# THE THAI JOURNAL OF VETERINARY MEDICINE

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This publication will be indexed and abstracted in Science Citation Index Expanded (SciSearch®), SCOPUS, CABI, Elsevier, ProQuest and EBSCO

**Printing:**
Chulalongkorn University Printing House (5305-128/600)  
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Contents

Review Article

Bone Cell Function: A Review
Nguyen Hoai Nam, Naruepon Kampa

Original Article

Short Term Echocardiographic and Clinical Effects of Ramipril in Dogs with Asymptomatic Degenerative Mitral Valve Disease
Prakit Kohkayasit, Sirilak Surachetpong

Effects of the Environmental Factors, Age and Breeds on Semen Characteristics in Thai Indigenous Chicken: A One-year Study
Pranjit Sonseeda, Thevin Vongpralub, Bunyut Laopaiboon

Development of Enzyme-Linked Immunosorbent Assay to Detect Antibodies against Chicken Infectious Anemia Virus
Niwat Chansiripornchai, Sangklae Pongthanes, Piyarat Chansiripornchai, Wisanu Wanasaawang

Effects of the Environmental Factors, Age and Breeds on Semen Characteristics in Thai Indigenous Chicken: A One-year Study
Pranjit Sonseeda, Thevin Vongpralub, Bunyut Laopaiboon

Development of Enzyme-Linked Immunosorbent Assay to Detect Antibodies against Chicken Infectious Anemia Virus
Niwat Chansiripornchai, Sangklae Pongthanes, Piyarat Chansiripornchai, Wisanu Wanasaawang

Genetic Diversity of Mycoplasma hyosynoviae Field Isolates in Thailand
Pacharee Thongkan, Sommai Yuwapanichsampan, Hideki Kobayashi, Pornpen Pathanasophon, Masato Kishima, Koshi Yamamoto

Effect of Improved Cooling on Daily Rhythmicity of Body Temperature in Crossbred Holstein Dairy Cows under Tropical Conditions
Siriwat Suadsong, Thuchadaporn Chaikhan, Junpen Suwimonteerabutr

Surgical Removal of Urethral and Bladder Stones in Female Asian Elephant (Elephas maximus) by Episiotomy and Urethrotomy
Nikorn Thongtip, Benjapol Lorsanyaluck, Manakorn Sukmak, Sirinart Chaichanathong, Naris Thengchaisiri, Pornchai Sunyathitiseree, Woraewidh Wajjwalku

Acute Pulmonary Toxicity Caused by Single Intratracheal Instillation of Various Doses of Colloidal Silver Nanoparticles in Mice: Pathological Changes, Particle Bioaccumulation and Metallothionen Protein Expression
Theerayuth Kaewamatawong, Wijit Banlunara, Pattapat Maneewattanapinyo, Chuchaa Thammacharoen, Sanong Ekagisit

Efficacy of Different Vaccination Programs against Velogenic Newcastle Disease Virus Challenge in 28-day-old Broiler Chickens
Tawatchai Pohuang, Nida Sirikobkul, Jiroj Sasipreeyagan

Comparison of Gentamicin Impregnated Polyethyleneterephlate Bead, Gentamicin Coated Native Calcium Sulfate Bead and Gentamicin Coated High Porous Calcium Sulfate Bead on Osteomyelitis Management in a Rat Model
Chaiyakorn Thitiyanaporn, Panaresu Udankumsonri, Naris Thengchaisiri

Inactivation of Infectious Bronchitis Virus with Various Kinds of Disinfectants
Pheneplint Bengthong, Thotsapol Thomrongswaanakij, Niwat Chansiripornchai

Evaluation of Hematology Profiles and Measurement of Serum Cardiac Troponin Level in Canine Monocytic Ehrlichiosis
Rungrato Osathanon, Wadasinee Moormarm, Naiyana Suksontilap, Pattaya Krajangrit, Piyanart Lekcharoensook, Pruksa Julapanthong, Nithakhulporn Wongrekngan

Discrimination between Tropical Bed Bug Cimex hemipterus and Common Bed Bug Cimex lectularius (Hemiptera: Cimicidae) by PCR-RFLP
Apisut Tavatsin, Kittithouch Lorlerthhum, Atchara Phumee, Lisanoved Thavara, Jotika Boon-Long, Rungrat Boonserm, Padet Siriysatien

Short Communication

Viability and Growth of Preantral Follicles Derived from Cryopreserved Ovarian Tissues of a Cheetah (Acinonyx jubatus) Post-mortem
Grinsarong Wonghandue, Nae Tanpradit, Daraka Thongthainun, Paveen Thawanut, Kayawadee Chatdarong

Detection of Genetic Variations Using RAPD Markers in Siberian Huskies Affected with Swimming Puppy Syndrome
Siriwadee Chomdej, Arphaphorn Dokphut, Waranee Pradit, Korakot Ngawongpanit
Endoscopic Evaluation of Gastric Mucosa to Determine Safety of Three Chondroprotective Drugs in Healthy Dogs
Korakot Nganvongpanit, Kittipong Kungprathum, Terdsak Yano, Kampanart Soontornvipart

Chemical Immobilization of Bornean Leopard Cats (Prionailurus bengalensis borneoensis) with Tiletamine and Zolazepam under Field Conditions in Borneo
Nájera Fernando, Cediel-Algavia Rafael, Hearn Andrew, Ross Jo, Dench Rosalie, Alcázar Paloma, Nathan Senthivel, de Gaspar Iñaki, Revuelta Luis

Investigation into Bacillus anthracis Spore in Soil and Analysis of Environmental Parameters Related to Repeated Anthrax Outbreak in Sirajganj, Bangladesh

Buccal Swab as a Source of Noninvasive Technique for Genomic DNA Collection in Felidae
Janjira Phavaphutanon, Sudtisa Laopium, Kawin Nanklang, Kaikamoke Sirinaramitr, Kornchai Kornkaewrat, Anuchai Pinyopummin, Jitrakorn Viriyarumpa, Piyawan Suthanmapinunt, Narathip Vorawattanatham

Diagnostic Forum
ECG Quiz
Chollada Buranakarl, Suesanakit Sawangkoon, Anusak Kijtawornrat, Thanusorn Phakhawambodee

Ophthalmology Snapshot
Nalinee Tuntivanich

Ultrasound Diagnosis
Phiwipha Kamonrat

What is your Diagnosis
Pranee Tuntivanich, Siwicha Chuthatep
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In the Journal’s Table of Contents, published articles will be shown under one of the appropriate Section titles listed below

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Clinical or Pathological Reports These are short reports of original clinical or pathological findings whose importance mean that they will be of interest to veterinarians.

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Bone Cell Function: A Review

Nguyen Hoai Nam 1,2* Naruepon Kampa 1

Abstract

Bone is dynamic tissue which is continuously formed and absorbed by its own cells in response to stimuli such as hormones, mechanical loading and growth factors. Understanding the function of bone cells is important, not only in orthopedic field but also in research study involving bone. Bone cells work in harmony to maintain a balance between bone formation and resorption, ultimately to control bone structure and function. Osteoblasts are cells, which contribute to deposition of organic components of bone extracellular matrix. They control recruitment, differentiation and maturation of osteoclasts that participate in resorption activity. In addition, osteoclasts associated with bone resorption also express several factors that regulate osteoblast function. Osteocytes, the terminally differentiated osteoblasts, act as the mechano-sensors and modulate both osteoblast and osteoclast activity, and regulate mineral homeostasis in bone tissue and mineral concentration in the blood. Similarly, bone lining cells are thought to play a role in regulation of calcium and phosphate metabolism in bone tissue, and aid osteoclasts and osteoblasts in bone remodeling.

Keywords: bone lining cells, cell interaction, osteoblasts, osteoclasts, osteocytes

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บทคัดย่อ

หน้าที่ของเซลล์กระดูก

Nguyen Hoai Nam 1,2

กระดูกเป็นเนื้อเยื่อที่มีการเปลี่ยนแปลงตลอดเวลา ทั้งในรูปแบบการสร้างใหม่และการสลายทดแทนกัน ซึ่งเป็นการตอบสนองต่อการกระตุ้นที่กระทบต่อเซลล์กระดูกเองได้แก่ ฮอร์โมน แรงที่มากระทบและปัจจัยที่ส่งผลต่อการเจริญเติบโต การทำความเข้าใจเกี่ยวกับหน้าที่ของเซลล์กระดูกมีความสำคัญ ไมเพียงเกี่ยวข้องในเรื่องของการทำงานออร์โธปิดิกส์ ยังรวมถึงงานวิจัยที่เกี่ยวกับกระดูก โดยทั่วไป เซลล์กระดูกจะทำงานประสานกันเพื่อให้การสร้างและการสลายทดแทนกันเป็นไปอย่างสมดุล เซลล์ออสติโอบลาสต์ (osteoblast) ที่หน้าที่คือ.secure the extracellular matrix. ที่หน้าที่คือ. ทำการนำแร่ธาตุเข้ามาเพื่อสร้างกระดูก และทำการควบคุมรูปแบบการเจริญเติบโตของเซลล์ออสติโอคลาสต์ (osteoclast) ซึ่งเป็นเซลล์ที่สร้างกระดูก เซลล์ออสติโอคลาสต์ที่เรียกว่ามาจากเซลล์ออสติโอบลาสต์ ซึ่งเป็นเซลล์กระดูกที่สร้างกระดูก ซึ่งมีหน้าที่ควบคุมการเจริญเติบโตของเซลล์ออสติโอคลาสต์และเซลล์ออสติโอคลาสต์ เซลล์ออสติโอคลาสต์บ่อยครั้งที่เรียกว่า bone lining ซึ่งมีหน้าที่ควบคุมการเจริญเติบโตของเซลล์ออสติโอคลาสต์และเซลล์ออสติโอคลาสต์. ซึ่งมีหน้าที่ควบคุมการเจริญเติบโตของเซลล์ออสติโอคลาสต์และเซลล์ออสติโอคลาสต์.

Introduction

Bone has a number of functions including protection, movement, leverage, mineral storage, and a source of hematopoietic cells and stem cells (Boyce and Xing, 2007). Bone metabolism is dynamic with continuous bone formation and resorption (Kwan Tat et al., 2004). A balance of these two opposing activities guarantees microstructure and function of the bone. Osteoblasts secrete bone extracellular matrix which is subsequently mineralized to build strength and hardness. In contrast, osteoclasts produce acids and enzymes to destroy the bone matrix and the structure of bone tissue (Nakamura, 2007). Although they act in two opposing directions, these two cell types interact to harmonize and modulate bone remodeling. Osteoblasts express several factors to regulate the differentiation and activity of osteoclasts (Phan et al., 2004). Conversely, osteoclasts also exert modulatory signals to control osteoblastogenesis (Karsdal and Henriksen, 2007). Moreover, these two cell types are ruled by osteocytes whose additional function is to maintain mineral equilibrium and to target distant organs such as kidney to adjust mineral excretion (Bonnwald, 2011). Bone remodeling may also be aided by bone lining cells (Kim et al., 2012) which were thought to make a negligible contribution to the process (Nakamura, 2007). This review describes the functions of bone cells, the interaction between osteoblasts and osteoclasts, and the control mechanisms asserted by osteocytes.

Osteoblasts Form Bone Matrix and Control Osteoclast Activity

Osteoblasts originate from multi-potential mesenchymal progenitors (Martin et al., 2011) and in metabolical active stage, osteoblasts are cuboidal and basophilic. However, they are flattened and less basophilic when resting (Samuelson, 2007). Their nuclei are located at the end of the cells where they are in contact with capillaries. Productive life of a lamellar osteoblast in human is about 3 months. Being specialized stromal cells, osteoblasts are exclusively responsible for the formation, deposition and mineralization of bone tissue (Phan et al., 2004). These cells secrete osteoid, the organic components of bone matrix, consisting of collagen and non-collagenous proteins such as glycoproteins and proteoglycans (Jubb et al., 2007). The organic bone matrix is subsequently mineralized by the deposition of calcium phosphate crystals and hydroxyapatite to create hardness and strength of the bone. Osteoblasts also produce several bone morphogenetic proteins (BMPs) and growth factors such as insulin-like growth factor (IGF), transforming growth factor-β (TGF-β), which are stored in the mineralized bone matrix (Nakamura, 2007). The fact that matrix metalloprotease-13 (MMP-13) is secreted by osteoblasts under regulation of parathyroid hormone suggests that these cells may also participate in degradation of collagen during bone resorption in...
Osteoblasts also regulate differentiation and the bone resorption activity of osteoclasts. Osteoblasts produce macrophage-colony stimulating factor (M-CSF) that is indispensable for both proliferation of osteoclast progenitors and their differentiation into mature osteoclasts, enhancing osteoclastogenesis. Lacking M-CSF, mice have very few osteoclasts and develop osteoporosis. The effect of M-CSF on osteoclasts seems to be phasic, since it is reported that M-CSF has negligible effect on the formation of osteoclasts and activity of mature osteoclasts, but it does influence the number of osteoclast progenitors. The secretion of M-CSF is up-regulated by the binding of parathyroid hormone to its receptor on the surface of osteoblasts.

Discovery of osteoprotegerin (OPG), a receptor activator of nuclear factor kappa-B ligand (RANKL) which is derived from osteoblasts, leads to further understanding of the mechanism of the cross-communication between osteoblasts and osteoclasts (Boyle et al., 2003). RANKL is a trans-membrane protein on the surface of osteoclastic cells that binds to its own receptor, RANK, which is on the surface of both osteoclast progenitors and mature osteoclasts (Hsu et al., 1999). By expressing RANKL, osteoblasts can promote the formation of osteoclasts (Boyce and Xing, 2007). The expression of RANKL is induced by bone resorption stimulating factors such as 1,25(OH)2D3, prostaglandin E2 (Singh et al., 2012), parathyroid hormone (Huang et al., 2004), and interleukin-1 (Nakamura, 2007). On the other hand, it can be down-regulated by estrogen (Srivastava et al., 2001). In contrast to the effect of RANKL, OPG protects the skeleton from excessive resorption by binding to RANKL, and thereby preventing it from binding to its receptor, RANK (Boyce and Xing, 2007). Collectively, the RANK/RANKL/OPG axis has a pivotal role in the control of osteoclastogenesis in which the RANKL/OPG ratio is an indispensable determinant of the formation of osteoclasts and bone resorption activity. The expression of OPG is up-regulated by estrogen, TGF-β and BMPs (Nakamura, 2007) and down-regulated by 1, 25(OH)2D3 (Horwood et al., 1998), while the osteoclast expression of RANK is induced by low intensity laser irradiation (Aihara et al., 2006).

Osteoblasts may control the osteoclast formation by other mechanisms. M-CSF expressed by osteoblasts binds to c-Fms receptors on the osteoclast surfaces (Suda et al., 1999). Interestingly, osteoblasts also produce interleukin-34 (IL-34), which is a ligand for c-Fms receptor (Lin et al., 2008). Similar to M-CSF, IL-34 promotes macrophage colony formation, but in a different way (Chihara et al., 2010). This cytokine is believed to be involved in the differentiation of hematopoietic progenitor cells into quiescent osteoclast progenitors, which subsequently circulate to find bone and finally differentiate into osteoclasts (Yamashita et al., 2012). Osteoblasts can also secrete and express several other cytokines such as IL-1α (Lomri et al., 2001), IL-6, IL-8, IL-10 (Hyzy et al., 2012), IL-11 (Sakai et al., 1999), and tumor necrosis factor alpha (TNF-α). Almost all of these factors promote osteoclastogenesis, and all these mechanisms are RANKL-independent (Bendre et al., 2003; Kudo et al., 2003), except IL-10, which inhibits the generation of osteoclasts (Evans and Fox, 2007). A series of bone morphogenetic proteins, i.e BMP2-9,15, are derived from osteoblasts as well (Sutrapreysri et al., 2006). BMP2, 4, 5, 6 are capable of promoting osteoclastic bone resorption (Kaneko et al., 2000; Wutzl et al., 2006), whereas BMP7 inhibits osteoclast generation (Maurer et al., 2012). Recently, a protein named Wnt5a expressed by osteoblasts has been found to promote the expression of RANK in osteoclast precursors, thereby enhancing osteoclastogenesis (Maeda et al., 2012). By contrast, another product of osteoblasts, semaphorin-3A, is reported to suppress osteoclast differentiation by binding to neuropilin-1 receptor and subsequently inhibiting the effect of RANKL (Hayashi et al., 2012). Thus, findings show that osteoblasts express several signals to control the formation of osteoclasts and the bone resorption activity.

**Osteoclasts Not Only Absorb Bone, But Also Control Osteoblast Activity**

Osteoclasts are giant cells with acidophilic cytoplasm and 2 to 100 nuclei. It was thought that osteoclasts were the result of the fusion of osteoblasts as they can dissociate again into osteogenic precursors. However, it is now widely accepted that osteoclasts are derived from myeloid progenitors of the monocyte-macrophage lineage. Osteoclasts have a unique ultrastructure called “ruffled border”, which is a complex interfolded finger-like structure that helps the cells in move during their bone resorption activity. Adjacent to and surrounding the “ruffled border” is the “sealing zone”, where the plasma membrane of the osteoclasts comes very close to the bone surface to ensure attachment (Martin et al., 2011). The life expectancy of human osteoclasts is dependent of the location and need, and varies from about 10 days to 6 weeks.

Osteoclasts are responsible for the bone resorption, and the differentiation and activity of osteoclasts are regulated by the expression of several factors by other bone cells. After being recruited, differentiated and mature, osteoclasts attach to the bone surface, and secrete lactic and citric acids to lower the pH and facilitate the dissolution of minerals in the bone matrix (Samuelson, 1997). The digestion of organic components of the bone matrix is conducted by lysosomal enzymes, i.e. cathepsin K and matrix metalloproteinase-9, which are in charge of degradation of collagen and gelatin, respectively (Nakamura, 2007). The inactivation of osteoclasts is attributed to calcitonin, a thyroid hormone which causes a decrease in the number of nuclei per osteoclast, the number of osteoclasts and the number of osteoclast progenitors (Jubb et al., 2007). This hormone also causes the destruction of actin filaments, the loss of clear zone, and the retraction of osteoblasts, and subsequent detachment of osteoclasts from the bone surface (Nakamura, 2007).
In addition to functioning as bone absorbing cells, osteoclasts are also involved in the control of osteoblast activity. One reported that osteoclasts synthesize and secrete hepatocyte growth factor (HGF), which supports osteoblasts entering their cell cycle and stimulates DNA synthesis in osteoblasts. This growth factor also enhances osteoblast differentiation on the hydroxyapatite surface (Hossain et al., 2005). However, HGF is also expressed by osteoclasts (Taichman et al., 2001). Therefore, the effect of HGF, which is expressed by osteoclasts, on the osteoblasts. Phan et al. (2004), who suggested that HGF secreted by surrounding osteoblasts might be as important as osteoclasts.

Sclerostin produced by mouse osteoclasts is also reported to negatively regulate the bone formation by repressing the differentiation and/or function of osteoblasts (Kusu et al., 2003). Recently, Ota et al. (2012) also suggested that murine osteoclasts expressed sclerostin in quantities that may impair the bone formation in an age-dependent manner. Interestingly, the expression of sclerostin by osteoclasts in 24-month-old mice is significantly elevated in conditioned media than that by osteoclasts from 6-week-old mice. In human, by contrast, osteoclasts do not produce sclerostin (Winkler et al., 2005). However, HGF is also expressed by osteoclasts in conditioned medium did not. More evidence concerning the interaction between osteoblasts and osteoclasts is now available since Zhao et al. 2006 reported that the molecule ephrin B2 present on the surface of osteoclasts expressed anabolic signals to the osteoblasts by binding to corresponding EphB4 receptors on the osteoclasts. The binding of ephrin B2 to EphB4 not only enhances bone formation, but also inhibits bone resorption (Zhao et al., 2006). By contrast, platelet derived growth factor-BB (PDGF-BB) produced by osteoclasts inhibits osteoblastogenesis (Kubota et al., 2002). This mechanism was elucidated by a discovery that PDGF-BB binds to PDGFB-B receptor on the surface of osteoblasts (Sanchez-Fernandez et al., 2008). In addition, osteoclasts positively modulate the osteoblast activity by producing BMP6, Wnt10b and sphingosine kinase-1 (Pederson et al., 2008).

Osteocyte Regulates Bone Remodeling and Mineral Homeostasis

Approximately 10-20% of osteoblasts are enclosed in the bone matrix, and become osteocytes (Franz-Odendaal et al., 2006). During this transformation time, there is substantial change in cell morphology. Nascent and mature osteocytes are about 30% and 70% volumetrically smaller than osteoblasts, respectively (Knothe Tate et al., 2004). Nascent osteocytes develop processes toward mineralization and subsequently towards vascularity when they are mature (Hekimsoy, 2008). Osteocytes are considered to be terminally differentiated and the most abundant cells in bone tissues. They have extremely large surface areas because of numerous cytoplasmic processes (Nakamura, 2007). Osteocytes of mature lamellar bone are flat or plump oval cells with more branching processes than those of woven bone. The life cycle of osteocytes can be up to 35 years in humans and many years in other animals (Jubb et al., 1993). The death of osteocytes is considered the consequence of senescence, degeneration, necrosis, apoptosis and/or osteoclastic engulfment (Knothe Tate et al., 2004).

Being the most abundant cells in bone tissues, osteocytes express various functions such as mechano-sensor, regulation of mineral metabolism, remodeling of perilacunar matrix and regulation of bone resorption and formation. The change in mechanical loading and PTH may result in the alteration of osteocyte activity, and modulation of bone resorption and formation. It is suggested that the mechanical loading imposes the interstitial fluid flow, which may deform osteocytes, their processes and cilia, and subsequently causes changes in cells activity (Hekimsoy, 2008). Consistently, Bonewald. (2006) proposed that osteocytes might sense the load through cell body processes and cilia. Mechanical loading stimulates dentin matrix protein 1 (DMP1) expression in osteocytes in vivo, resulting in alteration of the osteocyte matrix microenvironment by inducing formation of osteopontin, bone sialoprotein, etc. (Gluhak-Heinrich et al., 2003). Moreover, loading causes the release of nitric oxide, ATP, prostaglandin E2, and promotion of dendritic elongation (Bonewald, 2011). Furthermore, unloading up-regulates the expression of sclerostin from osteocytes (Kogianni et al., 2008), whereas PTH down-regulates (O’Brien et al., 2008). Similarly, osteocyte gene expression of Sost, which encodes sclerostin, is changed due to the change of mechanical loading (Robling et al., 2008).

Osteocytes may regulate phosphate homeostasis and mineralization. The mechanism in which osteocytes modulate mineral homeostasis is thought to be conducted through expressing their molecular products such as DMP1, fibroblast growth factor-23 (FGF-23), phosphate regulating neutral endopeptidase on chromosome X (PHEX) and matrix extracellular phosphoglycoprotein (MEPE) (Bonewald, 2007; Gluhak-Heinrich et al., 2007). DMP1 is pivotal for the normal osteocyte activity and mineralization since the absence of this protein causes defective osteocyte maturation and increased FGF-23 expression, leading to excessive excretion of phosphate in the kidney. In human and many animal species, rickets and osteomalacia, which are typically featured with soft bone and defective mineralization, are widely known as the cause of vitamin D deficiency (Dittmer and Thompson, 2011). In mice, these diseases are found in individuals who lack DMP1 (Feng et al., 2006). Increases in MEPE
expression result in the degradation of bone extracellular matrix and hypophosphatemia, which is due to phosphaturia (David et al., 2010). PHEX deficiency is necessary for the expression of FGF-23 and MEPE (Liu, 2006; David, 2009). In addition, healthy osteocytes are responsible for removal and replacement of the perilacunar matrix and potentially play a role in mineral homeostasis (Bonewald, 2011). Based on these observations, Bonewald (2011) proposed that the osteocyte network functioned as an endocrine system that acted beyond the bone tissues, targeting distant organs such as kidney.

Osteocytes can modulate both bone resorption and formation through their effects on osteoblasts and osteoclasts. Conditioned medium (CM) from osteocytes stimulates the proliferation of bone marrow stem cells and their differentiation into osteoblasts (Heino et al., 2004). Under physical contact, which is a prerequisite, osteocytes exposed to this fluid shear rapidly increase alkaline phosphatase activity of osteoblasts (Taylor et al., 2007). Furthermore, osteocytes produce low-density lipoprotein receptor related protein-5 (LRP-5) and LRP-6 in which the former protein promotes increased bone mass by enhancing osteoblast differentiation (Cui et al., 2011). In contrast, the latter protein inhibits RANKL expression, resulting in decreased osteoclastogenesis and bone resorption (Kubota et al., 2008). It is hypothesized that the expression of LRP-5 is inversely mediated by hormone serotonin since patients with a high bone mass phenotype due to the activation of a LRP-5 gene mutation have low plasma serotonin levels (Frost et al., 2010). By contrast, PTH signaling up-regulates the LRP-5 expression in osteocytes, and thereby increasing the osteoblast number and bone mass (O’Brien et al., 2008).

Osteocytes support the formation and activation of osteoclasts through the expression of large amounts of M-CSF and RANKL. Moreover, the RANKL/OPG ratio expressed by osteocytes is greater than those by osteoblasts and stromal cells (Zhao et al., 2002). On the other hand, osteocytes also produce TGF-β to inhibit osteoclastic bone resorption, and the expression of TGF-β is elevated if the osteocytes are treated with estradiol 17β (Heino et al., 2002).

Osteocytes also modulate bone remodeling through expression of osteoprotegerin (OPG) and sclerostin. OPG expression in osteocytes is stimulated by mechanical loading (Terai et al., 1999). Downregulation of OPG is in parallel with the depletion of Wnt/β-catenin in osteocytes, and thereby predisposing individuals to porous bone (Kramer et al., 2010). In contrast to OPG, which inhibits bone resorption, sclerostin produced by osteocytes inhibits bone formation (Poole et al., 2005). It is believed that sclerostin reduces the lifespan of osteoblasts by stimulating apoptosis (Sutherland et al., 2004). The effect of sclerostin on bone resorption is controversial. Li et al. (2008) reported that sclerostin had no effect on bone resorption. However, recently it has been denoted that sclerostin also promotes osteoclast formation (Wijenayaka et al., 2011). Its expression by osteocytes is reduced by mechanical stimulation (Robling et al., 2008) and parathyroid hormone (Bellido et al., 2005), and is stimulated by calcitonin (Gooi et al., 2010). In addition, sclerostin and Dickkopf-related protein-1 (Dkk1), which is also expressed by osteocytes, are two negative regulators of Wnt/β-catenin pathway (Bonewald, 2011).

Apoptotic osteocytes express apoptotic bodies that are responsible for initiating the osteoclastic bone resorption on quiescent bone surfaces. Unlike the case of healthy osteocytes, the mechanism in which the apoptotic osteocytes increase osteoclastogenesis is independent of the RANK/RANKL/OPG axis because the addition of OPG does not influence the osteoclastogenic activity of apoptotic osteocytes (Kogianni et al., 2008).

### Bone Lining Cells Aid Osteoclasts and Osteoblasts in Bone Remodeling

Bone lining cells (BLCs) are flattened in shape, with few cell organelles. With this morphological feature, BLCs are believed to have little or no involvement in bone formation (Nakamura, 2007). However, these cells are found to contribute to the bone remodeling, and to affect the concentration of minerals in blood and bone tissues. It is observed that mechanical loading stimulates bone formation by reactivation of BLCs to become active osteoblasts. Similarly, BLCs can be reactivated by intermittent treatment of parathyroid hormone (PTH) (Kim et al., 2012). The increase in bone formation with PTH treatment is not associated with cell proliferation, but most likely due to activation of preexisting quiescent BLCs to osteoblasts. PTH and calcitonin directly target BLCs, influencing Ca : PO4 ratios in mitochondria, suggesting that these two hormones act on BLCs to modulate mineral concentrations of blood and temporary storage of calcium at bone surfaces.

These cells are believed to participate in the bone resorption activity, thereby being partly responsible for the bone remodeling. Before the osteoclastic activity, BLCs digest non-mineralized collagen protruding from bone surfaces. Moreover, bone resorption by osteoclasts is not completed, and these cells leave remnants of demineralized non-digested bone collagens behind after their withdrawal. In their turn, BLCs digest collagens left by osteoclasts in the resorption lacunae. Interestingly, they further form a cement line and deposit a thin layer of fibrillar collagen on the cleaned bone surfaces that may facilitate the subsequent osteoblast activity (Everts et al., 2002).

### Conclusions

Mutual interaction among bone cells is strongly evident from this review. Osteoblasts control the differentiation and bone resorption activity of osteoclasts via several mechanisms in which RANK\RANKL\OPG axis is prevalent and dominant. In addition, many other factors such as M-CSF, Wnt5a, semaphorin 3A, ILs and BMPs also express their effects on osteoclasts either directly or indirectly. The expressions of ephrin B2 and PDGF-BB in osteoclasts, and the discovery of EphB4 and PDGF-
BB receptors in osteoblasts are undeniable evidence which proves that osteoclasts express signals to regulate the osteoblastogenesis and bone formation. In response to mechanical loading, PTH, I, 25(OH)2D3, and estrogen, osteocytes produce several factors to modulate bone formation and resorption, mineralization of the bone matrix, and mineral homeostasis in bone tissues. Bone lining cells aid both osteoclasts and osteoblasts in bone remodeling by absorbing remnants left by osteoclasts in the bone lacunae and secreting cement lines and fibrillar collagens to facilitate osteoblasts deposition and bone formation. Though various findings have partly explained the communication among bone cells, other mechanisms are believed to exist and in need of elucidation. Collectively, bone cells work in harmony, and mutually interact to ensure the balance between bone formation and bone resorption.

Acknowledgement

The authors thank Dr. Frank F. Mallory for reviewing the manuscript. The PhD study program is granted by Ministry of Education and Training, Vietnamese Government.

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Short Term Echocardiographic and Clinical Effects of Ramipril on Dogs with Asymptomatic Degenerative Mitral Valve Disease

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Abstract

Angiotensin converting enzyme (ACE) inhibitors have beneficial effects on degenerative mitral valve disease (DMVD) dogs with stages C and D (ACVIM classification) and on dogs with congestive heart failure. However, ACE inhibitors' effects on stage B2 or asymptomatic DMVD dogs have still been uncertain. Ramipril is an ACE inhibitor that has lipophilic effects and can suppress ACE in cardiac tissue effectively. We hypothesized that ramipril had beneficial effects on dogs with naturally occurring DMVD in stage B2. Twenty dogs with stage B2 DMVD, weighing between 3-12 kg and being older than 6 years, were recruited into the study. The dogs were single blinded randomized and divided into 2 groups. Owners made decisions whether or not to supplement their dogs with ramipril. Dogs in the ramipril group (n = 10) received ramipril once a day at dose of 0.22 mg/kg PO. The control group (n = 10) did not receive any drugs for 91 days. Complete physical examination, electrocardiography and echocardiography were performed on days 0, 28, 56 and 91. Echocardiographic examination was used to determine cardiac sizes and structural changes. Independent t-test was performed to compare differences between dogs in ramipril and control groups. Repeated ANOVA was used to compare differences within groups between days 0, 28, 56 and 91. $P < 0.05$ was considered statistically significant. Cardiac chamber size, systolic function and severity of mitral regurgitation were not significantly different between the 2 groups throughout the study period. In conclusion, ramipril did not affect cardiac size, severity of mitral regurgitation and systolic function in 91-day study period.

Keywords: angiotensin converting enzyme inhibitors, congestive heart failure, dog, mitral valve disease, ramipril

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ผลระยะสั้นของยารามิพริลต่อค่าคลื่นเสียงสะท้อนความถี่สูงและอาการทางคลินิกในสุนัขที่มีภาวะลิ้นหัวใจเสื่อมในกลุ่มที่ยังไม่แสดงอาการทางคลินิก

บทคัดย่อ

ผลระยะสั้นของยารามิพริลต่อค่าคลื่นเสียงสะท้อนความถี่สูงและอาการทางคลินิกในสุนัขที่มีภาวะลิ้นหัวใจเสื่อมในกลุ่มที่ยังไม่แสดงอาการทางคลินิก

Introduction

Degenerative mitral valve disease (DMVD) is a contribution of cardiovascular morbidity and mortality in dogs (Egenvall et al., 2006). This chronic progressive disease increases incidence in small breeds of old dogs and is found more frequently in males than females. (Häggström et al., 2009). DMVD is characterized by a long preclinical period. It has been found in about 3-7% of all canine population (Borgarelli and Buchanan, 2012). Some dogs may develop heart failure in a short period of time whereas some dogs may stay healthy and have no clinical signs or progress to heart failure for several years (Kvart et al., 2002). The incidence is particularly high and shows strong component to a polygenic mode of inheritance in some breeds, for example, Dachshund and Cavalier King Charles spaniel (CKCS) (Swift, 1996). The prevalence of DMVD is strongly associated with age and breed. In early stage, affected valves may function adequately without hemodynamic effects (Borgarelli et al., 2011). The clinical signs of DMVD vary from dogs to dogs including cough, anorexia, exercise intolerance, dyspnea to sudden death. DMVD involves complex connective tissue degeneration. The histological changes by excessive destruction and derangement of valve layer structure with accumulation of glycosaminoglycans in the leaflet and chordae tendinae (Fox, 2012). In later stage, mitral valves and chordae tendinae severely thicken and become redundant, causing improper leaflet coaptation and regurgitation of blood across the closed mitral valve during ventricular systole. The valve regurgitation leads to drop in forward stroke volume, cardiac output and increased intraatrial pressure resulting in renin angiotensin aldosterone system (RAAS) activation to maintain cardiac output. The compensatory process is able to maintain cardiac output for a long period of time. Once the heart fails to compensate, the decompensatory heart failure develops and death can occur.
Several reports have proven that angiotensin converting enzyme (ACE) inhibitors benefit management of congestive heart failure in dogs by increasing survival rate, improving quality of life in dogs with classes II and III International Small Animal Cardiac Health Council (ISACHC) classification heart failure (The BENCH Study group, 1999; Häggström et al., 2008). The beneficial effects of ACE inhibitors in other stages of heart failure particularly in asymptomatic dogs have been debated. Previous multicenter double blinded studies found that ACE inhibitors did not have significant effects in treatment asymptomatic dogs with DMVD (Kvart et al., 2002; Atkins et al., 2007). A retrospective study of Pouchelon et al. (2008) reported a possible benefit of early treatment with benazepril in asymptomatic dogs with DMVD. Thus, the beneficial effects of ACE inhibitors in asymptomatic DMVD dogs are uncertain and remain in the area of debate.

ACE is present in both plasma and tissues. In addition, there are differences in the relative tissue or circulating affinity of ACE inhibitors (Apurva et al., 2005). Ramipril is a long acting ACE inhibitor, which has a lipophilic property. Therefore, it can penetrate to local tissues and suppress ACE releasing there effectively. According to Ramakrishna et al. (1991) demonstrated that ramipril had high tissue affinity property and could reduce both tissue and plasma ACE activity. Ramipril is one of ACE inhibitors in dicarboxylate containing agent group. ACE inhibitors in this group are prodrugs or precursors that need to be converted to active metabolites by liver esterase enzyme before they can work. Because of a more lipophilic effect and a long acting duration, ramipril may be superior to other ACE inhibitors in treatment of DMVD dogs. Several reports show that use of ramipril is safer than the other ACE inhibitors in renal impairment patients (Hope study investigators, 2000; Lefebvre et al., 2006). The study of ramipril in naturally occurring DMVD is still lacking, particularly in asymptomatic dogs. We hypothesized that ramipril had beneficial effects on dogs with naturally occurring canine DMVD in stage B2 (ACVIM classification) or dogs with DMVD that have no clinical signs but have cardiac structural changes. The aim of this study was to evaluate short term echocardiographic and clinical effects of ramipril in naturally occurring asymptomatic stage B2 DMVD dogs (ACVIM classification).

Materials and Methods

This study was a single blinded randomized prospective study. All dogs enrolled into the study were patients of the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University. The protocol used in the present study was approved by Chulalongkorn University Animal Care and Use Committee. Dogs affected with stage B2 DMVD (ACVIM classification) (Atkins et al., 2009) presented at Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University were included in the study. Due to possible differences in the nature of DMVD between small and large breeds of dogs (Borgarelli et al., 2004), only small breeds that weighed between 3-12 kg and were older than 6 years were selected for study. All dogs had systolic murmur heard best over the left cardiac apex or the mitral area. The dogs underwent complete physical examination, cardiac auscultation, blood collection, radiography, echocardiography and electrocardiography. Results were collected as baseline data. Thoracic radiography was performed to assess hemodynamic significance of DMVD and rule out primary respiratory diseases as well as signs of pulmonary edema secondary to congestive heart failure. Echocardiography was used to confirm the diagnosis of mitral valve degeneration. Cardiac remodeling was determined by M-mode echocardiography including enlarged left atrium (increased left atrium to aorta dimension ratio more than 1.3 (Boon et al., 1983) and/or increased left ventricular chamber size with decreased left ventricular wall thickness. Blood samples were submitted to evaluate renal and liver status before starting the study and to assess clinical tolerance as well as undesirable side effects from ramipril after enrollment in the study. All dogs with acute and chronic renal insufficiency (defined as creatinine(Cr) above 1.8 mg/dl and BUN above 27 mg/dl) (Douglas et al., 2005) were excluded from the study. Due to teratogenic effects of ramipril, the study was not performed in pregnant and lactating bitches. Dogs with clinical signs of heart failure including ascites, dyspnea, exercise intolerance, cough or dogs receiving any drugs that might have effects on cardiovascular system and dogs with other kinds of heart diseases or abnormalities rather than DMVD were excluded from the study.

Because there have not been any standard protocols for treatment of dogs with stage B2 DMVD recently (Atkins et al., 2009), the owners themselves made decisions whether or not to supplement their dogs with the drug. In the ramipril group, dogs were supplemented with ramipril 0.125 mg/kg per OS once a day for 91 days. Dogs in the control group did not receive any drugs. Complete physical examination, electrocardiography and echocardiography were performed on days 0, 28, 56, and 91. Radiography and blood collection for hematology and biochemistry analysis were performed on days 0 and 91. Quality of life and clinical signs were evaluated by clinical score in Table 1. Cardiac structure remodeling was evaluated from two dimensional and M-mode echocardiography assessed by ultrasound machine (LogicTMS Pro) with multi-frequency 6-10 MHz microconvex and 5-6 MHz phase array transducers. Left ventricular internal diastolic diameter (LVId), left ventricular internal systolic diameter (LVIDs), left ventricular free wall thickness during diastole (LVWd) and systole (LWVs), ventricular septal thickness during diastole (VSt) and systole (VSts), and the ratio of left atrium to aorta dimension (LA/Ao) were measured from right parasternal short and long axis views. To evaluate the chamber size and wall thickness, the echographic indices including LVIDd (LVIDd/Body surface area (BSA)), LVIDs (LVIDs/BSA), VSdi (VSDi/BSA), VSsi (VSSi/BSA), LVWdi (LVWdi/BSA), and LVWsi (LVWsi/BSA) were used to reduce body weight variation between dogs. Fractional shortening was calculated by ((LVEDd- LVEDs)/LVEDd)x100.
Comparison differences within groups between days 0, 28, 56 and 91.

Statistically significant.

Control and ramipril groups. Paired ANOVA was used to compare differences within and control groups on days 0, 28, 56 and 91. Repeated compare echocardiographic values between ramipril (ranging from 0.14-0.22 mg/kg).

The average dose of ramipril in the ramipril group was 0.18 ± 0.03 mg/kg (ranging from 0.14-0.22 mg/kg).

Modified from: Häggström et al., 2008.

Regurgitant flow velocity (MR), area of regurgitant jet as a proportion to the area of left atrium (ARJ/LA) and Proximal Isovelocity Surface Area (PISA) were measured for evaluating severity of mitral valve regurgitation. All echocardiographic examinations were performed in conscious un-sedated dogs.

The clinical score was evaluated as descriptive analysis. Independent t-test was used to compare echocardiographic values between ramipril and control groups on days 0, 28, 56 and 91. Repeated ANOVA was used to compare differences within control and ramipril groups. Paired t-test was used to compare differences within groups between days 0, 28, 56 and 91. p-value less than 0.05 was considered statistically significant.

**Results**

A total number of 23 dogs enrolled in the study. Twelve dogs (9 males and 3 females) were assigned to the ramipril group while the other 11 dogs (5 males and 6 females) were in the control group. In the ramipril group, two dogs were excluded from the study due to an increase in creatinine concentration in one dog (Cr > 1.8 mg/dl) and death from car accident in the other. In the control group, one dog was excluded due to the car accident. Therefore, only 20 dogs were included for final analysis. The intensity of heart murmur of all dogs in the control group was grade IV. Two dogs had grade III and 8 dogs had grade IV murmur in the ramipril group. The control group consisted of 4 males and 6 females. Breeds included 7 Poodles, 2 mixed breeds and 1 Miniature Pinscher. Ten dogs in the ramipril group consisted of 7 males and 3 females, including 2 mixed breeds, 2 Chihuahuas, 2 Poodles and one each of Spitz, Shih tzu, Yorkshire Terrier and Dachshund. At baseline, age, weight, heart rate, respiratory rate were similar and not statistically different between the control and ramipril groups (Table 2).

**Effects of ramipril on echocardiographic values:** LVIDdi and LA/Ao parameters were used to evaluate cardiac chamber size. LVIDdi and LA/Ao were not statistically different between the control and ramipril groups on days 0, 28, 56 and 91 (Fig 1). To assess the degree of mitral regurgitation between the control and ramipril groups, the MR, ARJ/LA and PISA were determined. The results of PISA, MR and ARJ/LA showed insignificant difference between the control and ramipril groups on days 0, 28, 56 and 91 (Fig 2). %FS and LVIDdi were used to assess systolic function. Both values were not statistically different between the two groups throughout 91 days of the study (Fig 3). The echocardiographic values are presented in Table 3.

**Progressive of disease in control and ramipril groups:** The progressive of disease in the control and ramipril groups was studied on days 0, 28, 56 and 91. On the control group, PISA on days 28 and 56 was the same as day 0. In day 91, PISA was significantly different compared to day 0 (p = 0.01). ARJ/LA changed significantly on day 56 compared to day 0 and day 28 (p = 0.03) but not different from day 91 (Table 4). In the ramipril group, all echocardiographic values were not statistically different between days 0, 28, 56 and 91 (Table 5).

**Effects of ramipril on clinical signs, potential adverse reactions and causes of withdrawal:** Three dogs in the ramipril group showed an improvement of clinical status. Two dogs increased appetite and one dog reduced frequency of cough. One dog was withdrawn because of increase in Cr and BUN after two months of treatment with ramipril.

**Table 1 Scoring protocols for clinical signs**

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>Score</th>
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<tr>
<td>Appetite</td>
<td>I: decreased appetite, II: normal appetite, III: increased appetite</td>
</tr>
<tr>
<td>Cough</td>
<td>I: normal, II: few cough, III: cough all day</td>
</tr>
<tr>
<td>Exercise intolerance</td>
<td>I: dog is able to fully exercise, II: Dog is less active than normal, avoided long walk, III: Dog is inactive and will only get up to eat and drink</td>
</tr>
<tr>
<td>Attitude</td>
<td>I: alert and responsive, II: moderately lethargic, III: minimal responsive</td>
</tr>
<tr>
<td>Respiratory effort</td>
<td>I: normal, II: increase rate or effort, III: severe respiratory distress</td>
</tr>
</tbody>
</table>

**Table 2 Baseline characteristics of 20 dogs with degenerative mitral valve disease**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Ramipril (n = 10)</th>
<th>Control group (n = 10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>11.70 ± 2.41</td>
<td>12.10 ± 2.51</td>
<td>0.72</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>7.27 ± 3.17</td>
<td>6.59 ± 3.30</td>
<td>0.65</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>145.60 ± 15.85</td>
<td>137.00 ± 21.10</td>
<td>0.39</td>
</tr>
<tr>
<td>Respiratory rate (rpm)</td>
<td>39.00 ± 7.95</td>
<td>38.60 ± 7.89</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Data presented as mean ± SD (bpm: beat per minute, rpm: respiratory rate per minute)

**Figure 1** Effect of ramipril on LVIDdi and LA/Ao. These graphs show echocardiographic value on days 0, 28, 56 and 91 of ramipril and control groups. (A) LVIDdi (Left ventricular internal diameter diastolic index, (B) LA/Ao (Left atrial/Aorta).
Effect of ramipril on heart rate, respiratory rate, heart murmur grade and electrocardiography: Heart rate and respiratory rate were not significantly different between the ramipril and control groups throughout the study period (Table 6). Grade of heart murmur in the control group was grade IV in all dogs throughout 91 days. In the ramipril group, heart murmur was grade III in 2 dogs and grade IV in 8 dogs. On day 28, one dog in the ramipril group had decreased intensity of heart murmur from grade IV to grade III. On day 91, heart murmur in 2 dogs was increased intensity from grade III to grade IV. Electrocardiography was normal and no evidence of arrhythmia was seen in both control and ramipril groups.

Effects of ramipril on complete blood count and blood chemistry: SGPT in the control group was higher than in the ramipril group on day 0 ($p = 0.04$). However, the SGPT was still in the normal limit in both groups (Table 7). Values of other blood parameters between the control and ramipril groups were not significantly different on days 0 and 91. All blood hematology and blood chemistry values in the control group between day 0 and day 91 were not significantly different. In the ramipril group, platelet number on day 91 decreased compared to day 0 ($p = 0.03$) and white blood cell count on day 91 increased significantly compared to day 0 ($p = 0.01$) (Table 8).

Table 3 Baseline characteristics of 20 dogs with degenerative mitral valve disease

<table>
<thead>
<tr>
<th>Variables</th>
<th>Day0</th>
<th>Day 28</th>
<th>Day 56</th>
<th>Day 91</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDdi (mm/m²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.30 ± 1.60</td>
<td>0.74</td>
<td>8.60 ± 2.00</td>
<td>0.41</td>
</tr>
<tr>
<td>Ramipril</td>
<td>8.10 ± 1.20</td>
<td>0.70</td>
<td>7.90 ± 1.40</td>
<td>0.70</td>
</tr>
<tr>
<td>LA/Ao</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.80 ± 0.30</td>
<td>0.07</td>
<td>1.70 ± 0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>Ramipril</td>
<td>1.60 ± 0.10</td>
<td>0.07</td>
<td>1.60 ± 0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>PISA(cm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.98 ± 0.45</td>
<td>0.18</td>
<td>1.26 ± 0.37</td>
<td>0.78</td>
</tr>
<tr>
<td>Ramipril</td>
<td>1.56 ± 0.21</td>
<td>0.19</td>
<td>1.19 ± 0.59</td>
<td>0.78</td>
</tr>
<tr>
<td>MR (m/s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.50 ± 0.80</td>
<td>0.85</td>
<td>4.60 ± 0.90</td>
<td>0.55</td>
</tr>
<tr>
<td>Ramipril</td>
<td>4.70 ± 1.30</td>
<td>0.85</td>
<td>5.00 ± 1.10</td>
<td>0.55</td>
</tr>
<tr>
<td>AJR/LA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50.50 ± 20.10</td>
<td>0.05</td>
<td>43.70 ± 10.40</td>
<td>0.25</td>
</tr>
<tr>
<td>Ramipril</td>
<td>38.30 ± 18.00</td>
<td>0.05</td>
<td>37.30 ± 17.60</td>
<td>0.25</td>
</tr>
<tr>
<td>%FS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>43.30 ± 7.50</td>
<td>0.27</td>
<td>41.90 ± 6.40</td>
<td>0.11</td>
</tr>
<tr>
<td>Ramipril</td>
<td>39.80 ± 6.30</td>
<td>0.27</td>
<td>38.60 ± 4.80</td>
<td>0.35</td>
</tr>
<tr>
<td>LVIDsi (mm/m²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.50 ± 0.80</td>
<td>0.85</td>
<td>5.00 ± 1.10</td>
<td>0.55</td>
</tr>
<tr>
<td>Ramipril</td>
<td>4.70 ± 1.30</td>
<td>0.85</td>
<td>4.60 ± 0.90</td>
<td>0.55</td>
</tr>
</tbody>
</table>

LVIDdi: left ventricular diameter diastolic index; LA/Ao: left atrial /aorta ratio; PISA: Proximal Isovelocity Surface Area; MR: mitral regurgitant flow velocity; AJR/LA: atrial regurgitant jet/left atrial area; % FS: % fraction shortening; LVIDsi: Left ventricular internal diameter systolic index
activates sympathetic nervous system (SNS) and enzyme in the lungs. ATII, a potent vasoconstrictor, angiotensin II (ATII) by angiotensin-converting to angiotensin I (ATI). ATI is converted into apparatus. Renin, then, breakdowns angiotensinogen by releasing renin from juxtaglomerular apparatus. Renin-angiotensinaldosterone system (RAAS) is a complex neurohormonal compensatory system functioning to maintain blood pressure and tissue perfusion. In DMVD, the valvular structure deformation can cause ineffective valve coaptation resulting in regurgitation of blood back into the left atrium as well as decrease in forward stroke volume and blood pressure. This phenomenon activates RAAS by releasing renin from juxtaglomerular apparatus. Renin, then, breakdowns angiotensinogen to angiotensin I (ATI). ATI is converted into angiotensin II (ATII) by angiotensin-converting enzyme in the lungs. ATII, a potent vasoconstrictor, activates sympathetic nervous system (SNS) and increases the release of aldosterone and anti-diuretic hormone (ADH) to maintain cardiac output, blood pressure and tissue perfusion in normal state (Sisson, 2004). This mechanism is useful in acute phrase of hypotension. However, in chronic phrase, ATII releases growth factors that promote remodeling of vessels and myocardium resulting in decreased vascular compliance and increased afterload. In long term, ATII causes pathological ventricular hypertrophy, myocardial necrosis from the cytotoxic effect and loss of myocardium contractility causing cardiac systolic dysfunction. Moreover, chronic activation of SNS and RAAS will increase the cardiac preload by increasing blood volume and venous return to the heart leading to excessive volume retention and eccentric hypertrophy or cardiac dilatation. The chronic volume overload leads to ventricular remodeling, ventricular dysfunction and heart failure (Davila et al., 2005). ACE inhibitors have been used to reduce effect of ATII, RAAS stimulation and over compensatory mechanism which can cause heart failure.

**Discussion**

This study aimed to investigate the short-term effect of ramipril in dogs with stage B2 DMVD. The study demonstrated that ramipril did not affect cardiac chamber size, mitral regurgitation severity and systolic function assessed by echocardiography in 91-day period of treatment.

Renin-angiotensin-aldosterone system (RAAS) is a complex neurohormonal compensatory system functioning to maintain blood pressure and tissue perfusion. In DMVD, the valvular structure deformation can cause ineffective valve coaptation resulting in regurgitation of blood back into the left atrium as well as decrease in forward stroke volume and blood pressure. This phenomenon activates RAAS by releasing renin from juxtaglomerular apparatus. Renin, then, breakdowns angiotensinogen to angiotensin I (ATI). ATI is converted into angiotensin II (ATII) by angiotensin-converting enzyme in the lungs. ATII, a potent vasoconstrictor, activates sympathetic nervous system (SNS) and increases the release of aldosterone and anti-diuretic hormone (ADH) to maintain cardiac output, blood pressure and tissue perfusion in normal state (Sisson, 2004). This mechanism is useful in acute phrase of hypotension. However, in chronic phrase, ATII releases growth factors that promote remodeling of vessels and myocardium resulting in decreased vascular compliance and increased afterload. In long term, ATII causes pathological ventricular hypertrophy, myocardial necrosis from the cytotoxic effect and loss of myocardium contractility causing cardiac systolic dysfunction. Moreover, chronic activation of SNS and RAAS will increase the cardiac preload by increasing blood volume and venous return to the heart leading to excessive volume retention and eccentric hypertrophy or cardiac dilatation. The chronic volume overload leads to ventricular remodeling, ventricular dysfunction and heart failure (Davila et al., 2005). ACE inhibitors have been used to reduce effect of ATII, RAAS stimulation and over compensatory mechanism which can cause heart failure.
Table 7 Comparison of blood hematology and chemistry values obtained from dogs in control and ramipril groups at day 0 and 91

<table>
<thead>
<tr>
<th>Variables</th>
<th>Day 0</th>
<th>p-value</th>
<th>Day 91</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ramipril</td>
<td>Control</td>
<td>Ramipril</td>
</tr>
<tr>
<td>RBC (x 10^6)</td>
<td>6.39 ± 1.19</td>
<td>0.96</td>
<td>6.25 ± 1.57</td>
<td>0.15</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>6.41 ± 1.03</td>
<td></td>
<td>7.01 ± 0.87</td>
<td></td>
</tr>
<tr>
<td>Hct (%)</td>
<td>14.57 ± 2.52</td>
<td>0.51</td>
<td>14.60 ± 3.27</td>
<td>0.87</td>
</tr>
<tr>
<td>WBC (x 10^3)</td>
<td>46.00 ± 6.85</td>
<td>0.50</td>
<td>43.80 ± 8.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Neutrophil (x 10^3)</td>
<td>12.21 ± 7.11</td>
<td>0.07</td>
<td>10.91 ± 4.17</td>
<td>0.13</td>
</tr>
<tr>
<td>Band cell</td>
<td>21.90 ± 57.8</td>
<td>0.47</td>
<td>13.00 ± 41.10</td>
<td>0.34</td>
</tr>
<tr>
<td>Monocyte (x 10^3)</td>
<td>11.80 ± 12.3</td>
<td>0.33</td>
<td>12.81 ± 11.17</td>
<td>0.92</td>
</tr>
<tr>
<td>Eosinophil (x 10^3)</td>
<td>11.91 ± 15.51</td>
<td>0.33</td>
<td>15.44 ± 25.01</td>
<td>0.26</td>
</tr>
<tr>
<td>Lymphocyte (x 10^3)</td>
<td>2.34 ± 1.47</td>
<td>0.20</td>
<td>2.07 ± 11.43</td>
<td>0.86</td>
</tr>
<tr>
<td>Platelet (x 10^9)</td>
<td>29.51 ± 7.60</td>
<td>0.16</td>
<td>30.34 ± 74.08</td>
<td>0.72</td>
</tr>
<tr>
<td>SGPT (Unit/litre)</td>
<td>25.70 ± 7.28</td>
<td>0.04</td>
<td>66.00 ± 36.80</td>
<td>0.51</td>
</tr>
<tr>
<td>ALP (Unit/litre)</td>
<td>15.00 ± 130.00</td>
<td>0.77</td>
<td>120.30 ± 61.90</td>
<td>0.56</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>15.50 ± 4.93</td>
<td>0.50</td>
<td>24.60 ± 11.50</td>
<td>0.70</td>
</tr>
<tr>
<td>CREA (mg/dl)</td>
<td>0.89 ± 0.32</td>
<td>0.98</td>
<td>0.93 ± 0.23</td>
<td>0.69</td>
</tr>
</tbody>
</table>

*P < 0.05 indicates significant difference between control and ramipril groups (RBC : red blood cell, Hb : hemoglobin, Hct : hematocrit, WBC : white blood cell, SGPT : serum glutamic pyruvic transaminase, ALP : alkaline phosphatase, BUN : blood urea nitrogen, CREA : creatinine).

Table 8 Comparison of blood hematology and chemistry values obtained from dogs in control and ramipril groups

<table>
<thead>
<tr>
<th>Blood parameter</th>
<th>Day 0</th>
<th>p-value</th>
<th>Day 91</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (x 10^6)</td>
<td>6.39 ± 1.18</td>
<td>0.59</td>
<td>6.41 ± 1.03</td>
<td>0.14</td>
</tr>
<tr>
<td>Hb (g / dl)</td>
<td>6.41 ± 1.03</td>
<td></td>
<td>7.01 ± 0.87</td>
<td></td>
</tr>
<tr>
<td>Hct</td>
<td>14.57 ± 2.52</td>
<td>0.96</td>
<td>15.30 ± 2.35</td>
<td>0.59</td>
</tr>
<tr>
<td>WBC (x 10^9)</td>
<td>46.00 ± 6.85</td>
<td>0.50</td>
<td>43.80 ± 8.00</td>
<td>0.35</td>
</tr>
<tr>
<td>Neutrophil (x 10^3)</td>
<td>12.21 ± 7.11</td>
<td>0.66</td>
<td>10.37 ± 4.18</td>
<td>0.01</td>
</tr>
<tr>
<td>Band cell</td>
<td>21.90 ± 57.8</td>
<td>0.35</td>
<td>46.50 ± 80.24</td>
<td>0.30</td>
</tr>
<tr>
<td>Monocyte (x 10^3)</td>
<td>11.80 ± 12.3</td>
<td>0.54</td>
<td>7.27 ± 7.44</td>
<td>0.08</td>
</tr>
<tr>
<td>Eosinophil (x 10^3)</td>
<td>11.91 ± 15.51</td>
<td>0.35</td>
<td>6.65 ± 5.80</td>
<td>0.73</td>
</tr>
<tr>
<td>Lymphocyte (x 10^3)</td>
<td>2.34 ± 1.47</td>
<td>0.12</td>
<td>1.61 ± 0.96</td>
<td>0.25</td>
</tr>
<tr>
<td>Platelet (x 10^9)</td>
<td>29.51 ± 7.60</td>
<td>0.72</td>
<td>37.99 ± 16.35</td>
<td>0.03*</td>
</tr>
<tr>
<td>SGPT (Unit/litre)</td>
<td>25.70 ± 7.28</td>
<td>0.94</td>
<td>40.10 ± 22.05</td>
<td>0.08</td>
</tr>
<tr>
<td>ALP (Unit/litre)</td>
<td>15.00 ± 130.00</td>
<td>0.34</td>
<td>139.60 ± 135.76</td>
<td>0.62</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>15.50 ± 4.93</td>
<td>0.06</td>
<td>17.67 ± 8.84</td>
<td>0.17</td>
</tr>
<tr>
<td>CREA (mg/dl)</td>
<td>0.89 ± 0.32</td>
<td>0.71</td>
<td>0.89 ± 0.19</td>
<td>0.73</td>
</tr>
</tbody>
</table>

*p < 0.05 indicates significant difference between day 0 and 91 (RBC : red blood cell, Hb : hemoglobin, Hct : hematocrit, WBC : white blood cell, SGPT : serum glutamic pyruvic transaminase, ALP : alkaline phosphatase, BUN : blood urea nitrogen, CREA : creatinine).
Despite the common mechanism of action, ACE inhibitors differ with regard to properties such as half-life, duration of action, active metabolites, affinity to ACE, lipophily or hydrophilicity. These different properties may account for different response to various ACE inhibitors in the same patients. Ramipril is an ACE inhibitor that is widely used to treat heart failure in human medicine. However, clinical study of ramipril in veterinary medicine is still lacking. Ramipril is a precursor that has to metabolize into an active product, ramiprilat in the liver before it works. Ramipril has lipophilic and high affinity to ACE. Thus, it can penetrate tissues and suppress systemic and local RAAS more effectively than other ACE inhibitors that have low tissue ACE affinity such as enalapril and lisinopril (Wolfgang, 1992). Pilote et al. (2008) found that ramipril could decrease mortality rate in human with congestive heart failure more effectively compared to enalapril and captopril. A previous study in humans showed the beneficial effects of ramipril in lowering the risk of mortality in patients that had cardiovascular problem (Hope study, 2000). A study of Hartman et al. (1993) found that local angiotensin II might play a role as a growth factor that could promote cardiac hypertrophy in heart failure condition. Due to a high density of angiotensin II receptors and angiotensin converting enzyme (ACE) in canine myocardium (Dell’Italia et al., 1997), use of high lipophilic ACE inhibitors such as ramipril may suppress local renin angiotensin aldosterone system (RAAS) in the myocardium more effectively than other ACE inhibitors such as enalapril or captopril (Kvart et al., 2002). However, the present study did not show any beneficial effects of ramipril compared with the untreated group. Firstly, this lack of effect may occur from other angiotensin II forming pathways in cardiac tissues (McDonald et al., 2001). Chymase is another pathway that can trigger angiotensin II from angiotensin I (Balcels et al., 1996). Ramipril can suppress ACE pathway but not chymase pathway. In other words, ramipril cannot completely inhibit production of angiotensin II. Thus, hemodynamic changes secondary to RAAS stimulation can still occur. Secondly, RAAS may not be fully activated in asymptomatic DMVD dogs. A human study by Francis et al. (1990) showed that plasma renin in patients affected by left ventricular dysfunction without heart failure was not higher than normal patients, suggesting that RAAS had not been activated. Same as in people, the RAAS was suggested not to be stimulated in the heart diseased dogs without congestive heart failure (Häggström et al., 1997). However, the RAAS stimulation has not been studied in stage B2 DMVD dogs yet. Thirdly, the short duration of the study may not be enough to show changes of disease that have long progressive period like DMVD. Lastly, the small sample size may affect the power of the study.

For the clinical effects of ramipril, one dog in the ramipril group had decreased cough frequency and two dogs increased in appetite. These findings suggest that ramipril might improve clinical status of diseased dogs. The beneficial effects of ACE inhibitors on improvement in clinical signs of dogs with DMVD have been previously reported (The COVE study, 1995; The improve study, 1999). The effect of ramipril on blood chemistry values and complete blood count was determined before and after treatment. The blood chemistry values of dogs in the ramipril group were not significantly different on days 0 and 91. Most of the dogs were well-tolerated to ramipril except one dog that had azotemia which was transient and did not need specific treatment. This result indicates that ramipril is similar to other ACE inhibitors which may have side effects on renal function and can cause azotemia (Weinberg, 1993). Therefore, dogs that receive ramipril or other ACE inhibitors should be monitored renal function continually.

Echocardiography is a non-invasive technique that can investigate cardiac chamber size, structural abnormalities and function. Since echocardiography is a non-invasive technique, it is practical for a repeated, follow-up measurement. In this study, several echocardiographic parameters were used to assess cardiac enlargement, severity of mitral valve regurgitation and systolic function of the heart. The mitral regurgitant flow is dependent on left atrial pressure, systolic left ventricular function, and preload and systemic arterial pressure (Chetboul and Tissier, 2012). The regurgitant flow may decrease in case of systolic ventricular impairment and high left atrial pressure. Therefore, the mitral regurgitant flow velocity may not be a good indicating for evaluation the regurgitant severity. A previous study found that atrial regurgitant jet/left atrial area (ARJ/LA) and LA/Ao were correlated with the severity of mitral regurgitation (Gouni et al., 2007). Although ARJ/LA can subjectively differentiate mild and moderate degree of DMVD, difference between moderate and severe degrees is difficult to evaluate by this technique. PISA or flow convergence method is more reliable for the discrimination of mitral regurgitant severity compared to the color mapping technique or ARJ/LA (Chetboul and Tissier, 2012). However, PISA method has some limitations. First, PISA is accurate for regurgitant flow with circular orifice only. Second, some dogs with multiple regurgitant jet can cause PISA inaccuracy. Third, PISA is inaccurate if precise location of the orifice and the flow convergence shapes could not be determined. If misalignment or eccentric jet occurs, it will underestimate flow velocity and overestimate orifice area (Zoghbi et al., 2003). The regurgitant severity assessed by PISA, ARJ/LA and MR in the ramipril group was unchanged throughout the study period and similar to the control group suggesting a lack of short-term effects of ramipril in decreasing mitral regurgitant severity. The regurgitant severity in the control group was more varied during the period of the study. The severity changed at some points of time. ARJ/LA and PISA were significantly different on days 56 and 91, respectively, when compared to day 0 in the control group. These findings suggest that without any drug the severity of regurgitation may change within a short period of time. With ramipril, the progression of regurgitation severity may be controlled or delayed.
In conclusion, based on echocardiographic values, this study showed that ramipril did not affect cardiac size, mitral regurgitation severity and systolic function in short term treatment duration. However, due to the slow progression with long preclinical period of this disease, further studies with long-term treatment duration should be performed. Because the current available data from this clinical trial and other studies do not confirm or support that early treatment with ACE inhibitors is beneficial, dogs with stage B2 DMVD should be individually evaluated and treated on a case by case basis.

Acknowledgements
This study was supported by a grant from the 90th year Chulalongkorn Scholarship, Graduate School, Chulalongkorn University. The authors would like to thank Asst Prof Walasinee Moonarmard for providing statistical analysis assistance.

References


Effects of Environmental Factors, Ages and Breeds on Semen Characteristics in Thai Indigenous Chickens: A One-year Study

Pornjit Sonseeda 1,2  Thevin Vongpralub 1,2*  Banyat Laopaiboon 1

Abstract

Several reports on the semen characteristics of domestic fowl have indicated that environmental factors, breed and age significantly affect semen quality. There is, however, a paucity of data on effects of environmental factor, breed and age on semen characteristic in Thai indigenous chickens. The aims of the present study were to investigate the effects of annual ambient temperature and humidity, local breeds (i.e. Lueng hang kaow, Pradoo hang dam and Chee), and ages (10 and 18 months) on sperm production and semen quality among Thai native cocks. Thirty-six Thai native cocks were housed under natural environmental conditions, and semen was collected routinely twice a week using the massage method. Sperm production and semen quality were determined every two weeks for one year. Semen was evaluated for volume, mass movement, % total motility, % live normal, sperm concentration/ml and sperm number/ejaculate. Over the 12-month period, ambient temperature and humidity did not significantly affect sperm production and semen quality \( (p < 0.05) \). Breeds and ages also had no effect on semen characteristics among the Thai native cocks. The result of the present study indicates that the sperm production and semen quality of captive Thai native cocks were not influenced by environmental factors, breeds and ages.

Keywords: age, indigenous chicken, line, season, sperm

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บทคัดย่อ
ผลกระทบของปัจจัยจากสิ่งแวดล้อม สายพันธุ์ และอายุต่อคุณลักษณะของน้ำเชื้อในไก่พื้นเมืองไทย: การศึกษาในรอบ 1 ปี

พรจิตสอนสีดา 1,2 เทวินทร์วงษ์พระลับ 1,2* บัญญัติเหล่าไพบูลย์ 1

มีรายงานชี้ให้เห็นถึงผลกระทบของปัจจัยจากสิ่งแวดล้อม สายพันธุ์ และอายุต่อคุณภาพน้ำเชื้อในไก่พื้นเมืองไทย แต่ไม่พบรายงานการศึกษาเรื่องผลกระทบของสภาพแวดล้อม สายพันธุ์และอายุต่อคุณลักษณะน้ำเชื้อในไก่พื้นเมืองไทยดังนั้นการวิจัยในครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลกระทบของอุณหภูมิแวดล้อมและความชื้นในรอบ 12 เดือนต่อคุณลักษณะของน้ำเชื้อในฟันเมืองไทยสายพันธุ์ (เหลืองหางขาว, ประดู่หางดำ, และชี) ที่มีอายุเริ่มต้นแตกต่างกัน 2 ช่วงอายุ คือ 10 และ 18 เดือน ใช้พ่อพันธุ์ไก่พื้นเมืองไทยจำนวนทั้งสิ้น 36 ตัว เลือกในโรงเรือนแบบเปิดภายใต้สภาวะแวดล้อมตามธรรมชาติ วิธีเก็บน้ำเชื้อสัปดาห์ละ 2 ครั้งเป็นประจำ และเก็บข้อมูลคุณภาพน้ำเชื้อทุก 2 สัปดาห์ตลอดช่วงระยะเวลา 1 ปี โดยการวิเคราะห์น้ำเชื้อใช้วิธีการดูจากการลูบหลังและบีบที่บริเวณโคนหาง ประเมินคุณภาพน้ำเชื้อโดยการประเมินค่าปริมาตรของน้ำเชื้อ การเคลื่อนที่แบบกลุ่ม และร้อยละของการเคลื่อนที่รวมของอสุจิ ร้อยละของการดื้อที่วิวัฒนาและรูปร่างปกติ ความเข้มข้นของอสุจิต่อมิลลิลิตร และจุนจุนเนื้อเยือกหลั่งจากการศึกษาอย่างต่อเนื่องตลอดระยะเวลา 12 เดือนพบว่าอุณหภูมิแวดล้อมและความชื้นสัมพัทธ์ไม่มีผลต่อคุณภาพน้ำเชื้อในฟันเมืองไทย ส่วนสายพันธุ์และอายุนั้นพบว่าไม่มีผลต่อคุณลักษณะของน้ำเชื้อเช่นเดียวกัน ผลการศึกษาครั้งนี้ชี้ให้เห็นว่า ปัจจัยจากสิ่งแวดล้อม สายพันธุ์ และอายุไม่มีผลต่อคุณภาพน้ำเชื้อในฟันเมืองไทยที่เลี้ยงในโรงเรือนแบบเปิด

คำสำคัญ: อายุ, ไก่, สายพันธุ์, ฤดูกาล, อสุจิ

Introduction

For best results in AI, poultry breeders need to ensure the highest quality of collected semen (Alkan et al., 2002). The importance of raw semen assessment to identify males of different fertilizing abilities is routinely employed (Wishart, 2009). Avian semen quality significantly affects fertility which is in turn affected by breed (Tabatabaei et al., 2010) and environmental conditions (Elagib et al., 2012).

Seasonal variation in semen quality of domestic fowl has been documented (Santiago-Moreno et al., 2011). McDaniel et al. (1996) reported that an ambient temperature of > 31°C depressed rooster sperm motility, viability and fertilization potential. Even environmental temperature at ejaculation has an important effect on exogenous physiological factors influencing avian sperm motility (Ashizawa and Sano, 1990; Wishart and Wilson, 1999).

There are reports concluding that both breed and age affect the quality of fresh and stored semen (Kelso et al., 1996). Semen from avian species that has been studied, included turkeys, ganders, pheasants, drakes, and cocks (Wishart, 2009). It was found that semen of older birds had significantly lower motility, viability and mass movement than younger birds. They also reported that semen quality of poultry species declined with age (Kelso et al., 1996; Kotloska et al., 2005; Long et al., 2010). Reports on Iranian indigenous broiler (Tabatabaei et al., 2010) and 8 pedigreed lines (Long et al., 2010) indicated that among younger chickens there was greater motility.

Among Thai native chickens, only few have been studied. Therefore, there is a dearth of data on semen quality by season, breed and age. Consequently, the present study was conducted to characterize semen quality by environmental factors, breed and age of Thai native chickens. An understanding of these parameters would allow for design and optimization of semen cryopreservation of Thai indigenous chicken genetic resources.
Materials and Methods

Animals and Semen collection: The experiment was conducted on 36 mature, Thai native cocks kept in individual cages. There were three different breeds of birds (i.e. Lueng hang khoa, Pradoo hang dam and Chee) and two different ages (10 and 18 months) at the beginning of the experiment. Cockerels were fed 120-130 g/head/day and water was provided ad libitum. The animals were reared under natural environmental conditions, (5° 38’ N, 105° 37’ E), where they received a natural light : dark photoperiod (11.14L : 12.46D to 13.01L : 11.59D) throughout the experiment. Semen was collected (using the massage method) from each male twice a week for 1 year (July, 2009 - June, 2010).

Semen evaluation: Semen from an individual cock was collected in a 1.5 ml microtube containing 0.1 ml Schramm diluent comprising 2.85 g sodium glutamate, 0.5 g glucose, 0.25 g inositol, 0.5 g potassium acetate, 0.07 g magnesium acetate in 100 ml of double-distilled water. Immediately after collecting, semen characteristics were determined every two weeks. Volume: Ejaculate volume of semen was determined using 0.1 ml graduated tube rculin syringe (Abd El Ghany, 1997). Mass movement and motility: Mass movement, aspects of wave motion, was conducted examining a drop of semen on a warm slide under a microscope at 100x. The score was assigned between 0 (total sperm were motionless) and 5 (wave motion varied rapidly, eddies were present). Motility was assessed on the basis of percentage of sperm showing forward motion (Ax et al., 2000). % live normal sperm: The percentage of live normal sperm was evaluated according to Ax et al., (2000). Briefly, a drop of well-mixed semen was mixed with a drop of eosin-nigrosin stain on a glass slide. Another glass slide was used to prepare smear film and was evaluated immediately under a microscope. Sperm abnormalities were optimally detected using immersion oil at 1000x and a minimum of 200 cells were evaluated for abnormalities. The unstained and normal morphology spermatozoa were recorded as live normal sperm. Sperm Concentration: Concentration of the semen sample was assumed using a hemacytometer (Ax et al., 2000). Briefly, a 1:1000 dilution of semen (semen 4 µl : 3,996 µl 4%NaCl) was loaded into a hemacytometer. The count was performed at 200x under bright-field microscopy.

Temperature-humidity index (THI): Temperature and humidity were collected using digital thermometer. The temperature-humidity index (THI) was calculated using a mathematical model (Mader and Davis, 2004).

\[ \text{THI} : (0.8 \times \text{AT}) + \left[ \left( \frac{\text{RH}}{100} \right) \times (\text{AT}-14.4) \right] + 46.4 \]

AT : Ambient Temperature (°C)
RH : Relative Humidity (%)

Statistical analysis: Data collected on semen characteristics were subsequently subjected to a one-way analysis of variance (ANOVA) using SAS (1993).

Results

During 12 months, mean ambient temperature, humidity and THI were 27.6°C (ranging from 20.6-31.2°C), 69.1% (ranging from 53.5-82.2%) and 77.5% (ranging from 67.1-82.1%), respectively. Ambient temperature, humidity and THI did not significantly (p > 0.05) affect sperm production and semen quality of Thai native chicken (Table 1). The average of mass movement, % total motile, volume, %live normal, concentration, and total sperm/ejaculate were 4.3 score, 88.2%, 0.3 ml, 91.1%, 431.9x10⁷ cell/ml, and 147.0x10⁷ cell/ejaculate, respectively. These data suggest that temperature, humidity and THI have little measurable effect on semen characteristics of the native chickens.

Table 1 Effect of environmental factors on semen quality among Thai native chickens

<table>
<thead>
<tr>
<th>Annual</th>
<th>semen quality parameter</th>
<th>Climate</th>
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<tbody>
<tr>
<td></td>
<td>Mass movement</td>
<td>Motility %</td>
</tr>
<tr>
<td>July</td>
<td>4.28</td>
<td>87.5</td>
</tr>
<tr>
<td>August</td>
<td>4.31</td>
<td>89.5</td>
</tr>
<tr>
<td>September</td>
<td>4.37</td>
<td>88.3</td>
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<tr>
<td>October</td>
<td>4.31</td>
<td>88.5</td>
</tr>
<tr>
<td>November</td>
<td>4.30</td>
<td>88.8</td>
</tr>
<tr>
<td>December</td>
<td>4.28</td>
<td>88.9</td>
</tr>
<tr>
<td>January</td>
<td>4.31</td>
<td>88.7</td>
</tr>
<tr>
<td>February</td>
<td>4.32</td>
<td>88.5</td>
</tr>
<tr>
<td>March</td>
<td>4.37</td>
<td>87.3</td>
</tr>
<tr>
<td>April</td>
<td>4.35</td>
<td>87.4</td>
</tr>
<tr>
<td>May</td>
<td>4.29</td>
<td>87.5</td>
</tr>
<tr>
<td>June</td>
<td>4.28</td>
<td>87.1</td>
</tr>
<tr>
<td>Mean</td>
<td>4.3</td>
<td>88.2</td>
</tr>
<tr>
<td>SEM</td>
<td>0.05</td>
<td>1.30</td>
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</tbody>
</table>
For the effect of age on semen quality, the current study found that the age of Thai native cocks, either between 10 and 22 months (10 months group) or between 18 and 30 months (18 months group) had no significant (p > 0.05) influence on semen quality. The average of mass movement, % total motile, volume, % live normal, concentration, and total sperm/ejaculate of the 10-months group were 4.31 score, 88.3%, 0.328 ml, 90.5%, 413.89 x 10^7 cell/ml, and 147.70 x 10^7 cell/ejaculate, respectively. The result revealed that age had no effect on semen quality between the 10th and 30th month of age among Thai native cocks. The results suggest that Thai native chicken breeds have a long reproductive life time.

The comparative effect of breeds (i.e., Lueng hang kaoa, Pradoo hang dam and Chee) on the semen quality of Thai native cocks is presented in Table 2. The average of mass movement, % total motile, volume, % live normal, concentration, and total sperm/ejaculate of Lueng hang kaoa, Pradoo hang dam and Chee were 4.30 score, 88.62%, 0.342 ml, 91.2%, 438.43 x 10^7 cell/ml, and 149.71 x 10^7 cell/ejaculate; 4.31 score, 88.06%, 0.336 ml, 90.5%, 413.89 x 10^7 cell/ml, and 147.70 x 10^7 cell/ejaculate; and 4.31 score, 87.96%, 0.336 ml, 91.7%, 434.8 x 10^7 cell/ml, and 147.71 x 10^7 cell/ejaculate, respectively. The result revealed that age had no effect on semen quality between the 10th and 30th month of age among Thai native cocks. The results suggest that Thai native chicken breeds have a long reproductive life time.

The comparative effect of breeds and age on semen quality of Thai native cocks

Table 2. The average of mass movement, % total motile, volume, % live normal, concentration, and total sperm/ejaculate of 18 months group were 4.30 score, 88.08%, 0.335 ml, 91.4%, 429.13 x 10^7 cell/ml, and 149.06 x 10^7 cell/ejaculate, respectively. The result revealed that age had no effect on semen quality between the 10th and 30th month of age among Thai native cocks. The results suggest that Thai native chicken breeds have a long reproductive life time.

**Discussion**

Effect of environmental factor on semen quality can vary considerably over the year in seasonal mammalian species such as sheep, cattle and ibex (D’Alessandro and Martemucci, 2003; Koivisto et al., 2009; Coloma et al., 2010). Several studies on fowl showed that there was a seasonal effect on semen quality. Studies in Sudan (Elagib et al., 2012) and in Sahel region of Nigeria (Bah et al., 2001) on older birds showed a significant reduction in sperm concentration during the summer season, consistent with an earlier report by Elagib (2012) which suggested that high temperatures reduced sperm concentration. However, the abnormal percentage was not harmfully affected during summer months.

Semen quality of indigenous chickens was not significantly different throughout the year (Table 1). These data suggest that temperature, humidity and THI have little measurable effect on semen characteristics of the native chickens. The results of this study are similar to a report by Santiago-Moreno et al. (2011) which showed that the characteristics of fresh semen from native Spanish chicken breeds were not affected by season. In contrast, Bah et al. (2001) found that semen characteristics varied significantly (p < 0.001) during the rainy season. The highest sperm concentration was recorded in July, while the greatest volume and highest total sperm count was in August. Similarly, Elagib et al. (2012) reported that one-year-old cocks produced less semen volume during the summer months than during the autumn months.

Environmental factors such as temperature and photoperiod housing are thought to play an prominent role in semen quality in many countries (Peltoniemi et al., 1999). The effects of hot ambient temperature on the heterophil/lymphocyte ratio were determined for a comparison of high ambient temperature tolerance between Thai indigenous chickens, cross-bred Thai indigenous chickens and broilers. The Thai native chickens showed higher heat tolerance than cross-bred Thai indigenous chickens and broilers (Aengwanich, 2007). Duangdus (2008) assessed the effect of high environmental temperature in Thai native chickens (Chee) and commercial broilers on productive performance, respiratory rate, and rectal temperature. The results showed that the Thai native chickens (Chee) was more tolerant to high temperature than the broilers. These previous reports suggested the indigenous chickens are fully adapted to high environmental temperature. Floyd and Tyler (2011) determined the effect of different photostimulation photoperiods (8-18 hour) on age of first semen production, testis weight and semen characteristics among male broiler breeders. A large degree of variation was recorded, however, the trend in data suggests that a photoperiod of > 13 hours may be detrimental to testis development and subsequent sperm production. A study in Thailand determined that the average day length (photoperiod) was 12.1 hours (12 hours 7 min, ranging from 11 hour 14 min- 13 hour 1 min). The respective photoperiod in the hot, rainy, and cool season were 12.4 hours, 12.5 hour and 11.5 hours (Hydrographic Department, Royal Thai Navy, 2010). The effect of photoperiod in

### Table 2

<table>
<thead>
<tr>
<th>Breeds</th>
<th>Volume (ml)</th>
<th>Mass movement</th>
<th>Motility %</th>
<th>Live normal%</th>
<th>Concentration 1X10^7cell/ml</th>
<th>Total sperm 1X10^7cell/ejaculate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lueng hang kaoa</td>
<td>0.342</td>
<td>4.30</td>
<td>88.62</td>
<td>91.2</td>
<td>438.43</td>
<td>149.71</td>
</tr>
<tr>
<td>Pradoo hang dam</td>
<td>0.328</td>
<td>4.31</td>
<td>88.06</td>
<td>90.5</td>
<td>413.89</td>
<td>137.70</td>
</tr>
<tr>
<td>Chee</td>
<td>0.336</td>
<td>4.33</td>
<td>87.96</td>
<td>91.7</td>
<td>434.80</td>
<td>147.70</td>
</tr>
</tbody>
</table>

Age

|          | 0.336 | 4.31 | 88.3 | 90.8 | 434.74 | 145.01 |

**SEM**

|          | 0.012 | 0.025 | 0.69 | 0.67 | 17.34 | 7.86 |

The results suggest that Thai native chicken breeds have a long reproductive life time.
the northeastern region may be less important on the semen quality of Thai native chickens. The data gathered in the current research do not support the influence of seasonality on semen quality; perhaps because of the narrow range of photoperiod, different genetic backgrounds and different location of the chickens.

For the effect of breed on semen quality, the results of this study (Table 2) indicate that there is no statistically significant influence of breed on semen quality among native Thai cocks. A study of four breeds of chicken performed in Poland (Black Minorca, Green-Legged, Italian Partridge Partridge, and White Crested Black Polish) (Siudzinska and Lukaszewicz, 2008) confirmed that breed had no significant affect on semen quality. Furthermore, the volume of ejaculate and sperm concentrations collected from those breeds were not significantly different. Long et al. (2010) investigated fresh semen from 8 pedigreed layer lines and found no differences among poultry lines or strainsvis-à-vis semen quality. The reason for the non-effect of breed on semen quality maybe the close genetic backgrounds (Siudzinska and Lukaszewicz, 2008; Long et al., 2010). Among Thai native chickens, Aumpapron (2010) reported that Lueng hang kaow, Pradoo hang dam and Chee had close genetic backgrounds, which supports the results of the present study. In contrast, Peters et al. (2008) observed differences in strains of Nigerian indigenous cocksvis-à-vis semen volume, concentration and motility. The different results may due to the different genetic backgrounds.

For the effect of age on semen quality, the current study found that the age of Thai native cocks, either between 10 and 22 months or between 18 and 30 months, had no significant influence on semen quality. This finding agrees with the results obtained by Chotesangasa (2001) who reported on semen quality of Thai native cocks (between9 months and 2 years old) over a period of 2 months. In contrast, it was reported that ejaculate volume and sperm concentration are dependent on the age of the male (Bakst and Cecil, 1992; Kelso et al., 1996; Kotloska et al., 2005; Long et al., 2010). Moreover, Kotloska et al. (2005) reported that spermatozoa concentration of turkeys declines with age (from 31 to 51 wks). Cerolini et al. (1997) studied the effect of age on semen concentration and found that significantly increased from the 24th wk to the 39th wk of age; but did not vary substantially between the 39th and 54th wk; and was at the lowest concentration in the 72nd wk. The highest rate of sperm motility was observed in the first half of the reproductive period (i.e., in the 24th and 39th wk of life) and it significantly decreased in the second half of the reproductive period (i.e., by 35% at the 39th wk and 42% at the 45th wk). Tabatabaei et al. (2010) reported that age also affected semen quality of the indigenous broiler breeder rooster in Iran which was measured during the 26th to the 45th wk of life. The results of the present study, however, revealed that age had no effect on semen quality between the 10th and 30th month of age among Thai native cocks. The results suggest that Thai native chicken breeds have a longer reproductive lifetime than the commercial breeds and Iranian indigenous chickens previously reported.

Conclusions

The respective semen quality (mass movement, % total motile, volume, % live normal, concentration, and total sperm number/ejaculate) of the cocks within three Thai native breeds (i.e., Lueng hang kaow, Pradoo hang dam and Chee) was not significantly different. Moreover, age and environmental factors had no effect on semen quality. Taken together, the research strongly suggests that between 10 and 30 months of age indigenous cocks have similar quality of semen.

Acknowledgements

This research was partially supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education AG-BIO/PERDO-CHE) and Agricultural Biotechnology Research Center for Sustainable Economy, Khon Kaen University. As well as, a part of research was supported by Research and Development Network Center for Animal Breeding (Native Chicken), Khon Kea University. The authors would like to thank the animal care staff at the Faculty of Agriculture, Khon Kaen University for their assistance and Mr. Bryan Roderick Hamman and Mrs. Janice Helen-Hamman for assistance with the English presentation of the manuscript.

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Development of Enzyme-Linked Immunosorbent Assay to Detect Antibodies against Chicken Infectious Anemia Virus

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Abstract

A standardized and robust indirect enzyme-linked immunosorbent assay (ELISA) to detect antibodies against chicken infectious anemia virus (CIAV) was developed. Thai CIAV was isolated and used for the entirety of this study. For virus cultivation, a cell density of MDCC-MSB1 cells at 3 x 10^6 cells/ml and a fetal bovine serum concentration at 10 percent were the most appropriate for Thai CIAV. Then, crude virus of CIAV was prepared using ultracentrifugation technique. To develop the ELISA test, optimal dilution of goat anti-chicken IgG was 1 : 200 and antigen concentration in the ELISA was 1.00 µg/ml, as determined by checkerboard titration. In the present study, a comparison was made between commercial and in-house ELISA for the detection of antibodies to CIAV. Relative sensitivity, specificity and accuracy of in-house ELISA were 93%, 78% and 86%, respectively. Agreement between commercial and in-house ELISA was substantial (Kappa value = 0.71). Consequently, the in-house ELISA was as good as the commercial ELISA in screening chicken serum samples for antibodies against CIAV.

Keywords: chicken infectious anemia virus, ELISA, MDCC-MSB1 cells, test kit

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Introduction

Chicken infectious anemia virus (CIAV) is a causative agent of chicken infectious anemia (CIA) (Haillemariam et al., 2008). The CIAV is a ubiquitous and highly resistant chicken virus causing anemia and death in chicks less than 3 weeks old and immunosuppression in chickens older than 3 weeks old (Miller et al., 2003; Chansiripornchai et al., 2012). The disease is characterized by aplastic anemia and generalized lymphoid atrophy with concomitant immunosuppression with secondary viral, bacterial or fungal infections (Schat and van Santen, 2008). Worldwide, there are approximately 50 billion chickens raised every year (Kaiser, 2010). The CIA causes serious economic loss to commercial broiler industry (Simeonov and Doumanova, 2005). Furthermore, the disease also affects producers of specific-pathogen-free (SPF) eggs. As a consequence of seroconversion, the flock is considered positive and the eggs are no longer SPF (Schat, 2008). Therefore, it is necessary to ensure that those SPF chicken flocks that supplying eggs for vaccine production are free of CIAV infection (Todd et al., 1990). Vertical transmission of the virus plays an important role in infection among young chickens (Miller et al., 2003). Detection of seroconversion in breeder flocks should be conducted before egg production (Iwata et al., 1998). A vaccine-based strategy to control clinical and subclinical disease associated with CIAV infection is also necessary (Todd et al., 1995). Various serological assays have been developed including indirect immunofluorescence assay (IFA) (Simeonov and Doumanova, 2005), virus neutralization (VN) test, ELISA-based assays (Schatand van Santen, 2008) and indirect immunoperoxidase assay (IIP) (Lamichhane et al., 1992). The VN test is more sensitive than other assays (Otaki et al., 1991), but the procedure is time-consuming, expensive and laborious. The continuous passage of the virus makes the test too cumbersome for use in large serologic surveys (Lamichhane et al., 1992). The IFA requires expensive equipment and experienced personnel (Todd et al., 1990). The ELISA is quick and simple to perform and is suitable for large-scale testing (Todd et al., 1990). The advantage of the ELISA test may be the cost of antigen preparation (Lamichhane et al., 1990). Although various commercial ELISA kits are available, all of them have to be imported, making the cost too high for large scale screening of antibodies to CIAV in chickens. Therefore, the purpose of the present study was to prepare a reliable indirect ELISA for the detection of CIAV antibodies in monitoring infections or assessing vaccination in chickens.
Materials and Methods

**Virus and appropriate viral cultivation:** The CIAV used in this study was isolated from bone marrow of CIA-infected chickens in Thailand. A viral isolation was inoculated into MDCC-MSB1 cell line (CLS, Germany), which is a chicken-T cell line established from Marek’s disease lymphoma, and incubated at 37°C for 3 days. Cytopathic effects typical of CIAV were observed with the use of an inverted microscope. Appropriate cell density and concentration of fetal bovine serum (FBS) (Gibco BRL, MD) were determined.

**Cell cultures:** The CIAV field strain was cultivated for 5 passages in MDCC-MSB1 cells. The MDCC-MSB1 of 3x10^4 or 3x10^5 cells were cultured in RPMI 1640 medium containing 2 or 10% FBS in 5% CO₂ at 40°C to find the most suitable condition for the virus cultivation.

**ELISA antigens:** The infected cultures were harvested and stored at -70°C until use. Virus/cell lysate was frozen and thawed 3 times and left at 37°C for 3 days. Cellular debris was removed by refrigerated centrifuge (Centurion Scientific Ltd., UK) at 2,500 g for 30 min at 4°C. The supernatant was centrifuged at 80,000 g for 1.5 hour at 4°C in an ultracentrifuge (L-XP Series ultracentrifuge class: R (50.2 Ti), Beckman, USA). The crude virus pellets were thoroughly resuspended using phosphate buffered saline (PBS). Aliquots of the antigen were stored at -70°C.

**Protein analysis:** Protein concentration was determined by the method of Bradford using Quick start™ (Bio-Rad Lab, USA). Samples containing the CIAV virus were analyzed using SDS-PAGE by electrophoresis in gels containing 12.5% polyacrylamide as described by Laemmli (1970). Precision Plus Protein™ Standards (Bio-Rad Lab, USA) were included in the analysis. Gels were stained with Coomassie blue R for 1 hour and washed with destaining solution.

**Optimization of in-house ELISA test:** An ELISA protocol was modified from Todd et al. (1990). Briefly, CIAV-positive and negative reference sera (Biocheck, Holland) were dilute at 1 : 1000 dilutions and then added to a microtiter plate coated with CIAV antigen at different concentration 0.125, 0.250, 0.500, 1.00, 2.00 and 4.000 mg/ml of crude virus, followed by overnight incubation at room temperature. The fluid was then poured from the antigen-coated plates and the plates were tapped dry. A blocking (300 µl/well) solution containing 1 % bovine serum albumin in PBS was added and incubated for 60 min. After 3 times of washing with 300 µl of washing buffer, the goat anti-chicken IgG (H+L) horseradish peroxidase (Synbiotics Corporation, USA) at different dilutions, 1 : 50, 1 : 100, 1 : 200, 1 : 400 and 1 : 800, was added and then incubated at room temperature for 60 min. Following the incubation period, the unreacted conjugate was removed by 3 washing times. ABTS-Hydrogen peroxidase (Synbiotics Corporation, USA) substrate was added into the wells followed by 15 min incubation at room temperature. The reaction was stopped by adding 100 µl of the stop solution and the absorbance value was measured at 405 nm using a 6-well ELISA reader (Biotek Instruments, USA). The commercial CIAV ELISA test kits were obtained from BioChek Poultry Immunoassay (Holland).

**Determination of positive/negative cut-off and tested serum samples:** A mean of sample-to-positive (SP) ratio value plus 2 standard deviations (SD) of the control sera was calculated according to the formulation below as described by Crowther (2002).

\[
\text{Cut off} = \frac{(X + 2\text{SD (Negative)}) - (X - 2\text{SD (Positive)})}{2}
\]

Reference sera against infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), Pasteurella multocida (PM), Mycoplasma synoviae (MS) (Synbiotics Corporation, USA) were used for test kit analysis. CIAV antibody positive and negative sera were obtained from BioChek (Holland). The field serum samples of CIAV-infected flocks were collected from 180 commercial broiler chickens.

**Statistical analysis:** Cut-off value, relative sensitivity, specificity and accuracy of the in-house were calculated (Chansiripornchai, 2007). A Kappa test was also applied for agreement between in-house and commercial ELISA tests according to Goodwin et al. (1992). A Kappa value of greater than or equal to 0.40 represents good agreement.

**Results**

**Cultivation of virus:** An MDCC-MSB1 cells suspension containing 3.0 x 10^6 cells/ml was infected with the Thai CIAV strain. The cells showed typical cytopathic effects (CPE) (Fig 1).

**Determination of appropriate viral cultivation:** The present study indicated that a cell density of MDCC-MSB1 cells at 3 x 10^5 cells/ml and FBS concentration at 10% were the most appropriate for Thai CIAV cultivation. These cell density and FBS concentration revealed the highest 50% tissue culture infectious dose (TCID50) (Table 1).

![Figure 1: Cytopathic effects in MDCC-MSB1 cells after 3-day incubation. A. Uninfected cells. B. MDCC-MSB1 cells infected with CIAV.](image)

<table>
<thead>
<tr>
<th>No.</th>
<th>Cell density (cells/ml)</th>
<th>FBS concentration</th>
<th>TCID50/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 x 10^6</td>
<td>2 %</td>
<td>4 x 10^8</td>
</tr>
<tr>
<td>2</td>
<td>3 x 10^6</td>
<td>10 %</td>
<td>4 x 10^8</td>
</tr>
<tr>
<td>3</td>
<td>3 x 10^6</td>
<td>2 %</td>
<td>4 x 10^9</td>
</tr>
<tr>
<td>4</td>
<td>3 x 10^6</td>
<td>10 %</td>
<td>4 x 10^9</td>
</tr>
</tbody>
</table>

Note: FBS: fetal bovine serum; TCID50: 50% tissue culture infectious dose

**Statistical analysis:** Cut-off value, relative sensitivity, specificity and accuracy of the in-house were calculated (Chansiripornchai, 2007). A Kappa test was also applied for agreement between in-house and commercial ELISA tests according to Goodwin et al. (1992). A Kappa value of greater than or equal to 0.40 represents good agreement.
Protein analysis: The protein concentration of crude CIAV was 2.382 mg/ml. The SDS-PAGE analysis revealed a single band of 57.3 kDa of CIAV (Fig 2).

Optimization of ELISA test: Different concentrations of conjugate and antigen were titrated in combination using the CIAV-antibody positive and negative sera. The results were evaluated for the maximal positive-to-negative (S/N) ratio between positive and negative sera. One to two hundred of conjugate dilution and 1 µg/ml of antigen concentration were chosen throughout the study according to a subsequent dilution indicating a satisfactory difference between the references of positive and negative sera (Table 2).

Determination of positive/negative cut-off: A cut-off value calculated by the mean of sample-to-positive (SP) ratio and standard deviation (SD) of the control sera was set at 0.265. There was no cross reaction of the in-house ELISA test to various respiratory avian pathogens including IBV, ILTV, PM and MS (Table 3). The relative sensitivity, specificity, and accuracy of 180 CIAV collected serum samples were 93%, 78% and 86%, respectively. The positive and negative predictive values were 81% and 92%, respectively. Agreement between the commercial and in-house ELISA was highly significant (Kappa value = 0.71).

Discussion

Since the development of various commercially available ELISA kits for the detection and measurement of antibodies to CIAV, improvements to this technique have been carried out. These ELISA test kits are widely used by poultry diagnostic laboratories. The CIAV-specific antibodies to natural infection can be detected by ELISA within 2 to 3 weeks of initial exposure (Michalski et al., 1996). The principal aim of the study was to determine the value of in-house ELISA test using Thai CIAV isolate in detecting CIAV infection in chickens. In this study, the Thai CIAV was isolated and used for the entirety of the study. Recently, MDCC-MSB1 cell cultures have been preferred for in vitro cultivation, although sublines of MDCC-MSB1 differ in their susceptibility to infection (Schat, 2008). For the Thai CIAV isolate cultivation, a cell density of MDCC-MSB1 cells at 3 x 10^6 cells/ml and aFBS concentration at 10% gave the highest yield of virus. The crude virus was used to prepare the in-house ELISA test instead of purified preparations such as sucrose-density-gradient centrifugation because it would not be economically viable due to the losses incurred (Todd et al., 1990b).

In general, the antigens used for CIAV enzyme immunoassay were prepared from partially purified virus preparation grown in MDCC-MSB1 cells (Schat and van Santen, 2008). After ultracentrifugation, the crude CIAV was used for preparing a coating antigen for ELISA. The SDS-PAGE analysis revealed a single band of 57.3 kDa of CIAV. Previous reports had demonstrated that a 50 kDa viral protein (VP1) was the only protein detected in highly purified virus particles (Todd et al., 1990a). The molecular weights of VP1, VP2 or VP3 were 50, 30/27 and 16 kDa by SDS-PAGE, respectively (Iwata et al., 1998).

The ELISA using the crude lysate from Thai CIAV-infected cells as an antigen was examined. At present, the substantial agreement (Kappa value = 0.71) between the in-house ELISA and the commercial ELISA means that it is possible to use this ELISA to screen chicken serum samples for antibodies against CIAV. The in-house ELISA using Thai CIAV coating

### Table 2

<table>
<thead>
<tr>
<th>Conjugate dilution/ Antigen Concentration</th>
<th>1:50</th>
<th>1:100</th>
<th>1:200</th>
<th>1:400</th>
<th>1:800</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125 µg/ml</td>
<td>3.92</td>
<td>4.96</td>
<td>5.08</td>
<td>4.40</td>
<td>3.59</td>
</tr>
<tr>
<td>0.250 µg/ml</td>
<td>3.68</td>
<td>4.42</td>
<td>5.09</td>
<td>6.15</td>
<td>2.47</td>
</tr>
<tr>
<td>0.500 µg/ml</td>
<td>3.73</td>
<td>4.95</td>
<td>4.57</td>
<td>5.87</td>
<td>1.94</td>
</tr>
<tr>
<td>1.000 µg/ml</td>
<td>3.13</td>
<td>4.89</td>
<td>6.60*</td>
<td>6.39</td>
<td>2.43</td>
</tr>
<tr>
<td>2.000 µg/ml</td>
<td>1.29</td>
<td>4.30</td>
<td>5.89</td>
<td>5.37</td>
<td>2.23</td>
</tr>
<tr>
<td>4.000 µg/ml</td>
<td>3.00</td>
<td>4.55</td>
<td>4.26</td>
<td>4.57</td>
<td>2.30</td>
</tr>
</tbody>
</table>

Remarks: *represents the highest S/N ratio from this study (Biorad, USA). Lane 1 is the CIAV crude virus.

### Table 3

<table>
<thead>
<tr>
<th>Reference negative sera</th>
<th>Mean ± Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive sera</td>
<td>0.133 ± 0.013</td>
</tr>
<tr>
<td>IBV</td>
<td>0.172 ± 0.014</td>
</tr>
<tr>
<td>ILTV</td>
<td>0.115 ± 0.002</td>
</tr>
<tr>
<td>PM</td>
<td>0.234 ± 0.014</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Commercial ELISA</th>
<th>In-house ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>20</td>
</tr>
<tr>
<td>Negative</td>
<td>70</td>
</tr>
</tbody>
</table>

### Table 4 Contingency table for comparison of commercial and in-house ELISAs.

<table>
<thead>
<tr>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
<td>20</td>
<td>104</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>76</td>
</tr>
<tr>
<td>90</td>
<td>90</td>
<td>180</td>
</tr>
</tbody>
</table>
virus provides a simple means of detecting anti-CIAV antibodies for monitoring CIAV infections or evaluating vaccination in breeder farms in Thailand.

This in-house ELISA technique has the advantage of the same basic principle being able to be used for detecting antibodies against other chicken pathogens using the same reagent except the coating antigen.

Acknowledgements

This work was supported by the Thai Research Fund (TRF) and Office of Higher Education Commission 2010-2012, MRG 5380058.

References


Genetic Diversity of *Mycoplasma hyosynoviae* Field Isolates in Thailand

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Pornpen Pathanasophon\(^1\) Masato Kishima\(^3\) Koshi Yamamoto\(^4\)

**Abstract**

Pulsed-field gel electrophoresis (PFGE) and Random amplified polymorphic DNA (RAPD) analyses were performed to compare techniques and to investigate genetic diversity as an epidemiological data of Thai *M. hyosynoviae* isolates. A total of 42 isolates including a type strain S16 was typable and consisted of 39 different patterns by RAPD technique, whereas 37 isolates (97%) were typable and consisted of 22 different patterns by PFGE technique. Based on PFGE patterns, multiple clones of *M. hyosynoviae* were generally present in pig farms, whereas high genetic heterogeneity of *M. hyosynoviae* among the pig farms was shown. No identical PFGE pattern between the pig farms was found except two farms that were located in the same province. This finding might indicate the distribution of the organism from the same source. Monitoring the genetic diversity of *M. hyosynoviae* strains using PFGE analysis should be useful to elucidate the epidemiology of *M. hyosynoviae* infections in Thailand.

**Keywords:** genetic diversity, *Mycoplasma hyosynoviae*, PFGE, RAPD, Thailand

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บทคัดย่อ

ความหลากหลายทางพันธุกรรมของเชื้อมัยโคพลาสมาไฮโอซินโนวิเอ้ที่เพาะได้ในประเทศไทย

พัชรีทองคําคูณ1,4, สมหมายสมหมาย1,2, โคบายาชิฮิเดกิ3, พรเพ็ญพรพัฒนโสภณ1, มาซาโตะคิชิมา1,2, โคชิยามamoto4

เมื่อนำเทคนิคPFGEและRAPDมาเปรียบเทียบความสามารถในการแยกและศึกษาความแตกต่างทางพันธุกรรมเพื่อเป็นข้อมูลทางระบาดวิทยาของเชื้อมัยโคพลาสมาไฮโอวินโนวิอี้ที่เพาะได้ในประเทศไทยจำนวน41สายพันธุ์และเชื้อสายพันธุ์อ้างอิงS16พบว่าวิธีRAPDสามารถแยกความแตกต่างของเชื้อทั้ง42สายพันธุ์ของใน39รูปแบบในขณะที่PFGEพบการอยู่เป็นกลุ่มของเชื้อที่มาจากสายพันธุ์ต้นกำเนิดเดียวกันในแต่ละฟาร์มในขณะที่PFGEพบการกระจายของเชื้อที่มาจากต่างฟาร์มที่มีหลากหลายทางพันธุกรรมค่อนข้างสูงโดยเทคโนโลยีPFGEมีความสามารถในการแยกความแตกต่างของเชื้อทั้ง42สายพันธุ์โดยมีการดีเอ็นเอกลัพธ์ในรูปแบบPFGEทั้งหมด41รูปแบบโดยสิ้นเชิง

คำสำคัญ: ความหลากหลายทางพันธุกรรม, มัยโคพลาสมา, ไฮโอซินโนวิเอ้, ประเทศไทย

Introduction

*Mycoplasma hyosynoviae* is one of the porcine mycoplasma pathogens distributed worldwide. It commonly inhabits the upper respiratory airways and tonsils of adult pigs. Pigs are occasionally infected with and developed pneumatic lesion, non-suppurative arthritis and lameness. However, the development of pneumonia and/or arthritis might depend on many factors including variation of virulent factors and antigenicity of different strains (Hagedorn-Olsen et al., 1999). Nowadays, arthritis caused by *M. hyosynoviae* becomes increasing problem in many countries (Neilsen et al., 2001; Assuncao et al., 2005; Dahlia et al., 2009). Recently, the occurrence of three porcine mycoplasmas including the first demonstration of *M. hyosynoviae* infection in Thailand identified by isolation technique and PCR was reported (Makhanon et al., 2012). The semi-nested PCR was helpful in screening the presence of *M. hyosynoviae* in the farms. Consequently, the organisms could be isolated from the pigs in various farms (Thongkamkoon et al., 2012). Genomic characterization of *M. hyosynoviae* strains using various molecular typing methods is helpful in revealing the intraspecies genomic variations and facilitating epidemiological studies. Selection of molecular typing technique for mycoplasma species might depend on genetic basis of the species, typability and discriminatory power of the technique as well as laboratory facility. Amplified fragment length polymorphism (AFLP) has been used for typing various mycoplasma species of human and animal origin and *M. hyosynoviae* showed highly genetic difference among isolates which were observed by AFLP and pulsed-field gel electrophoresis (PFGE) analysis techniques (Kokotovic et al., 1999). These two techniques yielded the comparable results for differentiation of *M. hyosynoviae* strains obtained from different geographical locations. The identical patterns were detected only for the strain obtained from the same country suggesting the ability of the methods in monitoring the epidemiological relatedness of the strains. Moreover, the discriminatory power for differentiation of *M. hyosynoviae* isolates of AFLP was equal to PFGE analysis (Kokotovic et al., 2002). Random amplified polymorphic DNA (RAPD) analysis was developed and found to be useful to investigate the epidemiologically related strains of avian mycoplasmas. For *M. synoviae*, RAPD has been
used for differentiation of subspecies level (Fan et al., 1995). However, comparison between RAPD and PFGE was evaluated and found that typability and discriminatory power of RAPD were still greater than PFGE (Marois et al., 2001). For a genetically homogeneous species, M. pneumoniae, PFGE and RAPD had a little advantage over RFLP analysis of the P1 gene (Cousin-Allery et al., 2000). A high diversity was observed for the strains of M. bovis by RAPD technique (McAuliffe et al., 2004). Although it seemed to have lower discriminatory power for differentiation of M. pneumoniae strains (Stakenborg et al., 2006), the discrimination power of RAPD to M. hyosynoviae was doubtful since it have never been used for intraspecies study of M. hyosynoviae.

The aim of the present study was to compare PFGE and RAPD techniques for molecular typing of M. hyosynoviae and to investigate the genetic diversity as an epidemiological data of Thai M. hyosynoviae isolates. The relationship between the genetic patterns and partial sequence of 23S rRNA gene of the isolates was also demonstrated.

**Materials and Methods**

**Mycoplasma strains**: Forty one Thai isolates of M. hyosynoviae isolated from slaughtered pigs during 2008-2011 at National Institute of Animal Health, Thailand and kept at -80°C were used. Type strain S16 obtained from National Institute of Animal Health, Japan was also included. Of the Thai isolates, 18 were isolated from tonsils, synovial fluid and lung of pigs from 5 farms located in Chiang Mai (1), Buri Ram (2), Nakhon Sawan (1) and Nakhon Ratchasima (1) in 2008, 13 were isolated from tonsils of pigs that had more than 90% similarity were considered to be the same strain and 10 were isolated from lungs and tonsil of pigs from 3 farms located in Suphan Buri, Nakhon Ratchasima and Saraburi in 2011. Each of M. hyosynoviae isolates was propagated in 30 ml Hayflick’s broth with arginine and mucin (Friis et al., 1991) and incubated in 37°C for overnight. Consequently, the broth was centrifuged at 13000 xg for 5 min at 4°C (Model CR22GII, Hitachi, Japan). The packed cells were harvested by centrifugation at 10,000 xg for 30 min at 4°C (Himac CR22GII, Hitachi, Japan). The packed cells were washed three times in 2 ml washing buffer (50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, pH 7.2) and centrifuged at 13000 xg for 5 min at 4°C (Model CR22GII, Hitachi, Japan). The packed cells were washed three times in DW and DNA fragments were visualized and photographed under ultraviolet light in a gel documentation system (GelDoc-It, UVP, USA).

**Random amplified polymorphic DNA (RAPD)**: One ml of each M. hyosynoviae culture was harvested at 10,000 xg for 10 min (Model 1920, Kubota, Japan). The pellet was washed once with 1 ml PBS and mixed with Instagene matrix (Bio-Rad, USA) for DNA preparation following the manufacturer’s instruction. RAPD was performed for all samples in one single PCR using Ready-To-Go RAPD analysis kit (GE Healthcare, Life Sciences, USA) following the manufacturer’s instruction. Briefly, five µl of DNA template, 25 pmol of RAPD analysis primer 6-5’-[d(CCCGTCAGCA)]- 3’ and 15 µl of distilled water were added into a tube containing RAPD analysis bead. The contents were mixed gently by vortex. A sample was placed in a thermal cycler (Hybaid, Thermo electron, USA) using the following program: 1 cycle of 95°C for 5 min, 45 cycles of 95°C for 1 min, 36°C for 1 min and 72°C for 2 min. Amplification products were electrophoresed through 2% agarose gel containing 0.1 µg/ml ethidium bromide at 100 volts for 50 min. RAPD patterns were also visualized and photographed under ultraviolet light.

**Data analysis**: Dendrogram, based on the PFGE patterns, was constructed using BIO-PROFIL Bio-1D++ v11.11 software (Vil-Lourmat, Germany). Similar matrix between PFGE profiles was calculated using Dice similarity coefficient. Cluster analysis was performed with unweighted pair group method using average linkages (UPGMA). Isolates that had 100% similarity were considered to be the same strain and isolates that had more than 90% similarity were considered to be genetically related isolates and to be derived from a common parent. The dendrogram, based on the RAPD patterns, was also constructed with the same software.

**Results**

**PFGE**: Of 41 Thai M. hyosynoviae isolates, 37 isolates were typable by PFGE technique whereas 4 isolates which consisted of 2 isolates from farm B and one isolate each from farm D and F did not yield well-separated bands. The banding patterns of typable strains consisted of 6-12 fragments in the size range of 2-48 kb. Twenty two different patterns were detected among 37 Thai M. hyosynoviae isolates and the type strain S16 (Fig 1). Six isolates obtained from farm A
differentiated into 4 PFGE patterns including patterns 1, 2, 12 and 13. Similarity between pattern 1 and 2 was more than 90%. The same result was also shown between pattern 12 and 13. Of the 5 isolates obtained from farm B, 4 isolates had 100% similarity of PFGE pattern 9 whereas the other isolate showed PFGE pattern 17 which had 100% similarity to the isolate obtained from farm C. Three isolates obtained from farm E showed PFGE patterns 4 and 11. Eight isolates obtained from the first fattening pig herd in farm F showed PFGE patterns 3, 7, 8, 10 and 14, whereas 5 isolates obtained from another fattening pig herd that was raised two months later in the same farm showed the same PFGE pattern 10. Three isolates obtained from farm G showed PFGE patterns 5 and 15. Isolate obtained from different fattening pig herd that was raised four months later in the same farm showed PFGE pattern 16 which had 75% similarity to pattern 15. The isolate obtained from farm I showed the PFGE pattern 20. Five isolates obtained from farm H showed PFGE patterns 6, 18, 19 and 21. The *M. hyosynoviae* type strain S16 showed PFGE pattern 22, which was distinguishable from the local isolates.

![Figure 1](image.png)

**Figure 1** Dendrogram of PFGE fragments of 37 Thai *M. hyosynoviae* isolates and type strain S16. Cluster analysis was performed with UPGMA using Dice similarity coefficient and 2% interval of confidence for band matching.
Figure 2 Dendrogram of RAPD profiles of 41 Thai M. hyosynoviae isolates and type strain S16. Cluster analysis was performed with UPGMA using Dice similarity coefficient and 2% interval of confidence for band matching.

In this study, the typability of PFGE to 41 Thai M. hyosynoviae isolates was 91%. PFGE technique had a high reproducibility with the identical banding pattern obtained for replicate samples, even when the DNA extraction and electrophoresis were performed at different time.

RAPD: Five randomly selected Thai M. hyosynoviae isolates and the type strain S16 were typable by RAPD technique using 6 RAPD analysis primers and RAPD beads, however different discriminatory powers were found among the primers (result not shown). RAPD analysis primer 6 was selected due to the highest discriminatory power. The reproducibility was stable.
only when RAPD was performed using the RAPD beads since the results obtained from different pre-prepared commercial mastermix showed different fingerprints (result not shown). The banding patterns consisted of 1-12 fragments in the size range of 200-1500 bp. Thirty nine RAPD patterns were detected among 41 Thai M. hyosynoviae isolates and the type strain S16 (Fig 2). All isolates obtained from the different farms showed different RAPD patterns and almost all isolates obtained from the same farms showed different RAPD patterns. The exception was 4 isolates obtained from farm F showing 100% similarity pattern 33 which was in agreement with the result obtained from PFGE analysis. The three isolates from farm C, of which 2 isolates could not be typed by PFGE, showed their RAPD patterns (patterns 6, 7, 8) within the group with 67% similarity (Fig 2). The RAPD patterns 7 and 8 which had about 83% similarity belonged to the isolates obtained from the same farm whereas RAPD 3 and 4 which had about 77% similarity belonged to the isolates showing the same PFGE profile.

Relationship between genetic profiles and sequences of domains II and V of 23S rRNA : An acquired G745A transition in domain II was found in Thai M. hyosynoviae isolates including all 6 isolates from farm A: 4 isolates from farm G, of which 2 isolates showed an identical PFGE pattern 15 and 1 isolate showed the related PFGE pattern 16; 3 isolates from farm H, of which 2 isolates showed an identical PFGE pattern 18. In addition, 2 isolates from farm H showing an identical PFGE pattern 3 obtained an acquired C739T transition in domain II. 

Discussion

As revealed by PFGE, the clonal appearance was found in farm A, B, E, F, G and H. The same clone found in different organs of pigs including pattern 9 in farm B and pattern 12 in farm A might indicate the invasion ability of M. hyosynoviae strains from tonsil to either joint or lung of the pig. The 92% similarity between patterns 1 and 2, and the 95% similarity between patterns 12 and 13 in farm A demonstrated the alteration in genetic composition of one clone during its persistence in the herd. Diversity of Danish M. hyosynoviae strains, using AFLP analysis, consisted of 13 identical patterns or clonal appearance for 2 to 12 strains obtained from the same farms and the strains obtained from the same herds had either 1 or 4 different clonal lines (Kokotovic et al., 2002). Similar result was found in this study since 2 to 5 clonal lines were also found in farms A, B, E, F, G and H supporting the evidence of highly genetic heterogeneity of M. hyosynoviae. Sharing of identical pattern between two strains obtained the different geographic locations in Denmark was observed once in the previous study (Kokotovic et al., 2002). In contrast, we found only a sharing of the same clonal of pattern 17 obtained from different farms (farm B and farm C) within the same province. Thus, they might get the piglets from the same source. However, sharing the identical pattern of M. hyosynoviae obtained from different geographic location in Thailand might be observed following our investigation in the future.

In this study, RAPD revealed almost 100% of genetically heterogeneous M. hyosynoviae. Although the result could be complicated due to inconsistent band intensities, this finding might support that a high rate of changes in M. hyosynoviae genome was possible (Kokotovic et al., 2002). In contrast, many genetically heterogeneous M. hyopneumoniae isolates and few single clones distributed among the pigs herds. A single clonal line was common inside one herd (Stakenborg et al., 2005). Despite the fact that only specific clones were responsible for the outbreak among the farms in close geographic location (Mayor et al., 2007), strong evidence of multiple clones of M. hyopneumoniae circulating in a single pig or pigs within one herd was observed by RAPD analysis (Nathues et al., 2011). Those results might indicate that M. hyopneumoniae is a lower genetically heterogeneous species compared to M. hyosynoviae, resulting in a chance to have identical RAPD profiles of M. hyopneumoniae among pig population.

Although RAPD seemed to have typability and discriminatory potential greater than PFGE in this study, we preferred using PFGE to reveal genetic diversity of M. hyosynoviae in Thailand. The most important drawback of RAPD was the limited reproducibility of the technique although all standard reagents and materials were used. Number and intensity of the bands might vary due to more or less DNA template, resulting in analysis problems. In addition, RAPD result was not related to the farms or origins of the isolates, whereas PFGE results showed a good correlation of its patterns and the origins of the isolates. Interestingly, the relationship between the alteration of 23S rRNA gene and PFGE pattern of M. hyosynoviae isolates within farms A, G and H was demonstrated, which supported the reliability of PFGE results and obviously confirmed that PFGE would be a suitable and useful tool for epidemiological study of M. hyosynoviae.

In conclusion, the typability of RAPD and PFGE for Thai M. hyosynoviae isolates in this study was 100% and 91%, respectively. RAPD profiles showed very high genetic heterogeneity of M. hyosynoviae isolates inside the herd and among the herds, whereas PFGE profiles could be grouped and revealed a single or multiple clonal lines of M. hyosynoviae inside the herd and between the herds within the same province. Although M. hyosynoviae is considered a highly genetic heterogeneity, a longitudinal observation for their subspecies PFGE patterns among pig population throughout the country will be useful to elucidate the epidemiology of M. hyosynoviae infections in Thailand.

Acknowledgements

We would like to thank Miss Natthika Ratanapan, Miss Waranya Duangs-a-ad, Mr. Kamtorn Promto and Mrs. Ubonrat Tanklang for their technical assistance. We also would like to thank all the staff at bacteriology section for their assistance.
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Effect of Improved Cooling on Daily Rhythmicity of Body Temperature in Cross-bred Holstein Dairy Cows under Tropical Conditions

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Abstract

The objectives of this experiment were to study the effect of heat stress on daily rhythmicity of body temperature in dairy cows under hot and humid climatic conditions and to evaluate the efficacy of improved cooling system by monitoring body temperature. Sequential measurements of body temperature, air temperature, relative humidity and THI were monitored every 5 min. Four cross-bred Holstein-Frisian (93.75% HF) cows were assigned randomly to be used in two trial periods to determine the effects of heat stress and improved cooling system on body temperature. The experiment was conducted in the barn for a total of 6 days, 3 days without a supplemental cooling system (control) and 3 days with a supplemental cooling system (treatment). All cows were housed in the same barn throughout the experiment, but the treatment animals were exposed to the improved cooling system with sprinklers and fans in the holding pen 7 times a day. In this study, air temperature was negatively correlated ($r = -0.983$, $p < 0.0001$) with relative humidity, but positively correlated with THI ($r = 0.996$, $p < 0.001$). In addition, THI and air temperature were positively correlated ($r = 0.709$, $p < 0.0001$ and $r = 0.714$, $p < 0.0001$, respectively) with body temperature of the control animals. The mean level of body temperature of the control animals was higher ($p < 0.05$) than the treatment animals. Body temperature of the control animals consistently rose during the day, reaching a peak in the afternoon (1400 h), after which it remained relatively stable until midnight and then fell throughout early morning. The daily rhythmicity of body temperature had a mean level of 39.3±0.3°C with a maximum-minimum range of 0.9°C in the control animals. In contrast, body temperatures of the treatment animals fell by 0.3-1.0°C after the improved cooling occurred at 0500, 0900, 1100, 1400, 1600, 1900 and 2200 h. Thus, their body temperatures were maintained in the range of 37.9-39.4°C. The mean level of body temperature of these animals was 38.5±0.3°C. In conclusion, climatic conditions have a significant influence on body temperature. Body temperature of dairy cows under tropical conditions has a daily rhythmicity, with a period of rising body temperature and increasing heat load during the day (hyperthermia), followed by a period of heat dissipation and falling body temperature during the night. Improved intensive cooling with sprinklers and fans has the potential to decrease the severity of heat stress and maintain a normal body temperature of dairy cows under hot and humid climatic conditions.

Keywords: cooling cow, cross-bred Holstein dairy cows, heat stress, rhythmicity of body temperature, tropical condition

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Heat stress has a significant impact on dairy cattle in hot and humid climates. Environmental factors, which contribute to heat stress, include high air temperatures, radiant energy, and high humidity, all of which compromise the cow’s ability to dissipate body heat. When cows cannot dissipate sufficient heat to maintain thermal balance, rectal temperatures greater than 39.0°C and respiration rates greater than 60/min indicate that cows are undergoing heat stress sufficient to affect milk yield and fertility (Kadokawa et al., 2012). Air temperature is a major component of heat stress, however humidity must also be considered because evaporative heat loss is more effective when humidity is low. Temperature and humidity index (THI) combines temperature and humidity into an indicator of cow comfort. Cows begin to be stressed when THI exceeds 72, with increases in air temperature, THI, and body heat. When THI is above critical thresholds have been 80 and 90, respectively (Armstrong, 1994). In addition, heat stress can cause a decrease in fertility and represents a major factor of economic loss in dairy cows under hot climates.
In hot and humid climatic conditions, dairy cows cannot dissipate sufficient body heat to prevent a rise in body temperature. In addition, metabolic heat increments require effective thermoregulatory mechanisms to maintain body temperature in a thermoneutral zone and in physiological homeostasis. Dairy cows can suffer hyperthermia if they fail to maintain thermoneutrality (Kadzere et al., 2002). The measurement of body temperature is a reliable method for the evaluation of heat stress in dairy cows. Vaginal temperatures were associated with rectal measurement, and provided the advantage of capturing diurnal changes in body temperature (Vickers et al., 2010). This is a relatively accurate tool for determining the degree of heat stress. In healthy dairy cows, a body temperature range from 37.9°C to 39.6°C for primiparous cows (mean 38.8°C) and 37.9°C to 39.5°C for multiparous cows (mean 38.7°C) was determined by Wenz et al. (2011). Daily rhythmicity of body temperature has a mean level of 38.3°C, a range of excursion of 1.4°C (Piccione et al., 2003). The daily rhythmicity of body temperature is influenced both by changes in heat production and changes in heat loss. Animals have a thermoregulatory system to regulate body temperature by balancing rate of heat exchange between the animal and its environment. Heat loss by conduction and convection is increased by lowering air temperature. Vickers et al. (2010) found that body temperature was higher at night, and lower between 0800 to 1000 h for healthy cows (39.0±0.02°C). In tropical conditions, body temperature of dairy cows has been shown to decrease in cooled cows compared to non-cooled cows (38.7±0.5 and 39.7±0.6°C, respectively). In addition, over a variation of environmental conditions, the body temperature of non-cooled cows was found to fluctuate from 38.9±0.7 to 39.7±0.6°C (Chaiyabutr et al., 2008). However, sequential measurements of body temperature or daily rhythmicity in body temperature have not been studied in cross-bred Holstein dairy cows.

For dairy farms, farmers need information about how and why their animals respond to environmental challenges in order to make improved decisions on strategies and tactics for reducing losses during hot weather. These strategies involve minimizing heat gain by reducing solar heat load and maximizing heat loss by reducing air temperature around the animal or increasing evaporative heat loss directly from the animals. Measurements of body temperatures can be used to characterize stress levels of dairy cows and aid in further development of environmental modifications for improving their productivity. In addition, an understanding of the influences of climate change and the effectiveness of improved cooling systems on daily rhythmicity of their body temperature is necessary for control and management of dairy cattle housing. The objectives of this study were to examine the effect of heat stress on daily rhythmicity of body temperature in dairy cows under hot and humid climatic conditions and to evaluate the efficacy of utilizing an improved cooling system by monitoring internal body temperatures.

**Materials and Methods**

**Experimental animals and management:** This study was conducted in a commercial dairy farm that is located in the central part of Thailand. The experiment was performed in August, in which air temperature ranged from 25 to 33°C and air humidity ranged from 60 to 80%. In this farm, lactating dairy cows were housed in a naturally ventilated free stall barn without a supplemental cooling system and were fed a total mixed ration (TMR) and water ad libitum. Dairy cows were milked twice daily (0200 and 1400 h).

The environmental management system for cooling cows in this study involved intermittent cooling with sprinklers and forced air ventilation with fans in the holding pen. Cows were cooled in the holding pen for 45 min with intervals of 2-3 hours between cooling periods, beginning at 0500 hours up to until 2200 hours. The cooling system during the cooling period (45 min) consisted of sprinklers and fans, sequentially activated to repeat cooling cycles of wetting by sprinklers for 1 min and air forced ventilation by fans for 4 min.

Four multiparous (2-3 lactations), cross-bred Holstein-Frisian (93.75% HF), healthy early postpartum (30-45 days) dairy cows were assigned randomly to use in two trial periods to determine the effect of this improved cooling system on body temperature. The experiment was conducted for a total of 6 days, 3 days each in the barn without the supplemental cooling system (control) and in the same barn with the supplemental cooling system (treatment). All cows were housed in the same barn throughout the experiment, but treatment animals were exposed to the improved cooling system with sprinklers and fans in the holding pen 7 times a day at 0500, 0900, 1100, 1400, 1600, 1900 and 2200 hours.

**Data collection**

**Environmental conditions:** Environmental conditions in the barn such as air temperature and relative humidity were recorded every 5 min using data loggers (Easy log®, EL-USB-2; Lascar Electronics, Salisbury, UK) throughout the experiment. The temperature humidity index (THI) was calculated according to the formula: \[ \text{THI} = (0.8 \times T) + \left[ \frac{\text{RH}}{100} \times (T-14.3) \right] + 46.4 \] where \( T \) was air ambient temperature (°C) and \( \text{RH} \) was relative humidity (%) (Gaughan et al., 2010). Mean level of air temperature,
relative humidity and THI were calculated for 5-min blocks.

**Cow temperature:** A more comprehensive assessment of body temperature throughout the day was obtained by the use of a small computerized data logger attached to a blank CIDR device. The data logger (Thermocron®, iButton; Maxim, Sunnyvale, California, USA) was used in this study to monitor internal body temperature every 5 min. It was designed to continuously monitor the animal’s body temperature 24 hours a day. These devices were inserted into the vagina of the dairy cows. Vaginal temperatures were recorded for all cows in this study (both those exposed to and those not exposed to the supplemental cooling system) throughout the day to determine the daily rhythmicity in body temperature.

**Statistical analysis:** Sequential measurements of body temperature, air temperature, relative humidity and THI were monitored every 5 min. The level of mean was calculated for 5-min blocks throughout a day. Data were presented as mean±SD. Significant difference of each parameter between groups was tested by *t*-tests for matched samples by repeated-measures analyses of variance (ANOVA). Relationships between parameters were examined by simple correlation. The level of significance was set at *p* < 0.05.

**Results**

**Environmental conditions:** Climatic conditions in the barn (air temperature, relative humidity and THI) were plotted as a function of time of day in Figure 1. Each data point represented the mean air temperature, mean relative humidity and mean THI for a given 5-min interval throughout a day. The mean air temperature over the experiment was 29.3±2.3°C. Air temperature consistently rose during the day, reaching a maximum air temperature of 33.1°C in the afternoon (1300 h). It remained stable until the evening and then fell throughout the night. The minimum air temperature was 26.1°C and occurred at 0600 h. In contrast, relative humidity rose during the night, reaching a peak in the morning. The maximum relative humidity was 86.1%, which occurred at 0600 h, after this it fell during the day. The minimum relative humidity was 62.3% which occurred in the afternoon (1500 h). The mean relative humidity was 74.5±8.0%. In this study, air temperature was negatively correlated (*r* = -0.983, *p* < 0.0001) with relative humidity, but positively correlated with THI (*r* = 0.996, *p* < 0.0001). The mean THI in the barn was 80.6±2.3. The maximum THI was 84.6 and occurred in the afternoon (1300 h) and fell throughout the night. The minimum THI was 77.3 and occurred at 0600 h. In this study, THI exceeded the critical point of 72 throughout the experiment. The exposure to condition of severe heat stress (THI > 80) occurred in late morning through late evening. The condition of mild heat stress occurred in early morning. In addition, THI and air temperature were positively correlated (*r* = 0.709, *p* < 0.0001 and *r* = 0.714, *p* < 0.0001, respectively) with body temperature in the control animals. These results indicate that climatic conditions have a significant influence on body temperature of dairy cows in hot environments.

**Cow temperature:** The mean vaginal temperatures of four dairy cows were measured both with and without supplemental cooling system exposure. Internal body temperatures plotted as a mean of vaginal temperature of each 5-min interval for each trial period (control and treatment) are shown in Fig 2. The mean vaginal temperature of the animals without supplemental cooling system (control) was higher (*p* < 0.05) than the treatment animals. Body temperatures rose during the day and fell gradually during the night, reaching a daily low level early in the morning. The mean level of body temperature was 39.3±0.3°C for the control animals and 38.5±0.3°C for the treatment animals. The maximum and minimum of body temperature were 39.7 and 38.8°C, respectively for the control animals, and 39.4 and 37.9°C, respectively for the treatment animals.

In the control animals, body temperature consistently rose during the day, reaching a peak in the afternoon (1400 h) and then remained relatively stable until midnight after which it fell throughout the

![Figure 1](image莫过于). Daily changes in mean of air temperature, relative humidity and THI in the dairy farm barn throughout the experiment.
early morning. In contrast, the mean of body temperatures of the treatment animals fell by 0.3–1.0°C after the improved cooling occurred at 0500, 0900, 1100, 1400, 1600, 1900 and 2200 h, thus maintaining the body temperature of the cows in the range of 37.9–39.4°C.

A regular pattern of daily rhythmicity of body temperature occurred in both groups; however there was significant difference between the groups. In the control animals, the body temperatures rose during the day. Minimum body temperature usually occurred early in the morning (0600 h), and then steadily increased during the day. Maximum body temperature usually occurred in the afternoon (1400 h). The heat load built up during the day was dissipated at night such that body temperature fell gradually during the night, reaching a daily low level early in the morning. The daily rise in body temperature and sustained hyperthermia for long periods did not occur in the treatment animals. The body temperatures of the treatment animals slowly increased during the day and fell after cooling in the holding pen, and then steadily increased when the animals were out of the barn. However, the body temperature fell markedly when these animals returned to the holding pen again.

Discussion

As can be seen from the climatic conditions shown in Fig 1, the dairy cows in this study were subjected to moderate heat stress during the day and mild heat stress during the night throughout the experiment. A comfortable environmental temperature range for dairy cows is between 4°C and 25°C (Roenfeldt, 1998), but the climatic conditions in the central part of Thailand generally involve high temperature and high relative humidity throughout a year. Thus, heat stress is chronic in nature; there is often little relief from heat during the night until early morning. Increase in air temperature above 27°C causes elevation of body temperature, which is defined as hyperthermia in cattle (García-Iesperito et al., 2007). In this study it was found that air temperature and THI were highly correlated with body temperature in the dairy cows without a supplemental cooling system. These cows had a high body temperature during a significant portion of the day and their body temperature returned to normal only during late night and early morning (Fig 3). Maia et al. (2005) reported that when air temperature was greater than 30°C, cutaneous evaporation became the primary venue for heat loss, accounting for approximately 85% of the total heat loss, while the rest was lost by respiratory evaporation. In this study, the control cows generally needed several hours past sundown to dissipate heat and cool down from an extremely hot day. The increase in body temperature is a normal mechanism by which animals respond to the heat load from hot ambient conditions and the heat loss by convection and evaporation is effective only when the air temperature declines below 27°C, not under high environmental air temperature and air humidity during early night. Heat flow occurs through processes dependent on surrounding temperature and humidity. Hansen (2004) reported that heat loss by skin was dependent on the temperature gradient between the animal and the air. Burfeind et al. (2012) reported that during the hot period (THI 74.1±4.4), healthy dairy cows exhibited rectal temperature ≥39.5°C. Therefore, in order to minimize the effects of heat stress, modifications to dairy housing environments have been implemented to alleviate thermal stressors and improve cow comfort. In addition, a cow’s body temperature should be maintained within narrow limits in order to sustain its physiological processes. A variety of cooling systems are available for heat-stressed cows to maintain normal body temperature during the day (Flamenbaum and Galon, 2010). Intensive cooling systems with a combination of sprinklers and fans have been used for improving cow comfort, milk yield

![Figure 2](image-url)
and reproduction (Collier et al., 2006; Flamenbaum and Galon, 2010). This system enhanced the conductance of heat from the body core to the skin by wetting of skin and from the surrounding air by air forced ventilation. Gebremedhin et al. (2008) reported that wetting the skin surface and increasing air velocity profoundly increased evaporation rate by converting sensible heat to latent heat. The average body temperature of cooled cows in this study was most likely 38.5°C. According to this study, the range was found to be 37.9 to 39.4°C, and the proportion of hourly body temperature values above the threshold (> 39.0°C) was also lesser in the treatment cows compared with the control cows. These results suggest that the supplemental cooling in the holding pen for 7 times per day was effective in reducing body temperature of cross-bred Holstein dairy cows.

The dynamic responses of body temperature to environmental conditions shown in this study demonstrate the utility of environmental management. Environmental temperature had a significant influence on the body temperature of cross-bred Holstein dairy cows. Body temperatures of these cows in hot and humid conditions tend to increase during daylight hours, and then heat load is dissipated at night. The intensive cooling system used in this study had a significant effect on the decrease in body temperature and allowed the cows to maintain a normal body temperature of 38.5-39.3°C relatively easily throughout a day. As shown in Fig 2, the mean level of body temperature of the control animals was consistently higher than the body temperature in treatment animals. In addition, the duration of hyperthermia in the control animals was longer than in the treatment animals. Heat stress increases the body temperature of dairy cow which affects the reproductive functioning and production. Dry matter intake and milk yield can decrease about 20-25% in cows subjected to heat stress under tropical conditions (Suadsong et al., 2008; Chanpongsang et al., 2010). Previous research indicates that improved cooling systems for cross-bred Holstein dairy cows under hot and humid climatic conditions results in decreased body temperature and increased feed intake and milk production (Chaiyabutr et al., 2008; Suadsong et al., 2008). In addition, heat stress can cause infertility and represents a major source of economic loss in dairy cows under tropical conditions. High air temperature causes a significant reduction in fertility in cattle. Hyperthermia leads to disturbances in the functioning of the reproductive system (Rynkowska et al., 2011). Paula-Lopes et al. (2012) reported that exposure of bovine oocytes to elevated temperature affected the events required for successful oocyte maturation, fertilization and preimplantation embryonic development. The elevation of temperature decreasing oocyte function occurs due to a series of cellular alterations that affects nuclear and cytoplasmic compartments of the bovine oocyte. Previous research reported that the reproductive dysfunction caused by heat stress could be reduced by reducing the magnitude of heat stress that drove dairy cows into hyperthermia (Hansen and Arechiga, 1999). It’s difficult for dairy cows in hot environments to meet their full potential for either milk yield or fertility. The most common way to reduce the effects of heat stress on dairy cow is to change the cow’s environment to reduce the severity of heat stress. Therefore, the study of the daily rhythmicity of cow’s body temperature can increase the effectiveness of heat stress management by providing information as to how well cows regulate body temperature during the day.

In conclusion, in this study, climatic conditions were shown to have a significant influence on body temperature in cross-bred Holstein dairy cows under tropical conditions. Body temperature was found to have a daily rhythmicity in which there was a period of rising body temperature and increasing heat load or hyperthermia during the day, followed by a period of heat dissipation and falling body temperature during the night. The improved cooling system was shown to have the potential to decrease the severity of heat stress and to help maintain a normal body temperature in cross-bred Holstein dairy cows. Consequently, this study is particularly useful for the development of proactive tactical management guidelines for dairy cattle producers in tropical areas.

Acknowledgements

This study was funded by Thailand Research Fund, the Office of the Higher Education Commission and Faculty of Veterinary Science, Chulalongkorn University. We would like to thank Namphon dairy farm and Udom dairy farm for their support in providing animals and facilities in this study. Moreover, we would like to thank Mr. Philippe Marcou for his revision of the English text.

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Surgical Removal of Urethral and Bladder Stones in Female Asian Elephant (*Elephas maximus*) by Episiotomy and Urethrotomy

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Abstract

A 45-year-old female Asian elephant (*Elephas maximus*) was presented with urinary incontinence and solid masses at the perineal area. Vaginoscopy revealed stricture of the vaginal vestibule. Trans skin and rectal ultrasonography demonstrated presence of hyperechogenic uroliths. Episiotomy and urethrotomy were performed after standing sedation with xylazine and perineal infiltration with lidocaine. Total of 8 kg urethral calculi were retrieved manually. Calcium carbonate was identified as the main component of the stones. Due to acute urinary obstruction after the operation, an episiotomy was left open allowing urine to pass through the incision. Following urine scalding, necrotic tissues were detected along the tracts below the incision wound through umbilicus. Daily wound cleaning and antibiotics treatment for one month were performed. The episiotomy wound was left to heal by secondary intention and led to a 2 cm stricture. The healing process completed in 5 months post-operatively. The elephant can urinate via normal tract in 8 months after surgery.

Keywords: Asian elephant, episiotomy, surgery, urethral calculi, urethrotomy

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บทคัดย่อ

การผ่าตัดแก้ไขภาวะนิ่วในทางเดินปัสสาวะและกระเพาะปัสสาวะของช้างเอเชีย (Elephas maximus) เพศเมียด้วยวิธี episiotomy และ urethrotomy

นิกร ทองทิพย์1,2,3,4* เบญจพล หล่อสัญลักษณ์1 นามอนุช สุขมา3,4 ศิรินทร ข้อข้อง3,4 บริศ เด็จเจริญ5 พรชัย สัญฐิติเสรี2 วรรธิ์ วิชชวิศ6

ช้างเอเชีย (Elephas maximus) เพศเมีย อายุ 45 ปี เป็นการรักษาด้วยการผ่าตัดแก้ไขรูปแบบการปัสสาวะและกระเพาะปัสสาวะ ของช้างที่นำมาจากฝีเย็บ และจากการตรวจด้วยกล้องส่องตรวจช่องคลอดพบการตีบแคบของเวสติบูลส่วนที่ต่อกับช่องคลอด และจากการอัลตร้าซาวด์ผ่านทางผิวหนังและทวารหนักพบวัตถุที่สะท้อนคลื่นอัลตร้าซาวด์คล้ายก้อนนิ่ว ทำาการวิเคราะห์ซึ่งมีการเป็นของอิสระในบริเวณฝีเย็บ ทำาการผ่าตัดผ่านเนื้อเยื่อ (episiotomy) และผ่าเปิดท่อปัสสาวะ (urethrotomy) จากนั้นทำาการสั่งเสียก่อน ห้ามเกินคัน นำนิ่วออกจากฝีเย็บทั้งหมดเท่ากับ 8 กก. จากการวิเคราะห์ที่นำเสนอว่าเป็นสีส้มตัวหนึ่งในแอลกอฮอล์ ภาพหลัง การผ่าตัดและเหล็กการเปลี่ยน พบการตีบแคบของปัสสาวะบริเวณที่ผ่าตัด จึงทำาการเปิดแผลผ่าตัดเพื่อให้ปัสสาวะไหลออกผ่านแผลผ่าตัด ตลอดจนเนื้อเยื่อที่กระทำาการเป็นของอิสระที่ต่อกับแผลผ่าตัด เกิดการบั้นเลือดที่ผ่าตัดเนื้อเยื่อที่มีการติดเชื้อเป็นเวลา หนึ่งเดือนและทำาแผลเกิดการคั่งอุดตันที่สุทธิแบบsecondary intention ผลที่ได้จากวิเคราะห์และการวิเคราะห์หลังการผ่าตัด มีการหายออกและแผลเกิดการหายแบบsecondary intention แผลฝีเย็บใช้เวลาในการหายและเหลือนิ่วและความกว้างของแผลประมาณ 2 ซม. ในเวลา 5 เดือน ช้างสามารถปัสสาวะได้ทางช่องเปิดปกติภายใน 8 เดือนหลังการผ่าตัด

คำสำคัญ: การผ่าตัด ช้างเอเชีย นิ่วทางเดินปัสสาวะ

Introduction

Cystic and urethral calculi are found in domestic animals such as companion animals (Runge et al., 2011) more frequently than in elephant and wild animals. Calcium carbonate urolithiasis is the most common in horse (Mair and Osborn, 1986). Predisposing causes of stone in horse are not well understood. Tissue damage, cystitis, remaining suture material, supersaturation of urine with certain minerals and urine stasis have been proposed (Vengust, 2011). In human, the risk factors of urolithiasis are divided into intrinsic and extrinsic factors. Intrinsic factors include nationality, genetic, age and sex. Extrinsic factors include geography, climate, season, water, food and occupation (Tanthanuch, 2002). Urethral calculi infrequently occur in elephant. Two stones in the left ureter have been found during necropsy of an African elephant died from chronic renal failure (Morris et al., 1987). Calcium carbonate was noted as the type of both stones. Ruedi (1995) successfully identified bladder stone using endoscope. Surgical treatment of urethral calculi in elephant is infrequently done. There was a report cited by Hildebrandt et al. (2000) about urethrotomy for stone removing in male elephant by Lange et al. (1999). Their result revealed permanent urethral fistula similar to that in females for episiotomy for dystocia treatment. There are some manuscripts published about the episiotomy for removing dead body of the elephant calf during dystocia in Thailand (Yartboon et al., 2002; Thitaram et al., 2006). However, to our knowledge, there has been no report about urethral or bladder stones removal in female elephant. Surgical removal of urocystolith in large animal species has been described in horse. The surgical options included midline laparotomy and cystotomy (Weaver, 1968), pararectal cystotomy (Abuja et al., 2010), perineal urethrotomy (Hanson and Poland, 1995), urethral sphincterotomy (Firth, 1976) and parainguinal
laparocystotomy (Beard, 2004). In this present report, we describe the diagnosis, surgical procedure by using episiotomy and urethrotomy and post-operative care of urethral calculi in female Asian elephant (Elephas maximus).

Animal signalment and clinical signs: A 45-year-old female Asian elephant (Elephas maximus) exhibited signs of urinary incontinence and abnormal mass at perineal area. The estimated weight of the elephant was 3,000 kg. The elephant's body temperature was 99°F with pulse rate and respiratory rate of 35 beats per minute and 5 times per minute, respectively. The elephant was admitted to Kasetsart University, Kamphaeng Saen Veterinary Teaching Hospital on 14 September 2012. The peroneal mass was around 30x30 cm and continued to increase in size (Fig 1). The elephant became anorexic and strained to urinate and defecate. Further history taking revealed that the abnormal mass under anus has been identified one year earlier. However, the mass could be pushed back manually by mahout during that time. Rectal and topical palpation revealed the firm mass and the elephant showed mild pain when pressed.

Laboratory Tests: Pre-operative routine blood examination via auricular vein puncture was done. The samples were immediately sent to laboratory. Hematology and blood chemistry profiles are shown in Table 1 and 2. Hematological profiles, liver enzyme and kidney function were within normal limits.

Ultrasonography: Transrectal ultrasonography (ALOKA; SSD 500 with convex transducer probe; 3.5 MHz) was performed. Ultrasonography via transrectal and skin revealed circular masses in various sizes. The biggest size of the abnormal masses via ultrasonography image was 10 cm in diameter. These circular masses showed their hyperechogenic appearance similar to uroliths in horse (Hanson and Poland, 1995).

Vestibuloendoscopy and cystoureteroscopy: Vestibuloendoscopy and cystoureteroscopy were carried out using a fiber optic video endoscope with light source of PENTAX (EPM3300 and EC3830 fz). Vestibuloendoscopy was done on the first day before operation. Cystoureteroscopy was done one month

![Figure 1 Abnormal mass under anus](image-url)

Table 1 Hematology profiles of Kammoon before and after surgery

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-operation</th>
<th>Post-operation</th>
<th>Reference value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Day 0)</td>
<td>(Day 4th)</td>
<td>(Day 17th)</td>
</tr>
<tr>
<td>Hb (mg%)</td>
<td>10.3</td>
<td>10.3</td>
<td>9.7</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>27.6</td>
<td>26.9</td>
<td>25.4</td>
</tr>
<tr>
<td>RBC (x 10⁶/µl)</td>
<td>2.38</td>
<td>2.3</td>
<td>2.18</td>
</tr>
<tr>
<td>PLT (x 10⁵ g%)</td>
<td>220</td>
<td>254</td>
<td>240</td>
</tr>
<tr>
<td>PP (mg%)</td>
<td>7.4</td>
<td>7.8</td>
<td>7.8</td>
</tr>
<tr>
<td>WBC (x 10⁶/µl)</td>
<td>12.6</td>
<td>10.4</td>
<td>14.3</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>28.1</td>
<td>55.9</td>
<td>66.0</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>65.4</td>
<td>28.0</td>
<td>52.4</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>31.5</td>
<td>17.4</td>
<td>19.9</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>4.0</td>
<td>2.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Basophil (%)</td>
<td>0.7</td>
<td>0.6</td>
<td>0.33</td>
</tr>
</tbody>
</table>


Table 2 Blood chemistry profiles of Kammoon before and after surgery

<table>
<thead>
<tr>
<th>Blood chemistries</th>
<th>Pre-operation</th>
<th>Post-operation</th>
<th>Reference value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Day 0)</td>
<td>(Day 4th)</td>
<td>(Day 33th)</td>
</tr>
<tr>
<td>BUN (mg%)</td>
<td>7.5</td>
<td>7.0</td>
<td>5.2</td>
</tr>
<tr>
<td>Creatinine (mg%)</td>
<td>1.9</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>16</td>
<td>NA</td>
<td>26</td>
</tr>
</tbody>
</table>

BUN: blood urea nitrogen, AST: Aspartate aminotranferase, NA: Not available
after surgery. The vestibuloendoscopy revealed the non-infected vestibule. The vestibuloendoscopy could not reach the vaginal canal due to the stricture of the vestibule. The cystoureteroscopy did not reveal any stones in both urinary bladder and ureters.

**Fine needle aspiration:** The sample for cytological examination was collected by fine needle aspiration at perineal area. The fine needle aspiration with 18 gauge needle collected few amounts of necrotic tissue, pus and bloody exudates. Cytology examination revealed the presence of pus cells with 3+ cocci bacteria. However, the bacterial culture was not done.

**Clinical diagnosis and differential diagnosis:** Clinical diagnosis was based on rectal and topical palpation, fine needle aspiration, ultrasonography, vestibuloendoscopy, hematology and blood chemistry analysis. The list of differential diagnosis included neoplasia, abscess, dystocia and urethral calculi. From all of examinations, urethral stone was assumed as the clinical case.

**Treatment plans:** Due to the lack of information of surgical removal of urethral stone in female elephant, episiotomy for exploration was performed. Then, urethrotomy for removing stone was done. Closing of urethral muscle, vestibular wall and skin was also performed.

**Sedation, pre-operative and surgical procedure:** Before starting, the elephant was given fluid therapy by using NSS through ear vein. Flow rate of the fluid was around 2 liters per hour for 5 hours. The elephant was sedated by administering xylazine hydrochloride 0.08 mg/kg intramuscularly (Ilium Xylazil-100®, Troy Lab PTY Ltd, Smithfield NSW, Australia). Sixty ml of 2% lidocaine hydrochloride was infiltrated covering the incision area at perineum under anal opening with 1 ml per 1 cm². The elephant was calm and did not show signs of pain, then after 2 hours operation, she moved her back and front legs more frequently. Fifty mg of xylazine hydrochloride was given intravenously to sedate and provide additional analgesia. An episiotomy with a 15-cm incision line was performed at the perineum proximal to the anus. The vaginal vestibular muscle was deeply incised exposing the underlying mass. The urethrotomy toward the mass was then performed allowing the urine as well as urethral stones to come out. Various sizes of urethral stones ranging from 1-10 cm in diameter were removed. A total of 8 kg urethral calculi with various sizes were removed (Fig 2). During the calculi removal, the elephant urinated through the incisional wound all the time. The episiotomy was closed with chromic cat gut No. 2 (KRÜUSE, Langeskov, Denmark) by apposing the vaginal vestibular muscle. The skin was closed routinely with Supramide No.2 (KRÜUSE, Langeskov, Denmark). After the operation, the elephant was given yohimbine hydrochloride (Reverzine®, Parnell Lab Pty Ltd, NSW, Australia) 0.125 mg/kg intravenously.

**Acute complication and treatment:** Although the muscles and skin were completely closed, 3 hours post-operative period the elephant demonstrated a sign of stranguria without any urine coming out through normal tract. The urethral obstruction, as the consequence of soft tissue swelling and accumulation of urine under surgical area, was clinical detected. Consequently, all suture materials were cut letting the urine drained through the incision wound and the swelling disappeared subsequently. The elephant showed signs of bloating both sides of dorsal abdomen swelling with gas accumulation 5 hours post-operatively. The elephant was treated by enema with tap water to increase the gastrointestinal motility. The ear vein was cannulated for administering intravenous medication. Total of 300 mg of metoclopramide hydrochloride (MET-SIL®, T.P. Drug Lab, Bangkok, Thailand) were diluted in NSS intravenously. One hour later, the elephant defecated several times leading to the reduction in the size of abdomen. The elephant was persuaded to walk 20 min per hour. It took 5 hours for a full recovery with more than 20 attempts to defeate.

**Post-operative care:** Daily wound cleaning was done by using chlorhexidine scrub (O.R. Scrub, Spinpharm Co. Ltd., Bangkok, Thailand) on the skin and subcutaneous tissue. The bladder was lavaged with clean tap water and 10 liters of NSS using equine stomach tube. Topical of nitrofurazone ointment (Bactacin®, Osoth Inter Lab, Bangkok, Thailand) and insect repellent (Negazun®, Bayer CropScience Ltd.) were applied. A systemic control of bacterial infections was also performed by using long acting amoxycillin at (Longamox®, Vetoquinol, Lure, France) a total dose of 7,500 mg/day for 30 days intramuscularly. After 14 days of amoxycillin injection, administration of enrofloxacin at (Syvaquinol 100®, SYVA Lab, LeÓn, Spain) a total dose of 4,800 mg per day for 14 days intramuscularly was administered to the elephant. The total course of antibiotic injections was one month. Phenylbutazone (Butasy1®, Novartis Animal Health, Australia) at a total dose of 9000 mg daily was injected intramuscularly for 5 days. The injectable supplement containing butaphosphan and cyanoocobalamin (Calosal®, Bayer Health Care, Kansas, USA) at a total of 50 ml was intramuscularly administered every 2 days for a 30-day period. Thirty tablets of Serratiopeptidase were also daily given per

![Figure 2 Urethral stones removed from Kammoon](image-url)
oral for 30 days. Urethral canula modified from the re-used elephant endotracheal tube was inserted to the urethra canal. The elephant could urinate via the canula. However, the fitting of the canula on the elephant perineum was not entirely secured and was later removed unintentionally on the first day when the elephant wagged its tail on the canula. These resulted in a urine-contaminated surgical wound and an undermining of the urine through the subcutaneous tissue extending from perineal area to ventral part of abdomen including umbilicus (Fig 3). The accumulation of urine under the subcutaneous tissue and the urine scalding led to pustular exudates and necrotic tissues around the surgical wound. The wound was then cleaned and the necrotic tissues were removed daily. After the operation, the elephant had good appetite and was able to defecate normally.

However, the elephant was unable to lie down in lateral recumbency position for sleeping. The elephant slept only in standing position. She used her head and trunk to support her weight. The wound was healed by secondary intention and the healing significantly narrowed the surgical wound after one month (Fig 4). Cystoureteroscopy was also performed one month after surgery through the incisional wound and no stone was found inside the bladder (Fig 5) and both ureters (Fig 6). Then, the elephant was sent back to her facility. Five months after surgery, the examination showed that the incision was covered with some necrotic tissue and once the necrotic tissue was removed the examiner found a stricture with 2 cm in diameter (Fig 5). The elephant could still urinate through the stricture and through its normal tract via vestibule and vulva. The patency of vaginal vestibule lower than the incision was confirmed by putting 20 liters of sterile water via the modified equine stomach tube. However, the elephant could urinate via normal tract in 8 months after surgery.
Laboratory test during post-operative care and urolith analysis: Blood samples were also collected on 4th, 17th and 33th days post-operatively. Mid-stream urine samples were collected on the 4th and 92th days after the operation. Hematological findings revealed neutrophilia on the 4th-17th days after operation and normal level on the pre-operative period day 0 and day 33 after the surgery. The liver enzyme and kidney function were within normal limits. Urinalyses were done on the 4th and 92th days after surgery. On day 4, the results revealed alkaliniuria (pH 9), hemoglobinuria and pyuria. Calcium oxalate monohydrate and calcium oxalate dihydrate crystals were found in urine sediment. On the 92th day, the results revealed lower urine pH 8 compared to the previous analysis and hemoglobinuria was no longer found. Upon urine sediment, only calcium carbonate was found. Urine specific gravity (USG) of both days revealed isostenuria (Table 3). The stone were sent for composition analysis by using IR Spectrum technique (Uldall, 1981) at the Department of Surgery, Faculty of Medicine, Prince of Songkla University. The stone composition analysis with IR Spectrum revealed only calcium carbonate in both surface and nuclear regions.

Discussion

In this present report, the causes of urethral calculi in elephant remained unclear. Low water intake or high mineral water drinking and low amount of water in the food were considered. Due to the elephant’s translocation from mainland to the island, the water and food supply might not be ideal resulting in an opportune condition for calculus formation. It remains unknown whether renal pelvis of an elephant can produce mucus that can prevent crystal formation similar to a horse (Mair and Holt, 1994). Equine urine also contains other components such as pyrophosphate, citrate, magnesium, and glycosaminoglycans that also help inhibit urolith formation (Schott, 1998). However, such factors have not been elucidated in elephant yet. The predisposing factor studies of the stones formation in elephant need to be done in the near future.

Table 3 Urinalysis results of Kammoon on the 4th and 92th days after surgery.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Post-operation</th>
<th>Reference value (Weidner et al. 2009)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Yellow</td>
<td>Yellow pale yellow (54%), medium to dark yellow (45%)</td>
</tr>
<tr>
<td>Transparency</td>
<td>Cloudy</td>
<td>Cloudy clear (36%), cloudy (64%)</td>
</tr>
<tr>
<td>Sp. Gr.</td>
<td>1.022</td>
<td>1.010 - 1.036</td>
</tr>
<tr>
<td>Nitrite</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pH</td>
<td>9</td>
<td>6.8 - 8.6</td>
</tr>
<tr>
<td>Protein (mg/dl)</td>
<td>Negative</td>
<td>0.12 - 0.248</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Ketone</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>Negative</td>
<td>95% negative</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>+4</td>
<td>5% positive</td>
</tr>
<tr>
<td>Blood</td>
<td>Negative</td>
<td>4.5%</td>
</tr>
<tr>
<td>RBC</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Casts</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Crystals</td>
<td>Calcium oxalate monohydrate 100/hpf</td>
<td>Samples with crystals (100%)</td>
</tr>
<tr>
<td></td>
<td>Calcium oxalate dihydrate 10/hpf</td>
<td>Samples with multiple types of crystals (32%)</td>
</tr>
<tr>
<td>Cells</td>
<td>WBC 20/hpf</td>
<td>9.1%</td>
</tr>
<tr>
<td></td>
<td>Transition cell 1/hpf</td>
<td>4.5%</td>
</tr>
<tr>
<td></td>
<td>Bacteria +1</td>
<td>18%</td>
</tr>
</tbody>
</table>

Figure 7 Stricture of episiotomy wound at 5 month after surgery
limitations of diagnostic tools and surgical procedure. Episiotomy has only been performed to correct dystocia (Yartbantoong et al., 2002; Thitaram et al., 2006) and vaginal prolapse (personal data) in this species but perineal urethrotomy has not been done. The present study shows the surgical technique that could remove urethral stone in a female elephant. The complications after surgery to remove urethral calculi with the episiotomy technique were also found in the present study. In a normal episiotomy patient, when the incision reaches the hollow of the vaginal vestibule, there should be a space. Nevertheless, in the present case, the stones pressed firmly against the urethral muscles and were attached to the vestibule wall. As a consequence, no space was left inside the vestibule and excising through the deeper layer of the urethral muscles was the only option to remove the stones. A perineal urethrotomy in a male horse has been suggested as a surgical procedure of choice for removal of urethral calculi in the presence of urethral stricture (Hanson and Poland, 1995). In this study, similar to the perineal urethrotomy in a male horse, the episiotomy was allowed to heal by secondary intention. Urethral stricture or fistula formation may occur in the post-operative period. In this report, the stricture was formed and urine was allowed to pass through this opening. The prolonged term undermining of urine in the subcutaneous and skin occurred and led to bed sore, bacterial infection and drainage of exudates from the infected area for a period of 1 week post-operation. Even though immediately after the detection of the complication attempts to prevent the infection by draining the urine with 18 gauge needles were made, the infection occurred. The authors suggest that the drainage of urine from subcutaneous tissue by a more aggressive but systematic method such as making strategic incisions and placing at least passive drains allowing the fluid drainage through gravity flow must be considered to prevent serious infection. Wound cleaning and trimming of the necrotic tissues were performed on a daily basis. Despite the surgical wound being contaminated with urine every day, the granulation tissue at the opening occurred and caused a stricture with a diameter of 2 cm. The authors observed that when the wound was closed by necrotic tissue unexpectedly, the elephant could pass its urine through the vaginal vestibule. After the removal of necrotic tissue, the elephant could urinate through the stricture (Fig 7).

Another life threatening complication in this report was the 5-hours bloating after surgery. The bloating was corrected by enema with tap water, intravenous administering of diluted 300 mg metoclopramide hydrochloride in NSS and continuous walk for 20 min per hour. The previous dose of metoclopramide (250–400 mg per elephant, IV) as an antiemetic has been recommended (Cheeran et al., 1995). Metoclopramide has been shown to be effective in treating post-operative ileus in animals, thus it was helpful in preventing the gastro-intestinal stasis associated with bloating. In only one hour, she defecated several times. The decreasing abdominal size was detected. During 5 hours later, the elephant defecated more than 20 times and sometimes there was only flatulus expelled through the anus.

There are 2 other reports on the high percentage of calcium based crystals in healthy elephant urine (Kingsukon et al., 2006; Weidner et al., 2009). Moreover, 2 stones in the ureter, which were also calcium carbonate, were found to be the cause of the African elephant death from chronic renal failure (Morris et al., 1987). The finding of the present study confirms that calcium based crystals are common in elephant urine. The high level of calcium salts in the diet as well as limited water intake are the contributing factors of urolith in this elephant. In spite of the alkalinity of urine and the high concentration of calcium in urine, urolithiasis is an uncommon disease in elephant compared to other species.

**Conclusion**

In conclusion, this report exhibits the authors experience in the removal of urethral and bladder stones in a female elephant. This is the first case that the calculi have ever been removed ante-mortem surgically in female elephant. The authors are confident that the combination of episiotomy and urethrotomy can be used in future cases to manually retrieve urethral and bladder calculi in the female elephants.

**Acknowledgements**

We thank Assoc. Prof. Dr. Monthira Tanthanuch for calculus analysis. We gratefully thank Mr. Alongkot Chukaew and Dr. Boripat Siriaroonrat for supporting this work. We thank Dr. Wannasit Chantornvong for editing manuscript and our staffs at the Veterinary Teaching Hospital of Kasetsart University Kamphaeng Saen, for their support. We thank Dr. Sudarat Amornsak for some part of post operative care.

**References**


Hanson RR and Poland HM 1995. Perineal


Acute Pulmonary Toxicity Caused by Single Intratracheal Instillation of Various Doses of Colloidal Silver Nanoparticles in Mice: Pathological Changes, Particle Bioaccumulation and Metallothionien Protein Expression

Theerayuth Kaewamatawong 1* Wijit Banlunara1 Pattwat Maneewattanapinyo2 Chuchaat Thammacharoen2 Sanong Ekgasit2

Abstract

To study acute lung toxicity of various doses of colloidal silver nanoparticles (Ag-NPs), mice were intratracheally instilled with 0, 10, 100, 1000 or 10,000 ppm of Ag-NPs. Histopathology, autometallography (AMG) and immunohistochemistry were determined at 1, 3, 7 and 15 days post-exposure. Instillation of 100, 1,000 and 10,000 ppm Ag-NPs produced moderate to severe necrotizing bronchitis and alveolitis with hypertrophy and hyperplasia of alveolar epithelial cells. The severity of the pulmonary inflammation and damage increased in a dose-dependent manner. Concomitant laminin immunohistochemical findings generally correlated with pulmonary lesions. Interleukin 1- beta (IL-1β) and tumor necrotic factor-alpha (TNF-α) positive immunostaining were found in the inflammatory lesions in lungs of treated animals. Superoxide dismutase (SOD) and metallothioneine (MT) expression occurred in particle laden AMs and lung epithelial cells, which correlated with inflammatory sites and particle aggregated areas. AMG gains were found in particle laden AMs, alveolar epithelial cells and macrophages in hilar lymph nodes. These findings suggest that instillation of AgNPs causes acute lung inflammation and tissue damage in a concentration-dependent manner. IL-1β and TNF-α may involve in the pathogenesis of the acute lung toxicity. Oxidative stress may underlie the lung tissue injury. Moreover, the expression of MT in tissues responded to AgNPs accumulation.

Keywords: acute, colloidal silver nanoparticles, intratracheal instillation, lung toxicity, mouse

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Original Article
Introduction

Silver nanoparticles (Ag-NPs) have been known to have inhibitory and bactericidal effects as well as to be effective in retarding growth of mold, harmful spores and germs. Ag-NPs are used and applied in a wide range of applications, especially in health applications and textile industry (Chen and Schluesener, 2008). In vitro studies reveal cytotoxic effects of Ag-NPs including reducing cell viability, damaging cell membrane and interrupting the biological effects of theepithelial and mesenchymal cells. These toxic effects were related to generation of reactive oxygen species (ROS) that affects mitochondrial function (Medina et al., 2007). There are few studies of in vivo pulmonary toxicity of Ag-NPs. These reports demonstrated the distribution of Ag-NPs in the lung and systemic organs including liver, kidney, spleen, brain, and heart with rapid clearance from the organs then enter systemic pathway (Takenaka et al., 2001). For pathological effects, there were no significant health impacts of acute inhalation exposure to Ag-NPs. However, subacute exposure to Ag-NPs showed slight pulmonary inflammation and cytotoxicity (Stebounova et al., 2011). The underlying causes and pathogenesis of Ag-NPs toxicity are still largely unclear.

IL-1β and TNF-α, major proinflammatory cytokines involved in acute inflammation and tissue injury, often act synergistically in complex regulation on signaling molecules or protein expression in animal or human diseases (Kolb et al., 2001). Several studies have described important roles of IL-1β and TNF-α in lung injury caused by exposure to ambient...
particles. Alveolar macrophages that exposed particulate particles release TNF-α and IL-1β, which induce expression of proinflammatory mediators such as nuclear factor kappa-B (NF-kB) and activator protein 1 (AP-1). Both proinflammatory mediators activate binding of transcription factors to the enhancers of the mediator genes that is important to inflammatory process (Ishii et al., 2004; Mukhopadhyay et al., 2006).

Nanoparticles have been reported to cause oxidative stress as a result of the generation of ROS in a number of in vitro and in vivo studies (Dick et al., 2003; Donaldson and Stone, 2003; Kaewamatawong et al., 2006). In vivo study of nanosilver also revealed the cytotoxicity of particles that were related to the generation of ROS (Choi et al., 2010; Miura and Shinohara, 2009).

Metallothionein (MT), a low molecular weight and cystein-rich protein, can regulate essential metals such as Zn and plays an important role in detoxification of non-essential metal ions such as Ag, Cd, Pb and Hg (Nordberg and Nordberg, 2009). Several laboratory and field studies noted that metallothionein (MT) played an important role in heavy metal homeostasis and detoxification in animals. Expression of MT in tissues responded heavy metal exposure has been reported in various kinds of organisms and animals (Alvarado et al., 2006; Kaewamatawong et al., 2012). The protective role of MT to silver nanomaterials is still unknown. There is no report of MT expression in in vivo study caused by exposure to Ag-NPs.

To demonstrate pulmonary basement membrane damage caused by exposure to AgNPs, expression of laminin was used as representative by immunostaining. Laminin is an intrinsic component of all basement membranes and plays a central role in the formation, architecture, and stability of basement membranes as well as the control of cellular interactions. It can be used as a marker of pulmonary basement membrane injury because it is present along the alveoli throughout the lung (Aumailley and Smythe, 1998).

To study the deposition of our Ag-NPs, autometallography (AMG) was performed. Several toxicity studies of heavy metal used AMG technique to detect small amount of the metal in the cells of various kinds of animals (Danscher and Stoltenberg, 2006). In this study, we tried to use this technique to detect in situ AgNPs in lung and lymph node parenchyma.

The purpose of this study was to elucidate the potential toxic effects associated with single intratracheal instillation of Ag-NPs using mouse model for pulmonary histopathological changes during acute stage. The pathogenesis of pulmonary toxicity of Ag-NPs relating to the proinflammatory cytokines and reactive oxygen species, and the protective role of metallothionein were elucidated using immunohistochemistry. Moreover, the distribution and accumulation of Ag-NPs were investigated by histochemical autometallography.

**Materials and Methods**

**Experimental animal:** Seven to eight week-old male ICR mice were purchased from National Laboratory Animal Centre, Mahidol University, Thailand. The animals were kept in an animal facility that of which the light/dark cycle was control under 12:12 hr, temperature of 25±1°C and relative humidity of 55±10%. The mice were fed mouse chew and given filtered tap water ad libitum throughout the experiment. All protocols of animal experiments were approved by the ethics committee of Chulalongkorn University Animal Care and Use Committee (CU-ACUC; Approval No. 12310080).

**Particles:** Colloidal silver nanoparticle were acquired from Sensor Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Thailand. Preparation protocol of AgNPs was described in our previous study (Maneewattanapinyo et al., 2011). Briefly, a 0.094 M aqueous solution of silver nitrate (AgNO₃; Merck) was added dropwise to an aqueous solution of 0.07 M sodium borohydride (NaBH₄; Merck) under a vigorous stir. Soluble starch (Merck) was used as stabilizer and solvent in the mixing processes. Purification of the AgNPs was precipitated using centrifugation. Percentage of purity of the AgNPs measured by macro- and microelectrode was 99.96%. Ag ions concentration in Ag-NPs was less than 0.04%. The Ag-NPs had a spherical configuration with a relatively uniform size distribution approximately 10-20 nm on average. To obtain various concentrations of AgNPs, the Ag-NPs were suspended in distilled water prior to use.

**Experimental design:** Sixty male ICR mice were singly intratracheally instilled with 50 µl aqueous suspensions of 10, 100, 1000 or 10,000 ppm of Ag-NPs suspensions. The control group of mice was instilled with 50 µl of distilled water. One, 3, 7 and 15 days after instillation, the animals in each group were sacrificed. Lungs and hilar lymph nodes were collected in 10% buffered neutral formalin for routine histopathological evaluations and immunohistochemistry.

**Immunohistochemistry protocol:** Tissue samples from lungs and lymph nodes of the control and treated animals were immunostained to detect pulmonary basement membranes (laminin), proinflammatory cytokines (TNF-α, IL-1β), antioxidant enzymes (Cu/Zn SOD), and MT. After deparaffinization, the sections were treated with proteinase K for 30 min at 4°C (for detection of laminin) or with citrate buffer solution (pH = 5.4-6.0; for detection of TNF-α, IL-1β, Cu/Zn SOD and MT) for 20 min at 121°C by autoclave and microwave heat at 700 W for 5 min in the process of antigen retrieval. The sections were incubated with 3% H₂O₂ in methanol to quench endogenous peroxidase for 30 min at room temperature. The slides were then blocked with 10% normal goat serum (laminin, TNF-α and Cu/Zn SOD) for 5 min in microwave oven at 250 W or 1% bovine serum albumin (IL-1β and MT) for 30 min at 37°C. Thereafter, the sample and positive control sections were incubated overnight at 4°C with primary antibodies (monoclonal rabbit anti-laminin Ab, 1 : 200
dilution; polyclonal rabbit anti TNF-α Ab, Monosan, Uden, the Netherlands, 1 : 15 dilution; polyclonal rabbit anti-IL-1β Ab, Santa Cruz Biotechnology, Santa Cruz, CA, 1 : 200 dilution; and monoclonal mouse anti-MT Ab, Dako®, Glostrup, 1 : 50 dilution). For the negative control sections, they were incubated with phosphate buffered saline. The biotinylated anti-mouse IgG antibody and EnVision polymer (DakoREALESTM EnVisionTM detection system, Dako®, Denmark) was applied to the sections as a secondary antibody. Brown staining with the substrate 3,3’-diaminobenzidine tetrahydrochloride (DAB) was determined as positive result and the sections were counterstained with Mayer’s hematoxylin for 30 sec.

**Autometalllography staining:** Lung and hilar lymph nodes from the control and treated mice were investigated for the presence of Ag-NPs distribution and accumulation. After deparaffinization, the sections were incubated with 1% potassium cyanide for 2 hours to eliminate other metal residues and then rinsed well with tap water and distilled water (DW). For silver amplification, physical developer (50% Arabic gum, 50% citrate buffer, 5.6% hydroquinone and 17% AgNO₃) was applied to the sections 1 hr in automatic shaker at 26°C. Thereafter, the sections were reacted with 10% sodium thiosulfate and Farmer’s solution (20% sodium thiosulfate and 7.5% potassium ferric cyanide) to eliminate silver residues. The sections were rinsed in tap water and counterstained with Mayer’s hematoxylin. Positive reactions resulted in yellow-brown to black silver grains.

**Results**

**Clinical and gross findings:** In the control group and the 10 and 100 ppm of Ag-NPs treated groups, there were no exposure related clinical signs in any time. Forty percent of the mice in the 1,000 and 10,000 ppm treated groups showed a sign of dyspnea shortly after instillation. However, this sign recovered 6 hours post-exposure. Grossly, the instillation of 10 and 100 ppm Ag-NPs caused mild congestion and edema in the lungs. In both 1,000 and 10,000 ppm Ag-NPs treated animals, tiny pin-head sized to patchy black brown foci scattered in the lung lobes throughout the experiment (Fig 1). The degree of lesions described above in 10,000 ppm treated group was more severe than 1,000 ppm treated group.

**Histopathology:** One day after the instillation, accumulation of free aggregated particles was found in the alveoli and bronchiolar lumens of all treated groups. Some of the aggregated particles were present within alveolar macrophages, and occasionally present within alveolar epithelial cells with increasing number of cells in alveolar wall (Fig 2A). The animal instilled with 1,000 and 10,000 ppm Ag-NPs had severe accumulation of neutrophils, active alveolar macrophages (AMs) and Ag-NPs laden AMs in aggregated areas (Fig 2B). Three days after the instillation, the lungs from 1,000 and 10,000 ppm Ag-NPs treated groups revealed severe multifocal alveolitis characterized by accumulation of numerous active AMs, particle-laden AMs, and neutrophils with some necrotic cells. Proliferation of alveolar type II epithelial cells were also noted in the affected areas (Fig 2C). The 100 ppm Ag-NPs treated group showed similar lesions but less severe pulmonary lesions.

**Figure 1** Lung from 10,000 ppm Ag-NPs treated animals at 1 day post-exposure shows patchy black brown foci (Bold arrow) scattered in lung lobes. (Bar = 1 cm)

**Figure 2** Lung sections from various doses of Ag-NPs treated groups at various time points, H&E stain. (A) Distributions of DEPs in AMs (arrowheads) and alveolar epithelium (arrowheads); 1,000 ppm treated group at 1 day post-exposure, Bar = 600 μm. (B) Influx of neutrophils and accumulation of particle laden AMs in Ag-NPs (arrowheads) aggregated areas; 10,000 ppm treated group at 1 day post-exposure, Bar = 250 μm. (C) Focal necrotizing alveolitis with hyperplasia of alveolar type II epithelial cells; 10,000 ppm treated group at 3 day post-exposure, Bar = 700 μm
Moderate congestion with particle laden AMs and alveolar epithelium were seen in the lungs of the 10 ppm Ag-NPs treated groups. Seven days after the instillation of Ag-NPs, severe loss of lung architecture, cellular necrosis, neutrophil infiltration, and alveolar type II proliferation were noted in the lungs of the 1,000 and 10,000 ppm treated groups. Aggregated particles and particle laden AMs were also seen in some areas of lung parenchyma. The 100 ppm Ag-NPs treated groups induced similar histopathological patterns, but the lesions were milder and occupied a small area of the lung specimens. Mild congestion was observed in the lungs from 10 ppm Ag-NPs treated groups. The lungs of mice killed at 15 days post-exposure had similar appearances and severity of lesions to those observed at 7 days post-exposure. The lesions of adjacent lymph nodes were also elucidated. The hilar lymph nodes of treated animals at 1 and 3 days post-exposure were slightly enlarged with mild to moderate particle laden macrophages and neutrophil infiltration in subcapsular and medullary sinus. At 7 days post-exposure, multifocal necrosis of lymphoid cells and mild to moderate histiocytic infiltration were noted with mild to moderate particle laden macrophage infiltration. The lungs from the 15 day post-exposure groups showed similar but greater severe lesions than those from the 7 day post-exposure groups.

**Immunohistological evaluation**

**Laminin:** Thin lines of intense brown positive laminin stains were observed along the basement membranes of alveolar septa, bronchus, bronchioles, blood vessels and bronchial glands in the control mice (Fig 3A). Lung tissues from Ag-NPs treated mice showed extensive patchy areas of nonstain or weak positive reaction and discontinuous pattern of the alveolar basement membranes in site of the inflammatory foci (Fig 3B). The interruption and weak positive immunoreactivity were also seen in the alveoli enclosing to the Ag-NPs aggregation or particle laden AMs accumulation. The severity and distribution of the lesions were in dose dependent manner and showed no different intensity during the exposure dates.

**Inflammatory cytokine: IL-1 and TNF**

**IL-1β:** The expression of IL-1β was occasionally observed in some alveolar macrophages in the control group. However, the IL-1β expression was mainly in the cytoplasm of particle laden AMs and active AMs, and

![Figure 3](image1.png)

**Figure 3** Laminin immunohistochemistry in lungs of control and Ag-NPs-treated mice sacrificed at 3 days post-exposure. (A) Brown thin string-like positive staining along alveolar basement membranes (arrows) in control animals, Bar = 300 μm (B) Weak and discontinuous positive patterns of alveolar basement membrane at focal alveolitis in Ag-NPs-treated mice, Bar = 600 μm.

![Figure 4](image2.png)

**Figure 4** Intense brown positive immunostaining of IL1-β appears in a large number of particle laden AMs (arrows) in lungs of 10,000 ppm group killed at 7 days post-exposure, Bar = 600 μm.

![Figure 5](image3.png)

**Figure 5** Prominent brown positive immunostaining of TNF-a is found mainly in particle laden AMs (arrows) and occasionally in bronchiolar epithelial cell (arrowheads) associated with inflammatory nodules (N) in lungs of 10,000 ppm group killed at 3 days post-exposure, Bar = 600 μm.
occasionally in bronchiolar epithelium of the lungs from the mice exposed to Ag-NPs at 1 to 15 days post-exposure (Fig 4).

**TNF-α:** In the control group, the expression of TNF-α was barely detectable in both airway epithelium and lung parenchyma. In contrast, in the Ag-NPs-treated mice at 1-15 days post-exposure, positive staining for TNF-α appeared in a large number of cells associated with pulmonary inflammation. TNF-α was expressed mainly in the cytoplasm of particle laden AMs and active AMs, and occasionally in bronchiolar epithelium that associated with inflammatory nodules and necrotic areas (Fig 5).

**SOD:** The expression of SOD was seen in small number of AMs in the control animals. After 1-15 days, SOD expression was observed chiefly in particle laden and activated AMs, and moderately in bronchiolar epithelial cells that associated with the inflammatory sites and particle aggregated areas but minimally in alveolar epithelial cells (Fig 6).

**Metallothionine (MT-1 and MT-2):** In the Ag-NPs-treated mice at 1 to 15 days post-exposure, positive staining for MT appeared in a large number of cells associated with aggregated nodules and areas that enclose to particle laden AMs. MT was expressed mainly in the cytoplasm of particle laden and active AMs and occasionally detectable in bronchiolar and alveolar epithelial cells (Fig 7). In contrast, the immunohistochemical staining of MT was barely detectable in the cytoplasm of airway epithelium in the control animals.

**Autometallography:** The deposition of Ag-NPs characterized by black positive silver grains were observed in the cytoplasm of particle laden AMs and alveolar epithelium (Fig 8). The large number of positive cells and clump of free black grains appeared in the area of inflammatory nodules. Degrees and distribution of Ag-NPs positive staining showed a trend toward increasing in a dose-dependent manner, but not different among the exposure days. On the other hand, no evidence of positive silver grains was found in the lungs from the control group.

**Discussion**

The purpose of this study was to determine the pathological effects of intratracheal instillation of various doses of Ag-NPs on the lungs of mice in terms of dose response during the acute stage. Possible mechanisms for the pulmonary injury involving inflammatory changes and tissue damage were discussed. In addition, the distribution of the Ag-NPs in lung tissues and the protective role of MT were also elucidated.

Subacute inhalation study of 1 ppm commercial Ag-NPs revealed no pathologic changes including alveolitis, perivasculitis, lymphoid agglomerates, epithelial damage, granulomas, giant cells or fibrosis (Stebounova et al., 2011). In our pilot study, we also found that the single instillation of 1 ppm Ag-NPs revealed no significant pathological lesions in all observation times.
The adverse effects of nanoparticles on the pulmonary basement membranes were reported in nanotoxicity murine models (Kaewmatawong et al., 2005; Shimada et al., 2006). In mice exposed to Ag-NPs in this study, we showed the direct effects of Ag-NPs on the basement membranes as evidence of weak and discontinuous positive stainings of laminins along the alveoli that refer to the aggregation of Ag-NPs. The disturbance of the equilibrium between the synthesis and degradation changes of pulmonary extracellular matrix (ECM) including laminin may result in pulmonary fibrosis (Dunsmore and Rannels, 1996). The accelerated degradation of the ECM as present in the weakened positive immunolabeling for laminin in the acute inflammatory lesions may develop into fibrosis in the chronic stages.

Many kinds of nanoparticles can induce the production of IL-1β and TNF-α that are responsible for the induction and modulation of chemokines in the lungs (Pryhuber et al., 2003). Many types of pulmonary cell, mainly alveolar macrophages and airway epithelial cells, can release these two cytokines. In the current study, IL-1β and TNF-α were shown to be present in the inflammatory lesions in both alveolar macrophages and airway epithelial cells of the treated mice’s lungs, suggesting that these cytokines may be involved in the pathogenesis the acute lung toxicity induced by Ag-NPs.

To prevent oxidative harmful reaction, cells develop free-radical scavenging process by various kinds of antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase. Superoxide dismutase (SOD) are primary antioxidant enzymes that scavenges the ROS by catalyzing the dismutation reaction of the superoxide anion to hydrogen peroxide. Several in vitro nanotoxicity studies have been revealed the association between the free-radical generation and SOD scavenging activity. Dey et al. (2008) demonstrated increases in manganese superoxide dismutase (MnSOD) protein levels induced by nanosized alumina in mouse skin epithelial cells. Decrease in SOD and glutathione (GSH) level that is associated with generation of peroxy radicals after Ag-NPs exposure to human fibrosarcoma (HT-1080) and human skin/carcinoma (A431) cells was reported (Arora et al., 2008). In our study, the results of the positive Cu/Zn SOD immunoreactivity mainly in particle laden and activated AMs associated with the inflammatory sites and some free particle aggregated areas indicate a pulmonary response to oxidant stress generated by Ag-NPs in either direct effect of particles themselves or indirect effect of inflammatory reaction.

In vitro cytotoxicity study of astrocytes exposure to Ag-NPs showed upregulation of MT via activation of metal regulatory transcription factor 1 (MTF-1) (Luther et al., 2012). Certain mechanisms associated with the MT responses to Ag-NPs exposure remains unclear. Silver (Ag) can directly stimulate the production of MT via the initiation of thionien in the cells (Kim et al., 2009). In another mechanism, enhancement of MT induction is associated with their antioxidant role that response to an increase in oxyradicals (Haq et al., 2003). In the present study, MT immuno-expression was detected in the macrophages or airway epithelium that were associated with Ag-NPs aggregated nodules and areas adjacent to particle laden AMs. We, therefore, suggest that MT might have a protective role to Ag-NPs at acute stage. The underlying mechanism of the induction of MT caused by exposure to Ag-NPs should be elucidated.

Several inhalation studies demonstrated the translocation of Ag-NPs by inductively coupled plasma mass spectrometry (ICPMS) analysis. The silver was detected mainly in the lungs and adjacent lymph nodes, but minimally in the olfactory bulb, liver, kidney, spleen, brain, heart and blood (Takenaka et al., 2001). In our study, we used AMG histochemical techniques to demonstrate the in situ deposition and distribution of Ag-NPs in the lungs and hilar lymph nodes. To our knowledge, this is the 1st report to use this kind of technique to detect the nano metal particles. The positive AMG gains were found markedly in the cytoplasm of the particle laden AMs as well as the macrophages in the adjacent lymph nodes, and occasionally in the alveolar epithelial cells. These results indicated that the major clearance mechanism of instilled Ag-NPs might be via phagocytosis by alveolar macrophages and lymphatic circulation. Moreover, the evidence of Ag-NPs entering the alveolar epithelium might be another clearance path and also a possible way to gain access to the blood circulation.

In summary, this study demonstrated the pulmonary pathological responses after intratracheal instillation of various doses of Ag-NPs in mice during the acute stage. Ag-NPs produced the severity of pulmonary lesions in the treated animals in a concentration-dependent manner. IL-1β and TNF-α were proinflammatory cytokines involved in the pathogenesis of the acute lung toxicity induced by Ag-NPs. We also found pulmonary response to oxidative stress in the Ag-NPs treated animals, which may be one of the underlying causes of the lung tissue injury. Furthermore, our current study found that Ag-NPs could induce the expression of MT that might be one of the protective mechanisms of lung against nanoparticles.

Acknowledgements

This work was supported by a grant from The National Research Council of Thailand, 2008 and Thailand Research Fund (MRG5480115), 2011.

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Efficacy of Different Vaccination Programs against Velogenic Newcastle Disease Virus Challenged in 28-day-old Broiler Chickens

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Abstract

In the present study, the efficacy of different vaccination programs against a velogenic Newcastle disease virus (vNDV) challenged in 28-day-old broiler chickens was examined. The chickens were divided into 5 groups, 20 chickens in each. Group 1 did not receive any vaccine and served as the non-vaccinated control group. Groups 2-5 were vaccinated with different vaccination programs against vNDV. At 1-day-old, the chickens in all the vaccinated groups were vaccinated with live Newcastle disease (ND) vaccine, Ulster 2C strain, followed by different inactivated vaccines including vaccine A in groups 2 and 4 and vaccine B in groups 3 and 5. At 7-day-old, revaccination was performed in the vaccinated groups including live vaccine, Ulster 2C strain, in groups 2 and 3 and live vaccine, B1 strain, in groups 4 and 5. Finally, the chickens in every group were challenged with vNDV at 28-day-old. Results showed that NDV HI titer at the challenge inoculation time of all the vaccinated groups was significantly higher ($p < 0.05$) than that of the non-vaccinated group. At 14 days post-inoculation, mortality rate of the vaccinated groups was significantly lower ($p < 0.05$) than that of the non-vaccinated group. Body weights of all the vaccinated groups were significantly ($p < 0.05$) higher than those of the non-vaccinated group. The results indicate that all the vaccination programs used in this study could alleviate body weight loss and protect the chickens from lethal infection with vNDV.

Keywords: chickens, vaccine, Newcastle disease virus, efficacy

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Original Article

บทคัดย่อ

ประสิทธิภาพของโปรแกรมวัคซีนที่แตกต่างกันต่อการป้องกันการติดเชื้อไวรัสนิวคาสเซิลสายพันธุ์รุนแรงในไก่เนื้ออายุ 28 วัน

การศึกษาครั้งนี้เป็นการทดสอบประสิทธิภาพของโปรแกรมวัคซีนที่แตกต่างกันในการป้องกันเชื้อไวรัสนิวคาสเซิลสายพันธุ์รุนแรงในไก่เนื้ออายุ 28 วัน โดยแบ่งไก่ทดลองออกเป็น 5 กลุ่มๆละ 20 ตัว กลุ่มที่ 1 เป็นกลุ่มขอควบคุมที่ไม่ได้รับวัคซีน ส่วนกลุ่มที่ 2-5 ให้วัคซีนป้องกันเชื้อไวรัสนิวคาสเซิลสายพันธุ์รุนแรงในไก่เนื้ออายุ 28 วัน กลุ่มที่ 2 ไม่ให้วัคซีน กลุ่มที่ 3-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 4-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 6 ไม่ให้วัคซีน กลุ่มที่ 7-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 8 ไม่ให้วัคซีน กลุ่มที่ 9-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 10 ไม่ให้วัคซีน กลุ่มที่ 11-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 12 ไม่ให้วัคซีน กลุ่มที่ 13-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 14 ไม่ให้วัคซีน กลุ่มที่ 15-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 16 ไม่ให้วัคซีน กลุ่มที่ 17-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 18 ไม่ให้วัคซีน กลุ่มที่ 19-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 20 ไม่ให้วัคซีน กลุ่มที่ 21-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 22 ไม่ให้วัคซีน กลุ่มที่ 23-5 ให้วัคซีนฟักทองวัคซีนสายพันธุ์ Ulster 2C ใหม่ กลุ่มที่ 24 ไม่ให้วัคซีน กลุ่มที่ 25 ไม่ให้วัคซีน กลุ่มที่ 26 ไม่ให้วัคซีน กลุ่มที่ 27 ไม่ให้วัคซีน กลุ่มที่ 28 ไม่ให้วัคซีน กลุ่มที่ 29 ไม่ให้วัคซีน กลุ่มที่ 30 ไม่ให้วัคซีน กลุ่มที่ 31 ไม่ให้วัคซีน กลุ่มที่ 32 ไม่ให้วัคซีน กลุ่มที่ 33 ไม่ให้วัคซีน กลุ่มที่ 34 ไม่ให้วัคซีน กลุ่มที่ 35 ไม่ให้วัคซีน กลุ่มที่ 36 ไม่ให้วัคซีน กลุ่มที่ 37 ไม่ให้วัคซีน กลุ่มที่ 38 ไม่ให้วัคซีน กลุ่มที่ 39 ไม่ให้วัคซีน กลุ่มที่ 40 ไม่ให้วัคซีน

การศึกษาพบว่า NDV HI titer ในวันที่ไก่ได้รับเชื้อพิษทับของไก่กลุ่มที่ได้รับวัคซีนมีระดับสูงกว่าไก่ที่ไม่ได้รับวัคซีนอย่างมีนัยสำคัญทางสถิติ (p < 0.05) เริ่มตั้งแต่ 14 วัน ปรากฏว่าอัตราการตายของไก่ที่ได้รับวัคซีนกลุ่มที่ 2-5 ต่ำกว่าไก่ที่ไม่ได้รับวัคซีนกลุ่มที่ 1 (p < 0.05) น้ำหนักตัวของไก่ที่ได้รับวัคซีนกลุ่มที่ 2-5 มีน้ำหนักตัวมากกว่าไก่ที่ไม่ได้รับวัคซีนอย่างมีนัยสำคัญทางสถิติ (p < 0.05) ผลการทดลองแสดงให้เห็นว่าโปรแกรมวัคซีนที่ใช้ในการศึกษาครั้งนี้ทุกโปรแกรมสามารถลดการติดเชื้อไวรัสนิวคาสเซิลสายพันธุ์รุนแรงได้

คำสำคัญ: ไก่, วัคซีน, ไวรัสนิวคาสเซิล, ประสิทธิภาพ

Introduction

Newcastle disease virus (NDV), classified as member of the genus *Avulavirus* in the family Paramyxoviridae, is one of the most important infectious agents encountered in poultry industry worldwide due to the high mortality and production loss associated with the disease (Mayo, 2002). Major disease problems in many countries of the world, including Asian countries, are caused by velogenic NDV (vNDV) (Awan et al., 1994). Virulent NDV affects nervous, respiratory and gastrointestinal systems (Brown et al., 1999; Alexander, 2003). Clinical signs are characterized by listlessness, respiratory distress and weakness, followed later by prostration and death. Infected chickens may appear suddenly with high mortality occurring in the absence of other clinical signs. Morbidity and mortality rates of infected chickens vary from 1-100% (Alexander, 2003).

Maternally-derived antibodies (MDA) in young chicks are primary means of antigen-specific protection against many pathogens in the field (Hamal et al., 2006) but they may be at risk to infection when under protective level of MDA titer occurs. Previously, we found that MDA in 14- and 21-day-old chickens at level of 3.85±1.04 and 1.55±0.94, respectively, could not protect the chickens against challenge with vNDV. However, our vaccination programs designed for that experiment could induce protective efficacy against infection in the 14- and 21-day-old chickens (Sasipreeyajan et al., 2012). Therefore, chickens at 28-day-old which have MDA lower than those of 14- and 21-day-old are stated to be at higher risk of vNDV infection. Moreover, the risk will be increased if there are no effective methods of infectious disease prevention and control.

Due to serious economic loss caused by ND, effective methods of control are needed urgently. Prevention and control are through the use of live attenuated or killed vaccine and many of the vaccination programs have been used in commercial chicken flocks (Khalifeh et al., 2009). To achieve reasonable protection against ND, vaccination programs against ND have been designed to stimulate not only systemic but also mucosal immune responses (Takada and Kida, 1996). Inactivated vaccines have been used for inducing mainly systemic immunity (Rauwa et al., 2009). Live vaccines prepared
from lentogenic strains such as LaSota, Clone 30 and VG/GA are widely used because they provide high efficacy of protection through the induction of both systemic and local immunity (Seal et al., 2000; Rauwa et al., 2009). However, undesirable vaccine reactions may occur, especially after the application of live LaSota strain (Mast et al., 2005). To avoid vaccine reactions, mild ND vaccine, Hitchner B1 strain and avirulent ND vaccine, Ulster 2C strain, are commercially available (van Eck et al., 1991). Vaccination programs and procedures differ between countries and even between farms in the same country, depending on local circumstances (Rehmani, 1996). Therefore, it is necessary, in all countries, to compare the efficacy of vaccines that are available and to ascertain efficient methods of application. In the current study, the objective was to determine the protection afforded by different vaccination programs.

To ascertain efficient methods of application. In the current study, the objective was to determine the protection afforded by different vaccination programs against challenge with vNDV in 28-day-old broiler chickens.

**Materials and Methods**

**Chickens:** One hundred, one-day-old female broiler chickens (Cobb 500) were moved from a commercial hatchery (the Krungthai hatchery, a subsidiary company of GFPT, Chonburi province) to the University. The chickens were housed in the experimental animal facility at the Livestock Hospital at the Faculty of Veterinary Science, Chulalongkorn University, Nakhornpathom, Thailand. The chickens were divided into 5 groups of 20 chickens each. Groups 1, 2, 3, 4, and 5 were vaccinated with different regimes of vaccination program against NDV as shown in Table 1. Group 1 served as the non-vaccinated control group, which did not receive any NDV vaccine. Feed and water were provided *ad libitum*. Guidelines and legislative regulations on the use of animals for scientific purposes of Chulalongkorn University, Bangkok, Thailand were followed as certified in permission No. 12310005.

**Vaccines:** Four commercial vaccines were used in this study. Inactivated ND vaccine A (Chick i N-K, Fort Dodge, Campinas, Brazil) was given at 1-day-old by subcutaneous (S/Q) injection at the base of the skull (0.1 ml/bird, each 0.1 ml of the vaccine containing at least $10^{6.0}$ EID$_{50}$ of LaSota strain NDV). Inactivated ND vaccine B (Poulvac i N LaSota, Fort Dodge, Campinas, Brazil) was given at 1-day-old by S/Q injection at the base of the skull (0.1 ml/bird, each 0.5 ml of the vaccine containing at least $10^{6.0}$ EID$_{50}$ of LaSota strain NDV). Live ND, Ulster 2C strain (Poulvac NDW, Fort Dodge, Campinas, Brazil) was given intra-nasally (I/N) at 1- and 7-day-old (1 dose/bird, each dose of the vaccine containing at least $10^{6.5}$ EID$_{50}$ of Ulster 2C strain NDV). Live ND, B1 strain (Fort Dodge Animal Health, Fort Dodge, USA) was given I/N at 7-day-old (1 dose/bird, each dose of the vaccine containing at least $10^{6.3}$ EID$_{50}$ of B1 strain NDV).

**Challenge study:** Chickens from every group were vaccinated with vNDV at 28-day-old. Each chicken received approximately $10^{5}$ EID$_{50}$ of vNDV by oral drops (Chansiripornchai and Sasipreeyajan, 2006). Clinical signs and mortality were observed for 14 days post-inoculation (DPI). Dead chickens were necropsied and gross lesions were observed. To confirm the infection in dead chickens, they were necropsied and gross lesions of typical ND were recorded. Tracheas were collected for detection of NDV and typing as velogenic strain by using one-step RT-PCR followed by restriction endonuclease analysis (Creeelan et al., 2002).

**Body weight:** All the chicken were weighed individually at 28-day-old before challenge and at 42-day-old which was 14 DPI.

**Serological evaluations:** Thirty blood samples were randomly collected from all groups at 1-day-old. Ten, 10, and 20 blood samples/group were collected at 7-, 14- and 21-day-old. Before the challenge at 28-day-old, 20 blood samples from each group were collected. After challenge at 42-day-old (14 DPI), the remaining chickens in each group were bled. Sera were collected and tested for NDV antibody titers by the haemagglutination-inhibition (HI) test, micro method (Allan and Gough, 1974).

**Statistical analysis:** Body weights and antibody titers were analyzed and compared between groups using ANOVA and least significant difference (LSD) tests. Mortality was compared using Chi-square test. Significance was tested at a probability level of 0.05.

**Results**

**Body weights:** Before the challenge inoculation at 28 days old, the body weights of the vaccinated group 4 were the lowest and significantly different ($p < 0.05$) from the others. At 42-day-old (14 DPI), the body weights of all the vaccinated groups were higher ($p < 0.05$) than those of the non-vaccinated control group. Among the vaccinated groups, there was no significant difference ($p > 0.05$) in their body weights at 14 DPI (Table 2).

**Mortality rate:** After the challenge inoculation, the mortality rate of the vaccinated groups observed at 14

### Table 1 Vaccination programs and age of challenge.

<table>
<thead>
<tr>
<th>Group</th>
<th>1-day-old</th>
<th>7-day-old</th>
<th>28-day-old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live vaccine</td>
<td>Inactivated vaccine</td>
<td>Live vaccine</td>
</tr>
<tr>
<td>1</td>
<td>Ulster 2C strain - I/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Ulster 2C strain - I/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ulster 2C strain - I/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Ulster 2C strain - I/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Ulster 2C strain - I/N</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$I/N: intra-nasally; $^b$S/Q: subcutaneously
NDV HI titers: MDA at 1-day-old was 7.27±0.94. It declined to 1.65±0.81 at 28-day-old. At 14-day-old, the NDV HI titer of group 5 was the highest (5.10±0.82a), and it was significantly different (p < 0.05) from the others. Before the challenge inoculation, the NDV HI titers of the vaccinated groups at 7-, 21- and 28-day-old were not significantly different (p > 0.05) from each other. For the non-vaccinated control group, the NDV HI titer was not significantly different (p > 0.05) from the vaccinated groups at 7-, 14- and 21-day-old, but at the time of inoculation (28-day-old), the NDV HI titers of the non-vaccinated control group were significantly lower (p < 0.05) than those of the others (Table 3).

### Discussion

Previously, we observed a similar rate of protection after vNDV challenge in chickens which were vaccinated with live and, simultaneously, with inactivated vaccines at 1-day-old and revaccinated with live B1 or LaSota strains at 7-day-old (Sasipreeyajan et al., 2012). However, LaSota vaccine is reported to cause damage in the tracheal epithelium of vaccinated chickens. This predisposes them to secondary infections (Mast et al., 2005). Therefore, we decided to use a mild ND vaccine, B1 strain and the avirulent ND vaccine, Ulster 2C strain at the revaccination time. The challenge experiment demonstrated that all of the vaccination programs could alleviate weight loss and the mortality rate of the infected chickens, consistent with observations by other investigators who found that the body weight of the vaccinated and challenged chickens was significantly higher than that of the non-vaccinated and challenged chickens (Ezema et al., 2009;

### Table 2 Body weights before and after vNDV challenge (28- and 42-day-old), mortality rate and percentage of protection at 14 DPI

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (gm/bird)</th>
<th>Mortality</th>
<th>Percent</th>
<th>Percent of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28-day-old (0 DPI)</td>
<td>42-day-old (14 DPI)</td>
<td>Number</td>
<td>Percent</td>
</tr>
<tr>
<td>1</td>
<td>1,334.0 ± 73.98a,b</td>
<td>1,386.7 ± 476.90a</td>
<td>17/20b</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>(n=20)b</td>
<td>(n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1,326.5 ± 109.51a</td>
<td>1,981.1 ± 146.81b</td>
<td>1/20b</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td>(n=19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1,321.5 ± 86.59a,b</td>
<td>1,894.7 ± 296.95b</td>
<td>1/20b</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td>(n=19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1,276.0 ± 59.95b</td>
<td>1,802.5 ± 401.31b</td>
<td>0/20b</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td>(n=20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1,320.0 ± 68.06a,b</td>
<td>1,907.4 ± 434.27b</td>
<td>1/20b</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td>(n=19)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3 Mean HI antibody titers (log2) before and after vNDV challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>1-day-old</th>
<th>7-day-old</th>
<th>14-day-old</th>
<th>21-day-old</th>
<th>28-day-old</th>
<th>42-day-old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HI titers (Log2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.27 ± 0.94^a</td>
<td>5.90 ± 0.57^a</td>
<td>4.30 ± 0.82^b,c</td>
<td>3.25 ± 1.09^a</td>
<td>1.65 ± 0.81^a</td>
<td>10.00 ± 1.00^b,c</td>
</tr>
<tr>
<td></td>
<td>(n=30)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>2</td>
<td>5.60 ± 0.97^a</td>
<td>3.60 ± 0.70^a</td>
<td>3.60 ± 1.19^a</td>
<td>3.70 ± 2.11b</td>
<td>9.26 ± 1.41^a,c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=19)</td>
</tr>
<tr>
<td>3</td>
<td>5.50 ± 1.27^a</td>
<td>3.60 ± 0.97^a</td>
<td>3.75 ± 0.85^a</td>
<td>3.45 ± 1.10b</td>
<td>8.95 ± 1.39^a,b</td>
<td>9.85 ± 1.09^a,b</td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=19)</td>
</tr>
<tr>
<td>4</td>
<td>5.00 ± 0.82^a</td>
<td>3.80 ± 0.79^a</td>
<td>3.25 ± 1.16^a</td>
<td>3.30 ± 1.17b</td>
<td></td>
<td>10.47 ± 1.65^c</td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=3)</td>
<td>(n=19)</td>
</tr>
<tr>
<td>5</td>
<td>5.80 ± 1.14^a</td>
<td>5.10 ± 0.99^a</td>
<td>3.60 ± 1.14^a</td>
<td>3.60 ± 1.19b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=3)</td>
<td>(n=19)</td>
</tr>
</tbody>
</table>

^a,b The different superscript in each column means statistically significant difference (p < 0.05).

^A Mean±standard deviation (SD).
^B Number of chickens in the group.
^C Number of dead chickens / total chickens in the group.
The mortality rate of the vaccinated chickens was lower than that of the non-vaccinated chickens. This was consistent with other authors who have studied the efficacy of different vaccination programs against vNDV (Chansiripornchai and Sasipreeyajan, 2005; Chansiripornchai and Sasipreeyajan, 2006; Sasipreeyajan et al., 2012). Based on the results of alleviation in weight loss and mortality rate of vaccinated and challenged chickens, the protective efficacy of vaccination programs against challenge with vNDV was indicated.

The protection against challenge with vNDV occurring in this study may be due to the combined advantageous effects of using both live attenuated and inactivated vaccines and revaccination with live vaccine. This protection phenomenon is similar to the recovery of other researchers who found a high level of protection against challenge with vNDV in chickens vaccinated with live and inactivated vaccines (Chansiripornchai and Sasipreeyajan, 2005; Chansiripornchai and Sasipreeyajan, 2006; Sasipreeyajan et al., 2012). In vaccinated chickens, local IgA and IgM on the mucosal surface of the respiratory tracts plays a critical role as a primary barrier against viral infection, resulting in the protection of the chickens from subsequent systemic infection (Takada and Kida, 1996). Live vaccines could induce local antibody responses such as IgA production in the Harderian gland (Russell and Koch, 1993) along with lacrimal IgM following intraocular inoculation with NDV (Russell, 1993). Humoral immune response in the live vaccinated chickens was detected at 7 DPV, which was earlier than in the chickens vaccinated with inactivated vaccine, but at 28 DPV, lower titers than those observed in the group receiving inactivated vaccine were detected. However, live vaccine could induce cell-mediated immune response to vNDV superior to that received from the inactivated vaccine (Lambrecht et al., 2004). A single subcutaneous vaccination with inactivated vaccine was not effective in inhibiting infection of vNDV on mucosal surfaces but it could inhibit tissue infection mechanisms of the virus which contribute to protection from lethal systemic infection (Takada and Kida, 1996). Al-Garib et al. (2003) found that at 14, 21 and 28 days post-vaccination (DPV), serum IgM response was higher in birds that had been systemically immunized with inactivated vaccine than in the chickens vaccinated with live NDV vaccine. However, IgG was delayed in chickens that had been immunized with inactivated vaccine compared to the chickens immunized with live vaccine.

All of the vaccination programs used in this study provided a high level of protection against challenge with vNDV at 21 days post-revaccination. It may be that the vaccinated chickens had sufficient protective immunity at those times. Al-Garib et al. (2003) reported that the HI titers in the serum of chickens inoculated with live vaccine reached a plateau level at 14 DPV. IgG in the serum reached a plateau level at 14 DPV. Rauwa et al. (2009) found that live vaccines induced NDV-specific HI and IgG antibodies from 7 and 14 DPV, respectively. They could still be detected until 35 DPV. Systemic NDV specific IgA was first observed at 7 DPV and showed a peak value at 14 DPV. After that, it decreased but could still be detected until 35 DPV. NDV specific cell-mediated immunity after vaccination with live vaccines was observed from 7 to 35 DPV. Duodenal NDV specific IgA was detected from 14 to 35 DPV. Al-Garib et al. (2003) also reported that after vaccination with inactivated vaccine, NDV HI titer was detected from 7 DPV onwards. In serum, IgM was firstly detected at 14 DPV and remained at a high level until 28 DPV. IgG and low levels of IgA response were detected from 14 DPV onwards.

At the challenge inoculation time, although the NDV HI titer of the non-vaccinated control group remained (1.65±0.81), the mortality rate of the chickens in this group was the highest. This indicates that MDA at this level could not provide protection against challenge with vNDV. On the other hand, the NDV HI titer observed in the vaccinated chickens was composed of both MDA and an active immune response induced by vaccines. Therefore, the level was higher than that of the non-vaccinated control group and it gave significantly better protection than that of the non-vaccinated control chickens. These suggest that MDA may not be sufficient in the protection of offspring and a major concern is the necessity for vaccination at an early age in order to get the highest protective level against infection with vNDV. Herein, we found that the lowest NDV HI titer of 3.30±1.17 showed a protection of 100%. This was consistent with the report of Hamid et al. (1991), who observed no clinical signs in the immunized chickens with an HI antibody of 3 log₂ and above but the chickens with lower HI titres (≤3 log₂) showed depression and anorexia. The protection observed in the vaccinated chickens with low HI titres in their serum may be due to the effect of cell-mediated immune response and local immunity in the respiratory and intestinal tracts induced by vaccines (van Eck et al., 1991; Takada and Kida, 1996; Lambrecht et al., 2004; Rauwa et al., 2009).

In conclusion, the protection against vNDV was observed in all of the vaccination programs used in this work. Therefore, simultaneous vaccination with live Ulster 2C strain intra-nasally, inactivated LaSota strain subcutaneously injected into the base of the skull at 1 day old, and a booster vaccination with live Ulster 2C or B1 strain intra-nasally at 7-day-old was effective against the challenge with vNDV at 28-day-old.

**Acknowledgements**

This study was financially supported, in part, by the Avian Health Research Unit, the Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University.
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Comparison of Gentamicin Impregnated Polymethylmethacrylate Bead, Gentamicin Coated Native Calcium Sulfate Bead and Gentamicin Coated High Porous Calcium Sulfate Bead on Osteomyelitis Management in a Rat Model

Chaiyakorn Thitiyanaporn1,2,3 Pareeya Udomkusonsri 4 Naris Thengchaisri 3*

Abstract

Three types of gentamicin beads were compared in a rat osteomyelitis model. The gentamicin beads were prepared in cylinder shape (diameter 2 mm x height 4 mm). Gentamicin impregnated polymethylmethacrylate (GI-PMMA) beads were fabricated according to manufacturer’s instructions. Gentamicin coated native calcium sulfate (G-NCS) bead and gentamicin coated high porous calcium sulfate (G-HPCS) bead were prepared in laboratory. Osteomyelitis was induced in the rat’s tibias by using methicillin resistant Staphylococcus aureus (MRSA). After 3 weeks of infection, the infected tibias were implanted with GI-PMMA bead, G-NCS bead, G-HPCS bead or sham treatment (control). Radiographic change, white blood cell count and infection signs were weekly monitored for 6 weeks. At the end of the experiment, all tibias were collected for histopathologic examination and bone culture. Although white blood cell count and infection signs were not significantly different among different group of rats, the radiolucent area reduced significantly in GI-PMMA, G-NCS and G-HPCS compared to the control group. There was no significant difference in bacterial count among the groups, however, the histopathologic results revealed new bone development in G-NCS and G-HPCS groups, and a large bone defect in GI-PMMA group resulting from bead removal. This study suggests that the G-HPCS can be used as a local antibiotic carrier for management of osteomyelitis instead of calcium sulfate and polymethylmethacrylate beads.

Keywords: bead, gentamicin, osteomyelitis, rat

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บทคัดย่อ
การเปรียบเทียบเม็ดลูกปัดโพลีเมดทิลเมดอะคริเลทที่เอิบชุ่มด้วยเยนต้ามิซินเม็ดลูกปัดแคลเซียมซัลเฟตแบบดั้งเดิมเคลือบด้วยเยนต้ามิซินและเม็ดลูกปัดแคลเซียมซัลเฟตแบบมีรูพรุนสูงเคลือบด้วยเยนต้ามิซินในการจัดการภาวะกระดูกอักเสบในหนูแรท

ชัยกร ฐิติญาณพร 1,2,3 ปารียา อุดมกุศลศรี 4 นริศ เต็งชัยศรี 3*

เปรียบเทียบเม็ดลูกปัดเยนต้ามิซินสามชนิดในการจัดการภาวะกระดูกอักเสบของหนูแรท เตรียมเม็ดลูกปัดเยนต้ามิซินในรูปทรงกระบอก (เส้นผ่านศูนย์กลาง 2 มม. x สูง 4 มม.) ขึ้นรูปเม็ดลูกปัดโพลีเมดทิลเมดอะคริเลทที่เอิบชุ่มด้วยเยนต้ามิซินด้วยวิธีที่บริษัทแนะนำ เตรียมเม็ดลูกปัดแคลเซียมซัลเฟตแบบดั้งเดิมและแบบมีรูพรุนสูงเคลือบด้วยเยนต้ามิซินในห้องปฏิบัติการ ให้เชื้อสตาฟิลโลคoccus ออเรียสที่ดื้อยาเมททิซิล นอกจากนั้นแบ่งหนูออกเป็น 4 กลุ่ม ได้แก่ กลุ่มที่รักษาด้วยเม็ดลูกปัดโพลีเมดทิลเมดอะคริเลทที่เอิบชุ่มด้วยเยนต้ามิซิน กลุ่มที่รักษาด้วยเม็ดลูกปัดแคลเซียมซัลเฟตแบบดั้งเดิมเคลือบด้วยเยนต้ามิซิน กลุ่มที่รักษาด้วยเม็ดลูกปัดแคลเซียมซัลเฟตแบบมีรูพรุนสูงเคลือบด้วยเยนต้ามิซิน และกลุ่มควบคุม (ไม่ทำการรักษา) ตามลำดับ ประเมินความเปลี่ยนแปลงของภาพถ่ายรังสี จำนวนเม็ดเลือดขาว และอาการการติดเชื้อ ในทุกสัปดาห์จนครบ 6 สัปดาห์ เก็บตัวอย่างกระดูกหลังการทดลองสิ้นสุด แม้ว่าจำนวนเม็ดเลือดขาวและอาการการติดเชื้อไม่มีความแตกต่างในแต่ละกลุ่ม แต่จากภาพถ่ายรังสีพบพื้นที่โปร่งในกระดูกลดลงอย่างมีนัยสำคัญในกลุ่ม G-PMMA, G-NCS, G-HPCS เมื่อเปรียบเทียบกับกลุ่มควบคุม แม้ว่าจำนวนเบคทีเรียจะไม่มีความแตกต่างกันในแต่ละกลุ่ม แต่ผลจุลพยาธิวิทยาพบการสร้างกระดูกขึ้นใหม่ในกลุ่ม G-NCS และ G-HPCS และพบว่ามีการกระดูกแข็งตัวเกิดขึ้นเมื่อนำ G-PMMA ออกจากกระดูก จากการทดลองนี้แสดงให้เห็นว่า G-HPCS สามารถใช้เป็นยาทางปฏิชีวนะในการจัดการภาวะกระดูกอักเสบในหนูแรท มีความสามารถใช้แทนเม็ดลูกปัดแคลเซียมซัลเฟตและเม็ดลูกปัดโพลีเมดทิลเมดอะคริเลทได้

คำสำคัญ: เม็ดลูกปัด, เยนต้ามิซิน, กระดูกอักเสบ, หนูแรท

Introduction
Osteomyelitis is an inflammatory process accompanied by bone destruction and caused by microorganism infection (Lew et al., 2004). Treatments for osteomyelitis involve surgical debridement of necrotic tissue, irrigation, obliteration of dead space, bone repair, adequate soft tissue coverage and systemic antimicrobial administration for 4-6 weeks (Mader et al., 1999; Lazzarini et al., 2004). The necrosis of the infected bone tissue is a result of decrease in vascularity compromising the effectiveness of systemic antibiotic therapy (Mader et al., 2002).

Antibiotic impregnated polymethylmethacrylate (PMMA) beads are clinically used in various areas including joint replacement surgery and commonly used for standard treatment of local infected tissue, especially osteomyelitis (Kelsey et al., 1995; Roeder et al., 2000; Koo et al., 2001; Gondusky et al., 2009; Malizos et al., 2010). However, disadvantages of PMMA used in osteomyelitis management are requirement for surgical removal (Mader et al., 2002; Nelson et al., 2002), enhancement of bacterial colonization (Mader et al., 2002), higher cost of management and release of toxic substance during setting (Santschi et al., 2003). Unlike PMMA, calcium sulfate beads have been used in vitro and in vivo studies as a vehicle to deliver antibiotics, growth factors and other pharmacologic agents (Santschi et al., 2003; Ham et al., 2008; Kanellakopoulou et al., 2009; Thomas and Puleo, 2009; Xie et al., 2009). Moreover, antibiotic impregnated calcium sulfate beads have been used in medical practices, especially for the treatment for osteomyelitis (Ham et al., 2008; Kanellakopoulou et al., 2009). A local antibiotic delivery system has been employed because it
Native calcium sulfate beads were prepared as described before (Thitiyanaporn et al., 2012). Calcium sulfate beads were immersed in gentamicin sulfate injection solution 40 mg/ml (T.P. drug Lab, Thailand) for 5 minutes and dried under blower overnight.

G-HPCS: For preparation of high porous calcium sulfate beads, calcium sulfate hemihydrated and sodium chloride (Sigma, USA) were weighed and manually mixed in a ratio of 1:1 w/w. Sterile distilled water was added to the mixture in ratio 10:7 w/v of calcium sulfate hydrates and distilled water. After the mixture became homogenous slurry, it was poured into a cylinder mold. For salt leaching technique, the beads were placed with deionized water in an ultrasonic cleaner as described before (Thitiyanaporn et al., 2012). Calcium sulfate beads were immersed in gentamicin sulfate injection solution 40 mg/ml (T.P. drug Lab, Thailand) for 5 minutes and dried under blower overnight. GI-PMMA, G-NCS and G-HPCS beads were sterilized with ethylene oxide before use.

Animals: Male Wistar rats, approximately 3 months old, weighing 250-300 g, were used in this study. All rats were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. The rats were randomly sorted into 4 groups, control (n = 10), GI-PMMA bead (n = 10), G-NCS (n = 10) and G-HPCS (n = 10). The rats were kept in individual cage with unlimited food and water. This study was approved by the Kasetsart University Animal Use Committee ID number ACKU 02753.

Osteomyelitis model: Osteomyelitis was induced in the left proximal tibia. The rats were anesthetized with pentobarbital (0.6 mg/kg) intra-peritoneal injection. The osteomyelitis induction method was modified from previous studies (Monzon et al., 2001; Orhan et al., 2009). Briefly, then left proximal tibias were prepared by hair shave, chlorhexidine scrub and alcohol as routine preoperative preparation. One centimeter skin incision was performed at the

Materials and Methods

GI-PMMA, G-NCS and G-HPCS beads preparation:

GI-PMMA: Commercial polymethylmethacrylate (PMMA) with gentamicin sulfate 3.8% (GENTAFIX®3, Teknimed S.A., France) was used. Sterile powder and sterile liquid were mixed according to the manufacturer’s instructions. The mixture was poured into a silicone cylinder mold (2x4 mm, diameter x height) and left until the cement set. The PMMA beads were removed from the mold after setting.

G-NCS: Native calcium sulfate beads were prepared by mixing calcium sulfate hemihydrates powder (Sigma, USA) with sterile distilled water in ratio of 10:7 w/v. The mixture was poured into a silicone cylinder mold (2x4 mm, diameter x height). Calcium sulfate beads were immersed in gentamicin sulfate injection solution 40 mg/ml (T.P. drug Lab, Thailand) for 5 minutes and dried under blower overnight.

G-HPCS: For preparation of high porous calcium sulfate beads, calcium sulfate hemihydrated and sodium chloride (Sigma, USA) were weighed and manually mixed in a ratio of 1:1 w/w. Sterile distilled water was added to the mixture in ratio 10:7 w/v of calcium sulfate hydrates and distilled water. After the mixture became homogenous slurry, it was poured into a cylinder mold. For salt leaching technique, the beads were placed with deionized water in an ultrasonic cleaner as described before (Thitiyanaporn et al., 2012). Calcium sulfate beads were immersed in gentamicin sulfate injection solution 40 mg/ml (T.P. drug Lab, Thailand) for 5 minutes and dried under blower overnight. GI-PMMA, G-NCS and G-HPCS beads were sterilized with ethylene oxide before use.

craniomedian area of proximal tibia. Soft tissue and muscle were dissected through the bone. One millimeter hole at median cortex was created with Kirschner wire (K-wire) connecting to bone marrow. A K-wire (5.0 x 1.0 mm) coated with methicillin resistance _Staphylococcus aureus_ (MRSA) (National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand) biofilm was inserted into marrow cavity. Five hundred microliters of 4.0 x 10^9 CFU/ml MRSA was injected into the bone marrow cavity. The fascia and soft tissue were closed with polyglyconate (Maxon®, 4-0). Skin was sutured with nylon (Difilon®, 4-0) interrupted suture. After inoculation, the left tibia was monitored for osteomyelitis by gross appearance and radiographic examination weekly for 3 weeks (Fig 1).

**Surgical procedure:** After 3 weeks post-inoculation, the rats were anesthetized with pentobarbital (0.6 mg/kg) intra-peritoneal injection. The left proximal tibia was prepared by hair shave, chlorhexidine scrub and skin were closed routinely. A 5-scale clinical sign scoring system was modified from a previous study (Rissing et al., 1985). Blood samples (0.5 ml) were collected from tail vein in EDTA container before MRSA inoculation at the day of treatment and post-treatment (0±0) during the experiment in each group. Although the clinical signs were not significantly different between pre- and post-treatment (0±0) after two weeks. The infection sign scores of infected tibias were not significantly different between pre-MRSA inoculation and returned to normal (score 0) after two weeks. The infection sign scores of infected tibias were not significantly different between pre- and post-treatment (0±0) during the experiment in every group. Although the clinical signs were not present as osteomyelitis condition, the radiographic results revealed radiolucent area around the K-wire, indicating bone destruction.

**Bone culture:** The whole right tibias of the rats were collected with sterile procedure after euthanasia. All soft tissues were removed from the tibia samples. The tibia samples were weighed and pulverized with mortar under sterile condition. One milliliter of sterile normal saline solution was added to the bone samples and quantitatively cultured by ten consecutive 1 : 10 dilution in sterile normal saline. A 0.1 ml aliquot of each dilution was plated onto Mueller Hinton agar (Difco, USA) and incubated at 37°C for 18-24 hours. The colonies on the surface of agar plate were counted for calculating the colony forming unit (CFU) per weight (gram) of bone. Bacterial growth was expressed as log10 CFU/g tissue.

**Histological examination:** After euthanizing the rats with pentobarbital over dose, the tibias were stored in 10% neutral buffered formalin more than 24 hours. The samples were decalcified with 10% aqueous EDTA for a month and examined by a routine tissue processing for light microscopic procedure. The samples were stained with hematoxylin and eosin (H&E) for examining the cell types and Masson’s trichrome for examine bone collagen. Histopathological osteomyelitis results were evaluated according the parameters including 1. Abscess formation, 2. Sequestrum formation, 3. Enlargement of corticalis, 4. Destruction of corticalis, and 5. General impression. The parameters 1 to 4 were scored with 0 (absent) or 1 (present). Parameter 5 was scored from 0 (absent), 1 (mild), to 2 (severe) for each ROI (Lucke et al., 2003). In addition, bone destruction and callus formation were scored by 0 : absent, 1 : involvement of 1-25% of bone cortex, 2 : involvement of 26-50% of bone cortex, and 3 : involvement of 51-100% of bone cortex.

**Data analysis:** The clinical sign score, white blood cell count, and radiographic scores were analyzed with Kruskal-Wallis One-Way ANOVA. Histopathological scores were analyzed with Fisher’s exact test. Data was expressed as the mean±standard deviation. Statistical difference was accepted with a p < 0.05 level.

**Results**

**Infection signs:** Infected tibias were slightly swollen and painful (score 1-2) in the last three days after MRSA inoculation and returned to normal (score 0) after two weeks. The infection sign scores of infected tibias were not significantly different between pre-MRSA inoculation (0±0), pre-treatment (0.04±0.21) and post-treatment (0±0) during the experiment in every group. Although the clinical signs were not present as osteomyelitis condition, the radiographic results revealed radiolucent area around the K-wire, indicating bone destruction.

**White blood cell counts:** The total white blood cell count was not significantly different between pre-inoculation and treatment period in every group. The results are shown in the Table 1.

**Radiographic examination:** The infected tibias were evaluated by radiographic picture after treatment. The
osteomyelitis of tibia was scored as percentage of radiolucent area in different levels between the groups. Sample radiographic pictures of control, GI-PMMA, G-NCS and G-HPCS groups are shown in Fig 2. The percentage of radiolucent area scoring result is shown in Fig 3.

**Bone culture:** After 6 weeks of treatment, bacteria colony forming unit (CFU) per gram of tissue was examined. Average of log_{10} CFU/g is shown in Table 2. The average of bacterial growth was not different among the control, GI-PMMA, G-NCS and G-HPCS groups.

**Histopathological examination:** All histopathological findings of the control, GI-PMMA, G-NCS and G-HPCS groups showed typical signs of bone infection such as abscess in bone marrow, bone sequestrum, destruction of the bone cortex and periosteal elevation. In the control group, the infected tibias presented multiple pyogranuloma, sequestrum and destruction of corticalis (Fig 4A and 5A). In the GI-PMMA group, the infected tibias showed multiple pyogranuloma with callus formation surrounding the GI-PMMA implantation site and cortical bone destruction (Fig 4B and 5B). In the G-NCS and G-HPCS groups, the infected tibias showed multiple pyogranuloma and new bone formation at the bone cortex without bone destruction (Fig 4C, 5C, 4D, and 5D). Histopathological scores indicated that osteomyelitis scores in the control group were significantly higher than the GI-PMMA, G-NCS and G-HPCS groups. Bone destruction scores in the G-NCS group was significantly lower than the control group and bone destruction scores in both G-NCS and G-HPCS groups were significantly lower than the GI-PMMA group. Interestingly, bone formation scores in both G-NCS and G-HPCS groups were significantly higher than the control and GI-PMMA groups.

**Table 1** Total white blood cell count (x 10^3/µl) in pre-inoculation and treatment periods (6 weeks)

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>GI-PMMA</th>
<th>G-NCS</th>
<th>G-HPCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-inoculation</td>
<td>6.63±2.23</td>
<td>7.06±2.50</td>
<td>7.06±1.91</td>
<td>7.12±1.10</td>
</tr>
<tr>
<td>Week 0</td>
<td>5.83±2.56</td>
<td>6.48±1.92</td>
<td>7.54±2.39</td>
<td>6.57±2.72</td>
</tr>
<tr>
<td>Week 1</td>
<td>6.65±2.43</td>
<td>8.02±2.51</td>
<td>8.48±3.09</td>
<td>7.88±2.05</td>
</tr>
<tr>
<td>Week 2</td>
<td>6.98±1.08</td>
<td>6.25±1.26</td>
<td>7.21±2.38</td>
<td>7.93±2.36</td>
</tr>
<tr>
<td>Week 3</td>
<td>6.69±2.10</td>
<td>8.32±1.93</td>
<td>7.90±3.29</td>
<td>6.46±1.66</td>
</tr>
<tr>
<td>Week 4</td>
<td>7.58±1.43</td>
<td>7.09±1.38</td>
<td>7.10±1.46</td>
<td>6.77±2.35</td>
</tr>
<tr>
<td>Week 5</td>
<td>7.45±1.26</td>
<td>7.38±1.41</td>
<td>6.46±1.75</td>
<td>7.22±2.63</td>
</tr>
<tr>
<td>Week 6</td>
<td>5.25±2.18</td>
<td>4.93±1.48</td>
<td>5.10±1.43</td>
<td>6.82±3.81</td>
</tr>
</tbody>
</table>

Note: Pre-inoculation : Before inoculate MRSA, Week 0 : Treatment day, Week 1 : 7 days after treatment, Week 2 : 14 days after treatment, Week 3 : 21 days after treatment, Week 4 : 28 days after treatment, Week 5 : 35 days after treatment and Week 6 : 42 days after treatment.

**Table 2** Bacterial growth (mean±SD of log_{10} CFU/g) from infected tibias after 6-week treatment period

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>GI-PMMA</th>
<th>G-NCS</th>
<th>G-HPCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD of log_{10} CFU/g</td>
<td>3.64±0.38</td>
<td>3.59±0.63</td>
<td>3.24±0.37</td>
<td>3.43±0.66</td>
</tr>
</tbody>
</table>

**Table 3** Histopathological scores

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Osteomyelitis</th>
<th>Bone destruction</th>
<th>Callus formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>5±0</td>
<td>1.60±0.55</td>
<td>1.80±0.84</td>
</tr>
<tr>
<td>GI-PMMA</td>
<td>5</td>
<td>3.8±0.45</td>
<td>2.60±0.55</td>
<td>0.40±0.55</td>
</tr>
<tr>
<td>G-NCS</td>
<td>5</td>
<td>3.2±0.45</td>
<td>0.20±0.45</td>
<td>3±0</td>
</tr>
<tr>
<td>G-HPCS</td>
<td>5</td>
<td>3.2±0.45</td>
<td>0.44±0.50</td>
<td>3±0</td>
</tr>
</tbody>
</table>

a,b,c Different superscripts indicate a significant difference (p < 0.05) vs. control group.

Figure 2 Radiographic pictures of the left tibia present in both osteomyelitis induction period and treatment period in control (a), GI-PMMA (b), G-NCS (c) and G-HPCS (d) groups. 0b : MRSA inoculation day, 1b : 1 week after MRSA inoculation, 2b : 2 weeks after MRSA inoculation, 3b : 3 weeks after MRSA inoculation, 0a : treatment day, 1a : 1 week after treatment, 2a : 2 weeks after treatment, 3a : 3 weeks after treatment, 4a : 4 weeks after treatment, 5a : 5 weeks after treatment, 6a : 6 weeks after treatment.

Figure 3 Radiolucent area scores of control, GI-PMMA, G-NCS and G-HPCS group. *p* < 0.05 vs. control group.
Figure 4 Bone marrow picture of control (A), GI-PMMA (B), G-NCS (C) and G-HPCS (D) H&E staining at x 400. Pyogranuloma and sequestrum were found in bone marrow in every group. Large space of GI-PMMA implantation site is presented in a Fig B.

Discussion

The management of osteomyelitis is quite difficult because of poor vascularization at the infected area due to the presence of necrosis of the surrounding soft tissue. Localized antibiotic administration is used for providing high concentration of antibiotic at the infected site without systemic side effect. Commercial gentamicin impregnated PMMA is widely used as commercial local antibiotic provider. Calcium sulfate bead is also used as a local antibiotic provider. The high porous calcium sulfate (HPCS) bead was developed in this study to reduce the side effect of calcium sulfate and to be used as a local antibiotic provider as same as calcium sulfate bead. A study showed that the difference of the porosity level of calcium sulfate bead had an effect on antibiotic release in an in vitro study and that high porous calcium sulfate bead could provide antibiotic in the highest concentration when compared in weight to calcium sulfate (Thitiyanaporn et al., 2012). However, an in vitro comparison study of gentamicin impregnated PMMA bead with gentamicin coated PMMA bead, gentamicin coated native calcium sulfate bead and gentamicin coated high porous calcium sulfate bead showed highly different patterns of gentamicin release and osteoblast attachment (Thitiyanaporn et al., 2013). However, the osteomyelitis management result of this study was similar in the GI-PMMA, G-NCS and G-HPCS groups. The GI-PMMA bead is a non-absorbable material; it had to be removed after a period of time, while G-NCS and G-HPCS are absorbable material. After GI-PMMA was removed, the wide space of the implanted bone cortex still remained. This space may be a result of the bone fracture from instability of the affected bone.

There are numerous reports of rat osteomyelitis model developed for studying in pathogenesis, diagnosis and osteomyelitis management. In our study, the rat osteomyelitis was modified from the experiment of Orhan et al. (2010) and Monzón et al. (2001). Orhan et al. (2010) developed the rat osteomyelitis model in rat tibia by injecting 200 µl of methicillin resistance S. aureus (MRSA) containing 1.0 x 10^7 CFU/ml and implanting Kirschner wire (5.0 x 1.0 mm) to the bone marrow cavity. The bone hole was sealed with dental gypsum and left for 3 months for development of the chronic osteomyelitis (Orhan et al., 2009). The experiment of Monzón et al. (2001) developed the osteomyelitis model by implanting the Kirschner wire coated with biofilm of the S.aureus into the bone marrow and allowed 42 days of chronic osteomyelitis development (Monzon et al., 2001). Our study used 500 µl of MRSA 4.0 x 10^7 CFU/ml and implanted a Kirschner wire (5.0 x 1.0 mm) coated with biofilm of MRSA. The osteomyelitis was allowed to develop for 21 days. The osteomyelitis was developed in only bone marrow cavity and did not affect the other parts of bone. In a study of Fukushima et al. (2005), they injected S. aureus strain BB in Wistar rat tibia in many concentrations consisting of control : 0 CFU/5 µl, G1 : 6 x 10^2 CFU/5 µl, G2 : 6 x 10^3 CFU/5 µl, G3 : 6 x 10^4 CFU/5 µl, G4 : 6 x 10^5 CFU/5 µl and G5 : 6 x 10^6 CFU/5 µl and closed the tibial hole with bone wax. The osteomyelitis was allowed to develop within 1 week and the osteomyelitis was assessed in radiographic and histopathological examination. The recommended dose was G3 : 6 x 10^3 CFU/5 µl, sufficient to develop osteomyelitis condition in rat tibia (Fukushima et al., 2005). Last decade studies showed that the necrotic agent was not necessary for developing osteomyelitis condition in rat model (Nelson et al., 1990; Gisby et al., 1994; Monzon et al., 2001; Fukushima et al., 2005; Orhan et al., 2009) as the previous studies (Norden, 1970; Rissing et al., 1985; Mendel et al., 1999). The osteomyelitis model in rat was not created only in tibia but also in femur (Chen et al., 2005), mandible (Shvyrykov et al., 1981; Chistov, 1989) and hematogenous route (Kadyrov et al., 1966; Hienz et al., 1995).

The rat osteomyelitis model in the current study did not affect the weight, infection signs score...
and white blood cell count. This result was due to the limited infection only in the marrow cavity. The deterioration of bone structure may happen if the osteomyelitis condition was allowed to continue for 42 days (Monzon et al., 2001; Brin et al., 2008) or 3 months (Orhan et al., 2009) as the previous experiments. However, after 3 weeks of osteomyelitis, the induction in this study showed osteomyelitis signs in the radiographic examination. The radiographic study showed the radiolucent area in the bone marrow cavity surrounding the inoculation site. After treatment, the radiolucent area in the control group was continuously widen, while radiolucent area in the GI-PMMA, G-NCS and G-HPCS groups were significantly decreased. This result suggested that the GI-PMMA, G-NCS and G-HPCS could be used for managing osteomyelitis.

High porous calcium sulfate bead was synthesized utilizing salt leaching technique to increase the porosity level and reduce the amount of calcium sulfate. G-HPCS is a new fabricated antibiotic bead that can be used as a local antibiotic provider for osteomyelitis. Histopathological study with H&E and Masson’s trichrome staining revealed sequestrums, pyogranulomatous abscess and bone cortex deterioration in the control group, in contrast, new bone regeneration was found at the implantation site in the G-NCS and G-HPCS groups (Fig 4, 5 and Table 3). These results suggest that calcium sulfate beads could facilitate the new bone regeneration. Although the results of GI-PMMA on the treatment for osteomyelitis in this study were satisfying, the unabsorbable property of the PMMA could interfere with the bone healing process as found in this study.

Conclusion

High porous calcium sulfate bead was synthesized utilizing salt leaching technique to increase the porosity level and reduce the amount of calcium sulfate. G-HPCS is a new fabricated antibiotic bead that can be used as a local antibiotic provider for management of osteomyelitis comparable to the commercial antibiotic impregnated bone cement and the calcium sulfate coated antibiotic. In addition, G-HPCS has lower amount of carrier materials, thus helps reduce the inflammatory response to an implant material.

Acknowledgements

This research was financially supported by Kasetsart University Research and Development Institute (KURDI), and Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate School and Research Development Office, Office of Higher Education Commission, Ministry of Education. (AG-BIO/PERDO-CHE).

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Inactivation of Infectious Bronchitis Virus with Various Kinds of Disinfectants

Pheemphat Bengtong 1  Thotsapol Thomrongsuwannakij 1  Niwat Chansiripornchai 1,2*

Abstract

This study aimed to perform an in vitro testing of the efficacy of various kinds of disinfectant on infectious bronchitis virus (IBV). Four kinds of disinfectants including Virusnip, Omnicide, CID2000 and Virkon S were used for the virus inactivation test. After challenge with each strain of IBV for each contact time (30 seconds, 1, 5 and 30 minutes), we recorded number of dead embryo/6 inoculated eggs after incubation. Results showed that there was no significant difference among the contact times except 1:800 Virusnip tested for Tha07 (p < 0.05). For Tha03, there was a significant difference among the disinfectants at 30 seconds and 1 minute of contact times (p < 0.05). For Tha08, there was a significant difference among the disinfectants at 1 minute of contact time (p < 0.05). For Tha09, there was a significant difference among the disinfectants at 5 and 30 minutes of contact times (p < 0.05). For Tha10, there was a significant difference among the disinfectants at 5 minutes of contact time (p < 0.05). Virusnip revealed the ability to inactivate the activity of 9 IBV strains in all exposure times, especially at dilutions of 1 : 100 and 1 : 200.

Keywords: disinfectants, inactivation, infectious bronchitis virus

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Introduction

Infectious bronchitis virus (IBV) is an enveloped coronavirus which is characterized by respiratory signs and may result in renal damage, decreased egg production and decreased quality of eggs (Dolz et al., 2012). IBV is acquired via horizontal transmission; for example, inhalation or direct contact with contaminated poultry, litter, equipment or other fomites (de Wit et al., 1998). Although vertical transmission of the virus within the embryo has never been reported, the virus may be present on the shell surface of hatching eggs via shedding from the oviduct or the alimentary tract (Gallardo et al., 2011).

Development of a disease’s prevention system is the most important part of poultry disease control. Biosecurity, good management and efficacy of disinfectant application are key points to the success of poultry disease prevention. The principle of biosecurity is based on reducing the risk of infection (Fasina et al., 2012). Although IBV is considered to be sensitive to common disinfectants, in vitro testing of disinfectants is an important guideline to apply to the disinfectants in field conditions (McDonnell and Russell, 1999). The disinfectants used in this study were composed of 4 kinds. Firstly, Virusnip contains two active ingredients, which are potassium peroxymonopersulphate (PMP) as an oxidizing agent and sodium dichloroisocyanurate (SDIC) as an organic releasing chlorine. Secondly, Virkon S contains two active ingredients, which are sodium dodecyl benzene sulphonate as an anionic surfactant and potassium monopersulphate as an oxidizing agent. Thirdly, CID 2000 contains two active ingredients, which are hydrogen peroxide and per oxyacetic acid as oxidizing agents. Lastly, Omnicide contains two active ingredients, which are glutaraldehyde and alkyl benzyl dimethyl ammonium chloride as a subclass of quaternary ammonium compounds (QACs). These disinfectants are commonly used in Thailand’s poultry farm. Therefore, the aim of this study was to perform the in vitro testing of the efficacy of various kinds of disinfectants on IBV.

Materials and Methods

Embryonated eggs: Seven-day-old embryonic eggs were bought from Kasetsart University and immediately transported for incubation in a hatchery of the Avian Health Research Unit, Chulalongkorn University. Nine- to eleven-day embryonic eggs were used in our experiments. Six embryonic eggs were taken to test virucidal activity of disinfectant in each contact time (Table 1).

Infectious bronchitis virus: Nine isolates of Thai IBV were used for the testing of virus inactivation by disinfectants. A dosage of virus was prepared in a solution containing approximately 1.0 x 10^6 ELD50. A half ml of virus was also mixed with a half ml of disinfectant.

Disinfectants: All tested disinfectants were registered by the Department of Livestock Development,
The disinfectants used in this study were composed of 4 kinds including Virusnip (Novartis Animal Health, Switzerland), Omnicide (Metrex, USA), Virkon S (Antec International Ltd, UK) and CID 2000 (CID LINES, Belgium). Test protocol was modified from Suarez et al. (2003). Briefly, all the disinfectants were diluted with distilled water following the manufacturers’ recommendation for each product. Omnicide, Virkon S and CID 2000 were diluted and tested at concentrations of 1 : 150, 1 : 200 and 2%, respectively, except that Virusnip was diluted and tested at concentrations of 1 : 100, 1 : 200 1 : 400 and 1 : 800. All diluted disinfecants were aliquoted and kept at 25°C. A half ml of IBV containing approximately 1.0 x 10⁶ ELD₅₀ was mixed with 0.5 ml of diluted disinfectants and incubated for 30 sec, 1, 5 and 30 min at room temperature. In addition, 0.5 ml of IBVs was mixed with 0.5 ml of phosphate buffered saline (PBS), and 0.5 ml of disinfectant was mixed with 0.5 ml of distilled water, serving as positive and negative controls, respectively (Table 1). The positive control and the negative control was inoculated into nine- to eleven-day old chicken embryonic eggs in six replications and candled twice a day for 7 days. Inoculated embryonic eggs dying prior to 24 hours were discarded. Allantoic fluid was harvested from each egg on day 7 post inoculation or upon death. Secondary egg inoculations were performed to determine the presence of the virus.

Statistical analysis: The Kruskal-Wallis and Mann-Whitney U test were used to define difference between strains of IBV in each contact time of a disinfectant and among all disinfectants of each IBV strain.

Results

Results of IBV inactivation with various kinds of disinfectants and exposure times are shown in Table 2. In each strain of IBV, we compared the results at each contact time of different disinfectants and at each contact time we compared the results from different strains of IBV. From all groups of these tests, there was no significant difference among their durations (30 sec, 1 min, 5 min and 30 min) except 1 : 800 Virusnip tested for Tha07 which had a significant difference among their durations (p < 0.05).

For the Tha03 strain of IBV, at 30 seconds of contact time, the number of embryonic deaths of 1 : 200 Virusnip, CID 2000 and Virkon S was significantly less than 1 : 800 Virusnip (p < 0.05) and at 1 minute of contact time, the number of embryonic deaths of 1 : 100 Virusnip, CID 2000, Omnicide and Virkon S was significantly less than 1 : 800 Virusnip (p < 0.05).

For the Tha08 strain of IBV, at 1 minute of contact time, the number of embryonic deaths of 1 : 100, 1 : 200, 1 : 400 and 1 : 800 Virusnip and Omnicide was significantly less than Virus S (p < 0.05).

For the Tha09 strain of IBV, at 5 minutes of contact time, the number of embryonic deaths of 1 : 100, Virusnip and Virkon S was significantly less than 1 : 400 and 1 : 800 Virusnip (p < 0.05) and at 30 minutes of contact time, the number of embryonic deaths of 1 : 100, 1 : 200 and 1 : 400 Virusnip, Omnicide and Virkon S was significantly less than 1:800 Virusnin (p < 0.05).

For the Tha10 strain of IBV, at 5 minutes of contact time, the number of embryonic deaths of 1 : 100, 1 : 200, 1 : 400 and 1 : 800 Virusnin and Virkon S was significantly less than Omnicide (p < 0.05).

Discussion

In this study, we investigated the in vitro testing of the efficacy of various kinds of disinfectants including Virusnin, Omnicide, CID 2000 and Virkon S on nine strains of IBV. In this study, we used a concentration of disinfectants according to the recommendation of the manufacturers except for Virusnin whose concentration we varied at 1 : 100, 1 : 200, 1 : 400 and 1 : 800. The results indicated that the proper concentration and contact time to inactivate nine strains of IBV was Virusnin 1 : 200 for 1 minute. The virucidal activity of Virusnin was derived from both the oxidizing agent (PMP) and the organic releasing chlorine (SDIC). The oxidizing agent (PMP) probably denatures proteins and enzymes and increases cell wall permeability by disrupting sulfhydryl (-SH) and sulfur (S-S) bonds and organic releasing chlorine (SDIC) which provides a higher concentration of available chlorine is less susceptible to be inactivated by organic matter than sodium hypochlorite (McDonnell and Russell, 1999). In water, SDIC generates hypochlorites that are ready to disinfect instantly, destroying the cellular activity of proteins and inhibiting DNA synthesis of microorganisms (McDonnell and Russell, 1999) and

<table>
<thead>
<tr>
<th>Group</th>
<th>Contact time</th>
<th>30 seconds</th>
<th>1 minute</th>
<th>5 minutes</th>
<th>30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IBV + PBS</td>
<td>IBV + PBS</td>
<td>IBV + PBS</td>
<td>IBV + PBS</td>
<td>IBV + PBS</td>
</tr>
<tr>
<td>2</td>
<td>Disinfectant + PBS</td>
<td>Disinfectant + PBS</td>
<td>Disinfectant + PBS</td>
<td>Disinfectant + PBS</td>
<td>Disinfectant + PBS</td>
</tr>
<tr>
<td>3</td>
<td>IBV+ Virusnin (1:100)</td>
<td>IBV+ Virusnin (1:100)</td>
<td>IBV+ Virusnin (1:100)</td>
<td>IBV+ Virusnin (1:100)</td>
<td>IBV+ Virusnin (1:100)</td>
</tr>
<tr>
<td>4</td>
<td>IBV+ Virusnin (1:200)</td>
<td>IBV+ Virusnin (1:200)</td>
<td>IBV+ Virusnin (1:200)</td>
<td>IBV+ Virusnin (1:200)</td>
<td>IBV+ Virusnin (1:200)</td>
</tr>
<tr>
<td>5</td>
<td>IBV+ Virusnin (1:400)</td>
<td>IBV+ Virusnin (1:400)</td>
<td>IBV+ Virusnin (1:400)</td>
<td>IBV+ Virusnin (1:400)</td>
<td>IBV+ Virusnin (1:400)</td>
</tr>
<tr>
<td>6</td>
<td>IBV+ Virusnin (1:800)</td>
<td>IBV+ Virusnin (1:800)</td>
<td>IBV+ Virusnin (1:800)</td>
<td>IBV+ Virusnin (1:800)</td>
<td>IBV+ Virusnin (1:800)</td>
</tr>
<tr>
<td>7</td>
<td>IBV+ Omnicide (1:150)</td>
<td>IBV+ Omnicide (1:150)</td>
<td>IBV+ Omnicide (1:150)</td>
<td>IBV+ Omnicide (1:150)</td>
<td>IBV+ Omnicide (1:150)</td>
</tr>
<tr>
<td>8</td>
<td>IBV+ CID 2000 (2%)</td>
<td>IBV+ CID 2000 (2%)</td>
<td>IBV+ CID 2000 (2%)</td>
<td>IBV+ CID 2000 (2%)</td>
<td>IBV+ CID 2000 (2%)</td>
</tr>
</tbody>
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Table 2 Number of dead embryo/6 inoculated eggs after incubation with each IBV strain.

<table>
<thead>
<tr>
<th>Disinfectants</th>
<th>Dilution</th>
<th>Exposure times</th>
<th>Number of dead embryo/6 inoculated eggs of each IBV strains</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Tha03 Tha04 Tha05 Tha07 Tha08 Tha09 Tha10 Tha28 Tha29 Total</td>
</tr>
<tr>
<td>Virusnip</td>
<td>1 : 100</td>
<td>30 sec</td>
<td>1 0 0 0 0 0 0 0 0 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 min</td>
<td>0¹ 0 0 0 0 0 1 0 0 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
<td>0 0 0 0 0 0 0 1 0 1</td>
</tr>
<tr>
<td></td>
<td>1 : 200</td>
<td>30 sec</td>
<td>0 0 0 0 1 2 0 0 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 min</td>
<td>1 0 0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
<td>0 0 0 0 2 0 0 0 1 3</td>
</tr>
<tr>
<td></td>
<td>1 : 400</td>
<td>30 sec</td>
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<tr>
<td></td>
<td></td>
<td>1 min</td>
<td>0 1 0 0 0 0 0 0 0 3</td>
</tr>
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<td></td>
<td></td>
<td>5 min</td>
<td>0 0 0 0 2 0 0 0 0 0</td>
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<tr>
<td></td>
<td>1 : 800</td>
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<td>0 3 0 3 2 4 0 2 18</td>
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<td></td>
<td></td>
<td>1 min</td>
<td>2 0 0 0 0 4 0 0 1 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
<td>1 0 0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>Omnicide</td>
<td>1 : 150</td>
<td>30 sec</td>
<td>2 2 1 3 3 2 3 2 2 17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 min</td>
<td>0¹ 3 2 1 0 1 1 2 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
<td>0 1 0 1 3 4 2 1 13</td>
</tr>
<tr>
<td>CID 2000</td>
<td>2%</td>
<td>30 sec</td>
<td>0 2 0 2 2 2 0 1 11</td>
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<tr>
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<td></td>
<td>1 min</td>
<td>1 0 1 1 3 3 3 3 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
<td>0 0 0 0 1 3 3 3 13</td>
</tr>
<tr>
<td>Virkon S</td>
<td>1 : 200</td>
<td>30 sec</td>
<td>0 0 0 0 1 1 0 1 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 min</td>
<td>0 1 0 1 0 0 1 0 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
<td>1 0 0 0 0 2 0 0 4</td>
</tr>
<tr>
<td>Positive</td>
<td>NA</td>
<td>6 6 6 6 5 6 6 6 6 52</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>NA</td>
<td>0 0 0 0 0 0 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

NA: not applicable

are immediately regenerated back into new active SDIC by PMP, providing continuous action until all the monopersulphate is used up (manufacturer’s information) which causes a higher performance than with only oxidizing agents (Virkon S, CID 2000). The Virusnip dilution of 1 : 200 also completely killed classical swine fever virus, porcine reproductive and respiratory syndrome virus and pseudorabies virus in 30 seconds to 5 minutes (Bunpapong et al., 2010). This is in contrast to a study in Japan which indicated that 0.1% benzalkonium chloride (BC) was an effective virucidal agent against avian bronchitis virus in the allantoic fluid of chicken eggs. Nonetheless, disinfectant effectiveness depends on many factors, for example, contact time, temperature, activity in organic matter or protein-containing material, type of chemical, and concentration and quantity of the chemical (Kennedy et al., 2000).

In conclusion, Virusnip revealed the best inactivating activity in all exposure times, especially at dilutions of 1 : 100 and 1 : 200. At the dilution of 1 : 100, all 9 IBV strains were inactivated within 30 min, except for Tha28. At the dilution of 1 : 200, most 9 IBV strains were inactivated within 30 min, except for Tha07 and Tha29. Virkon S seemed to be in the second rank of the inactivated efficacy to IBV. At the dilution of 1:200 of Virkon S, most of the IBV strains were killed within 30 min, except for Tha08 and Tha28. CID2000 and Omnicide were the third and fourth ranks of inactivated efficacy to IBV. In conclusion, Virusnip revealed the best inactivated efficacy to 9 strains of Thai IBV isolates.

Acknowledgements

This study was financially supported by Novartis Animal Health Inc. We would like to thank Dr. Nisit Chansong and Dr. Kai Sievert, Novartis Animal Health, for their crucial suggestion.

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Evaluation of Hematology Profiles and Measurement of Serum Cardiac Troponin Level in Canine Monocytic Ehrlichiosis

Rungrote Osathanon 1* Walasinee Moonarmart 1 Naiyana Suksantilap 2 Nattiya Krajangpit 2 Piyanart Lekcharoensook 2 Pruksa Julapanthong 3 Nutthakulporn Wongrerkngam 3

Abstract

Canine monocytic ehrlichiosis (CME) is an important multisystemic disease in dogs which is caused by Ehrlichia canis (E. canis). Systemic inflammatory response syndrome (SIRS) and severe anemia from E. canis might be the potential causes of myocardial damage. Cardiac troponin T (cTnT) is considered to be a sensitive and specific biomarker for myocardial damage in many mammalian species. The objectives of this study were to evaluate the effect of CME on serum cTnT levels in dogs and to determine the relationship between serum cTnT and values obtained from hematology. Fifty-two client-owned dogs were recruited into this study and clinical data were thoroughly recorded. Blood samples were collected from 52 dogs and tested for antigen of E. canis by multiplex PCR. The cTnT levels were measured by Elecsys®/cobas e™ cTnT fourth-generation assay. The dogs were divided into 4 groups. The control group consisted of 15 healthy dogs. The negative group included 20 dogs presented with at least 2 inclusion criteria but yielded negative results for Multiplex PCR. The E. canis infected group consisted of 10 dogs, which were positive for E. canis only. Lastly, the other blood parasite infected group included 7 dogs with multiplex PCR positive for Babesia spp. and/or Hepatozoon canis. Results showed that serum cTnT concentration was not different among the 4 groups (p = 0.70) and did not significantly increase in dogs with natural E. canis infection. However, there was a negative correlation between cTnT and RBC, and a correlation of cTnT with age. These implied that anemia and aging might cause myocardial injury, consequently, increased serum cTnT level in both normal and E. canis infected dogs.

Keywords: Cardiac troponin T, Ehrlichia canis, hematology profiles, myocardial damage

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บทคัดย่อ
การประเมินค่าโลหิตวิทยาและการตรวจวัดระดับของซีรั่ม Cardiac Troponin T ในสุนัขที่ป่วยด้วยโรค Canine Monocytic Ehrlichiosis

รุ่งโรจน์ โอสถานนท์1*, วลาสินี มูลอามาตย์2, นัยนา สุขสันติลาภ2, ณัฏฐิญา กระจ่างพิศ2, ปิยนาถ เล็กเจริญสุข2, พฤกษา จุฬพันธ์ทอง3, ณัฐกุลภรณ์ วงษ์ฤกษ์งาม3

Canine monocytic ehrlichiosis (CME) เป็นโรคที่สำคัญที่ส่งผลกระทบต่อหลายระบบในสุนัขและมีสาเหตุมาจากเชื้อ Ehrlichia canis (E. canis) ซึ่งอาจทำให้เกิดการเสียหายของกล้ามเนื้อหัวใจได้ รวมถึงภาวะอักเสบทั่วร่างกายและภาวะโลหิตจางแบบรุนแรงในสัตวเลี้ยงลูกด้วยนม ซึ่งการตรวจ cardiac troponin T (cTnT) เป็นวิธีที่ไวและจำเพาะต่อการวินิจฉัยภาวะการเสียหายของกล้ามเนื้อหัวใจ การทดลองในครั้งนี้มีการประเมินค่าโลหิตวิทยาและการตรวจวัดระดับของซีรั่ม cTnT ในสุนัขที่มีอาการผิดปกติ 52 ตัว บนกลุ่มหลักในการตรวจ cTnT โดยที่พบการตรวจลบต่อการตรวจ antigen ของ E. canis 15 ตัว กระทำโดยการตรวจ multiplex PCR และวัดระดับ cTnT โดยใช้ Elecsys®/cobas e™ cTnT fourth-generation assay ทำการแบ่งกลุ่มสุนัขออกเป็น 4 กลุ่ม กลุ่มควบคุมมีสุนัขจำนวน 15 ตัว กลุ่มที่ให้ผลลบมีสุนัขจำนวน 20 ตัวที่มีการตรวจลบ 2 ชนิดจากข้อกําหนดของการทดลอง กลุ่มที่เป็น E. canis มีสุนัขจำนวน 10 ตัวที่มีการตรวจบวกต่อการตรวจ multiplex PCR และกลุ่มที่เป็นพยาธิในเม็ดเลือดชนิดอื่น ๆ มีสุนัขจำนวน 7 ตัวที่มีการตรวจบวกต่อการตรวจ Babesia spp. และ/หรือ Hepatozoon canis ด้วยวิธี multiplex PCR จากการทำงานตรวจวัดระดับซีรั่ม cTnT ไม่มีความแตกต่างระหว่าง 4 กลุ่ม (p = 0.70) และไม่พบสูงขึ้นอย่างไม่ซักซ้อมในสุนัขที่ติดเชื้อ E. canis ทางธรรมชาติ นอกจากนี้ยังพบความสัมพันธ์ระหว่าง cTnT กับอายุและความสัมพันธ์กับจำนวนเม็ดเลือดแดง จึงเป็นไปได้ว่าภาวะเสี่ยง และอายุที่มากขึ้นอาจส่งผลให้สามารถตรวจพบซีรั่ม cTnT ในกระแสเลือดเพิ่มขึ้น

คำสำคัญ: การเสียหายของกล้ามเนื้อหัวใจ, คาร์ดิแอคโทรโปนิน, ค่าโลหิตวิทยา, โรคพยาธิในเม็ดเลือด

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Introduction

Canine monocytic ehrlichiosis (CME) is firstly described by Donation and Lestoquard in Algeria in 1965 (Harrus et al., 1999). It is now recognized as an important canine disease that can be found throughout the world (Kelly, 2000; McQuiston et al., 2003; Harrus and Waner, 2011). CME is caused by gram-negative obligate intracellular cocci bacteria, named Ehrlichia canis (Cohn, 2003; Rikihisa, 2010). This bacterium is a tick-borne organism and transmitted by Rhipicephalus sanguineus (Groves et al., 1975). CME infected dogs is presented with asymptomatic or multisystemic clinical signs (Harrus and Waner, 2011). Examples of acute or chronic clinical signs are lethargy, obtundation, weight loss, fever, loss of appetite, lymphadenopathy, splenomegaly, and bleeding disorders (Harrus et al., 1999; Procajlo et al., 2011). CME can be diagnosed using several techniques, for example, presentation of intracytoplasmic E. canis-morulae in blood smear, hematology, cytology, serology, isolation and molecular detection (Harrus and Waner, 2011). Molecular biology approach using polymerase chain reaction (PCR) to detect experimental E. canis infection was introduced (Iqbal et al., 1994). This molecular technique is commonly employed as a definitive diagnosis for E. canis infection (Harrus and Waner, 2011). The E. canis DNA detection using PCR technique illustrated a high sensitivity and specificity for the detection of concurrent ehrlichial infections (Baneth et al., 2009). The most common target genes are p30-based PCR and 16S rRNA. However, the p30-based PCR assay is more sensitive than the 16S rRNA-based PCR assay (Stich et al., 2002). A multiplex polymerase chain reaction (PCR) was developed for simultaneous detection of canine blood parasites, including E. canis, Babesia spp and Hepatozoon canis,
Troponins are myofibrillar proteins that are present in both skeletal and cardiac muscles (Babuin and Jaffe, 2005). It regulates the interaction between myosin and actin (DeFrancesco et al., 2002). Three types of troponins are reported, which are troponin T, troponin I, and troponin C (Babuin and Jaffe, 2005). Different types of troponins have different functions. It has been reported that troponin T is bound to tropomyosin. In addition, troponin C is bound to calcium, while, troponin I inhibits coupling of myosin and actin. Several cardiac troponin (cTn) isoforms have been identified. They are cardiac troponins C, I, and T (cTnC, cTnI, and cTnT) (Adams et al., 1993-a). The cTnI and cTnT cardiac isoforms are specific to cardiac muscle and exhibit a high percentage of the conservation between humans and dogs (Adams et al., 1993-b; O’Brien et al., 1997). cTnT binds the troponin-tropomyosin complex to actin filament (Adams et al., 1993-a). It has 260 amino acids with a molecular weight of 37 kDa. Increase in serum cTn levels in humans correlates with histopathological changes in cardiac muscle such as from ischemic injury and toxicity (O’Brien, 2006). Loss of membrane integrity of damaged cardiac myocytes causes release of cTn into blood circulation. After acute myocardial injury, initial increase in cTn levels in blood is caused by release of cytosolic pool. Consequently, the sustained blood cTn levels are due to the release of structurally bound troponin. Therefore, cTn is considered to be the myocardial leakage marker (Katus et al., 1991; Adams et al., 1994; Jaffe et al., 1996). cTnI was measured for diagnosis of cardiovascular diseases in dogs by an automated immunoassay method. A mouse monoclonal anti-troponin I antibody and a goat polyclonal anti-troponin I antibody were used for capture and detection of troponin I molecules directed against a unique 31 amino acid extension of the cTnI N-terminus, respectively (Spratt et al., 2005). This method has been validated using human serum samples which has been shown to be highly reliable and cardiospecific (Collinson et al., 2001). cTnI levels were significantly elevated in dogs with acquired mitral valve disease, dilated cardiomyopathy and pericardial effusion (Spratt et al., 2005). In cats with congestive heart failure from hypertrophic cardiomyopathy (HCM), the serum cTnI was significantly higher than in normal cats. This result may indicate that cats with HCM have ongoing myocardial damage (Herndon et al., 2002). Moreover, Brazilian dogs with acute E. canis infection had higher serum cTnI concentrations than healthy dogs. This suggested that acute E. canis infection might cause myocardial injury and systemic inflammatory response syndrome (Diniz et al., 2008).

C.TnT has a high sensitivity and specificity for myocardial damage, leading to the potential utilization as a marker for cardiomyocyte injury (Adams et al., 1993-b; Ohman et al., 1996; Fredericks et al., 2001). In rat and canine myocardial infarction models, cTnT concentration was significantly increased and highly correlated with the size of infarction within 3 h of injury (O’Brien et al., 1997). Taken together, these data imply the correlation of serum cTn levels and myocardial damage (Freda et al., 2002).

Recently, CME has been investigated extensively (Harrus et al., 1999). The main examination is focused on the pathogenesis clarification of the diseases (Harrus et al., 1999). Increasing evidences illustrate the involvement of immune mechanisms in the pathogenesis of acute CME associated with vasculitis (Cohn, 2003). These conditions include polymyositis (Shaw et al., 2001), glomerulonephritis (Avery and Avery, 2007), hepatitis (Mylonakis et al., 2010), uveitis (Komenou et al., 2007), meningoencephalitis (Grindem et al., 2002) and polyarthritis (Weiss et al., 1999). The evidence illustrates that the naturally infected E. canis may cause myocardial damage in Brazilian dogs (Diniz et al., 2008). Moreover, the serum cTnI levels in dogs with non-myelosuppressive and myelosuppressive ehrlichiosis are significantly increased (Koutinas et al., 2012). Taken together, these results imply the relationship of CME, cardiac damage and serum troponin levels. Therefore, the aims of this study were to evaluate the effect of CME on serum cTnI levels in dogs and to determine the relationship between serum cTnT and values obtained from hematology.

Materials and Methods

Case Materials: The study was performed in client-owned dogs that visited Prasu-Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University during a two-month period (April 2012 to May 2012). Thirty-seven dogs with at least two inclusion criteria were recruited into the potential CME group. The inclusion criteria were tick infestation, bleeding, nervous sign, inflammatory ocular disease, anemia (packed cell volume less than 35%), leucopenia (white blood cell less than 6000 cells/µl), hyperthermia (body temperature over than 102.9°F), hyperproteinemia (total protein over than 7.8 g/dl), and thrombocytopenia (platelet less than 150,000 cells/µl). For the control group, fifteen healthy dogs were recruited. Dogs were excluded from the study if they had the evidences of trauma, antirickettsial drug administration (less than 30 days), and heart diseases. The protocol used in this study was approved by Faculty of Veterinary Science Animal Care and Use Committee (FVS-ACUC).

Data collection: Comprehensive data including sex, breed, age, body weight, body condition score, medical history, medication and external parasite infestation history of all dogs recruited into this study were completely recorded. Blood (7 ml) was taken from cephalic or saphenous veins and further collected into K3-EDTA-treated tube (Becton Dickinson) and plain tube (Becton Dickinson). A single drop of blood in K3-EDTA-treated tube was tested by WITNESS® EHLRICHA IgG antibody test. A small amount of EDTA anti-coagulated blood was employed for hematology by a VetABC hematology

from blood samples in a single reaction. This multiplex PCR primers were specific to E. canis VirB9, Babesia spp 16S rRNA and H. canis 16S rRNA that yielded 100% identity to the sequences of these blood parasites (Kledmanee et al., 2009).

Multiplex PCR primers were specific to 16S rRNA that yielded 100% identity to the sequences of these blood parasites (Kledmanee et al., 2009).
analysers (SCIL Animal Care Company, IL, USA). All blood samples in K3-EDTA-treated tube were stored at -80°C for subsequent analysis of DNA extraction and multiplex polymerase chain reaction (PCR) amplification. Blood samples in plain tubes were centrifuged at 4°C and 3000 rpm for 10 minutes and serum were collected and stored at -80°C for subsequent cTnT analysis.

Multiplex PCR: *E. canis* antigen in the specimens was evaluated using multiplex PCR that has been developed by Kledmanee et al. (2009). The multiplex PCR primers were specific to *E. canis* VirB9, *Babesia spp.* 16S rRNA and *Hepatozoon canis* 16S rRNA (Kledmanee et al., 2009). Parasite DNA was extracted by proteinase K digestion and phenol-chloroform : isoamyl (25 : 24 : 1). Primer designed by GenBank was used for multiple alignments using Bio Edit v 7.0.4 software. The multiplex PCR amplification was performed in thermocycler (PCT-200). The amplicons were further separated by electrophoresis in 2.5% agarose gel and visualized under ultraviolet light. All selected samples were detected at Laboratory Department of the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University. This method from multiplex PCR could 100% identify the sequences of *E. canis*, *Babesia spp.* and *Hepatozoon canis*, providing a highly specific tool for diagnosis of tick-borne parasitic co-infections.

**Serum cTnT:** Serum cTnT was measured by Elecsys®/cobas e™ cTnT fourth-generation assay (Roche Diagnostics) on Elecsys 2010/cobas e 411 and Modular® Analytics E170/cobase 601 immunoanalyzers (Roche Diagnostics), according to the manufacturer’s instruction. All samples were measured at Clinical Pathology Unit, Siriraj Hospital, Mahidol University, Thailand. Principles and protocol of the assay were described (Giannitis et al., 2010). Briefly, the assay uses fragment antigen-binding (FAB) fragments of 2 cTnT-specific mouse monoclonal antibodies in a sandwich format. The antibodies recognize epitopes located in the central part of the cTnT molecule (amino acid positions 125–131 and 135–147, respectively). Detection is based on an electrochemiluminescence immunoassay (ECLIA), using a Tris(bipyridyl)-ruthenium(II) complex as label. This assay was performed based on human cTnT that yielded 99% sensitivity and 98% specificity.

**Statistical Analyses:** All data were analyzed by computerized statistical software (SPSS 18.0 for Windows, Chicago, IL, USA). The data in each group were tested for normality by using Shapiro-Wilk test. Comparisons between groups were tested by Kruskal-Wallis test, followed by post hoc Mann-Whitney U test. Spearman’s rank correlation coefficient was used to assess the correlation between dependent variables cTnT and independent variables: age and values obtained from hematology. *P* value less than 0.05 was considered statistically significant.

### Results

Fifty-two dogs were recruited into this study, consisting of 29 males (6 neutered, 22 entired, and 1 no data) and 23 females (12 spayed, 9 entired, and 2 no data). There were 16 breeds, including 15 mixed breeds, 9 Thai breeds, 6 Golden retrievers, 4 Siberian huskies, 3 Beagles, 3 Labrador retrievers, 3 Poodles, 1 Cocker spaniel; 1 Bangkaew, 1 German shepherd, 1 Pitbull, 1 Pug, 1 Shih Tzu, 1 St. Bernard, 1 Welsh Corgi and 1 unrecorded. The dogs were classified according to the positive results for *E. canis* or other parasites based on multiplex PCR results. They were divided into 4 groups (Table 1), which were control group, negative group, *E. canis* infected group, and other blood parasite infected group. The control group consisted of 15 healthy dogs. The negative group including 20 dogs presented with at least 2 inclusion criteria but negative results for Multiplex PCR. The *E. canis* infected group included 10 dogs which were positive for *E. canis* only. Lastly, the other blood parasite infected group included 7 dogs with multiplex PCR positive for *Babesia spp.* and/or *Hepatozoon canis*. Clinical variables obtained from the 52 dogs are illustrated in Table 2.

There was no statistical difference in age, WBCs, monocytes, lymphocytes, eosinophils, basophils, band neutrophils, RBCs, MCV, MCH, MCHC, RDW, and plasma protein among all 4 groups (Table 3). The neutrophils of dogs were significantly different among the 4 groups (*p = 0.039*). The neutrophils of the negative group were significantly higher than those of the *E. canis* infected group (*p = 0.025*) and the other blood parasite infected group (*p = 0.027*). Medians of the neutrophil in the control and the negative groups were in normal range, whereas medians in the *E. canis* infected group and the other blood parasite infected group were neutropenia (Table 3).

RBCs of the dogs in all groups were significantly different (*p = 0.002*). The RBC of the *E. canis* infected group and the other blood parasite infected group was significantly lower than the control group (*p < 0.001* and *p = 0.001*, respectively). Moreover, the RBC of the *E. canis* infected group was also significantly lower than the negative group (*p = 0.016*) (Table 3).

### Table 1 Group distribution of 37 dogs according to multiplex PCR results and 15 healthy dogs

<table>
<thead>
<tr>
<th>Dog groups</th>
<th>N</th>
<th>PCR positive Organism (s)</th>
<th>N</th>
<th><em>E. canis</em> Seroreactivitya (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Negativeb</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td><em>E. canis</em> infected</td>
<td>10</td>
<td><em>E. canis</em></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Other blood parasite</td>
<td>7</td>
<td><em>Babesia spp.</em></td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>infected</td>
<td>7</td>
<td><em>Hepatozoon canis</em></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Hepatozoon canis</em></td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

*aWITNESS® EHRLICHIA*  
*bDogs presented with at least 2 inclusion criteria but negative results for Multiplex PCR*  

---

Hematocrit of the dogs in all groups was significantly different ($p = 0.003$). The hematocrit of the *E. canis* infected group was significantly lower than the control group ($p < 0.001$) and the negative group ($p = 0.025$). Medians of the RBC in the control group and the negative group were in normal range. However, medians in the *E. canis* infected group and the other blood parasite infected group were significantly different ($p < 0.001$). In addition, the hematocrit of the *E. canis* infected group was significantly lower than the negative group ($p = 0.016$) (Table 3).

Hemoglobin of the dogs in all groups was significantly different ($p = 0.002$). The hemoglobin of the *E. canis* infected group was significantly lower than the control group ($p < 0.001$). In addition, the hemoglobin of the *E. canis* infected group was significantly lower than the negative group ($p = 0.016$) (Table 3).

**Table 2** Descriptive statistics of clinical variables of 52 dogs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low</th>
<th>Median</th>
<th>High</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>2.0</td>
<td>4.08</td>
<td>7.0</td>
<td>0.25 - 12.42</td>
</tr>
<tr>
<td>WBC (10^3 cells/μl)</td>
<td>8.3</td>
<td>10.5</td>
<td>13.0</td>
<td>0.9 - 23.4</td>
</tr>
<tr>
<td>Neutrophil (10^3 cells/μl)</td>
<td>5.9</td>
<td>7.7</td>
<td>9.8</td>
<td>0 - 16.4</td>
</tr>
<tr>
<td>Band-neutrophil (10^3 cells/μl)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 - 0.3</td>
</tr>
<tr>
<td>Lymphocyte (10^3 cells/μl)</td>
<td>1.4</td>
<td>2.0</td>
<td>3.2</td>
<td>0 - 7.0</td>
</tr>
<tr>
<td>Monocyte (10^3 cells/μl)</td>
<td>0</td>
<td>0.12</td>
<td>0.28</td>
<td>0 - 13.3</td>
</tr>
<tr>
<td>Eosinophil (10^3 cells/μl)</td>
<td>0</td>
<td>0.19</td>
<td>0.78</td>
<td>0 - 1.7</td>
</tr>
<tr>
<td>Basophil (10^3 cells/μl)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 - 0.2</td>
</tr>
<tr>
<td>RBC (10^6 cells/μl)</td>
<td>4.60</td>
<td>5.46</td>
<td>6.91</td>
<td>0.80 - 8.50</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>10.40</td>
<td>13.10</td>
<td>16.00</td>
<td>1.70 - 19.50</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>32.0</td>
<td>38.6</td>
<td>48.0</td>
<td>5.4 - 57.1</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>67</td>
<td>69</td>
<td>71</td>
<td>62 - 74</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>22</td>
<td>23.10</td>
<td>24</td>
<td>19 - 25</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.80</td>
<td>33.30</td>
<td>34.20</td>
<td>30 - 36</td>
</tr>
<tr>
<td>Pt (10^3 cells/μl)</td>
<td>68</td>
<td>126</td>
<td>232</td>
<td>7 - 386</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>14.50</td>
<td>15.30</td>
<td>16.50</td>
<td>11.50 - 22.10</td>
</tr>
<tr>
<td>Plasma protein (g/dL)</td>
<td>8.60</td>
<td>9.20</td>
<td>10</td>
<td>6 - 12</td>
</tr>
<tr>
<td>cTnT (pg/ml)</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>0 - 370</td>
</tr>
</tbody>
</table>

**Table 3** Comparisons of control group (n = 15), negative group (n = 20), *E. canis* infected group (n = 10), and other blood parasite infected group (n = 7). Statistical differences were evaluated by the Kruskal-Wallis test, followed by Mann-Whitney U post hoc analysis. Results are in frequencies or medians (interquartile range)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 15)</th>
<th>Negative (n = 20)</th>
<th><em>E. canis</em> infected (n = 10)</th>
<th>Other blood parasite infected (n = 7)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>4.3 (17.7, 7.7)</td>
<td>4.0 (2.1, 7.7)</td>
<td>3.0 (1.0, 6.2)</td>
<td>6.0 (4.0, 9)</td>
<td>0.618</td>
</tr>
<tr>
<td>WBC (10^3 cells/μl)</td>
<td>11.3 (8.9, 14.0)</td>
<td>12.0 (8.98, 13.72)</td>
<td>8.95 (7.18, 12.5)</td>
<td>8.3 (6.6, 12.2)</td>
<td>0.153</td>
</tr>
<tr>
<td>Neutrophil (10^3 cells/μl)</td>
<td>8.18 (6.37, 10.14)</td>
<td>8.20 (7.08, 10.40)</td>
<td>6.67 (3.35, 8.60)</td>
<td>5.48 (4.54, 9.27)</td>
<td>0.039*</td>
</tr>
<tr>
<td>Band-neutrophil (10^3 cells/μl)</td>
<td>0</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0.514</td>
</tr>
<tr>
<td>Lymphocyte (10^3 cells/μl)</td>
<td>2.00 (1.51, 2.27)</td>
<td>1.93 (1.49, 3.39)</td>
<td>2.98 (1.10, 3.64)</td>
<td>1.95 (1.12, 2.83)</td>
<td>0.855</td>
</tr>
<tr>
<td>Monocyte (10^3 cells/μl)</td>
<td>0.25 (0.28)</td>
<td>0 (0, 0.19)</td>
<td>0.22 (0, 0.80)</td>
<td>0.27 (0, 0.36)</td>
<td>0.099</td>
</tr>
<tr>
<td>Eosinophil (10^3 cells/μl)</td>
<td>0.29 (0.09, 0.78)</td>
<td>0.28 (0.02, 0.95)</td>
<td>0.04 (0, 0.19)</td>
<td>0.57 (0.05, 0.82)</td>
<td>0.120</td>
</tr>
<tr>
<td>Basophil (10^3 cells/μl)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0.309</td>
</tr>
<tr>
<td>RBC (10^6 cells/μl)</td>
<td>6.50 (6.20, 7.40)</td>
<td>6.27 (4.53, 7.15)</td>
<td>3.95 (2.43, 5.15)</td>
<td>5.20 (4.70, 7.00)</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>15.30 (14.10, 17.00)</td>
<td>14.25 (10.78, 16.45)</td>
<td>9.45 (5.93, 12.28)</td>
<td>12.90 (10.40, 16.30)</td>
<td>0.003**</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>45.8 (42.7, 51.1)</td>
<td>43.2 (32.2, 49.8)</td>
<td>27.8 (17.8, 36.6)</td>
<td>37.4 (32.0, 48.5)</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>69 (68, 72)</td>
<td>69 (67, 71)</td>
<td>68 (66, 71)</td>
<td>69 (65, 72)</td>
<td>0.825</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>23.4 (22.6, 24.1)</td>
<td>22.8 (20.2, 23.7)</td>
<td>23.2 (21.4, 24.4)</td>
<td>23.0 (21.5, 24.7)</td>
<td>0.749</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>33.8 (32.7, 34.3)</td>
<td>33.1 (32.6, 33.8)</td>
<td>33.3 (32.8, 34.6)</td>
<td>33.3 (33.0, 34.4)</td>
<td>0.649</td>
</tr>
<tr>
<td>Pt (10^3 cells/μl)</td>
<td>270 (232, 334)</td>
<td>99 (72, 133)</td>
<td>54 (28, 114)</td>
<td>127 (67, 172)</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>15.1 (14.7, 15.6)</td>
<td>15.3 (14.1, 16.1)</td>
<td>17.2 (14.8, 17.5)</td>
<td>15.4 (14.3, 16.6)</td>
<td>0.208</td>
</tr>
<tr>
<td>Plasma protein (g/dL)</td>
<td>9.0 (8.1, 9.2)</td>
<td>9.4 (8.3, 10.4)</td>
<td>10.1 (8.8, 11.6)</td>
<td>9.6 (9.0, 10.6)</td>
<td>0.080</td>
</tr>
<tr>
<td>cTnT (pg/ml)</td>
<td>0.004 (0.003, 0.007)</td>
<td>0.004 (0.003, 0.005)</td>
<td>0.005 (0.004, 0.015)</td>
<td>0.003 (0.003, 0.005)</td>
<td>0.703</td>
</tr>
</tbody>
</table>
Platelets of the dogs in all groups were significantly different \((p < 0.001)\). The platelets of the negative group \((p < 0.001)\), *E. canis* infected group \((p < 0.001)\) and the other blood parasite infected group \((p = 0.01)\) were significantly lower than the control group. Median of the platelets in the control group were in normal range. In contrast, medians of the platelets in the negative, *E. canis* infected group and the other blood parasite infected group were thrombocytopenia (Table 3).

There was no difference in serum cTnT concentrations among the control group, negative group, *E. canis* infected group, and other blood parasite infected group \((p = 0.70)\). Median and interquartiles of serum cTnT concentrations in each group are shown in Table 3.

Correlation between cTnT and age and hematology values are displayed in Table 4. Serum cTnT was correlated with age in 52 dogs (Spearman’s correlation coefficients \(r_s = 0.42, p = 0.002\)) and the correlation was stronger in the control dogs exclusively (Spearman’s correlation coefficients \(r_s = 0.75, p = 0.001\)).

Serum cTnT was weakly negatively correlated with lymphocytes in all dogs (Spearman’s correlation coefficients \(r_s = -0.32, p = 0.02\)). This negative correlation was stronger in the control dogs exclusively (Spearman’s correlation coefficients \(r_s = -0.77, p = 0.001\)) and was weaker in the dogs with *E. canis* exclusively (Spearman’s correlation coefficients \(r_s = -0.65, p = 0.04\)) (Table 4). Serum cTnT was weakly negatively correlated with RBC in all dogs (Spearman’s correlation coefficients \(r_s = -0.29, p = 0.03\)).

**Discussion**

Troponin T is detected in the blood at 4 to 12 hours after acute myocardial infarction. The mean hours to peak is between 10 to 24 hours, and return to baseline at 5 to 10 days (Goldmann et al., 2001). The serum half-life of cTnT is around 4 hours. Thus, the prolong detection of serum cTnT may suggest a continuing release due to myocardial damage (Herman et al., 1999). In human, cTnT has been employed as a biomarker for myocardial injury due to its sensitivity and specificity (Babuin and Jaffe, 2005). In veterinary medicine, increase in serum troponin level can be detected in patients with various cardiac and noncardiac diseases such as gastric dilatation and volvulus (Schober et al., 2002), pyometra (Hagman et al., 2007), cardiac contusion (Schober et al., 1999), babesiosis (Lovett et al., 2002), acquired and congenital cardiac diseases (Oyama and Sisson, 2004; Spratt et al., 2005), arrhythmogenic right ventricular cardiomyopathy in boxer (Baumgart et al., 2007), experimental infarction (Ricchiuti et al., 1998), and pericardial effusion (Shaw et al., 2004; Spratt et al., 2005; Linde et al., 2006).

In human medicine, the high-sensitivity cardiac troponin T (hs-cTnT) has been introduced as a gold standard to diagnose myocardial infarction (Santalo et al., 2013). In addition, the high value of hs-cTnT is related with increasing risk of cardiovascular problems in patients with hypertrophic cardiomyopathy (Kubo et al., 2013). In acute ischemic stroke patients, the serum cTnT levels were increased above the normal limit about 36.4% of the patients in the study group (Kral et al., 2013). Furthermore, both cTnT and cTnI are effectively used for identifying doxorubicin-induced myocardial injury (Reagan et al., 2013). Unfortunately, the hs-cTnT cannot improve the diagnostic performance of acute coronary syndrome, yet the acute myocardial infarction detection sensitivity is considerably good (Borna et al., 2013). Together, these informations demonstrated the appropriate utilization of cTnT for the diagnosis of myocardial problem in human.

In a previous study, cTnI was employed as a myocardial damage indicator in Brazilian dogs with an acute *E. canis* infection (Diniz et al., 2008). The results suggested that *E. canis* infection was a risk factor for cardiac injury associated with severe anemia.

**Table 4** Spearman’s correlation coefficients \((r_s)\) of cTnT and age, hematological values. Correlation coefficients were illustrated for all dogs, control dogs, negative dogs, *E. canis* infected dogs and other blood parasite infected dogs.
and systemic inflammatory response syndrome (SIRS) (Diniz et al., 2008). Together, it was hypothesized that the pathogenesis of a canine cardiac injury with ehrlichiosis involved the vasculitis, myocardial hemorrhages, tenticul hypoperfusion, and intense inflammation (Diniz et al., 2008). However, there is no evidence of E. canis infected cardiomyocytes, thus, the mechanism of cardiac cell injury and the troponin release into the bloodstream is still unknown (Diniz et al., 2008).

In the present study, the serum cTnT levels in CME were determined. The results showed that serum cTnT concentrations were not different among the 4 groups (p = 0.70), implying that there is no evidence of severe myocardial injury in dogs with natural occurring E. canis infection. However, the serum cTnT was correlated with age of the dogs, similar to one study in humans (Chapelle et al., 2002). This suggested that patients in advanced age might develop subclinical myocardial lesions and continuous release of the cTnT. This evidence may be applied to that occurred in dogs.

Interestingly, serum cTnT was negatively correlated with lymphocytes and RBCs. Indeed, previous studies showed that serum cTnT was increased in anemic dogs because myocardial ischemia induced myocardial injury (Ohman et al., 1996; O’Brien et al., 1997 a). However, the relationship between lymphocyte count and serum cTnT level remains unclear and needs further investigation.

We hypothesized that the severity of myocardial damage might be related to the phase of CME because dogs infected with E. canis in acute phase and anemic dogs had a higher risk of myocardial injury (Diniz et al., 2008). The lack of circulating cTnT suggests that although some myocardial disease is present, it may not severe enough to cause an increase in serum cTnT concentrations (DeFrancesco et al., 2002) or it may not cause ongoing cardiac injury (Serra et al., 2010). Furthermore, due to the serum half-life of cTnT, the length of time after myocardial injury may be involved (Alpert et al., 2000). Further studies are indeed required to confirm the association of E. canis infection and myocardial injury in dogs.

**Conclusion**

In conclusion, serum cTnT levels were not increased in dogs naturally infected with E. canis. However, it was positively correlated with age and negatively correlated with RBC. This suggest that anemia may cause an increase in serum cTnT level, and it should be treated with caution in older dogs infected with CME.

**Acknowledgements**

We gratefully acknowledge animal patients and owners for the results and would like to thank all staff of the Monitoring and Surveillance Center for Zoonotic Disease in Wildlife and Exotic Animals (MoZWE) for their help. This project was supported by the Faculty of Veterinary Science, Mahidol University, Thailand and the Zoetis (Thailand) Ltd.

**References**


DeFrancesco TC, Atkins CE, Keene BW, Coats JR and Hauck ML 2002. Prospective clinical


Discrimination between Tropical Bed Bug *Cimex hemipterus* and Common Bed Bug *Cimex lectularius* (Hemiptera: Cimicidae) by PCR-RFLP

Apiwat Tawatsin 1  Kittitouch Lorlertthum 2  Atchara Phumee 3  Usavadee Thavara 1  Jotika Boon-Long 1  Rungfar Boonserm 2  Padet Siriyasatien 2,4*

Abstract

Bed bugs, *Cimex hemipterus* and *Cimex lectularius*, are common blood-sucking ectoparasites of human and currently found in many countries around the world. In Thailand, both species have been found mostly in hotels in tourist attraction areas and the insecticide resistance of these insects was also documented. To date, identification of these two bed bug species is based on morphological taxonomy, a technique which requires expertise and in some instance is difficult especially for immature bed bugs or incomplete bed bug samples. In this study, we analyzed the cytochrome c oxidase subunit I (COI) gene of bed bugs, *C. hemipterus* and *C. lectularius* collected from various regions of Thailand. PCR-RFLP and phylogenetic analysis demonstrated that the COI gene could significantly differentiate between the two bed bug species. Moreover, the phylogenetic tree could separate clusters of insecticide resistant from insecticide susceptible *C. lectularius* strains. However, sequence analysis of *C. hemipterus* showed no significant intra-specific variation from different geographical regions of Thailand. Data obtained from this study will be valuable for epidemiological distribution of bed bugs in Thailand and subsequently for the most effective control of these insects.

Keywords: bed bug, cytochrome c oxidase subunit I gene, phylogenetic tree, PCR-RFLP

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บทคัดย่อ

การจำแนกตัวเรือดสายพันธุ์ Cimex hemipterus และ Cimex lectularius โดยวิธี PCR-RFLP

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ตัวเรือดชนิด Cimex hemipterus และ Cimex lectularius เป็นปรสิตภายนอกที่ดูดเลือดรบกวนมนุษย์และพบได้ในหลายประเทศทั่วโลก ยังพบในประเทศไทยในโรงแรมที่เป็นแหล่งท่องเที่ยวที่สำคัญและมีรายงานว่าตัวเรือดตัวต่อมีการติดต่อกันและมีการกระจายในพื้นที่ที่มีการบินของแมลง การจัดจำแนกชนิดของตัวเรือดดังกล่าวไม่สามารถทำได้โดยอาศัยลักษณะทางสัณฐานวิทยา เทคนิคนี้ต้องการผู้เชี่ยวชาญและในบางกรณีเกิดความยุ่งยาก เช่นในระยะตัวอ่อน หรือตัวอย่างที่ไม่สมบูรณ์ รายงานนี้จึงทำให้การศึกษาในตัวเรือดมีความน่าสนใจ การศึกษาจะต้องใช้วิธีบินการตรวจสอบยีนในตัวเรือดชนิด C. hemipterus และ C. lectularius ที่เก็บตัวอย่างมาจากภูมิภาคต่างๆของประเทศไทย และพบว่าโดยวิธี PCR-RFLP และแผนภูมิต้นไม้ในยีน COI สามารถแยกความแตกต่างระหว่างตัวเรือดชนิดได้อย่างมีนัยสำคัญ นอกจากนี้ยีน COI สามารถแยกตัวเรือดชนิด C. lectularius กลุ่มที่มีการต้านทานกับสารเคมีกีฏ쁨ออกจากกลุ่มที่ไม่ตัวต่อม สารเคมีได้อย่างมีนัยสำคัญ ตัวเรือดชนิด C. hemipterus มีความหลากหลายทางพันธุกรรมอยู่ในภูมิภาคต่างๆของประเทศไทย ข้อมูลที่ได้จากการศึกษาจะมีประโยชน์ในด้านระบาดวิทยาของตัวเรือดในประเทศไทยและช่วยให้การควบคุมตัวเรือดดำเนินไปอย่างมีประสิทธิภาพสูงสุด

คำสำคัญ: ตัวเรือด ยีนไซโตรโครม ซีออกซิเดส หน่วยย่อยที่ 1 (COI) แผนภูมิต้นไม้ PCR-RFLP

Introduction

Bed bugs (Hemiptera: Cimicidae) are important blood-sucking ectoparasites of human. Two major bed bug species feed on human blood; Cimex hemipterus, the tropical bed bug and Cimex lectularius, the common bed bug (Harlan et al., 2008). The first report of bed bug was from England in 1583 (Kemper, 1936). During the second half of the 20th century bed bugs were rare in North America and Western Europe (Ryan et al., 2002); however, in recent years they have increased in many parts of the world (Krueger and Paul, 2000; Bates, 2000; Potter et al., 2010; Criado et al., 2011). This may be due to the increase in human migration especially tourism industry and development of insecticides resistance of the insects (Romero et al., 2007; Krause-Parello and Sciscione, 2009). It is likely that the bed bugs were transported on clothes in luggages of travelers (Delaunay and Pharm, 2012).

Bed bugs require blood for development of nymphs to the next developmental stages (Johnson, 1941) and for reproduction of adults. Female bed bug produces 5-7 eggs per week with approximately 200-500 eggs in her lifetime, and adults can survive for up to a year without feeding (Pinto et al., 2007). Bed bugs live in cracks and crevices around bed or wooden furniture in hotels, hostels, private homes, trains, and cruise ships (Delaunay et al., 2011) and they can spread easily from shelter to shelter (Stephen et al., 2005). They are notorious as pests that crawl out at night to bite and feed on human blood. Although there has been no scientifically-based evidence showing that bed bugs transmit diseases (Dolling, 1991), people who are bitten may suffer from intense itch, inflammation, allergic symptoms and psychological effects (Usinger, 1966; O’Neill et al., 1997; Doggett and Russell, 2009).

In Thailand, bed bugs had disappeared from the country for decades. Until recently, bed bugs were found in hotels in tourist attraction areas in different regions of the country and these bed bugs were resistant to various insecticides, especially those in the pyrethroid group (Tawatsin et al., 2011). In fact, C. hemipterus was resistant to DDT since 1970s (WHO, 1976) and C. lectularius showed resistance to bifenthrin and α-cypermethrin recently (Suwannayod et al., 2010).

Identification of bed bugs in Thailand has been based on insect morphology. Although this
procedure can identify adult stage easily, it is very difficult in immature stages or eggs (Kolb et al., 2009). Moreover, taxonomic identification requires highly experienced person and complete samples of bed bugs. Nowadays, molecular techniques have been developed for taxonomic identification such as nucleotide sequence analysis, phylogenetic tree, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Kress and Erickson, 2008). These techniques are fast, accurate and highly sensitive; moreover, it can be performed for species identification in immature stages, cast skins or incomplete samples of bed bugs from the fields. In this study, we demonstrate the utility of mitochondrial cytochrome c oxidase subunit I (COI) gene for discrimination by PCR-RFLP and phylogenetic analysis of these two main important gene for discrimination by PCR-RFLP and strains were also reported.

Materials and Methods

Bed bug collections and rearing: Bed bugs, C. hemipterus and C. lectularius, in this study were collected from hotels in different parts of Thailand; Central (Bangkok), Northern (Chiang Mai and Phitsanulok), North-Eastern (Ubon Ratchathani), and Southern (Phuket and Krabi). Insecticide susceptible strain (from Tokyo) and insecticide resistant strain (from Chiba) of C. lectularius (provided by Dr. Mamoru Watanabe) were also used in this study. The bed bugs were identified using morphological keys described by Pratt and Stojanovich (1967). The insects were maintained in laboratory of the Biology and Ecology Section, National Institute of Health, Department of Medical Sciences, Thailand. Environmental conditions of the rearing room were set at 26-28°C, 60-80% RH, and a photoperiod of 12 : 12 (L : D) hour. The bed bugs were reared in plastic cups covered with fine mesh chiffon cloth. A piece of cardboard (4 x 8 cm) was put inside the cups for the bed bugs to crawl up and insert their mouthparts through the mesh top to feed. For blood feeding, the bed bugs had access to artificial feeding unit for 30 minutes, using almost expired donated-blood through the mesh top to feed. For blood feeding, the bed bugs had access to artificial feeding unit for 30 minutes, using almost expired donated-blood received from Blood Bank, Thai Red Cross. This method was modified from that developed by Montes et al. (2002).

DNA extraction: Individual bed bug of each sample was lysed by lysis buffer and placed in liquid nitrogen for 1 minute and then ground with a sterile plastic pestle. Genomic DNA was isolated using DNA extraction kits: Invisorb® Spin Tissue Mini Kit (STRATEC Molecular GmbH, Germany) according to the manufacturer’s instructions. The extracted DNA was eluted in 100 µl of elution buffer; the fraction of extracted DNA was spectrophotometrically quantitated using a Nanodrop 2000c (Thermo-scientific, USA). The extracted DNA samples were kept at -80°C for long term storage.

PCR amplification: Sequences of the COI gene of C. hemipterus and C. lectularius were obtained from GenBank with Accession number GU985538.1 and GU985525.1, respectively (Balvin and Vilimova, 2010). The sequences were aligned using the multiple alignment programs ClustalX version 1.81 (Thompson et al., 1997). Degenerate oligonucleotide primers were designed as forward primer (5’ GMCAACCTGGCTCATTATGG 3’) and reverse primer (5’ ATAAAGTTGTYAWAGWARAGG 3’). Primers were synthesized by 1st BASE Oligonucleotide (Oligo) Synthesis services company (1st BASE Laboratories, Malaysia). The amplification reaction was set up in a final volume of 25 µl, containing approximately 100 ng of extracted DNA. Polymerase chain reactions (PCR) were performed in a GeneAmp PCR system 2400; Applied Biosystems®, USA. The reaction conditions are as follows: denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 48°C for 1 min, and 72°C for 1 min and final extension at 72°C for 7 min. Aliquots of the amplicons were analyzed on a 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized with Quantity One quantification analysis software version 4.5.2 Gel Doc EQ system (Bio-Rad, CA, USA).

DNA sequencing and RFLP patterns prediction: The PCR amplicons were ligated into pGEM-T Easy Vector (Promega, USA). The ligated vectors were transformed into DH5α strain competent cells, and then the chimeric plasmids were screened by blue-white colony selection system. The suspected positive colonies were cultured and used for further plasmid DNA extraction by using Invisorb® Spin Plasmid Mini kit (STRATEC Molecular GmbH, Germany) following the manufacturer’s instructions. Purified plasmids were sent to sequence by 1st BASE DNA sequencing services (1st base laboratories, Malaysia) using universal forward T7 primer. Nucleotide sequences were analyzed using BioEdit Sequence Alignment Editor Version 7.0.9.0 (Hall, 1999) and the consensus sequences were BLAST search (available at http://www.ncbi.nlm.gov/BLAST) for species identification. The nucleotide sequences of COI gene obtained from this study were submitted to the GenBank database. The resulting sequences were used for prediction of species-specific restriction sites by using NEBcutter V2.0 web-based program (available at http://www.neb.com/NEBcutter2/index.php). From restriction prediction data, BfaI restriction enzyme recognize 5′...C/T A G...3′ sites were chosen for PCR-RFLP.

PCR-RFLP: The PCR products were digested in separate reaction with BfaI (Thermo-scientific, USA). The reaction mixture was incubated at 37°C for 15 min followed by heat inactivation at 65°C for 5 min. The restriction products were electrophoresed through 8% native polyacrylamide gel electrophoresis run at 100 V for 70 min (MiniProtein 3 cell; Bio-Rad®, USA), followed by ethidium bromide staining and visualized on a Quantity One quantification analysis software version 4.5.2 Gel Doc EQ system (Bio-Rad, CA, USA).

Sequence variation and Phylogenetic tree construction: The nucleotide sequences of each
species from various regions were aligned for variation positions. Phylogenetic tree were constructed by Maximum-likelihood method using Kimura’s 2-parameter model implemented in MEGA© version 5.1 (Tamura et al., 2011). The reliability of an inferred tree was tested by 1000 bootstrap. *Triatoma dimidiata* (Kissing bug) accession no. JQ575031 as outgroup.

**Results**

In this study, 15 bed bugs were collected from 6 different regions of Thailand and 2 samples were provided from Japan. PCR amplicons of the COI gene from *C. hemipterus* and *C. lectularius* were approximately 580 bp in size (Fig 1). Amplified COI gene sequences obtained from this study varied from 576 to 581 bp. Consensus COI gene sequences of *C. hemipterus* and *C. lectularius* were blast in the GenBank database and showed the percentage identity range from 99 to 100. The nucleotide sequences showed maximum intra-specific variation approximately 0.8% in *C. hemipterus* and 0.6% in *C. lectularius*; nevertheless, the minimum inter-specific variation showed approximately 19.6% (data not shown). The nucleotide sequences of COI gene from the bed bugs were submitted to GenBank and accession numbers of JX826468 to JX826482 were assigned (Table 1).

The COI sequences of *C. hemipterus* collected from various parts of Thailand could be grouped into three groups (CH1, CH2 and CH3). The sequences variation was found at position 380 and deletion of three bases of COI gene between positions 445-447 were found in CH1 and CH3 (Fig 2). COI gene sequence of *C. lectularius* collected from Ubon Ratchathani was 100% identical to the insecticide susceptible *C. lectularius* from Tokyo isolated. Variations of COI gene sequences of *C. lectularius* between insecticide resistant and susceptible strains were found at position 96, 146 and 204 in this study (Fig 3).

<table>
<thead>
<tr>
<th>Location</th>
<th>Isolated code</th>
<th>Bed bug species</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chiang Mai</td>
<td>C.H.agri1</td>
<td><em>C. hemipterus</em></td>
<td>JX826468</td>
</tr>
<tr>
<td></td>
<td>C.H.gate2</td>
<td><em>C. hemipterus</em></td>
<td>JX826469</td>
</tr>
<tr>
<td></td>
<td>C.H.sarin3</td>
<td><em>C. hemipterus</em></td>
<td>JX826470</td>
</tr>
<tr>
<td></td>
<td>C.H.cm1-5</td>
<td><em>C. hemipterus</em></td>
<td>JX826471</td>
</tr>
<tr>
<td></td>
<td>C.H.cm2-6</td>
<td><em>C. hemipterus</em></td>
<td>JX826472</td>
</tr>
<tr>
<td>Phuket</td>
<td>C.H.phu7</td>
<td><em>C. hemipterus</em></td>
<td>JX826473</td>
</tr>
<tr>
<td>Krabi</td>
<td>C.H.kb8</td>
<td><em>C. hemipterus</em></td>
<td>JX826474</td>
</tr>
<tr>
<td>Bangkok</td>
<td>C.Hbk 9-1</td>
<td><em>C. hemipterus</em></td>
<td>JX826476</td>
</tr>
<tr>
<td>Phitsanulok</td>
<td>C.H.plh1</td>
<td><em>C. hemipterus</em></td>
<td>JX826478</td>
</tr>
<tr>
<td>Tokyo (susceptibility)</td>
<td>C.L.Tokyo</td>
<td><em>C. lectularius</em></td>
<td>JX826480</td>
</tr>
<tr>
<td>Chiba (resistance)</td>
<td>C.L.Chiba</td>
<td><em>C. lectularius</em></td>
<td>JX826481</td>
</tr>
<tr>
<td>Ubon Ratchathani</td>
<td>C.L.2</td>
<td><em>C. lectularius</em></td>
<td>JX826482</td>
</tr>
</tbody>
</table>

**Figure 1** 8% native polyacrylamide gel electrophoresis shows PCR-RFLP patterns of COI product digested with BfaI restriction enzyme. Lane1-2: undigested PCR products amplified from *C. lectularius* and *C. hemipterus*, respectively. RFLP patterns of *C. lectularius*: 120 and 459 bp (lane 3-5); *C. hemipterus*: 57, 168, and 351 bp (lane 6-7) and mixed DNA of both species: 57, 120, 168, 351 and 459 bp (lane 8) from BfaI digestion. Lane M: 25 bp DNA standard marker.

**Figure 2** Nucleotide sequence comparison of COI genes of *C. hemipterus*, based on these sequences they can be classified into three groups: CH1 represented sequence of *C. hemipterus* isolates C.H.sarin3, CMU4 and agr1 from Chiang Mai; CH2 represented sequence of *C. hemipterus* isolates C.H.cm2-6, gate 2 and C.H.cm1-5 from Chiang Mai, *C. hemipterus* isolates C.H.kb8 from Krabi, *C. hemipterus* isolate C.Hbk 9-1 from Bangkok, and *C. hemipterus* isolates C.H.phu7 from Phuket; CH3 represented sequence of *C. hemipterus* isolates C.Hbk10-2 and C.H.12 isolate from Bangkok and *C. hemipterus* isolate C.H.plh1 from Phitsanulok.
Figure 3 Nucleotide sequence comparison of COI genes of insecticide resistant and susceptible C. lectularius strains; C.L.Tokyo: C. lectularius isolate C.L.Tokyo (susceptibility); C.L.2 Ubon: C. lectularius isolate C.L.2 from Ubon Ratchathani; C.L.Chiba: C. lectularius isolate C.L.Chiba (resistant).

Figure 4 Maximum-likelihood trees were constructed using GTR + G + I evolution model of COI gene in 12 isolates of C. hemipterus and 3 isolates of C. lectularius. T. dimidiata accession no. JQ575031 sequences were used as outgroup.

RFLP pattern of these two species were predicted by the NEBcutter V2.0 web-based program and BfaI restriction was selected. PCR products of each species were digested with the BfaI restriction enzyme and the fragments separated by 8% native polyacrylamide gel electrophoresis. The results demonstrated the PCR product of approximate 579 bp in length for both species, C. hemipterus (57, 168, and 351 bp) and C. lectularius (120 and 459 bp) and mixed DNA of both species (57, 120, 168, 351 and 459 bp) (Fig 1).

The constructed phylogenetic tree could clearly separate two major clades of C. hemipterus and C. lectularius although they belonged to the same genus. However, all of the C. hemipterus isolates clustered together showing no significant difference between different regions because of minor nucleotide variations between the same species. On the contrary, in C. lectularius, the insecticide resistant strain from Chiba was separated from Tokyo (insecticide susceptibility) and Ubon Ratchathani strains by the phylogenetic construction (Fig 3).

Discussion

Nowadays, there are increasing reports of bed bug infestation and resistance to various insecticides has also been documented. Two species of bed bugs, C. hemipterus and C. lectularius, are found in Thailand and they become resistant to various insecticides (Tawatsin et al., 2011). Ghauri (1973) revealed that two species of bed bugs can be distinguished by looking at the first segment of the thorax, which expanded more laterally and of which the extreme margins are more flattened in C. lectularius than C. hemipterus. Several reports suggested that the type of insecticide resistance were different between bed bug strains (Karunaratne et al., 2007; Romero et al., 2007; Kilpinen et al., 2011). Therefore, taxonomic identification is important with necessary specialized taxonomic expertise. Molecular techniques are commonly used to apply in research labs worldwide for species identification such as sequence, phylogenetic tree analysis and PCR-RFLP in order to identify reliably and practically. This study used DNA-based identification by application of COI sequences for differentiation of bed bugs. COI is a mitochondrial gene which is conserved in arthropods,
species specific and has relatively high degree of genetic variation. We demonstrated the value of PCR-RFLP to differentiate two bed bug’s species. This result showed that intra-specific polymorphism was not observed here by digestion with BfaI restriction enzyme as well as RFLP could be used for mix samples of two bed bugs. This benefit can help the survey of the bed bugs, when only cast skins or eggs as well as carcass damage of bed bugs are found. The PCR-RFLP can potentially lead to supersede taxonomic misidentification errors. In addition, phylogenetic tree showed the monophyletic clade in each species. According to intra-specific variation analysis, we found intra-variation in C. hemipterus (approximately 0.8%) because the sequence showed indeling of TAT base position, which could be confirmed by pick individual 10 colonies for sequencing. C. hemipterus can be grouped into 3 groups based on sequence variations. CH1 was found only in Chiang Mai, CH 2 was found in Chiang Mai, Bangkok, Krabi and Phuket, and CH3 was found in Bangkok and Phitsanulok (Fig 2). The study of C. lectularius indicated minor nucleotide variations between the insecticide resistant and susceptible strains in Japan as well as insecticide resistant isolates single branch of insecticide susceptible and C. lectularius in Thailand. This study is the first to report that the COI gene sequences were different between insecticide resistant (Chiba) and susceptible (Tokyo) strains of common bed bugs, C. lectularius collected from Ubon Ratchathani used in our study was susceptible to various insecticides (unpublished data) and the COI sequences were 100% identical to the insecticide susceptible isolates from Tokyo (Fig 3). Susceptible strain of C. hemipterus was unavailable in our laboratory, so it was not included in this study. Furthermore, this study investigated only 2 cosmopile species, therefore, studies in other species such as C. columbarius, C. pipistrelli, C. dissimilis, and Oeciacus should be conducted as well as more collected samples from different geographical regions.

In conclusion, we demonstrated the ability of PCR-RFLP to discriminate between common bed bug and tropical bed bug. The sequence data obtained from the study showed minor variation between the same bed bug species. However, COI sequences of C. lectularius were different between insecticide resistant and insecticide susceptible strains. The sequence data from this study will be useful for epidemiological studies and for proper planning for effective bed bug control in the future.

Acknowledgements

This work was supported by Integrated Innovation Academic Center (IIAC), Chulalongkorn University Centenary Academic Development Project, the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR1160A-56), Ratchadapiseksompoch Fund, Faculty of Medicine, Chulalongkorn University (Grant No. RA (MF) 02/56) and Thailand Research Fund to W. Choochote (TRF Senior Research Scholar: RTA5480006).

References


Viability and Growth of Preantral Follicles Derived from Cryopreserved Ovarian Tissues of a Cheetah (*Acinonyx jubatus*) Post-mortem

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**Abstract**

This study aimed to investigate freezing effects of ovarian tissues on survival of preantral follicles and observing in vitro growing of preantral follicles retrieved from cryopreserved ovarian cortical tissues of a cheetah post-mortem. After 29-hour cold storage, ovarian cortices were cut into small pieces (2.0 x 2.0 x 1.0 mm³) and allocated to be frozen using a passive cooling container (n = 3 pieces) or vitrification (n=3 pieces). After one year of storage, 43 (10/23) and 21% (12/58) follicles isolated from ovarian tissues cryopreserved using a passive cooling device (slow freezing rate) and vitrification, respectively, were viable (positively stained with neutral red). Thereafter, the viable follicles were in vitro grown in a culture medium containing M199 supplemented with growth hormone (GH), follicular-stimulating hormone (FSH), insulin-like growth factor I (IGF-I) and activin A for 7 days. Diameters and diameter gains were examined on Days 0, 3 and 7. Follicle viability was assessed on Days 0 and 7. Diameters of follicles frozen by the slow freezing decreased gradually from 53.5±14.2 µm on Day 0 to 50.9±17.1 µm with 2 out of 10 viables, whereas those frozen using vitrification maintained their diameters between 50.7±15.6 µm and 50.5±17.9 µm on Days 0 and 7, respectively, with 2 of 12 viable. In conclusion, the passive cooling container is suggested to perform a slow freezing rate for ovarian tissue cryopreservation. Although the cheetah ovarian follicles obtained from cryopreserved tissues can be grown in vitro for 7 days, optimization of culture medium is required to improve the viability and growing rate.

**Keywords:** feline, freezing, gamete rescue

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Introduction

Preantral follicle culture is a promising approach to restore fertility in human and endangered animals (Smitz et al., 2010). This tool provides great opportunity to preserve child-bearing potential in cancer patients, particularly pre-pubertal women prior to receiving a radiation or chemotherapy for treatment of cancers (Rodriguez-Wallberg and Oktay, 2012). Restoration of fertilizable follicles from cryopreserved ovarian tissues is feasible through a tissue re-implantation or follicle in vitro culture. Although ovarian implantation has exhibited achievements with birth of 18 human babies (Andersen et al., 2012; Wiedemann et al., 2012), risk of reintroduction of malignant cells back to recipients has been concerned (Kim, 2010). In rare animal species, female gametes may be recovered when animals die accidentally or underwent spaying for medical reasons (Jewgenow and Paris, 2006). Rescue of gamete and ovarian cryopreservation followed by in vitro follicle culture offers promising approach for fertility preservation in these valuable animals. Live birth has been reported from in vitro culture of cryopreserved ovarian follicles in mice (Wang et al., 2011).

Decrease in populations mainly by habitat destruction and poaching, cheetah (*Acinonyx jubatus*) is classified as vulnerable by International Union for Conservation of Nature (IUCN, 2012). Although practical efforts with assisted reproductive techniques...
(ART) including induction of folliculogenesis using hormones, in vitro oocyte maturation (IVM), in vitro fertilization (IVF), artificial insemination (AI) and embryo transfer (ET) have been utilized to improve reproductive capacity in captive population, success in giving live offspring of this vulnerable species is low (Pelican et al., 2006). Therefore, alternative approaches by conserving immature follicles in ovarian tissues to enhance propagation of the cheetah are essential to ensure existence of the species in the future. Current protocols for ovarian tissue cryopreservation have been categorized into 2 methods; conventional slow freezing and rapid freezing or vitrification (Isachenko et al., 2007). While the slow freezing has been employed as a standard protocol for preserving oocytes and various somatic tissues, vitrification is an alternative technique requiring lesser steps of freezing and freezing equipment, consequently simplifying cryopreservation process. Comparative outcomes of slow freezing and vitrification promoting ovarian graft survival after transplantation have been exhibited in monkeys (Yeoman et al., 2005) and mice (Kim et al., 2011). In felids, successful ovarian cryopreservation has been demonstrated in domestic cats (Lima et al., 2006) and lions (Wiedemann et al., 2012) using programmatic slow freezing. Recently, a passive freezing container has replaced a programmable freezer to convey the slow freezing rate. However, application of the device has not been investigated in cats.

In vitro follicle culture in felids has been reported in the domestic cats indicating beneficial effects of culture medium, proteins, gonadotropins and ovarian growth factors on promoting preantral follicle growth and viability (Jewgenow and Pitra, 1993; Jewgenow, 1996; Wongbandue et al., 2013). In addition, secondary follicles were shown capable of developing to antral stage (Jewgenow and Pitra, 1993). However, these investigations limited only in preantral follicles collected from fresh ovarian tissues of the domestic cats, and have not been studied in other wild species. This study aimed to: 1) compare two freezing methods for ovarian tissues retrieved from a cheetah post-mortem, and 2) perform in vitro growing of preantral follicle extracted from cryopreserved ovarian tissues.

Materials and Methods

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Company (St. Louis, MO, USA), unless otherwise stated.

Animal and collection of ovaries: A pair of ovary was obtained from a cheetah which died in captivity at Khaokew Open Zoo (Chonburi, Thailand). Ovaries were removed after 3 hours of necropsy, stored in 0.9% (v/w) normal saline solution (NSS) at 4°C, and shipped to the laboratory in 29 hours. Upon arrival, connective tissues and blood vessels were trimmed off and ovaries were washed 3 times in NSS supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Each ovary was cut into small pieces of 2.0x2.0x1.0 mm³. Pieces of cortical tissues were then allocated to be frozen using a passive cooling container (n = 3) or vitrification (n = 3).

Cryopreservation

Slow freezing and thawing: Ovarian cortices were incubated in cryopreservation medium containing 1.5 M dimethyl sulphoxide (DMSO) and 0.1 M sucrose in phosphate buffered solution (PBS) at 4°C for 15 min. Thereafter, the tissues were placed into cryovials and incubated for 15 min at 4°C. Next, vials containing ovarian cortices were transferred into pre-cooled passive cooling device (Coolcell, Biocision, Lakspur, CA, USA) and placed in -80°C freezer for 24 hours to achieve cooling rate of -1°C/min. Afterward, frozen vials were stored in liquid nitrogen (-196°C) until analyzed. Thawing of cryopreserved tissues was performed according to Cleary et al. (2001). In brief, cryovials were removed from liquid nitrogen and placed in a water bath at 37°C for 3 min. The tissues were then incubated in a thawing medium (PBS added with 0.75 mol/l DMSO and 0.2 mol/l sucrose) for 10 min at room temperature before being transferred to a dissection medium (M199 supplemented with 25 mM HEPES, 0.23 mM sodium pyruvate, 2 mM L-glutamine, 0.3% (w/v) bovine serum albumin (BSA), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin).

Vitrification and warming: Vitrification of ovarian tissues was modified from a previous report using this method for freezing testicular tissue (two-step freezing technique) (Thuwanut and Chatdarong, 2012). The tissues were incubated in an equilibration medium containing HEPES M199 supplemented with 7.5% (w/v) DMSO, 7.5% (w/v) ethylene glycol (EG) and 20% (v/v) fetal calf serum (FCS) at room temperature for 15 min, followed by transferring into a vitrification medium (HEPES M199 supplemented with 15% (w/v) DMSO, 15% (w/v) EG and 0.5 M sucrose) at 4°C for 15 min. Subsequently, the ovarian tissues were immersed in liquid nitrogen. For thawing, cryovials were immersed in a waterbath at 37°C for 3 min. Thawed tissues were then placed into a warming medium containing 1 M sucrose and 20% FCS in HEPES M199 at 37°C for 10 min.

Preantral follicle isolation and selection: Preantral follicles were isolated from ovarian tissues by mechanical technique. In brief, frozen-thawed ovarian tissues were placed on a petridish containing the dissection medium. Thereafter, they were held with surgical forcesps and finely sliced with surgical blades and needles. Isolated preantral follicles of normal morphology, characterized by an intact basement membrane, round or oval in shape, and absence of pigmented granulosa cells were selected under a stereomicroscope (SMZ645, Nikon, Japan) at x 50 magnification. Follicle viability was determined by staining with 50 μg/ml neutral red (38°C, 20 min) (Wongbandue et al., 2013). Only morphologically normal and viable follicles stained red were chosen for in vitro culture.

Culture of preantral follicles: Selected preantral follicles were washed in the dissection medium before
transferring to a culture medium that composed of M199 supplemented with 12.5 mM HEPES, 0.23 mM sodium pyruvate, 2 mM L-glutamine, 0.3% (w/v) BSA, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 1% (v/v) insulin-transferrin-selenite solution (ITS), 2.13 µg/ml follicle stimulating hormone (FSH), 10 ng/ml insulin-like growth factor I (IGF-I), 1.0 mIU/ml growth hormone (GH) and 10 ng/ml activin A (rhAct A; R & D Systems, Abingdon, UK) (Wongbandue et al., 2013). The follicles were individually placed into 20-µl drops of medium, which were prepared in tissue culture dishes and pre-equilibrated for 2 hours at 38.5°C, 5% CO₂ in air. Thereafter, the droplets containing follicles were overlaid with mineral oil and incubated at 38.5°C, 5% CO₂ in air for 7 days. Every two days, half of the medium was replaced with freshly prepared medium.

Assessment of preantral follicle growth: To allow assessment of follicle growth, photographs of each follicle were taken on Days 0, 3 and 7 using an inverted microscope (CKX41, Olympus, Japan) installed with a digital microscopic camera (DP20, Olympus, Japan). Mean diameters of follicles were determined with assistance of the program DP2-BSW (Olympus, Japan) by measuring 2 maximum diameters perpendicularly through the center of each follicle. In addition, diameter gains were calculated by subtractions of the follicle diameters between Days 0 and 3, and Days 3 and 7.

Viability assessment: On Day 7, follicles were examined for viability by staining with 50 µg/ml neutral red for 20 min and immediately assessed under an inverted microscope (CKX41, Olympus, Japan) at ×400 magnification. Follicles with >50% granulosa cells stained red in cytoplasmic lysosome were classified as viable.

Results and Discussion

A total of 23 and 58 preantral follicles were isolated from cryopreserved ovarian tissues underwent slow freezing and vitrification, respectively. The pattern of preantral follicle growing is represented in Fig 1. The preantral follicles retrieved from slow freezing exhibited viability of 43% (10 of 23) compared to 21% (12 of 58) of those vitrified (Table 1). On Day 0, the initial diameters of the selected follicles were 53.4±4.5 µm and 50.7±4.5 in slow freezing (n = 10) and vitrification group (n = 12), respectively (Fig 2A). The follicles in the slow freezing group showed decreased diameter during Days 3 to 7 compared to the vitrification group (Fig 2B). After frozen-thawed, follicles recovered from ovarian tissues cryopreserved using the slow freezing method presented higher percentages of viability than the vitrification group (Table 1). However, after 7-day culture, the percentages of viability were similar between the two groups.

The present study is the first to demonstrate the survival of cheetah preantral follicles after ovarian tissue was cryopreserved and cultured for 7 days. Ovarian tissue cryopreservation has been performed in wildlife post-mortem, including elephants (Gunaseha et al., 1998), wombats (Cleary et al., 2004) and lions (Wiedemann et al., 2012). The constraints of this technique in wild animals usually involve health of the animals prior to ovarian recovery (Johnston et al., 1991), duration of organ transportation and storage temperature. Moreover, time delay between animal’s death and ovarian recovery resulted in dramatic decrease in survived follicles (Cleary et al., 2001). Miao et al. (2007) revealed an increase in granulosa cell apoptosis leading to follicle and oocyte degeneration when excision of ovaries was delayed up to 30 minutes in mice carcasses. Duration and temperature during transportation affecting viability and morphology of preantral follicles was demonstrated in dogs (Lopes et al., 2009). In line with the above reports, health status, duration of time from animal’s death to ovarian recovery and transportation of the cheetah in this study contributed to initial poor condition of preantral follicle which might account for small numbers of follicles recovered from cryopreserved ovarian tissues.

Figure 1 Vitrified cheetah preantral follicle after frozen-thawed (A), after 3 days (B) and 7 days of in vitro (C) and stained red with neutral red on Day 7 of culture (D). Bar = 50 µm.

Figure 2 Diameter (A) and diameter gain (B) of cryopreserved cheetah preantral follicles during 7 days of in vitro culture.
In the present study, the proportion of viable cheetah follicles in the slow freezing group (43%) was comparable to that in the lions (37-59%) (Wiedemann et al., 2012) and the domestic cats (39%) (Lima et al., 2006). In addition, the survival rates of preantral follicles isolated from vitrified-warmed ovarian tissues were slightly higher than the previous study in the cats (21% vs 18%, respectively) (Galiguis et al., 2012). However, assessment of follicle viability in those studies was based on histological morphology and sizes of preantral follicles within ovarian tissues, whereas the follicle viability in our study indicated physiological function by cell uptake of non-toxic dye neutral red. The ovarian tissues frozen using the slow freezing resulted in a higher post-thawed survival rate of preantral follicles compared to the vitrified-warmed ovarian tissues in this study. Although a recent histological study in monkey indicated an advantage of vitrification preserving ovarian stromal integrity and intact follicle morphology above slow-freezing (Ting et al., 2011), high cryoprotectant concentration was used. The decrease in follicle viability and estradiol production has been reported in the vitrified-warmed ovarian tissues compared to the slow freezing group (Milenkovic et al., 2012). Taken together, the lower number of viable follicles in this study was likely contributed to cryoprotectant toxicity in the vitrification medium.

Advances in in vitro preantral follicle culture have been reported in various species including human (Telfer et al., 2008), primate (Xu et al., 2009a), ovine (Arunakumari et al., 2010) and murine (Wang et al., 2011) whereas developments in felids are limited. In felid species, achievements of in vitro follicle culture were demonstrated only in the domestic cats (Jewgenow and Pitra, 1993; Wongbandue et al., 2013). In addition, this study revealed the use of FSH and growth factors (IGF-I and activin A) supplements in the culture medium according to Wongbandue et al. (2013). These effects influence physiological function of preantral follicle compartments and could account for the low survival and growth rate of cryopreserved ovarian follicles culture in vitro in this study.

In conclusion, the preantral follicles retrieved from ovarian tissues of the cheetah 29 hours post-mortem survived after cryopreservation using the slow freezing and vitrification. In addition, the technique of slow freezing with passive cooling container supported viability of the follicles post-thawing better than vitrification. This study also presents the ability of cheetah preantral follicles to grow in vitro up to 7 days. The report represents possibility of female gamete rescue in the felid species post-mortem.

**Acknowledgements**

This present study was financially supported by the Royal Golden Jubilee (RGJ) Ph.D. program (PHD/0252/2551). The authors would like to thank Research Unit for Obstetrics and Reproduction in Animals, Chulalongkorn University for providing laboratory equipment.

**References**


Detection of Genetic Variations Using RAPD Markers in Siberian Huskies Affected with Swimming Puppy Syndrome

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Abstract

Swimming puppy syndrome (SPS) is a curious disease found in dogs. The causes and pathology of this disease are still unknown, although heredity is usually considered to be one of the underlying factors. The objective of this study was to investigate the association between genetics and SPS in a Siberian husky model. Four Siberian husky puppies diagnosed with SPS were subjects of this study, while three other healthy Siberian husky puppies served as controls. Blood samples were collected for DNA isolation, using random amplified polymorphic DNA (RAPD) technique with 16 random primers. No genetic variation was found between affected puppies and healthy puppies, which indicated that swimming puppy syndrome is not controlled by genetics.

Keywords: dog, RAPD, Swimming puppy syndrome

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บทคัดย่อ
การตรวจสอบความหลากหลายทางพันธุกรรมของกลุ่มอาการขากระถางในลูกสุนัขในสุนัขสายพันธุ์ไซบีเรียนฮัสกีด้วยเทคนิค RAPD marker

สิริวดีชมเดช1,2 อาภาพรดอกพุฒ1,2 วรรณีประดิษฐ1,2 กรกฎงานวงศ์พาณิชย์2,3

กลุ่มอาการขากระถางในสุนัข (Swimming puppy syndrome; SPS) เป็นความผิดปกติที่น่าสนใจในสุนัข จนถึงปัจจุบันสาเหตุและการเกิดโรคยังคงไม่ทราบแน่ชัด แต่เชื่อว่าพันธุกรรมอาจเป็นสาเหตุหนึ่งของความผิดปกตินี้ การศึกษาครั้งนี้มีจุดประสงค์เพื่อตรวจสอบความสัมพันธ์ระหว่างพันธุกรรมและอาการขากระถางในสุนัขสายพันธุ์ไซบีเรียนฮัสกี จำนวน 7 ตัว ที่มีอาการขากระถาง ได้แก่ 4 ตัวผู้ชายและ 3 ตัว ผู้หญิง จากการสกัดดีเอ็นเอจากเลือด 16 ตัวที่มีความสัมพันธ์กับกลุ่มอาการขากระถาง และการเกิดกลุ่มอาการขากระถางในสุนัขไม่ได้มีความเกี่ยวข้องกับพันธุกรรม

คำสำคัญ: RAPD กลุ่มอาการขากระถางในสุนัข สุนัข

1 ภาควิชาวิทยาการสัตวศาสตร์ มหาวิทยาลัยเชียงใหม่ อ.เมือง จ.เชียงใหม่ 50200
2 ศูนย์วิจัยวัสดุศาสตร์ มหาวิทยาลัยเชียงใหม่ อ.เมือง จ.เชียงใหม่ 50200
3 ห้องปฏิบัติการวิจัยโรคกระดูกและข้อในสัตว์ ภาควิชชีววิทยาการสัตวศาสตร์และสัตวแพทยศาสตร์มหาวิทยาลัยเชียงใหม่ อ.เมือง จ.เชียงใหม่ 50200

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Introduction

Swimming puppy syndrome (SPS) is an as-yet-unexplained disease mostly occurring in some neonatal dogs. This syndrome is also known as swimmer syndrome, flat pup syndrome, splay leg (paraparesis), splay weak (tetraparesis), and myofibrillar hypoplasia. Physiological changes and pathogenesis of this disease are unclear, and the limited number of published reports is a major obstacle to increasing knowledge of this disorder. Although some publications have presented methods for the treatment of this disease (Verhoeven et al., 2006; Yardimci et al., 2009), only one research report in 2012 published the results of serum biochemistry analysis (Nganvongpanit, 2012). Ngavingpanit (2012) reported that complete blood counts and blood chemistry in four Siberian husky puppies diagnosed with swimming puppy syndrome, when compared with four healthy Siberian husky puppies, only serum creatine kinase in affected puppies was significantly higher than in normal puppies. In 1977, Lorenz and colleague identified certain underlying factors of this syndrome. In the early stage of the disease, newborn puppies seem normal: they gain weight quickly, suck well and appear to be completely healthy. Signs begin to appear when the puppy learns to walk (2nd-3rd week), exhibiting spread-out legs like a swimmer. Heredity has often been attributed to be the primary underlying factor of SPS. Thus, the important question is: is this syndrome controlled by genetics? So far, scientists and veterinarians have not been able to answer this question. This case report investigates

<table>
<thead>
<tr>
<th>Table 1 Case and control puppy information</th>
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<tbody>
<tr>
<td><strong>Puppies affected with swimming puppy syndrome</strong></td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Age (weeks)</td>
</tr>
<tr>
<td>Weight (kg)</td>
</tr>
<tr>
<td>Limb affected</td>
</tr>
<tr>
<td>Number of puppies/litter</td>
</tr>
<tr>
<td><strong>Control puppies</strong></td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Age (weeks)</td>
</tr>
<tr>
<td>Weight (kg)</td>
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<tr>
<td>Number of puppies/litter</td>
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</table>
the relationship between genetics and SPS in dogs, using random amplified polymorphic DNA (RAPD) technique. This technique is a simple and rapid technique which requires very small quantity of genomic DNA and no sequencing, cloning and hybridization representing distinct advantages over other molecular techniques generally used in genomic characterization. Presently, RAPD is wildly use as technique of choice to study genetic variations in many aspects (Ben Abdeljelil et al., 2011; Nathues et al., 2011; Dione et al., 2012; Taha, 2012).

**Case history**

Four Siberian husky puppies diagnosed with swimming puppy syndrome based on clinical signs were subjects in this study, while three other healthy Siberian husky puppies served as controls. The differential diagnosis of SPS was used 1) clinical signs; walrus-swimming movements on the belly, extended limbs and 2) time of disease presented; within 2 months old. Moreover, all puppies recovered within 1 month treatment program; limb bandage and physical therapy. Three SPS puppies were affected at both hind limbs, while the other was affected at the fore and hind limbs. All puppies recovered and walked normally within 1-2 months after being treated using bandage technique and rehabilitation program without any medication. This is a proof that all puppies included in this study were affected with SPS. Puppy information and pedigrees are presented in Table 1 and Fig 1. Two ml blood samples were collected from the cephalic vein for DNA isolation. The isolation of total genomic DNA for molecular marker analysis was carried out utilizing the phenol-chloroform method, as described in a previous work (Chomdej et al., 2011). Genomic DNA from each sample was diluted to a concentration of 10 ng/ml for use in RAPD technique with 16 random primers (Table 2).

PCR was performed in a total volume of 25 µl volume containing: 1x reaction buffer (500 mM KCl, 15 mM MgCl2, 100 mM Tris- HCl, 1 mg/ml BSA, 100 mM (NH4)2SO4, RBC Bioscience), 2 mM MgCl2 (RBC Bioscience), 0.2 mM dNTP (Vivantis Technologies), 0.4 µM primers (Operon Technologies), 1 U Taq DNA polymerase (RBC Bioscience), 10 ng/ml genomic DNA and deionized distilled water. PCR was performed by MJ Mini Personal Thermal Cycler (Bio-Rad) with the cycling profile as follow: 95°C for 5 min for 1 cycle, 94°C for 30 sec, 45°C for 60 sec and 72°C for 90 sec for 35 cycles and 72°C for 5 min. After the PCR completed, the amplified samples were evaluated using agarose gel electrophoresis.

To verify the association of RAPD bands with SPS, the presence and absence of bands from all primers were statistically calculated using a chi-square test. The appearances of polymorphic fragments were scored as 0 and 1 for the occurrence and the absence, respectively. The data were used to calculate the p-value to receive p-value at < 0.05.

**Results and Discussion**

In this study, only one breed of dog was used to eliminate the genetic variation among breeds. However, no significant difference was observed between the genetics of SPS and healthy puppies. This result correlated with the pedigrees of the five Siberian husky families in the study, which had not previously presented any indications of heredity to this syndrome, as shown in Fig 1. Thus, the results from this study, both RAPD result and pedigrees, appear to indicate that SPS in dogs is not controlled via genetics. Nevertheless, this study is only a brief report with a low number of samples, especially SPS samples. In addition, the lack of data for the grandparent generation is one of the limitations for pedigree analysis in this study. Furthermore, RAPD is not the only detection technique for genetic variation. Other techniques to identify the DNA fingerprint such as restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) (Vos et al., 1995) could be applied for analysis of the relationship of heredity and SPS. However, RAPD technique was chosen for use in this study for several reasons: we are more familiar with this well-established technique; and, using RAPD, we have recently found an association between genes and patellar luxation in dogs (data not yet published).

**Table 2** Sixteen random primer sequences used in this study

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence (5’→3’)</th>
<th>G+C percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>GCATCAATCT</td>
<td>40</td>
</tr>
<tr>
<td>AP42</td>
<td>AAGCCGGCAAC</td>
<td>60</td>
</tr>
<tr>
<td>OPB04</td>
<td>GCATCGGAGT</td>
<td>60</td>
</tr>
<tr>
<td>OPB05</td>
<td>TGGCCCCCTTC</td>
<td>70</td>
</tr>
<tr>
<td>OPB06</td>
<td>TGCTCTCCGCC</td>
<td>70</td>
</tr>
<tr>
<td>OPB07</td>
<td>GTGCACCAGG</td>
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</tr>
<tr>
<td>OPB08</td>
<td>GTCCACACGG</td>
<td>70</td>
</tr>
<tr>
<td>OPB10</td>
<td>CTGCTGGGAC</td>
<td>70</td>
</tr>
<tr>
<td>OPB17</td>
<td>AGGGAAAGAG</td>
<td>60</td>
</tr>
<tr>
<td>OPB18</td>
<td>CCGACACAGT</td>
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</tr>
<tr>
<td>OPS11</td>
<td>AGTCGGGTGG</td>
<td>70</td>
</tr>
<tr>
<td>OPS16</td>
<td>AGGGGGTTTCC</td>
<td>70</td>
</tr>
<tr>
<td>OPW09</td>
<td>GTGACCCGAG</td>
<td>60</td>
</tr>
<tr>
<td>R37</td>
<td>GAGTCACCTG</td>
<td>60</td>
</tr>
<tr>
<td>R55</td>
<td>GCCATCCCGC</td>
<td>70</td>
</tr>
<tr>
<td>R105</td>
<td>GCAACCGAACG</td>
<td>70</td>
</tr>
</tbody>
</table>

**Figure 1** Pedigrees of 7 puppies participating in the study (4 puppies with SPS and 3 unaffected controls). Blood samples were collected from dogs from five Siberian husky families. F1–F4 = litter number, male/female puppies per litter, and SPS characteristics.
Other risk factors, including environment and nutrition, and especially musculoskeletal abnormalities as mentioned in the studies of Nganvongpanit (2012) and Lorenz (1997), should be evaluated to clarify the cause(s) of this syndrome, if possible. Increasing the number of affected puppies studied would increase confidence in the results; but this is very difficult because SPS cases in dogs are rare. Data from Nganvongpanit in 2013 from a 6-year retrospective study of 2,443 puppies (1,183 males and 1,260 females) in Thailand, from October 2006 through September 2012, found that only 2.13% of puppies were affected with this disease. Finally, finding the answer to the question of whether SPS is controlled by genetics remains an exciting prospect; but so far all data and knowledge support that SPS is not a genetic disorder.

Acknowledgements

This study was financially supported by the National Research University Project under Thailand’s Office of the Higher Education Commission, year 2012-2013. We also thank Metta Pet Hospital, Chiang Mai, Thailand, for their kind assistance.

References


Endoscopic Evaluation of Gastric Mucosa to Determine Safety of Three Chondroprotective Drugs in Healthy Dogs

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Kumpanart Soontornvipart 5

Abstract

This randomized, double-blind, placebo-controlled study investigated the effects of glucosamine/chondroitin sulfate, chondroitin sulfate, and diacerein on the gastric mucosa of dogs, and on clinical signs of vomiting and diarrhea. Twenty-one healthy adult dogs were included in the study, and were randomly assigned to seven treatment groups, 3 dogs per group. The effect of those medicines on the gastric mucosa was evaluated by endoscopy on day 3 and day 14, and compared with pre-treatment. Clinical signs, including vomiting and diarrhea, were recorded every day during the study period. The results showed a non-significant effect of glucosamine/chondroitin sulfate, chondroitin sulfate, and diacerein on gastric mucosal lesions in healthy dogs. One dog vomited after receiving glucosamine/chondroitin sulfate, but only for the first 2 days. All dogs receiving diacerein showed symptoms of diarrhea during the 14-day trial. In conclusion, glucosamine/chondroitin sulfate, chondroitin sulfate, and diacerein at doses of 1,500 and 3,000 mg/day are gastric-safe for use in dogs.

Keywords: chondroitin sulfate, diacerein, dog, gastric mucosa, glucosamine

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Introduction

Medications for treatment of osteoarthritis (OA) can be classified into two groups: symptom-modifying and disease-modifying drugs (McNamara et al., 1997). Symptom-modifying drugs include non-steroidal anti-inflammatory drugs (NSAIDs), while disease-modifying or chondroprotective drugs include glucosamine, chondroitin sulfate, diacerein and tetracycline. These chondroprotective drugs are commonly prescribed for treatment of OA because they are able to control metabolism in OA-joint by decreasing catabolism and increasing anabolism. A number of research studies have presented the effects of these drugs on improving the pathology of OA in humans and in animals as well (Olsen, 2011; Davies et al., 2013). However, there are many questions that arise when using these drug, for example, which is the most effective, over how long a period of time should they be used, how often should they be taken, and at what concentration. Although some of these questions have been partially answered, no drug has been clearly demonstrated to be more effective than the others; the drug must also be used for a lifetime, at a concentration depending on clinical signs. Thus far, clinical studies have found little evidence of adverse side effects from the use of chondroprotective drugs (Leffler et al., 1999; Brandt et al., 2005; Nganvongpanit et al., 2009).

In a human study that graded the efficacy of chondroprotective drugs, glucosamine, chondroitin sulfate and diacerein were classified as ‘platinum’, which indicated that there were good evidence for their effectiveness in the treatment of OA (Bruyère et al., 2008). Glucosamine is an aminosaccharide, acting as a preferred substrate for the biosynthesis of glycosaminoglycan chains and, subsequently, for the production of aggrecan and other proteoglycans of cartilage. It can increase matrix structural protein synthesis (Reginster et al., 2005). Chondroitin sulfate is a sulfated glycosaminoglycan (GAG) and acts as a preferred substrate for the biosynthesis of matrix of connective tissues. The most effective property of chondroitin sulfate in OA-joint is pro-anabolic and anti-catabolic effect on, although chondroitin sulfate also increases cell viability and demonstrates anti-inflammatory properties (Hochberg et al., 2013). Diacerein (9, 10-dihydro-4, 5-bis (acetoxy)-9, 10-dihydro-9, 10-dioxo-2-anthracene carboxylic acid) acts as an IL-1β blocker, inhibiting the IL-1β-stimulated MMP-3 and collagenase activity (Martel-Pelletier and Pelletier, 2010).
has focused on the effect of these drugs on gastric mucosa when used in high dose; previous studies reported only clinical signs after receiving drugs. Therefore, this study aimed to investigate the effect of two different doses (1,500 and 3,000 mg per dog) of three chondroprotective drugs including glucosamine, chondroitin sulfate and diacerein on the gastric mucosa by clinical signs and endoscopic evaluation.

**Materials and Methods**

The study protocol was approved by the ethics committee of the Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand, in 2012. Twenty-one healthy adult dogs (male = 10, female = 11) volunteered for the study, based on a normal physical examination, unremarkable serum chemistry (liver and kidney) and complete blood count (Table 1). All dogs participating in the study had no history of gastrointestinal disorders, for example, vomiting and diarrhea for at least 2 months prior to the study. The dogs did not receive any medicine for 1 month before entering the study. The pool of 21 dogs were randomly double-blind assigned to seven treatment groups (Table 2). Three chondroprotective drugs, glucosamine/chondroitin sulfate (Synoquin®; Vet plus, England), chondroitin sulfate (Fortiflex®; Virbac, France), and diacerein (Artrodar®; TRB Chemedica, Switzerland), were used in the study, while gelatin capsules served as a placebo (control). Medicines were administered once a day, after evening meal. All dogs in the study were fed only dry food twice a day, morning and evening. Additional food and medicine were restricted during the study period.

The effect of these chondroprotective drugs on the gastric mucosa was evaluated using endoscopy (Schölly Fiberoptic, Germany) on day 3 and day 14, and the results were compared with pre-treatment. Moreover, clinical signs, including vomiting and diarrhea, were recorded every day during the study period. Gastroscopy was performed under general anesthesia. All animals first received 0.04 mg/kg atropine sulfate (TP Drug Lab, Thailand) by intramuscular injection as a preanesthetic agent. Propofol® (B. Braun, Germany) was administered at a 3 mg/kg intravenous dosage as a general anesthesia inducer. The animals were left under spontaneous and mechanical ventilation with oxygen at 100 vol%, maintained with 1% to 3% isoflurane inhalation anesthetic (Terrell™; Minrad, USA). During evaluation of the stomach, veterinarians were blinded to the group classification of dogs.

Gastric mucosal lesions were scored on the basis of a 12-point scale, as described in Baan et al, (2011) (Table 3). The stomach was divided endoscopically into four anatomical regions: 1) pylorus and pyloric antrum; 2) incisura angularis, extending along the lesser curvature; 3) greater curvature from the cardia to the pyloric antrum; and 4) cardia, extending from the greater curvature region to the lesser curvature that was not included with the incisura angularis (Moreau et al., 2005). R statistical software was used to analyze the study results. Fisher test function was applied for analysis of the relationship between different treatments and endoscopic scores, as well as instances of vomiting and diarrhea.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Range of complete blood counts and blood chemistry pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>37-55</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>12-18</td>
</tr>
<tr>
<td>RBC (x106 cells/mm3)</td>
<td>5.5-8.5</td>
</tr>
<tr>
<td>WBC count (cell/µl)</td>
<td>6,000-17,000</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>60-77</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>12-30</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>3-10</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>2-10</td>
</tr>
<tr>
<td>Basophil (%)</td>
<td>0-1</td>
</tr>
<tr>
<td>Alkaline Phosphatase (mg/dl)</td>
<td>20-120</td>
</tr>
<tr>
<td>ALT (mg/dl)</td>
<td>5-50</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>10-22</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.4-1.5</td>
</tr>
</tbody>
</table>

* Standard value from Veterinary Central Laboratory, Veterinary Diagnostic Laboratory, Chiang Mai, Thailand

---

**Table 2** Experimental groups in the study

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>N (male/female)</th>
<th>Weight (kg)</th>
<th>Age (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-1500</td>
<td>Glucosamine/ chondroitin sulfate 1,500 mg/day</td>
<td>3 (2/1)</td>
<td>19.30±3.20</td>
<td>44.33 ± 8.02</td>
</tr>
<tr>
<td>GC-3000</td>
<td>Glucosamine/ chondroitin sulfate 3,000 mg/day</td>
<td>3 (1/2)</td>
<td>21.33±4.04</td>
<td>45.33 ± 15.95</td>
</tr>
<tr>
<td>C-1500</td>
<td>Chondroitin sulfate 1,500 mg/day</td>
<td>3 (1/2)</td>
<td>20.33±3.21</td>
<td>40.33 ± 12.58</td>
</tr>
<tr>
<td>C-3000</td>
<td>Chondroitin sulfate 3,000 mg/day</td>
<td>3 (1/2)</td>
<td>20.67±2.08</td>
<td>45.00 ± 13.75</td>
</tr>
<tr>
<td>DAR-1500</td>
<td>Diacerein 1,500 mg/day</td>
<td>3 (2/1)</td>
<td>19.77±3.42</td>
<td>44.33 ± 16.26</td>
</tr>
<tr>
<td>DAR-3000</td>
<td>Diacerein 3,000 mg/day</td>
<td>3 (2/1)</td>
<td>20.77±2.80</td>
<td>41.33 ± 13.61</td>
</tr>
<tr>
<td>Control</td>
<td>Placebo</td>
<td>3 (1/2)</td>
<td>19.90±4.08</td>
<td>37.67 ± 16.07</td>
</tr>
</tbody>
</table>
**Results and Discussion**

Significant level was set at $p < 0.05$. During the study, vomiting was observed on days 1 and 2 in one member (1/3) of the GC-3000 group, which received 3,000 mg/day glucosamine/chondroitin sulfate ($p > 0.05$), but this clinical sign disappeared after day 3; none of the dogs in the other four groups showed this side effect. In a human study, vomiting was reported in 0.83% of patients receiving glucosamine/chondroitin sulfate (Kelly, 1998), while the side effects of the other two medicines were not reported.

Soft stools or diarrhea were found in the diacerein groups, which the dose of 1,500 or 3,000 mg/day (DAR-1500 and DAR-3000) were administered. All dogs in the DAR-1500 group had diarrhea from day 1 to day 9 ($p < 0.01$); on day 10, diarrhea was found in 2 dogs ($p < 0.01$), and on days 11-14 in 1 dog ($p > 0.01$). In the DAR-3000 group, 3 dogs showed symptoms of diarrhea during days 1-10 ($p < 0.01$), and 2 dogs during days 11-14 ($p > 0.01$). This side effect was not found in the other two groups. A previous study reported that 2.48% of human patients receiving glucosamine (Kelley, 1998) had diarrhea. The cause of diarrhea after receiving diacerein is not well understood; however it is believed that this may be due to the chemical structure of diacerein and rhein, which are anthraquinone derivatives (Nicolas et al., 1998). Anthraquinaone is a laxative agent, and for this reason diacerein has a laxative effect as well. In humans, a high percentage of patients receiving diacerein initially showed symptoms of diarrhea. However, this side effect decreased over long-term use.

Total gastroscopy scores for all groups on days 3 and 14 were not significantly different ($p > 0.05$) compared to day 0. Only 1 dog receiving glucosamine/chondroitin sulfate had a gastric mucosal lesion score of 1.3 on day 14 and 1 dog receiving chondroitin sulfate 3,000 mg/day mucosal lesion score of 2 on day 3 of the experiment, glucosamine/chondroitin had a gastric 0.05) compared to day 0. Only 1 dog receiving

This randomized, double-blind, placebo-controlled study showed the non-significant effect of glucosamine/chondroitin sulfate, chondroitin sulfate and diacerein on the gastric mucosal lesions in healthy dogs. Moreover, a high dose (3,000 mg/day) of glucosamine/chondroitin sulfate could irritate the stomach and cause vomiting, but this occurred only for the first few days, after which this side effect disappeared. Another side effect found during the study was that the use of a high dose of diacerein could cause soft stools. However, the overall results of this study demonstrated the safety of glucosamine/chondroitin sulfate, chondroitin sulfate, and diacerein when administered in doses of 1,500 and 3,000 mg/kg, after which no gastric mucosal lesions were observed.

### Table 4 Gastroscopy scoring scale

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-1500</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>GC-3000</td>
<td>1.3 ± 0.6</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>C-1500</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>C-3000</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>DAR-1500</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>DAR-3000</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Control</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

| P value | nd    | 1.00  | 1.00  |

### Acknowledgements

The authors gratefully acknowledge financial support via research grants from the Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand, and the National Research University Project under Thailand’s Office of the Higher Education Commission year 2012.

### References


Chemical Immobilization of Bornean Leopard Cats
(*Prionailurus bengalensis borneoensis*) with Tiletamine and
Zolazepam under Field Conditions in Borneo

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Alcázar Paloma 1 Nathan Senthivel 3  de Gaspar Iñaki 2  Revuelta Luis 2

Abstract

Nine wild Bornean leopard cats were anesthetized using a combination of tiletamine and zolazepam (Zoletil©) after being captured in humanely-designed live traps in Sabah, Malaysian Borneo, for the purpose of fitting radio-collars. For five leopard cats (group 1) a single dose of 6.92±1.06 mg/kg of Zoletil© was administered. The mean induction time from the initial Zoletil© dose was 7.9 ± 1.77 minutes, and the mean anesthesia time was 47.2 ± 25.1. For 4 leopard cats (group 2) after an initial mean dose of 6.92±1.06 mg/kg of Zoletil©, it was necessary to administer a second dose (or booster) of Zoletil© (mean dose 2.6±0.33 mg/kg) or ketamine (mean dose 3.5± 0.05mg / kg) to achieve complete immobilization. There were differences between the periods of anesthesia resulting from these boosters, which were 43.5 ± 2.1 minutes for ketamine and 89.5 ± 6.36 minutes for Zoletil©. We conclude that an initial dose of Zoletil© of 6.92 mg/kg can produce an adequate plane of anaesthesia without needing additional or booster injections of anaesthetic; if a booster is required, the use of ketamine in preference to Zoletil© has the benefit of shorter release times (245 minutes for ketamine booster compared to 350 minutes for Zoletil© booster) whilst providing adequate anesthetic times (mean 43.5 minutes for ketamine booster).

Keywords: Chemical immobilization, Ketamine, leopard cat, *Prionailurus bengalensis borneoensis*, Tiletamine, Zolazepam

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การใช้ยา Tiletamine และ Zolazepam ในการควบคุมแมวดาวสายพันธุ์บอร์เนียว (Prionailurus bengalensis borneoensis) ในการปฏิบัติงานในท้องที่เกาะบอร์เนียว

Nájera Fernando1,2 Cediel-Algovia Rafael 2 Hearn Andrew1,3 Ross Jo 1,3 Dench Rosalie1 Alcázar Paloma1 Nathan Senthivel3 de Gaspar Ihaki2 Revuelta Luis2

การศึกษาได้ว่ายาผสมเวลา สยายพักผันระยะเวลานาน 9 ตัว ด้วยการใช้ Zoletil® ซึ่งเป็นตัวยี่ที่ผสมระหว่างไทล์ทามีน (tiletamine) กับโซลาซีแปม (zolazepam) หลังจากที่จับไดจากการวางกันตั้งที่สัตว์ชื่นชอบในรูปแบบการ บนเกาะบอร์เนียวของมาเลเซีย เพื่อให้สัตว์ฟื้นตัวตามแนวปลอดภัย โดยกลุ่มที่ 1 ประกอบด้วยเวลาจำนวน 5 ตัว วางยาตัวยาให้ Zoletil® ขนาดยา 6.92±1.06 มก./กก. เพียงขนานเดียว ค่าเฉลี่ยระยะเวลาการนรกลา (ค่าให้ยา) ขั้นต่ำค่าเฉลี่ย 7.9±1.77 นาที และค่าเฉลี่ยระยะเวลาการสลบ คือ 47.2±25.1 นาที กลุ่มที่ 2 ประกอบด้วยเวลาจำนวน 4 ตัว หลังจากที่จับให้ Zoletil® ในปริมาณ 6.92±1.06 มก./กก. ในครั้งแรกแล้ว จำนวนตีไม่มีการเติมยาเสริมเข้าไป ด้วยการใช้ Zoletil® (ค่าเฉลี่ยขนาดยา 2.6±0.33 มก./กก.) หรือ ketamine (ค่าเฉลี่ยขนาดยา 3.5±0.05 มก./กก.) เพื่อให้เกิดการสลบโดยสมบูรณ์ พบว่ามีความแตกต่างกันของระยะเวลาการสลบ โดยการใช้ ketamine ทำให้สลบนาน 43.5±2 นาที และ Zoletil® ทำให้สลบนาน 89.5±63.6 นาที ผู้ศึกษาจึงมีความเห็นว่า การใช้ Zoletil® ในครั้งแรกที่ระดับ 6.92±1.06 มก./กก. กับเพียงพอสำหรับการให้สลบโดยไม่ต้องมีการเติมยาเพิ่มเพื่อการสลบอีก ถ้าเป็นการมีการเติมยาเติมยา ควรใช้ ketamine แทนการใช้ Zoletil® เพราะสามารถทำให้สัตว์พักผันและปล่อยได้เร็วกว่า (245 นาที เมื่อเติมยาด้วย ketamine เรียบเรียงเก็บ 350 นาที เมื่อใช้ Zoletil® เป็น booster) แต่จะพิจารณาการให้เกิดการสลบโดยอย่างสมบูรณ์ (ค่าเฉลี่ย 43.5 นาที เมื่อใช้ ketamine เป็น booster)

คำสำคัญ: การใช้ยาในงาน บ่อยใช้ยาPrionailurus bengalensis borneoensis ไทเทลามีน ketamine

Introduction

Tiletamine is an anesthetic agent chemically related to ketamine, both are considered dissociative agents. Zolazepam is a diazepinone minor tranquilizer. Pharmacology of this drug combination is similar to that shown by the combination of ketamine and diazepam (Plumb, 2005). Tiletamine and zolazepam have been used extensively for chemical restraint of several species of non-domestic felines (Deem et al., 1998; Shindle and Tewes, 2000; Kreeger, 2002; Grassman et al., 2004). Among the advantages reported by authors using Zoletil® to perform anesthesia in wild cats in field conditions, two important features are their wide safety margin and the short induction period (Shindle and Tewes, 2000; Grassman et al., 2004). Recovery time when using this drug can be decreased using flumazenil (Spelman et al., 1997). Bornean leopard cat (Prionailurus bengalensis borneoensis) is the only species of the Bornean felids which remains listed as Least Concern by the IUCN (IUCN, 2011). Within the techniques to study the ecology of leopard cats in the wild, radiotelemetry is widely used, by means of attaching a collar with a radio transmitter to the target animal after its chemical immobilization (Sunquist and Sunquist, 2002).

There are few publications with focus on the field anesthesia of leopard cats in the wild (Grassman et al., 2004). In this study we assessed the use of Zoletil® to achieve chemical immobilization of wild leopard cats and compared the effects of booster doses of Zoletil® or ketamine on both anesthesia and release times.

Materials and Methods

The capture and chemical immobilization of leopard cats was carried out as part of an ecological study of this species in the Ulu Segama Forest Reserve, Sabah, Malaysian Borneo between May 2008 and March 2009. We used live-traps of various sizes, all were cage-style and triggered by a treadle...
booster injections of either Zoletil® or ketamine plane of anesthesia within 14 min, we administered Barcelona, Spain) in case booster anesthetic doses Zoletil® and or ketamine (Imalgene® 1000, Merial, sized cats (Shindle and Tewes, 2000).

Once a leopard cat was trapped, we covered the cage with a cloth to reduce stress while we assessed the animal’s suitability for anesthesia, estimated the animal’s weight and determined the volume of drug to inject. Zoletil® (Kreeger et al., 2002) intramuscular, an estimated dose of 7 mg/kg, was used for the anesthesia of the leopard cats trapped, based on previous publications in the species (Grassman et al., 2004; Rajaratnam et al., 2007) and other publications related to medium-sized cats (Shindle and Tewes, 2000).

Booster doses of ketamine and Zoletil® have been used by other authors in wild carnivores following an initial dose of Zoletil®(Kreeger et al., 1990). Therefore, we were prepared with additional Zoletil® and or ketamine (Imalgene® 1000, Merial, Barcelona, Spain) in case booster anesthetic doses were required. If the animal did not reach the desired plane of anesthesia within 14 min, we administered booster injections of either Zoletil® or ketamine (depending on availability) at a dose of 3 mg/kg (Kreeger et al, 2002) intramuscular.

Leopard cats were injected into the hindquarters by hand. We recorded the induction time (time from injection of the drug until the head rests on the floor), anesthesia time (time from the head resting on the floor until the animal is able to lift it again) and the release time (time from the animal lifting its head after anaesthetic until full normal behavior returns with no evidence of drug action, and the animal is able to be released). We also recorded the handling time during which the animals were measured and weighed, radio-collars were fitted, body temperature, respiratory rate, heart rate were recorded, samples of hair were taken for genetic studies and a blood sample was taken for hematology and biochemistry analysis. Once the captured leopard cats were weighed, we completed the calculation of the actual dose received by the animal given in Table 2 Induction, anesthesia, release and effective working times for animals in each of the two study groups. † Animals group 1; *Animals group 2.

<table>
<thead>
<tr>
<th></th>
<th>Zoletil†</th>
<th>Zoletil + Zoletil*</th>
<th>Zoletil + Ketamine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoletil® (mg/kg)</td>
<td>6.92 ± 1.06</td>
<td>6.92 ± 1.06</td>
<td>6.92 ± 1.06</td>
</tr>
<tr>
<td>Booster (Zoletil or Ketamine) (mg/kg)</td>
<td>2.6 ± 0.33</td>
<td>2.6 ± 0.33</td>
<td>3.05 ± 0.05</td>
</tr>
<tr>
<td>Induction Time (min)</td>
<td>7.9 ± 1.77</td>
<td>9.5 ± 2.12</td>
<td>7.5 ± 2.12</td>
</tr>
<tr>
<td>Anesthesia time (min)</td>
<td>47.2 ± 25.1</td>
<td>89.5 ± 6.36</td>
<td>43.5 ± 2.1</td>
</tr>
<tr>
<td>Release Time (min)</td>
<td>236.2 ± 19.69</td>
<td>350.0 ± 70.7</td>
<td>245.0 ± 91.9</td>
</tr>
<tr>
<td>Effective Working Time (min)</td>
<td>36.0 ± 6.52</td>
<td>42.5 ± 3.54</td>
<td>40 ± 0.00</td>
</tr>
</tbody>
</table>

Table 2 Induction, anesthesia, release and effective working times for animals in each of the two study groups. † Animals group 1; *Animals group 2.

In five leopard cats (group 1), the average Zoletil® dose used was 6.92±1.06 mg/kg. This dose was enough to manage and perform all the required procedures in the animals. In four leopard cats (group 2), however, an initial mean dosage of Zoletil® of 6.92±1.06 mg/kg was insufficient to produce the complete muscle relaxation and loss of consciousness necessary for the planned procedures within 14 minutes post-injection (Kreeger, 2002). We, therefore, decided to inject an extra dose of 3 mg/kg of Zoletil® in two animals and 3 mg/kg of ketamine in another two animals. The results are shown in Table 2. For group 2, the induction time started since the animal rested it’s head on the floor after the booster was injected.

In all cases the signs of drug effects were observed during the induction time and were similar to those previously reported for ocelots (Shindle and Tewes, 2000) such as licking the nose and lips, loss of control of head and neck and limb paralysis. However, in group 2 up to 14 min after the injection of Zoletil®, the leopard cats were still responsive to low levels of environmental stimulation (e.g. slight noise), indicating that the level of anesthesia was not adequate for their safe removal from the trap and subsequent handling. Therefore, we injected the extra dose of 2.6±0.33 mg/kg of Zoletil® or 3.05±0.051 mg/kg ketamine (both intramuscular).

All statistical analyses were performed with the software program SPSS program for Windows (SPSS 15.0; SPSS Inc., Chicago, IL, USA). Relationships between measures of drug effect and drug dosages were tested with a one-way ANOVA test and confirmed with a Welch and Brown-Forsythe Tests. Significance was accepted at p < 0.05

Results and Discussion

We successfully trapped 9 leopard cats. The estimated weights ranged between 2.0-2.45 kg in males (n = 6) and 1.70-1.90 kg in females (n = 3). The mean actual weight for males was 2.1±0.12 kg and 1.72±0.10 kg for females.
The ketamine boosters did not cause seizures in any cats. Seizures have been reported in wild cats (Kreeger, 2002; Grassman, 2004) and non-domestic cats in captivity with the use of ketamine. Of the cases in which a booster anesthetic injection was administered, we found a statistically significant difference (p < 0.001) in the anesthesia and release times in those leopard cats immobilized with Zoletil® followed by a booster of the same drug compared to those receiving a booster of ketamine. Although the induction times obtained in our study differ from other previous research with free-ranging leopard cats, we find major differences in the anesthesia times, where using Zoletil® at a higher dose or Zoletil® plus a booster of Zoletil® increases the immobilization times.

In view of these results, we conclude that a relationship exists between the dose of Zoletil® and the times of anesthesia and release, being significantly longer when Zoletil® is used in higher doses or in those cases where a booster of Zoletil® is administered. For non-painful procedures in which animal handling is minimal, the estimated dose to use of 6.92 mg/kg appears to be adequate. In situations where the Zoletil® primary dose does not achieve an adequate anesthetic plane, we recommend a booster dose of 3 mg/kg of ketamine after the initial estimated dose of Zoletil® of 6.92 mg/kg if the planned procedure can be performed within the anesthesia time (mean 43.5 min). This protocol does not significantly lengthen the time of anesthesia nor the release time of the animal. Due to the extended anesthesia and release times resulting from administration of a booster dose of Zoletil®, we recommend the use of ketamine in preference to Zoletil® for booster doses after an initial dose of Zoletil®. In this study Zoletil® proved to be a useful and safe drug for chemical restraint of free-ranging Bornean leopard cats.

**Acknowledgements**

We are indebted to our research assistants, Glen Reynolds and the Royal Society’s South East Asia Rainforest Research Programme (SEARRP) for logistical support. We thank Yayasan Sabah, Sabah Wildlife Department, Sabah Forestry Department, Danum Valley Management Committee, FACE foundation, the State Secretary, the Sabah Chief Minister’s Department, and the Prime Minister’s Department (EPU) for permission to conduct research. We thank Siew Te Wong for providing advice regarding trapping and immobilization protocols. This research was funded by the Darwin Initiative, Felidae Conservation Fund, and the Royal Society HG Wills International Trust for Nature Conservation, Panthera and the Royal Society’s SEARRP. Lastly, we thank to Zoo Atlanta, Idea Wild, Point Defiance Zoo & Aquarium, Point Defiance Zoo Society, the Point Defiance American Association of Zoo Keepers (AAZK) chapter, the Clouded Leopard Project and The Rufford Foundation for keeping on supporting the veterinary team on the field in Borneo.

**Table 3** Anesthesia and induction times in groups 1 and 2 in comparison with previous studies. *Grassman et al., 2004; Group 1: Zoletil + Zoletil®; ZH + KH (Zoletil + ketamine)*

<table>
<thead>
<tr>
<th></th>
<th>Mean Dose (mg / kg)</th>
<th>Induction Time (min)</th>
<th>Anesthesia time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoletil®</td>
<td>12.3 ± 2.8</td>
<td>4.2 ± 2.8</td>
<td>67.0 ± 30.06</td>
</tr>
<tr>
<td>Zoletil®</td>
<td>6.92 ± 1.06</td>
<td>7.9 ± 1.77</td>
<td>47.2 ± 25.1</td>
</tr>
<tr>
<td>ZH + ZH</td>
<td>6.92 ± 2.6</td>
<td>9.5 ± 2.12</td>
<td>89.5 ± 6.36</td>
</tr>
<tr>
<td>ZH + KH</td>
<td>6.92 ± 3.05</td>
<td>7.5 ± 2.12</td>
<td>43.5 ± 2.12</td>
</tr>
</tbody>
</table>

**References**


Investigation into *Bacillus anthracis* Spore in Soil and Analysis of Environmental Parameters Related to Repeated Anthrax Outbreak in Sirajganj, Bangladesh

Md. Murshidul Ahsan¹ ² Mohammad Ferdousur Rahman Khan¹ Md. Bahanur Rahman¹ Jayedul Hassan¹ Shah Md. Ziqrul Haq Chowdhury³ Md. Shafiullah Parvej¹ Mueena Jahan¹ K. H. M. Nazmul Hussain Nazir⁴*

**Abstract**

The study was conducted for the isolation and detection of *Bacillus anthracis* spores from soil collected from Sirajganj district (a north-western district of Bangladesh), and to assess the parameters that may relate to the repeated anthrax outbreak. A total of 48 soil samples were collected from the study area during January to November 2012. Endospores were extracted from soil and the *Bacillus anthracis* was identified using conventional bacteriological, biochemical and sensitivity test against Penicillin-G. The viable *B. anthracis* spores could be detected from 14 (29.17%) soil samples. Moisture content, pH, calcium and organic carbon contents of the soils were measured and the values of the endospore positive samples ranged from 6.31-28.37%, 5.17-7.22, 484.35-1372.35 ppm and 0.15-2.35%, respectively. All the endospore positive soil samples were of loamy type, while none of the clay type soil was found to be positive for *B. anthracis*, suggesting the influence of soil type on the occurrence of anthrax endospore in studied area. The mean pH of anthrax positive soil was weakly acidic (6.38±0.15), indicating that a suitable pH range for anthrax spore was present in the soil of Sirajganj. During the disease outbreak period (May and June) the average temperature of this area was 32°C and the average rainfall was 158 mm and 90 mm, respectively. Although the temperature variation had no significant influence on the occurrence of anthrax spore, rainfall was found to be significant.

**Keywords:** anthrax, *Bacillus anthracis* spore, Bangladesh, ecology

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บทคัดย่อ

การสำรวจสปอร์ของ Bacillus anthracis ในดิน และการวิเคราะห์ภาวะสภาพแวดล้อมที่สัมพันธ์กับการระบาดของโรคแอนแทรกซ์ใน Sirajganj ประเทศบังคลาเทศ

Md. Murshidul Ahsan1,2 Mohammad Ferdousur Rahman Khan1 Md. Bahanur Rahman1 Jayedul Hassan1 Shah Md. Ziqrul Haq Chowdhury3 Md Shafiullah Parvej1 Mueena Jahan1 K. H. M. Nazmul Hussain Nazir4*

ท่าทีแยกและการตรวจหาสปอร์ของ Bacillus anthracis จากดินที่เก็บจากอำเภอ Sirajganj (เขตทางตะวันตกเฉียงเหนือของประเทศบังคลาเทศ) และประเมินทราบด้านที่เกี่ยวข้องกับการระบาดของโรคแอนแทรกซ์ซ้า ตัวอย่างดินทั้งหมด 48 ตัวอย่างถูกเก็บรวบรวมจากพื้นที่การศึกษาในช่วงเดือนมกราคมถึงพฤศจิกายนปีค.ศ. 2012 มาทำการทดสอบพบว่าในสปอร์ของ Bacillus anthracis โดยใช้การตรวจสอบแบบวิทยาการ ชีวเคมี และความไวต่อ Penicillin G ตรวจสอบสปอร์ของ B. anthracis ที่มีชีวิตจำนวน 14 ตัวอย่าง (ร้อยละ 29.17) ทำการวัดค่าความชื้น ความเป็นกรดด่าง ปริมาณแคลเซียมและอินทรีย์คาร์บอนของดิน รวมไปถึงการวัดค่าความชื้นที่ที่เหมาะสมในการดักจับสปอร์ พบว่ามีค่าเท่ากับ 6.31-28.37% 5.17-7.22 ppm และ 0.15-2.35% ตามลำดับ ดัชนีของที่พบมีสปอร์เป็นตัวอย่างสปอร์ที่เป็นตัวอย่างเป็นตัวอย่างที่ดีในดินที่ไม่พบสปอร์ของ B. anthracis พบว่าเป็นในดินที่สปอร์ของ B. anthracis ซึ่งมีเหตุผลที่เป็นประโยชน์ในการเกิดโรคแอนแทรกซ์ขึ้นที่ดินที่ทำการศึกษา ค่าความเป็นกรดด่างของดินที่ตรวจสอบแล้วพบว่า B. anthracis ค่าความเป็นกรดด่างเป็นตัวอย่างที่เหมาะสมในการเจริญเติบโต (6.38±0.15) ซึ่งพื้นที่ที่มีความเป็นกรดด่างที่เหมาะสมในการเจริญเติบโตของ B. anthracis ในช่วงระยะเวลาที่เกิดการระบาดของโรค (เดือนพฤษภาคมถึงมิถุนายน) อุณหภูมิเฉลี่ยของพื้นที่นี้คือ 32ºC และปริมาณน้ำฝนเฉลี่ยคือ 158 มม. และ 90 มม. ตามลำดับ แม้ว่าการเปลี่ยนแปลงอุณหภูมิไม่มีอิทธิพลสําคัญ แต่พบว่าปริมาณน้ําฝนสําคัญต่อการเกิดของสปอร์แอนแทรกซ์

คำสำคัญ: แอนแทรกซ์ สปอร์ Bacillus anthracis บังคลาเทศ อาหารแอนแทรกซ์

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Introduction

Anthrax (popularly known as - Torka, Duckmina, Duckshat, Dhash or Dharash in Bangladesh) is an acute disease caused by a soil-borne, spore forming bacterium, Bacillus anthracis. When the vegetative form of the bacterium is exposed to the atmosphere and conditions are unfavorable for the continued multiplication, it forms spores which are resistant to heat and chemical disinfectants (Hirsh and Zee, 1999; OIE, 2004), and this dormant stage may persist for years in soil as viable (Dragon et al., 2001). These viable spores, acting as an important factor in the epidemiology, are transmitted to herbivorous animal through ingestion of soil contaminated feed and water (Tibball et al., 1991). Several environmental parameters like geographical location, soil type, ambient temperature, rainfall, relative humidity etc. are potential associates for the survival of bacterial spore and maintaining the ecological conditions of repeated outbreak of anthrax (Dragon and Rennie, 1995).

As a common problem, the disease naturally occurs around the globe. Within last 10 years, the disease has been reported in the USA (Mongoh et al., 2008), Australia (Durrheim et al., 2009), Sweden (Lewerin et al., 2010), Italy (Fasanella et al., 2010) and many places in Europe at various frequencies. However, the disease is especially found in tropical and sub-tropical countries (Biswa et al., 2011). In many African and Asian countries, especially in countries having poor vaccination coverage among susceptible livestock, anthrax outbreak occurs periodically in animals, and subsequently transmits to human (WHO, 2008). Until 2009, the disease was periodically reported in animals and human in Bangladesh (Ahmed et al., 2010). However, in recent years, the disease has occurred repeatedly exerting panic to farmers; the outbreaks speculate that it is no longer sporadic rather than enzootic in Bangladesh (Ahmed et al., 2010; Fasanella et al., 2013). In
Bangladesh, the outbreak is mostly prevalent in Sirajganj (a north-western district located about 141 km far from the capital city) which is considered as one of the top most Cattle belt areas (Ahmed et al., 2010; Biswas et al., 2011). Investigation into anthrax in Bangladesh was limited to field observation during active epizootics focusing mainly on the host leaving the environmental factors untouched. To date, there is no report that describes the soil and weather related factors responsible for the repeated anthrax outbreak in Bangladesh. Therefore, the present study was undertaken to investigate the association of anthrax spore in soils in Sirajganj district, and to evaluate the environmental parameters that might have positive influence on the survival of the bacterial spores in the soil.

**Materials and Methods**

**Site selection and sample collection:** The study was conducted in 3 Upazillas (sub-district) of Sirajganj (a north-western district of Bangladesh) (Figure 1) namely Shahzadpur, Belkuchi and Ullapara over a period of January to November 2012. A total of 48 soil samples were collected randomly from anthrax reported areas. The place of sample collection in the study area was selected based on suspected carcass disposal or burial sites, comparatively low-lying area, livestock habitats and livestock pasturing sites. Approximately 400-gm of surface soil from a maximum depth of one-foot was collected in double layered plastic bags and transported to laboratory as early as possible.

**Isolation and identification of Bacillus anthracis:** Isolation and identification of the bacteria were carried out at the Bacteriology and Molecular Microbiology Laboratory of the Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University (BAU). Physical and chemical parameters of the collected soils were examined at the Department of Soil Science, BAU. The isolation and identification of *B. anthracis* from the soil samples was performed according to the procedures described in the *Manual for Laboratory Diagnosis of Anthrax* (WHO, 2003) and OIE.

**Terrestrial Manual 2008** (OIE, 2008). In brief, one gram of soil sample was blended in 10 ml of sterile distilled water and placed in a water bath at 62.5±0.5°C for 30-60 min. The heat will destroy all non-spore-forming bacteria. 10-fold dilution to 10⁻² or 10⁻³ was then prepared. From each dilution, 250-300 µl was plated on to Polymyxin B - Lysozyme - EDTA -Thallous acetate agar (PLET agar, Sigma-Aldrich, Switzerland) and incubated at 37°C for 40-48 hours. The PLET agar is a selective medium for *B. anthracis* that inhibits all Gram-negative and most Gram-positive bacteria including *B. cereus* (Dragon and Rennie, 2001). For confirmatory identification, the colonies were grown on Blood agar, Nutrient agar and Gelatin stab agar to observe the characteristic morphology (WHO, 2008). Microscopic examination was done after staining the bacteria by Gram’s Method and MacFadyean reaction (WHO, 2008). In addition, the bacteria were subjected to biochemical tests and antibiotic susceptibility test against Penicillin-G (10 IU/disc; Oxoid, UK) (Dragan et al., 2005; WHO, 2008).

**Physical and chemical analysis of soil:** Soil type (e.g. sandy, loamy or clay) was determined by gross examination. Soil moisture content was determined by Gravimetric method (Wagner et al., 1999). Soil pH was determined by glass electrode pH meter as described by Eckert and Thomas Sims (1995). Soil calcium content was determined according to the outlines of Wolf and Beegle (1995). Organic carbon was determined by wet-oxidation method (Grewal et al., 1991).

**Collection of the data regarding weather parameters (temperature and rainfall):** Weather related data of the study was collected from the website of Accuweather (http://www.accuweather.com).

**Data analysis:** Statistical analysis was performed using Statistical Package for Social Science (SPSS) commercial software packages (version 17). Frequency tables and cross tables were produced to present study findings. One-way ANOVA was used to see association among soil parameters followed by Dunn’s Multiple Range Test (DMRT). Cramer’s V test was used to measure possible association between different subareas for presence of *B. anthracis* spore in the soil. A *p* value of < 0.05 was considered significant in all analysis.

**Results and Discussion**

Among the 48 soil samples, 14 (29.17%) were found to be positive for the presence of spore of *B. anthracis* in selective PLET agar medium. The frequency of positive samples among the study areas is mentioned in Table 1. The bacteria formed rough, creamy-white, 2-3 cm in diameter and tacky colonies on PLET agar, non-hemolytic and grey colonies on blood agar, Medusa headed colony on Nutrient agar, inverted fir tree like growth in Gelatin stab culture, and cotton wool like growth in Nutrient broth. All the positive isolates were susceptible to Penicillin-G and liquefied gelatin slowly. The isolates were found positive for catalase, Voges-Proskauer (VP) and Methyl-Red (MR) and negative for indole test. The bacteria fermented Dextrose, Sucrose and Maltose.
producing only acid and did not ferment Lactose and Mannitol. Gram-positive bacilli arranging single, pair or chain were observed under microscope. Blue-black bacilli were seen with pink amorphous capsule under microscope (100X objectives) with immersion oil through staining with 1% Polychrome Methylene Blue.

Different parameters of the soil samples like soil type, moisture, pH, calcium content and organic carbon, and presence of spore in the collected samples are summarized in the Table 2. Among the 48 soil samples, 77.08 and 22.92% were loamy and clay type, respectively. All the positive soils (29.17%) were loamy type (Table 1). The association between pH and calcium contents were highly significant ($p < 0.05$), but moisture content and organic carbon content did not vary significantly ($p > 0.05$) among the places (Table 2). The mean moisture content of the positive soil samples was 16.69±2.06%, whereas the pH, calcium content and organic carbon contents were 6.38±0.15, 831.77±62.16 ppm and 0.86±0.17%, respectively (Table 2). The pH level differed significantly ($p < 0.05$) between anthrax spore positive and negative soils, whereas moisture content, Ca level and organic content did not vary significantly ($p > 0.05$) (Table 2). The association between the subareas and the presence or absence of spore in the soil was not significant (Table 3). The monthly average temperature in Sirajganj district ranged from 19-32°C; the highest average temperature (32°C) was recorded in May-June, and the lowest average temperature (19°C) was recorded in December and January, respectively. The monthly average precipitation of rain in Sirajganj district ranged from 0-310 mm; the highest average rainfall (310 mm) was recorded in July, and the lowest average rainfall (0 mm) was recorded in March.

The endospores of $B. anthracis$ are highly resistant to environmental stress and in favorable conditions the spores may exist as dormant for decades between epidemics. Sirajganj, a northwestern district, is considered as one of the important milk pocket areas in Bangladesh. In recent years, the outbreak of anthrax has repeatedly occurred in this area. Through this study, different factors including survival of $B. anthracis$ spore in soil and various environmental parameters were investigated to reveal the possible causes of the repeated outbreak. Here, out of 48 soil samples, 14 (29.17%) were found to be positive for the presence of $B. anthracis$ endospore.

Shahzadpur, Ullapara and Belkuchi are the most prevalent upazillas (sub-district) in Sirajganj, accordingly soil samples were collected from these areas. The highest prevalence of soil association with the spore was found in Shahzadpur (50%), however, the association between different areas for the presence of spores was not significant. In 2010, an investigation into the association between anthrax spore and the soil of Shirajganj district was done, in which soil and turbinate bone samples were collected only from the suspected places located within the anthrax affected farm compound (Fasanella et al., 2013). However, in our study, we focused on the versatile places such as the low-lying areas, livestock habitats, livestock pasturing sites and suspected burial site of the dead animal in open environment. Thus, the findings of our study widen the existing report on the occurrence of anthrax spore in the Shirajganj district. There are several reports on the examination of anthrax bacteria in soil in the world. After the deliberate contamination of the Gruinard Island during the World War II, sampling was done regularly over 40 years and the spores were, almost without exception, isolated from the top 6 cm of soil (Manchee et al., 1981). Similar results also found in northern Canada, south Sudan, and Isfahan in Iran (Dragon et al., 2001; Moazen-Jula et al., 2004). In a study, Moazen-Jula et al. (2004) could isolate 9 (15%) isolates of the bacterium from 60 soil specimens. Dragon et al. (2005) described the sensitivity of isolation of $B. anthracis$ from soil using PLET medium. However, none of the negative samples could be declared to be free from anthrax spore. To overcome this constraint in confirmatory identification, we performed sugar fermentation, biochemical and antibiotic sensitivity tests.

### Table 1

<table>
<thead>
<tr>
<th>Sample sources</th>
<th>Soil samples examined (n = 48)</th>
<th>Positive samples (%)</th>
<th>Soil type of positive sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Loamy type</td>
<td>Clay type</td>
<td></td>
</tr>
<tr>
<td>Shahzadpur,</td>
<td>12</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>Sirajganj</td>
<td></td>
<td></td>
<td>(50.00)</td>
</tr>
<tr>
<td>Belkuchi</td>
<td>13</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Sirajganj</td>
<td></td>
<td></td>
<td>(22.22)</td>
</tr>
<tr>
<td>Ullapara,</td>
<td>12</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Sirajganj</td>
<td></td>
<td></td>
<td>(22.22)</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>(77.08%)</td>
<td>(22.92%)</td>
<td>(29.17%)</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Shahzadpur, Sirajganj</th>
<th>Belkuchi, Sirajganj</th>
<th>Ullapara, Sirajganj</th>
<th>Overall</th>
<th>$p$ value</th>
<th>$B. anthracis$ positive</th>
<th>$B. anthracis$ negative</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>11.57±1.06</td>
<td>17.77±2.37</td>
<td>17.04± 2.21</td>
<td>16.69±2.06</td>
<td>15.64±1.60</td>
<td>0.711 NS</td>
<td>6.38±0.15</td>
<td>5.96±0.09</td>
</tr>
<tr>
<td>pH</td>
<td>6.41±0.15</td>
<td>5.64±0.11</td>
<td>6.34±0.09</td>
<td>6.38±0.15</td>
<td>5.96±0.09</td>
<td>0.000 S</td>
<td>831.77±62.16</td>
<td>911.05±35.93</td>
</tr>
<tr>
<td>Ca (ppm)</td>
<td>671.06±35.75</td>
<td>782.60±47.92</td>
<td>1137.83±70.40</td>
<td>831.77±62.16</td>
<td>911.05±35.93</td>
<td>0.414 NS</td>
<td>0.86±0.17</td>
<td>1.00±0.10</td>
</tr>
<tr>
<td>Organic Carbon (%)</td>
<td>0.93±0.21</td>
<td>0.82±0.14</td>
<td>1.12±0.11</td>
<td>0.305 NS</td>
<td>0.440 NS</td>
<td>0.711 NS</td>
<td>6.38±0.15</td>
<td>5.96±0.09</td>
</tr>
</tbody>
</table>

NS: Non-significant ($p > 0.05$), S: Significant ($p < 0.05$)
The moisture content of soil mainly depends on its type, which may influence in the long time persistency of anthrax spore. In this study, 100% of the positive soils were loamy type. Although the influence of loamy type soil on anthrax outbreak is not clearly known, it is thought that loamy type soil increases anthrax outbreak (Fox et al., 1977). The mean moisture content of the anthrax positive soil specimens was 16.6±2.06, which was higher than that of the negative samples (p > 0.05) suggesting that a moisture range between 6.31-28.37% might be favorable for the viability of B. anthracis spore in the soil.

The soil pH controls the availability of many nutrients in soil (Pabian and Brittingham, 2012). Alkaline soil containing high nitrogen, Ca, and organic matter gives favorable condition to the spore for growing in soil (Dragon and Rennie, 1995; Jula et al., 2007; Hugh-Jones and Blackburn, 2009). Besides, an alternative hypothesis has been tested by Dey et al. (2012) who found that a kind of moist soil amoeba (Acanthamoeba castellanii) might take part in germination and intracellular multiplication of B. anthracis spores. The weakly acidic soil may provide good condition to the spore (Artenstein et al., 2004). In our study, the mean pH of the anthrax positive soil was slightly acidic (6.38±0.15) and differed (p<0.05) from the anthrax negative samples (5.96±0.09). In contrast, Moazen-Jula et al. (2004) found that a slight alkaline pH range (7.2-8.7) was suitable. The soil that is rich in organic matter and calcium promotes the survival of resilient B. anthracis spores (Dey et al., 2012). We found that the calcium and organic carbon contents in the anthrax positive specimens were lower compared to the negative samples, but this difference was not statistically significant (p > 0.05).

In addition to adequate Ca, nitrogen and organic matter in the soil, anthrax outbreak requires favorable seasonal changes such as warm weather followed by heavy rain (Moazen-Jula et al., 2004; Dey et al., 2012). The bacteria are thought to undergo a vegetative cycle when the above conditions are fulfilled. By this process, anthrax spores could be concentrated in top soil to cause disease in grazing animals, occurring outbreak separated by disease-free intervals. In May-June 2012, anthrax outbreak occurred in Shahzadpur, Ullapara and Belkuchi upazillas (IEDCR, 2012). At that time period, the highest monthly average temperature in these areas was 32°C indicating that a moderate high temperature provides a microenvironment that promotes cycling of anthrax spore. All the studied areas were low-lying areas, thus enhancing the possibility of having anthrax spore (Hugh-Jones and Blackburn, 2009). During March 2012, the average rainfall was 0 mm with an average warm temperature (29°C), whereas a heavy rainfall occurred in the following months, suggesting that a favorable condition for the germination and accumulation of anthrax spore (Hugh-Jones and Blackburn, 2009).

Table 3 Association between subareas and B. anthracis status

<table>
<thead>
<tr>
<th>Subareas</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shahzadpur</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Sirajganj</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>Belkuchi</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>Ullapara</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>Creamer’s V</td>
<td>0.265</td>
<td>(p = 0.186*)</td>
</tr>
</tbody>
</table>

*There is no significant association between subareas and presence of B. anthracis spores in soil (p > 0.05)

Conclusion

The repeated anthrax outbreak in livestock (mostly cattle) and subsequent infection to human has been considered as a nationwide alarming issue in Bangladesh. The study revealed, for the first time in Bangladesh, certain ecological factors that might be responsible for survival of anthrax spore in soil, e.g. soil type, Ca content, organic carbon content and soil pH.

Acknowledgements

This study was financially supported by Bangladesh Agricultural Research Council (BARC), Dhaka, Bangladesh under Core Research Projects 2011-12 (No. 24; to Nazir, KHMNH). The authors are grateful to Dr. Md. Tanvir Rahman, Associate Professor, Department of Microbiology and Hygiene, Bangladesh Agricultural University for his critical review and suggestions on the manuscript.

References


Buccal Swab as a Source of Noninvasive Technique for Genomic DNA Collection in Felidae

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Abstract

This study was conducted to determine whether buccal cells obtained by swabbing could be a DNA source for genetic analysis, and to compare two preservative media, distilled water and Tris-EDTA (TE), pH 8.0. Buccal cell samples were collected from 30 domestic cats (Felis catus), 4 captive fishing cats (Prionailurus viverrinus) and 8 captive tigers (Panthera tigris). The total concentration of DNA and purity were not statistically different between the preservative media. All samples could be graded as a minimum as high quality DNA (DNA found with A260/A280 ratio >2.0 or < 1.8). In addition, the quality of DNA extracted from both media was successfully amplified for PCR. However, when compared to whole blood samples, total DNA concentration from buccal swab was significantly lower ($p < 0.05$). Despite the decrease in DNA yield, buccal swab could be an alternative source for obtaining DNA for genetic analyses. This non-invasive, simple and inexpensive technique eases genetic studies in this family especially in domestic cat from which whole blood samples are difficult to collect.

Keywords: buccal swab, felid, genomic DNA, non-invasive technique

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Introduction

Among these felidae, tiger (Panthera tigris) and fishing cat (Prionailurus viverrinus) which are endangered large and medium-sized wild cats (IUCN 2012 status-EN) are of interest to researchers for genetic study (Luo et al., 2004; Xua et al., 2005; Bhagavatula and Lalji, 2006). Owing to their dwindling number, it is difficult to get their DNA samples in the wild. DNA samples used for genetic studies are usually obtained from whole blood or tissue in order to get large amount of DNA. However, the method to handle wild or young animals and obtain blood samples is difficult especially animal in the felid family. Therefore, captive felids are alternative sources of DNA samples and because they are easily inclined to feel stressed non-invasive technique is recommended.

Non-invasive samples from feces, urine or shed hair are widely used in wildlife or threatened animal. However, these samples encounter problems of contamination, degradation, low amounts of DNA and PCR inhibitor (Bellemain and Taberlet, 2004; Cheng et al., 2010). Buccal swab or mouthwash is an alternative technique to collect exfoliated buccal epithelial cells in human. There are different types of buccal swab techniques, e.g. cotton swabs and cytobrushes that could obtain the same DNA yields as buccal swab techniques, e.g. cotton swabs and PCR inhibitor (Bellemain and Taberlet, 2004; Garcia-Closas et al., 2001). Buccal swab or mouthwash is an alternative technique to collect exfoliated buccal epithelial cells in human. There are different types of buccal swab techniques, e.g. cotton swabs and cytobrushes that could obtain the same DNA yields as buccal swab techniques, e.g. cotton swabs and PCR inhibitor (Bellemain and Taberlet, 2004; Garcia-Closas et al., 2001).

Hence, buccal swab technique could be applied to felids with acceptance of their owners. There are many commercial buccal DNA test kits such as Buccal DNA Collection and Preservation Kit (NorgenBiotek Corporation), Isohelix DNA Buccal Swabs and Isolation Kits (Cell Projects Ltd). These test kits contain all components required for collecting, preserving and transporting a DNA sample. They are easy and convenient to use, however they are considerably expensive. Alternative preservative media are distilled water and Tris-EDTA. Tris-EDTA can solubilize and protect DNA from degradation and can solubilize and protect DNA from degradation and can solubilize and protect DNA from degradation and can solubilize and protect DNA from degradation and can solubilize and protect DNA from degradation and can solubilize and protect DNA from degradation.
DNA samples (Smith and Morin, 2005).

The purpose of this study was to compare buccal swab in distilled water and TE buffer preservative media and blood for DNA extraction by using domestic cats and wild felids as models.

Materials and Methods

Sample collection

Buccal cell samples: Experiment I: Thirty domestic cats (Felis catus), 4 captive fishing cats (Pnomiaurus viverrinus) and 8 captive tigers (Panthera tigris) were used as animal models for sample collection. The domestic cats came from 3 cat kennels, the fishing cats and tigers came from a zoo. Buccal swab and blood samples of the domestic cats were collected without sedation. The fishing cats and tigers were anesthetized with 15 mg/kg of ketamine-HCl (Katamil, Troy Lab, Smithfield, NSW, Australia) and 0.5 mg/kg of xylazine-HCl (Lium xylaxil, Troy Laboratories) and maintained as necessary with isoflurane inhalant anesthesia (Aerane, Baxter Health Care Corp., Deerfield IL, USA; 1-2%, v/v). One sterile isoflurane inhalant anesthesia (Aerane, Baxter Health Laboratories) and maintained as necessary with isoflurane inhalant anesthesia (Aerane, Baxter Health Care Corp., Deerfield IL, USA; 1-2%, v/v). One sterile.

Whole blood samples

Experiment II: In order to evaluate DNA quality of whole blood DNA, samples in the suitable preservative medium were further compared with samples from whole blood DNA samples. These samples was composed of 172 buccal samples (30 samples from the first experiment and 142 new samples) and whole blood samples were collected by venipuncture from the cephalic vein of 111 domestic cats. One milliliter of blood samples were collected by venipuncture from 111 domestic cats. One milliliter of whole blood DNA samples. These samples were further compared with samples from whole blood DNA samples.

DNA extraction method: Two buccal swabs per animal were isolated using EZNA® Tissue DNA Kit (Omega Bio-Tek, Inc) following the manufacturer’s instructions with slight modification. After centrifugation at 13,000 rpm for 5 min, 0.2 ml of buccal sediment sample within preservative medium was used for each DNA extraction. The protocol started by adding 200 µl of TL buffer and 25 µl of OB protease into 0.2 ml of buccal sediment sample, vortexing to mix the sample and incubating the sample at 55°C to complete cell lysis. The sample was vortexed every 5-10 min, average lysis time was 1 hour. The sample was centrifuged at 13,000xg for 5 min and the supernatant was carefully aspirated and transferred to a sterile microfuge tube. Then, the

DNA concentration and purity determination: Total DNA concentration was determined by spectrophotometer at 260 nm. DNA quality was evaluated by spectrophotometer at A260/A280 ratio (Cheng et al., 2010). DNA quality of samples were divided into 3 grades: grade 1, the highest quality DNA, DNA found with A260/A280 ratio between 1.8-2.0; grade 2, high quality DNA, DNA found with A260/A280 ratio < 1.8 or > 2.0; and grade 3, the poor quality DNA, no DNA found.

PCR amplification success rate: Extracted DNA from every buccal swabs and blood samples was used as a template for PCR amplification of two cat microsatellite markers (F124 and FCA298). Amplification reaction was performed in a 20 µl volume using 100 ng of genomic DNA. The PCR condition was amplified with a thermal cycle program; initial denaturation for 5 min at 94°C followed by 10 cycles of denaturation for 15 sec at 94°C, annealing for 15 sec at 55°C, and extension for 30 sec at 72°C, followed by 20 cycles of denaturation for 15 sec at 94°C, annealing for 15 sec at 55°C, and extension for 30 sec at 72°C and final extension for 30 min at 72°C. The amplified DNA fragments were analyzed in 1.5% agarose gel electrophoresis and DNA bands were visualized with GelStar® Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc). The results of the PCR were scored as positive if there was amplification or negative if there was no amplification.

Results and Discussion

Buccal cells DNA concentration and purity: DNA was successfully extracted from all buccal samples. The DNA concentration and purity from the domestic cats, fishing cats and tigers are shown in Table 1. The DNA concentration obtained from buccal swab in the domestic cats ranged from 13.0-67.9 ng/µl and 12.4-66.1 ng/µl in distilled water and TE preservative medium, respectively. The DNA purity of domestic cats was in the highest grade (grade 1) and high
quality DNA (grade 2), the value of A260/A280 ratio ranged from 1.51-2.84 in distilled water and 1.54-3.02 in TE (Table 1). The range of DNA concentration was 36.2-51.2 ng/µl (distilled water) and 30.6-59.7 ng/µl (TE) in the fishing cats and 42.0-158.9 ng/µl (distilled water) and 53.7-97.8 ng/µl (TE) in the tigers. Their DNA purity was lower than that of the domestic cats and was graded as only high quality DNA (grade 2).

From all the samples, 50% (15/30) of the domestic cat DNA preserved in distilled water were in grade 1 and 50% (15/30) in grade 2. Similarly 53.33% (16/30) of the cells preserved in TE were in grade 1 and 46.67% (14/30) in grade 2. In contrast, all of the DNA samples of the fishing cats and tigers were in grade 2. The mean purity ratio of buccal DNA of the domestic cats, fishing cats and tigers was 1.97-1.98, 1.14-1.16 and 1.18-1.25, respectively (Table 1). The total concentration and purity of DNA were not statistically different between the preservative media (p > 0.05). Therefore, distilled water was chosen as the preservative medium to compare with whole blood samples in the second experiment.

To determine the quality of DNA preserved in the chosen medium, 172 buccal swab samples preserved in distilled water, 30 samples from the first experiment and 142 new samples, were analyzed with 111 DNA samples from whole blood. When compared DNA purity from buccal swab with whole blood, less than 50% (29/111) of the samples from whole blood were in grade 1. T-test showed that total DNA concentration from the whole blood yielded significantly higher DNA concentration than the buccal swab (p < 0.05). The concentration values ranged from 1.20-8.45 µg in the whole blood and 0.56-5.21 µg in the buccal swab samples (Table 2). While neither of the DNA samples from these 2 sources had poor quality (grade 3) as shown in Fig 1, there was no statistical difference in purity of the DNA extracted from these 2 methods.

There was more distribution of total DNA concentration from the whole blood than the buccal swab samples. The highest yield of the buccal swab samples was 5-6 µg compared to 8-9 µg of the whole blood samples. More than 80% of the buccal swab samples had DNA concentration 1-2 µg. The adequacy and quality of DNA were assessed by the success rate of amplifying DNA fragment of two microsatellite markers. PCR amplification success was observed in both 111 whole blood and 172 buccal swab samples.

From the first experiment, the DNA yield preserved in distilled water obtained per swab in this study was 0.39-2.04 µg (domestic cats), 1.09-1.54 µg (fishing cats) and 1.26-4.77 µg (tigers). Compared to buccal DNA concentration from domestic dog (0.15-1.69 µg) and human (0.54-1.05 µg), the amount of DNA per swab was in the same range (Woo et al., 2007; Mitsouras and Erica, 2009). In the second experiment, when the DNA concentration from whole blood and buccal swab samples were compared, the distribution of whole blood DNA concentration per animal varied more than buccal swab DNA. This difference may come from the amount of white blood cells in the samples.

Figure 1 DNA quality of 111 whole blood and 172 buccal swab from domestic cat samples were divided into 3 grades: grade 1, the highest quality DNA, DNA found with A260/A280 ratio 1.8-2.0; grade 2, high quality DNA, DNA found with A260/A280 ratio < 1.8 or > 2.0; and grade 3, the poor quality DNA, no DNA found.

Table 1 Concentration and purity ratio (A260/A280) of DNA from domestic cats, fishing cats and tigers preserved in distilled water (H2O) and TE

<table>
<thead>
<tr>
<th>Species</th>
<th>Total DNA conc. (µg)</th>
<th>A260/A280</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Range</td>
</tr>
<tr>
<td>Domestic cat</td>
<td>1.08±0.46 0.39–2.04</td>
<td>1.98±0.29 1.51–2.84</td>
</tr>
<tr>
<td>Fishing cat</td>
<td>1.31±0.21 0.92–1.79</td>
<td>1.14±0.12 1.04–1.23</td>
</tr>
<tr>
<td>Tiger</td>
<td>2.65±1.21 1.26–4.77</td>
<td>1.25±0.09 1.12–1.38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Total DNA conc. (µg)</th>
<th>A260/A280</th>
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<td></td>
<td>Mean±SD</td>
<td>Range</td>
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<tr>
<td></td>
<td>Mean±SD</td>
<td>Range</td>
</tr>
<tr>
<td>Domestic cat</td>
<td>0.92±0.37 0.37–1.98</td>
<td>1.97±0.29 1.54–3.02</td>
</tr>
<tr>
<td>Fishing cat</td>
<td>1.36±0.39 0.92–1.79</td>
<td>1.14±0.08 1.04–1.23</td>
</tr>
<tr>
<td>Tiger</td>
<td>2.29±0.50 1.61–2.93</td>
<td>1.18±0.08 1.11–1.34</td>
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</tbody>
</table>

Table 2 Compared mean concentration and purity of DNA from 111 whole blood and 172 buccal swab samples preserved in distilled water of domestic cats

<table>
<thead>
<tr>
<th>Method of collection</th>
<th>Total DNA conc. (µg)</th>
<th>A260/A280</th>
<th>DNA grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood (n = 111)</td>
<td>4.25±1.64 1.20–8.45</td>
<td>1.70±0.16 1.28–2.09</td>
<td>29 82</td>
</tr>
<tr>
<td>Buccal swab (n = 172)</td>
<td>1.67±0.88 0.56–5.21</td>
<td>2.0±0.17 1.55–2.50</td>
<td>69 103</td>
</tr>
</tbody>
</table>
cells and buccal cells in each sample. A study in human indicated that the protein contamination of buccal DNA was more than blood DNA. In human buccal DNA, the average purity of A260/A280 ratio was 1.3 (Livy et al., 2012) and 1.6-2.0 (Satia-Abouta et al., 2002). We found the low A260/A280 ratio result in buccal DNA from the fishing cats and tigers, which indicated protein contamination. However, in the domestic cats the average purity was 2.0 (A260/280 ratio), which was in the range of good DNA purity (1.8-2.0 A260/A280 ratio) (Wang et al., 2011). The different ratios may result from different plasma protein concentration in different species that contaminates the purity of DNA.

The result revealed that both distilled water and TE could be used as preservative media of buccal cells. DNA samples from buccal swab preserved in distilled water provided enough quantity and purity for PCR reactions that identified short DNA fragment revealed by 100% of PCR amplification successful rate. When compared the cost, distilled water should be more suitable than TE because it is less expensive. PCR reaction in this laboratory used 100 ng of genomic DNA. When we observed the yield of genomic DNA from buccal swab technique preserved in distilled water of domestic cats, fishing cats and tigers, this technique provided sufficient DNA for an estimation of 4-20 PCR reactions in domestic cats, 11-15 PCR reactions in fishing cats and 12-47 PCR reactions in tigers. This amount of DNA is enough for diagnosis of genetic disease such as polycystic kidney disease in domestic cat or blood parasite detection by using PCR technique. However, genetic study such as DNA fingerprint, parentage testing identified more than 10 loci for the analysis. Therefore, it is necessary to collect more than one cotton swab per animal. Awareness of buccal cell samples collection is food intake and milk sucking in young animals. The particle of food or milk remaining in the mouth might be contaminated with DNA of other animal. To avoid this problem, we recommend the same protocol with human to wait at least 45 minutes after smoking, drinking or eating before sample collection (Cheng et al., 2010). These animals should rinse their mouth after eating and wait at least 45-60 minutes prior to DNA sample collection.

The advantage of buccal cells over blood is the method to get the samples especially in young or very small animal. It is easy and decreases the risk of anesthesia or stress to handle the animal. In captive felid such as tiger and lion, zoo keeper can take the buccal samples without sedating the animals. This non-invasive source of DNA is widely accepted by patients for genetic studies and clinical disease diagnosis (Cheng et al., 2010). Moreover, samples stored in a freezer at -20°C for up to 4 months still contain genomic DNA as found in this study. Therefore, it should be suitable for sample collection in field study where laboratory is not available for sample processing immediately after sample collection. However, there was a report on storage duration that affected the quality of DNA in human DNA (Nedel et al., 2009). Degradation of DNA occurred after storing the buccal samples. Therefore, processing the samples as soon as possible is the better way to receive good yield and quality of DNA samples.

Researchers are always in search of appropriate noninvasive techniques to get DNA samples for their studies. Whole blood is the ideal samples but it is not suitable for some studies especially in the case of wildlife, very small or young animals. The result of this study revealed that the collection of DNA from buccal swab preserved in distilled water is a cost-effective and practical method in felid species. Even though the yield of DNA is lower than peripheral blood, the quality, concentration of genomic DNA and success rate of PCR amplification are sufficient for genetic studies. The success of research studies is not only on the quality of DNA, but also on the number of participants in the project. This method is noninvasive and decreases stress of captive animal, therefore it encourages owners to participate in a research and increases participant samples in the field study.

Acknowledgements

The authors would like to thank Kasetsart University Research and Development Institute for the research fund.

References


Strip 1, 2 and 3 were recorded from a 12 years old spayed female Golden retriever weighing 32 kilograms. In August, 2012, the dog had history of conjunctivitis due to entropion. At the end of the year, multiple nodules were found underneath the skin especially at the right cervical area. One soft tissue mass was ruptured at the ventral abdomen. Fine-needle biopsy revealed basal cell tumor in origin. The ECG was recorded prior to surgery and respiratory sinus arrhythmia was found. Thoracic radiograph showed normal heart size and normal lung appearance. All masses were surgically removed; pilomatricoma was histologically diagnosed. Six months later, small masses were detected again at the area of left hip, left body trunk and right elbow. The owner complained that the dog had frequent coughing at night. Thoracic radiograph was re-evaluated. Normal heart size (VHS = 9.7) with bulging of the left ventricle, thickening of the lateral mediasternum and mild interstitial pattern of caudodorsal lung fields were found. The abdominal radiograph revealed two subcutaneous masses, of which the size was 2.7x2.3 cm² on the back area, dorsal to last rib and 4.3x3.4 cm² located at the caudal lumbar to proximal tail area. During this time, the dog had no syncope. The pulse was strong without pulse deficit. Complete blood counts as well as the liver and kidney profiles were within normal limits. The ECG was re-evaluated. The results were shown in strip 1, 2 and 3 with two different paper speeds.

Please answer before turning to the next page.

1Department of Veterinary Physiology, 2Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University
Interpretation

**Ventricular preexcitation**

Heart rate was approximately 120 beats per minute. One normal P-QRS complex was seen (curve arrow) along with normal PR interval and negative Q wave deflection (strip 3). However, most of QRS complexes had variation of shape and height which indicates impulse differently travelling through the ventricles.

Ventricular pre-excitation occurs in this case when impulses originate from sinoatrial node or atrium and prematurely activate the portion of the ventricles through an accessory pathway. Impulses are able to reach the ventricles initially without going through the AV node. Most of the complexes had P-wave appearing closely just before the R waves (small straight arrows) and were followed by an early slur to the QRS complexes, which is call “a delta wave” (hollow arrows). This wave is caused by the portion of ventricle prematurely activated while the sinus impulse is conducted through the accessory pathway without delay. The remainder of the ventricles is then activated from both normal and accessory pathway. Many factors affecting the shape of the ECG waveforms include the location of the accessory pathway, the conduction time required inside the atria, the normal AV node - bundle of His pathway and the accessory pathway.

It was possible that the accessory pathway may cause the reentry circuit. If the ventricular pre-excitation occurs with episode of paroxysmal supraventricular tachycardia, the Wolff-Parkinson-White (WPW) syndrome will be diagnosed.

The anatomic locations of ventricular preexcitation were demonstrated as bundle of Kent (accessory AV conductions), James fiber (AV nodal bypass tracts) and Mahaim fibers (nodoventricular tract). Previous study in a dog using epicardial mapping showed that the bundle of Kent was the most important location.

Small waves (big straight arrows) represented normal P-waves that is originated from the SA node and travelled through atria via the normal tract. However, these normal P-waves were not followed by the normal QRS complexes suggesting that they did not reached the ventricle through the normal AV conduction pathway and were replaced by the competitive ectopic rhythm.

The ectopic impulses travelling through accessory pathway may occur temporary or may be sustained depending upon the patient’s heart condition. The ventricular pre-excitation without tachycardia does not require treatment. However, if supraventricular tachycardia was manifested, treatments including ocular or carotid sinus compression, medication or direct current shock may be necessary.
Ophthalmology Snapshot

Nalinee Tuntivanich

History

A 9 year-old, female Miniature Pinscher was presented at the Ophthalmology Clinic, Animal Teaching Hospital, Chulalongkorn University with a chief complaint of rapid impaired blindness and white eye. Menace responses were negative while dazzle reflexes were strongly detected. Degree of conjunctival hyperemia was noticed on both eyes. Intraocular pressures were 5 mmHg.

The dog had had diabetic mellitus and been treated for over two years. Fasting blood glucose (FBG) at the first visit was 464 mg%, while it had been ranged 200-350 mg% during the past 2 years. Intraocular pressures measured from both eyes every visits were, for the whole time, below 10 mmHg.

Question
What is your diagnosis?

Figure 1 The female Miniature Pinscher presented with white eyes.
(For better quality, figures can be viewed in the TJVM website)

Please turn to the next page for answers ……….
Diabetic cataract

Figure 2 Front views of the left eye revealing aggressive lens resorption (2A) and precipitation of lens protein in the anterior chamber (2B).

(For better quality, figures can be viewed in the TJVM website)

Comments

A concern of Diabetes mellitus (DM) should be taken when dogs are presented with rapid onset of cataract and impaired vision. Ophthalmoscopic examinations initially reveal very opaque, swollen lenses known as intumescent cataract. As diabetic cataract rapidly progresses, hypermature cataract characterized by resorbed lens is usually noticed.

When more glucose enters sorbitol pathway in hyperglycemic dogs, accumulation of sorbitol, fructose and dulcitol occurs upon metabolism of aldose reductase in the lens. Increased lens cell membrane permeability leads to fluid ingress, accumulation of osmotic products, glycosylation of lens protein and shortly, dense cataract formation. Rapid swollen lens, resulting in an increased porosity of intact lens capsule, can cause small tear or spontaneous lens capsule rupture in canine cases associated with DM.

Leakage of lens protein into the aqueous elicits phacoclastic uveitis; immune-mediated mechanism of the body to lens protein, which is normally isolated inside the lens capsule. Topical NSAID is recommended to control lens-induced uveitis. Cataract surgery is advised with high success prior to the development of uveitis as well as other secondary complications.

References


ULTRASOUND DIAGNOSIS

Phiwipa Kamonrat

History

A seven-month-old, male, Shih Tzu dog was referred to the Chulalongkorn University, Small Animal, Veterinary Teaching Hospital for solving a chronic problem of demodex infestation. The dog was alert, had a normal appetite and showed no other clinical signs. Water intake and urination were normal. Physical examination revealed pink mucous membranes and a slightly tense abdomen on palpation. The result of a complete blood count was within a normal range and a blood parasite was not found. The serum biochemical analyses indicated a mild increase in alkaline phosphatase (ALP) (113 IU) and markedly elevated blood urea nitrogen (104.8 mg%) and creatinine (2.8 mg%) concentrations. Urinalysis was unremarkable. Survey radiography of thorax and abdomen were within normal limits. An abdominal ultrasonography was performed to obtain more specific information of the urinary tract.

Ultrasoundographic Findings

Real-time, ultrasonographic images were obtained using an 8 MHz microconvex, phased array transducer with the dog in dorsal recumbency. The overall renal tissue appeared hyperechoic, relative to hepatic and splenic parenchyma, compared at approximately the same depth. Both kidneys had significantly poor corticomedullary distinction and uneven cortical contour, especially the left one (Figure 1 and 2). Linear measurements of right and left kidneys were 2.9x4.3 and 2.5x4.1 cm, respectively. Each kidney contained multifocal, more than 5 cystic lesions throughout the renal parenchyma, mostly in the cortex. These renal cysts appeared as well-defined, 3-15 mm-sized, round-to oval-shaped, anechoic structures, with distal acoustic enhancement. There was a renal pelvic dilation, characterized as an anechoic space around the renal crest, measuring 4.5 and 3.5 mm of the right and left kidneys, respectively. A ureteral dilation was not evident. A thin layer of anechoic fluid was present at the periphery of the cortex of each kidney, indicative of a subcapsular perirenal fluid. The urinary bladder was seen markedly distended with anechoic urine. The urinary bladder wall was smooth, echoic, and 1 mm thick. Liver and spleen were normal in echotexture.

Ultrasoundographic Diagnosis

Bilateral polycystic renal disease
Figure 1 Sagittal and transverse ultrasonographic images of the right (A) and left (B) kidneys of a seven-month-old, male, Shih Tzu dog in dorsal recumbency. Both kidneys had significantly poor corticomedullary distinction and contained multifocal, small, anechoic cysts throughout the renal cortices. Renal pelvic dilation and subcapsular perirenal fluid were also found.

Figure 2 Schematics of the relative positions of the structures scanned in figure 1. C - anechoic cyst; P - anechoic renal pelvis; F - subcapsular perirenal fluid.

Comments

The cause of polycystic renal disease in dogs and cats may be congenital or acquired. Inherited polycystic kidney disease has been reported in Bull Terrier dogs (O’Leary et al., 1999) and Persian cats (Reichle et al., 2002). Acquired cysts are associated with various types of nephropathies. Not all animals with polycystic kidney disease develop clinical signs. Ultrasonography is a noninvasive, sensitive and specific technique for diagnosing a polycystic kidney disease. Ultrasonographic findings that are consistent in appearance with polycystic kidney lesions include multifocal, spherical to ovoid, anechoic structures that are delineated by smooth, thin, echogenic wall, with distinct far-wall border. These cysts are usually bilateral, vary in size and occur throughout the renal cortex and medulla. If they lie near the periphery of the cortex, they can distort the renal outline. Ultrasound artifacts that are helpful in differentiating cystic from solid structures include a strong acoustic enhancement distal to the anechoic cyst and an edge shadowing distal to the lateral margins of the cyst (Penninck, 2002).

In this dog, there had been no clinical signs of renal failure present. However, additional ultrasonographic findings, included a diffuse increase in renal echogenicity, poor corticomedullary differentiation, renal pelvic dilation and subcapsular perirenal fluid, concurrent with marked elevation of BUN and creatinine concentrations were supportive of nephropathy.

References


WHAT IS YOUR DIAGNOSIS

Pranee Tuntivanich¹ Suwicha Chuthatep¹

Signalment
A 5-year-old female Domestic short hair cat

History
The cat had had stranguria and had not improved after medical treatment. Urinary catheterization was performed to relieve urine retention as a palliative treatment. Reddish-colored urine was observed. Owner did not recognize any previous traumatic history.

Clinical Examination
The cat was in pain during abdominal palpation, from which distended urinary bladder (UB) was detected. Slight increase of renal blood panel and panleukopenia were detected from hematological examination.

Radiographic Examination
Ventrodorsal and right lateral abdominal radiographs were taken to evaluate urinary tract. Positive contrast urethrocystography was performed by using non-ionic iodine contrast (Iohexol 300 mg/l/ml: the same volume as the volume of urine taken out of the bladder after UB catheterization) to get more detail of the UB and urethral abnormality.

Figure 1A, B Plain ventrodorsal and right lateral abdominal radiographs
Figure 2 Positive contrast urethrocystograph

Give your diagnosis and turn to the next page.

¹ Department of Veterinary Surgery, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
**Radiographic findings**

Plain abdominal radiographs (Fig.1A, B) showed marked UB distension. Cranial displacement of the UB silhouette was found on the lateral radiograph (1B). Retrograde positive contrast cystograph (Fig.2) revealed a filling defect in urethral lumen and neck of UB. The vesicourethral junction was dilated so small amount of contrast was refluxed into the ureter. Positive contrast could not get passed through the body of UB. There was no evidence of any pelvic bone or lumbosacral vertebral fracture. Other abdominal organs were in normal appearances.

**Radiographic diagnosis**

Stenosis of the neck of UB

**Discussion**

Stenosis of the UB is caused by hyperactivity of mucosal and muscular layer healed after UB rupture or cystitis. To investigate this abnormality, positive contrast urethrocystography is recommended to identify UB luminal lesion. If lumen stenosis is suspected, case should be taken for an estimation of contrast volume used in this special technique to avoid iatrogenic UB rupture. Palpation of UB during examination or radiographic positioning should gently perform to avoid increasing pressure to the UB. Vesicourethral reflux can occur when using large volume of positive contrast medium or high pressure contrast injection during retrograde urethrocystography in normal cat. In a case of cystitis that is associated with dysfunction of the vesicoureteral valves, vesicourethral reflux can also be seen via retrograde urethrocystography.

**Figure 3** Ultrasound showed a dilated neck of UB with a thin hypoechoic septum separating between the body and the neck of UB (black arrow). The small hyperechoic particles (white arrows) represented air bubbles, which were retained after urinary catheterization.

**Figure 4** Neck of UB (black arrows) was separated from the body by thin connective tissue band and presented as a small chamber connecting between ureter and urethra.

**Reference**


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ต้องเอกสารแนบ

ขอแสดงความนับถือ

(…………………………........)

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