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Pathology and Molecular diagnosis of Paramyxovirus infection in Boidae and Pythonidae in Thailand

Miss Piyaporn Kongmakee

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By	Miss Piyaporn Kongmakee
Field of Study	Veterinary Pathobiology
Thesis Advisor	Associate Professor Wijit Banlunara, DVM., Ph.D.
Thesis Co-Advisor	Assistant Professor Somporn Techangamsuwan, DVM., M.Sc., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, DVM.,M.Sc.,Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Anudep Rungsipipat, DVM., Ph.D.)

.....Thesis Advisor

(Associate Professor Wijit Banlunara, DVM., Ph.D.)

.....Thesis Co-Advisor

(Assistant Professor Somporn Techangamsuwan, DVM., M.Sc., Ph.D.)

.....External Examiner

(Assistant Professor Sompoth Weerakhun, DVM., Ph.D.)

ปิยพร คงเมคี : การวินิจฉัยการติดเชื้อพารามิกโซไวรัสในงูวงศ์บอและวงศ์หลามด้วยวิธีทางพยาธิวิทยาและอณูชีววิทยาในประเทศไทย . (Pathology and Molecular diagnosis of Paramyxovirus infection in Boidae and Pythonidae in Thailand) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร. วิจิตร บรรลุนารา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. สพ.ญ. ดร. สมพร เตชะงามสุวรรณ, 58 หน้า.

เชื้อไวรัสวงศ์พารามิกโซ เป็นเชื้อก่อโรคที่สำคัญกลุ่มหนึ่งในสัตว์เลื้อยคลาน โดยเฉพาะในงูวงศ์บอ (Boidae) และวงศ์หลาม (Pythonidae) ที่สำคัญประกอบด้วย ซันไชน์ไวรัส เฟอร์ลาไวรัส และ กลุ่มอื่นๆ โดยพบว่า งูที่มีการติดเชื้อไวรัสดังกล่าวมักแสดงอาการผิดปกติทางระบบประสาท ทางเดินหายใจ หรือไม่แสดงอาการ ในประเทศไทยพบมีสัตว์ป่วยสงสัยการติดเชื้อจำนวนมาก แต่เนื่องจากข้อจำกัดในการตรวจทางห้องปฏิบัติการ จึงทำให้ไม่สามารถวินิจฉัยการติดเชื้อชนิดนี้ได้

วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อตรวจหาและแยกเชื้อไวรัสวงศ์พารามิกโซจากตัวอย่างอวัยวะและเยื่อเมือกในช่องปากและรูเปิดร่วมของสัตว์ที่สงสัยการติดเชื้อ ทำการเก็บตัวอย่างจากงูตาย 35 ตัวอย่าง และงูป่วย 40 ตัวอย่าง ไม่พบรอยโรคทางมหกายวิภาคที่มีความจำเพาะต่อการติดเชื้อไวรัส รอยโรคทางจุลพยาธิวิทยาพบอินคลูชันบอดีสะสมแทรกในไซโตพลาสมของ ตับ ตับอ่อน ไต ปอด ม้ามและสมอง การศึกษาทางอณูชีววิทยา พบว่าจากตัวอย่างร้อยละ 7.69 (2/26) ซึ่งเป็นตัวอย่างจากงูวงศ์บอให้ผลการวิเคราะห์ลำดับนิวคลีโอไทด์สอดคล้องกับซันไชน์ไวรัส (Sunshine virus) ในขณะเดียวกันตัวอย่างร้อยละ 42.31 (11/26) จากงูทั้งวงศ์บอและวงศ์หลาม ให้ผลวิเคราะห์สอดคล้อง กับไวรัสพารามิกโซอื่นๆ ที่ก่อโรคในสัตว์เลื้อยคลาน (reptilian paramyxovirus group) นอกจากนี้ยังพบว่ามีกลุ่มตัวอย่างที่แสดงลำดับนิวคลีโอไทด์คล้ายกันในกลุ่ม แต่ไม่สามารถจำแนกให้เข้ากับไวรัสพารามิกโซในทั้งสองกลุ่มที่พบจากการศึกษาครั้งนี้ ซึ่ง กลุ่มตัวอย่างดังกล่าวมีความเป็นไปได้ที่จะเป็นไวรัสสายพันธุ์ใหม่หรือสายพันธุ์ที่มีการเปลี่ยนแปลง ซึ่งจำเป็นต้องใช้เทคโนโลยีที่มีความก้าวหน้า เช่น เทคโนโลยีเอ็นจีเอส (Next generation sequencing) เพื่อช่วยในการวิเคราะห์ลำดับนิวคลีโอไทด์ที่มีความแม่นยำและหลากหลายเพิ่มขึ้น

จากผลการศึกษา เป็นครั้งแรกที่มีการตรวจพบ การติดเชื้อไวรัสพารามิกโซที่ก่อโรคในงูวงศ์บอและวงศ์หลามในประเทศไทย ซึ่งก่อโรคที่มีความรุนแรงและเป็นอุปสรรคในการจัดการสุขภาพงูอย่างมาก โดยการพัฒนาห้องปฏิบัติการเพื่อตรวจหาการติดเชื้อยังเป็นการเพิ่มความสามารถให้กับสัตวแพทย์ในการวินิจฉัยการติดเชื้อ เพื่อให้เกิดการควบคุมและการป้องกันโรคได้อย่างมีประสิทธิภาพสูงสุด

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต	.....
สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
ปีการศึกษา	2557	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....

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KEYWORDS: INCLUSION BODY, PATHOLOGY, PCR, SNAKES, SUNSHINE VIRUS, REPTILIAN PARAMYXOVIRUS

PIYAPORN KONGMAKEE: Pathology and Molecular diagnosis of Paramyxovirus infection in Boidae and Pythonidae in Thailand. ADVISOR: ASSOC. PROF. DR. WIJIT BANLUNARA, DVM., Ph.D., CO-ADVISOR: ASST. PROF. SOMPORN TECHANGAMSUWAN, DVM., M.Sc., Ph.D., 58 pp.

Reptilian paramyxovirus (rPMV) is one of the most important viral infection occurring worldwide in reptiles especially in Boidae and Pythonidae snakes. The members of rPMV are Sunshine virus and Ferlavirus. The infected snakes may show clinical signs related with neuro-respiratory disorder. There are many suspected clinical cases that could not specify the causes. So far, there is no information and available diagnostic laboratory to identify these viruses in Thailand. Thirty-five necropsied snakes and 40 oral and cloacal swabs from snakes with history of neuro-respiratory problems were collected for histopathology and molecular study. Microscopically, no remarkable lesions in the other visceral organs were observed except generalized congestion. Histopathologically, most distinguished lesion of the suspected cases was numerous varied sizes of eosinophilic intracytoplasmic inclusion bodies in liver, pancreas, kidney, spleen, lung and brain. Molecular study found 2 from 26 (7.69%) positives products showed specific amplicons with high nucleotide identity with Sunshine virus. Eleven of 26 (42.31%) positive products showed 91-96% homology sequence to reptilian paramyxovirus group. Sunshine virus and other rPMV were successfully identified by using RT-PCR and genetic sequencing. This study is the first report of rPMV infection in Boidae and Pythonidae snakes in Thailand. The other five sequences shared homology in group but not to Sunshine virus. This may refer to new strain of rPMV. However, advance technology such as next-generation sequencing may obligatory for further study.

Department:	Veterinary Pathology	Student's Signature	.....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	.....
Academic Year:	2014	Co-Advisor's Signature	.....

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CONTENTS

Page

Page

List of Figures

List of Tables

List of Abbreviations

º C	=	degree Celsius (centrigrad)
bp	=	base pair (s)
DNA	=	deoxyribonucleic acid
FDLV	=	Fer-de-lance virus
IBD	=	Inclusion body disease
H&E	=	hematoxylin and eosin staining
HI	=	hemeagglutination inhibition
hr.	=	hour (s)
mm^3	=	cubic millimater
PB	=	Phosphate buffer
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcription polymerase chain reaction
Sec	=	second (s)
TEM	=	Transmission electron microscopy

CHAPTER I
INTRODUCTION

Reptilian Paramyxovirus (rPMV) is an important pathogen in reptile species especially snakes and lizards which have been reported the incidence of infection worldwide in both captive and free ranging snakes. The evidence of rPMV infection were found in Europe (Manvell et al., 2000; Papp et al., 2009; Pees et al., 2010), North America (Jacobson et al., 1981; Jacobson et al., 1980; Jacobson et al., 1997), South America (Kolesnikovas et al., 2006; Marschang et al., 2002) and Australia (Hyndman et al., 2012[a,b]). During the decade of 1970s, there were a number of case reports and researches in rPMV leading to the classification of rPMV into two groups; Ferlavirus and Sunshine virus. Both viruses are member of family *Paramyxoviridae* which is a negative sense single stranded RNA virus with helical nucleocapsid in lipid envelop. Ferlavirus is a member in genus *Ferlavirus*, subfamily *Paramyxovirinae*. Sunshine virus is a novel paramyxovirus ((ICTV), 2012) which was firstly isolated in Australia but could not identify into any subfamily in *Paramyxoviridae* family (Hyndman et al., 2012a; Hyndman et al., 2012b). The RNA genome of paramyxovirus contains six common genes consisted of nucleocapsid (N), phosphoprotein (P), large RNA polymerase (L), matrix (M), hemagglutinin (H) and fusion (F) genes. Ferlavirus, interestingly, contains there is a unique gene (U) which remains unknown its function but is used to identify into genus Ferlavirus (Kurath et al., 2004). The host range of rPMV infection includes Boidae, Colubridae, Crotalidae, Elapidae, Viperidae and Pythonidae (Jacobson et al., 1980). Moreover, Ferlavirus could infect lizards and chelonians (tortoises and turtles), while Sunshine virus infects mostly Pythonidae (Hyndman et al., 2012a).

The rPMV infected snakes show varied clinical symptoms ranging from no visible sign to neuro-respiratory syndromes; pneumonia, open-mouth breathing, nervous disorders, abnormal posturing and inability to right the body. The infected colony usually shows high mortality rate. Following post-mortem examination, there are no specific gross lesions but various histopathological changes in neurological and respiratory systems.

The distinguished finding is inclusion bodies either intranuclear or intracytoplasmic in epithelium of various organs such as lungs, kidneys, brain, pancreas and liver. This particular lesion needs to be differentiated from other viral infections inducing neuro-respiratory disorder and inclusion bodies in reptile species such as Adenoviridae, Retroviridae or Inclusion body disease (IBD) and Reoviridae. Therefore, the advanced molecular technique and genetic sequencing are important tools for diagnosis the rPMV infection.

In Thailand, there are a number of captive snakes belonging to private owners or private breeders. These groups import snakes as breeder and also export snakes to different part of the world. Meanwhile, the veterinary medical research groups and zoological parks take care the native species and captive breeding species. Some animals show suspected clinical symptoms such as pneumonia, head tremor, open-mouth breathing, cannot correct position. Besides the limitation on laboratory diagnosis, some snakes die with unknown causes and do not perform necropsy to find out the cause of death. Based on this fact, the evidence of infectious diseases in snakes in Thailand remains obscure.

Nowadays, there is no information of rPMV infection and available diagnostic laboratory in Thailand. The aims of this study were to investigate the pathology of infected animals and to establish the molecular diagnostic methods for the detection and differentiation between Ferlavirus and Sunshine virus. The advantages of this study would be benefit to not only private owners and animal keepers, but also veterinarians for accurate diagnosis, rightful management and prevention of rPMV infection in snakes.

Objectives of Study

1. To develop the reverse transcription-polymerase chain reaction (RT-PCR) technique for the detection of rPMV infection in Thailand.
2. To study the pathological changes in rPMV infected snakes.
3. To investigate the incidence of rPMV infection in snakes in Thailand during 2010 - 2014.

CHAPTER II
LITERATURE REVIEW

Virus is one of pathogens that play an important role in human and animal health. In reptile, even they are not a popular pet species or have no economic significance; there are a number of studies on virus infection in reptile especially in snakes. Virus that has been identified as a causative agent in snakes includes Adenovirus, Reovirus, Retrovirus and Paramyxovirus.

Reptilian Paramyxovirus (rPMV) is an important pathogen in reptiles which has been reported in snakes from different regions of the world including Switzerland where the first case was revealed (Folsch and Leloup, 1976), United Kingdom (Manvell et al., 2000), Spain(Oros et al., 2001), Mexico (Marschang et al., 2002), Brazil (Kolesnikovas et al., 2006), USA(Allender M. C., 2008), Germany (Abbas et al., 2011; Papp et al., 2010; Pees et al., 2010), Hungary (Papp et al., 2013), Hong kong (Woo et al., 2014) and Australia (Hyndman and Shilton, 2011; Hyndman et al., 2012a; Hyndman et al., 2012b) where a novel paramyxovirus called Sunshine virus was identified.

Since the first report of rPMV or Ferlavirus or Fer de Lance virus (FDLV) infection in Fer-de-Lance viper, there were numbers of infected cases in many snake species including viperid, pythonid and colubrid (Ariel, 2011; Hyndman et al., 2012a; Jacobson et al., 1981). In addition, other species such as iguana, caiman, Mexican lizards and tortoise were susceptible to this virus as well (Gravendyck M., 1998; Marschang et al., 2002; Papp et al., 2009).

Virus taxonomy

Paramyxoviridae is a single stranded negative sense RNA with pleomorphic envelope and comprises approximately between 15,000 – 19,000 nucleotide lengths. This virus family has two subfamilies: *Paramyxovirinae* and *Pneumovirinae*. Subfamily *Paramyxovirinae* composes of seven genera in which the members are pathogens in

humans and animals such as Nipah virus, Measles virus, canine distemper virus, Newcastle disease virus and Ferlavirus or reptilian paramyxovirus. Subfamily *Pneumovirinae* has only two genuses and they are pathogens in humans and animals such as human respiratory syncytial virus and avian pneumovirus.

Most members of subfamily *Paramyxovirinae* have genome following "rule of six" or contain six genes in order 3' N-P/V-M-F-HN-L 5' that encode corresponding proteins. The structural proteins include N protein (encapsid RNA for nucleocapsid formation), P protein (a component of active polymerase complex), M protein (organize and maintain viral structure), F protein (induce cell membrane and viral fusion), HN protein (cell attachment protein) and L protein (catalytic subunit of RNA-dependent RNA polymerase) (Figure 1).

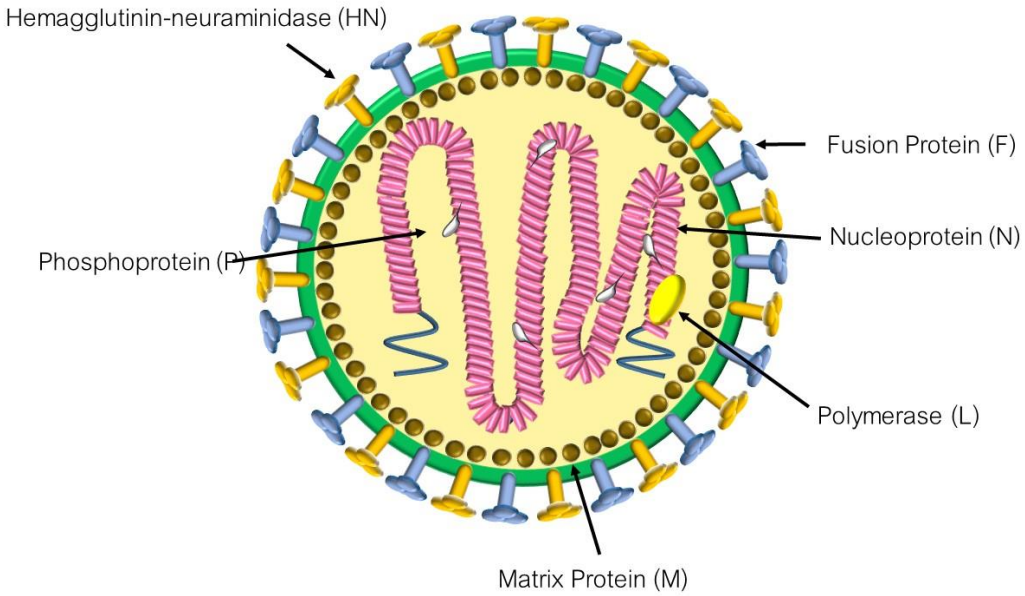

Figure 1 A schematic present the structure of Paramyxoviridae containing genome following "rule of the six"

Since Fölsch and Leloup (1972) discovered the Ferlavirus or Fer-de-Lance virus (FDLV) infection in Fer-de-Lance viper from Switzerland, more than 40 pathogens have been isolated from different reptile species later. Until 2004, the complete genome sequence of FDLV was achieved (Kurath et al., 2004). The FDLV genome consists of seven genes in order 3' N-U-P/V-M-F-HN-L 5' that encode ten proteins including six variant proteins, three accessory proteins and a unique protein. FDLV shows a novel gene presenting between N and P genes, named unique (U) gene (Figure 2), that has dissimilarity with other viruses in *Paramyxoviridae*. According to unique characteristic of the gene, FDLV is listed as a member in a new genus of subfamily *Paramyxovirinae* named Ferlavirus (Figure 3) ((ICTV), 2012).

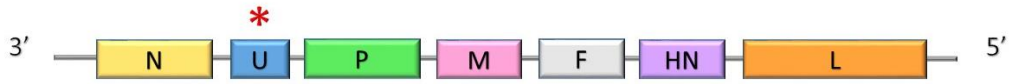

Figure 2 Genome of Fer-de-Lance virus or Ferlavirus

In 2012, another reptilian paramyxovirus was identified from Australian pythons and named according to the place discovered, Sunshine virus (Hyndman et al., 2012a; Hyndman et al., 2012b). Sunshine virus belongs to family *Paramyxovidae*, order *Mononegavivales*, but could not identify to any subfamily ((ICTV), 2012) (Figure 3). Moreover, as the development of technologies facilitated the scientist to discover and diagnosed a novel member of reptilian paramyxovirus infection in anaconda from Hong Kong. This novel virus called anaconda paramyxovirus, a member of genus Ferlavirus (Woo et al., 2014).

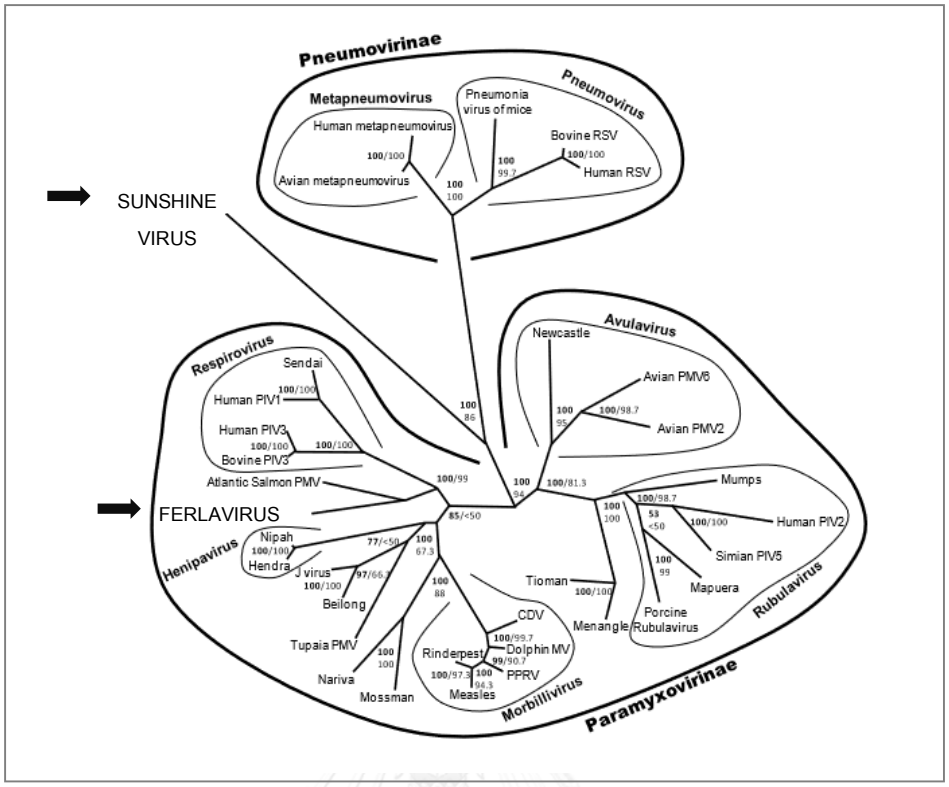

Figure 3 Bayesian phylogenic tree shows a relationship of Ferlavirus in subfamily Paramyxovirinae and Sunshine virus (Modified from Hyndman et al., 2012)

Additionally, paramyxovirus can expand its host range and cause disease in chelonians and lizards. There are some reports on clinical infection and genetic characterization of PMV in these two species. The genetic characterizations are closely related between PMV from lizards and snakes than a chelonian group (Marschang et al., 2009). These findings propose the possibility of cross species infection from paramyxovirus induction.

Clinical signs

Even though member of *Paramyxoviridae* is identified as causative agents in snake but the clinical signs of infected animals are not specific such as depression, loss appetites, regurgitation, respiratory distress, open-mouth breathing. They might even show no clinical sign and died suddenly (Jacobson et al., 1981). Some snakes may show neurological signs which are related with paramyxovirus infection such as abnormal

posturing, loss cutaneous sensation, inability to right itself and complete flaccid paralysis (West et al., 2001).

Viral transmission

The previous researches showed that paramyxovirus is detectable from oral or respiratory secretion and cloacal excretion which reveal the possible routes of transmission including aerosol and direct contacts. These findings are related with many clinical cases that animal died or showed clinical signs after contact or had new animals move into the same (Hyndman et al., 2012a; Papp et al., 2013). Recently, there reported an evidence of vertical transmission from Sunshine virus positive dam to the embryos of non-viable eggs in Australian python proving by polymerase chain reaction (PCR) assay (Hyndman et al., 2014).

In infected colony, virus causes high mortality and several episodes of infections are detected from new animals that introduce into the group, so the prevention is the most important strategy for disease control. Pre-shipment and quarantine protocol should be settled for new coming animals. Clinical observation even necropsy should be done in all new members or after death, respectively.

Gross pathology and histopathology

Pathological findings of infected snakes are variable and non-pathognomonic. In Ferlavirus and Sunshine virus infected cases, macroscopic findings are significantly seen in respiratory systems composing of frothy exudates, mucoid or caseous exudate in lung, pulmonary congestion and edema (Folsch and Leloup, 1976; Hyndman et al., 2012a; Jacobson et al., 1980; Kolesnikovas et al., 2006; Oros et al., 2001) as well as blood in oral cavity and hemorrhagic pneumonia (West et al., 2001). While other systems showed unspecific lesions, such as white nodules on hepatic surface and parenchyma (Jacobson et al., 1992), enlargement of liver and spleen (Abbas et al., 2013) even no remarkable gross pathology. On the other hand, gross lesions of the positive paramyxovirus Anaconda showed necrotizing inflammation of multi-organs such as kidney, liver, spleen,

pancreas, and intestine (Woo et al., 2014) which were far different from Ferlavirus and Sunshine virus infection.

Histopathologically, the related findings from both Ferlavirus and Sunshine virus infection are broncho-interstitial pneumonia with diffuse pneumocytes type 2 hyperplasia, epithelial cells of bronchi primary septa and flaveola hyperplasia (Homer et al., 1995), lymphoid aggregation (Hyndman et al., 2012a; Jacobson et al., 1997) or heterophilic granulocytes infiltration in lung (Papp et al., 2013) and few pale eosinophilic intracytoplasmic inclusion bodies in pulmonary epithelial cells (Jacobson et al., 1997). Infected brain may show severe spongiosis, gliosis, meningoencephalitis, syncytial cell formation and low number of eosinophilic intracytoplasmic inclusion bodies in glial cells (Hyndman et al., 2012a; Jacobson et al., 2001). However, histopathological findings of PMV infected anaconda were related with gross pathology. Kidney showed high severity of histopathological lesion with multifocal necrotizing, heterophilic nephritis with multinucleated syncytial cells (Woo et al., 2014). Furthermore, some bacterial co-infection such as *Aeromonas* spp. or *Pseudomonas* spp. could be identified and produced non-specific lesions on histopathology (Papp et al., 2013).

Based on the fact that the presence of neuro-respiratory disorders and inclusion bodies in reptile species could be found in other virus infections such as Adenoviridae, Retroviridae or Inclusion body disease (IBD) and Reoviridae. Therefore, the advance and specific laboratory methods are in need and considered important for the diagnosis of the rPMV infection.

Laboratory diagnosis

As there is no specific lesion from pathological aspects, other laboratory methods are the important tool for rPMV detection and diagnosis. There are many different methodologies for viral detection and identification. Viral isolation and transmission electron microscopy (TEM) are goal standard for viral structural identification (Hyndman et al., 2012a; Manvell et al., 2000); however, their applications are limited because of the time-consuming, requirements of specific instruments and technical supports.

Serological diagnosis is helpful for large numbers of samples but it remains low potency for detection. However, hemagglutination inhibition test (HI), which detects antibodies titer against virus antigen, is the reliable method for diagnosis and disease monitoring in live animals (Allender M. C., 2008; Hyndman et al., 2012a; Jacobson et al., 1980; Jacobson et al., 1997).

For viral antigen detection, immunohistochemistry (Homer et al., 1995; Oros et al., 2001) and complementary DNA: RNA *insitu* Hybridization (Sand et al., 2004) are methods that use antibodies for localization viral antigen in target cells on histopathology. In addition, reverse transcription polymerase chain reaction (RT-PCR) technique that detect antigen using specific primers are reported (Ahne et al., 1999; Franke et al., 2001; Hyndman et al., 2012a; Hyndman et al., 2012b; Jacobson et al., 1997; Kurath et al., 2004; Marschang et al., 2009; Papp et al., 2013; Papp et al., 2010). RT-PCR is the most appropriate method for rPMV detection. Previous investigations have been done on RNA dependent RNA polymerase (L), hemagglutinin-neuraminidase (HN), fusion protein (F) and unique (U) genes (Abbas et al., 2011; Ahne et al., 1999; Clark et al., 1979; Franke et al., 2001; Hyndman et al., 2012a; Hyndman et al., 2012b; Papp et al., 2010; Sand et al., 2004). However, those studies are performed to detect Ferlavirus, but could not detect Sunshine virus, based on the unique genetic characteristic. Therefore, a new RT-PCR protocol with more specific primers is need for Sunshine virus detection and differentiation between these two viruses (Hyndman et al., 2012a). The advantages of such molecular analysis are antemortem detection which are applicable for cloacal or oral swabs in animals with or without clinical signs as well as postmortem diagnosis using fresh tissue samples (Papp et al., 2010).

CHAPTER III
MATERIALS AND METHODS

1. Animals

This study focused on Paramyxovirus infected snakes in Boidae and Pythonidae species. Sample were collected from necropsy cases and clinical cases of Boidae and Pythonidae species during January 2011 to March 2015. Forty live snakes and 35 necropsied cases were collected from Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Kasetsart Animal Hospital, Faculty of Veterinary Medicine, Kasetsart University, Mahidol University, private veterinary clinics, zoo animal hospitals and private owners. General information, clinical history, signalments and clinical examinations were recorded as follows;

- Signalments: species, age and gender of animal.
- Management information: source of animal, number of animals in group, duration of parenting, environment and food management.
- Clinical history: quarantine and heath management program, clinical signs and changing of behavior.
- Clinical examination: general appearance of each animal was noted for rule out other underlying problems.

For necropsy cases, snakes that died with unknown causes or showed suspected clinical signs or had clinical history on neuro-respiratory system were used in the study.

2. Sample collection

2.1 A antemortem sampling

Thirty-five oropharyngeal and cloacal swabs of snakes that clinically suspected to Paramyxovirus infection were included. Sterilized rayon tipped applicators were soaked with sterile phosphate saline buffer (PBS) before swab into oral cavity and cloaca of snakes. For oral swab, cleaned mouth gag was used for opening the mouth with disinfectant between each snake. Moisten applicator was rolled on oral surface, glottis and trachea. During swab, appearances of oral cavity and secretion or saliva were noted. Pre-moisten applicator cloacal swab was applied into cloaca about 2-5 cm depth depending upon size of animals. Then, they were kept in sterile PBS in 1.5 ml eppendrorf tube and freezed at -80°C until further assayed.

2.2 The postmortem sampling

Thirty-five carcasses were necropsied systemically with completely macroscopic lesion record. The tissue samples were collected including brain, lungs, liver, kidney, spleen pancreas and others. Tissue samples were divided into 2 parts. Individual fresh tissue from each organs were kept separately at -80°C for molecular study while another part was fixed in 10% neutral buffered formalin for histopathological study.

3. Microscopic examination

3.1 Histopathology

Formalin fixed tissues were routinely processed by automatic tissue processor and embedded in paraffin. Four microns thickness of tissue sections were cut, depariffinized and stained with Hematoxylin and Eosin (H&E) for light microscopic examination.

3.2 Histochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue sections were subjected to special staining for identify type of the genomic viral inclusion bodies (DNA or RNA virus). Methyl green-Pyronin Y stain was applied for RNA detection, while Feulgen method was applied for DNA detection. Briefly, after deparaffinization and rehydration steps, four microns thick sections were stained with Methyl green-Pyronin Y solution for 5 minutes. Then, sections were rinsed with distilled water, alcohol-graded dehydration and mounted with permount (Carson, 1997).

For the Feulgen method, tissue sections were rinsed with M-HCl at room temperature then preheated at 60°C for 8 min. The sections were transferred to Schiff's reagent for 20 min, rinsed with bisulphate solution and water. One percent of light green in 1% acetic acid solution was used as counterstaining for 20-30 second (Sheehan and Hrapchak, 1980).

Microscopically, RNA stain will show bright pink to red color by pyronin stain while DNA stain with methyl green shows green to blue green color.

4. Transmission electron microscopy (TEM)

Formalin fixed tissue were trimmed into 1-2 mm thickness, washed and re-fixed in 2.5% glutaraldehyde in phosphate buffer (PB), pH 7.4 for 24 hr. Glutaraldehyde-fixed tissues were chopped into size 1x1 mm^3, re-fixed in 2.5% formaldehyde for 3 hr., then washed in PB. The fixed tissues were submitted to Scientific and Technological Research Equipment Centre, Chulalongkorn University for embedding and cut for TEM. Viral inclusion bodies were observed under JEOL model JEM-1400 (Japan).

5. Reverse - transcription polymerase chain reaction (RT-PCR)

5.1 RNA extraction and cDNA amplification

The RNA was extracted from oropharyngeal and cloacal swabs by using viral DNA/RNA Extraction Kit II™ (Geneaid Biotech, Taiwan) following product's protocol. For tissue sample, the frozen tissue was chopped into small pieces. Two hundred microliters (µL) of steriled PBS were added with 20 µL of proteinase K; tissues were incubated at 56°C and mixed by vortex every 20 – 30 min for 3 hr or until the solution appeared clear. The homogenized tissue was spin down at 15,000 round per minute (RPM) for 1 min for supernatant collection. Two hundred µL of supernatant were used for nucleic acid extraction with the same protocol of swab samples.

The RNA concentration and purity were quantified by NanoDrop™ Lite Spectophotometer (Thermo Scientific, USA). Viral RNA genomic was reverse transcribed to cDNA using Omniscript RT kit™ (Qiagen, Germany) which was incubated at 37°C for 60 min.

The positive control for Sunshine virus and Ferlavirus were kindly provided by Dr. Tim H. Hydnman (Murdoch University, Australia) and Dr. Rachell Marschang (University of Hohenheim, Stuttgart, Germany), respectively.

5.2 Polymerase chain reaction (PCR)

PCR reactions were done using Thermocycler (Dynamica C-master ™, Dynamica Scienctific Ltd., Hong Kong) with GoTaq® Green Mastermix (Promega, USA). PCR primers were selected from previous studies (Hyndman et al., 2013)

For Sunshine virus, four primers were used as follows;

Sunshine S1: 5'GGAAAGGGAGGTCTATG 3'

Sunshine AS1: 5' ATTCAACATCTGGGGTC 3'

Sunshine S2: 5' TTCAAGGAGATAACCAGG 3'

Sunshine AS2: 5' CGGGATTCCCATAGAC 3'

Three pairs of primers (set 1: S1-AS1, set 2: S2-AS2 and set 3: S2-AS1) were utilized with conditions as indicated: first heat at 94°C for 2 min, 40 cycles of 94°C for 20 sec of denaturation, 45°C for 45 sec of annealing and 72°C for 30 sec of extension.

For Ferlavirus, two primers were utilized as follows;

qS2: 5'GTTATGGCAAATCATGCTGCGATACCTTA 3'

qAS2: 5'CTGATGGGAGATAATGCCTTGTCCTTCAT3'

The condition for PCR was achieved as follows: first heat at 94°C for 2 min and 40 cycles of 94°C for 20 sec of denaturation, 55°C for 45 sec of annealing and 72°C for 30 sec of extension.

The amplicons from Sunshine virus primer sets (set 1: 153 bp, set 2: 230 bp and set 3: 357 bp) and Ferlavirus (157 bp) were visualized by 2% agarose gel electrophoresis in 0.5% Tris-borate-EDTA (TBE) after staining with 10% ethidium bromide under an UV illuminator and compared with positive controls.

6. Phylogenetic analysis

The products were purified using NucleoSpin Extract II™ (Macherey-Nagel, Düren, Germany) and submitted to Solgent™ (Korea) for genetic sequencing. Nucleotide alignments and phylogenetic tree were analyzed using Mega 6 software and compared with published data deposited in GenBank.

7. Data analysis

General information of infected snakes (both live and necropsy cases) composed of source of animal, clinical history or clinical signs, and general management were discussed in descriptive analysis. Gross pathology and histopathology of necropsy cases were described in descriptive analysis consisted of affected organs, location and severity of the lesions. PCR results and genetic sequencing were compared and discussed.

CHAPTER IV
RESULTS

1. Animal information

Totally 75 snakes were studied in this study. Forty alive snakes were Ball python (*Python regius*, 17/40), Bat-eater python (cross breed python, 1/40), Red tailed boa (*Boa constrictor*, 4/40), Jungle carpet python (*Morelia spilota,* 1/40), Golden python (*Molur bivittatus*, 8/40), Green anaconda (*Eunectes murinus*, 6/40), Green tree python (*Morelia virids*, 1/40) and Burmese python (*Python bivittatus*, 2/40). The 35 dead snakes were Ball python (9/35), Red tailed boa (13/35), Golden python (2/35), Green anaconda (2/35), Yellow anaconda (*Eunectes notaeus*, 1/35), Olive python (*Liasis olivaceus*, 3/35), Burmese python (1/35), Viper boa (*Candoia aspera*, 1/35) and non-specified python (3/35). Animal age were 5 month to 10 years old. Snakes were fed with frozen mice and chicken legs. All snakes were kept indoor separately in plastic containers or lived in exhibitions where environment were designed similar to natural requirement on each snakes. All snakes received ultraviolet light source mainly lamp or sunbath occasionally. Moreover, animal showed clinical signs in rainy to winter season or when was kept in high humidity with low ventilation area.

2. Clinical Data

Clinical signs of alive snakes were categorized into chronic respiratory problem with unresponsiveness to antibiotic treatment (21/40), open mouth breathing and hyper-salivation (6/20), chronic anorexia and emaciation with unknown causes (2/40). In addition, some ill snakes had history of exposure to the suspected cases (20/40)

Thirty-five necropsied snakes displayed chronic respiratory signs with depression (20/35), chronic anorexia with emaciation (6/35) and no detectable clinical signs (12/35), (Table 1).

Table 1 Clinical history of alive and necropsied snakes

Case No.	Ref. Code	Species	Clinical symptoms
		Necropsied animal	
1	TH01/2011	Red tailed boa	Chronic anorexia
2	TH02/2011	Red tailed boa	Chronic anorexia
3	TH03/2011	Red tailed boa	Chronic anorexia
4	TH04/2012	Red tailed boa	Chronic anorexia
5	TH05/2012	ND	ND*
6	TH06/2012	Red tailed boa	ND
7	TH07/2012	Ball python	ND
8	TH08/2012	Red tailed boa [A]	Chronic anorexia
9	TH09/2012	Red tailed boa [A]	Emaciation with weakness, chronic anorexia
10	TH10/2013	Red tailed boa [A]	ND
11	TH11/2013	Red tailed boa	ND
12	TH12/2013	Viper boa [B]	Chronic anorexia
13	TH13/2013	ND	ND
14	TH14/2013	ND	ND
15	TH15/2014	Olive python [B]	Chronic respiratory signs; opened-mouth breathing
16	TH16/2014	Ball python [B]	ND
17	TH17/2014	Red tailed boa [C]	Chronic respiratory signs, depression, weakness
18	TH18/2014	Red tailed boa [C]	Chronic respiratory signs, depression, weakness
19	TH19/2014	Red tailed boa [C]	Chronic respiratory signs, depression, weakness
20	TH20/2014	Ball python [B]	Depression with chronic respiratory signs
21	TH21/2014	Olive-golden python [B]	Depression with chronic respiratory signs
22	TH22/2014	Red tailed boa [B]	Depression with chronic respiratory signs
23	TH23/2014	Ball python [B]	Depression with chronic respiratory signs
24	TH24/2014	Ball python [B]	Depression with chronic respiratory signs
25	TH25/2014	Golden python [B]	Depression with chronic respiratory signs

Table 1 Clinical history of alive and necropsied snakes (continued)

Case No.	Ref. Code	Species	Clinical symptoms
26	TH26/2014	Ball python [B]	Depression with chronic respiratory signs
27	TH27/2014	Ball python [B]	Depression with chronic respiratory signs
28	TH28/2014	Olive python [B]	Depression with chronic respiratory signs
29	TH29/2014	Burmese python [B]	Depression with chronic respiratory signs
30	TH30/2014	Green Anaconda [B]	Depression with chronic respiratory signs, chronic anorexia
31	TH31/2014	Yellow Anaconda [B]	Depression with chronic respiratory signs, stomatitis
32	TH32/2014	Ball python	Chronic respiratory signs
33	TH33/2014	Ball python	Chronic respiratory signs
34	TH74/2015	Green anaconda [B]	Chronic respiratory signs
35	TH75/2014	Burmese python [B]	Chronic respiratory signs
Live animal			
1	THS34/2014	Golden python	Chronic respiratory signs
2	THS35/2014	Ball python	Chronic respiratory signs
3	THS36/2014	Golden python	Chronic respiratory signs
4	THS37/2014	Golden python	Chronic respiratory signs
5	THS38/2014	Red tailed boa	ND
6	THS39/2014	Ball python	ND
7	THS40/2014	Ball python	ND
8	THS41/2014	Red tailed boa	Chronic respiratory signs
9	THS42/2014	Red tailed boa	Chronic anorexia, emaciation
10	THS43/2014	Ball python	Chronic anorexia, emaciation
11	THS44/2014	Ball python	Hypersalivation
12	THS45/2014	Green tree python	Hypersalivation
13	THS46/2014	Ball python	Recurrent respiratory signs
14	THS47/2014	Ball python	Adopted from flocks that had snake with severe respiratory signs
15	THS48/2014	Red tailed boa	ND
16	THS49/2014	Ball python	ND
17	THS50/2014	Bat-eater python	ND
18	THS51/2014	Burmese python[B]	ND
19	THS52/2014	Ball python	ND
20	THS53/2014	Ball python	ND

Table 1 Clinical history of alive and necropsied snakes (continued)

Case No.	Case No.	Case No.	Case No.
21	THS54/2014	Ball python	Hypersalivation with mild stomatitis and recurrent respiratory signs
22	THS55/2014	Ball python	Hypersalivation with moderate stomatitis and recurrent respiratory signs
23	THS56/2014	Ball python	Hypersalivation with recurrent respiratory signs
24	THS57/2014	Ball python	Hypersalivation with recurrent respiratory signs
25	THS58/2015	Green anaconda [B]	ND **
26	THS59/2015	Green anaconda [B]	ND **
27	THS60/2015	Green anaconda [B]	ND **
28	THS61/2015	Green anaconda [B]	ND **
29	THS62/2015	Green anaconda [B]	ND **
30	THS63/2015	Burmese python [B]	Severe chronic respiratory signs**
31	THS64/2015	Carpet python [B]	Severe chronic respiratory signs**
32	THS65/2015	Green tree python [B]	Severe chronic respiratory signs**
33	THS66/2015	Ball python	Severe chronic respiratory signs**
34	THS67/2015	Ball python	Severe chronic respiratory signs**
35	THS68/2015	Ball python	Severe chronic respiratory signs**
36	THS69/2015	Golden python	Severe chronic respiratory signs**
37	THS70/2015	Golden python	Severe chronic respiratory signs**
38	THS71/2015	Golden python	Severe chronic respiratory signs**
39	THS72/2015	Golden python [B]	Severe chronic respiratory signs**
40	THS73/2015	Green anaconda [B]	Severe chronic respiratory signs**

* ND: No data; ** The animals present in the same colony of severe chronic respiratory signs ; [A] : Snakes from colony A; [B] : Snakes from colony B; [C] : Snakes from colony C

3. Pathology

Pathological findings of 35 snakes were described in Appendix 1. Gross pathology showed no specific lesions for the disease. Selected tissue samples from twenty-seven cases were further processed for histopathologic examination and revealed suspected intracytoplasmic inclusion bodies (ICIB). Nine out of this 27 cases were subjected for further molecular analysis.

For brain, gross pathology showed no remarkable lesion. Histopathologically, many eosinophilic intracytoplasmic inclusion bodies were seen in neurons at cerebrum, brain stem, hippocampus and in epithelial cells of choroid plexus (3/27: TH11/2013, TH17/2014, TH18/2014), (Figure 5). Moderate degree of non-suppurative perivascular cuffing were observed by 2-3 layers of mononuclear cells localization in cerebral cortex.

Macroscopic lesions of respiratory system consisted of varied degree of pulmonary edema and congestion, with or without white to cloudy yellow sticky mucus discharge. Focal pulmonary necrosis accompanied with severe chronic pneumonia was noted in some snakes (5/27). Microscopically, severe interstitial pneumonia with granulocytic cells infiltration in faveola and eosinophilic to amphophilic intranuclear and intracytoplasmic inclusion bodies were evident in faveolar epithelial cells (3/27; TH17/2014, TH18/2014 and TH19/2014), (Figure 6).

Grossly, the intestines showed predominantly necrotic enteritis in various degree (10/35). Histopathologically, catarrhal to necrotic with or without hemorrhage enteritis were characterized by sloughed off or necrotic villi with lymphoid cells aggregation in submucosa and lamina propria.

Liver were marked dark red color with white multifocal foci in gross pathology. Hepatic congestion and fatty degeneration with granulocytic cell infiltration were seen. Numerous various size of bright eosinophilic intracytoplasmic inclusion bodies were located in hepatocytes (Figure 7) and bile duct epithelium (9/27).

The kidney, spleen and pancreas mainly showed grossly organ congestion. Microscopically, remarkable lesions were renal congestion, tubular congestion with diffuses inflammatory cell infiltration. Ten of 27 snakes showed eosinophilic intracytoplasmic inclusion bodies in renal tubular epithelial cells (Figure 8). Spleen showed mild degree of necrotic splenitis without presence of inclusion bodies. The pancreas displayed many eosinophilic intracytoplasmic inclusion body in exocrine acinar cells with many inflammatory cell infiltration (4/27).

In addition, bacterial culture were done in some snakes from lung lavage during treatment or lung tissue during post mortem examination. Resistant *Pseudomonas spp.* was displayed as the main pathogen from bacterial culture.

4. Histochemical results

The tissue section of 7 cases (TH08/2012, TH09/2012, TH10/2013, TH11/2013, TH17/2014, TH18/2014 and TH19/2014) containing prominent inclusion bodies were selected for methyl green pyronin Y stain and Feulgen method. Intracytoplasmic inclusion bodies in all cases showed positively bright magenta with methyl green pyronin Y stain indicating RNA aggregation in the inclusion bodies (Figure 9 and 10). The Feulgen method was stained negative in all section.

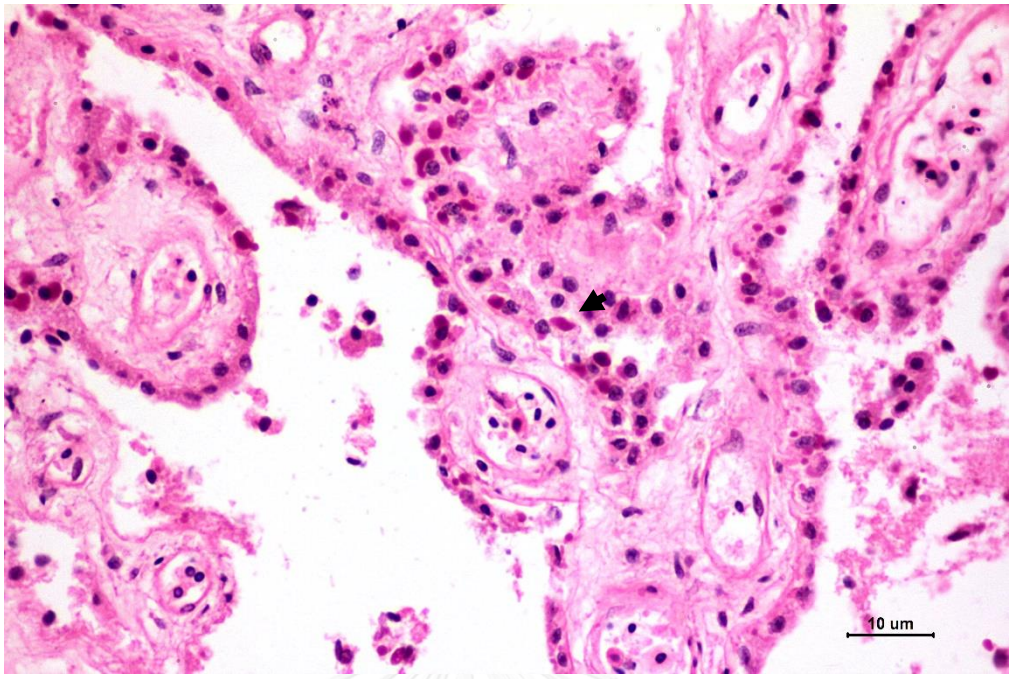

Figure 5 Histopathology of brain, choroid plexus epithelium cells showed eosinophilic intracytoplasmic inclusion bodies (arrow head); inset, eosinophilic intracytoplasmic inclusion bodies, Red tailed boa, (TH17/2014; H&E stain).

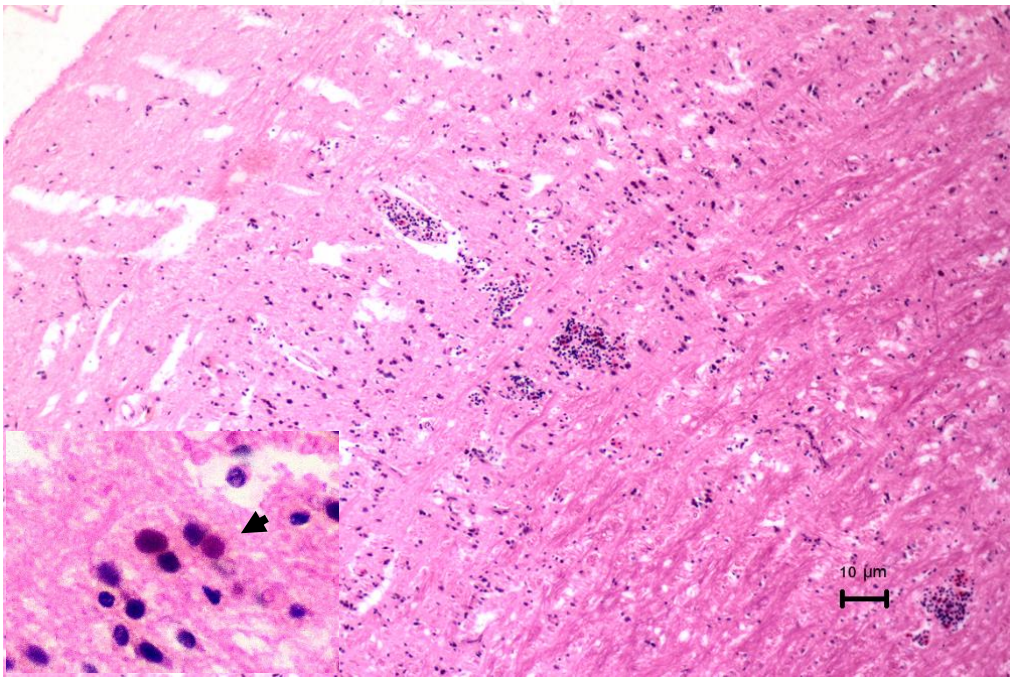

Figure 4 Histopathology of brain stem, moderate diffuse non suppurative encephalitis showed by mononuclear cells perivascular cuffing brain stem (*) (10x). (inset) Eosinophilic intracytoplasmic inclusion bodies (arrow head) in neurons. Red tailed boa, (TH17/2014; H&E stain).

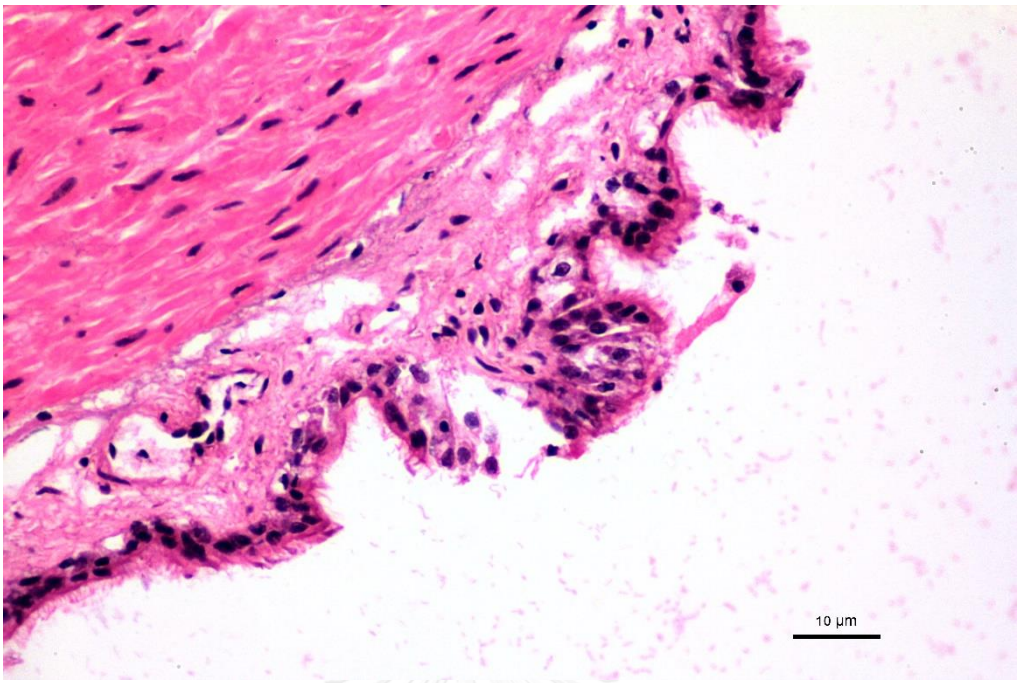

Figure 6 Histopathology of lung, Intracytoplasmic inclusion bodies in faveolar epithelial cells, Red tailed boa, (TH18/2014; H&E stain).

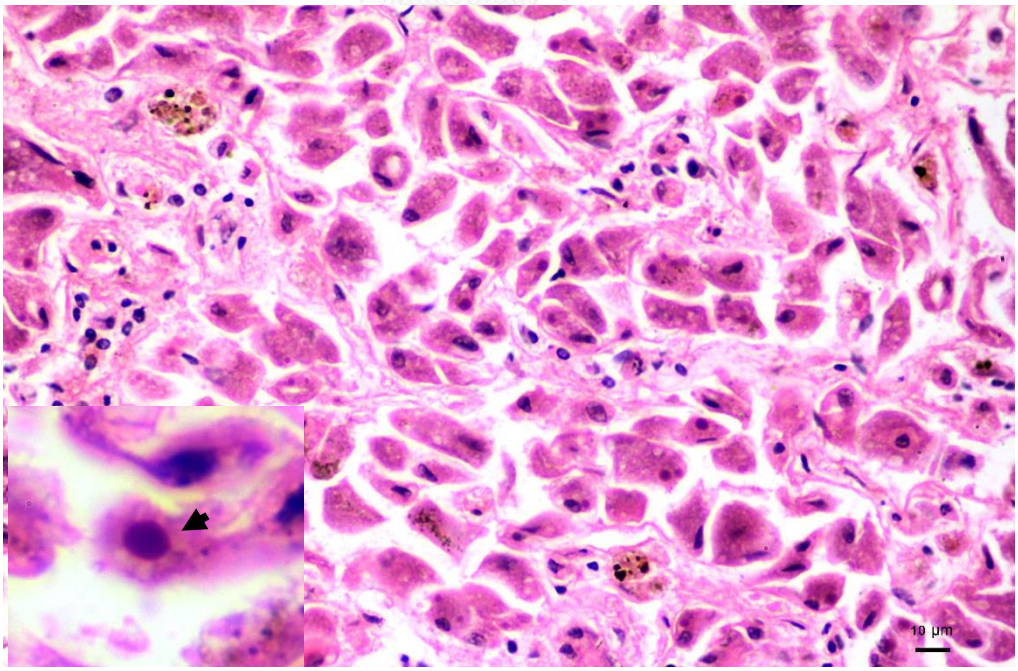

Figure 7 Histopathology of liver, diffuse non-suppurative hepatitis showed mononuclear cells infiltration in hepatic sinusoid. (Inset) (40x), eosinophilic intracytoplasmic inclusion bodies (arrow) in hepatocytes, Red tailed boa (TH08/2013; H&E stain).

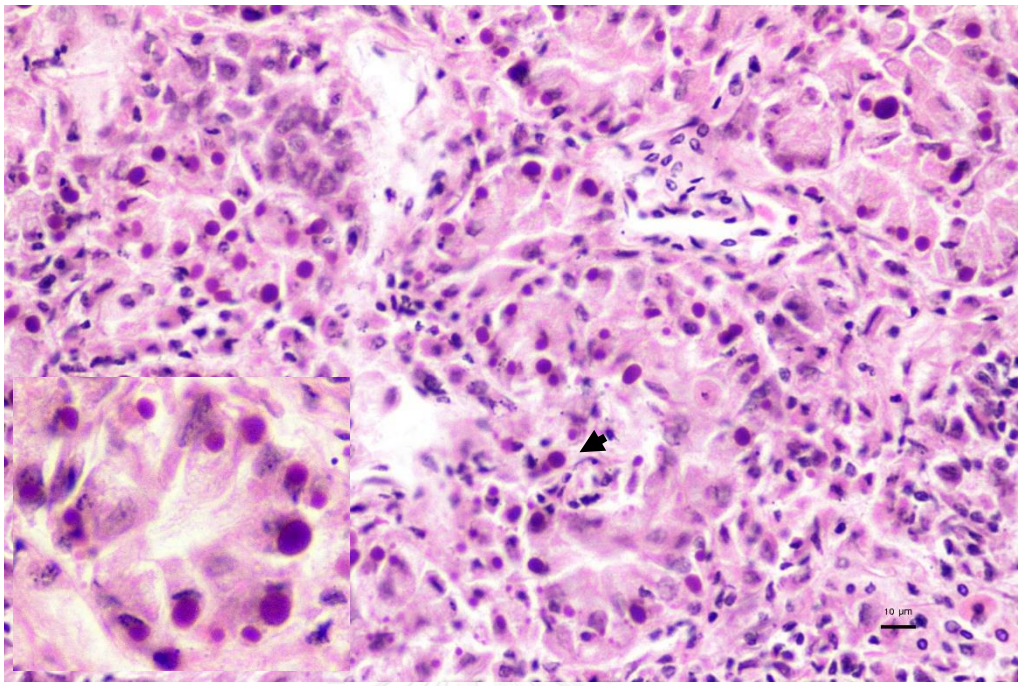

Figure 8 Histopathology of kidney, non-suppurative interstitial nephritis with mononuclear cells infiltration in interstitial tissue (arrow) Inset; eosinophilic intracytoplasmic inclusion bodies in renal tubular cells. Red tailed python (TH08/2013; H&E stain)

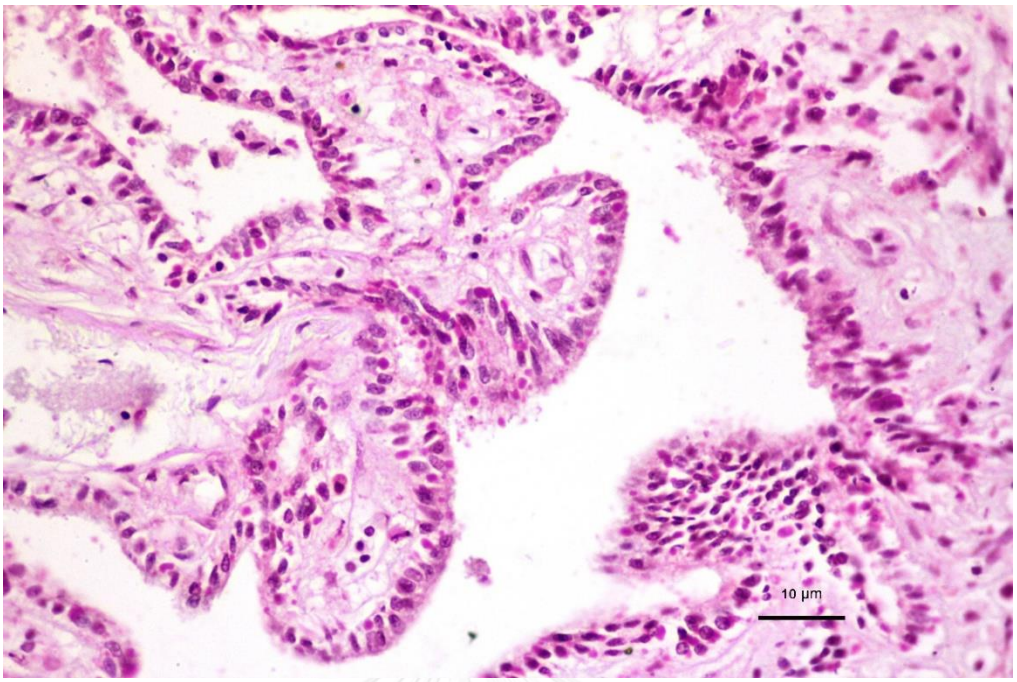

Figure 9 Histochemistry of choroid plexus, epithelium cells, intracytoplasmic inclusion bodies presented bright margenta color; Red tailed boa, (TH17/2014; Methyl green-pyronin stain)

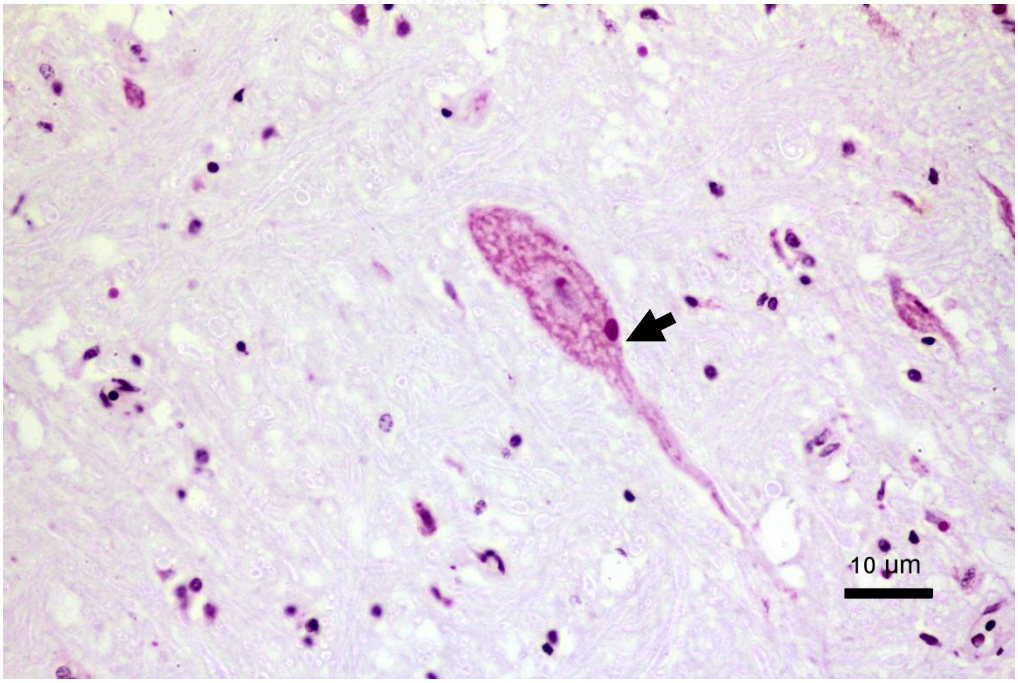

Figure 10 Histochemistry of neuron, intracytoplasmic inclusion bodies in neuron showed bright magenta from pyronin Y stain (arrow head), Red tailed boa, (TH17/2014; Methyl green-pyronin stain).

Table 2 Pathological diagnosis, inclusion bodies related organs and PCR results from each case.

Case No.	Ref. Code	Presence of inclusion bodies							PCR	Pathological diagnosis
		Brain	Lung	Heart	Liver	Kidney	Spleen	Pancreas		
1	TH01/2011				+					Hepatitis and necrotic enteritis with eosinophilic intracytoplasmic inclusion bodies
2	TH02/2011									Myocarditis associated with necrotic enteritis
3	TH03/2011			+		+			(+)^{S}	Tubulo-nephropathy with cardiac congestion
4	TH04/2012				+					Interstitial pneumonia with hepatic degeneration
5	TH05/2012				+	+			(+)^{S}	Necrotic hepatitis and nephritis
6	TH06/2012									Interstitial pneumonia
7	TH07/2012									Hemorrhagic pneumonia and suppurative nephritis
8	TH08/2012				+	+			(+)^{S}	Tubular degeneration with suppurative hepatitis
9	TH09/2012				+	+		+	(+)^{S}	Interstitial pneumonia, tubulonephrosis, nephritis and necrotic splenitis
10	TH10/2013				+	+		+	(+)^{S}	Tubular degeneration and nephritis with hepatic degeneration and diffuse hepatitis
11	TH11/2013	+	+		+	+		+	(+)^{S}	Necrotic splenitis, fatty liver, tubular degeneration
12	TH12/2013									Non suppurative endocarditis with pulmonary congestion with tubular and hepatic degeneration

+: intracytoplasmic inclusion bodies were found; (+) PCR result positive; ^{S}: Sunshine virus positive; ^{F}: Ferlavirus positive; N/A Histopathology were not available

Table 2 Pathological diagnosis, inclusion bodies related organs and PCR results from each case (cont.)

Case No.	Ref. Code	Presence of inclusion bodies							PCR	Pathological diagnosis
		Brain	Lung	Heart	Liver	Kidney	Spleen	Pancreas		
13	TH13/2013									Pneumonia with necrotic splenitis and catarrhal enteritis
14	TH14/2013									Interstitial pneumonia with tubular and hepatic degeneration
15	TH15/2014					+				Interstitial nephritis with tubular congestion
16	TH16/2014									Interstitial pneumonia with necrotic spenitis and enteritis
17	TH17/2014	+	+			+	+		(+) [F]	Interstitial pneumonia with spenitis and pancreatitis
18	TH18/2014	+	+		+	+	+	+	(+) [F]	Interstitial pneumonia with necrotic spenitis and pancreatitis
19	TH19/2014								(+) [F]	N/A
20	TH20/2014				+					Chronic pneumonia with hepatitis and necrotic enteritis
21	TH21/2014								(+) [S]	Necrotic hemorrhagic pneumonia with cerebral congestion
22	TH22/2014									Non-suppurative endocarditis, pneumonitis with chronic hepatitis
23	TH23/2014		+							Necrotic pneumonia, hepatitis, pancreatitis and enteritis
24	TH24/2014									N/A

+: intracytoplasmic inclusion bodies were found; (+) PCR result positive; [S]: Sunshine virus positive; [F]: Ferlavirus positive; N/A Histopathology were not available

Table 2 Pathological diagnosis, inclusion bodies related organs and PCR results from each case (cont.)

Case No.	Ref. Code	Presence of inclusion bodies							PCR	Pathological diagnosis
		Brain	Lung	Heart	Liver	Kidney	Spleen	Pancreas		
25	TH25/2014									Tubular degeneration with necrotic hepatitis, pancreatitis
26	TH26/2014									Interstitial nephritis with necrotic gastritis and enteritis
27	TH27/2014									Interstitial pneumonia
28	TH28/2014									N/A
29	TH29/2014								(+)[F]	N/A
30	TH30/2014								(+)[F]	N/A
31	TH31/2014									N/A
32	TH32/2014								(+)[F]	N/A
33	TH33/2014									Interstitial pneumonia with hepatitis
34	TH74/2015								(+)[F]	N/A
35	TH75/2015								(+)[F]	N/A

+: intracytoplasmic inclusion bodies were found; (+) PCR result positive; [s]: Sunshine virus positive; [F]: Ferlavirus positive; N/A Histopathology were not available

5. Transmission electron microscope (TEM)

The liver and spleen sections from a red tailed boa (TH10/2013, TH18/2014) that showed intra cytoplasmic inclusion bodies from histopathology were submitted for TEM examination. The results showed various sizes of inclusions or dark amorphous like material with unclear border in cytoplasm of hepatocytes and splenocytes. Some structure arranged in parallel like pattern (Figure 11). In higher magnification, the ultrastructure of the amorphous material displayed in cluster and could not be identified as viral particles or virus structure (Figure 12-13).

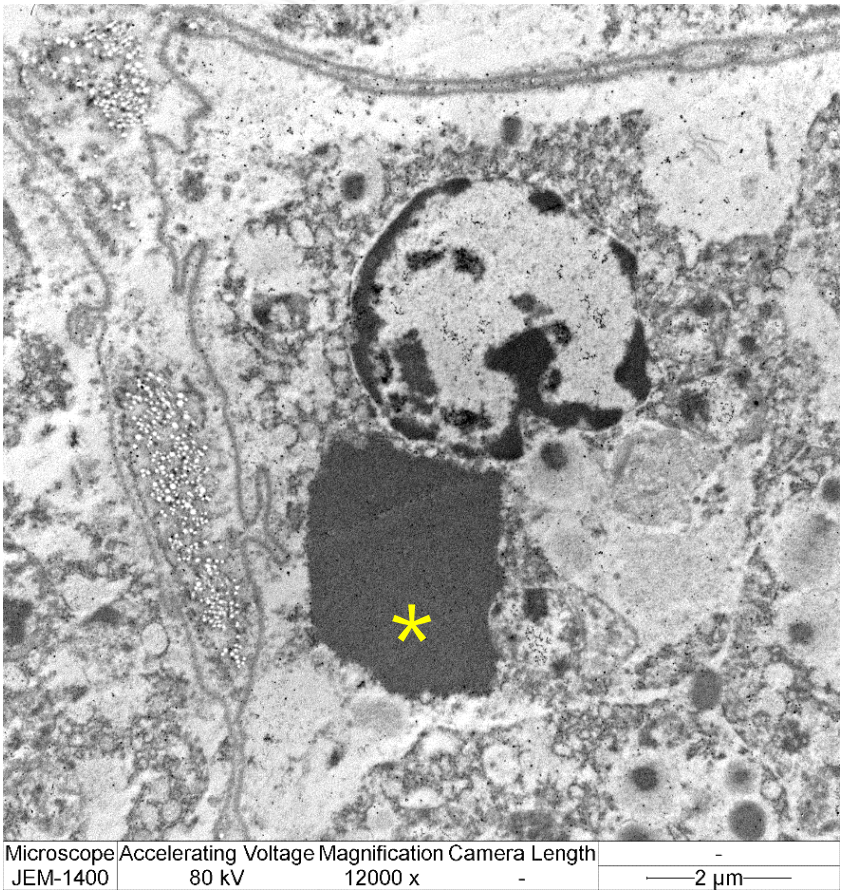

Figure 11 TEM of spleen

Red tailed boa (08/2012), spleen, Transmission electron microscopic view of inclusion body (*) in splenocyte that compatible with intracytoplasmic inclusion bodies from histological examination.

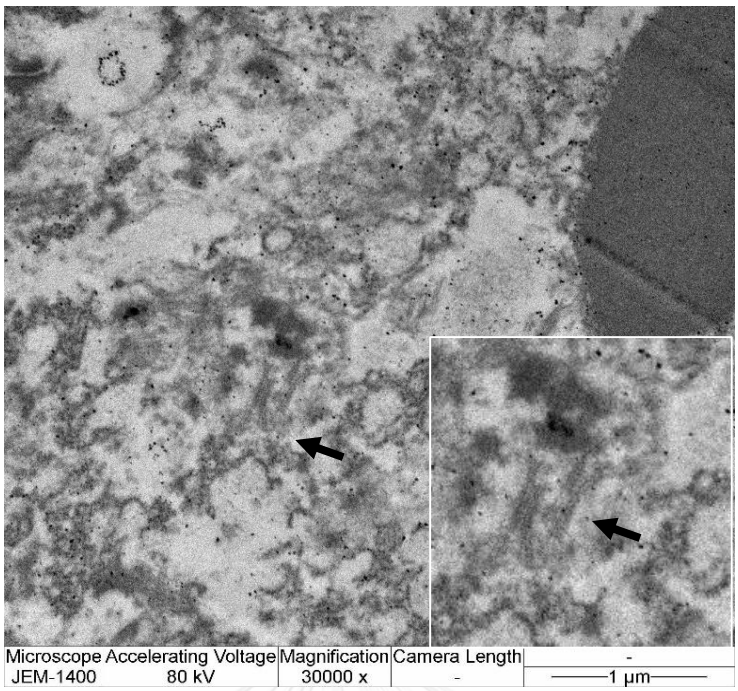

Figure 12 TEM of spleen; Red tailed boa (TH08/2012), spleen, TEM view of inclusion body showed particle arranged in parallel at border of inclusion and surrounded area. Inset, structure align like herring bone characteristic (arrow)

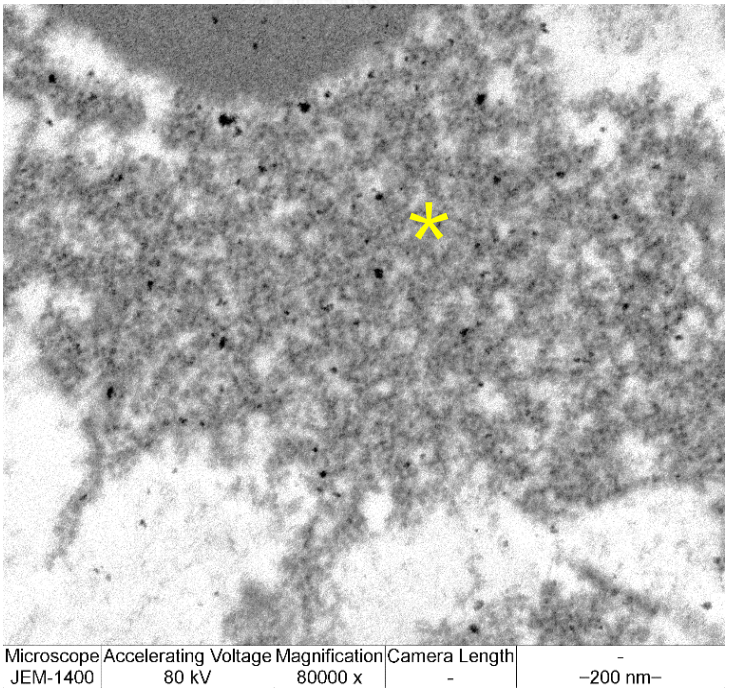

Figure 13 TEM of spleen; Red tailed boa (TH08/2012), spleen, high magnification displayed cluster of amorphous like structure which could not identified the structure of the particles (*).

6. Reverse-transcription polymerase chain reaction (RT-PCR)

Viral nucleic acid was extracted from tissue and swab samples and transcribed to cDNA. Particular fragment of Sunshine virus and Ferlavirus were further amplified using different primers. The Sunshine virus positive cases showed the 153 bp of primer set 1, 230 bp of primer set 2 and 357 bp of primer set 3. The result from this study showed appropriated product for Sunshine virus from primer set 2 and 3 while no product were detected using primer set 1. For Ferlarvirus positive cases showed 157 bp of amplicon size. Seven of 35 cases (20 %) of the tissue samples were positive for Sunshine virus and six (17.14%) for Ferlavirus. For swab samples eight of forty samples (20%) were positive for Ferlavirus. Eighteen purified products of Sunshine virus and Ferlavirus positive cases were submitted for nucleotide sequencing with the same primers set for RT-PCR.

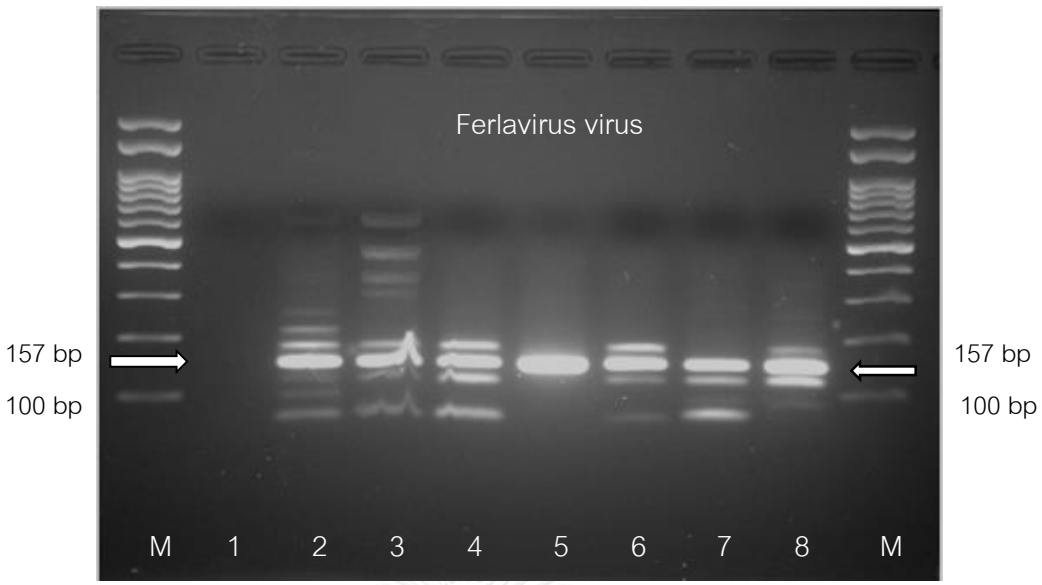

Figure 14 RT-PCR positive results of 157bp- Ferlavirus; M: 100 bp DNA marker, lane 1: negative control, lane 2: THS55/2015, lane 3: THS56/2015, lane 4: THS57/2015, lane 5: Ferlavirus positive control, lane 6: THS58/2015, lane 7: THS59/2015, lane 8: THS60/2015.

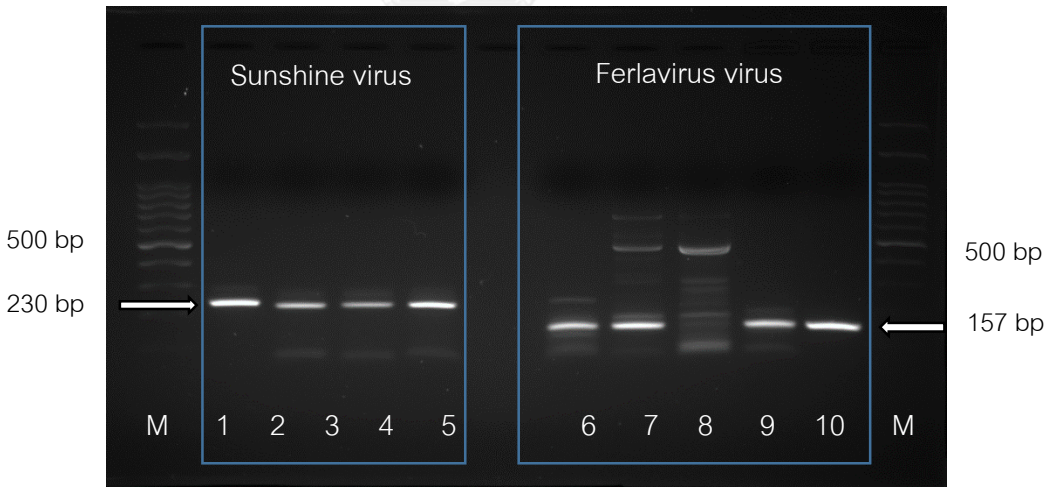

Figure 15 PCR result of Sunshine virus and Ferlavirus, RT-PCR positive results of 230bp- Sunshine virus and 157bp- Ferlavirus; M: 100 bp DNA marker, lane 1: Sunshine virus positive control, lane 2: TH29/2014, lane 3: TH30/2014, lane 4: TH30/2014, lane 5: blank, lane 6: TH29/2014, lane 7:THS66/2015, lane 8: THS67/2015, lane 9: TH19/2014, lane 10: Ferlavirus positive control.

Table 3 RT-PCR results for Sunshine virus and Ferlavirus detection of tissue samples.

Case No.	Ref. Code	Result		Case No.	Ref. Code	Result	
		Sunshine virus	Ferlavirus			Sunshine virus	Ferlavirus
1	TH01/2011	-	-	19	TH19/2014	-	+
2	TH02/2011	-	-	20	TH20/2014	-	-
3	TH03/2011	+	-	21	TH21/2014	+	-
4	TH04/2012	-	-	22	TH22/2014	-	-
5	TH05/2012	+	-	23	TH23/2014	-	-
6	TH06/2012	-	-	24	TH24/2014	-	-
7	TH07/2012	-	-	25	TH25/2014	-	-
8	TH08/2012	+	-	26	TH26/2014	-	-
9	TH09/2012	+	-	27	TH27/2014	-	-
10	TH10/2013	+	-	28	TH28/2014	-	-
11	TH11/2013	+	-	29	TH29/2014	-	+
12	TH12/2013	-	-	30	TH30/2014	-	-
13	TH13/2013	-	-	31	TH31/2014	-	-
14	TH14/2013	-	-	32	TH32/2014	-	-
15	TH15/2014	-	-	33	TH33/2014	-	-
16	TH16/2014	-	-	34	TH74/2015	-	+
17	TH17/2014	-	+	35	TH75/2015	-	+
18	TH18/2014	-	+		Total	7	6

Table 4 RT-PCR results for Sunshine virus and Ferlavirus detection of swab samples.

Case No.	Ref. Code	Result		Case No.	Ref. Code	Result	
		Sunshine virus	Ferlavirus			Sunshine virus	Ferlavirus
1	THS34/2014	-	-	21	THS54/2014	-	+
2	THS35/2014	-	-	22	THS55/2014	-	-
3	THS36/2014	-	-	23	THS56/2014	-	-
4	THS37/2014	-	-	24	THS57/2014	-	-
5	THS38/2014	-	-	25	THS58/2015	-	+
6	THS39/2014	-	-	26	THS59/2015	-	+
7	THS40/2014	-	-	27	THS60/2015	-	+
8	THS41/2014	-	-	28	THS61/2015	-	+
9	THS42/2014	-	-	29	THS62/2015	-	+
10	THS43/2014	-	-	30	THS63/2015	-	+
11	THS44/2014	-	-	31	THS64/2015	-	-
12	THS45/2014	-	-	32	THS65/2015	-	-
13	THS46/2014	-	-	33	THS66/2015	-	+
14	THS47/2014	-	-	34	THS67/2015	-	-
15	THS48/2014	-	-	35	THS68/2015	-	-
16	THS49/2014	-	-	36	THS69/2015	-	-
17	THS50/2014	-	-	37	THS70/2015	-	-
18	THS51/2014	-	-	38	THS71/2015	-	-
19	THS52/2014	-	-	39	THS72/2015	-	-
20	THS53/2014	-	-	40	THS73/2015	-	-
					Total	-	8/40

7. Sequencing and phylogenetic analyses

The sequences of the study mainly presented a group of reptilian paramyxovirus which based on published data in GenBank. Sequences were divided into two groups; Sunshine virus and other reptilian paramyxovirus. Two tissue samples of red tailed boa (2/5; TH09/2012 and TH10/2013) showed 97 – 98% nucleotide identity and displayed high similarity to Sunshine virus accession no. NC 025345 (Figure 16).

On other paramyxovirus groups, twelve of fourteen tissue samples of three red tailed boa (TH17/2014, TH18/2014 and TH19/2014), a golden python (TH29/2014), a green anaconda (TH74/2015) and seven swabs samples from five green anacondas (THS58/2015, THS59/2015, THS60/2015, THS61/2015, THS62/2015) a golden python (THS63/2015) and a ball python (THS66/2015) showed of 91 - 96 % nucleotide identity to group of reptilian paramyxovirus nucleotides following; Lizard paramyxovirus isolate Xeno-USA99 (28xpc/99) L protein (L) gene (accession no. GQ277614), a groups of Anaconda paramyxovirus isolate (accession no. KJ956407, KJ956406, KJ956404), reptilian paramyxovirus subgroup a RNA dependent RNA polymerase (L) gene (accession no.GU726898), and a group of snake paramyxovirus isolate Pyt-2 clone 7 RNA dependent RNA polymerase (L) gene (accession no. GU393346). (Figure 16). Moreover, the estimates of evolutionary divergence between sequences showed in Appendix 3.

Moreover, three samples presented similar PCR product sizes with positive control of Sunshine virus in electrophoresis but these could not be grouped within any viral species after nucleotide sequencing and analysis (Appendix 4).

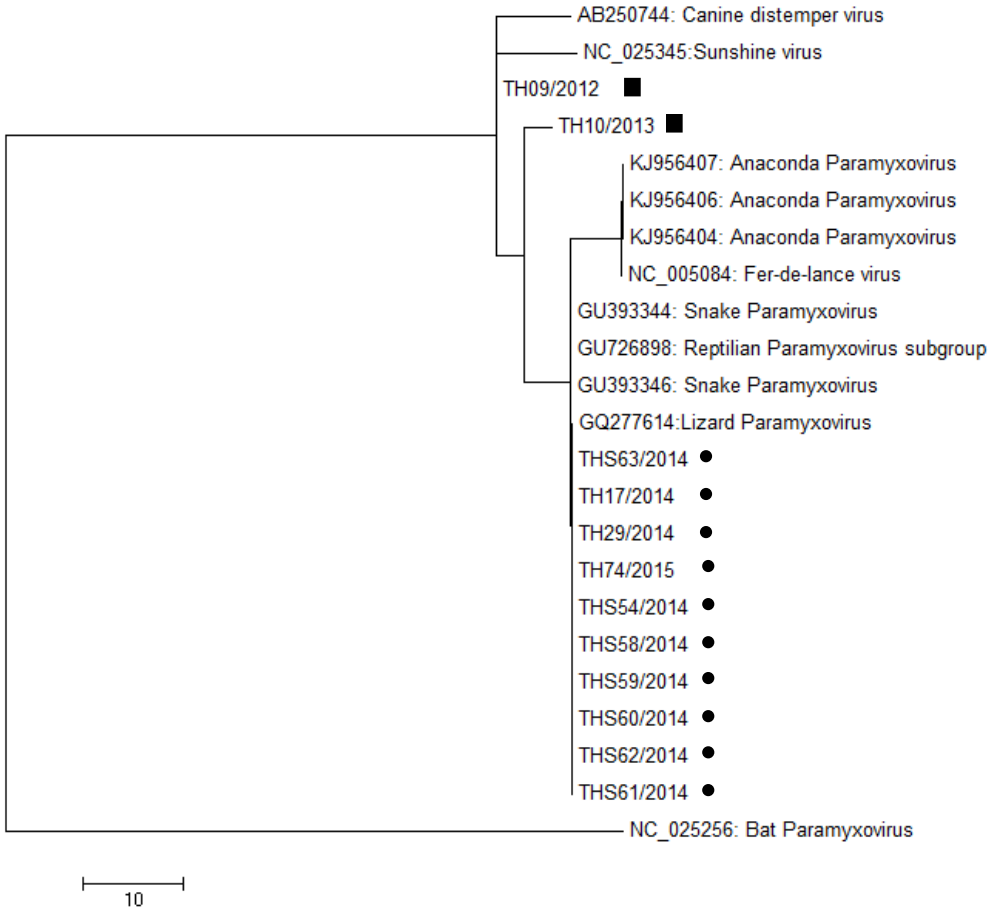

Figure 16 The phylogenetic tree of reptilian paramyxovirus and other paramyxovirus

The phylogenetic tree of reptilian paramyxovirus based on nucleotide sequence that showed high similarity published database on Genbank. The phylogenetic tree of reptilian paramyxovirus based on nucleotide sequence that showed high similarity published database on Genbank; Sunshine virus (NC 025345), Fer-de-lance virus (NC_005084) Anaconda paramyxovirus isolate (KJ956404, KJ9564076 and KJ956407), Reptilian paramyxovirus subgroup (GU726898), Snake paramyxovirus (GU393346). The symbol (●) indicates 10 reptilian paramyxovirus positive sequences and symbols (■) indicates 2 Sunshine virus positive sequences analyzed in this study.

CHAPTER V
DISCUSSION AND CONCLUSION

Reptilian paramyxovirus, the emerging infectious disease that showed high mortality in reptile species especially in snakes. This report developed diagnostic techniques and tools for disease detection and identified as causative agent in many parts of the world. In Thailand, Boidae and Pythonidae are popular snake species that raise as pet. There are many health problems in this species especially chronic respiratory problems. A large number of snakes showed clinical signs of respiratory system, which are incurable and acute death after treatment.

Nowadays, suspected snakes were clinically diagnosed based on clinical signs and some laboratory data such as hematology or bacterial culture. These observations may not identify the precise causative agent of the problems especially viral infection. This study focused on the rPMV infection in snakes that had related history or showed suspected clinical signs.

Clinically illed snakes presented predominant clinical signs on hypersalivation, open mouth breathing, stomatitis and no even clinical signs that related with previous studies regarding paramyxovirus infected snakes (Jacobson et al., 1980; West et al., 2001). However, snakes that had no clinical signs, the history taking of snakes without clinical signs is an important which will be facilitate veterinarians to investigate the disease in the affected snakes. In this study, a group of apparently green anacondas (colony B, n=6) with history of contact to the suspected cases showed PCR positive results to reptilian paramyxovirus. This finding was in agreement with previous reports showing resident snakes become ill when moving a new one to colony without disease quarantine (Folsch and Leloup, 1976; Hyndman et al., 2012a).

In addition, environmental information also should be listed or checked for classify clinical cases. Changes of environmental conditions such as humidity, temperature and ventilation may affect on captive snake's health status due to stress. All

snakes in this study showed clinical signs during rainy to winter season which was improper condition for snakes, low ventilation high humidity and induced distress that made snakes susceptible to diseases.

Base on history, Necropsy cases were divided into two groups. Firstly, a group of snakes with severe chronic purulent pneumonia and no response to any antibiotic treatment and another group were dead snakes with non-specific clinical signs or unknown history.

Lungs presented pulmonary congestion and mucoid to caseous pneumonia or necrotic pneumonitis. These pulmonary lesions might caused by secondary bacterial infection which induced severity of disease and probably the cause of death. In this study, the resistant strains of *Pseudomonas* sp. is a bacterial pathogen that was cultured from oral and lung swabs from alive and dead animals, respectively. Moreover, there are other gram negative bacterials that isolated from rPMV infected snakes e.g. *Aeromonad, Enterobacter, Escherichia* or *Proteus*. (Jacobson et al., 1997; Oros et al., 2001). Other lesions were studied such as hepatic, renal and splenic congestion with enlargement similar with previous reports (Folsch and Leloup, 1976; Hyndman et al., 2012a; Jacobson et al., 1980; Kolesnikovas et al., 2006; Papp et al., 2013).

Histopathologically, the predominant lesion of positive viral infected cases was presence eosinophilic intracytoplasmic inclusion bodies in epithelial cells of visceral organs mainly in hepatocytes, renal tubular epithelium and apocrine acinar cells with or without inflammatory reaction. These organs were commonly susceptible infected cells that found intracytoplasmic inclusion bodies in rPMV infected snakes. (Sand et al., 2004).

In addition, the brain and lung were also found intracytoplasmic inclusion bodies in epithelium of red tailed boas that positive to rPMV (TH11/2013, TH17/2014 and TH18/2014). Inclusion bodies located in cytoplasm of bronchial and faveolar epithelium of rPMV positive snakes. Sunshine virus positive in red tailed boa did not showed any

inclusion bodies in lung epithelium. The other lesions of lung were severe congestion, alveolitis. Moreover, these rPMV positive snakes presented intracytoplasmic inclusion bodies in neuron, brain stem and choroid plexus with non-suppurative perivascular cuffing. These two organs; brain and lung are common location that reported from previous studies on ferlavirus infection snakes. (Jacobson et al., 2002).

Even though the animals were classified from clinical signs especially in respiratory system, but histopathological especially intracytoplasmic inclusion bodies were remarked in various organs e.g. liver, kidney, spleen and pancreas rather than in lung tissue. Moreover, the lesions were similarly to other *paramyxovirus*; canine distemper virus, reptilian paramyxovirus is epitheliotropism and presented eosinophilic intracytoplasmic inclusion bodies in epithelial cells with non- suppurative inflammation of various organs.

Intracytoplasmic inclusion bodies of Sunshine virus and other rPMV positive cases showed same size, located site and staining pattern with other viral infections such as Retrovirus or Adenovirus. Immunohistochemistry stained with specific viral antibody, in-situ hybridization and PRC for detection of specific viral nucleic acid should performed for make a final diagnosis.

Under TEM, tissue sections from suspected cases showed intracytoplasmic dense amorphous electron dense structures surrounding with material arrange in chain-liked structure. These structure were multifocally distributed in cytoplasm of the infected cells. However, in a high magnification the picture were not clear enough to identify viral family. As same as in Sunshine virus study by Hyndman (2012), TEM showed un-identified ICIB without viral particle were detected. However, there is a controversial study noted that inclusion body from Sunshine virus infection may not be formed viral structure (Hyndman et al., 2012a).

Nucleotide sequencing and phylogenetic analysis of this study were divided into two main groups; Sunshine and other rPMV group. In Sunshine virus group, two of seven PCR positive samples showed nucleotide sequence homology to Sunshine virus while the others were not compatible with other sequences submitted in GenBank. However, these unidentified sequences (5/7) shared their homologies within group. This finding might be due to the specificity of primer that previous study designed for detect Sunshine virus from cell culture which nucleic acid was more purified (Hyndman et al., 2012a; Hyndman et al., 2012b). In the other hand, this unidentified group may represent the other novel subgroups of Sunshine virus. So high nucleotide sequencing technology or complete genome sequencing may need for viral identification. These was similar to a previous study in Anaconda paramyxovirus, a novel paramyxovirus in genus Ferlavirus, that was identified using complete genome sequencing from a cluster of fatal anaconda (Woo et al., 2014).

The comparison between genetic analysis results with clinical signs and pathological results represented that genetic analysis more related with pathological changed than clinical sign of animal. So suspected clinical sign may not good criteria to classify infected snakes. These also related with results of the non-clinical sign Anacondas in this study that showed genetic analysis closely related with paramyxovirus. Moreover, genetic analysis results were most related with histopathologic results base on inclusion bodies formation in each organs than gross pathology. So genetic analysis can be used as confirmation tool in dead snakes but should be used for disease surveillance and monitoring in lived snakes.

Interestingly, the positive Sunshine virus snakes in this study were red tailed boa (*Boa constrictors*), a member of family Boidae which had non published report in this species (Hyndman et al., 2012a; Hyndman et al., 2012b; Hyndman et al., 2013). This is

the first non- Pythonidae cases that infected with Sunshine virus. Moreover, this is also the first report on Sunshine virus infection in snakes in Thailand.

For reptilian paramyxovirus group, four necropsied and seven clinical cases showed nucleotide sequence homology to reptilian paramyxovirus group such as Lizard paramyxovirus isolate Xeno-USA99 (28xpc/99) L protein (L) gene, Anaconda paramyxovirus isolate, Reptilian paramyxovirus subgroup a RNA dependent RNA polymerase (L) gene and Snake paramyxovirus isolate Pyt-2 clone 7 RNA dependent RNA polymerase (L) gene. According to the species of infected snake and viral reference species demonstrated that both family Boidae and Pythonidae can be infected by various reptilian paramyxovirus species even paramyxovirus from lizard. In this study, snakes that infected rPMV were taken from different colonies. There as lack information about origin of red tailed boa from private owner. This snake colony showed chronic anorexia with no specific respiratory problem. This two snakes were treated with antibiotic but not response to any treatment and died later.

Another colony had various species of snakes including Ball python, Burmese python, red tailed boa and green anacondas. These group of animals were housed separate terrarium in the same building and same caretaker. The origin of animal in this colony were from different sources such as donated animal, in-house breeding from imported parents and bought from private breeders. An important point for this colony is the animal started to showed clinical problems since one female green anaconda were adopted in to the colony. Moreover, the building of this colony had low ventilation, low shedding day light and high humidity especially in rainy to winter season. These are related factors that impact to animal health as well as hygiene of the keeper. Personal hygiene is very important for snake caretaker and who handle equipment in infected or suspected area.

Conclusion

This study demonstrated pathological changes in a group of paramyxovirus infected snakes; the reptilian paramyxovirus and Sunshine virus. The remarkable histopathology from infected cases were eosinophilic intracytoplasmic inclusion bodies in various organs. However, the pathological changes could not identified the causative agent that induced inclusion bodies formation. Therefore, molecular diagnosis is play an important role for pathogen identification.

The phylogenetic analysis in this study demonstrated that there were an incidence of reptilian paramyxovirus in Thailand since 2011 and consisted of Sunshine virus, anaconda paramyxovirus, Lizard paramyxovirus and reptilian paramyxovirus in different subgroup. These virus were diagnosed from Boidae and Pythonidae snakes that consider as popular pet snakes with high market value. Furthermore, this study shows the first report on paramyxovirus infection in snakes in Thailand. The red tailed boa in this study is the first Sunshine virus infection in non-pythonidae species.

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APPENDICES

Appendix 1: Histopathological diagnosis

Ref. code	Organ	Histopathological diagnosis
TH01/2011	Liver	Diffuse hepatitis with eosinophilic intracytoplasmic inclusion bodies in bile duct epithelium and hepatocytes
	Intestine	Necrotic enteritis
TH02/2011	Heart	Non suppurative myocarditis and congestion
	Lung	Mild to moderate pulmonary congestion
	Kidney	No remarkable lesion (NRL)*
	Liver	Congestion
	Intestine	Severe necrotic enteritis
TH03/2011	Heart	Congestion with intracytoplasmic inclusion bodies in myocytes
	Kidney	Tubular degeneration with intracytoplasmic inclusion bodies in tubular epithelium
	Intestine	Necrotic enteritis
TH04/2011	Heart	Congestion (autolysis)
	Lung	Mild interstitial pneumonia
	Kidney	Congestion
	Liver	Hepatic degeneration with intracytoplasmic inclusion bodies in hepatocytes
TH05/2012	Brain	Cerebral congestion
	Heart	Congestion
	Kidney	Multifocal necrotic nephritis with intracytoplasmic inclusion bodies in tubular epithelium
	Liver	Necrotic hepatitis and intracytoplasmic inclusion bodies in hepatocytes and bile duct epithelium
TH06/2012	Lung	Interstitial pneumonia
	Liver	Hepatic congestion
	Pancreas	NRL
	Intestine	NRL
TH07/2012	Heart	Mild congestion
	Lung	Diffuse suppurative hemorrhagic pneumonia

Ref. code	Organ	Histopathological diagnosis
	Kidney	NRL
	Liver	Multifocal non-suppurative hepatitis
TH08/2012	Kidney	Congestion with eosinophilic intracytoplasmic inclusion bodies in tubular epithelium
	Liver	Diffuse suppurative hepatitis and congestion with intracytoplasmic inclusion bodies in hepatocyte and bile duct epithelium
	Intestine	NRL
TH09/2012	Lung	Mild interstitial pneumonia
	Kidney	Mild interstitial nephritis and tubular degeneration with intracytoplasmic inclusion bodies in tubular epithelium
	Spleen	Focal necrotic splenitis
	Liver	Diffuse hepatic fatty degeneration with intracytoplasmic inclusion bodies in hepatocyte
	Pancreas	Congestion with intracytoplasmic inclusion bodies in exocrine acinar cells
TH10/2013	Lung	Interstitial pneumonia
	Kidney	Diffuse interstitial nephritis with intracytoplasmic inclusion bodies in tubular epithelium
	Liver	Diffuse plasmocytic hepatitis with intracytoplasmic inclusion bodies in hepatocyte and bile duct epithelium
	Pancreas	Mild pancreatitis with intracytoplasmic inclusion bodies in exocrine acinar cells
TH11/2013	Brain	Congestion with intracytoplasmic inclusion bodies in neurons
	Heart	Non-suppurative endocarditis
	Lung	Pulmonary congestion with intracytoplasmic inclusion bodies in flaveolar epithelium
	Kidney	Tubular degeneration with intracytoplasmic inclusion bodies in tubular epithelium
TH11/2013	Spleen	Necrotic splenitis
	Liver	Multifocal hepatic degeneration with intracytoplasmic inclusion bodies in hepatocytes

Ref. code	Organ	Histopathological diagnosis
	Pancreas	Necrotic with intracytoplasmic inclusion bodies in exocrine acinar cells
	Intestine	Necrotic enteritis
TH12/2013	Brain	NRL
	Lung	Necrotic purulent pneumonia
	Kidney	Congestion
	Spleen	Necrotic splenitis
	Intestine	Catarrhal enteritis with villous atrophy with intra-lesional parasite
	Brain	NRL
TH13/2013	Heart	Congestion
	Lung	Mild interstitial pneumonia
	Kidney	Diffuse tubular degeneration
	Liver	Diffuse hepatitis with fatty degeneration
	Pancreas	Necrotic pancreatitis
	Intestine	Mild catarrhal enteritis
TH14/2013	Heart	Severe myocardial congestion and hemorrhage
	Lung	Suppurative pneumonia
	Kidney	Multifocal tubular degeneration
	Liver	Necrotic hepatitis
	Pancreas	NRL
TH15/2014	Heart	NRL
	Lung	Mild congestion
	Kidney	Interstitial nephritis, intracytoplasmic inclusion bodies in tubular epithelium
	Liver	Multifocal congestion
	Pancreas	Marked congestion
	Intestine	Chronic enteritis
TH16/2014	Lung	Interstitial pneumonia
	Kidney	Congestion
	Spleen	Purulent necrotic splenitis

Ref. code	Organ	Histopathological diagnosis
	Liver	Fatty degeneration
	Intestine	Necrotic enteritis
TH17/2014	Brain	Mild congestion with intracytoplasmic inclusion bodies in neurons
	Lung	Mild interstitial pneumonia with intracytoplasmic inclusion bodies in flaveolar epithelium
	Kidney	Interstitial nephritis with intracytoplasmic inclusion bodies in tubular epithelium
	Spleen	Multifocal necrotic splenitis with intracytoplasmic inclusion bodies in splenocyte
	Liver	Eosinophilic hepatitis with intracytoplasmic inclusion bodies in hepatocyte
	Pancreas	Diffuse necrosis with intracytoplasmic inclusion bodies in exocrine acinar cells
	Intestine	Necrotic hemorrhagic eosinophilic enteritis
TH18/2014	Brain	Mild congestion with intracytoplasmic inclusion bodies in neurons
	Lung	Mild interstitial pneumonia with intracytoplasmic inclusion bodies in flaveolar epithelium
	Kidney	Interstitial nephritis with intracytoplasmic inclusion bodies in tubular epithelium
	Spleen	Multifocal necrotic splenitis with intracytoplasmic inclusion bodies in splenocyte
	Liver	Eosinophilic hepatitis with intracytoplasmic inclusion bodies in hepatocytes
	Pancreas	Diffuse necrotic with intracytoplasmic inclusion bodies in exocrine acinar cells
	Intestine	Necrotic hemorrhagic eosinophilic enteritis
TH20/2014	Heart	Congestion
	Lung	Chronic pneumonia
	Kidney	NRL
	Liver	Severe chronic diffuse hepatitis with INIB in hepatocyte?
	Intestine	Necrotic enteritis

Ref. code	Organ	Histopathological diagnosis
TH21/2014	Brain	Cerebral congestion
	Heart	Diffuse congestion
	Lung	Necrotic hemorrhagic pneumonia
	Kidney	Diffuse congestion
TH22/2014	Lung	Necrotic purulent pneumonia
	Kidney	Diffuse congestion
	Spleen	NRL
	Liver	Necrotic hepatitis
	Pancreas	Necrotic pancreatitis
	Intestine	Catarrhal enteritis
TH23/2014	Brain	NRL
	Heart	Interstitial non-suppurative endocarditis
	Lung	Diffuse non-suppurative pneumonitis and tracheitis
	Liver	chronic hepatitis congestion
	Lung	Necrotic purulent pneumonia with INIB in flaveolar epithelium
TH25/2014	Kidney	Tubular degeneration with congestion
	Spleen	NRL
	Liver	necrotic hepatitis with severe congestion
	Pancreas	Supurative pancreatitis
	Intestine	Necrotic enteritis
TH26/2014	Brain	NRL
	Lung	NRL
	Kidney	Interstitial nephritis
	Liver	Multifocal purulent hepatitis
	Stomach	Diffuse necrotic gastritis
	Intestine	Severe necrotic enteritis

Case No.	Organ	Histopathological diagnosis
TH27/2014	Lung	Suppurative pneumonia
	Kidney	Congestion
	Liver	Congestion
	Pancreas	NRL
TH33/2015	Brain	Congestion
	Lung	Severe diffuse interstitial pneumonia with congestion and edema
	Liver	Non-suppurative hepatitis

*NRL: No remarkable lesion

Appendix 2 Nucleotide sequences

Nucleotide sequence of Sunshine virus

>TH09/2012

TTTCAAGGAGATAACCAGGTAATCGCCGTCATCTTCTCTCCACCATCAAGGTCAAACT
AGAGAAAAAATAAATCATGAACATTTGGACTCAGTTAAGAGATTTTTGAAGACTTTCACT
GAGTTTAACAGTGCAATGGGACATGAGTTAAAAGTAGAGGAGACAATAATATCTAGAG
AGATCTTTGTATATTCCAAGAAGGAATGGTATAGGAAAGGGAGGTCTATGGGAATCCC
GA

>TH10/2013

TTTCAAGGAGAAACCAGGTAATCGCCATCGGGACTTCTCTCCACCATCAAGGTCAAAC
TAGAGAAAAAATAAATCATGAACATTTGGACTCAGTTAAGAGATTTTTGAAGACTTTCAC
TGAGTTTAACAGTGCAATGGGACATGAGTTAAAAGTAGAGGAGACAATAATATCTAGA
GAGATCTTTGTATATTCCAAGAAAATATATAGGAAAGGGAGGTCTATGGGAATCCCGG
GAAAGAAAGTAATGAAATCCTTTTGTTTTGACCCTGATGCAAAGATGAATTTCCTCTCAA
GTGTTGCACTCTATGAGTCATGTATTAGCTCAGTGGTATTAAAGAAGGCTTGACCCCA
GATGTTGAATC

Nucleotide sequence of reptilian Paramyxovirus

>TH17/2014

GTTATGGCAAATCATGCTGCGATACCTTATGAATTAAGTGTCAATAATTGGGAATCTTTT
ATAGGGTTCAAATTTGACAAATTCGAGGAAGTTAATCTTGACGAGGATTTGACTATATT
CATGAAGGACAAGGCATTATCTCCCATCAGA

>TH29/2014

GTTATGGCAAATCATGCTGCGATACCTTATGAATTAAGNGTCAATAATTGGGANTCTTTT
ATAGGGTTCAAATTTGACAAATTCGAGGAAGTTAATCTTGACGAGGATTTGACTATATT
CATGAAGGACAAGGCATTATCTCCCATCAGA

>THS54/2015

GTTATGGCAAATCATGCTGCGATACCTTATGAATTAAGTGTCAATAATTGGGAATCTTTT
ATAGGGTTCAAATTTGACAAATTCGAGGAAGTTAATCTTGACGAGGATTGTGACTATAT
TCATGAAGGACAAGGCATTATCTCCCATCAGA

>THS58/2015

GTTATGGCAAATCATGCTGCGATACCTTATGAATTAAGTGTCAATAATTGGGAATCTTTT
ATAGGGTTCAAATTTGACAAATTCGAGGAAGTTAATCTTGACGAGGATTTGACTATATT
CATGAAGGACAAGGCATTATCTCCCATCAGA

>THS59/2015

GTTATGGCAAATCATGCTGCGATACCTTATGAATTAAGTGTCAATAATTGGGAATCTTTT
ATAGGGTTCAAATTTGACAAATTCGAGGAAGTTAATCTTGACGAGGATTTGACTATATT
CATGAAGGACAAGGCATTATCTCCCATCAGA

>THS60/2015

GTTATGGCAAATCATGCTGCGATACCTTNTGAATTAAGTGTCAATAATTGGGAATCTTTT
ATAGGGTTCAAATTTGACAAATTCGAGGAAGTTAATCTTGACGACGGATTTGACTATAT
TCATGAAGGACAAGGCATTATCTCCCATCAGA

>THS61/2015

GTTATGGCAAATCATGCTGCGATACCTTATGAATTAAGTGTCAATAATTGGGAATCTTTT
ATAGGGTTCAAATTTGACAAATTCGAGGAAGTTAATCTTGACGAGGATTTGACTATATT
CATGAAGGACAAGGCATTATCTCCCATCAGA

>THS62/2015

GTTATGGCAAATCATGCTGCGATACCTTTGAATTAAGTGTCAATAATTGGGANTCTTTTA
TAGGGTTCAAATTTGACAAATTCGAGGAAGTTAATCTTGACGAGGATTTGACTATATTC
ATGAAGGACAAGGCATTATCTCCCATCAGA

>THS63/2015

GTTATGGCAAATCATGCTGCGATACCTTTGAATTAAGTGTCAATAATTGGGANTCTTTTA
TAGGGTTCAAATTTGACAAATTCGAGGAAGTTAATCTTGACGAGGATTTGACTATATTC
ATGAAGGACAAGGCATTATCTCCCATCAGA

>TH74/2015

GTTATGGCAAATCATGCTGCGATACCTTATGAATTAAGTGTCAATAATTGGGAATCTTTT
ATAGGGTTCAAATTTGACAAATTCGAGGAAGTTAATCTTGACGAGGATTTGACTATATT
CATGAAGGACAAGGCATTATCTCCCATCAGA

Appendix 3 Estimate of evolutionary

The estimates of evolutionary divergence between sequences

AB250744: Canine distemper virus																						
NC_025256:Bat Paramyxovirus	11.17																					
NC_005084: Fer-de-lance virus	7.05	10.33																				
GQ277614:Lizard Paramyxovirus	6.83	9.70	3.08																			
KJ956404-Anaconda Paramyxovirus	6.80	10.73	0.16	2.99																		
KJ956406-Anaconda Paramyxovirus	6.80	10.73	0.16	2.99	0.00																	
KJ956407-Anaconda Paramyxovirus	6.80	10.73	0.16	2.99	0.00	0.00																
GU393344: Snake Paramyxovirus	6.47	10.14	2.81	0.11	2.76	2.76	2.76															
GU393346: Snake Paramyxovirus	6.47	10.14	2.81	0.11	2.76	2.76	2.76	0.00														
GU726888: Reptilian Paramyxovirus subgroup	6.47	10.14	2.81	0.11	2.76	2.76	2.76	0.00	0.00													
TH17/2014	6.79	9.69	3.15	0.06	3.06	3.06	3.06	0.12	0.12	0.12												
TH29/2014	6.79	9.69	3.15	0.06	3.06	3.06	3.06	0.12	0.12	0.12	0.00											
TH74/2015	6.79	9.69	3.15	0.06	3.06	3.06	3.06	0.12	0.12	0.12	0.00	0.00										
THS54/2014	6.79	9.69	3.15	0.06	3.06	3.06	3.06	0.12	0.12	0.12	0.00	0.00	0.00									
THS58/2014	6.79	9.69	3.15	0.06	3.06	3.06	3.06	0.12	0.12	0.12	0.00	0.00	0.00	0.00								
THS59/2014	6.79	9.69	3.15	0.06	3.06	3.06	3.06	0.12	0.12	0.12	0.00	0.00	0.00	0.00	0.00							
THS60/2014	6.79	9.69	3.15	0.06	3.06	3.06	3.06	0.12	0.12	0.12	0.00	0.00	0.00	0.00	0.00	0.00						
THS61/2014	6.79	9.69	3.15	0.06	3.06	3.06	3.06	0.12	0.12	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
THS62/2014	6.79	9.69	3.15	0.06	3.06	3.06	3.06	0.12	0.12	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
THS63/2014	6.79	9.69	3.15	0.06	3.06	3.06	3.06	0.12	0.12	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
NC_025345:Sunshine virus	6.75	9.71	5.25	5.49	5.17	5.17	5.17	5.85	5.85	5.85	5.49	5.49	5.49	5.49	5.49	5.49	5.49	5.49	5.49	5.49		
TH09/2012	4.84	10.23	6.70	3.09	6.40	6.40	6.40	3.41	3.41	3.41	3.18	3.18	3.18	3.18	3.18	3.18	3.18	3.18	3.18	3.18	6.56	
TH10/2013	7.25	10.20	7.61	6.66	9.77	9.77	9.77	6.31	6.31	6.31	6.58	6.58	6.58	6.58	6.58	6.58	6.58	6.58	6.58	6.58	12.25	3.56

Appendix 4 Alignment of nucleotide sequences

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TH21/2014  T - - - - - - - - - C T T T C A A G G A G A T A A
TH11/2013  . - - - - - - - - - - - . . . . . . . . . . . . .
TH03/2011  C - - - - - - A A G G . . . . . . . . . . . . . .

TH21/2014  C C A G G C C T T A A G C T G - T G T A C G C A C
TH11/2013  . . . . C . . . . . . . . . . - . . . . - . . . T
TH03/2011  . . . . . . T C . . . . . . . - . . C . - . . . T

TH21/2014  T - - - - - T A C C T T T T A A A A C T C A T C T
TH11/2013  . - - - - - . . . . . . . . . . . . . . . . . . .
TH03/2011  C - - - - - . . . . . C . . . . . . . . . . . . .

TH21/2014  T T A A A A C T C C T T G C T A C - - - - - C T T
TH11/2013  . . . . . . . . . . . . . . . . . - - - - - . . .
TH03/2011  . . . . . . . . . . . . . . . . . - - - - - . . .

TH21/2014  T T T C A A T C A T T T - T T T T T A A T T C C C
TH11/2013  . . . . . . . . . . . . - C . . . . . . . . . . .
TH03/2011  . . . . . . . . . . . . - C . . . . . . . . . . .

TH21/2014  A T T A A T A G G A A C A G C A T G T C T C C C C
TH11/2013  . . . . . . . . . . . . . . . . . . . . . . . . .
TH03/2011  . . . . . . . . . . . . . . . . . . . . . . . . .

TH21/2014  A A C T A T T C G T A A A A G T T C T T G G G G G
TH11/2013  . . . . . . . . . . . . . . . . . . . . . . . . .
TH03/2011  . . . . . . . . . G . . . . . . . . A G . . . . .

TH21/2014  C T T G C T T T C T T T A T A G C A G A A A T G C
TH11/2013  . . . . . . . . . . . . . . . . . . . . . . . . .
TH03/2011  . C . A . . . . . A . . . . . A . . . . . . . . A

TH21/2014  T G C T G T T G C A T G C A T T A T T C T C T G A
TH11/2013  . . . . . . . . . . . . . - - . . . - . . . . . .
TH03/2011  . . . . . G . . . . . . . - - . . . - . . . . . .

TH21/2014  T G - C T G G T T A G T C T A T G G G A A G C C C
TH11/2013  . . - . . . . . . . . . . . . . . . . . . T . . .
TH03/2011  . . - . . . . A . . . . . . . . . . . . . T . . .

TH21/2014  G A - - - - -
TH11/2013  . . C C C G -
TH03/2011  . . C C C G A
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VITA

Miss Piyaporn Kongmakee was born on December 22, 1983 in Songkhla, Thailand. She graduated Bachelor Degree of Veterinary Science (DVM) since 2007 from Faculty of Veterinary Medicine, Chiangmai University, Thailand. She was granted by the 90th Anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund), Chulalongkorn University during studying for Master degree in Veterinary Pathobiology program, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. She is currently a pathologist at Bureau of Conservation Research and Education, Zoolgical park organization Under the Royal patronage of H.M. the King since 2008 . During study, she joined international seminars with poster and oral presentation in the topics that related with her research. In 2013, in the 6th meeting of Asian Society of Zoo and Wildlife Medicine/ Conservation (ASZWM) in Singapore, the poster in topic of inclusion body disease in a Boa constrictor was presented. In 2014 she also joined the 7th (ASZWM) in Vietnam with oral presentation in topic of Reptilian Paramyxovirus in Boidae and Pythonidae. Moreover, she also submitted other cases reports to share disease information in exotic and wildlife medicine in other seminars in Thailand during 2012-2015.