THE CLINICOPATHOLOGICAL STUDY AND ASSOCIATED CAUSES OF PLEURAL EFFUSIONS IN CATS

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การศึกษาทางพยาธิวิทยาคลินิกและโรคที่เกี่ยวข้องกับภาวะน้ำในช่องอกของแมว

น.ส.ฮซู มน ฮลา

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ฮซู มน ฮลา : การศึกษาทางพยาธิวิทยาคลินิกและโรคที่เกี่ยวข้องกับภาวะน้ำในช่องอกของแมว . (THE CLINICOPATHOLOGICAL STUDY AND ASSOCIATED CAUSES OF PLEURAL EFFUSIONS IN CATS) อ.ที่ปรึกษาหลัก : อารยา รัตนกถิกานนท์, อ.ที่ปรึกษาร่วม : เกษม รัตนภิญโญพิทักษ์

ภาวะของเหลวในช่องเยื่อหุ้มปอดเป็นการสะสมของของเหลวมากผิดปกติในบริเวณช่องว่างของเยื่อหุ้มปอด ซึ่งเป็นผลมาจากแรงดันภายในหลอดเลือดเพิ่มขึ้น หรือมีการซึมผ่านของเหลวผ่านเส้นเลือดฝอยเพิ่มขึ้น ซึ่งภาวะนี้มักนำไปสู่การเสียชีวิตอย่างมีนัยสำคัญในแมว วัตถุประสงค์ของการศึกษานี้คือเพื่อศึกษาตัวแปรทางพยาธิวิทยาคลินิกจากการตรวจทางเซลล์วิทยาและค่าชีวเคมีของของเหลวในช่องเยื่อหุ้มปอด และประเมินความสัมพันธ์ระหว่างความผิดปกติของตัวแปรเหล่านี้ กับสาเหตุที่นำไปสู่ภาวะของเหลวในช่องเยื่อหุ้มปอด การศึกษานี้ดำเนินการในแมวที่มีภาวะของเหลวในช่องเยื่อหุ้มปอดจำนวน 127 ตัว ที่เข้ารับการรักษา ณ โรงพยาบาลสัตว์เล็ก คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในช่วงปีพ.ศ. 2563 - 2564 ตัวอย่างของเหลวในเยื่อหุ้มปอดจะถูกวิเคราะห์และแบ่งออกเป็น 4 ชนิด รวมทั้งมีการวัดความเข้มข้นของเอนไซม์แลคเตทดีไฮโดรจีเนส และวิเคราะห์ปริมาณโปรตีนในของเหลวตัวอย่างด้วยวิธีการโปรตีนอิเล็กโตรโฟรีซิส แมวแต่ละตัวจะได้รับการวินิจฉัยสาเหตุที่ทำให้เกิดภาวของเหลวในช่องเยื่อหุ้มปอดซึ่งประกอบไปด้วย โรคเนื้องอก โรคหัวใจ โรคเยื่อบุช่องท้องติดเชื้อในแมว และหนองในช่องอก ในบรรดาสาเหตทั้งหมด เนื้องอกโดยเฉพาะอย่างยิ่งมะเร็งต่อมน้ำเหลืองเป็นกลุ่มผู้ป่วยที่ใหญ่ที่สุด (38.58%) รองลงมาคือโรคหัวใจ (21.26%) โรคเยื่อบุช่องท้องติดเชื้อในแมว (17.32%) และ และหนองในช่องอก (12.6%) ความเข้มข้นของเอนไซม์แลคเตทดีไฮโดรจีเนส ในของเหลวในเยื่อหุ้มปอด มีค่าสูงที่สุดในกลุ่มหนองในช่องอก และต่ำที่สุดในกลุ่มโรคหัวใจ ไม่มีความแตกต่างอย่างมีนัยสำคัญของความเข้มข้นของเอนไซม์แลคเตทดีไฮโดรจีเนสและความเข้มข้นของโปรตีนทั้งหมดในพลาสมาในเลือดระหว่างกลุ่มโรค ความเข้มข้นของโปรตีน α1 และ α2 โกลบูลินสูงกว่าอย่างมีนัยสำคัญในกลุ่มหนองในช่องอก และโรคเยื่อบุช่องท้องติดเชื้อในแมว เมื่อเปรียบเทียบกับกลุ่มโรคหัวใจ ความเข้มข้นของโปรตีน β globulin ในกลุ่มโรคเนื้องอก กลุ่มหนองในช่องอก และโรคเยื่อบุช่องท้องติดเชื้อในแมว มีค่าสูงกว่าในโรคหัวใจอย่างมีนัยสำคัญ ความเข้มข้นของโปรตีน γ-globulin ในกลุ่มกลุ่มหนองในช่องอก และโรคเยื่อบุช่องท้องติดเชื้อในแมว มีค่าสูงกว่ากลุ่มโรคหัวใจอย่างมีนัยสำคัญ อัตราส่วนโปรตีนชนิดอัลบูมินต่อโกลบูลิน ในกลุ่มโรคเยื่อบุช่องท้องติดเชื้อในแมว แตกต่างอย่างมีนัยสำคัญจากกลุ่มโรคหัวใจและเนื้องอก ($P < 0.0001$) โดยพบความเข้มข้นของโปรตีน γ โกลบูลินสูงสุดและอัตราส่วนโปรตีนชนิดอัลบูมินต่อโกลบูลิน ต่ำสุดในแมวกลุ่มโรคเยื่อบุช่องท้องติดเชื้อ การศึกษานี้เป็นครั้งแรกที่มีการวิเคราะห์ปริมาณโปรตีนในของเหลวในเยื่อหุ้มปอดในแมวด้วยวิธีการโปรตีนอิเล็กโตรโฟรีซิส ความเข้มข้นของโปรตีนแต่ละชนิดในของเหลวในเยื่อหุ้มปอดนั้นสัมพันธ์กับสาเหตุที่แท้จริงของการเกิดภาวะของเหลวสะสมในเยื่อหุ้มปอด ความเข้มข้นของเอนไซม์แลคเตทดีไฮโดรจีเนสและความเข้มข้นของโปรตีนที่ตรวจได้จากของเหลวในเยื่อหุ้มปอดมีความเกี่ยวข้องกับสาเหตุของความผิดปกติมากกว่าค่าที่ตรวจได้จากเลือด ข้อมูลที่ได้จากการศึกษานี้ได้ให้ข้อมูลใหม่ และเพิ่มความเข้าใจเกี่ยวกับสภาวะปัจจุบันที่ส่งผลต่อการเกิดเยื่อหุ้มปอดในแมวในกรุงเทพมหานคร

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Hsu Mon Hla : THE CLINICOPATHOLOGICAL STUDY AND ASSOCIATED CAUSES OF PLEURAL EFFUSIONS IN CATS . Advisor: Inst. ARAYA RADTANAKATIKANON Co-advisor: Inst. KASEM RATTANAPINYOPITUK

Pleural effusion is an excessive and abnormal accumulation of fluid in the pleural space resulting from increased hydrostatic pressure, increased permeability of vascular capillary, and often leading to significant mortality. The objectives of this study were to investigate the clinicopathological parameters obtained by cytological and biochemical examination of pleural effusion and evaluate the correlations between the abnormality of these parameters and underlying causes of the pleural effusion in cats. This study was carried out in 127 cats with pleural effusion visiting the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University during the year 2020–2021. Pleural fluid samples were routinely analyzed and classified into 4 groups. Evaluation of lactate dehydrogenase (LDH) concentration and protein electrophoresis in the fluid samples were performed. Underlying causes which included neoplasia, cardiac disease, feline infectious peritonitis (FIP) and pyothorax were identified. Among the underlying causes of pleural effusion, neoplasms especially lymphoma represented the largest group of the patients (38.58%), followed by cardiac disease (21.26%), FIP (17.32%), and pyothorax (12.6%). The LDH concentration in pleural effusion was highest in the pyothorax group and lowest in the cardiac disease group. There was no significant difference in LDH and total protein concentration in blood plasma between disease groups. The α1 and α2 globulin concentration is significantly higher in pyothorax and FIP when compared to the cardiac disease group. The β globulin is significantly higher in neoplasia, pyothorax and FIP than in cardiac disease. Cats with FIP, and pyothorax showed significantly higher γ-globulin concentration than in cardiac disease. The albumin to globulin ratio (A/G) in FIP was significantly different from cardiac and neoplasia ($P < 0.0001$). The highest γ globulin concentration and the lowest A/G ratio were found in FIP cats. In conclusion, this study evaluated protein electrophoresis of pleural effusion in cats for the first time. The concentrations of each protein fraction in pleural effusion are related to the underlying causes. The LDH and total protein levels in the pleural fluid are more related to the underlying causes than those in the blood. This study contributes new information and understanding on current underlying conditions that cause pleural effusion in cats in Bangkok.

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LIST OF ABBREVIATIONS

A/G	=	albumin to globulin ratio
AGE	=	agarose gel electrophoresis
AGP	=	alpha1-acid glycoprotein
ALP	=	alkaline phosphatase
ALT	=	alanine transaminase
APP	=	acute-phase protein
BSA	=	bovine serum albumin
BUN	=	blood urea nitrogen
°C	=	degree Celsius
CBC	=	complete blood count
CD	=	cardiac disease
CD	=	cluster of differentiation
CHF	=	congestive heart failure
Cr	=	creatinine
CRP	=	C-reactive protein
dl	=	deciliter
DNA	=	deoxyribonucleic acid
FCoV	=	feline coronavirus
FeLV	=	feline leukemia virus
FIP	=	feline infectious peritonitis
FIV	=	feline immunodeficiency virus
g	=	gram
HG	=	haptoglobin
IG	=	immunoglobulin
IGH	=	immunoglobulin heavy chain
IHC	=	immunohistochemistry
LDH	=	lactate dehydrogenase
NLR	=	negative likelihood ratio
nRPA	=	nested recombinase polymerase amplification

NTproBNP	=	N-terminal pro-B-type natriuretic peptide
PE	=	pleural effusion
PBS	=	phosphate buffered saline
Pla	=	plasma
PLR	=	positive likelihood ratio
RBC	=	red blood cell
RNA	=	ribonucleic acid
ROC	=	receiver operating characteristic
RTPCR	=	reverse transcriptase polymerase chain reaction
SAA	=	serum amyloid A
SpGr	=	specific gravity
TCRG	=	T-cell receptor gamma chain genes
TP	=	total protein
TNCC	=	total nucleated cell count
WBC	=	white blood cell

CHAPTER 1

INTRODUCTION

Pleural effusion is a condition of fluid accumulation in the pleural space that results from increased fluid formation or reduced fluid resorption. The accumulation of the fluid is observed in pulmonary, cardiac, or other systemic diseases depending on underlying causes and often leading to significant mortality (Medford and Maskell, 2005). This condition is a common medical emergency in veterinary medicine as well. Cats usually come to veterinary practices with remarkable signs of thoracic effusion such as dyspnea often as emergencies (Probo et al., 2018). Determining the disease etiology is important for proper treatment and a systematic approach for investigation is necessary because the differential diagnosis for pleural effusion is broad.

Classification of pleural effusion is important for the diagnosis. In human medicine, pleural effusions are categorized into two types: transudates which are caused by high hydrostatic pressure or low osmotic pressure and exudates resulting from increased vascular permeability (Valdés et al., 1991). The evaluation of biochemical parameters such as lactate dehydrogenase (LDH), total protein (TP) and triglyceride in both serum and pleural fluid have been shown as reliable parameters to identify the pathophysiological formation of a pleural effusion (Romero et al., 1993). The serum albumin concentration indicates whether the formation of effusion is caused by a low colloid osmotic pressure or high hydrostatic pressure in the transudate case (Zoia et al., 2009). In veterinary medicine, pleural effusions were originally categorized as transudate and exudate using specific gravity, protein concentration, the number and type of cells (Kirk, 1968). However, the cut-off parameters were frequently overlapping between exudate and transudate. Modified transudate group was introduced by Perman to veterinary medicine (Perman, 1971).

The author defined modified transudate as closely similar to an exudate based on cellularity and protein content, but it comes from rising hydrostatic pressure. However, the connection between the primary pathophysiological processes and characteristics of the fluids are not described very well and there are often overlaps between fluid types and causes (Beatty and Barrs, 2010a).

Until today, there have been limited studies investigating the prevalence of underlying diseases which are causing different types of pleural effusion in cats. In this study, protein electrophoresis of plasma and pleural fluid was successfully investigated and compared for the first time. The concentration of biochemical parameters in the plasma and pleural fluid including TP, LDH, .and triglyceride were also accessed. A comprehensive investigation of cytological and biochemical parameters of pleural fluid provides valuable information of the disease etiology or at the leastwise benefit clinicians to generate a list of differential diagnoses, as well as make therapeutic plans for the life-threatening condition.

Hypothesis

Current underlying conditions causing feline pleural effusion have been shifted. The abnormal changes in the concentration of LDH, triglyceride and protein electrophoresis patterns are associated with the underlying causes of the pleural effusion in the cat.

Objectives of the Study

(1) To investigate the clinicopathological parameters obtained by cytological and biochemical examination of pleural effusion

(2) To evaluate the correlations between the abnormality of clinicopathological parameters and underlying causes of pleural effusion

Keywords (Thai): การวิเคราะห์ทางชีวเคมี แมว เซลล์วิทยา น้ำในช่องอก

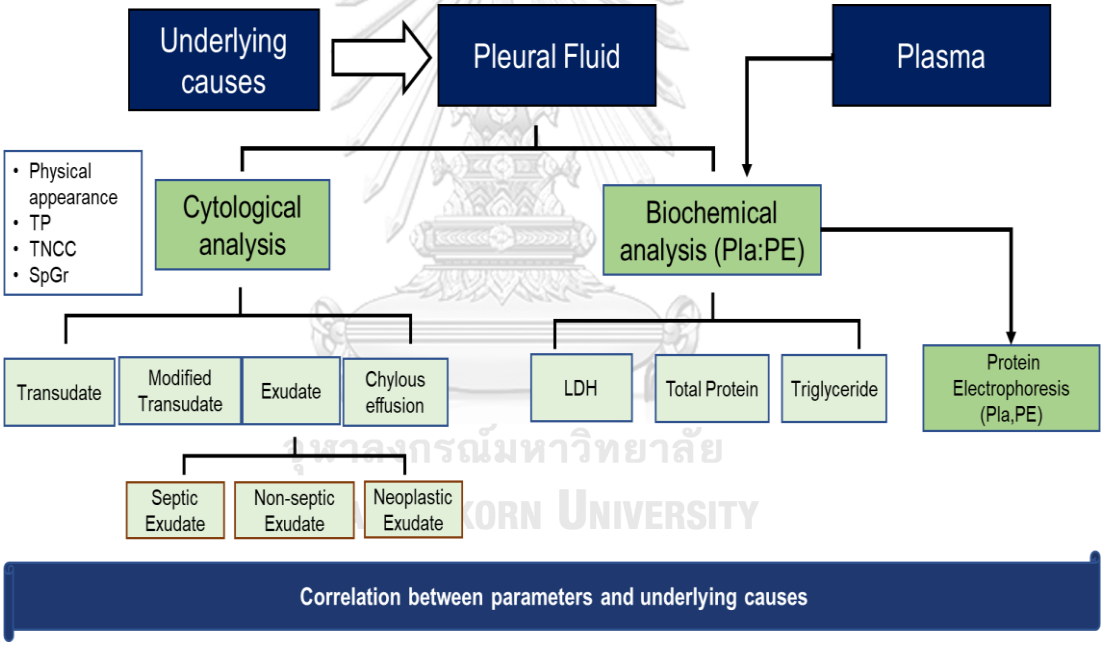
Keywords (English): biochemical analysis, cat, cytology, pleural effusion

Research questions

(1) What are the causes of pleural effusion in cats?

(2) How are these causes related to biochemical and cytological parameters in pleural effusion in cats?

Conceptual framework



Abbreviations

PE - pleural effusion

Pla - plasma

TP - total protein

TNCC - total nucleated cell count

SpGr - specific gravity

LDH - lactate dehydrogenase

CHAPTER 2

LITERATURE REVIEW

Abnormal fluid accumulation in body cavities results from a variety of underlying conditions (O'Brien and Lumsden, 1988; Dempsey and Ewing, 2011). An excess of fluid in the pleural cavity, so-called pleural effusion, is usually caused by an imbalance in the normal rate of pleural fluid production and reabsorption. Increasing capillary hydrostatic pressure resulting from congestive heart failure (CHF) or decreasing colloid osmotic pressure from hypoalbuminemia can cause pleural effusion. Moreover, it can also result from obstruction or dysfunction of the draining lymphatic ducts (Noone, 1985; Davies and Forrester, 1996). In veterinary medicine, the types of effusion are classified into transudate, modified transudate, exudate and other forms including chylous and hemorrhagic effusions based on physical examination, radiographic findings, cytological and biochemical analysis of the pleural fluid (Forrester et al., 1988). The most frequent clinical sign in cats suffering from pleural effusion presented to veterinary practitioners is dyspnea (Humm et al., 2013). The main reasons for hospitalization were breathing difficulty and anorexia. In some cases, coughing was also recorded. Chronic heart failure cats with pleural effusion had a significantly lower rectal temperature when they came to the hospital than the cats with pleural effusion from other causes (Ruiz et al., 2018).

As pleural effusion is a serious clinical condition in cats, identification of the underlying cause is critical for further therapeutic plans. The common underlying causes that lead to pleural effusion in cats included but were not limited to, CHF, neoplasia, infectious pleuritis and feline infectious peritonitis (FIP). Other causes were reported uncommonly (Zoia et al., 2009). Left or right-sided heart failure can cause pleural effusion appearing as a transudate, a modified transudate or a chylous effusion. The increase of hydrostatic pressure in the capillaries of the systemic or

pulmonary circulation causes elevation of ventricular diastolic pressure. In both dogs and cats, the visceral pleural veins and lymphatics drain into the pulmonary veins such that elevated pulmonary venous pressure causes the formation of the pleural effusion. The pleural effusion from left congestive heart failure can be found mostly in cats when compared to dogs (Kittleson and Kienle, 1998). In the dogs, effusions resulting from CHF are usually serosanguinous and slightly turbid. This fluid contains variable numbers of macrophages, lymphocytes, and erythrocytes, with a few numbers of mesothelial cells and non-degenerate neutrophils. Most of the feline thoracic effusions related to cardiac disease have been classified as modified transudates and chylous effusions (Beatty and Barrs, 2010; Smith et al., 2015). The increased pressure in the lymphatic ducts that ends in the left external jugular veins also cause chylothorax. Sixty to seventy-nine per cent of neoplasia-associated pleural effusions were reported as mediastinal lymphoma (Creighton and Wilkins, 1975; Gruffydd–Jones and Flecknell, 1978). In a study which included 306 cats with pleural effusion, the common underlying conditions were: cardiac disease (35.3%), neoplasia (30.7%), pyothorax (8.8%), FIP (8.5%), chylothorax (4.6%) and more than one etiology group (8.5%) (König et al., 2019).

The prevalence of feline leukemia virus (FeLV) is negatively related to mediastinal lymphoma in Australia because the FeLV negative Siamese cats showed a higher risk of developing mediastinal lymphoma. When mediastinal lesions are identified, other neoplastic and non-neoplastic mediastinal masses including thymoma and thymolipoma should also be considered (Louwerens et al., 2005). However, in Brazil, FeLV plays a direct role in lymphomagenesis through insertional mutagenesis and that increases the risk of lymphoma (Lingard et al., 2009). Sternal, cranial mediastinal and tracheobronchial lymph nodes can be enlarged because of various causes including tumors and virus infections such as FeLV (Louwerens et al.,

2005). The modified transudate and exudate are possibly caused by mediastinal, bronchopulmonary diseases or primary pleural neoplasia (Hirschberger et al., 1999). Over 80% of infectious pleuritis cases which results in an accumulation of purulent exudate or pyothorax, is caused by facultative and obligate anaerobes localized in the oropharyngeal area. Parapneumonic spreading of these oropharyngeal flora leads to its colonization in lung tissue (Barrs and Beatty, 2009). Several types of oropharyngeal flora including *Peptostreptococcus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Mycoplasma* spp., and *Actinomyces* spp., can cause pyothorax (Pidgeon, 1978; Thompson et al., 1992; Gulbahar and Gurturk, 2002).

Feline infectious peritonitis is a deadly disease caused by the feline coronavirus (FCoV) (Vennema et al., 1998). There are two forms of FCoV namely enteric form and FIP form which causes FIP. The enteric form shows self-limiting gastrointestinal signs. Once the virus is ingested, it enters enterocytes, replicates, and destroys the infected cells leading to diarrhea. The virus is transmitted by the fecal-oral route so feces can be used for viral antigen detection. Unlike the enteric form, FIP cannot be infectious by the fecal-oral route and emerges by mutation from the non-virulent enteric form in a small percentage of infected cats. The FIPV enters blood circulation and replicates in the macrophages that subsequently allows viremia throughout the body. The FIP consists of two forms depending on the host immune system namely: wet or effusive form and dry or non-effusive form (Addie et al., 2009). The disease may not develop in cats with strong cellular immunity, while those with a weak cellular immunity develop the non-effusive form and others without a cellular response have the effusive FIP (Pedersen and Black, 1983; Pedersen, 1995). In contrast, the increase of humoral immunity enhances the disease condition. Past studies documented that anti-FCoV antibodies promote the virus intake by macrophages (Hohdatsu et al., 1994; Pedersen, 1995). Additionally, a type III

hypersensitivity reaction mediated by the accumulation of immune complexes can cause fibrinoid necrosis of the vessel wall and increased capillary permeability that eventually results in protein and fluid leaking to the cavities (Pastoret and Bourtonboy, 1991). The complete blood count abnormalities in FIP include lymphopenia and anemia. Non-degenerate neutrophils are predominantly observed in body effusions. A diagnosis of FIP in the blood cannot be entirely reliable on polymerase chain reaction (PCR) because the positive only stands for the viral antigen but it does not mean that there is an association between FCoV infection and clinical disease (Meli et al., 2004). One of the reliable methods includes reverse transcriptase (RT) PCR using effusion fluids to detect FCoV messenger ribonucleic acid (mRNA) in peripheral blood mononuclear cells (Valenciano and Cowell, 2019). Feline infectious peritonitis infection is highly suggestive when TP is more than 8 g/dl in effusions, globulin concentration is more than 5 g/dl in both serum and effusions, albumin to globulin ratio (A/G) is less than 0.45 in both serum and effusions, and antibody titer of FCoV in the effusions is more than 1:1280 (Rahman et al., 2004). The specific tool for the confirmation of FIP is direct staining of FCoV within the macrophages by immunofluorescence in cytocentrifuged effusions (Hartmann, 2005). Most of the FIP patients are found at younger ages than those with neoplasia or CHF (Davies and Forrester, 1996). Apart from FIP, neoplasia and cardiac disease, there are other uncommon causes of pleural effusion which include trauma that usually results in hemorrhage and thoracic duct rupture that leads to chylous effusion (Störk et al., 2003).

There are many references to classify types of pleural fluid in terms of cellularity and protein content. Rakich PM & Latimer KS classified pleural effusions in dogs and cats into five groups: chylothorax, malignant pleural disease, exudative, transudate or modified transudate and hemorrhagic effusions according to the

clinicopathological and histopathological evaluation (Latimer, 2011). The effusion is defined as a malignant pleural disease when histopathology and cytology of pleural effusion revealed neoplastic diseases. When the fluid has a total nucleated cell count (TNCC) of more than 7,000 cells/µl and TP concentration of more than 3 g/dl, the effusion is called an exudate. The fluid that has TNCC less than 1,500 cells/µl and TP concentration lower than 2.5 g/dl are defined as transudate and the fluid that has TNCC between 1,000-7,000 cells/µl and TP concentration between 2.5-5 g/dl is defined as modified transudates. Chylothorax is characterized by the accumulation of chyle within the thoracic cavity and frequently appeared as white or pink opaque color due to a high concentration of triglyceride (>100 mg/dl). Cytologically, predominant small lymphocytes and lipid droplets are observed in chylous effusion. In human medicine, causes of chylothorax are classified as congenital, traumatic, neoplastic, and other various factors (DeMeester, 1983). In the traumatic type, patients present with coughing, dyspnea, and chest pain. The increased amount of chylous effusion in the chest cavity can cause more severe symptoms (Bessone et al., 1971). Hemorrhagic effusion is reported when the fluid contains pack red blood cell volume at a minimum of 25% of the patient's peripheral blood (Latimer, 2011). If a vessel is punctured, the pack cell volume of fluid will be equal to that of peripheral blood. It can be caused by rupturing of vessels or changes in vascular endothelial integrity which is normally maintained by the interaction of platelets and various clotting factors (Lighthouse Veterinary Consultants). Another reference of pleural fluid classification was published by Beatty and Barrs. According to the authors, there were four types of pleural effusions. The effusions are classified as transudates when TP is less than 2.5 g/dl and TNCC is less than 1,000 cell/ul, modified transudate when TP is 2.5-3.5 g/dl and TNCC is 500-10,000 cell/ul, exudates when TP is more than 3 g/dl and TNCC is more than 5,000

cell/ul and chylous that may have the same number as in exudate or modified transudate with predominant lymphocyte population (Beatty and Barrs, 2010).

Lactate dehydrogenase is an enzyme that catalyzes the last step of pyruvate conversion to lactate during anaerobic glycolysis. The LDH exists in 5 isoenzyme forms (LDH-1 to LDH-5) and can be found in different concentrations in different tissues (Zhao et al., 2014). The subunits: muscle (M) and heart (H) proteins which are encoded by LDHA and LDHB genes respectively are included in the composition of LDH tetrameric enzyme (Adeva-Andany et al., 2014). The isotype LDH-5 (4M) also known as LDHA is found predominantly in skeletal muscle. By contrast, LDH-1 (4H) also called LDHB is the predominant isoform found in heart muscle (Liang et al., 2016). Other isoforms: LDH-2 (3H1M), LDH-3(2H2M) and LDH-4 (1H3M) are found mainly in the reticuloendothelial system, lung and kidney, placenta and pancreas respectively (Dawson et al., 1964). Various pathological processes can cause the change in LDH isoenzyme fractions in many domestic animal species. The LDH-5 level was detected in acute muscle dystrophy in lambs (Sobiech and Kuleta, 2002), the LDH-2 and LDH-3 levels were elevated in dogs with lymphoma (Abate et al., 1997) and the levels of LDH-2 and LDH-4 were significantly increased in the urine than in the plasma in the case of bovine bladder cancer (Dawra et al., 1991). Biochemical analysis of serum LDH has been widely used to diagnose lymphoma, anemia, lung, and liver diseases in humans. Serum LDH level and its isoenzyme have been investigated in different kinds of cancers. Malignant tumor tissues release LDH into circulation and cause an abnormal increase in enzyme levels (Mohajertehran et al., 2019). Since red blood cells (RBC) contain a high amount of isoenzyme LDH-2, hemolysis of the blood sample can affect LDH level resulting in a false higher value (Farhana and Lappin, 2020). In human medicine, serum LDH level indicates an ongoing inflammatory process, and the increased concentration is used to

differentiate between exudates and transudates (Light, 2007). The LDH level is also high in patients with pulmonary tuberculosis. Most malignant and inflammatory conditions show a higher LDH in the effusion than in the blood plasma (Horrocks et al., 1962).

The acute phase response is a reaction that responds to both acute and chronic inflammatory conditions such as infection, inflammation, trauma, and neoplasia. There are three main groups of acute-phase proteins (APPs): the major APPs such as C-reactive protein (CRP) and serum amyloid A (SAA), the moderate APPs such as haptoglobin (HG) and α globulin protein (AGP) and the negative APPs such as albumin. The response of the APPs varies and depends on species (Petersen et al., 2004). Generally, CRP and SAA are the common acute phase response in any species (Baumann and Gauldie, 1994). In the cats, SAA expresses in the highest amount in response to inflammation, as opposed to CRP as observed in other species, followed by haptoglobin and AGP (Rosa and Mestrinho, 2019). Blood SAA level is an indicator for inflammatory conditions and infection in cats such as FIP, caliciviral infection, and chlamydiosis. It can also be increased in other non-infectious diseases for example diabetes mellitus and cancer (Tizard, 2013). Haptoglobin usually elevates 2- to 10-fold and is especially high in FIP (Tizard, 2013). The APPs have a high molecular weight that is generally more than 45 kDa, thus they have a long half-life in the plasma (Salgado et al., 2011). The APP level should be interpreted together with white blood cell and neutrophil counts because they are sensitive in the detection of inflammation and infection in their initial phase (Cerón et al., 2008; Alves et al., 2010). Human SAA turbidimetric immunoassay has been successfully optimized to detect SAA in feline serum (Hansen et al., 2006) and the application of various human biochemical parameters to use in feline effusion samples has been reported (Konig et al., 2019).

The total protein test measures the total amount of protein in the serum, specifically the amount of albumin and globulin. Protein electrophoresis is a reliable method that can determine protein composition precisely. In veterinary medicine, it is generally applicable to investigate hyperproteinemia and hypoproteinemia when monoclonal or polyclonal gammopathy is suspected (Gerou-Ferriani et al., 2011). In domestic cats, abnormal patterns of electrophoretogram are mainly related to infectious or inflammatory conditions (Taylor et al., 2010). The protein compositions in plasma or serum samples will migrate on a thin agarose gel with electrical current according to their charge and size. The separated bands appearing on the gel can be quantified by densitometry and numerous samples can be analyzed simultaneously. Albumin and four fractions of globulins including alpha (α) 1, α2, beta (β), and gamma (γ) can be determined by protein electrophoresis (Keren, 2003). Serum protein electrophoresis is one of the most useful tools for the diagnosis of FIP that shows increasing serum total protein concentration with decreasing A to G ratio (Pedersen, 2014). An increased concentration of the α2 and the γ globulin fraction in serum is a typical characteristic of the FIP electrophoretic pattern (Pedersen, 2009). The electrophoretograms also showed the increased concentration of α2 and the γ globulin fractions in serum and effusion of FIP cats (Giori et al., 2011). In the condition of naturally occurring FIP and unknown duration of the infection, abnormalities of protein fractions are variable, however, polyclonal gammopathies are usually found in the majority of FIP cats (Sparkes et al., 1991; Paltrinieri et al., 2001). Some APP like haptoglobins is included in the α globulin fraction and they are elevated in the presence of acute inflammation, malignancy, and nephrotic syndrome. Lipoproteins, immunoglobulin (Ig) A, IgM, and the negative APP such as transferrin are included in β globulin fraction. The increase of the β globulin fraction is found in inflammation, neoplasia, and various metabolic conditions. Increased γ

globulin is related to chronic antigenic stimulation because this fraction contains immunoglobulins especially IgG (O'Connell et al., 2005). These elevations can be described as polyclonal gammopathy when the increase in immunoglobulins is produced by a heterogeneous population of B cells. The monoclonal gammopathy appearing as a narrow peak of a single protein on the electropherogram is associated with malignant plasma cell disorders such as multiple myeloma, extramedullary plasmacytoma and lymphoma in dogs (Font et al., 1994). However, multiple myeloma is a rare condition in cats, approximately less than 1% of feline hemopoietic neoplasms (Eastman, 1996). In cats with lymphoma, the elevation of γ globulin fraction is not common, except when secondary infection or B-cell lymphoma is presented. Lymphoma cats showed lower albumin and higher β globulin concentrations compared to healthy control cats (Gerou-Ferriani et al., 2011). Serum electrophoresis of cats infected with feline immunodeficiency virus (FIV) showed polyclonal hypergammaglobulinemia in electrophoretic pattern and cats with FeLV infection showed various γ globulin levels and significantly higher α2 globulins (MacEwen, 1977). In the study including 155 cats, polyclonal increase in γ globulins was the most common and infectious and inflammatory diseases occupied the largest population among these cases. Monoclonal increase in globulins was uncommon and found associated with neoplasia and a few FIP cases (Taylor et al., 2010).

Lymphoma is the proliferation of neoplastic lymphoid cells, representing 90%of all tumors of hemopoietic origin in domestic cats (Valli et al., 2017). The differentiation of reactive and neoplastic lymphoid proliferations is challenging. If the microscopic evaluation is inconclusive, molecular clonality assays or PCR for antigen receptor gene rearrangement (PARR) to evaluate the diversity of lymphocyte antigen receptor gene rearrangement can be useful. Clonality assays are commonly used to

access atypical, mixed, or mature lymphoid proliferation in tissue, cell smears and body cavity fluid (Van Dongen et al., 2003). Once the differentiation of small cell lymphoma and mature lymphocytes cannot be verified by histomorphological examination alone, complementary tools like PCR-based immunoglobulin (IG) and T-cell receptor (TCR) clonality testing is suggested for confirmation. Rearrangement of the variable segments on immunoglobulin heavy chain (IGH) and T-cell receptor gamma chain (TCRG) genes through random recombination of variable, diversity, and joining regions occurs in the early stages of lymphoid differentiation (Blom et al., 1999; Jung et al., 2006). B- and T-cell lymphomas are the result of a clonal proliferation originating from a single neoplastic cell; because all tumor cells contain a unique clonal IGH or TCRG gene rearrangement, hence, clonal pattern or a certain size of the PCR product is detected (Van Dongen et al., 2003; Keller et al., 2016). The clonality assays are not stand-alone tests. The tests are useful when clinical, morphology, and immunophenotyping data are integrated. Clonality assays are species-specific, and currently available for dogs, cats, and horses in veterinary medicine (Keller et al., 2016).

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethical statements

The present study was approved by Chulalongkorn University Animal Care and Use Committee (Approval No. 2131016) in accordance with the faculty regulations. Client consent form is available in Appendix.

3.2. Cases and sample collection

Cats with pleural effusion in any age, sex, and breed that visited the Chulalongkorn University Small Animal Hospital during the year 2020–2021 were included in the study (n=127). Among the total of 127 cats, 114 cats with definitive causes were included in the statistical analysis. Number of cases in each analysis group is agreed upon according to the sample size calculator (G*Power 3.1.9.4). The pleural fluid was collected by ultrasound-guided thoracocentesis, and the blood was obtained from the cats on the same day of thoracocentesis. The K3EDTA tube (FL medical Co., Ltd, Italy) was used to collect 1 ml of the blood sample and a plain microcentrifuge tube was used to collect 1.5 ml of the fluid sample. Routine body fluid analysis was performed within 24 hours after the thoracocentesis. Fluid supernatants, pellets and plasma were kept at -80˚C for biochemical evaluation and further investigation.

3.3. Identification of underlying causes

Case signalment and clinical data including physical examination, thoracic radiographs, hematology parameters, bacterial culture results and molecular techniques that were applied were reviewed from electronic medical records to identify the underlying causes of pleural effusions. When the data from clinical records was limited or inconclusive, fluid pellets kept at -80˚C and cytology slides

from the routine body fluid analysis were used for further investigation of their suspected causes such as FIP and lymphoma. Neoplasia cases were diagnosed by cellular morphology. In confirmed lymphoma and lymphoma suspected cases, immunophenotyping and PCR for antigen receptor gene rearrangements (PARR) were performed. Cardiac diseases were diagnosed by echocardiography, X-ray, and N-terminal pro-B-type natriuretic peptide (NTproBNP) test according to the medical records. Feline infectious peritonitis was diagnosed by the predominance of non-degenerate neutrophils on cytological examination of the effusion and TP concentration. All the FIP cases were confirmed by RT-PCR of FCoV using the pellet of the pleural fluid. Pyothorax was verified by the presence of neutrophils and macrophages with intracytoplasmic bacteria in cytology examination and positive bacterial culture results. Feline leukemia virus infection was confirmed by the antigen test kit (Witness FeLV-FIV, Zoetis, USA).

3.4. Laboratory examination

3.4.1. Pleural fluid analysis and classification

Routine fluid analysis including physical appearance, TP concentration, TNCC, and specific gravity was performed. The TNCC per µl was evaluated in the hemocytometer chamber (Boeco Co., Ltd, Germany) by mixing the pleural fluid with a diluent (2% acetic acid) to lyse RBCs. The mixture was loaded onto the hemocytometer, counted the total number of cells in 4 squares of the counting chamber and obtained the number of the cells. TNCC/µl was calculated as the number of cells counted multiplied by 100 and divided by 4. The TP and specific gravity were measured by using the handheld refractometer (ATAGO Co., Ltd, Japan).

The pleural fluid was classified as transudate, modified transudate, exudate, and chylous effusion based on TNCC in cells/µl and the concentration of TP in g/dl

according to Rakich PM, Latimer KS. Briefly, the fluid sample is a transudate when the TNCC is <1,500 cells/µl and the TP concertation is <2.5 g/dl, a modified transudate when the TNCC is 1,000-7,000 cells/µl and the TP concentration is 2.5-5 g/dl, and an exudate when the TNCC is >7,000 cells/µl and TP concentration is >3g/dl. The fluid samples that predominantly contain small lymphocytes and lipid droplets on cytology and show physical characteristics as milky appearance was classified as chylous effusion (Latimer, 2011).

3.4.2. Cytological examination

After the routine fluid analysis was performed, the pleural fluid was promptly centrifuged at 3,500 rpm for 10 minutes using a microcentrifuge (Labnet International, Inc., Germany). The fluid sediment was applied on a clean glass slide for a thin smear preparation and air-dried. Then the smeared slide was fixed in absolute methanol solution (Sigma-Aldrich, Inc., Germany) for 2 minutes and air-dried. The methanol fixed smeared slides were stained with 1% Giemsa's solution (Sigma-Aldrich, Inc., Singapore) for 25 minutes. Cell morphology was observed under a compound light microscope by a veterinary pathologist. A microscopic description was made, and the fluid types were determined in the samples that were categorized as exudative effusion. The septic type was given when degenerate neutrophils and intracytoplasmic bacteria were observed in cytological examination or bacterial culture was suggestive of the septic condition. The neoplastic type was given when potential malignant cells are observed in the cytological examination. The samples were considered as a non-septic type when none of the two criteria was presented.

3.4.3. Biochemical examination

The fluid supernatant and plasma samples were subjected for biochemical examination using BS-800 Mindray Chemistry Analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China). The concentration of LDH and TP in every case of pleural effusion and triglyceride in cases which suspected chylous effusion was determined using the Mindray Chemistry LDH kit, TP kit, and TG kit, respectively (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China).

3.4.4. Protein electrophoresis

The protein electrophoresis was performed in both pleural fluid supernatant and plasma. Samples from fluid supernatant and plasma were prepared and separated by agarose gel electrophoresis (AGE) system with SPIFE® split beta SPE kit (Helena Laboratories, TX, USA). Both samples were electrophoresed for 6 min in 400 V. The gel was dried and stained with acid blue staining solution. Then it was destained in the citric acid solution. The procedures were performed by an automated machine (Spife®3000, Helena Laboratories, TX, USA) in all the steps. The density of each band of the samples in the electrophoresis pattern was analyzed and visualized using Quick Scan Touch software (Helena Laboratories, TX, USA). Protein fractions were determined according to their absorbance and the concentration of each fraction in g/dL was calculated. Albumin to globulin (A/G) ratios was also automatically calculated.

3.4.5. Immunohistochemistry (IHC)

Immunohistochemistry was performed as a preliminary in 7 FeLV positive cats with FeLV positive. Three cytology slides from the fluid pellet of each cat were prepared. The air-dried cytology slides were fixed in acetone for 2 minutes,

incubated in quenching buffer (3.33% hydrogen peroxide in 10X phosphate-buffered saline) for 10 minutes and washed with phosphate-buffered saline (PBS). One per cent bovine serum albumin (BSA) was used for blocking the non-specific binding of antibodies to the cells for 20 minutes at room temperature. For T lymphocyte identification, rat monoclonal CD3 antibody (1:10) (Leukocyte antigen biology lab, UC Davis, USA) were used as a primary antibody. For the B lymphocyte identification, rabbit-polyclonal CD20 antibody (1:300) (Abcam, UK) and mouse-monoclonal CD79α antibody (1:200) (Abcam, UK) were used as primary antibodies. The primary antibodies were incubated for 30 minutes at room temperature. For the secondary antibody, ImmPRESS anti-rat Ig (Vector laboratories, Inc, USA), ImPRESS anti-mouse Ig (Vector laboratories, Inc, USA), and Envision polymer (DAKO, Japan) for CD3, CD79α and CD20 antibodies, respectively, were applied and incubated for 30 minutes at room temperature. The Nova red solution (Vector Laboratories, Inc, USA) and diaminobenzidine (DAB) were used as substrates for and ImmPRESS kits and Envision polymer, respectively. The reaction was stopped by running tap water, then the slides were counterstained with hematoxylin for 10 seconds. The impression smear of normal cat lymph nodes obtained from necropsy was used as a positive control.

3.4.6. Clonality test (PCR for antigen receptor gene rearrangement: PARR)

In this study, 14 cats with inconclusive diagnoses of lymphoma were investigated. The pleural fluid pellet was summitted to Companion Animal Cancer Research Unit (CAC-RU), Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. The PARR approach has been described elsewhere (Radtanakatikanon et al. 2021). The PCR products were visualized by QIAxcel advanced automated electrophoresis analyzer (Qiagen, Germany) and analyzed using the QIAxcel ScreenGel software (1.6.0) (Qiagen, Germany).

3.4.7. Feline infectious peritonitis virus detection

Ten cats with suspecting FIP due to predominance of non-degenerate neutrophils with TP concentration in fluid cytology were tested for the FCoV antigen. Two hundred microliters of pleural fluid pellets kept at -80 °C were summitted to Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University, for the application of RT-PCR.

3.5. Statistical analysis

SPSS statistics version 22.0 (https://www.it.chula.ac.th/en/spss-v22) was used. Descriptive statistics were used to report the characteristics of cytological examination. Kolmogorov-Smirnov and Shapiro-Wilk normality test was used for verification of normal distribution of the data. Independent-samples Kruskal-Wallis test was used for the analysis of data which were not normally distributed. For unpaired data, Spearman's rank correlation test was used. To establish the optimal cut-off points of fluid to serum ratio of LDH, TP, albumin, α1, α2, β and γ globulins, receiver operating characteristic (ROC) curve analysis was used to plot sensitivity and specificity values while distinguishing between the underlying causes. Optimal cut-off points were established by selecting the points of test values that provided the greatest sensitivity and specificity. Positive likelihood ratio (PLR) and negative likelihood ratio (PLR) were defined by using sensitivity and specificity as an alternative way according to the formula: PLR = sensitivity / (100 – specificity) and NLR = (100 – sensitivity) / specificity. This diagnostic accuracy test was done using the significant pairs found in the Kruskal-Wallis comparison. The unknown, trauma, pneumonia and more than one etiology group were not included in the statistical comparison. The result was significantly different if the p-value was less than 0.05.

CHAPTER 4

RESULTS

4.1. Patient

Among the total of 127 cats, 114 cats with definitive causes were included in the statistical analysis. Comparison between the age of the cats, body temperature and underlying causes are described in Table 1. The pyothorax group was significantly younger than the cardiac one (P=0.001). With a median age of 1.25 years, cats diagnosed with FIP were significantly younger compared with cats with cardiac disease (P=0.016) and neoplasia (P<0.0001). There was no significant difference in body temperature between the groups. The most affected breed was domestic shorthair (105/127, 82.68%), others included Persian (6/127, 4.72%), American short hair (3/127, 2.36%), Scottish fold (4/127,3.15%), and Maine coon (1/127, 0.79%). Eight cats were documented as a mixed breed (6.30%) (Appendix Table 1). According to their discharge status on the electronic record, 23 of 127 cats died (18.11%).

Table 1 Signalment and clinical parameter in cats with the underlying condition of pleural effusion

Etiologies / Parameters	Neoplasia (a)	CD (b)	FIP (c)	Pyothorax (d)	Comparison between groups	P-value
Age (year)	n=49 3 (0.33-16)	n=27 5 (1-14)	n=22 1.25 (0.33-13)	n=16 1.25 (0.33-7)	d-b c-a c-b	0.001 0.016 <0.0001
Body Temperature (˚F)	n=38 100.7 (94.5-102.2)	n=24 100.8 (94-104.2)	n=21 100.6 (95.2-103.4)	n=13 100 (85- 104)	#	

Data are median (range)

CD = cardiac disease, FIP = feline infectious peritonitis.

The overall test does not show significant differences across samples.

4.2. Fluid type classification and underlying causes

Based on the fluid analysis, 6 types of fluid were classified: transudate, modified transudate, exudate (subgroups: septic, non-septic, and neoplastic), and chylous effusions in 127 cats. In the classification of the fluid, modified transudate was the most common form of fluid, and the least common form was transudates (Table 2). The percentage of underlying causes of effusions in 127 cats are listed in Table 3. Among the underlying causes of the pleural effusion, neoplasms represented the largest group of the patients (38.58%), followed by cardiac disease (21.26%), FIP (17.32%), and pyothorax (12.6%) (Table 3). The most common neoplasm found in this study was lymphoma (40/49, 81.63%), followed by epithelial cell tumor (2/49, 4.08%), feline fibrosarcoma (1/49, 2.04%) and unclassified neoplasms which were confirmed by X-ray findings showing nodules and masses (4/49, 8.16%). The relationship between underlying causes and types of fluid are shown in Table 4. Transudative effusions were observed only in cardiac disease (4/4, 100%). Feline infectious peritonitis effusions were mostly found as modified transudate (17/53, 32.07%). Chylous effusion was found both in neoplastic and cardiac disease. Cytology of the septic, non-septic and neoplastic exudates are shown in Figure 1.

Table 2 Types of fluid in 127 cats with pleural effusion

Type of fluids	Number of cats
Modified transudate	62 (48.82%)
Neoplastic exudate	30 (23.62%)
Septic exudate	14 (11.02%)
Non-septic exudate	10 (7.87%)
Chylous effusion	6 (4.72%)
Transudate	5 (3.94%)
Total	127

Table 3 Underlying conditions in 127 cats with pleural effusion

Underlying Causes	Number of cats
Neoplasia	49 (38.58%)
Cardiac Disease	27 (21.26%)
Feline Infectious Peritonitis	22 (17.32%)
Pyothorax	16 (12.60%)
Unknown	7 (5.51%)
Pneumonia	4 (3.15%)
More than one	1 (0.80)
Trauma	1 (0.80)
Total Number	127

Table 4 Relationship between underlying causes and types of pleural fluid

Type of fluid	Causes				Total
	Neoplasia	CD	FIP	Pyothorax	
Transudate	0	4	0	0	4
Modified transudate	16	20	17	2	53
Septic exudate	0	0	0	14	14
Neoplastic exudate	30	0	0	0	30
Non-septic exudate	0	2	5	0	9
Chylous effusion	3	1	0	0	4
Total	49	27	22	16	114

CD = cardiac disease, FIP = feline infectious peritonitis

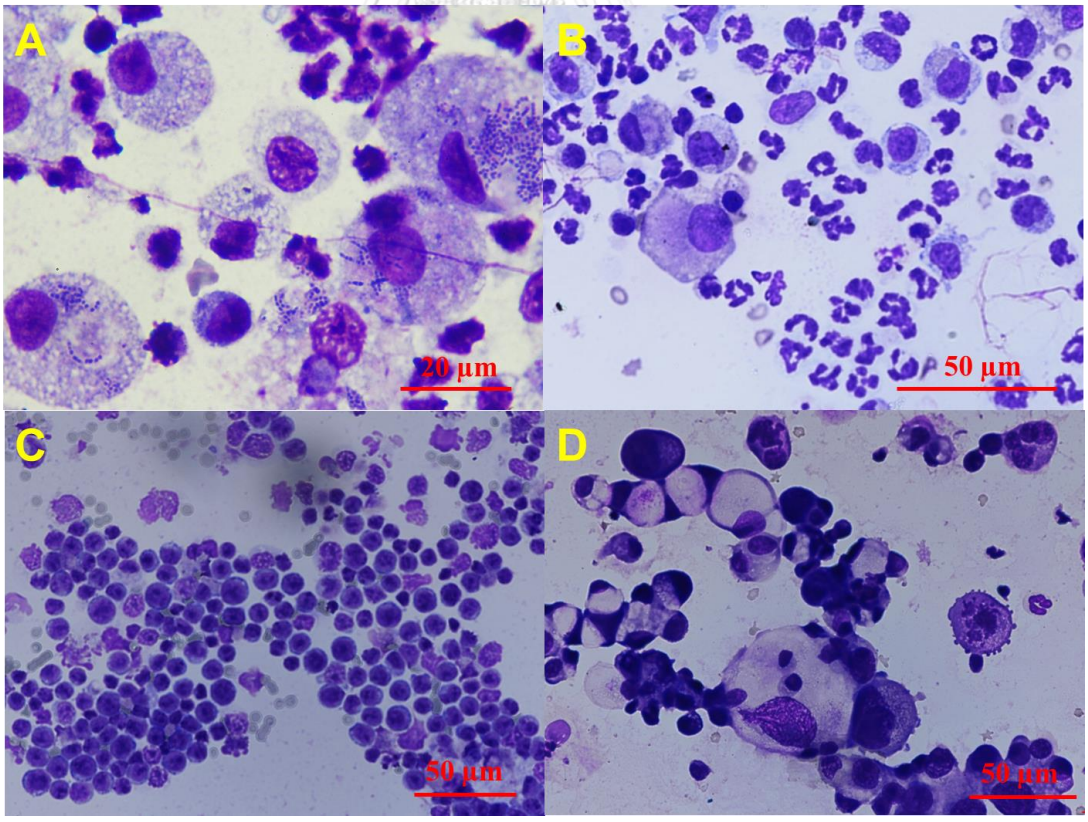


Figure 1 Cytology of pleural effusion (A) Pyogranulomatous effusion showing foamy macrophages with clusters of intracytoplasmic cocci and rod-shaped bacteria with

degenerated neutrophils (Septic exudate). (B) Feline infectious peritonitis effusion showing numerous numbers of non-degenerate neutrophils, foamy macrophages, and a few small lymphocytes (non-septic exudate). (C) Numerous numbers of large lymphoblasts suggestive of lymphoma (neoplastic exudate). (D) Numerous clusters of atypical epithelial cells showing various malignancy criteria including pleomorphism and mitosis suggestive of a malignant epithelial tumor (neoplastic exudate). Giemsa staining. Scale bars are indicated.

4.3. Effusion parameters and blood parameters

The statistical comparison between pleural effusion parameters [TNCC, specific gravity, TP and LDH concentration], complete blood count [red blood cell (RBC), hematocrit value, platelet count, total white blood cell (WBC), neutrophil, eosinophil, monocytes, lymphocytes] and serum biochemistry parameters [alanine transaminase (ALT), alkaline phosphatase (ALP), creatinine (Cr) and blood urea nitrogen (BUN)] and underlying causes are listed in Table 5. The number of cats that were analyzed for each parameter was varied and indicated in the table. Nucleated cell count was significantly lower in cardiac disease than in neoplastic diseases and pyothorax ($P<0.0001$). The TNCC in neoplasia ($P=0.019$) and pyothorax ($P<0.0001$) were also significantly higher than in FIP effusion. The specific gravity was significantly lower in cardiac disease than neoplasia ($P=0.016$), pyothorax ($P=0.042$) and FIP ($P<0.001$). The neoplasia group was significantly lower than in FIP effusion ($P=0.014$). The total protein concentration in pleural effusion was significantly lower in cardiac group than in the pyothorax ($P=0.035$), neoplasia ($P=0.001$) and FIP ($P<0.001$). The total protein concentration in FIP effusion was significantly higher than in pyothorax ($P<0.041$) and neoplasia ($P<0.004$). The LDH concentration in pleural effusion was highest in the pyothorax group and lowest in the cardiac disease group. There was no

significant difference of LDH and TP concentration in blood plasma between disease groups.

There was no significant difference of RBC count between the groups but the hematocrit value in neoplasia is significantly higher than in FIP (P=0.043) group. No significant. difference of total WBC count between the groups but eosinophil count of neoplasia group was significantly higher than FIP group (P=0.020). No significant difference of ALT and ALP levels was observed between the groups. Creatinine concentration in neoplasia (P=0.013) and cardiac disease was significantly higher than in the FIP group (P=0.001). Blood urea nitrogen in FIP was also lower than in cardiac disease (P=0.001) and pyothorax (P=0.011) (Table 5).

Table 5 Effusion parameters, CBC, and serum biochemistry parameters in cats with different etiologies for pleural effusion

Etiologies / Parameters	Neoplasia (a)	CD (b)	FIP (c)	Pyothorax (d)	Comparison between groups	P-value
Effusion Parameters						
TNCC (cells/µl)	n=48 12350 (75- 20000)	n=27 850 (50- 12250)	n=21 2925 (225- 18600)	n=16 20000 (550- 20000)	b-a b-d c-a c-d	<0.0001 <0.0001 0.019 <0.0001
SpGr	n=49 1.02 (1.010-1.037)	n=27 1.02 (1.012- 1.078)	n=22 1.034 (1.00-1.047)	n=16 1.028 (1.015-1.040)	b-a b-d b-c a-c	0.016 0.042 <0.0001 0.014
TP (g/dl)	n=49 4.1 (1.5-12.6)	n=27 2.5 (0.8- 8.4)	n=22 6.35 (3.2-10.8)	n=16 4.35 (0.6-10.6)	b-d b-a b-c	0.035 0.001 <0.0001

					d-c a-c	0.041 0.004
LDH (U/L)	n=49 873.30 (48- 8141)	n=27 94.60 (25-1539)	n=22 2358.20 (79-6587)	n=16 4203.65 (55- 34949)	b-a b-c b-d	<0.0001 <0.0001 <0.0001
CBC Parameters						
RBC ($x10^6$cell/ul)	n=48 7.12 (2.7-10.63)	n=26 6.87 (2.93-9.77)	n=22 6.75 (2.51- 10.66)	n=13 7.98 (3.63-10.81)	#	
Hematocrit (%)	n=48 33.2 (16.0-53.6)	n=26 30.0 (12.1-38.6)	n=22 29.35 (10.9-39.5)	n=13 35.0 (16.0-44.7)	c-a	0.043
Platelet ($x10^3$cell/ul)	n=48 134.0 (9.0-33)	n=26 135.0 (15.0-33)	n=22 80.0 (27.0-46)	n=13 140.0 (51.0-32)	#	
Total WBC ($x10^3$ cell/ul)	n=48 15.76 (5.80- 151.68)	n=26 13.61 (5.62-55.49)	n=22 18.77 (0.400-88.13)	n=13 19.56 (4.59- 75.59)	#	
Neutrophil ($x10^3$ cell/ul)	n=48 12.45 (1.98-141.06)	n=26 10.74 (3.36-48.61)	n=20 18.72 (4.71-80.90)	n=13 16.98 (2.02-70.60)	#	
Eosinophil (cell/µl)	n=48 387 (0- 1668)	n=26 236 (0- 1609)	n=20 150 (20- 1192)	n=13 216 (42- 544)	c-a	0.029
Monocyte (cell/µl)	n=48 375 (93-5612)	n=26 355 (37-2719)	n=20 294 (68-2732)	n=13 974 (121-9747)	#	
Lymphocyte (cell/µl)	n=48 1528	n=26 1396	n=20 1525	n=13 1542	#	

	(271-17374)	(281-4697)	(109-4897)	(444-4842)		
Serum Chemistry						
ALT(U/l)	n=46 37 (10-1361)	n=24 63.5 (8-502)	n=21 56 (12-261)	n=10 37.5 (10-121)	#	
ALP (U/l)	n=37 25 (8-268)	n=21 21 (5-219)	n=18 19.5 (5-164)	n=7 16 (5-72)	#	
Cr (mg%)	n=46 1.3 (0.5-18.7)	n=23 1.4 (0.7-29.4)	n=22 0.9 (0.4-4.4)	n=10 1.15 (0.7-6.3)	c-a c-b	0.013 0.001
BUN (mg%)	n=46 28.95 (13.2-257.0)	n=23 39.8 (15.0-274.6)	n=22 21.75 (13.0-99.3)	n=10 35.3 (25.0-104.5)	c-b c-d	0.001 0.011
TP (g/dl)	n=20 5.9 (4.1-7.1)	n=14 5.85 (4.7-11.4)	n=12 7.25 (4.7-8.7)	n=9 5.9 (3.7-7.3)	#	
LDH (U/L)	n=17 404.6 (173.9- 1736.7)	n=12 371.95 (115.9- 1286.3)	n=7 340.5 (216.8-1463.1)	n=6 427.2 (264.6- 2646.6)	#	

Data are median (range)

CD = cardiac disease, FIP = feline infectious peritonitis, TNCC = total nucleated cell count, SpGr = specific gravity, TP = total protein, LDH = Lactate dehydrogenase, CBC = complete blood count, RBC = red blood cell, WBC = white blood cell, ALT = alanine transaminase, ALP = alkaline phosphatase, Chol = cholesterol, Cr = creatinine, BUN = blood urea nitrogen

Only the significant pairs are described. # = No significant differences across the groups.

4.4. Protein electrophoresis in pleural effusion

Protein fractions of pleural effusion in the different disease groups were listed in Table 6. Cats diagnosed with cardiac disease had significantly lower albumin concentration in pleural effusion compared to neoplasia (P<0.0001). The α1 globulin concentration is significantly higher in pyothorax (P=0.017) and FIP (P<0.0001) when compared to the cardiac disease group. The α2 globulin concentration in the neoplasia and FIP group are significantly higher than in the cardiac group (P=0.001). The β globulin is significantly higher in neoplasia (P=0.011), pyothorax (P<0.0001) and FIP (P<0.0001) than in cardiac disease. The β globulin is significantly higher in FIP compared to in the neoplasia group (P=0.018). Cats with FIP (P<0.0001) and pyothorax (P=0.035) showed significantly higher in γ globulin concentration. than in cardiac disease. The γ globulin concentration in FIP was significantly higher than in neoplasia (P<0.0001). The albumin to globulin ratio (A/G) in FIP was significantly different from cardiac and neoplasia (P<0.0001). The highest γ globulin concentration (2.25 g/dl) and the lowest A/G ratio (0.4) were found in FIP cats and the polyclonal gammopathy was observed in the electrophoretic pattern (Figure 2).

Table 6 Protein fractions in pleural fluid of cats with different etiologies of pleural effusion

Etiologies / Proteins	Neoplasia (a)	CD (b)	FIP (c)	Pyothorax (d)	Comparison between groups	P-value
	n=49	n=27	n=22	n=14		
Albumin (g/dl)	2.07 (0.85-7.25)	1.17 (0.40-2.8)	1.66 (1.08-4.33)	1.58 (0.54-3.11)	b-a	<0.0001
α1 globulin (g/dl)	0.12 (0.03- 1.3)	0.09 (0.02-0.26)	0.15 (0.09-0.68)	0.15 (0.04-0.33)	b-d b-c	0.017 <0.0001
α2 globulin	0.54	0.25	0.73	0.47	b-a	0.001

(g/dl)	(0.1- 1.75)	(0.06-0.87)	(0.14-1.68)	(0.08-2.75)	b-c	0.001
β globulin (g/dl)	0.52 (0.22-2.09)	0.33 (0.11-0.92)	0.76 (0.4-1.79)	0.94 (0.23-2.47)	b-a b-d b-c a-c	0.011 <0.0001 <0.0001 0.018
γ globulin (g/dl)	0.74 (0.16-2.38)	0.52 (0.02-5.08)	2.25 (0.54-5.99)	1.26 (0.2-3.13)	b-d b-c a-c	0.035 <0.0001 <0.0001
A/G	1.1 (0.6-1.7)	0.9 (0.3-1.6)	0.4 (0.2-0.7)	0.5 (0.2-1.3)	c-b c-a d-b d-a	<0.0001 <0.0001 0.019 <0.0001

Data are median (range). Protein fractions were analyzed by protein electrophoresis. CD = cardiac disease, FIP = feline infectious peritonitis, A/G = albumin to globulin ratio

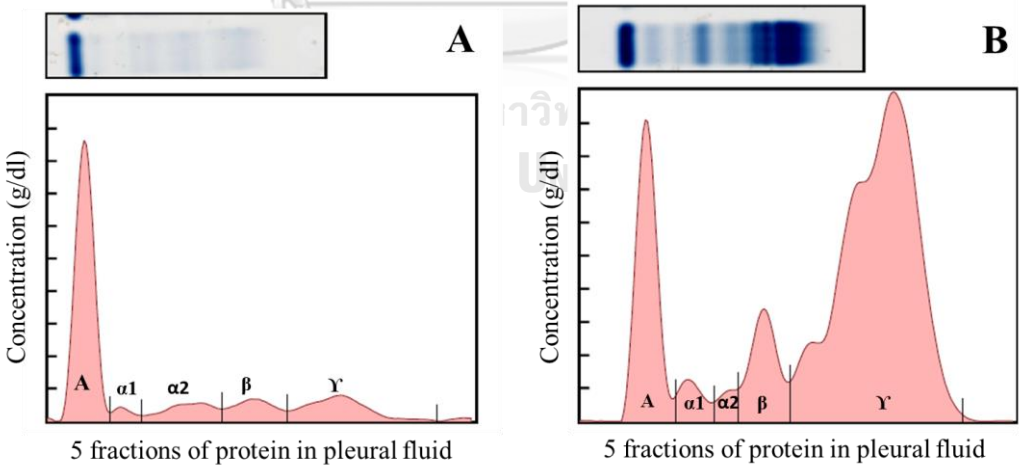


Figure 2 SPIFE® split beta gel and electrophoretogram of pleural fluid (A) Electrophoretogram found in cardiac effusion, (B) A polyclonal pattern was seen in the γ globulin region in the cat with FIP effusion

4.5. Protein electrophoresis in blood plasma

Protein fractions of blood plasma in the same cats with pleural effusion in different disease groups were listed in Table 7. Albumin concentration in blood plasma was significantly higher in the neoplasia group than in pyothorax (P=0.002), FIP (P=0.027) and cardiac disease (P=0.048). No significant difference between underlying causes was found in α1 globulin, α2 globulin, and β globulin concentration in blood plasma. The γ globulin concentration in FIP was significantly higher than in neoplasia (P<0.0001). The A/G ratio in FIP (P<0.0001) and pyothorax (P=0.001) are significantly lower than in neoplasia. A similar pattern of electrophoretogram in pleural effusion and blood plasma of the same cat with FIP was shown in Figure 3. with the γ concentration 5.06 g/dl and 4.08 g/dl respectively with the same A/G ratio (0.3).

Table 7 Protein fractions in the blood plasma of cats with different etiologies of pleural effusion

Etiologies / Parameters	Neoplasia (a)	CD (b)	FIP (c)	Pyothorax (d)	Comparison between groups	P-value
	n=20	n=14	n=12	n=9		
Albumin (g/dl)	2.38 (1.66-3.16)	1.86 (1.23-2.82)	1.79 (1.23-2.58)	1.72 (0.96-2.29)	d-a c-a b-a	0.002 0.027 0.048
α1 globulin (g/dl)	0.36 (0.16-1.46)	0.30 (0.13- 0.53)	0.28 (0.20- 0.55)	0.24 (0.14-0.55)	#	
α2 globulin (g/dl)	1.03 (0.34-1.37)	0.95 (0.21-1.37)	1.22 (0.14-1.62)	1.23 (0.15-1.54)	#	
β globulin (g/dl)	0.75 (0.58- 1.70)	0.97 (0.713- 2.28)	0.85 (0.65- 2.68)	0.78 (0.57-2.3)	#	

γ globulin (g/dl)	1.18 (0.66- 2.28)	1.83 (0.75- 6.60)	2.68 (1.47- 5.92)	1.64 (0.80- 2.82)	a-c	<0.0001
A/G	0.70 (0.50- 1.20)	0.50 (0.20- 0.70)	0.30 (0.20- 0.50)	0.40 (0.30- 0.70)	c-a d-a b-a	<0.0001 0.001 0.016

Data are median (range). Protein fractions were analyzed by protein electrophoresis CD = cardiac disease, FIP = feline infectious peritonitis, A/G = albumin to globulin ratio, # = No significant different between groups.

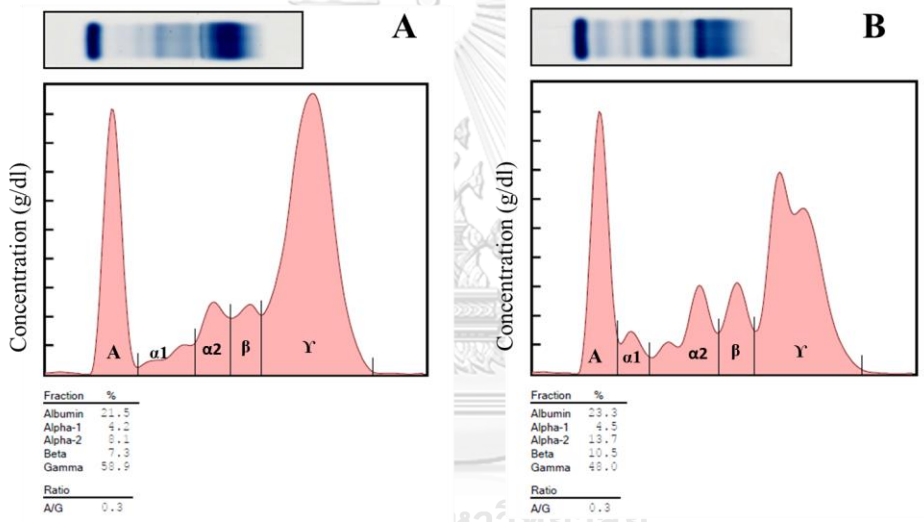


Figure 3 SPIFE® split β gel and electrophoretogram of (A) in pleural effusion and (B) in the blood plasma of the same cat with FIP effusion. Hypoalbuminemia (data not shown), the elevated α2 globulins and broad-based peak in the γ region, consistent with acute and chronic inflammation can be seen.

4.6. Comparison of parameters between pleural fluid and plasma

The correlation of LDH, TP concentration and A/G ratio between fluid and plasma was described In Appendix Table 2. The Spearman correlation illustrated as scattered plots was shown in Figure 4. The number of samples analyzed for LDH was 42, TP were 56 and A/G were 55. There was a weak positive correlation between LDH

concentration in pleural effusion and plasma concentration (r=0.312, P=0.044) and a weak positive correlation between fluid total protein and plasma total protein (r=0.472, P<0.0001). There was a strong positive correlation of the A/G ratio between fluid and plasma (r=0.798, P<0.0001). The Kruskal-Wallis test showed differences in LDH concentration between disease groups (P<0.001) in pleural effusion, but not in the blood plasma (Figure 5).

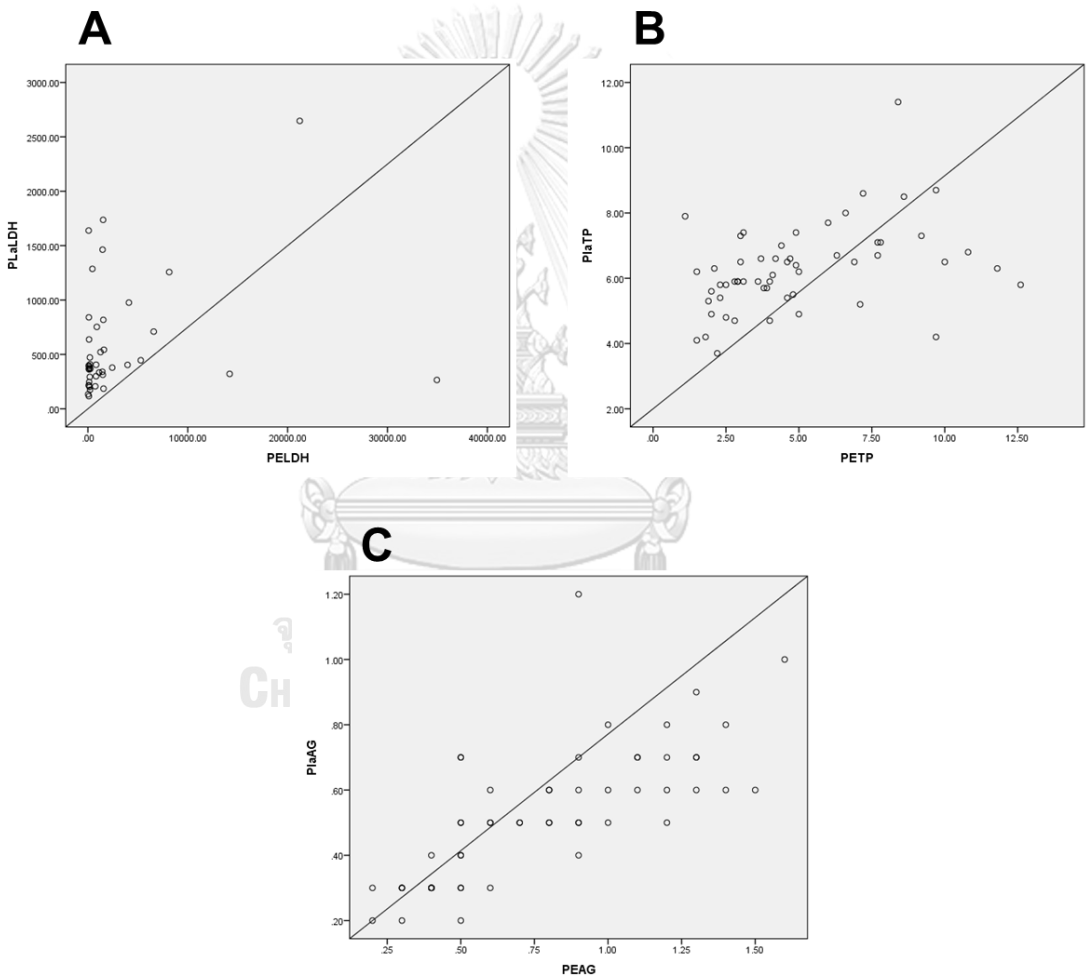


Figure 4 Spearman correlation between LDH, TP and A/G in pleural effusion and blood plasma. (A) weak positive correlation between LDH concentration in pleural effusion and plasma concentration (r=0.312, P=0.044). (B) Weak positive correlation between fluid total protein and plasma total protein (r=0.472, P<0.0001). (C) Strong positive A/G correlation between fluid and plasma (r=0.798, P<0.0001). PELDH =

concentration of LDH in pleural effusion, PlaLDH = concentration of LDH in blood plasma. PETP = total protein in pleural effusion, PlaTP = total protein in blood plasma, PEAG = albumin to globulin ratio in pleural effusion, PlaAG = albumin to globulin ratio in blood plasma.

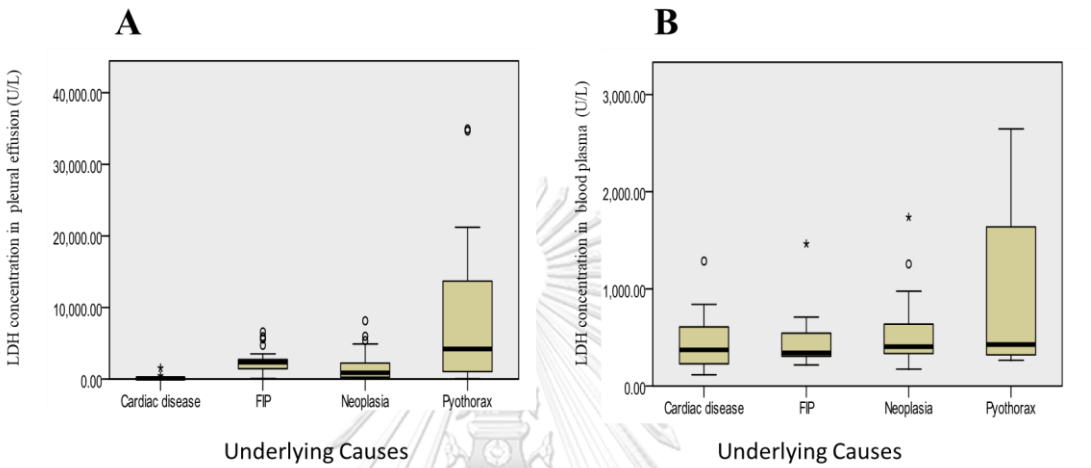


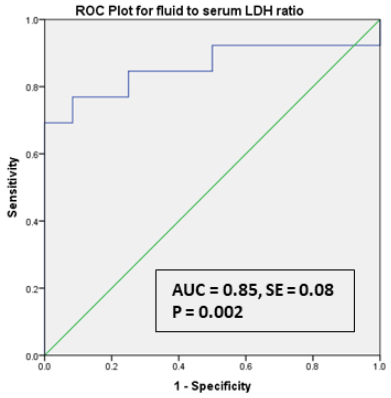
Figure 5. Box plot of LDH concentration in (A) pleural effusion and (B) blood plasma. Median values of LDH concentration in pleural effusion are shown in Y-axis. The ends of the whiskers represent the minimum and maximum values of the data.
LDH = lactate dehydrogenase, FIP = feline infectious peritonitis

4.7. Receiver operating characteristic (ROC) analysis of pleural fluid to serum ratio of LDH, total protein and the concentration of each fraction in differentiating between the underlying causes

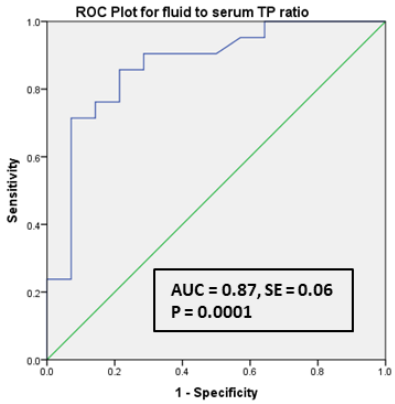
Data for LDH in the total of 12 cats with the underlying cause of cardiac disease, 13 cats with infectious causes and 20 cats with neoplasia were assessed for ROC analysis (Appendix Table 3 and 4). Receiver operating characteristics curve of pleural fluid to serum of LDH, TP, albumin, α1, α2, β, and γ globulin concentrations to differentiate cardiac from infectious diseases which included both FIP and pyothorax was shown in Figure 6. The ROC plots of pleural fluid to serum ratio of LDH, TP, and β globulin concentration to differentiate neoplasia from other causes

was described in Figure 7. The sensitivity, specificity, positive likelihood ratio, negative likelihood ratio of the best cut-off values was described in Table 8. The fluid to serum ratio of LDH concentration in the cardiac disease group had significantly lower than in the infectious group with the sensitivity, specificity, PLR and NLR of 85%, 75%, 3.4, and 0.2, respectively at a cut-off level of 0.51 (Fig. 6 A). The fluid to serum concentration ratio of TP, albumin, β, and γ globulin showed excellent separation (AUC>0.8) with P-value, 0.0001, 0.002, 0.001, and 0.003 respectively for differentiating cardiac from infectious causes. The fluid to serum ratio of LDH and β globulin concentration can be used to differentiate cardiac from neoplasia causes at the AUC score of more than 0.8 with P value 0.001 and 0.002 respectively (Fig. 7 A and C).

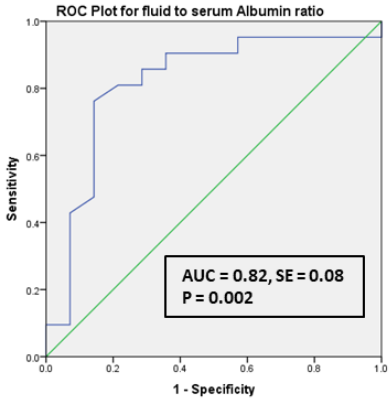
A


ROC Plot for fluid to serum LDH ratio
Sensitivity
1 - Specificity
AUC = 0.85, SE = 0.08
P = 0.002

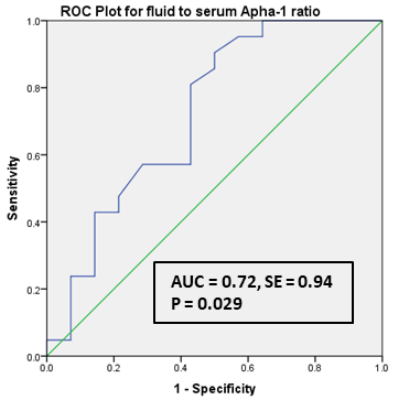
B


ROC Plot for fluid to serum TP ratio
Sensitivity
1 - Specificity
AUC = 0.87, SE = 0.06
P = 0.0001

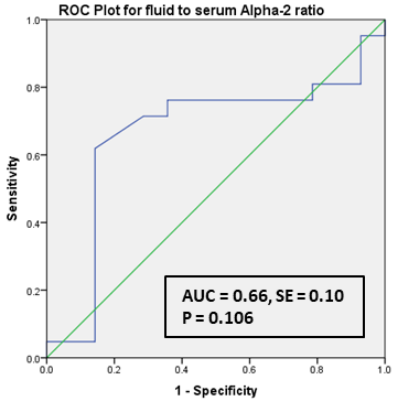
C


ROC Plot for fluid to serum Albumin ratio
Sensitivity
1 - Specificity
AUC = 0.82, SE = 0.08
P = 0.002

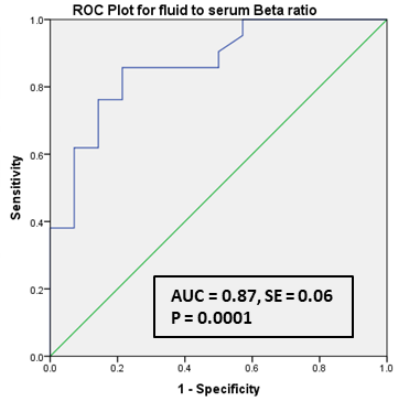
D


ROC Plot for fluid to serum Apha-1 ratio
Sensitivity
1 - Specificity
AUC = 0.72, SE = 0.94
P = 0.029

E


ROC Plot for fluid to serum Alpha-2 ratio
Sensitivity
1 - Specificity
AUC = 0.66, SE = 0.10
P = 0.106

F


ROC Plot for fluid to serum Beta ratio
Sensitivity
1 - Specificity
AUC = 0.87, SE = 0.06
P = 0.0001

G

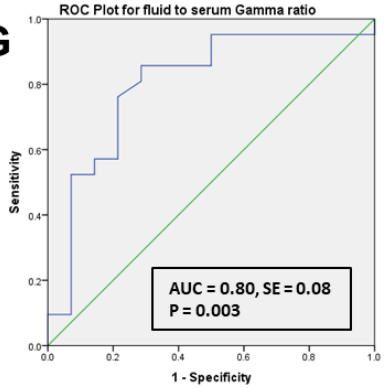

ROC Plot for fluid to serum Gamma ratio
Sensitivity
1 - Specificity
AUC = 0.80, SE = 0.08
P = 0.003

Figure 6 ROC plots of pleural fluid to serum ratio concentrations of LDH (A), TP (B), albumin (C), α1 (D), α2 (E), β (F), and γ (G) globulins. The optimum cut-off of fluid to serum ratios for LDH, TP, albumin, α1, β, and γ globulins were 0.51, 0.57, 0.77, 0.41, 0.56, 0.50, respectively. LDH = lactate dehydrogenase, TP = total protein, AUC = area under the curve, SE = standard error

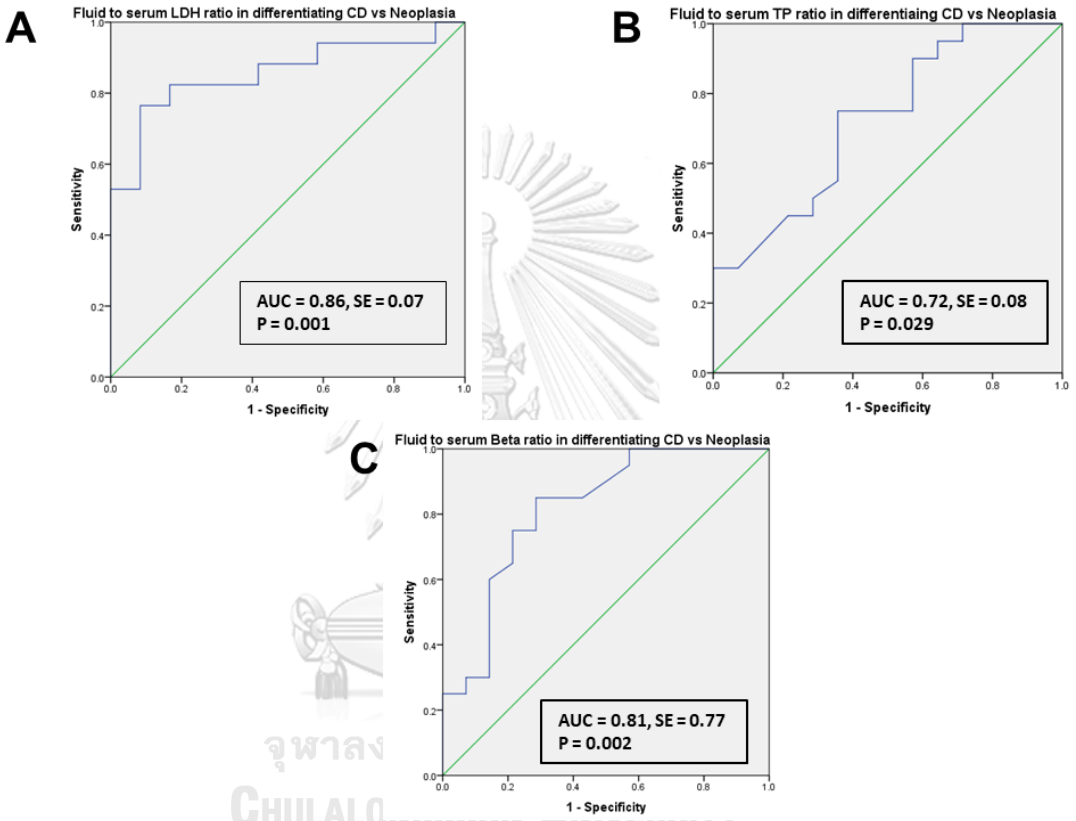


Figure 7 ROC plots of pleural fluid to serum ratio for LDH (A), TP (B), and β globulin (C) concentration. The optimum cut-off of fluid to serum ratios for LDH, TP, and β globulin concentration were 0.64, 0.61, 0.45, respectively. LDH = lactate dehydrogenase, TP = total protein, AUC = area under the curve, SE = standard error

Table 8 Results of the ROC analysis of fluid to serum ratio of LDH, TP, and its fractions in differentiating between the diseases

Factors tested	Accuracy (AUC±SE)	P-value	Best cut-off	Sensitivity %	Specificity %	PLR	NLR
LDH ratio (CD vs Infectious)	0.85±0.08	0.002	0.51	85	75	3.4	0.2
LDH ratio (CD vs Neoplasia)	0.86±0.07	0.001	0.64	82	84	5.12	0.21
TP ratio (CD vs Infectious)	0.87±0.06	0.0001	0.57	90	72	3.21	0.14
TP ratio (CD vs Neoplasia)	0.72±0.08	0.029	0.61	75	65	2.14	0.38
Albumin ratio (CD vs Infectious)	0.82±0.08	0.002	0.77	86	72	3.07	0.19
α1 globulin ratio (CD vs Infectious)	0.72±0.94	0.029	0.41	81	58	1.93	0.33
α2 globulin ratio (CD vs Infectious)	0.66±0.10	0.106	ns	-	-	-	-
β globulin ratio (CD vs Infectious)	0.87±0.06	0.0001	0.56	86	79	4.10	0.18
β globulin ratio (CD vs Neoplasia)	0.81±0.77	0.002	0.45	85	72	3.03	0.20
γ globulin ratio (CD vs Infectious)	0.80±0.08	0.003	0.50	86	72	3.07	0.19

AUC = area under the curve, SE = standard error, PLR = positive likelihood ratio, NLR = negative likelihood ratio, CD = cardiac disease, ns = not significant, LDH = lactate dehydrogenase, TP = total protein

4.8. Immunohistochemistry (IHC)

The unstained slides kept from the fluid pellet were used for IHC. No reactions on the slides were found. The control slide showed positive results with all antibodies tested.

4.9. Clonality test

Among 14 lymphoma suspected cases submitted for PARR, 6 samples showed clonal rearrangement in T cell assay and polyclonal rearrangement in B cell assays. One sample had clonal rearrangement in B cell assays and polyclonal rearrangement in T cells assay. Polyclonal rearrangement in T cell and B cell assay was shown in 7 samples.

4.10. Feline infectious peritonitis virus detection

The FCoV antigen was detected by RT-PCR in all the samples (n=10) that were submitted to the diagnostic laboratory.

CHAPTER 5

DISCUSSION

In this study, we investigated and classified 127 pleural effusion samples collected from cats that were clinically diagnosed with different diseases. Similar to the previous study, the median age of the cats suffering from pleural effusion caused by FIP, was significantly younger than those caused by cardiac disease and neoplasia (König et al., 2019). In our study, the median age of the FIP cats was 1.25 years old. Age is one of the most helpful parameters to make an initial supposition regarding the etiology of effusions. It may be due to non-immunocompetence and high susceptibility to the infection at a young age. The A/G ratio in our study was slightly lower than in the previous study showing that the median values of 0.4 in pleural effusion and 0.3 in the blood plasma (Valenciano and Cowell, 2019). Cats with cardiac disease displayed a significantly lower body temperature compared with cats of all other groups in the previous study (König et al., 2019). However, in our study, the body temperature was not different between each group and hypothermia was not observed. In contrast to age, the temperature is not helpful to differentiate the etiologies of the groups in the present study. The most affected breed was domestic short hair likely that it is the most popular breed in Thailand.

In this study, neoplasia represented the largest group of patients followed by cardiac disease. Among neoplastic cases in this study, 81.63% (40/49) of neoplasm causing pleural effusion was lymphoma. The other neoplasms were rare including adenocarcinoma, epithelial cell tumor, fibrosarcoma and unclassified neoplasm. This finding contrasts to a previous study in Germany which reported that cardiac disease comprised the highest percentage of the underlying causes (König et al., 2019). In the past 20 years, the widespread use of FeLV vaccination has reduced the incident rate of FeLV infection in Europe (Shelton et al., 1990). The higher number of neoplastic

cases in our study is likely due to the high prevalence of FeLV associated lymphoma in Thailand. In our study, 62.5% (25/40) of the cats diagnosed as mediastinal lymphoma based on fluid analysis was FeLV positive. Our study used the commercial FeLV test kit (immunochromatography assay) which detect the p27 antigen of the virus to confirm FeLV infection. In fact, the number of FeLV positive cats might be higher. The most recent study using nested recombinase polymerase amplification (nRPA) to detect exogenous FeLV DNA provirus and RT-PCR to detect FeLV RNA, showed that the FeLV detection rate in the regression phase was increased up to 45.8% compared to the rapid immunochromatographic assay (Lacharoje et al., 2021). Among the 40 morphologically diagnosed lymphoma cases, 14 cases were initially inconclusive and clonality testing for both T and B cell were performed. Forty-two per cent of them (6/14) had clonal rearrangement of T cell receptor gene, indicating T cell lymphoma which agreed with previous studies that FeLV infection is associated with mediastinal lymphoma commonly T cell origin (Neil and Onions, 1985). Only one case (1/14, 7%) showed clonal rearrangement for B cells. Fifty per cent of the cases (7/14) showed polyclonal rearrangement although radiographs revealed masses in the thoracic. This is likely due to the limited sensitivity of the clonality testing.

The failure of clonal detection in the presence of lymphoma can be explained in 3 main reasons; (1) inadequate primer coverage which can be suspected if negative clonality results disagree with clinical, morphologic, and immunophenotyping results, (2) primer site mutation in B cell lymphoma with natural somatic hypermutation in antigen receptor gene, (3) high polyclonal background from a large proportion of non-neoplastic lymphocyte population that can obscure a signal of clonal lymphocyte population (Amara et al., 2006; Gameiro et al., 2012; Lenze et al., 2012; Kokovic et al., 2015). The integrative diagnosis of lymphoma should be started from morphology, then immunophenotyping and molecular assay as an adjunctive tool

when other methods give an ambiguous result. Tissue fragments, cell smears, and fluid samples such as cerebrospinal fluid can be used as specimens for clonality testing (Keller et al., 2016). Our study suggests that pleural effusion is also a good quality sample for clonality when the specimen is well kept at -80°C. However, the air-dried slides that are kept at room temperature cannot be used for IHC. This suggests that storage time and method are crucial for protein antigen detection.

Another possibility of the failure to detect lymphoma is due to the other types of neoplasms located in the thoracic cavity such as mesothelioma and metastatic mammary gland tumor. Feline mammary gland tumors are the most common neoplasm in female cats, accounting for 12% of all neoplasms. More than 80% of the tumor is malignant and extremely aggressive with evidence of metastasis to the lung and regional lymph nodes (Rosen et al., 2020). Other epithelial cell tumors in cats are also common and usually malignant. The primary sites include the mammary gland, gastrointestinal and respiratory tracts, and skin (Graf et al., 2015). Other neoplasms located in the anterior mediastinum including the thymus, and parietal pleura can also lead to fluid accumulation due to lymphatic flow blockage (Thrall, 2020). These can result in the failure of detecting lymphoma in some cases.

The transudative effusion in this study contained low TP concentration (<3 g/dl), low TNCC (<1,000 cells/µl) which was found as a few small lymphocytes and neutrophils, similar to the earlier study (Valenciano and Cowell, 2019). In our case, all transudates developed from cardiac disease caused by changes in oncotic and hydrostatic pressure. Modified transudates were found in many underlying causes of the pleural effusion, thus in this study, the presence of modified transudate is not specific to the causes. We recommend that when modified transudate is given to a pleural effusion, types of the cells, gross characteristics of the fluid, together with clinical information should be further examined to support the tentative diagnosis.

Feline infectious peritonitis resulted in modified transudate and non-septic exudate. The modified transudate, so call protein-rich transudate, is a result of increased vascular permeability. Cytologically, the typical FIP effusion has a prominent proteinaceous background and shows primarily of nondegenerate neutrophils and lesser numbers of macrophages and small lymphocytes in the previous findings, similar to FIP cytology in our study (Rohrbach et al., 2001; Hartmann et al., 2003). The modified transudate from FIP effusion is caused by vasculitis and containing a few nucleated cells. However, some FIP effusions in our study were classified as non-septic exudate which contained >7,000 nucleated cell count cells per µl. It might be due to chronic irritation of the fluid to the pleural cavity (Tasker, 2018). Previous literature reported that the use of RT-PCR for the detection of FCoV using effusion fluids and peripheral blood mononuclear cells is a reliable technique (Hartmann et al., 2003). In our study, the FCoV was detected by RT-PCR using pleural effusion which was in accordance with the clinical presentation and cytological results suggestive of FIP.

According to Kruskal-Wallis comparison, the concentration of LDH in pleural effusion is highest in pyothorax agreeing with earlier investigations in which pleural fluid LDH of >1,000 U/L was suggestive of pyothorax in human pleural effusions (Sahn, 2007; Davies et al., 2010). The LDH concentration was also higher in FIP effusion, likely it is the highest in infectious than in degenerative diseases such as cardiac disease. A previous study indicated that most malignant and inflammatory conditions show a higher LDH concentration in the effusion than in the blood plasma (Horrocks et al., 1962). Our finding agrees with that study, LDH concentration in pleural effusion is more related to the underlying causes than in the blood plasma which showing no difference among disease groups. In the previous study, LDH concentration in pleural effusion was significantly lower in human with transudates

with a cut-off value of <200 U/l or pleural effusion to serum LDH ratio <0.60 was used to separate transudates from exudates (Light et al., 1972). In our study, LDH concentration in pleural fluid was significantly lower in cats with cardiac disease which developed as transudative effusion than those with infectious causes. By using ROC analysis, the concentration of fluid to serum LDH ratio of 0.51 can be used as a cut-off value to differentiate cardiac disease from infectious diseases. Slightly difference in the optimum cut-off value between our study and Light's study could be due to the use of different assays to measure LDH or the different inter-species levels of tissue LDH. Also, the concentration of fluid to serum ratio of total protein, albumin, β and γ globulins can differentiate cardiac disease from infectious diseases with their best cut-off values at 0.57, 0.77, 0.56, and 0.50, respectively. The concentration of fluid to serum ratios of LDH and β globulin can differentiate cardiac disease from neoplasia at their cut-off values of 0.64 and 0.45, respectively.

The use of protein electrophoresis in pleural effusion in cats was already optimized and protein fractions were analyzed successfully in this study. Cats diagnosed with cardiac disease had significantly lower albumin concentration in pleural effusion compared with neoplasia. It is due to the nature of transudative effusion developing in cardiac disease usually comes from hypoalbuminemia and fluid overload (Alleman, 2003; Dempsey and Ewing, 2011). Both α1 and α2 globulin concentration in pyothorax, neoplasia and FIP group is significantly higher than in the cardiac group suggesting that the positive APPs such as serum amyloid A (SAA), α1 antitrypsin, α1 acid glycoprotein (AGP), α2 macroglobulin, haptoglobin (Hp) and ceruloplasmin are elevating in pleural effusion (Skeldon, 2018). One study found that increased AGP concentrations in effusions were more useful (sensitivity and specificity of 93%) in differentiating FIP and non-FIP cases than AGP levels in the serum (Hazuchova et al., 2017). In our study, the increase of β globulin fraction in pleural

effusion was found in pyothorax and neoplasia suggesting that the negative APPs including transferrin and the pro-inflammatory protein (complement) are increasing in the pleural effusion causing by these underlying conditions (Skeldon, 2018). It is also similar to a previous study that documented the increase of the β globulin fraction in plasma in inflammation, neoplasia, and various metabolic conditions (O'Connell et al., 2005). Cats with FIP showed the highest γ globulin concentration and the lowest A/G ratio which is also likely due to a higher concentration of γ globulin. Increased γ globulin is related to chronic antigenic stimulation because this fraction contains immunoglobulins (Ig) especially IgG. These elevations can be described as polyclonal gammopathy when a high amount of Ig is produced by a heterogeneous population of B cells (Font et al., 1994). Polyclonal gammopathies were observed in the majority (68%) of FIP cases. The most common protein electrophoresis pattern of the pleural fluid in cats with infectious pleuritis (viral and bacterial causes) revealed a polyclonal gammopathy. Monoclonal gammopathy which is indicative of B cell neoplasms was not observed in this study. This might be due to the high prevalence of FeLV-induced lymphoma which is mostly derived from T-cell lymphoma (Tompkins and Tompkins, 1985). In our study, protein electrophoresis of the blood plasma only showed significant difference in the concentration of albumin, γ globulin fraction and A/G ratio between groups. However, protein electrophoresis of the pleural fluid reveals elevation of α2 and γ globulin concentration and showing polyclonal gammopathy in FIP effusions which are similar to the previous study conducted in the serum of 155 cats (Taylor et al., 2010). The reason for no significant difference shown in the plasma samples across underlying disease groups might be due to the restricted samples size used for statistical comparison in our study.

According to Spearman's rank correlation and non-parametric Kruskal-Wallis test, the significant differences between disease groups was shown in LDH

concentration in pleural effusion but not in the blood plasma. It is suggesting that LDH concentration in fluid is unlikely related to blood plasma. The same pattern was observed in the TP concentration which showed a weak positive correlation between fluid and plasma. This suggested that LDH and TP concentration in the pleural fluid can be indicative of a relating underlying disease when those parameter in blood plasma cannot. The A/G ratio had a strong correlation between the fluid and blood plasma especially in FIP cases and almost similar A/G ratio and electrophoretic patterns of the fluid and blood plasma in FIP was seen.

The study has some limitations because of its retrospective character. There was a number of undiagnosed cases that caused of pleural effusion could not be identified because full diagnostic work-up was not available for all patients. Moreover, some patients died during the treatment and necropsy was not performed. The second limitation was that the air-dried smeared slides from the pleural effusion could not be used for immunohistochemical study. Prolonged storage of the cytology slide at room temperature could alter cell surface protein antigen causing no reactions with the antibodies. The last limitation, although over 100 cats with pleural effusion were investigated, the population in each disease group used for statistical analysis were rather small. The data obtained from the study might not be representative of all forms of underlying diseases. All of the samples in this study were submitted to the Small Animal Hospital, Chulalongkorn University, so this can be represented only Bangkok and its vicinity areas but not Thailand.

In conclusion, this study investigated protein electrophoresis of pleural effusion in cats for the first time. Our result is proving that the concentration of protein fractions in the pleural fluid likely reflects their concentration in the blood. The concentrations of each protein fraction in pleural effusion are related to the

underlying causes. Underlying causes of pleural effusion in cats are different across global regions. The cytology, LDH measurement and protein electrophoresis of pleural effusion can aid in establishing a diagnosis in cats with pleural effusion. Lactate dehydrogenase and protein electrophoresis should be added in the routine diagnostic work-up of feline patients with pleural effusion. The fluid to serum ratios of albumin, β and γ globulin concentration can help discriminating cardiac disease from other infectious causes and the fluid to serum ratio of β globulin concentration can discriminate cardiac disease from neoplasia. When the diagnosis given as a modified transudate which is determined by total protein and nucleated cell count is insufficient to identify the underlying cause of the effusion, fluid to serum ratio of total protein, LDH, albumin, β and γ globulin concentration can be used as adjunctive parameters. This study contributes new information and understanding on current underlying conditions that cause pleural effusion in cats in Thailand. The presence of abnormalities in cytological and biochemical parameters are related to these conditions and used to determine disease etiology. Information obtained from this study provides information on the disease etiology and benefits clinicians to generate a list of differential diagnoses and therapeutic plans. Ultimately, the outcome of this study can be applied as a reference for the diagnosis of feline pleural effusion.

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Appendix

Consent Form

คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ใบยินยอมให้ใช้สัตว์เพื่อโครงการวิจัยและการเรียนการสอนทางสัตวแพทย์

.....

ข้าพเจ้า.....อยู่บ้านเลขที่ .....ขอลงนามในใบอนุญาตนี้ให้ไว้เพื่อแสดงว่าข้าพเจ้าอนุญาตให้ อาจารย์ สพ.ญ.ดร. อารยา รัตนกถิกานนท์ เป็นหัวหน้าหรือเจ้าของโครงการ ใช้ตัวอย่างเลือดและน้ำในช่องอก จากสัตว์ชื่อ.....ของข้าพเจ้าในการวิจัยโครงการ การศึกษาทางพยาธิวิทยาคลินิกและโรคที่เกี่ยวข้องกับภาวะน้ำในช่องอกของแมว โดยทั้งนี้ข้าพเจ้าเข้าใจดีว่า หัวหน้าโครงการวิจัยจะปฏิบัติต่อสัตว์ของข้าพเจ้าเป็นไปตามจรรยาบรรณของการใช้สัตว์

ลงนาม .....

(.....)

เจ้าของสัตว์

ลงนาม .....

(.....)

หัวหน้าโครงการ

ลงนาม .....

(.....)

พยาน

วันที่.....

หมายเหตุ : 1) เอกสารนี้ออกให้นักวิจัยหรืออาจารย์ผู้ดูแลการเรียนการสอนที่กระทำต่อสัตว์ เป็นมาตรฐานเดียวกันทั้งคณะสัตวแพทยศาสตร์ จุฬาฯ

2) เอกสารนี้ให้อยู่ในความรับผิดชอบของนักวิจัยหรืออาจารย์ผู้ปฏิบัติการ

3) เอกสารควรมีสำเนาอีก 1 ชุด

Table 1 Signalments and causes of 127 cats.

ID	Age (year)	Sex	Nutering Status	Breed	Temp ˚F	Causes
1	1	M	Intact	DSH	85	Pyothorax
2	1	M	Sterile	DSH	102	Neoplasia
3	3	F	Sterile	Mixed breed	NR	Neoplasia
4	7	M	Sterile	DSH	98	Neoplasia
5	5	M	Intact	Persian	NR	Cardiac disease
6	2	M	Sterile	DSH	97	Cardiac disease
7	8	M	Sterile	DSH	94	Unknown
8	3	M	Intact	DSH	99	Neoplasia
9	1	F	Sterile	DSH	101	Neoplasia
10	0.17	M	Intact	DSH	98	Pneumonia
11	7	F	Unknown	DSH	NR	Pyothorax
12	1	F	Sterile	DSH	100	Pyothorax
13	2	M	Sterile	American Short Hair	102	FIP
14	10	F	Intact	DSH	NR	FIP
15	11	F	Intact	DSH	97.4	Cardiac disease
16	13	F	Sterile	DSH	101	Neoplasia
17	3	M	Intact	Persian	NR	Unknown
18	2	M	Intact	DSH	96	Pyothorax
19	4	M	Unknown	DSH	NR	Neoplasia
20	2	F	Intact	Maine Coon	101	Cardiac disease
21	4	M	Sterile	DSH	98.6	Cardiac disease
22	0.42	F	Intact	DSH	99.8	FIP
23	1	F	Sterile	DSH	101.6	Pneumonia
24	0.58	F	Intact	Mixed breed	102	Trauma
25	6	M	Intact	Persian	NR	Neoplasia
26	0.58	M	Intact	DSH	100	Neoplasia

27	13	F	Sterile	DSH	100.6	FIP
28	4	F	Intact	DSH	101.4	Neoplasia
29	0.33	M	Intact	DSH	NR	Neoplasia
30	10	F	Sterile	DSH	101	Neoplasia
31	2	M	Intact	DSH	NR	Pyothorax
32	2	F	Sterile	DSH	103	Cardiac disease
33	4	F	Sterile	DSH	101.4	Neoplasia
34	1	F	Intact	DSH	102.2	FIP
35	10	F	Sterile	DSH	NR	Neoplasia
36	11	F	Intact	DSH	100	Neoplasia
37	5	M	Sterile	DSH	102	Neoplasia
38	13	F	Sterile	DSH	NR	Cardiac disease
39	1	M	Intact	DSH	99.8	Pyothorax
40	4	M	Sterile	DSH	101.8	FIP
41	10	F	Intact	DSH	99	Unknown
42	4	M	Intact	American Short Hair	100	Neoplasia
43	14	F	Sterile	DSH.	94	Pneumonia
44	12	M	Sterile	Mixed breed	94	Cardiac disease
45	4	M	Sterile	DSH	101	Neoplasia
46	3	M	Sterile	DSH	NR	More than one
47	10	F	Sterile	DSH	95	Unknown
48	3	M	Sterile	DSH	98	Cardiac disease
49	1	F	Intact	Mixed breed	100	FIP
50	1	F	Sterile	Mixed breed	99	Pyothorax
51	12	M	Sterile	DSH	101.2	Cardiac disease
52	5	F	Sterile	DSH	98.6	Neoplasia
53	1	M	Intact	DSH	98.8	Neoplasia
54	10	F	Intact	Persian	101	Neoplasia
55	3	M	Sterile	DSH	101	Cardiac disease

56	2	M	Intact	DSH	95.2	FIP
57	3	M	Intact	DSH	101.4	Neoplasia
58	2	M	Intact	DSH	100.4	Pyothorax
59	10	M	Sterile	DSH	99	Cardiac disease
60	6	M	Sterile	DSH	99.4	Neoplasia
61	10	F	Sterile	DSH	94	Cardiac disease
62	7	F	Sterile	DSH	97	Pyothorax
63	2	F	Sterile	DSH	100	Pyothorax
64	0.33	M	Intact	American Short Hair	101.2	FIP
65	14	M	Sterile	DSH	98	Cardiac disease
66	2	F	Intact	DSH.	101	Cardiac disease
67	16	M	Sterile	DSH	101	Neoplasia
68	10	M	Intact	DSH	NR	Pneumonia
69	14	M	Sterile	DSH	NR	Unknown
70	1	M	Intact	DSH	100	FIP
71	3	M	Sterile	DSH	100	FIP
72	4	M	Sterile	DSH	101	Cardiac disease
73	1	F	Intact	DSH	99	FIP
74	1	M	Intact	Mixed breed	101	FIP
75	5	M	Sterile	DSH	99	Cardiac disease
76	1	F	Sterile	DSH	100	Neoplasia
77	8	M	Sterile	DSH	101	Cardiac disease
78	6	F	Intact	Persian	100	Cardiac disease
79	4	F	Sterile	DSH	97.4	FIP
80	0.33	M	Intact	DSH	103	FIP
81	1	M	Intact	DSH	98.4	FIP
82	7	M	Sterile	DSH	100.6	Cardiac disease
83	4	M	Sterile	DSH	100.4	Neoplasia
84	7	F	Sterile	DSH	NR	Neoplasia

85	0.42	M	Intact	DSH	101	Pyothorax
86	0.92	M	Intact	Scottish Fold	102	FIP
87	0.33	M	Intact	DSH	100	Pyothorax
88	1	F	Sterile	DSH	NR	Pyothorax
89	1	M	Sterile	DSH	102	Neoplasia
90	3	F	Sterile	DSH	102.2	Neoplasia
91	3	F	Sterile	DSH	99.4	Neoplasia
92	2	F	Sterile	DSH	NR	Neoplasia
93	7	M	Intact	DSH	101	Cardiac disease
94	8	F	Sterile	DSH	95	Unknown
95	3	F	Sterile	DSH	101.4	Neoplasia
96	1	F	Intact	Scottish Fold	101	Cardiac disease
97	10	F	Sterile	DSH	96.4	Neoplasia
98	1	M	Sterile	Scottish Fold	100.8	Pyothorax
99	3	M	Sterile	DSH	NR	Cardiac disease
100	7	M	Intact	DSH	94.5	Neoplasia
101	2	M	Sterile	DSH	100.4	FIP
102	1	F	Sterile	DSH	95	Cardiac disease
103	2	F	Sterile	DSH	101.4	FIP
104	10	M	Unknown	DSH	100.6	Unknown
105	1	F	Sterile	Mixed breed	100.6	Neoplasia
106	1	F	Sterile	DSH	100	Neoplasia
107	1.5	M	Intact	DSH	104	Pyothorax
108	1.5	M	Intact	Scottish Fold	103.4	FIP
109	5	F	Sterile	DSH	101	Cardiac disease
110	1	M	Unknown	DSH	95.5	FIP
111	10	F	Unknown	DSH	101	Cardiac disease
112	1	M	Sterile	DSH	101	Neoplasia
113	1.75	F	Intact	DSH	101	FIP

114	8	M	Sterile	Mixed breed	NR	Neoplasia
115	2	M	Sterile	Mixed breed	99	Neoplasia
116	2	F	Sterile	DSH	99	Neoplasia
117	2	F	Unknown	DSH	NR	Neoplasia
118	2	M	Unknown	DSH	NR	Neoplasia
119	3	M	Sterile	DSH	NR	Neoplasia
120	1	M	Sterile	DSH	100.8	Neoplasia
121	1	F	Intact	DSH	99	Neoplasia
122	1	F	Unknown	DSH	101.2	Neoplasia
123	1	M	Intact	DSH	101.6	Neoplasia
124	2	M	Sterile	DSH	102.2	Neoplasia
125	1	F	Intact	DSH	100	Neoplasia
126	8	M	Sterile	Persian	104.2	Cardiac disease
127	3	M	Sterile	DSH	100.4	Pyothorax

Table 2 Correlation between LDH, TP and A/G in pleural effusion and blood plasma.

Parameter	**Type of sample**	**Correlation coefficient** (r)	**Sig.**
LDH	PELDH	0.312	0.044
	PlaLDH		
TP	PETP	0.472	<0.0001
	PlaTP		
A/G	PE A/G	0.798	<0.0001
	PlaA/G		

LDH = lactate dehydrogenase, TP = total protein, A/G = albumin to globulin ratio, PELDH = concentration of lactate dehydrogenase in pleural effusion, PlaLDH = lactate dehydrogenase in blood plasma, PETP = total protein in pleural effusion, PlaTP = total protein in blood plasma, PE A/G = albumin to globulin ratio in pleural effusion, Pla A/G = albumin to globulin ratio in the blood plasma

Table 3 The concentrations of LDH in the fluid and serum in cardiac and infectious causes

Cardiac diseases				Feline infectious peritonitis				Pyothorax			
Patie-nt	PELDH	PlaLDH	PELDH/ PlaLDH	Patie-nt	PELDH	PlaLDH	PELDH/ PlaLDH	Patient	PELDH	PlaLDH	PELDH/ PlaLDH
1	83.1	398.4	0.21	1	6587	709.6	9.28	1	212.7	408.5	0.52
2	95.5	367.4	0.26	2	1445.6	340.5	4.25	2	75.3	1638.4	0.05
3	183.4	293	0.63	3	817.1	299.6	2.73	3	5292.1	445.9	11.87
4	109.8	389.4	0.28	4	1465.7	1463.1	1.00	4	21211.3	2646.6	8.01
5	94.6	115.9	0.82	5	1489.5	312.4	4.77	5	14191.8	320.8	44.24
6	106.6	212	0.50	6	79.4	216.8	0.37	6	34948.8	264.6	132.08
7	443.7	1286.3	0.34	7	2435.2	378.2	6.44				
8	1539.4	817.6	1.88								
9	141.9	376.5	0.38								
10	90	840.6	0.11								
11	125	245.6	0.51								
12	25.2	134	0.19								

PELDH = concentration of lactate dehydrogenase in pleural effusion, PlaLDH = concentration of lactate dehydrogenase in blood plasma

Table 4. The concentrations of fluid to serum ratio of total protein and its fractions in cardiac disease and neoplasia

Patient	Cardiac disease						Neoplasia					
	TP ratio	Albumin ratio	α1 ratio	α2 ratio	β ratio	γ ratio	TP ratio	Albumin ratio	α1 ratio	α2 ratio	β ratio	γ ratio
1	0.36	0.56	0.30	0.49	0.15	0.32	0.67	0.82	0.34	0.53	0.69	0.55
2	0.53	0.63	0.81	1.82	0.26	0.46	0.41	0.53	0.23	0.09	0.69	0.35
3	0.60	0.86	0.56	0.34	0.66	0.41	1.37	2.01	0.45	0.53	1.42	1.03
4	0.56	0.77	0.56	2.02	0.42	1.80	0.67	0.90	0.34	0.42	0.63	0.56
5	0.24	0.38	0.14	0.28	0.23	0.18	0.68	0.79	0.34	0.64	0.68	0.56
6	0.68	0.95	0.18	0.17	1.29	0.61	0.49	0.58	0.23	0.44	0.47	0.45
7	0.46	0.70	0.17	0.35	0.44	0.32	0.63	0.84	0.02	0.69	1.16	0.46
8	0.33	0.36	0.67	0.66	0.24	0.30	0.49	0.52	0.17	0.47	0.42	0.56
9	0.74	0.82	0.57	0.64	0.50	0.77	0.64	0.87	0.28	0.35	0.69	0.57
10	0.36	0.52	0.15	0.24	0.27	0.32	1.10	1.24	0.45	0.31	2.25	1.00
11	1.02	1.55	0.27	0.61	0.95	0.80	0.47	0.62	0.17	0.39	0.46	0.37
12	0.42	0.66	0.17	0.29	0.30	0.26	0.43	0.55	0.24	0.35	0.36	0.37
13	0.40	0.55	0.37	0.24	0.44	0.32	2.17	2.73	1.03	1.70	2.30	1.77
14	0.43	0.53	1.04	0.30	0.39	0.37	0.37	0.51	0.53	0.16	0.39	0.22
15							1.87	2.33	0.89	1.83	1.30	1.35
16							0.71	0.93	0.25	0.56	0.66	0.67
17							1.54	1.96	0.86	1.02	1.60	1.26
18							1.15	1.36	1.02	1.04	0.59	1.18
19							1.06	1.40	0.43	1.01	0.83	0.93
20							0.52	0.44	0.27	0.47	0.87	0.58

VITA

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