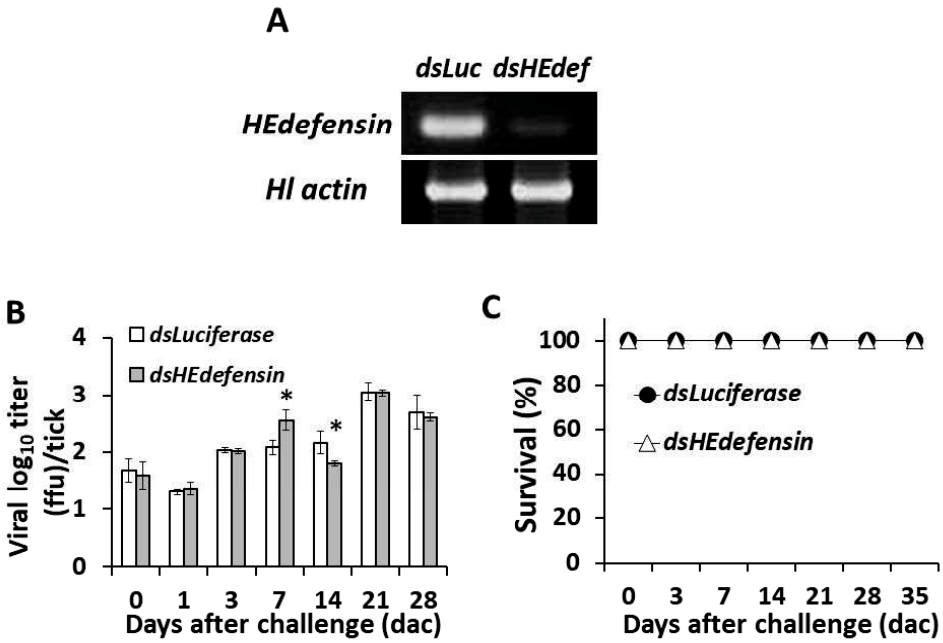

Fig. 4.10 Gene-specific silencing in ticks from each group at 28 d post-dsRNA inoculation. (B) Virus titers and (C) tick mortality were monitored after injecting LGTV into 4-day dsRNA-inoculated ticks. Values for the percentage of survival (%) were expressed as the percentage of live remaining ticks to the number of ticks at the start of the experiment. Error bars indicate SD in the mean values of five ticks. *$P < 0.05$, *dsHEdefensin* vs. *dsLuciferase*.

SUMMARY AND CONCLUSION

Despite of the important role of ticks in the transmission of viruses of public health importance and being second to mosquitoes in transmitting human diseases [3, 8], the diversity of tick-borne viruses has been less thoroughly studied than that of mosquito-borne viruses [9]. Even until now, detection of new pathogenic viruses are still being reported and known viruses continuously spread to new geographical locations [3]. Another aspect of TBVs that is still at an early stage of understanding is the tick-virus interaction, most especially on our knowledge about antiviral immunity pathways in ticks [33, 34]. This dissertation presents my studies on the survival dynamics of Langat virus and Thogoto virus in *H. longicornis*, focusing on the vector competence. In addition, I also conducted studies to broaden our understanding on the possible role of AMPs in the antiviral immunity of ticks.

In Chapter 1, I presented evidences which showed that *H. longicornis* can be efficiently infected with Langat virus (LGTV) through anal pore microinjection. One of the most important determinants of vector competence is the susceptibility of midgut cells to virus infection and in this study, the tick's midgut was observed to be the primary replication site. The virus also remained detectable for at least 120 days post-inoculation of the virus. More importantly, infected ticks can effectively transmit

LGTV to a susceptible host. However, demonstration of LGTV transmission through co-feeding between an infected adult and immature naïve ticks are needed to demonstrate the possible mechanism on how LGTV can circulate in the tick population, especially that no transovarial transmission was observed in the study using anal pore microinjection.

In Chapter 2, I demonstrated that *H. longicornis* is a competent vector or possibly the main tick carrier of the Thogoto virus (THOV) recently isolated in Japan as shown by the successful transfer of the virus to mice. I also observed that the virus inoculated via anal pore microinjection, readily transfers from the midgut to the salivary gland during feeding to facilitate the transmission of the virus to the host. The current study also managed to establish that naïve nymphs can be infected by THOV through natural routes of infections, particularly via co-feeding the naïve nymphs with an infected adult. However, additional studies are needed to identify which animals potentially serve as the reservoir host of the virus and to elucidate animal susceptibility and the geographic distribution of THOV infections in Japan.

In Chapter 3, I established the extracellular virucidal activity of longicin P4 against LGTV in vitro, and to our knowledge, this is the first report of an antiviral activity of a native or synthetic antimicrobial peptide derived from *H. longicornis*.

However, co-incubation of the peptide with an adenovirus failed to reduce infectivity and virus yield as compared with the control group. Moreover, no significant difference in mortality and virus titer in general was observed on both *longicin* gene-silenced and Luc dsRNA-injected ticks subsequently challenged with LGTV, suggesting that the role of the endogenous tick longicin in the antiviral defense of *H. longicornis* still remains to be demonstrated. Nonetheless, longicin P4 can be a potential therapeutic agent for tick-borne pathogens.

In Chapter 4, I characterized the recently identified HEdefensin gene from the hemolymph EST database of *H. longicornis*. The predicted amino acid sequence of HEdefensin showed the conserved cysteine residues normally found in arthropod defensins and was shown to have high amino acid sequence identity with some known tick defensins using BLAST analysis. The co-incubation of the LGTV with HEdefensin peptide at 37°C for 2 hours produced a 99.2% foci reduction and a five-fold lower virus yield as compared to cells infected with a medium-treated virus. However, the HEdefensin peptide has no antiviral activity against a non-enveloped virus and *HEdefensin* gene silencing showed no significant effect on tick mortality and virus titer after LGTV challenge. Nevertheless, the significant extracellular antiviral activity of the peptide against LGTV in vitro offers a new potential therapeutic agent for tick-borne

pathogens, particularly flaviviruses.

Taken altogether, the results in this dissertation indicate that *H. longicornis* is a competent vector of LGTV and the most likely vector of the Japanese isolate THOV. Moreover, the results of the evaluation of the antiviral activity of longicin and HEdefensin peptides elucidated the possible role of antimicrobial peptides in the innate antiviral immunity of ticks. The novel methods and evidences in my studies provided additional information to our current understanding of tick-virus interactions and will hopefully provide new control strategies against ticks and alternative therapeutic agents against tick-borne viruses.

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